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Conditional gene targeting of *TP53* in pig - a model for Li-Fraumeni disease and gastric cancer

Simon Leuchs

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The most exciting phrase to hear in science,
the one that heralds the most discoveries,
is not "Eureka!" but

"That's funny..."

–[Isaac Asimov]

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Abstract

The central tumour suppressor gene TP53 is frequently mutated in human cancers. Several rodent animal models with TP53 defects have been generated and provided important insight into carcinogenesis and the central functions of TP53 in cell cycle regulation. However, on a physiological level they are insufficient to establish novel treatments and methods for early cancer diagnostics in humans. Here the pig can serve as a model with suitable size and a closer genetic and physiological relationship.

This work reports the modification of the porcine TP53 locus by gene targeting in primary mesenchymal stem cells. It is part of a larger research project for gastrointestinal cancer in conjunction with an APC-modified porcine model. In detail, a conditional transcription stop cassette (LSL) was introduced into intron 1 of TP53, effectively acting as a gene knockout reversible by Cre-mediated recombination. Furthermore, the latent oncogenic mutation TP53^{R167H} was inserted into exon 5, which corresponds to the human hot-spot TP53^{R175H} mutation.

The modified cells were analysed in *in vitro* assays. The excision of the LSL lead to expression of the mutated allele and an accumulation of the mutant protein. Homozygous TP53^{R167H} cells were p53 deficient and exhibited abnormal proliferatory behaviour, indicating first steps towards a transformed phenotype through p53 loss and mutation.

Subsequently, heterozygous TP53^{R167H} cells were used to clone live genetically modified animals via somatic cell nuclear transfer (SCNT). These animals can serve as powerful models to develop cancer treatment and help to find curative approaches transferable to humans.

A complementing porcine reporter model was designed, that allows research of promoter functions and *in vivo* Cre-application techniques. For this, a dual reporter cassette was constructed with a loxP flanked beta-geo gene exchangable with a mCherry gene in case of Cre-activity. These cells were evaluated *in vitro* and transgenic fetuses generated by SCNT and analysed by lacZ staining.

Zusammenfassung

Menschliche Krebserkrankungen tragen häufig Mutationen im Tumor-Suppressor-Gen *TP53*. Es wurden mehrere Nager-Modelltiere mit defektem *TP53* Gen erstellt und sie ermöglichten wichtige Einblicke in menschliche Tumorigenese und *TP53*-vermittelte Zellzyklus Regulation. Diese Modelle sind allerdings aus physiologischen Gesichtspunkten unzureichend für die Entwicklung neuer Behandlungen und Frühdiagnostik im Menschen. Das Schwein bietet sich hierfür als geeinetes Modelltier an, insbesondere durch die vergleichbarere Grösse und nährere genetische und physiologische Verwandtschaft zum Menschen.

Diese Arbeit beschreibt die Modifikation des porzinen *TP53* Locus mittels "gene targeting" in primären mesenchymalen Stammzellen. Dies ist ein Teilprojekt für gastrointestinale Krebserkrankungen in Kombination mit einem APC-modifizierten Schweinemodell. Im Detail wurde eine Transkriptions-Stop Kassette (LSL) in Intron 1 von *TP53* eingefügt. Dieser faktische Gen Knockout kann durch Cre-vermittelte Rekombination rückgangig gemacht werden. Darüberhinaus wurde in Exon 5 die $TP53^{R167H}$ Mutation eingefügt. Diese entspricht der humanen hot-spot Mutation $TP53^{R175H}$.

Die modifizierten Zellen wurden *in vitro* Analysen unterzogen. Das Entfernen der LSL Kassette führte zu einer Expression des mutierten Allels und einer Akkumulation des mutierten Proteins. Homozygote $TP53^{R167H}$ Zellen waren p53-defizient und zeigten abnormale Proliferation, erste Anzeichen für einen transformierten Phänotyp.

Im Anschluss wurden heterozygote $TP53^{R167H}$ Zellen verwendet, um mittels somatischem Zellkerntransfer (SCNT) lebende genetisch modifizierte Tiere zu erstellen. Diese Tiere können als Modelle für die Entwicklung von Krebs-Therapien dienen und helfen, Heilungsansätze zu identifizieren, die auch auf den Menschen übertragbar sind.

Ein Reporter-Schweinemodell wurde entworfen, um das *TP53* Modell zu komplementieren. Es ermöglicht Studien über Promotor-Funktionen und über *in vivo* Anwendbarkeit von Cre-Rekombinase. Zu diesem Zweck wurde eine Doppel-Reporter-Kassette in Zellen integriert, die ein loxP-flankiertes beta-geo Gen beinhaltet, welches bei Cre-Aktivität gegen ein mCherry-Gen ausgetauscht wird. Diese Zellen wurden *in vitro* Analysen unterzogen und verwendet um transgene Föten mittels SCNT zu erstellen, die ebenfalls mittels lacZ-Färbung untersucht wurden.

1. Introduction

1.1. Introduction into the topic

Cancer is the forth most common cause of death and emerging even more prominently in the western world where it ranks third (World Health Organisation). The lifetime cancer risk has been statistically determined to approximately 40% in the current population [118]. With the increase in life expectancy, cancer is turning into an even more severe health risk. Medical progress has accelerated significantly with the rise of molecular biology and its revolutionary treatment options from monoclonal antibodies to somatic gene therapy. They depend on established methods of early diagnoses and specific molecular markers. However, these prerequisites are still quite rare in a disease so variable as cancer.

Therefore, research clarifying early cancer development, identifying new markers and ultimately leading to improved treatments is urgently required. An important part of this research takes place in *in vivo* models, mimicking the human disease. Information drawn from these experiments has provided much insight into cancer biology and proven valuable for therapeutic developments and understanding of oncogenesis.

It is now known, that in cancer defunct cell cycle regulation or faulty cell signalling ultimately cause continuous proliferation of tissues resulting in pathogenic phenotypes. These aberrations generally stem from genomic mutations acquired through contact with carcinogens, inheritance and ageing. In healthy cells so called tumour suppressor genes counteract these influences by activating DNA repair or apoptotic mechanisms. *TP53* is the most prominent member of this family, and of central interest in this work.

This thesis focusses on the modelling of two severe human cancerous pathologies in a porcine animal model: Li-Fraumeni syndrome (LFS) and gastric cancer (GC). *TP53* mutations play major roles in both. They are the main cause for LFS and when combined with second hit defects in other tumour suppressor or oncogenes can lead to cancer. The context in this work is the combination with a mutated adenomatous polyposis coli gene (APC), and its participation in gastric carcinogesesis [99, 258]. Animals carrying the latter modification are available for crossing experiments [73]. A parallel approach in this work is the mimicking of inflammatory processes in the stomach through tissue specific expression (compare [228]).

1.2. The tumour suppressor p53

The tumour suppressor gene *TP53* plays a central role in cell biology and tumour development. The so-called guardian of the genome acts as a key transcription factor in DNA damage response, cell cycle regulation and apoptosis. To date it is also the tumour suppressor gene lost or impaired in the majority of human cancers [240]. It is highly conserved across species both in structure and function and at the center of a large amount of research. There are more than 50.000 research articles listed in online libraries that cover or touch the field of roughly 40 years of p53 research.

1.2.1. Discovery

The protein was first identified in 1979 [155, 146, 176] as an associated to the simian virus 40 (SV40) large T antigen. It was taken for a virally produced protein and only later realized that it was produced by the host cell itself. The murine gene was cloned in 1982 and 1983, with the human sequence following shortly after in 1984 and 1985 in full length. At first it was mistaken for an oncogene, since it was found so frequently mutated in cancers and the transfection of cells with isolated p53 mRNA caused the cells to transform to a more oncogenic phenotype. As it turned out, the transforming mRNAs were isolates from carcinoma cell lines and not healthy tissue.

The sequence homologues in other species were isolated, but did not show the transforming functions *in vitro*, on the contrary, leading to an arrest of proliferation. After more and more contradicting results emerged, the role of p53 was corrected in 1989 by the group of Bert Vogelstein [16]. It started off an era of research on tumour suppressor genes, while at the same time unknowingly demonstrating the possibility of gain of function mutations.

Since that time the *TP53* gene has turned into one of the most extensively studied genes of all. However, there are still some key questions unanswered.

1.2.2. The p53 protein

The p53 protein is encoded by the 19.2 kb long *TP53* gene on chromosome 17p13.1 in humans (12q12-q14 in pigs) consisting of 11 exons that encode a full length transcript of 1182 bp (1161 bp in pigs). The sequence and structure are conserved across many species [110, 314]. The expressed protein is comprised of 393 amino acids (AA) (386 AA in pig) with a molecular weight of 43.6 kDa before posttranslational modifications and running at a size of 53 kDa on SDS gelelectrophoresis, hence the name p53.

The structure of this protein is not yet fully solved, as it was not possible to crystallise

it as a whole. However, the structure of singular domains, like the DNA-binding domain have been solved [110].

Its folding follows the boundaries of several seperate protein domains, shown in figure 1.1 (next page) a central DNA binding domain (DBD)(residues 102 - 292) is flanked by two distinct transactivation domains TAD1 and TAD2 (residues 17 - 25 and 48 -56 respectively), followed by proline rich domain (prD) a p53 multimerisation domain (residues 325 - 356), a bipartide nucleus localisation signal (NLS) (residues 305 -321) and a nucleus export domain (residues 339 - 350).

It binds to DNA as a homotetramer to a tandem repeat of the consensus pattern of purine (Pu) and pyrimidine (Pyr): 5-Pu-Pu-Pu-C-(A/T)-(T/A)-G-Pyr-Pyr-Pyr-3. The repeats are spaced 0-13bp apart [66]. Each subunit binds to one zinc ion as a cofactor between the residues 176, 179, 238 and 242.

It is expressed in 9 different isoforms from alternate promoter usage and alternate splicing. The isoforms are expressed with different tissue specificity and also conveying variable functions. They are enumerated 1-9 or alpha, beta and gamma with more specific annotations of their deletions, for example Del40-p53alpha.

Figure 1.1 gives an overview of the isoforms, and their respective deletions.

1.2.3. Gene function

The p53 protein is a transcription factor with influence in a wide range of cellular responses. It ranges from DNA damage response, across cell cycle regulation into apoptosis and other physiological functions. Some of the functions even seem oppositional: for example the cell cycle arrest and DNA damage repair in contrast to the induction of apoptosis.

Figure 1.2 on the next page gives a basic overview of *TP53* functions.

The role in tumour suppression is thought to be a more recent adaptation of the gene. It can be understood as a subtype of cell cycle regulation in long lived, multicellular organisms with the necessity to keep their cells regenerative potential under control (reviewed in [163]). The regenerative stem cells niches have to be kept intact throughout the accumulation of somatic mutations during the aging process.

The type of p53 response is dependent on the type and intensity of the stress, the genetic predicaments and cellular characteristics. Interactions between the p53 pathway and the survival signaling or the retinoblastoma pathway can lead to the different p53 signaling into growth arrest or apoptosis. It was also proposed, that the choice between cell cycle arrest and apoptosis is dependent on the p53 dose. Low doses lead to arrest and high doses to apoptosis. This is mirrored in the high binding affinity of p53 to the promoters of arrest promoting genes of the p21 pathway and the low affinity of p53 to pro-apoptotic

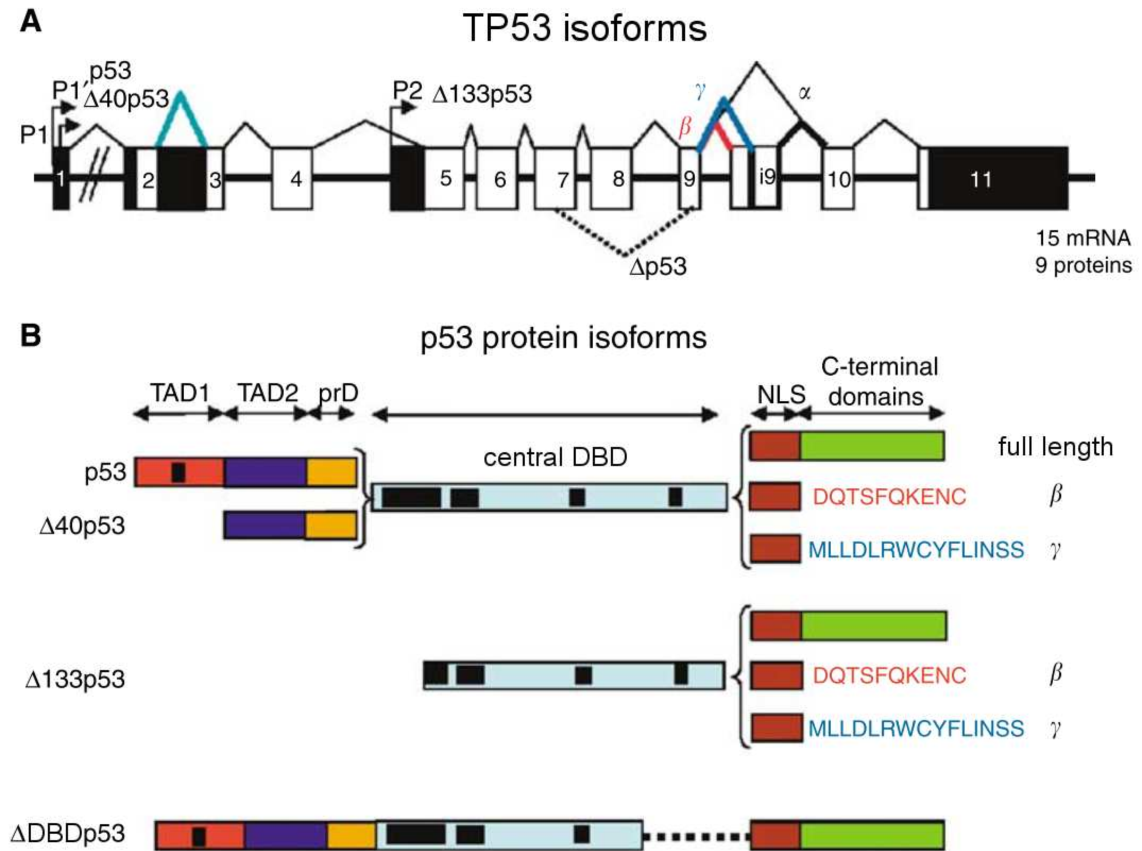

Figure 1.1.: *TP53* and p53 isoforms. A: Human *TP53* gene schematic with promoters P1 and P2 and splice forms α β and γ. B: p53 protein isoforms with transactivation domains (TAD), proline rich domain (prD) DNA binding central domain, nucleus localisation signal (NLS) and C-terminal domains. The N-terminally truncated forms are indicated as δp53 and C-terminally truncated forms are shown with their alternative amino acid endings β and γ. Figure from [26].

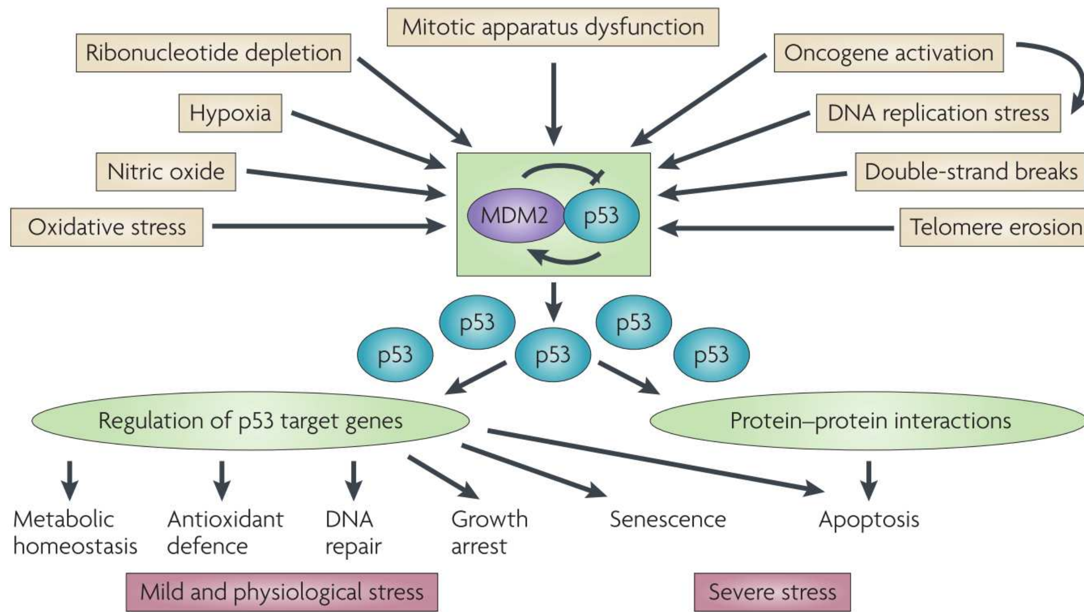

Figure 1.2.: Schematic of p53 functions with the p53–MDM2 feedback loop in the center. Various triggers for p53 response lead to the accumulation of p53 and depending on the severity of the stress a range of outcomes from simple DNA repair to apoptosis. Figure taken from [163].

genes like p53 upregulated modulator of apoptosis (PUMA) [214] and phorbol 12-myristate 13-acetate induced protein 1 (NOXA) [268].

However, this model is incomplete: p53 levels were also observed to rise and fall in a discrete oscillation after DNA damage. The number of p53 pulses has been linked to the severity of the DNA damage. Repairable damage only causes few pulses of p53. Irreparable damage however leads to a p53 response of several pulses followed by a constant high p53 dose. The latter then leads to apoptosis and shows the interlinking of both decision mechanisms [345].

P53 mediates its apoptotic functions in part by triggering the expression of apoptotic effectors. The most important of these are the B-cell lymphoma 2 (Bcl-2) homology domain 3 (BH3)-only factors PUMA and NOXA. These genes have been knocked out in cell lines and mice and certain cell populations appear to be protected against p53 mediated apoptosis to the same extent as p53 knockout lines [129, 203].

p53 also can promote apoptosis without its transactivation domains, so not acting as a transcription factor [103]. It was shown, that p53 translocation into mitochondria triggers an immidiate apoptotic reaction through cytochrome C release and caspase cascade activation [188]. Here, it seems to function as an BH3-only analogue. Therefore, one refers to the transcription-dependent and transcription-independent pathway of p53 me-

diated apoptosis. It is interesting to note, that tumour-derived p53 is unable to induce transcription-independent cell death [298, 242].

The major triggers of p53 activity are generally grouped into a DNA-damage response and tumour suppressive functions of p53. How strict this separation actually is, is still subject to debate. The following paragraphs provide an overview across both fields and the intermediate discussion.

DNA-damage response

Exposure to ultraviolet light, ionizing radiation or chemical agents among others can cause DNA damage like double strand breaks (DSB). This can lead to the accumulation of mutations, through incomplete repair. These mutations can be the point of origin of cancerous growths by activating oncogenes or inactivating tumour suppressors.

The p53 pathway is very responsive to DSBs but also single strand nicks [121]. The initial activation is mediated by ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) protein kinases, which are involved in DNA damage signaling. They phosphorylate p53 at diverse post-translational modification sites, varying according to type and intensity of the damage (reviewed in [199]). This stabilises the p53 protein and provokes a corresponding cell cycle arrest, senescense or apoptosis.

In blocking the cell cycle, p53 provides a timing window to repair the DNA damage. It can also trigger apoptotic signalling, in case of persistent or extensive DNA damage. This permanently inactivates or removes fatally damaged cells from an organism.

A p53 DNA damage response activation is also what leads to mortality from exposure to high amounts of radioactive radiation. The resulting extensive and systemic DNA damage leads to widespread apoptosis and finally collective organ failure. This explains, that avoiding the p53 response directly after irradiation mitigates this pathological effect drastically in mice [44].

It is interesting to note, that an increased DNA damage response is frequently found in the early development of tumours even before mutations in the p53 pathway or other chromosomal aberrations. Cells that proliferate abnormally in early neoplasias show activated DNA synthesis. An abnormal proliferation also leads to more frequent break off of the replication fork and subsequent DNA damage. This is not the case in other fast-proliferating tissues. Research shows this is probably due to aberrant expression of cyklins. This inhibits and terminates replication forks [18].

It could also be shown *in vitro*, that DNA damage response is necessary for oncogene induced senescence. A knockdown of the DNA damage regulator ATM eliminates this re-

sponse. Moreover, the expression of oncogenic Harvey rat sarcoma viral oncogene homolog (HRAS) was found to induce DNA damage foci [18].

This shows a close connection between the DNA damage response and the tumour suppressive functions of *TP53*. DNA damage induced senescence provides a safety measure that inhibits abnormal growth independent from known tumour suppressive pathways. It is however not sufficient on its own, and p53 tumour suppression does use other pathways for its effect.

One model explains the tumour suppression of p53 in part through its DNA response mechanism. The activated checkpoint kinases phosphorylate p53 and activate its DNA damage response. The model states, that the increased frequency of DNA damage in abnormally proliferating cells could be the cause for p53 mediated tumour suppression through cell cycle arrest [95].

Tumour suppression response

Tumour suppression requires a fine distinction between normal and neoplastic cells, since both rely on similar endogenous or exogenous proliferative and angiogenic signaling. The difference lies mainly in the persistence and self sufficiency of signaling in neoplasias [131]. It is interesting to note, that p53 itself does neither sense, nor is it activated by aberrant cell signaling. The role of p53 in tumour suppression is dependent on its activation through mediators. Only after activation p53 mediates cell cycle arrest or senescense/apoptosis, thereby effectively suppressing the tumour.

The most prominent tumour dependent activator of p53 is the p19/ARF protein pathway. This pathway can be activated by oncogenes and inhibits mouse double minute 2 homolog (MDM2), the negative regulator of p53 [44, 65]. It is induced by aberrantly high proliferative signals, a frequent but not the only trait of cancer. Continuous low level expression of oncogenes like c-Myc and HRAS however is able to circumvent this and still cause tumours [262, 212]. This means, that p53 tumour suppression is dependent on signaling intensity and not the actually oncogenic persistence.

The effectors of p53 mediated tumour suppression are still subject to research. The tumour suppression effect is mediated by a small subset of p53 activated genes. Not all transactivation domains of p53 are needed for tumour suppressive function [27] and several knockout models of p53 target genes still could not fully replicate the tumour susceptibility of p53 loss (reviewed in [182]).

A basic overview of the *TP53* pathway is shown in figure 1.3 on the next page. A more detailed overview is included in the appendix chapter A.1.3 in figure A.2.

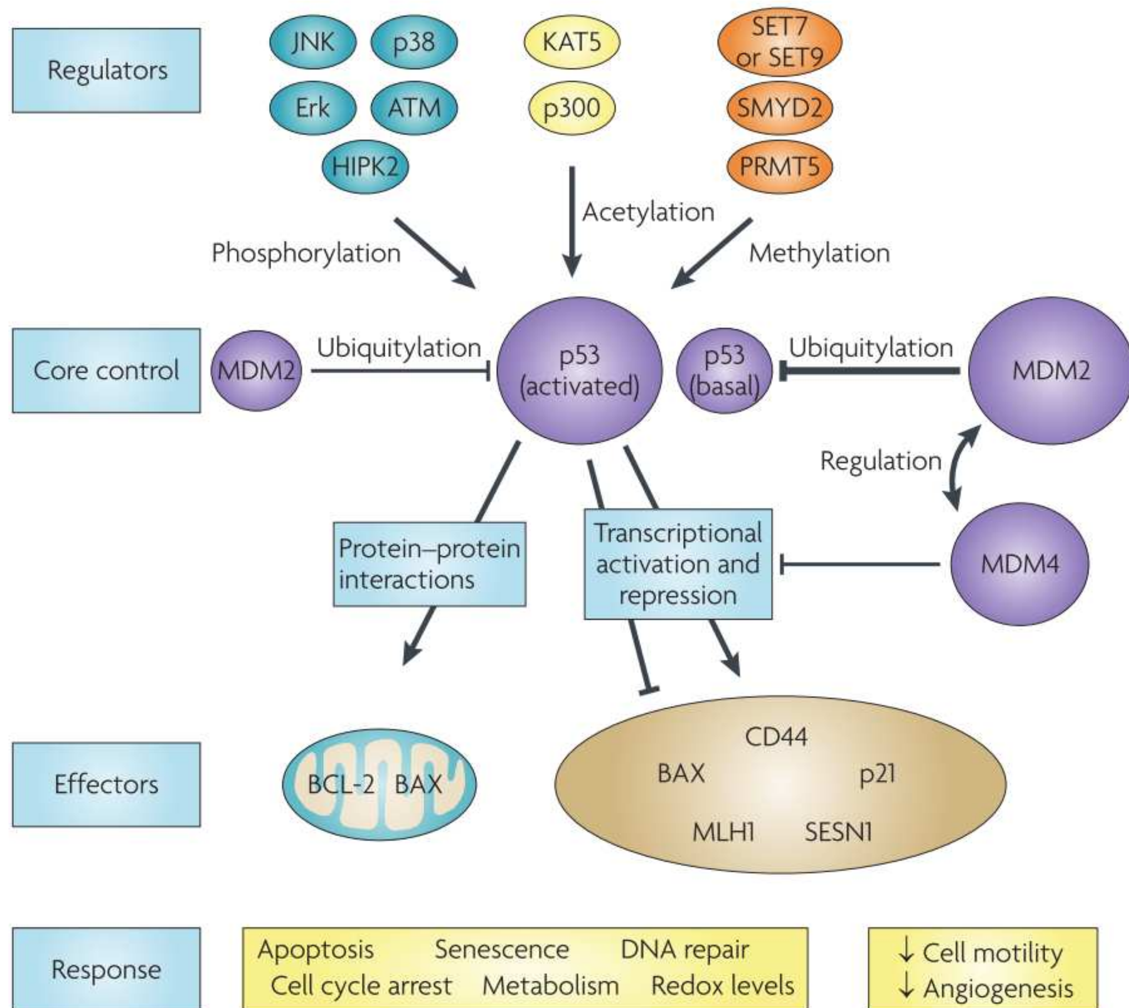

Figure 1.3.: Schematic of p53 pathway with the p53–MDM2 feedback loop in the center. The core regulators are noted together with their mode of posttranslational modifications. Also the p53 effectors and their mode of activation can be seen on the bottom. Again the range of outcomes is indicated. Figure taken from [324].

Additional functions of p53 *TP53* also plays a role in cell and organismal aging. The activity of p53 in cells can lead to cell cycle arrest senescence or apoptosis. With time, this slowly depletes tissues of the pluripotent regenerative cells and cell niches that have also taken DNA damage. This is basically an endogenous damaging of the tissue, which finally results in the degeneration that characterises the aging process. Some evidence suggests, that hightened p53 activity even promotes the aging process. Activation of p53 promotes cell ageing via the mTOR pathway [161].

The *TP53* gene shows more functionality beyond its role in cell cycle regulation. In its inactive state it also influences physiological functions like fertility [119], cell metabolism [133], mitochondrial respiration [193], autophagy [290], cell adhesion [89], stem cell maintenance [85] an development [51]. Mitochondrial p53 protein shows RNAse activity and degrades exonucleolytic RNA in the cytoplasm. This enhances the accuracy of DNA synthesis in mitochondria and is indeed lost in some oncogenic *TP53* mutants [17].

1.2.4. Gene regulation

Under physiological conditions p53 activity is ubiquitous but low. These expression levels are sufficient for mediating physiological functions. The half life of the protein is a mirror to its flexible function: a short 5 – 20 min allow for quick adaptations.

The *TP53* gene with its central role in cell cycle regulation and DNA integrity is understandably tightly regulated. Its main regulator is the ubiquitin ligase murine double minute 2 (MDM2). It acts in a complex with murine double minute 4 (MDM4) and leads to p53 ubiquitinylation and subsequent degradation [120]. The two proteins are linked in a negative regulatory feedback loop.

The interaction of p53 protein to MDM2 occurs between a C-terminal helix in the transactivation domain (TAD) of p53 (residues 18 - 26) and more weakly between p53 nascent turn motifs (residues 40 - 56). Mutation located in these TADs can inhibit the interaction and thereby distort the p53-MDM2 regulatory feedback loop [42, 174].

Figure 1.4 on the next page shows a simplified schematic of the direct regulatory network of p53 by proteasomal degradation.

The fine tuning of the activity is mediated by several posttranslational modifications. The p53 protein is the target of phosphorylation, acetylation, ubiquitinylation, neddylation and methylations. The complex pattern of modification sites and corresponding enzymes is reviewed in [296].

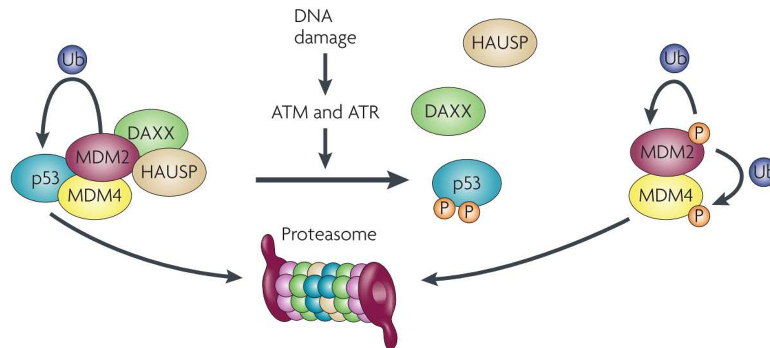

Figure 1.4.: The p53 protein is degraded via ubiquitylation (Ub) and the proteasom. This is mediated by E3 ubiquitin ligases, mainly by MDM2. p53 and MDM2 are connected in a negative feedback loop as p53 induces MDM2 expression. Furthermore, MDM2 ubiquitylates both itself and MDM4. MDM4 itself inhibits p53-mediated transcription. The herpesvirus-associated ubiquitin-specific protease (HAUSP; also known as USP7) deubiquitylates MDM2 and p53. DAXX interacts with both HAUSP and MDM2 and directs HAUSP activity towards MDM2 and MDM4, thus promoting p53 turnover. DNA damage phosphorylates (P) p53 by the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) protein kinases. This separates the protein complex, thereby stabilising p53 and leading to MDM2 and MDM4 degradation. Figure taken from [199].

1.2.5. Mutations of the *TP53* gene

There is a strong selection against p53 function in the majority of human cancers. Therefore, indirect or direct alteration of the p53 pathway is a frequent event [239]. This occurs through a range of mutations either within the *TP53* gene itself or in its pathways targets. These mutations are evolutionary selected for during cancer development, due to its central role for tumour suppression.

It is interesting to note, that inactivating p53 mutations differ from tumour type to tumour type. The reason behind this is still not completely understood. In lung cancer common hot-spot mutants are replaced by mutations caused by the benzo(a)-pyrene in cigarette smoke, which frequently causes G>T transversions [239]. Brain and breast cancers are more prone to carry mutations within DNA binding domain, while rarely found in adrenocortical carcinoma [222]. This possibly reflects the tumour specific selective pressure on certain p53 up- or downstream targets.

A selection of *TP53* mutations will be covered in the following paragraphs.

Loss of function mutations of *TP53*

The most straightforward mutations of p53 cause a loss of its function by indirect or direct inactivation [239] and is the underlying defect in Li-Fraumeni multiple cancer syndrome [13]. Naturally, this can be the effect of a physical loss or a mutation of the *TP53* locus.

The location, type and frequency of *TP53* somatic mutations have been surveyed across a wide range of human cancers [223]. 86% of *TP53* mutations lie between codons 125 and 300, broadly corresponding to the DNA binding region. Most are missense mutations that lead to p53 protein species with altered conformation or lacking specific DNA binding function [276, 75, 271].

Apart from mutations in the *TP53* gene itself, the most common mutations in the *TP53* pathway happen in the direct p53 regulators. The most prominent example is the deregulation of the ubiquitin ligase MDM2 [81]. Its upregulation leads to accelerated p53 ubiquitinylation. This destabilises the negative feedback loop (compare figure 1.4) and p53 gets degraded.

This loss of function facilitates tumour development and influences global gene expression because the physiological functions are also lost [219].

Gain of function mutation of p53

Certain mutations exhibit a dominant negative effect and may impart 'gain-of-function' oncogenic properties that are incompletely understood (reviewed in [225]). They may cause faster proliferation [265] or immortalisation of cells [32], in addition to the loss of wildtype p53 function. Some p53 mutants aid tumour development by enhancing vascularization via a reactive oxygen species (ROS)-mediated activation of the HIF1/VEGF-A pathway [134], or provide chemoresistance [301].

A prominent human hot spot mutation is the *TP53*$^{\mathrm{R273H}}$ contact mutant. This means, the exchanged amino acid is in direct contact with DNA [43]. *In vitro* it mediates resistance to apoptosis caused from the chemotherapeutics doxorubicin and methotrexate [331], promotes invasiveness and migration of endometrial cancer cells [60] and enhances proliferation in a prostrate cancer cell line [310]. This mutation has also been modelled in mice and shown gain of function effects also *in vivo* [221].

A more severe mutation is *TP53*$^{\mathrm{R175H}}$, a hot spot gain of function mutation identified in humans. In contrast to *TP53*$^{\mathrm{R273H}}$, it is a structural p53 mutation that mitigates p53 tetramerisation [271]. It is particularly potent in promoting carcinogenesis or supporting its foundations.

R175H mutant p53 inhibits wild-type p53 interaction with promoter elements [329, 313]. This in turn prevents the transcription-dependent p53 functions referred to in chapter 1.2.3.

It has also been found to advance angiogenesis in tumours, thereby securing the oxygen and nutrient supply of the proliferating cells [134]. It also promotes epithelial mesenchymal transition, a process that is believed to play an important role for tumour metastases and tissue invasion [142]. It has shown immortalization effects in *in vitro* experiments [32]. Like *TP53*$^{\mathrm{R273H}}$, it has also been transferred onto a murine background and shown *in vivo* effectiveness [221]. Based on these characteristics, it was chosen as the target mutation in this work.

1.2.6. Rescue of wildtype p53 function

Wildtype p53 is a potent inhibitor of tumorous growth. This can be seen by the strong selection against p53 function in cancer. It is interesting to note, that recent research has managed to reactivate the wildtype *TP53* allele or protein within tumours. This can lead to a profound decrease of tumour size and cancer regression [309]. There are trials to transfer this finding into human treatment. The reactivation was mediated by small molecules stabilising p53 [322] or immunologically by antibodies targeting a common epitope of mutant p53 [226]. Naturally, this whole approach necessitates a remaining functional *TP53* allele and no dominant negative *TP53* mutations present.

1.3. Gastric cancer and Li Fraumeni disease

This work was part of a large project to model gastrointestinal diseases in porcine animal models. The *TP53* gene knockout would predestine the animal model into a wide range of cancers, but in combination with molecular techniques should serve as a model of gastric cancer and alternatively Li-Fraumeni disease.

The scientific background for this stems from research of human pathologies. It is known, that *TP53* mutations or modifications play a role in gastric cancer and are the major cause for Li-Fraumeni syndrome [13, 252]. Further knowledge has been gained in rodent models, that support the viability of this works approach. Here, p53 knockout and mutated mice have shown increased susceptibility to stomach or intestinal carcinogenesis and Li Fraumeni like syndromes [109, 221, 306, 335].

1.3.1. Gastric cancer

Gastric cancer is one of the leading causes of cancer related deaths worldwide. This is not due to inoperability like cancer in other organs might be. Even a complete resection of the stomach is feasible and even conducted just for dietary restriction. Possibly the main reason this cancer is so deadly is, that its early stages are often overlooked or misinterpreted.

This proves to be fatal, as complete resection and cure can practically only be achieved in pre-metastatic early phases of tumour development.

Disease in numbers

Gastric cancer cases are unequally distributed across the globe, but overall is the fourth most common cancer worldwide. High incidence regions like northern Asia with 69 cases per 100.000 people stand in contrast to low risk regions in Africa or Australia with only 4-10 cases per 100.000 people. The incidence of mid- and distal intestinal gastric carcinoma is declining worldwide. Cancer in the cardia is on the rise however. This may be due to decreasing *Helicobacter pylori* (*H. pylori*) infections but at the same time increasing obesity. The latter is suspected to promote gastric cardia- and esophageal carcinoma. These changing trends are also expected to emerge in South America and Asia (review see [3, 204]) There are already more new cases diagnosed each year in China than in any other country. In Japan it was the most common type of cancer for men. In Western countries, the most common sites of gastric cancer are the proximal lesser curvature, cardia, and gastroesophageal junction [3]. In the United States there were 37,600 new diagnoses and 25,150 deaths from upper gastrointestinal cancers in 2009, making it one of the least common cancers in North America, yet.

In Japan and Korea, where screenings are widespread and common, early detection is often possible. In other countries, gastric cancer is often diagnosed at an advanced stage. Early gastric cancer is mostly overlooked or misinterpreted as dysplasia. However, these lesions already show lymph-node metastases of 2-5% for mucosal and even higher (11-20%) for submucosal locations [338]. Therefore, the rate of survival differs greatly between Japan (∼52%), in comparison to survival in the USA, Europe, and China (∼28%) (http://seer.cancer.gov/). Survival in patients with unresectable gastric cancer is low, but even if the cancer is resectable lies below 50% in developed countries (excluding Japan) [101].

Therefore, there is a demand for predictive biomarkers, that can be used to detect the early and resectable GC stages. Current biomarkers can give indications about disease progression and severity of prognosis. Large amounts of research have identified biomarkers through serum proteomics [249, 183] or comparative genome analysis [297]. Mutations in the p53 gene are also an event used for gastric cancer prognosis [252].

Risk factors

Risk factors in cancers usually involve hereditary as well as environmental components. In gastric cancer, since the stomach is an organ heavily contested by the environment,

genetic predisposition only accounts for 1% - 3% of all cases. Within these the predominant mutation is a defect in the E-cadherin locus correllated to 25% of hereditary gastric cancers [72]. Loss of this cell adhesion molecule facilitates tissue disorganization and thus cancerous dedifferentiation. Mutation carriers have a lifetime gastric cancer risk of 67% in men and 83% in women. Therefore usually a prophylactic removal of the stomach is advised if there is a known familial history [123]. Moreover, men are more susceptible to intestinal gastric cancer than women, possibly for hormonal reasons [35].

Risk factors for non-hereditary gastric cancer are known to be smoking, high salt intake and obesity. Over time, they cause long term damage to the stomach and facilitate tissue transformation. Therefore, the most successful way to avoid GC is actually a healthy life style and diet.

Apart from this, gastric infection with *H. pylori* is able to promote gastric carcinogenesis. These bacteria were classified as carcinogens by the World Health Organisation in 1994 as about one in five infected patients develops gastric ulcers. In one in hundred cases infection leads to gastric adenocarcinomas [279]. This development was also modeled successfully in animals [141]. *H. pylori* is specialised in long term colonisation of the gastric mucosa, thus it eventually provokes a long term immune reaction and inflammation. The infection progresses through chronic superficial (non-atrophic) to atrophic gastritis, intestinal metaplasia, and dysplasia toward GC.

It has been shown, that the genetic background of vacA and cagA alleles of *H. pylori* influences cancer development [180, 337]. The patients immune response [49, 83], mucosal and oxidative stress protection [33] and detoxification processes [300] also influence GC risk. Therefore, the correlation of *H. pylori* infection and gastric cancer risk in a population is not universal. Not all regions with frequent *H. pylori* infections, such as Africa, show elevated cancer risk. Nevertheless, H. pylori eradication can have a prophylactic effect against GC in high risk regions (reviewed in [132])

Oncogenesis

In general, GC is divided into adenocarcinomas of two histologic entities: the intestinal and the diffuse type, with distinct epidemiological and genetic patterns.

The intestinal subtype is characterised by a well differentiated tumour. Here the disease progresses along from gastritis to gastric intestinal metaplasia and dysplasia to cancer. This type mainly develops in elder people with a history of chronic gastritis.

Younger patients with gastric cancer predominantly show the undifferentiated diffuse type of tumour. This lesion typically develops directly from inflammatory foci, skipping

atrophic gastritis and metaplasia. This type of cancer shows stronger hereditary influence and multifocal distribution in the stomach.

Inflammatory processes in the mucosa are understood to be the basis of both lesions. Inflammation and the associated cytokines leads to macrophage accumulation, higher tissue turnover and predisposes for abnormally elevated proliferation. Also genetic stability is compromised, as mutagenic substances like reactive oxygen [15, 70] or nitrogen species [80] can arise during gastric inflammation. This initially promotes apoptosis of cells, the exact opposite of a carcinoma. The mutation frequency has been shown to be 4 fold higher in a H. pylori infected mouse model compared to wildtype conditions [299]. The persistence of these mutagens over time leads to an accumulation of DNA damage, loss of tumour suppressor genes, activation of oncogenes and thus cancer.

1.3.2. Li Fraumeni syndrome

Li Fraumeni syndrome (LFS) is an extremely rare pathology in humans that is defined by a hereditary early onset of cancers, often with several tumours developing within one patient. There are only about 400 cases in total reported in the literature [175]. The first report of the disease in literature was after a widespread epidemiological study in childhood sarcoma [165–167]. It follows an autosomal dominant inheritance and usually affects multiple members of a family. These tumours are not confined to a certain tissue of origin like GC but rather show diverse types. The most common LFS type tumours are soft tissue sarcomas and osteosarcomas, breast cancer, brain tumors, leukemia and adrenocortical carcinoma. It has also been linked to cases of both diffuse and intestinal gastric cancer [191]. Figure 1.5 shows a pedigree of a family with LFS [187].

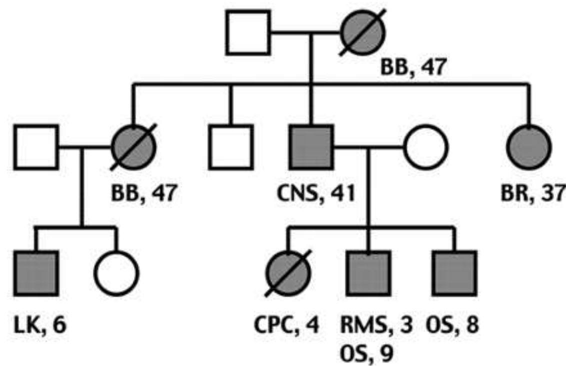

Figure 1.5.: Schematic pedigree of a family with LFS. Affected family members are marked as black circles and squares; deceased cases are crossed out. The age of confirmed cancers are indicated next to the abbreviations of cancer type: BB = bilateral breast cancer; BR = unilateral breast cancer; CNS = tumour in the central nervous system; LK = leukemia; CPC = choroid plexus carcinoma; RMS = rhabdomyosarcoma; OS = osteosarcoma. Figure from [187]

This heterogenous trait is embodied in the definition of LFS: "a proband with a sarcoma before the age of 45 years and a first-degree relative with any cancer before the age of 45 years and one additional first- or second-degree relative in the same lineage with any cancer before the age of 45 years or a sarcoma at any age".

The related Li-Fraumeni-like syndrome (LFL) is defined less stringently through a patient with any cancer during childhood, or a sarcoma, brain tumor, or adrenocortical tumor before 45 years of age. Again with an additional first- or second-degree relative in the same lineage with a typical LFS tumor at any age, and another first- or second-degree relative in the same lineage with any cancer before the age of 60 years [22]. This definition is sometimes defined more broadly as 2 different LFS-related tumors in first- or second-degree relatives at any age [64].

Both LFS and LFL are closely linked to germline mutations within the tumour suppressor gene *TP53*. Mutations of the gene are found in approximately 70% of LFS patients and 40% of the LFL cases [13]. This type of LFS is referred to as LFS1.

Non-p53 LFS/LFL cases could in parts be linked to mutations in the checkpoint kinase 2 (CHEK2) or a locus on chromosome 1q23 [13]. These disease subtypes are referred to as LFS2 and LFS3 respectively.

Understandably, the lifetime cancer risk of affected patients is very high. For males it rises with age to a maximum of 27% under the age of 45 and 54% beyond. In women the risk is significantly higher with 82% before the age of 45 and 100% beyond [332]. This discrepancy stems from the high rate of breast cancer.

The treatment of LFS is actually the treatment for each emerging type of cancer. A cure for the hereditary predisposition would require a systemic somatic gene therapy, a goal that is and probably will always be out of reach.

1.4. Animal models

Research for novel therapeutic approaches and the molecular understanding of oncogenesis is carried out both in *in vitro* and *in vivo* models. Cellular *in vitro* test systems, while convenient to set up and access, are subject to severe limitations. They fail to replicate the natural cellular environment and interactions and are only applicable to cell types that can be kept in culture. If the research in question demands more of its test system it has to be moved into a more complex environment: the animal model. In current cancer research animal models therefore play an important role. They are used to identify early disease stages that are still operable but untraceable in humans. They are also paramount in pre-clinical trials and testing of novel therapies. They allow for high case numbers under

defined conditions in contrast to studies in humans. Also the detailed observation of the whole timeline of disease development is possible.

1.4.1. Rodent cancer models

Rodents are at present the most common species used to model human disease conditions, having the advantage of low cost, short generation interval, easy handling and facility of genetic modification. Multiple cancers and their molecular background have been researched in rodents.

In some cases the cancerous disease occurs naturally and does not require additional interference. Mice for example tend to die of naturally occurring age dependent cancers. Hepatocellular carcinomas and adenomas are the most prevalent neoplasms. Also reticulum cell sarcomas, lymphomas and lung tumours are found [245]. Inbred mice from the CE/J strain develop tumours similar to the LFS spectrum mentioned in chapter 1.3.2 [68].

The use of naturally occurring cancers in research however is limited. They are not guaranteed to occur and the type of cancer can not be predicted beforehand. This leads to higher numbers of animals that need to be observed and no directed research. The advent of molecular biology and genetic modification technologies lead to an increase in artificially induced or even genetically defined rodent models for human cancerous diseases. These models have the advantage of high incidence and prevalence of predefined cancers, thereby reducing research effort and animal experiments. In the following paragraphs a subset of these will be shown in more detail.

Simple, unspecific models are based on the administration of carcinogenic substances to the animals. For example, mutagenic and nonmutagenic exposure of rats lead to changes of p53 and K-ras in rat lung tumours [20]. It was also found, that a diabetic mouse model is more susceptible to eternal carcinogenesis by N-methyl-N-nitrosourea (MNU) [340]. However, carcinogen induced tumour models are always less specific and the information gained therefore less valuable.

More specific models arose with the technologies for directed genomic modification. The most prominent cancerous mutations found in humans were re-created in rodents. The known oncogene K-ras with its most prominent mutation K-rasG12D is a commonly used gene for inducing cancer in an animal model. Mice with conditionally activatable K-rasG12D mutants were generated and the mutations effects shown in various tissues. Ubiquitous expression of the mutation from its endogenous promotor lead to gastric hyperplasia [192]. When the expression was directed by the keratin 5 promotor, the mice showed squamous cell carcinogenesis [312].

The tumour suppressor *TP53* was another important target. Mice and rats with hetero-

and homozygous *TP53* knockout were generated [58, 306], as well as mice carrying *TP53* mutations homologous to human hot spots [221]. Mice deficient in p53 were found to be highly susceptible to MNU primed carcinogenesis compared to heterozygous and wild type controls [335]. Also crossings of several deficient genotypes were performed e.g. combining *TP53* with K-ras mutations, thus promoting pancreatic cancerous lesions [109].

Of interest in this work were mouse models generated for gastric cancer research.

Oshima et al. have generated a transgenic mouse model for stomach cancer by artificially expressing inflammation and proliferation inducing factors. The expression was directed into the gastrointestinal tract by the cytokeratin 19 (KRT19) promotor. They observed the development of stomach cancer through intermediate spasmolytic polypeptide-expressing metaplasia with high penetrance and of severe phenotype in triple transgenic mice with KRT19-Wnt1, KRT-cyclooxygenase 2 (COX2) and KRT-microsomal prostaglandin E synthase 1 (mPTGES1) expression constructs [228]. Mice lacking the Wnt1 transgene would form hyper- and metaplasia through chronic inflammation in the stomach but leave out the abundant proliferation promoted by Wnt1 [126, 229, 227]. Only preneoplastic lesions with no malignant transformations were found in singular Wnt1 transgenic mice [228]. This indicates the interplay of inflammation and defective proliferation, which in the end leads to gastric cancer.

Another inflammation based model was demonstrated in mice with gene knockouts. Intestinal gastric cancer developed in mice deficient for estrogen-responsive gene trefoil protein (pS2) [160] or lacking the Src homology protein 2 (SHP2) binding site on the interleukin 6 family receptor gp130 [130].

A third approach was the generation of mice with artificial directed overexpression of potentially oncogenic factors. In one mouse model gastrin was expressed in pancreatic beta cells and secreted into the circulation, causing hypergastrinemia and lastly gastric carcinogenesis [317]. Directed expression by stomach specific promoters was used to cause gastric cancer by the simian virus 40 (SV40) large tumour antigen expressed from the ATPase, H+/K+ exchanging, beta polypeptide (ATP4b) proton pump promoter [286] or interleukin 1 beta as a procarcinogen [302].

Finally mouse models with mutations in cadherins were generated to model the common human hereditary mutations. Gastric signet ring cell carcinoma were found in cadherin 1 (Cdh1) +/- mice challenged with the carcinogen MNU [122]. Mice with complete E-cadherin loss were generated [205] and in combination with a loss of p53 the animals developed diffuse gastric cancer with 100% penetrance within a year [269].

1.4.2. The need for large animal models

Overall, mouse models develop cancerous lesions with varying degrees of similarity to the human counterparts. They were crucial for a better understanding of gastric oncogenesis.

But there are also severe constraints regarding the applicability of rodent models for human diseases (reviewed in [5]): Some rodent models simply fail to replicate the human disease phenotype rendering research on the topics impractical e.g. for familial adenomatous polyposis (FAP). Major differences in human and rodent diet distort the background of gastrointestinal research. And most obvious, the small size of mice and other rodents hinders translation of results. Scaling of treatment routines like chemotherapeutic dosage is very problematic, also given the differences in metabolism [201]. Obviously endoscopic visualization and surgical procedures have to be fitted / developed anew.

There is therefore an urgent need for disease models in species that more closely resemble humans in scale, anatomy and general physiology. In biomedical research this role can be fulfilled by pigs. They also have the advantage of relatively short gestation, large litter sizes and well-established husbandry procedures compared to other large animals. Anatomy and organ sizes in pigs are comparable to humans and thus pigs are commonly used in transplantation, or surgical research [284, 303]. For example, pigs are widely used for the training of endoscopic dissections [87, 114, 234, 282].

Humans and pigs are also closely related at the molecular level: The genomic sequences are highly homologous [21] and the pigs metabolome has been found to be comparable to the human with few differences [201].

It is interesting to note, that the porcine pregnane X receptor protein is more similar to the human form than the murine protein. This is a regulator of the important drug metabolising p450 cytochrome CYP3A and can help provide better results in screenings of drug metabolising effects [334].

Similarities were also found in tumour biology: Telomerase expression is low under physiological conditions and gets reactivated in cancerous tissues both in humans [278] as well as in pigs [235]. Also, spontaneous myelogenous leukemia development has been observed in inbred minipigs with pathological characteristics comparable to humans [62].

1.4.3. Pigs in biomedicine

Pigs have been used in medical and scientific research for a long time. Their above mentioned advantages and similarities to human make them such valuable models. The wide applicable range of pigs in biomedicine will be introduced in the following paragraphs.

Externally induced models

A dietary model of human diabetes type 2 was successfully generated in minipigs [37]. This again shows the similarity of pig and human regarding gastrointestinal phenotypes. Diabetes type 2 – so called "prosperity diabetes" – is emerging as a prominent problem in the Western world and case numbers are expected to rise.

Simplified cancer models are derived by ectopic inductions of pathologies. Carcinogen treatment (e.g. with MNU [335]) has been used in rats, resulting in cancer development. For pigs however, this approach might be impractical due to their large size and dosage.

There is a dietary model of gastric ulcers in specifically fed pigs. They can be induced merely by a small particle size of the diet and might serve as a medical training model for early gastric irritations [12]. Later disease stages are simulated by surgically inflicting wounds in the animal. For example, bleeding peptic ulcers were simulated by cutting pigs stomachs in a non survival training models [41].

A more straightforward approach was used by Adam et al. by the transplantation of *in vitro* modified cells. These cells were pre-treated to overexpress oncogenic factors: SV40 large T, c-myc and oncogenic activated K-ras [1]. They did show tumorous growth and could also be used for treatment developments. However, it completely lacks the natural early stages of cancer development, and it relies on several very severe oncogenes to drive proliferation.

The major drawback of all of these ectopic methods is the lack of natural disease development in an endogenous environment. Genetic modification facilitates and defines the disease outbreak as genetic predispositions are causative in a variety of cancers. Ideally, this reduces screening intensity and quantity since disease specifics are known beforehand and the cancer develops with high penetrance.

The following chapter provides an overview across genetically modified pig models that strive to better mimic human pathologies.

Genetically modified models

Several genetically modified pigs have been generated for biomedicine. They are based on engineered gene expression or inhibition and most are generated through somatic cell nuclear transfer (SCNT).

Some animal simply express visible marker genes like enhanced green fluorescent protein (EGFP) (initially transduced into oocytes [30] or fibroblasts [154]), red fluorescent humanised Kusabira-Orange [194] and other combinations [320]. They could be used for example in cell tracking / transplantation experiments. In parallel, porcine models are generated to provide utility tools for molecular biology. Cre-recombinase expressing animals or tet-

on/off systems are vital to move the field toward the complexity of genetic modifications now available in rodent models [38, 138].

Xenotransplantation The first animal with clinical applicability was produced to facilitate pig-to-human xenotransplantation. The motivation for this was to combat the shortage of donor organs. However, xenotransplantation is accompanied with severe immunological problems. The humoral and cell mediated rejection of foreign organs (reviewed in [137]) has to be circumvented. Current research tries to achieve this either by humanising the tissue [143, 153], artificially expressing complement regulators [82, 251] or, in the future, combinations thereof.

Regarding this topic, there exists the disputed risk of porcine endogenous retroviruses (PERVs) being transmitted to humans during the transplantation [56, 236]. The issue has been addressed with anti PERV RNA interference (RNAi) [236]. However, to date no *in vivo* infection in immunosuppressed primates with PERVs was observed [277].

Transplantation studies are also carried out in the reverse human-to-pig xenotransplantation. Here, cell transplantation techniques like stem cell treatments should be established for the human clinic. This research of course also has to deal with immuno-rejection. Therefore, an important breakthrough in this field has been the generation of immunodeficient pigs with an interleukin 2 receptor gamma gene knockout [283]. These pigs exhibit severe combined immunodeficiency (SCID) and can be used in pre-clinical trials of these therapies.

Cardiovascular Another aspect of clinical research in pigs is the cardiovascular system. Due to its high similarity it provides an excellent model for human phenotypes. Porcine models were generated with altered fatty acid ratios, believed to be beneficial for the cardio vasculature [152], or altered endothelial signal molecules nitric oxide (NO) [100, 326] and hydrogen peroxide [327] to clarify their role in vasculature.

Alzheimer's disease The similarity in size and physiology of the human and porcine brain [145] sparked the interest of Alzheimer's disease (AD) researchers. In humans AD leads to neurodegeneration due to toxic accumulation of A-beta peptides [315]. Researchers have generated a pig expressing a dominant mutation of A-beta [145] in the brain. The authors presume it will take 1-2 years for the phenotype to develop [92].

Diabetes As mentioned above, pigs were also used as models for diabetes research. This disease has also been modeled by genetic modifications. The current transgenic porcine model for type III diabetes is based on a mutated human hepatocyte nuclear factor 1-alpha (HNF1 alpha) gene [305]. Type II diabetes was modelled in transgenic pigs by expressing an impaired glucose-dependent insulinotropic polypeptide (GIP) receptor. These pigs showed impaired beta-cell proliferation and low oral glucose tolerance [257].

Cystic fibrosis Recently pigs were able to prove their necessity as a large animal model

when researchers were able to replicate the phenotype for a human disease, which the mouse models were unable to recreate. It was a porcine model for cystic fibrosis (CF) by modifications of the CFTR gene [139, 260, 261]. Existing mouse models with the same gene mutant did not recapitulate the human disease development in the lung. In contrast, the pigs did exhibit lung pathology similar to humans and helped resolve a question of the order of events in CF development. The initiating step is a mitigated removal of bacteria due to the more viscous mucous. This is later followed by severe inflammation [202, 243].

Cancer For cancer research the defined models are still lacking. The initial model utilised transplanted oncogenic cells [1], however without the ability to accurately model human cancers. Recent research has generated a porcine model for breast cancer by a BRCA 1 gene knockout via adeno-associated virus (AAV) [185]. However, the liveborn piglets all died within 18 days, so no *in vivo* insight has been gained yet. Within our own lab we modified the APC gene. APC is a tumour suppressor gene in the Wnt pathway that is commonly mutated in cancers. It inhibits the Wnt/beta-catenin signaling pathway by promoting beta-catenin degradation. We introduced the two most common truncated protein mutants found in FAP that result in reduced beta-catenin degradation. The same as in the case of cystic fibrosis, these animals reflect the human phenotype more closely than the mouse [73]. Also these animals are viable and initial *in vivo* experiments will soon commence.

Other diseases and applications Another porcine model has been developed for spinal muscular atrophy (SMA) a common genetic cause of infant mortality. The causative gene mutations in survival motor neuron (SMN1) have been transferred into a pig genomic background and animals generated to test efficacies of SMA medication. Now efforts are underway to ectopically express the SMN2 gene in pigs, which is only conserved in humans, to recreate the SMA phenotype [181]. Macular degeneration was modeled in pigs [275] as well as immunodeficiency e.g. by a kappa light chain KO [200, 250]

Pigs are also utilised as bioreactors for pharmaceuticals – so called "pharming" [136]. Porcine mammary glands could serve for large scale production of 2-3 liters of milk per day and are suitable for the synthesis and processing of proteins. This is of particular interest, since modern pharmaceuticals extend into the field of antibodies or macromolecules. Approximations regarding the yield of protein of interest and transgenic pigs needed for production show the immense potential of the method. Only 60 transgenic pigs would suffice to cover the needs of the United States for factor IX [308]. However, other animals like cows are of course even better suited for this type of pharming, due to higher milk yield.

Still, several transgenic pigs were generated, overexpressing pharmaceuticals like human factor VIII [230] and IX [307], haemoglobin [285], human protein C [307] and erythropoietin

[231], human granulocyte-macrophage stimulating factor [232] and van Willebrand factor [159].

Agricultural considerations

Genetically modified pigs could also play an important roles in agriculture, regarding feeding efficiency and waste / pollution reduction [91], meat production [169, 323] and heat stress resistance e.g. by overexpression of heat shock protein 70 [39].

1.4.4. Technologies for genome modification

The technologies needed for the genome modification in pigs are described in the following paragraphs. From their beginning in 1985 they have become more and more refined, but still do not match the toolkit available in mouse.

Introducing non-directed modifications

Early experiments were carried out in fertilised oocytes. The first genetic modifications in pigs was generated by pronuclear microinjection of DNA into an oocyte [97]. Later on, methods with higher efficiency like oozyte transduction with replication deficient viruses [30, 112] and sperm mediated DNA transfer were applied [157].

More recent methods are based on modifying the genome of somatic cells *in vitro*.

Generally speaking, the desired DNA sequences are introduced into the cells. A wide array of transfection methods are used to physically transfer the DNA sequences into the nucleus, for example by complexation with chemicals, nano-particles or lipid vesicles. The electroporation of cell membranes is also widely used to allow DNA access.

In some cases, the introduced sequences randomly integrate into the genome. Selection processes enrich for these events and the diverse random cell clones are screened for their suitability. DNA sequences sometimes integrate as multimeric concatemeres, in random orientations and also potentially in multiple loci.

This principle has been used in conjunction with SCNT to generate porcine models with artificial transgene expressions, several of which are mentioned above in chapter 1.4.3: for example for Alzheimers disease [145] and diabetes [257].

Introducing site specific modifications

Some disease models can require site directed modifications of the genome. Specific mutations were necessary for example to generate the models for CFTR [139] and FAP [73].

A well established method to achieve this is "gene targeting".

This technique modifies an endogenous gene by means of homologous recombination (HR). It relies on "gene targeting vectors" – plasmids containing the homologous region

of the target gene and the desired modification. Large constructs of homologous DNA sequence that are transfected into cells can lead to HR between itself and the "host genome" by utilising the cells endogenous DNA repair mechanisms. Modifications that are engineered in between homologous sequences can be introduced along with it. This targeted gene modification can also introduce expression cassettes with low copy numbers (1 per targeted allele) in contrast to random integration.

The main obstacle of this method is the low efficiency of HR. Most of the times it requires a complex experimental design to allow gene targeting. Due to the low efficiency of gene targeting, the targeting vectors usually carry features to enrich for positive gene targeting event. For example, resistance genes without promoters or polyA signals (so called promoter or polyA traps), relying on the endogenous features of the targeted site to take over these functions. The addition of nucleus localisation sequences (NLS) also enhances DNA uptake into the nucleus and thereby HR [171, 53] and cell cycle synchrony can also play an important role [206].

Gene targeting is more efficient in embryonic stem cells (ESC) (e.g. 78% in mouse ESCs without promoter trap [291]) than in somatic cells (1-2% in fetal fibroblasts with promoter trap [250]; 1.6% in primary porcine kidney cells with bacterial artificial chromosome (BAC) targeting [139]). However, ESCs are not yet established for large animal model systems like the pig. The induced pluripotent stem cell (iPSC) technology has emerged as an alternative for gene targeting in species with no ESCs available. These cells are reprogrammed ideally into a pluripotent state through the artificial expression of factors like Octamer binding transcription factor 4 (Oct4), sex determining region Y-box 2 (Sox2), Krueppel-like factor 4 (Klf4) and avian myelocytomatosis viral oncogene homolog c-myc. They are known to show ESC-like properties and might also allow for more efficient HR [287]. Yet, again no iPSCs independent from exogenous factors are available for pigs.

Apart from cell type, the accessibility of the genomic locus in heterochromatin can influence the efficiency. Here, the ROSA26 locus has been identified as especially susceptible to genomic modifications in mice and humans, and allowing for stable expression patterns of integrated constructs [124]

The most recent development has been the use of zinc fingers linked nucleases (ZFN) and Transcription Activator-Like Effector Nucleases (TALEN) technology. These engineered nucleases fused with specifically designed DNA recognition sites are able to introduce site specific double strand breaks (DSB). These are then repaired by non-homologous end joining (NHEJ) and can lead to DNA point mutations and thus gene knockouts. It has been in used to generate gene knockouts in pigs both in proof of principle (EGFP [319]) and functional models, e.g. a alpha 1,3-galactosyltransferase (GGTA1) knockout even with biallelic effects [104]. This method is very efficient if the recognition sites are designed

properly. Otherwise the high off target activity of the nucleases can greatly reduce cell viability. A recent study by Fahrenkrug has shown remarkable effectiveness of TALENs in primary cells and oozytes on a variety of targets, thus establishing this technology strongly in the field of genetically modified large animals.

It is interesting to note, that these nucleases can be very effectively combined with gene targeting. The site directed DSBs prime the cells repair mechanism and the engineered homologous construct with the desired modification is co-introduced as a repair template. This can drastically raise the HR frequency and thus gene targeting efficiency in somatic cells. There have been reports of efficiencies of up to 29% of gene replacement and 50% of gene addition in humans [179] and also great effectiveness in rabbits [74].

Other ways to improve gene targeting efficiency can be the use of adeno associated viruses AAVs. This has already been used successfully in pigs [127, 185, 260]. Also, a partially directed integration of sequences has been achieved by utilising transposases with integration hot spots [84].

1.4.5. Generating genetically modified pigs

The technology to artificially generate live animals from *in vitro* cell culture has emerged over the last 40 years and sparked worldwide interest with the cloning of "Dolly" the sheep [264, 330]. Generally speaking, it is based on *in vitro* manufacturing of an embryo that can be implanted into a foster mother and develop to birth. The method of its cloning determines, if that embryo consists entirely or in part of genetically modified cells.

Chimeras can be generated by injecting modified ESCs into a blastocyst. The ESCs partake in the development of the embryo, but do not form the whole animal. This approach requires stable ESCs of the species of interest. There have been no conclusive reports of ESC culture in pigs to date. However, current research is focussing on this topic, and has reported LIF-dependent, pluripotent stem cells established from the inner cell mass of porcine embryos [292]. Also, transgenic pigs have been developed to facilitate the generation of porcine ESCs. They carry a fluorescent EGFP reporter gene under the control of an Oct4 promoter, known to be active in ESCs and thus help to identify ESC-like cell populations [217].

As no ESCs are currently available, alternative technologies are applied to generate genetically modified pigs. Porcine germ cells were xenografted into immunodeficient mice and have produced functional transgenic porcine sperm for intracytoplasmatic sperm injection (ICSI) [150]. However, to actually generate transgenic animals from this, the germ cells have to be modified.

The established method today is the SCNT, which also was used in this work. The

implantable reconstructed embryo – mostly a two cell stage blastomere – is generated *in vitro*, by fusing an enucleated oocyte with a single somatic cell. This provides the oocyte with a diploid genome. This cell is then artificially activated to divide and can develop into a viable embryo. This method produces 100% transgenic offspring since the animals are all derived from modified cells.

The method of nuclear transfer (NT) was first suggested for pigs in 1985 [97] and successfully conducted with embryonic cells [247]. After the proof that NT can produce viable offspring in large animals from wildtype and genetically modified somatic cells [197, 264, 330], this also proved to be true for pigs (for example [73, 224, 244]).

However, this method has some drawbacks compared to the blastocyst injections with ESCs. The somatic cell carries an already differentiated genome with corresponding epigenetic changes. It has to undergo major dedifferentiation processes to support the transcriptional programms necessary for totipotency. This results in a low efficiency of life born animals compared to fused cells and implanted blastocysts. Oftentimes, it also causes complications in the newborn, which result from incomplete epigenetic reprogramming during development. Examples for this are the large offspring syndrome, macroglossia or an impaired immune system [115, 162, 248].

1.5. Tissue and timing specificity

Biomedical information derived from artificial models *in vitro* or *in vivo* are only as good as they are able to mimic the actual disease phenotype. New technologies in molecular biology allow for more specific effects to be introduced into model animals. They are based on a carefully regulated timing of gene expression or repression, just like in the actual disease development. The techniques to achieve this are summarized in the following chapters.

1.5.1. Ectopic or inducible expression systems

The most intuitive approach for generating specificity in time and location is an ectopic induction. A straightforward approach to this is *in vivo* electroporation of expression constructs, which has proven effective in large animals [255]

Another way of achieving specificity lies in chemically inducible or sensitive promoter structures driving the gene of interest. Transgenic animals carrying these constructs can be challenged by the effective substrate in the desired tissue at a defined timing. The most widely used example of this would be the TET on/off system to either repress or activate gene expression from doxycyclin inducible promotor structures. The latter has frequently been used in murine and recently even in porcine background (for example [4, 96, 138, 147]).

Another possibility is the modulation of expression from promoters sensitive to physical effects. The first experiment for light inducible gene expression was conducted in neurons expressing light sensitive rhodopsin [172]. In another approach, a transactivator was designed to respond to blue light and promote gene expression *in vitro* and *in vivo* [318]. Specific transgene expression was also achieved by utilising the endogenous heat shock response of cells. Heat shock protein (hsp) promoter coupled gene expression can be induced or repressed by modulation of the temperature surrounding the tissue. An example for this is the hsp 70B promoter driven herpes simplex virus - thymidin kinase (HSV-tk) system for an *in vitro* assay. This gene product is cytotoxic and could assist in a chemotherapeutic treatment approach [125].

1.5.2. Recombinase dependent systems

More advanced systems generate specificity by direct modification of the genomic DNA by recombination. A number of DNA recombinases have been utilized for targeted excision / introduction / inversion of DNA *in vitro* and *in vivo*. These recombinases recognize characteristic binding sequences and catalyse DNA crossovers between two sites, to either loop out (excise), introduce or invert intermediate sequences. The recognition sites mostly show a defined orientation within the sequence determining the outcome after recombination.

Most recombinases will continue to bind and recombine recognition sites that are still present as long as they remain active. This leads to an equilibrium of recombination events. To counteract this, recombinases with less stringent sequence recognition have been used. They can still recombine between two minimally mutated sites which can be designed to lead into one correct and one defunct site, that is removed from the equilibrium pulling the reaction that way. The most commonly used application of recombinases is the excision of DNA sequence. The cellular defence mechanism degrades free dsDNA in the cytoplasm and thus leads to a loss of the excised sequence.

The Cre-recombinase (Cre = causes recombination) derived from *E. coli* and its respective loxP recognition sites is among the most commonly used tools in this system. It recombines DNA between two 34bp so called loxP recognition sequences. The intermediate (flanked by loxP = "floxed") DNA is looped out and excised or flipped in its orientation. The outcome depends on the orientation of the directional loxP sequence, where same orientations lead to excision and opposite to an inversion. It can also mediate translocations between loci. Figure 1.6 on the next page depicts the schematics of Cre-mediated recombination, that are generally applicable to a wider range of recombinases.

Another common recombinase is the Flp-recombinase with its 34bp Flp-recombinase-target (FRT) recognition sites and similar excision/inversion characteristics. Combina-

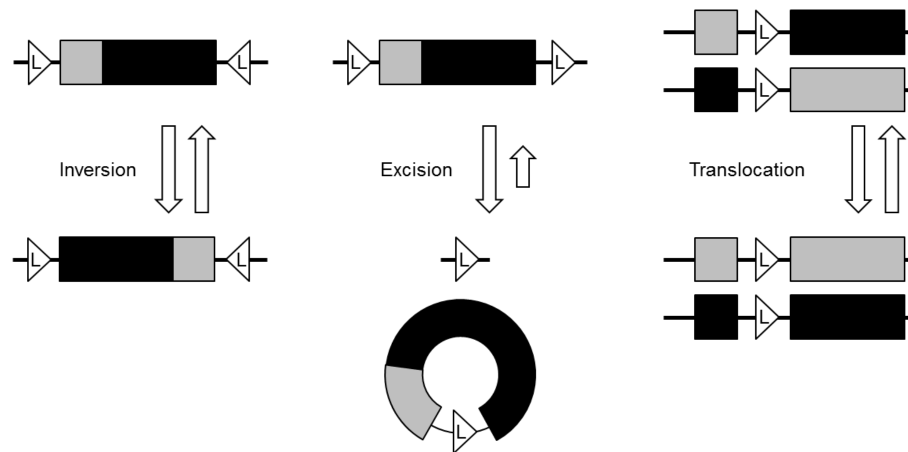

Figure 1.6.: Schematics of Cre-mediated recombination between loxP sites "L". Left: Inverted loxP orientations lead to an inversion of intermediate sequence. Middle: LoxP site with aligned orientations lead to a circular recombination of intermediate sequence, which is usually lost in the cell. Therefore the equilibrium indicated by the arrows favors the circular DNA with a residual loxP site left in the sequence of origin. Right: Translocations between two separate loXp containing sequences can also be mediated by Cre-recombinase.

tions of these recombinases can also be used to engineer multifaceted conditional genomic modifications.

Recombinases can be delivered or activated through a variety of approaches. Animals carrying a recombinase transgene are commonly bred into a background that carries the recombinase recognition sites, for example by Zhao in reporter lines with gastric [347, 348] or neural, and germline specificity [135]. Recombinases could also be applied via viral vectors like self-deleting lentiviral [241] or retroviral vectors [272] or directly as permeable fusion protein [328].

1.5.3. Tissue specific promoters

Tissue and timing specificity can also be achieved by using known endogenous promoters, utilising their regulation mechanisms / expression patterns and timings. This common approach couples transgenes to promoters with the desired functions and specificity. These fusion constructs are then integrated into the target genome either randomly or directed through gene targeting.

It has been used extensively in mice to model diverse genotypes. For this work is was of interest to specify expression into the stomach to activate the latent oncogenic mutation.

There are several known promoters expressing more or less specifically within the stomach. This chapter introduces the most relevant.

The first promoter that comes to mind is the one of the proton pump H, K- ATPase

that generates the low pH environment in the stomach. It has been successfully used to direct transgene expression to the stomach in mice. In detail, the transgene for diphteria toxin was used to ablate the gastric parietal cells [170]. The second "famous" gastric gene is gastrin, the regulator of gastric acid secretion (reviewed in [57]). Its promoter was also used to drive transgene expression in a gastric cancer cell line [349].

More interesting for this work was the cytokeratin 19 (KRT19) promoter is expressed in endothelial cells [198] and was already used to drive a mutated K-ras transgene [253]. This promoter was utilised by Oshima et al. for a mouse model of gastric cancer [228], and is described in detail in chapter 1.4.1.

1.6. Reporter animal models

The tissue and timing specific expression of endogenous promoters can be deduced to some extent from their function or by direct measurements and observations. However, the activity of a promoter is not always observable throughout all development stages or in all tissues. In these cases an *in vivo* test system – a reporter animal – can help define promoter specificity.

The scientific utility of reporter models is the detection expression from an unknown promoter via the coupling of a detectable reporter gene. These reporter genes usually encode for proteins that are visibly detectable either by staining or fluorescence. Common genes used for this are the beta-galactosidase gene lacZ (can be stained blue), and the green, red and yellow fluorescing proteins (GFP/RPF/YFP) with several commercially available modifications like reduced toxicity [311].

A direct way to use reporter genes to identify promoter expression patterns is to put the reporter gene under the control of the latter, and observe [86].

Another frequently used approach is the indirectly linking of the two via recombinase systems [169, 168, 184, 216]. In this case, the promoter in question drives a recombinase gene. The reporter gene gets activated in every cell after recombinase activity. This can be achieved by the recombination and excision of a transcription stop cassette in front of the reporter. This linked approach has the advantage, that the reporter signal stays active in a cell and all cells of its descent. Therefore, the activity of the promoter can be seen *in vivo* in all tissues the promoter showed activity throughout organismal development.

A more specialised version of the recombinase reporter systems is the use of two reporter genes. This will be referred to as a “dual reporter system”. It utilises a ubiquitously expressed reporter gene, that gets excised by recombinase activity and exchanges its expression with the second reporter gene located downstream. Figure 1.7 on the next page shows a schematic of this exchange process. The advantage of this system is the assurance,

that an absence of a visible reporter gene is not in fact a silencing of the whole expression cassette. The ubiquitous primary reporter gene should be detectable in all unrecombined tissues. This allows to search for off target- or other unexpected effects of the promoter in all tissues.

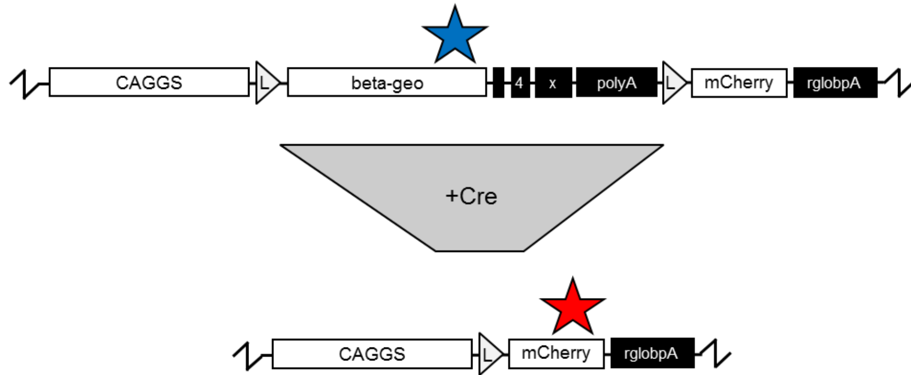

Figure 1.7.: Schematics of Cre-mediated recombination of the dual reporter cassette designed in this work. Top: The CAGGS promoter drives the loxP flanked ("L") beta-geo fusion gene containing a neo-resistance and a lacZ reporter gene, visible after lacZ stain in blue (star). The 3' mCherry fluorescent gene is not expressed. Bottom: After Cre-mediated recombination the beta-geo cassette, a singular loxP site remains. The CAGGS promoter now drives the mCherry gene, which results in red fluorescence (star).

There exist a variety of mouse models that express these dual reporter constructs. For example the Z/AP and Z/EG mice [178, 216]. They carry a floxed version of the lacZ gene followed by an alkaline phosphatase gene (Z/AP) or an enhanced green fluorescent protein gene (Z/EP) as secondary reporter. Their use has been vital to identify novel promoter expression patterns (reviewed in [164])

The dual reporter system has also already been extended into the porcine background. The chinese group of Li et al. report the generation of a porcine fetal fibroblast (poFF) cell line [168] and live reporter pigs [169]. The construct used, consists of a floxed neo-resistance gene with an enhanced GFP gene in a downstream activatable position. However, further experiments in this system have not been reported yet.

1.7. Aim of this work

The aim of this work was to establish porcine models for Li-Fraumeni syndrome (LFS) and for the early detection of gastric neoplasias that in human disease often stay unnoticed and could develop into gastric cancer.

This goal was to be achieved through the following measures. Firstly, the porcine tumour suppressor gene *TP53* should be modified into a conditional knockout with a latent activatable oncogenic mutation into exon 5 by gene targeting. This mutation should mimic a common human hot-spot mutation R175H, with severe dominant negative function. The conditional knockout should be achieved through a loxP flanked transcription stop cassette, and functionality of knockout and re-activation should be shown in *in vitro* assays. Subsequently, these cells should be used in somatic cell nuclear transfer to generate genetically modified animals. The resulting animals should model the frequent *TP53* deficiency in LFS.

However, *TP53* mutations alone only facilitate cancer development and necessitate a second hit mutation. Therefore, in future experiments the *TP53* knockout animals should also be crossed into an APC mutant background to serve as models for gastrointestinal cancer. This phenotype could be even further enhanced by activating the oncogenic mutation.

A secondary approach should be followed as a backup to the potentially ineffective gene targeting experiments. This was the modelling of the mouse gastric cancer model of Oshima [228] in pigs. Here the inflammatory factors COX2 and mPTGES1 and the proliferatory Wnt1 should be overexpressed with gastric specificity and thus mimic the natural disease development. This specificity should be achieved with a suitable promoter, the KRT19 promoter used in the original mouse model.

Spanning the gastric neoplasia topic stands the concept of tissue specific expression. Therefore, a reporter system should be designed and evaluated that allows for *in vitro* and *in vivo* testing of promoter activity. For more convenient analyses it should be coupled with dual visual markers indicating both an active and inactive promoter. The Cre-loxP system should provide the necessary molecular switches.

A successful introduction of the genetic modifications should be followed by the generation of live animals through SCNT.

2. Material and Methods

2.1. Material

2.1.1. Chemicals

Acetic acid	Fluka Laborchemikalien GmbH, Seelze, D
Anti-digoxigenin-AP Fab fragments	Roche Diagnostic GmbH, Mannheim, D
Boronic acid	Fluka Laborchemikalien GmbH, Seelze, D
Bromphenol blue	Sigma, Steinheim, D
CDP Star reagent	Roche Diagnostic GmbH, Mannheim, D
Chloroform	Aldrich-Chemie GmbH, Steinheim, D
Digoxygenin-triphosphate	Roche Diagnostic GmbH, Mannheim, D
N,N-Dimethylformamid (DMF)	SIGMA, Steinheim, D
Dithiotreitol (DTT)	Omnilab Life Science, Bremen, D
Ethanol (denatured)	Riedel de Haen, Seelze, D
Ethidium bromide	SIGMA, Steinheim, D
Ethylenediaminetetraacetic acid (EDTA)	SIGMA, Steinheim, D
Formalin (10%)	SIGMA, Steinheim, D
Glacial acetic acid	Fluka Laborchemikalien GmbH, Seelze, D
Glycerol	SIGMA, Steinheim, D
Isopropanol	Carl Roth GmbH, Karlsruhe, D
beta-Mercaptoethanol	SIGMA, Steinheim, D
Methanol	J.T. Baker, Griesheim, D
Potassium acetate	Riedel de Haen, Seelze, D
Potassium hexacyanoferrat (III)	AppliChem, Darmstadt, D
Potassium hexacyanoferrat (II)-trihydrat	AppliChem, Darmstadt, D
Sodium acetate	Carl Roth GmbH, Karlsruhe, D
Sodium chloride	Riedel de Haen, Seelze, D
Sodium dodecyl sulfate (SDS)	Omnilab, Bremen, D

Saccharose	SIGMA, Steinheim, D
TRIZMA Base	SIGMA, Steinheim, D
TRIzol reagent	Invitrogen, Karlsruhe, D
Tris hydrochloid (Tris HCl)	SIGMA, Steinheim, D

2.1.2. Buffers and Solutions

Annealing buffer (10x)	Tris 100 mM pH 7,5; NaCl 1 M; EDTA 1 mM in H_2O
DNA loading buffer (5x)	50% Glycerin (v/v); EDTA 0,2 M; Bromphenol blue (traces); in H_2O
DNase I buffer (10x)	New England Biolabs (NEB), Frankfurt a. M., D
Fast Digest buffer (10x)	Fermentas, St. Leon-Rot, D
Miniprep solution I	Saccharose 5 mM; EDTA 10 mM; Tris pH 8,0 25 mM; in H_2O
Miniprep solution II	NaOH 0,2 N; 1% SDS (w/v); in H_2O
Miniprep solution III	3 M Natrium-Acetat pH 5,3; in H_2O
Nanofectin Diluent	PAA, Pasching, D
Sodium chloride solution	NaCl 5 M; in H_2O
NEB buffer 1-4 (10x)	New England Biolabs (NEB), Frankfurt a. M., D
Passive lysis buffer (5x)	Promega, Mannheim, D
Rinse buffer (lacZ stain)	$MgCl_2$ 2 mM; Na_2HPO_4 100mM; NP40 Igepal 0.02% (v/v); Sodium Deoxycholate 0.01%; pH 7.3 (adj. with HCl); in H_2O
T4-DNA-ligase buffer (10x)	New England Biolabs (NEB), Frankfurt a. M., D
TAE-buffer (50x)	Tris 2 M; EDTA 50 mM; 5,71% glacial acetic acid (v/v); in H_2O
TBE-buffer (10x)	Tris 0,9 M; Boronic acid 0,9 M; EDTA 20 mM; in H_2O
Tris-EDTA	Tris 10 mM; EDTA 1 mM; in H_2O
Tris-low EDTA	Tris 10 mM; EDTA 0,1 mM; in H_2O
Tween20	SIGMA, Steinheim, D
X-Gal staining solution	100 mg X-Gal; 1 ml DMF

2.1.3. Bacterial strains, cell lines and primary cell cultures

Bacterial strains	
Emax (DH10B); bacterial strain	Invitrogen, Karlsruhe, D
Genotype:	F- mcrA (mrr-hsdRMS-mcrBC) 80lacZM15 lacX74 recA1 endA1 ara139 (ara, leu) 7697 galU galK - rpsL (StrR) nupG
GM2163; bacterial strain	New England Biolabs (NEB), Frankfurt a. M., D
Genotype:	F- 2-ara-I4 leuB6 tonA31lacY1 tsx-78 supE44 galK2 galT22 hisG4 rpsL136 xyl-5 mtl-1 thi-I daml3::Tn9 dcm-6 hsdR2 mcrB1 mcrA

Cell lines	
HEK293; human cell line	Human embryonic kidney cells; American Type Culture Collection (ATCC)
AGS	American Type Culture Collection (ATCC)
Kato III	American Type Culture Collection (ATCC)
Primary cell cultures	
Mesenchymal stem cells (MSC)	Isolated during this work
Adipose tissue derived mesenchymal stem cells (AdMSC)	Isolated during this work
Kidney fibroblasts (KDNF)	Isolated during this work

2.1.4. Bacterial culture materials

Ampicillin	SIGMA, Steinheim, D
Kanamycin	SIGMA, Steinheim, D
LB0-medium	Difco, Voigt Global Distr., Lawrence, KS, USA
LB-agar	Difco, Voigt Global Distr., Lawrence, KS, USA
Petridishes	Corning Incorporated, NY, USA

2.1.5. Cell culture materials

10 cm culture dish	Corning Incorporated, NY, USA
12-well-plate	Corning Incorporated, NY, USA
6-well-plate	Corning Incorporated, NY, USA
Accutase	PAA, Pasching, D
Advanced DMEM culture medium	Gibco BRL, Paisley, UK

Amphotericin B	PAA, Pasching, A
Blasticidin S	Invivogen, Toulouse, F
Basic fibroblast growth factor	PromoCell, Heidelberg, D
Chicken serum	PAA, Pasching, A
Dimethyl sulfoxide (DMSO)	SIGMA, Steinheim, D
DMEM culture medium	PAA, Pasching, D
Dulbecco's PBS (1x) without Mg2+/Ca2+	PAA, Pasching, D
Filter paper 1 mm	Carl Roth GmbH, Karlsruhe, D
Fetal calf serum	PAA, Pasching, D
Geneticin (G418)	PAA, Pasching, D
GlutaMAX (100x)	Invitrogen/Gibco Corp., Paisley, UK
Hank's buffered salt solution	PAA, Pasching, D
Hypoosmolar electroporation buffer	Eppendorf, Hamburg, D
Lipofectamine 2000	Invitrogen, Karlsruhe, D
Lymphocyte separation medium 1077	PAA, Pasching, A
Nanofectin	PAA, Pasching, D
Non-essential amino acids (100x)	PAA, Pasching, D
Opti-MEM	Gibco BRL, Paisley, UK
Penicillin/Streptomycin 10,000U, 10μg/μl in 0.9% NaCl	Sigma-Aldrich, Deisenhofen, D
T25-cell culture flask (25 cm^2)	Corning Incorporated, NY, USA
T75-cell culture flask (75 cm^2)	Corning Incorporated, NY, USA
T150-cell culture flask (150 cm^2)	Corning Incorporated, NY, USA
Cell culture water EP-Grade	PAA, Pasching, D
X-Gal (5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside)	SIGMA, Steinheim, Germany

2.1.6. Cell culture media

DMEM+ medium	DMEM	500 mL
	Fetal calf serum	10% (v/v)
	Non-essential amino acids	1% (v/v) (filtered through sterile filter 0,22 μm)
	GlutaMAX	final concentration 2 mM (filtered through sterile filter 0,22 μm)

ad/bmMSC medium	Advanced DMEM	500 mL
	Fetal calf serum	10% (v/v)
	Non-essential amino acids	1% (v/v) (filtered through sterile filter 0,22 μm)
	GlutaMAX	final concentration 2 mM (filtered through sterile filter 0,22 μm)
	FGF	final concentration 5 ng/ml
KDNF medium	DMEM	500 mL
	Fetal calf serum	10% (v/v)
	Non-essential amino acids	1% (v/v) (filtered through sterile filter 0,22 μm)
	Sodium pyruvate	1% (v/v)
	GlutaMAX	final concentration 2 mM (filtered through sterile filter 0,22 μm)
Freezing medium	Culture medium	60% (v/v)
	Fetal calf serum	30% (v/v)
	Dimethylsulfoxid	10% (v/v)
	Before use filter through sterile filter 0,22 μm	

2.1.7. Oligonucleotides

All oligonucleotides acquired from biomers.net GmbH (Ulm, D) or Sigma (Steinheim, D) and dissolved in H_2O to a stock concentration of 100μM.

Name	**Sequence 5' - 3'**	**Anneal [°C]**
Cherry XbaI ATG for	TCTAGAATGGTGAGCAAGGGCGAGGA	59
Cherry EcoRI Stop rev	GAATTCTTACTTGTACAGCTCGTCCA	
COX2 F	CATTCAGAAGCCGACTCACC	59
COX2 R	AAAACTGCTTTCCCCTTTCC	
Cre +XbaI for	TCTAGACCTTGCCACCATGGCACCCAAGA	59
Cre +BglII rev	AGATCTATCGCCATCTTGCAGCAGGCGCA	
EGFP+ATG +XbaI	TCTAGAATGGTGAGCAAGGGCGAGGA	59
EGFP+Stop +EcoRI	GAATTCTTACTTGTACAGCTCGTCCATGC	
LacZ intern for	GACGTCTCGTTGCTGCATAA	58
neo intern rev	GCTCTTCGTCCAGATCATCC	
NLSflank for	ACGCGTGCTGTGGAATGTGTGTCAGTTAG	60
NLSflank rev	ACGCGTGGAGTTAGGGGCGGGACTA	

p53mut for p53mut rev	GTACTCCCCTGCCCTCAATA GGGGTAACCCATCTGCTCTA	58
P53 targ for 1 (F1) p53 targ for 2 (F2) p53 targ for 3 (F3) p53 targ for 4 (F4) p53 targ SA rev p53 targ BS rev p53 targ neo rev p53 Int1_5 rev	TATCTTTTCACCCCATGTGTTTC CCAGGGAGTCCATCTAAAAGTG TTCTTGTCACCTGCCATCAG CCTTAACCAGTAGGCCACCA GAAAGACCGCGAAGAGTTTG ACATTGACACCAGTGAAGATGC GTCGGTCTTGACAAAAAGAACC TTCCACCAGTGAATCCACAA	59
p53probe for p53probe rev	GCAATGGAGGAGTCGCAGT CTGCCAGGGTAGGTCTTCTG	59
p53Int1 for p53Ex4 rev	CTGCCTTGTTTCTTCCCAGA CTGCCAGGGTAGGTCTTCTG	58
p53Int1_2 for p53Int1 rev	CTACACCAGAGCCACAGCAA GGCAGAGGCAGGAATATGAA	58
p53Ex1 for p53Int1_2 rev	TGGGTTGTCGAATTTCTTCA GGTACTGAAATGGGGTGGTG	58
PTGE1 F PTGE1 R	CCTGCGCTCGGAGACTTAG GTCAGTGGCTGGTCACAGGT	58
Wnt1 F Wnt1 R	CAGGGCTGTTAGAGCCAGAC AATGCTCCTAAGGCGAGTCC	59

2.1.8. Instrumentation

Amaxa Nucleofector	Lonza, Basel, CH
Analytical balance	Denver Instruments, Göttingen, D
BioPhotometer	Eppendorf, Hamburg, D
Centrifuge miniSpin	Eppendorf, Hamburg, D
Centrifuge Mikro200	Hettich Zentrifugen, Tuttlingen, D
Centrifuge 5810	Eppendorf, Hamburg, D
Coolable centrifuge 1-15K	SIGMA, Osterode, D
Coolable centrifuge 4K-15C	SIGMA, Osterode, D
Elektrophoresis power supply EPS301	Amersham Biosciences, Piscataway, NJ, USA
Electroporator Multiporator	Eppendorf, Hamburg, D
Forma Orbital Shaker	Thermo Electron GmbH, Dreieich, D
Freezer (-20 °C)	Liebherr International, Bulle, CH
Freezer (-80 °C)	Thermo Electron GmbH, Dreieich, D

Fridge (4 °C)	Beko Technologies GmbH, Neuss, D
Gelelectrophoresis-apparatus EC105	Thermo Electron GmbH, Dreieich, D
Gelphotoapparatus Gene Genius	Syngene, Cambridge, UK
Gelphotoprinter UP-D895MD	Syngene, Cambridge, UK
Gelchamber Classic CSSU78	Thermo Electron GmbH, Dreieich, D
Gelchamber Classic CSSU1214	Thermo Electron GmbH, Dreieich, D
Glomax 20/20 Luminometer	Promega, Mannheim, D
Heatingblock VLM2Q	Gefran, Seligenstadt, D
HeraSafe Incubator cell culture	Thermo Electron GmbH, Dreieich, D
Incubator (37 °C) Binder	Binder GmbH, Tuttlingen, D
Inversionmicroskope AxioVert25	Carl Zeiss MicroImaging GmbH, Göttingen, D
membraPure waterfilter	membraPure GmbH, Bodenheim, D
Microwave NN-E202 W	Panasonic, Hamburg, D
Neubauer improved cell counter	Brand, Wertheim, D
Peltier Thermal-Cycler DYAD	Bio-Rad, Munich, D
Pipet 0,2 - 2 μL	Mettler Toledo GmbH, Gießen, D
Pipet 2 - 20 μL	Mettler Toledo GmbH, Gießen, D
Pipet 20 - 200 μL	Mettler Toledo GmbH, Gießen, D
Pipet 100 - 1000 μL	Mettler Toledo GmbH, Gießen, D
Pipetting assistant Falcon Express	Becton Dickinson & Co., Sparks, USA
Pipetting assistant Handy Step	Brand, Wertheim, D
qPCR Mastercyler ep realplex	Eppendorf, Hamburg, D
Scale	Sartorius, Göttingen, D
Shaker Unitwist 3D	UniEquip GmbH, Munich, D
Sterile bench class II HeraSafe	Heraeus, Kendro, Hanau, D
Thermal printer DPU-414	Seiko Instruments Incorporated, Neu-Isenburg, D
Trans-Blot SD Cell	Bio-Rad, Munich, D
UV-Transluminator NU72K	Benda, Wiesloch, D
Vortex Mixer ZX Classic	VELP Scientifica, Mailand, I
Waterbath	Memmert GmbH & Co. KG, Schwabach, D

2.1.9. Enzymes

Restriction enzymes	New England Biolabs (NEB), Frankfurt a. M., D Fermentas, St. Leon-Rot, D

Polymerases

Platinum Taq DNA Polymerase	Invitrogen, Karlsruhe, D
GoTaq DNA polymerase	Promega GmbH, Mannheim, D
Phusion High Fidelity DNA polymerase	New England Biolabs, Frankfurt, D
Superscript III One-Step RT-PCR with Platinum Taq Kit	Invitrogen, Karlsruhe, D
Superscript III Reverse Transcriptase	Invitrogen, Karlsruhe, D

Other:

Blunting enzyme DNA-polymerase I large fragment (Klenow)	New England Biolabs (NEB), Frankfurt a. M., D
DNase I	New England Biolabs (NEB), Frankfurt a. M., D
RNase A	SIGMA, Osterode, D
T4-DNA-ligase	New England Biolabs (NEB), Frankfurt a. M., D
Proteinase K	SIGMA, Steinheim, D

2.1.10. Expendables

0,22 μm sterile filter	Zefa, Munich, D
10 mL Terumo syringe	Terumo Europe N.V., Leuven, B
Bacterial culture tubes 14 mL	Becton Dickinson & Co., Sparks, USA
BioPhotometer cuvettes UVette	Eppendorf, Hamburg, D
Centrifuge tube 15 mL	Corning Incorporated, NY, USA
Centrifuge tube 50 mL Cellstar	Greiner Bio-One GmbH, Frickenhausen, D
Costar Stripettes 2, 5, 10, 25 mL	Corning Incorporated, NY, USA
Cryotubes	Nunc, Wiesbaden-Biebrich, D
Electroporation cuvettes	PEQLAB Biotechnology GmbH, Erlangen, D
GenAgarose LE	GENAXXON bioscience, Biberach, D
PCR-reaction tube	Brand, Wertheim, D
Pipet Filter tips 0,2 - 20 μL SR-L10F	Mettler Toledo GmbH, Gießen, D
Pipet Filter tips 20 - 200 μL SR-L200F	Mettler Toledo GmbH, Gießen, D
Pipet Filter tips 100 - 1000 μL SR-L1000F	Mettler Toledo GmbH, Gießen, D

Pipet tips 0,2 - 20 µL RC-L10	Mettler Toledo GmbH, Gießen, D
Pipet tips 20 - 200 µL RC-L250	Mettler Toledo GmbH, Gießen, D
Pipet tips 100 - 1000 µL RC-L1000	Mettler Toledo GmbH, Gießen, D
Plastibrand PD Tips 2,5 mL	Brand, Wertheim, D
Plastibrand PD Tips 5 mL	Brand, Wertheim, D
Plastibrand PD Tips 12,5 mL	Brand, Wertheim, D
reaction tube 0,5 mL	Brand, Wertheim, D
reaction tube 1,5 mL	Paul Böttger OHG, Bodenmais, D
reaction tube 2 mL	NerbePlus, Winsen/Luhe, D

2.1.11. Kits

DualGlo Luciferase-Assay	Promega, Mannheim, D
Fast SYBR Green Master Mix	Applied Biosystems, Woolston, UK
GenElute Mammalian Genomic DNA Miniprep Kit	SIGMA, Steinheim, D
NucleoBond Xtra Plasmid Purification Kit	Macherey-Nagel GmbH & Co. KG, Düren, D
NucleoSpin Plasmid Quick Pure Kit	Macherey-Nagel GmbH & Co. KG, Düren, D
VenorGeM Mycoplasm testkit	Minerva Biolabs, Berlin, D
Wizard SV Gel & PCR Purification Kit	Promega, Mannheim, D

2.1.12. Subcloning plasmids

pBluescriptIISK+ (pBL)	Stratagene, La Jolla, USA
pGEM-T Easy Vector System I (pGEM)	Promega, Mannheim, D
pJET1.2/blunt (pJET)	Invitrogen, Karlsruhe, D
pSL1180 Amersham (pSL1180)	Amersham Bioscience, New Jersey, USA

2.1.13. Software

Axiovision 3.1	Carl Zeiss MicroImaging GmbH, Göttingen, D
Basic Local Alignment Search Tool (BLAST)	Altschul et al. http://www.ncbi.nlm.nih.gov/blast/Blast.cgi
GeneSnap 6.01	Synoptics Ltd., Cambridge, UK
Image J 1.39 U	National Institutes of Health, USA

Microsoft Excel	Microsoft Corporation, Unterschleißheim, D
Primer 3	Whitehead Institute for Biomedical Research http://frodo.wi.mit.edu/
qPCR-Software	Eppendorf, Hamburg, D
VectorNTI 10.3.0	Invitrogen, Karlsruhe, D

2.1.14. Additional material

Bovine serum albumin (BSA)(100x)	New England Biolabs (NEB), Frankfurt a. M., D
DNA size marker 100 bp (M100bp)	New England Biolabs (NEB), Frankfurt a. M., D
DNA size marker 1 kb (M1kb)	New England Biolabs (NEB), Frankfurt a. M., D
DNA size marker 1 kb Plus (M1kb+)	Invitrogen, Karlsruhe, D
dNTP 10 mM	New England Biolabs (NEB), Frankfurt a. M., D
Glass bottles	Simax, Sazava, CZ
RNase free water	Qiagen, Hilden, D
RNase inhibitor	PEQLAB Biotechnologie GmbH, Erlangen, D
Scalpel	B. Braun Melsungen AG, Melsungen, D

2.2. Methods

2.2.1. Molecular methods

Isolating genomic DNA from eucaryotic cells

Isolation of DNA from eukaryotic cells was performed using the GenElute Mammalian Genomic DNA Miniprep Kit (SIGMA) according to the manufacturers protocol. Subsequent storage at -20 °C assured long term DNA stability.

Determining DNA- and RNA-concentration

Concentrations of DNA- and RNA-solutions were measured utilising the BioPhotometer (Eppendorf). The extinction of nucleic acids at 260 nm was detected and an optical density of 1 was correlated to a nucleic acid amount of 30 μg/ml (DNA) and 50 μg/ml (RNA) respectively. Aliquots were diluted in water or a corresponding buffer and compared to a reference measurement without nucleic acids.

Restriction digest of DNA

Enzymatic cleaving of DNA was conducted using different restriction endonucleases according to the manufacturers protocol, differing only in the amount of enzyme used. Instead of 10 U/μg of DNA 3-5 U/μg were applied. DNA-amounts of up to 200 μg were treated in batches of 20 to 400 μl.

Gelelectrophoresis of DNA fragments

For electrophoretic separation of differently sized DNA-fragments was accomplished on agarose gels with concentrations ranging from 0.8 to 2% (w/v) according to the requirements. Low percentage gels allowed faster separation with less accurate size distinction. Visualisation of the DNA was achieved by adding ethidium bromide (1 mg/ml) diluted 10000-fold to the agarose. Fragments were electrophoretically dissociated in electric fields of 80 to 120 V up to the desired distance. Lower strength resulting in longer run times yet higher acuity and vice versa.

Ethidium bromide stained DNA-bands were examined and recorded using Gene Genius (Syngene).

Purification of DNA from agarose blocks

Following gelelectrophoresis DNA-bands could be excised from the agarose gel to isolate the contained DNA. A purification of the DNA was conducted by means of the Wizard SV Gel & PCR Purification Kit (Promega) according to the manufacturers protocol.

Precipitation of DNA by sodium-chloride and ethanol

Sodium-chloride (NaCl) and ethanol precipitation of in-sterile DNA-preparations was used to obtain sterilely solved samples e.g. for transfections.

After subsequent adding of 1/10 the volume of 5 M NaCl-solution and double the volume of ice cold 100% ethanol (v/v) to the sample, the solution was vortexed and kept over night at -20°C. The following day the sample was spun down for 10 min at 16000x**g** and 500 μl of 70% ethanol (v/v) were added under a sterile workbench. The precipitated DNA was spun down in a second step for 5 min at 16000x**g**, the supernatant discarded and the pellet dried and solved under sterile conditions. The volume of the solvent was chosen regarding the desired final DNA-concentration.

Fill in of single stranded DNA-overhangs (*blunting*)

In order to fill in single stranded DNA-overhangs, resulting for example from endonuclease activity, the enzyme DNA-polymerase I large fragment (Klenow) was applied. The steps were executed according to the manufacturers protocol, differing only in the final concentration of dNTPs which were elevated from 33 μM to 50 μM.

Ligation of DNA-fragments

Ligation of DNA-Fragmenten was carried out employing T4-DNA-Ligase according to the manufacturers protocol. In short ligation was conducted 2 h at room temperature or over night at 4°C.

Variable DNA-ratios were used, adjusting for an excess of the smaller fragment ranging from 3:1 to 10:1. In general 200 ng of total DNA were ligated in a volume of 20 μl.

Sequencing of DNA

Sequencing was carried out by eurofins / MWG / Operon as an external service provider.

Annealing of single stranded oligonucleotides

In order to anneal to complementary oligonucleotides to a DNA double strand 1 μg of each nucleotide was heated to 95°C for 10 min in 1x annealing buffer. Following the heating step the sample was gradually cooled down to room temperature. Successful annealing was confirmed via gelelectrophoresis on a 3% agarosegel (w/v). The motility of single stranded versus annealed oligos was compared.

Degradation of DNA with DNase I

A DNase I treatment was conducted in order to preclude a contamination of DNA in e.g. RNA solutions. Per 50 μl of volume 5,66 μl DNase I buffer (10x) und 1 μl DNase I (20 U/μl) were added. A 10 min incubation time at 37 °C was followed by addition of 0,56 μl of 0,5 M EDTA to a final concentration of 5 mM.

Isolation of RNA from eukaryotic cells

On average 1x10^6 cells were detached from their culture dishes and spun down at 320x**g** for 5 min. The supernatant was discarded and the cells resuspended in TRIzol. Following a 5 min incubation at room temperature the samples were processed directly or could be stored at -80°C.

Further purification of RNA was conducted according to the manufacturers protocol. The RNA was usually treated with DNase I to exclude a possible DNA contamination.

For higher purity RNA the RNA isolation kit (Roche) was used according to the manufacturers protocol.

RNA isolates were subsequently stored at -80°C until further use.

Polymerase chain reaction (PCR)

DNA fragments flanked by two selected oligonucleotides (primers) can be amplified, applying the polymerase chain reaction developed by Mullis. Differing polymerases were used according to the prerequisites at hand, following the manufacturers protocol. In the course of this work PCR was applied to problems ranging from screening for positive cloning or selection results to isolating DNA fragments or labeling of Southern blot probes.

For proof-reading amplifications Phusion Taq (NEB) was utilised. For control or screening PCRs and Dig labeling of Southern blot probes GoTaq (Promega) was applied. The average thermo-cycler settings are summarised in table 2.15.

Table 2.15.: Thermo-cycler settings

Temperature [°C]	Duration [min : s]	
94 – 98	2:00–5:00	
94 – 98	0:15–1:00	repeat 25
58 – 62	0:15–1:00	to
72	1:00 / kb	40 times
72	3:00–10:00	
4	forever	

One-step reverse transcriptase - polymerase chain reaction (RT-PCR)

Reverse transcription of RNA into cDNA with immediate subsequent PCR amplification was performed using Superscript III One-Step RT-PCR with Platinum Taq (Invitrogen). The manufacturers protocol was followed. To exclude possible DNA contamination of the RNA a separate PCR lacking reverse transcriptase was conducted with Platinum Taq DNA Polymerase (Invitrogen). The thermo-cycler settings are summarised in table 2.16 on the next page.

Table 2.16.: Thermo-cycler settings for RT-PCR

Temperature [°C]	Duration [min : s]	
55	30:00	
94	2:00	
94	0:30	repeat 30
58	0:30	to
68	1:00 / kb	35 times
68	5:00	
4	forever	

Primer design for PCR and RT-PCR

All primers were designed with the online tool Primer3 (http://frodo.wi.mit.edu) and ordered via Biomers or SIGMA. They were dissolved in ddH_2O to a final stock concentration of 100μM. Prior to use 20μM working solutions were separately diluted from the stock.

Southern blot

ScaI (NEB) digested genomic DNA from cell clones or piglet ear tip samples was electrophoresed, membrane bound, hybridized and probe detected with anti-digoxigenin antibody Fab fragments conjugated with alkaline phosphatase (Roche) by standard methods. The 517 bp *TP53* hybridization probe was generated using primers probeF (5' GCAATG-GAGGAGTCGCAGT 3') and probeR (5' CTGCCAGGGTAGGTCTTCTG 3') incorporating alkali labile digoxigenin-11-dUTP (Roche). Thermal cycling parameters were: 2 min, 95°C; then 35 cycles of: 30 sec, 95°CC; 30 sec, 62°C; 40 sec, 72°C; followed by 8 min, 72°C. The location of the hybridization probe is indicated in Figure 3.13.

2.2.2. Protein chemical methods

Cre transduction of eucaryotic cells

Cre protein production and transduction was established and performed by Cornelia Brönner under the supervision of Anja Saalfrank. The protein was produced in an *in vitro* system with the pTriEx-HTNC vector (Addgene) according to the methods described by Peitz [237] and Münst [211]. The protein was used for *in vitro* Cre-mediated recombination of eucaryotic cells. In short, cells were cultured with 5 μM purified Cre recombinase in

medium with reduced serum (0.5%) for 6-8 hours. Afterwards, culture was continued as normal.

Protein isolation from eucaryotic cells

Eucaryotic cells were dissociated mechanically in IP buffer (50 mM HEPES, 150 mM NaCl, 1mM EDTA, 0.5% IGEPAL-630, 10% glycerol, pH 7.9, 1x phosphatase inhibitor, 1x complete mini protease inhibitor (Roche)) and quantified using advanced protein assay reagent (Cytoskeleton Inc.).

Western blot detection of p53

An amount of 40 μg total protein was separated by 15% SDS-PAGE with 5% collection gel over 120 min and blotted to an Immobilion-P Transfer Membran (Millipore) by semidry blotting. The membrane was blocked for 1 h with 5% BSA (PAA) in TBS and incubated with the primary antibody (mouse anti-p53 (DO-1, Sigma; 1:5000); anti-GAPDH antibody (Clone-GAPDH-71.1, Sigma, 1:5000). Subsequent washing steps were followed by incubation with secondary horse radish peroxidase-labeled rabbit anti-mouse antibody (Abcam; 1:6000). Bands were detected using enhanced chemiluminescence Western blotting substrate (Pierce). Signal intensities on film were quantified using ImageJ (http://rsb.info.nih.gov/ij/).

2.2.3. Microbiological methods

Transformation of *Escherichia coli* (*E. coli*)

During the line of this work mainly E. coli safety strands Emax and GM2163 were transformed by electroporation. The amount of DNA was varied according to the employed vector, namely 10 to 50 ng of circular DNA and 2 μl of a ligation reaction.

In short, 50 μl electro-competent E. coli were thawed on ice and the electroporation cuvette pre-cooled. Above mentioned DNA amounts were added to the bacteria and the mixture transferred into the cuvette. A voltage of 2500 V was applied for 5 ms by the electroporation equipment. Immediately 600 μl LB0-medium were added and the whole solution cultured for 30 min at 37°C in a 14 ml reaction tube.

Subsequently the bacteria were plated on agar plates containing adequate antibiotics and incubated over night at 37°C. For selection purposes the antibiotic ampicillin was applied at a final concentration of 100 μg/ml and kanamycin at 50 μg/ml. This last step was carried out under a class II sterile bench.

Isolation of single cell clones

Adequately distinguishable colonies grown after transformation were sterilely picked off the agar plate, separately re-plated and put directly into suspension culture. The bacteria were thus cultured in antibiotic containing LB0-medium and propagated over night at 37°C. Re-plated clones were incubated over night at 37°C and afterwards stored up to 4 weeks at 4°C as backup.

Plasmid-isolation from *E. coli* suspension cultures

Purification of high purity plasmid DNA from up to 5 ml E. coli suspension cultures, e.g. for sequencing or transfections, was carried out with the NucleoSpin Plasmid Quick Pure Kit (Macherey-Nagel). Larger culture volumes of up to 300 ml were processed utilising the NucleoBond Xtra Plasmid Purification Kit (Macherey-Nagel) resulting in higher amounts of DNA.

If the DNA was to be used just for verification of intermediate steps, one of two other protocols was consulted, namely alkaline or non-alkaline plasmid preparation.

Alkaline plasmid preparation An initial 5 ml suspension culture in LB0 medium was spun down in 2 ml reaction tubes at 18000 x**g** for 1 min and resuspended in 100 μl miniprep-solution I. For cell lysis 200 μl miniprep-solution II was added and mixed by inversion. Following 3 min incubation at room temperature, lysis was stopped by the addition of 150 μl miniprep-solution III. Precipitation and removing of proteins and genomic DNA was achieved via incubation on ice for 15 min and subsequent spinning down at 18000x**g** for 15 min. The supernatant was transferred into a new reaction tube and the plasmid DNA precipitated by adding 1 ml of 95% ethanol (v/v) and spinning at 18000x**g** for 15 min. After discarding the supernatant the DNA pellet was washed in 0.5 ml 80% ethanol (v/v) and spinning at 18000x**g** for 10 min. A similar final washing procedure in 95% ethanol (v/v) was followed by drying of the pellet at room temperature. In the end the plasmid DNA was solved in 50 μl H_2O containing 20 μg/ml RNase A and was either stored at -20 °C or directly processed.

Non-alkaline plasmid preparation As an alternative to alkaline plasmid preparation, which necessitates an additional RNase A treatment to purify the DNA, a non-alkaline preparation method could also be used.

A desired volume of bacterial suspension culture was spun down in 2 ml reaction tubes at 18000 x**g** for 1 min and resuspended in 10% of the original LB0 volume. Equal amount of non-alkaline miniprep solution was added and sequentially incubated at room temperature

for 5 min and spun down at 10000 x**g** for 10 min. The supernatant is transferred into a new reaction tube and intermingled with 0.8 volumes of isopropanol. Following a second spinning step at 10000 x**g** for 10 min the pellet is washed in 1 ml of 70% ethanol (v/v) and spun down at 18000 x**g** for 1 min. Resulting plasmid DNA pellets are dried at room temperature and solved in ~1% of the original culture volume of a desired buffer solution.

Cryoconservation of bacterial cultures

Storage and durability of plasmid constructs in E. coli cultures was enhanced and made independent from stability of agar plates or bacterial solutions, by establishment of cryoconserved cultures in glycerol.

In short 500 μl sterile 99% glycerol (v/v) was prepared and mixed thoroughly with 500 μl E. coli suspension culture. This stock mixture was immediately frozen and stored at -80°C. For re-propagation of the bacteria, the stock was kept frozen and only a small aliquot was forwarded into a 37°C suspension culture.

2.2.4. Tissue culture methods

The following methods and techniques were carried out in an S1 cell culture laboratory under a class II sterile hood (HeraSafe; Heraeus), unless stated otherwise.

Culturing of eucaryotic cells

The cells were kept in HeraSafe incubators at 37°C and under an atmosphere with 100% humidity and 5% CO2. All used cell culture media and detachment agents were brought to 37°C prior to use, unless stated otherwise. The amount of medium or agent used for the different culture sizes is summarised in table 2.17.

Table 2.17.: Culture sizes

Dish size	Medium [ml]	Detaching agent [ml]
12-well-plate	1	0,2-1
6-well-plate	2-3	1-2
10 cm dish	10	3-4
T25-flask (25 cm^2)	4-8	3
T75-flask (75 cm^2)	25	4-6
T150-flask (150 cm^2)	50	10

In regular intervals the culture supernatant was checked for contamination by my-

coplasms. Therefor the VenorGeM mycoplasm test (Minerva Biolabs) was used according to the manufacturers protocol.

Cryoconservation

Freezing Cryocultures of all cell lines and pools derived during the course of this work were generated at regular intervals. Thereby it was secured that, should the need arise, earlier passages of the cell lines could be accessed e.g. after screening processes.

The medium was aspirated and the cells washed once with PBS. After that, the cells were detached with Accutase or Trypsin (PAA) and spun down for 5 min at 400x**g**. The supernatant was discarded and the pellet resuspended in a corresponding volume of freezing medium. Aliquots of 1 ml were devided into cryotubes and the tubes put into "Mr. Frosty" freezing blocks filled with 2-propanole. At a rate of -1 °C/min the cells were cooled to -80 °C and subsequently transferred into liquid nitrogen.

Thawing In contrast to the gradual freezing of cells, the thawing procedure was conducted in one fast step.

In short, the cryotubes were warmed for 5 min in a 37 °C water bath and the cells directly transferred into 10 ml of prepared, pre-tempered medium. Thereafter the cells were spun down at 400 x**g** for 5 min and the supernatant discarded. Following resuspension of the cell pellet in their corresponding medium, the cells were cultured under standard conditions. A medium exchange was arranged the next day. In case of antibiotic resistant cell clones the selective pressure was omitted during thawing and first reapplied the following day.

Passaging of cells

Cells were passaged at a cell layer confuence of 90% either into larger culture volumes or proportionately kept in similar sized cultures.

In short the medium was aspirated and the cells washed once with PBS. According to the culture sized differing amounts of detaching agent (Accutase or Trypsin(EDTA)) were applied (see table 2.17). Following incubation at 37°C for 5 min complete detachment was confirmed under a microscope and if required incubation time prolonged. In parallel pre-tempered medium was prepared in the consecutive culture vessels. The thoroughly detached cells were then transferred in total (for expanding) or proportionately passaged.

Determining the cell count

Cell count was determined using a haemocytometer. Cells were counted to obtain defined cell-seeding densities e.g. before transfections. The cell count per ml was calculated with the following formula:

$$\text{cell count} = \left(\frac{\text{number of cells counted}}{(\text{proportion of chamber counted})(\text{volume of chamber})} \right) \frac{\text{cells}}{\text{ml}}$$

Transfection of eukaryotic cells

Electroporation For the generation of stably transfected cell lines or pools the DNA-transfer by electroporation was used. Electroporation of eukaryotic cells was carried out utilising the Multiporator (Eppendorf) or the Amaxa nucleofector (Lonza) according to the manufacturers protocol.

In short, for the Multiporator, 10μg of DNA were transfected into 5x10^6 cells. For Amaxa, 2 μg linearised DNA was transfected applying the electroporation protocol C-17 into 5x10^5 cells. These were then cultured in a T75 flask and cultured under standard conditions.

Nanofection Test transfections of DNA constructs for functionality or overall transfection efficiency were conducted with the transfection reagent Nanofectin (PAA) or Lipofectamin 2000 (Invitrogen) according to the manufacturers protocol. In short 1x10^5 to 3x10^5 cells were transfected in a 50-60% confluent 6-well dish. The DNA (up to 3μg) and 9.6μl of Nanofectin / Lipofectamin 2000 were separately mixed with 150μl of Nanofectin Diluent each. The latter is added to the DNA solution, mixed and incubated for 30 min at room temperature. Meanwhile the cells medium is exchanged. The tranfection mixture is added to the medium and incubated for 4 h under standard conditions, after which another exchange with standard medium takes place.

Selection with G418 and Blasticidin S

Cells were challenged with the antibiotic selection process 48 h after tranfection. The resistance genes in use were either a amino glycoside phosphotransferase gene (neo) for G418 resistance or a blasticidin deaminase gene (BS) for Blasticidin S resistance. The antibiotic concentrations were 600 bis 800 μg/ml G418 and 5 bis 6 μg/ml Blasticidin S. The selection intensity was kept constant by regular medium exchange for $\sim$2 weeks and untreated cells kept in parallel culture as a control of selection progress.

Isolating single cell clones after selection

Single cell clones were generated by localized dissociation with cloning rings or small pieces of filter paper soaked in Accutase. Alternatively, transfections were dilution in multiwell plates prior to start of selection and the clonality assessed microscopically.

Cell populations of interest were expanded gradually in max. 1:5 splits to larger cell numbers. Cells were cryopreserved from a 6-well dish at the earliest time possible and only afterwards expanded to generate material for subsequent testing.

Soft agar growth assay

To investigate anchorage-independent growth, samples of 1 x 10^3 cells were seeded into a layer of 0.4% Noble agar in culture medium (DMEM, 20% fetal calf serum, 2% Pen/Strep) overlying 0.6% bottom agar in culture medium. Culture proceeded for 4 weeks, then macroscopically visible colonies $>50\mu$m were counted.

Doxorubicin assay

For each assay, 5 x 10^5 cells were plated on a 10 cm culture dish and treated with 1 μg/ml doxorubicin-hydrochloride for 24 h. Cells were then cultured under standard conditions with regular exchange of medium. After 14 days colony growth was visualized by staining with 0.5% crystal violet in 20% methanol.

lacZ stain of transgenic fetuses

The fetuses were isolated and dissected manually. The organs were fixated in 4% paraformaldehyde over night at 4°C on a shaker and subsequently made permeable by treatment with rinse buffer for 3 x 30min on a shaker. Then they were put lacZ staining solution for 48h at 37°C. Finally, the organs were postfixed in 10% formalin for 48h and lastly permanently stored in 70% ethanol.

2.2.5. Somatic cell nuclear transfer (SCNT) experiments

Nuclear transfer and embryo transfer were performed as previously described [138] by Barbara Kessler from the external laboratory of Prof. Eckhart Wolf (TUM Oberschleißheim). Between 80-120 reconstructed embryos were transferred to each recipient sow.

Animal experiments were approved by the Government of Upper Bavaria (permit number 55.2-1-54-2532-34-09) and performed according to the German Animal Welfare Act and European Union Normative for Care and Use of Experimental Animals.

3. Results

3.1. Conditional gene targeting of *TP53*

Modifications of the tumour suppressor gene *TP53* in mice have provided valuable insight into its central role for tumour development. However, the translation of results from mouse to human is restricted and must be resolved. For this purpose, a porcine model with a modified *TP53* gene would provide a powerful tool. Therefore in this work, two types of alterations were to be introduced into the porcine *TP53* gene: a gene knockout and an oncogenic mutation.

The *TP53* gene was modified in the following ways: Firstly, a gene knockout was introduced by insertion of a floxed transcription termination cassette (loxP-stop-loxP: LSL) into the first intron. This modified locus is referred to as $TP53^{LSL}$. Secondly, an oncogenic gain of function mutation R167H in exon 5, 3' downstream of the LSL. The location of the latent oncogenic mutation was chosen to be one of the most potent of a set of *TP53* hotspot mutants in human cancers – the $TP53^{R167H}$ G to A single base pair exchange homologous to the human $TP53^{R175H}$. This modified allele carrying both modifications is referred to as $TP53^{LSL*}$. In the absence of Cre, neither allele is predicted to express full length *TP53*, but rather the truncated form of exon 1 to the LSL. After removal of the LSL cassette by Cre recombination, $TP53^{LSL}$ is predicted to again express wild-type p53. Cre-mediated removal of the LSL cassette from $TP53^{LSL*}$ activates mutant p53-R167H expression.

Regarding function, $TP53^{LSL}$ is a reversible knockout while $TP53^{LSL*}$ is a Cre-inducible, latent mutant allele (see figure 3.1 next page).

3.1.1. Construction of *TP53* gene targeting constructs

Isolation of porcine *TP53* sequences

Several sequence fragments of *TP53* were isolated as a base stock for the construction of the vectors in this work. They were obtained from a BAC clone containing the porcine *TP53* gene (GenBank: AC127472.4). A PCR amplification of the isogenic sequences was

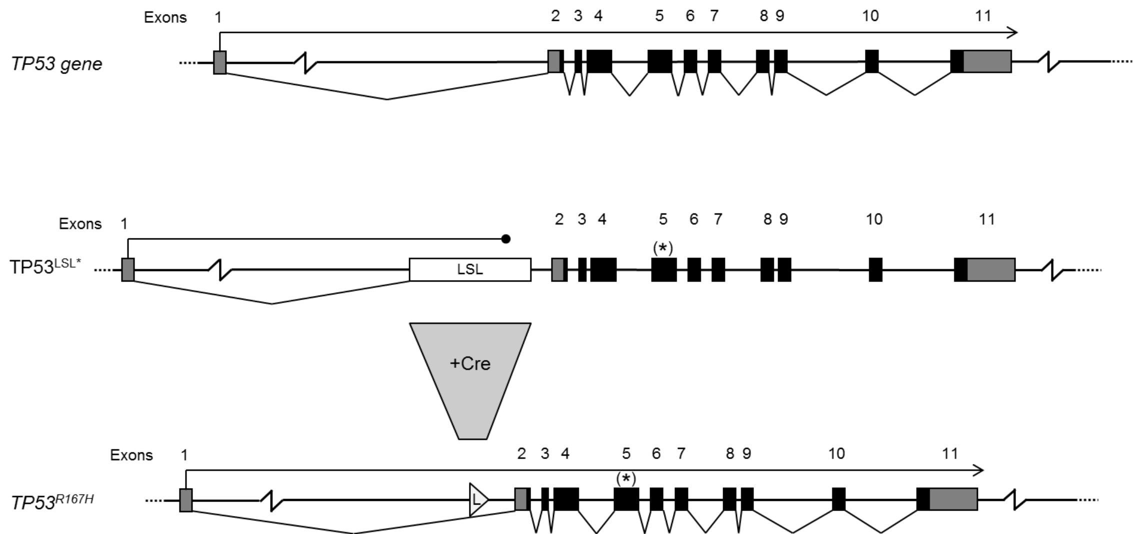

Figure 3.1.: Schematic overview of the wildtype and the modified *TP53* locus. *TP53*^{LSL*} contains both the LSL and the mutation, while the Cre-recombined locus only contains the remnant loxP site (L) and the mutation (*).

not possible, as the porcine genome was not sequenced at the beginning of this work. The exact means of subcloning are summarised in the appendix (see chapter A.1.1).

Preliminary tests of transcription stop cassette

One of the key components of the *TP53* gene targeting constructs was the generation of a functional floxed/loxP flanked transcription stop cassette (LSL). This cassette was designed and cloned by Noushin Rezaei to interrupt gene expression by alternative splicing into a resistance gene (either against Blasticidin S or G418) followed by three transcription terminating polyA signals. Figure 3.2 shows the LSL cassettes components and design in detail.

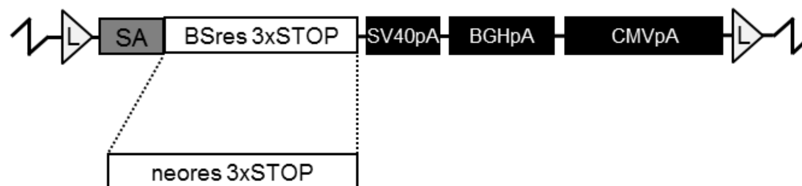

Figure 3.2.: Schematic overview of the floxed transcription stop cassette (LSL); L = loxP site; SA = splice acceptor; BSres/neores = resistance genes with triple stop codons; polyA signals from SV40 (SV40pA), bovine growth hormone (BGHpA) and cytomegalovirus (CMVpA)

Two characteristics were of particular functional importance and were tested before

the actual gene targeting experiments: the ability to stop transcription with the desired efficiency i.e. the "tightness" of the LSL, and the functionality of the splice acceptor within the desired genomic environment. These preliminary tests were carried out in human embryonic kidney 293 (HEK293) cells, due to their simple *in vitro* culture and transfectability. Test constructs were cloned, and are described in detail in the following paragraphs. A more detailed description of the subcloning is outlined in the appendix (see chapter A.1.1).

Transcription stop test vector The ability of the LSL to stop transcription was tested *in vitro* using a dual luciferase system. The psiCHECK2 vector (Promega) was used as a platform for this. It carries both a renilla and a firefly luciferase with promoters suited for eucaryotic expression. The activity of the renilla luciferase can be modified by introducing the LSL between promoter and gene. The activity of the firefly luciferase is used to normalise the measurements for transfection efficiency and cell toxicity. A schematic overview of the experiment is shown in figure 3.3.

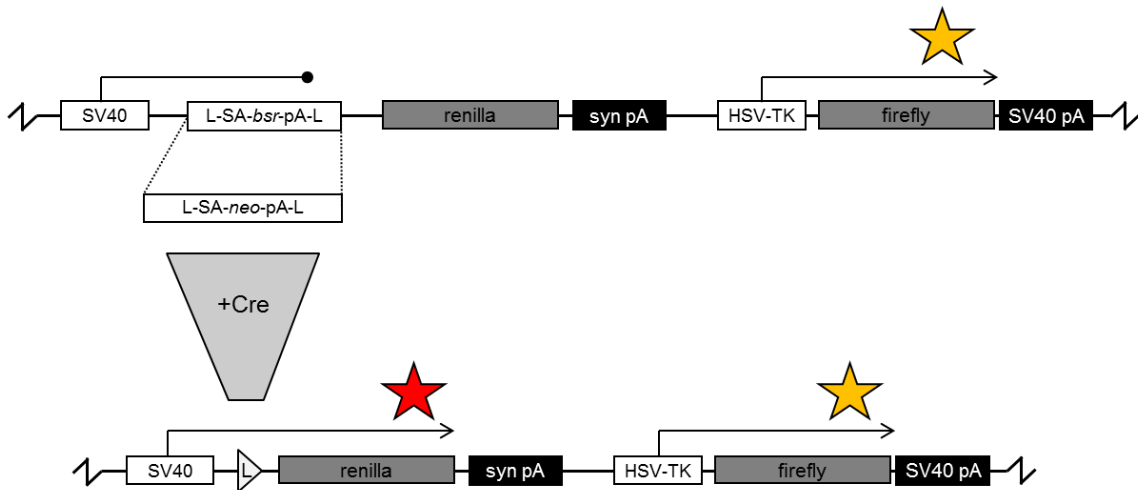

Figure 3.3.: Schematic overview of the dual luciferase assay experiment. The ubiquitous expression of the firefly luciferase is used as a normalisation control. The activity of the renilla luciferase is modified and put in relation to the firefly activity. After Cre-recombination the remnant loxP site (L) does no longer inhibit renilla expression.

The LSL (BS and neo) was cloned 5' of the renilla luciferase to check for the inhibition of expression by the LSL. The constructs were cotransfected into HEK293 cells with a tenfold amount of Cre-expression (pPGK-Cre) or mock control (puc19) constructs. The original psiCHECK2 plasmid and a similarly constructed plasmid from an external group (PD Saur - Klinikum rechts der Isar) served as controls. After 48h the difference between renilla activity relative to firefly activity was compared. Figure 3.4 (next page) shows the

ability of the LSL to prevent transcription in this assay. The relative luciferase activities are compared to constructs with either no LSL or no luciferase promoter.

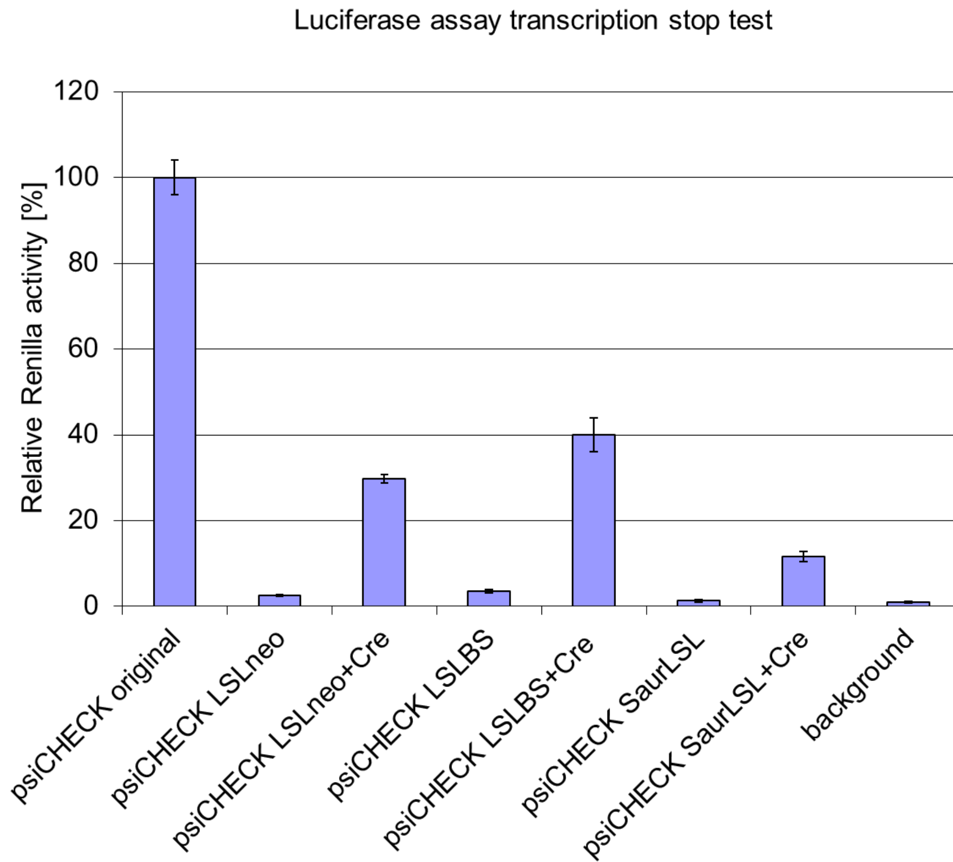

Figure 3.4.: Luciferase assay of psiCHECK-LSL test constructs with Cre-activation; the measure of the positive control plasmid was set to 100% relative activity; constructs with integrated LSL show only a background levels of Renilla activity, comparable to the external control plasmids; the Cre-mediated excision of the LSL re-enables Renilla expression, although only to approximately a third of the unmodified plasmid.

The relative renilla activity was reduced to background levels by the integrated LSL and thus comparable to the desired gene knockout. In addition, the cotransfection with pPGK-Cre lead to an excision of the LSL and reactivated the renilla expression to a level of 30 to 40% of the unmodified "psiCHECK original" vector. This incomplete reactivation was expected since the experiments were carried out transiently without selection of stable Cre-expressing cell clones. This could result in residual unrecombined psiCHECK-LSL, since the transient Cre-activity can not be externally quantified. Also, the loxP remnant was left in the Cre-recombined constructs. However, this minor sequence difference to the positive control vector can be neglected.

Splice acceptor test vector The functionality of the LSL splice acceptor was also tested *in vitro*. Constructs were cloned to model the splicing from the region of exon 1 into the LSL-BS/neo at its designated location in intron 1. The endogenous sequence of exon 2 to exon 6 served as competing splice accepting sites. For the sake of simplicity the approximately 8kb of the 9kb intron 1 sequence was omitted and a CAGGS promoter cloned 5' of the exon 1 sequence to drive resistance gene expression (see figure 3.5). The human background and transformed linage of the HEK293 cells made the use of the actual porcine *TP53* promoter impractical for this preliminary test. This construct was transfected into HEK293 cells and selection pressure was applied. Cells continued to proliferate, thus proving successful splicing from a promoter into the LSL cassette even in the presence of the splice acceptors of competing endogenous exons.

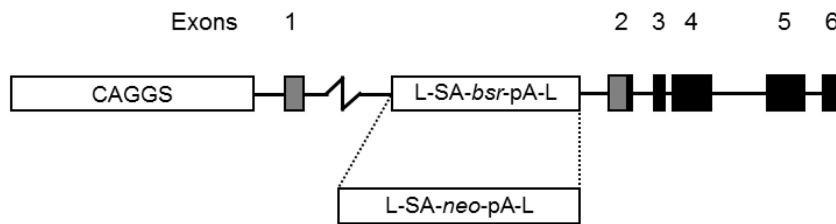

Figure 3.5.: Schematic overview of the splice acceptor test construct

Taken together, the LSL-tightness, re-activatability and the functional splice acceptor all confirm the applicability of the designed LSLBS/neo for a conditional gene knockout.

TP53 gene targeting vector

The construction of the *TP53* gene targeting vectors was started, after establishing the functionality of the LSL. Large sequence homology of approximately 10kb is needed to modify a genome via homologous recombination [54]. The desired modification is usually placed between two so called homology arms: a shorter part of homology to simplify the subsequent screening procedure by PCR – the "short arm" – and the majority of the homologous sequence – the "long arm".

The finished construct (see figure 3.6 next page) should contain the conditional transcription stop LSL cassette and the latent oncogenic R167H mutation, flanked by a 5' short- and a 3' long homology arm. These "gene targeting vectors" should carry a splice acceptor in front of a promoterless resistance genes to enrich for correctly integrated constructs – a so called promoter trap system. After integration into intron 1 of genomic *TP53* the resistance would utilise the endogenous promoter by alternative splicing into the LSL to disrupt the wildtype gene.

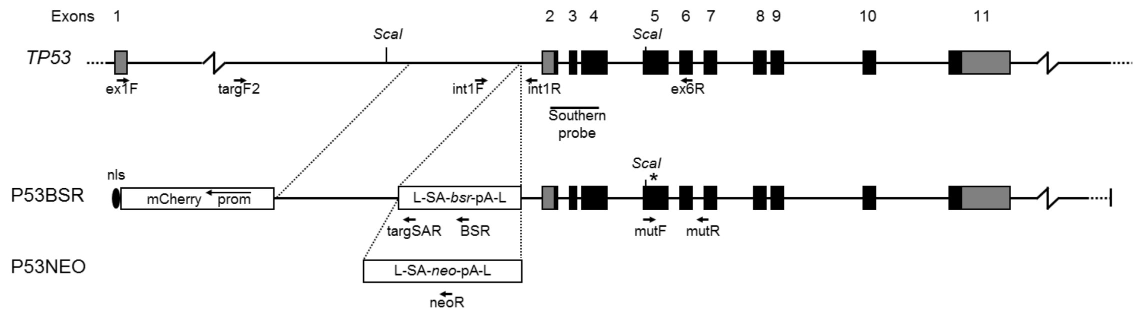

Figure 3.6.: Schematic overview of the wildtype *TP53* locus and the design of the targeting vectors. The latter only differ in the resistance gene in the LSL cassette.

It is also important that these arms are sufficiently specific for the locus and do not contain large amounts of repetitive sequence. This could lead to rearrangements at the targeted locus or unwanted additional integrations at other repetitive loci.

Lastly the desired modifications have to be incorporable into the construct: the modification for a conditional gene knockout of *TP53* ideally had to be placed upstream of any coding exons and the location of the mutation was predefined.

The aforementioned guidelines were applied to design DNA constructs with homology to the porcine *TP53* gene. The selected homology region covers a part of the porcine *TP53* intron 1 and the exons 2 - 11 with additional 3' flanking sequence. A detailed description of the cloning steps can be found in the appendix A.1.1.

Eliminating short arm promoter activity The first gene targeting experiments in porcine mesenchymal stem cells (MSC) gave rise to an unexpectedly large number of antibiotic resistant colonies. However, the subsequent PCR screen showed no sign of gene targeting and only hinted at random integrations. This could be explained by promoter activity inherent in the short arm region, which would drive the resistance gene expression independent of the integration site. In humans it is known, that a promoter region resides within the first intron of *TP53* [256]. However, it is mapped to the 5' 1.2kb of the intron, more than 7kb upstream of the homology region used here, making it a very unlikely to be the cause for this promoter activity.

The presence of promoter activity in the short homology arm was confirmed *in silico* and *in vitro*. Firstly, by sequence analysis with the online tool "Promoter 2.0 Prediction Server" [140] indicated putative promoter elements (see figure in appendix A.1). Secondly, an *in vitro* assay confirmed that a renilla luciferase gene is expressed in HEK293 cells when put 3' of the short arm sequence. A distinct Luciferase activity was detectable, compared to

a promoterless vector and untransfected cells. Figure 3.7 shows Renilla luciferase activity, driven by promoter elements within the short arm, thereby confirming the *in silico* findings.

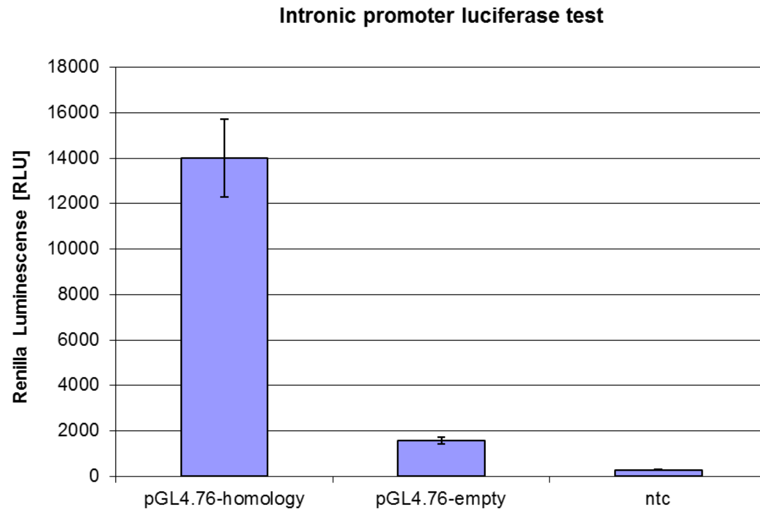

Figure 3.7.: Renilla luciferase in the pGL4.76 vector driven by the short homology arm. Empty vector and non-transfected cells (ntc) serve as negative controls. The homology arm shows intrinsic promoter activity.

To circumvent this activity, the short arm was modified to eliminate the promoter activity. The LSL cassettes (BS/neo) were newly cloned into a PmlI restriction site 385bp downstream of the inital ClaI site. Additionally the short homology arm was truncated down to 1212bp. This shortening of the short homology arms was expected to exclude the majority of promoter activity from the targeting vectors. However, it was also expected to mitigate the efficiency of homologous recombination [54, 102], leading to a trade off between successful homologous recombination and increased screening effort. Test-transfections showed greatly reduced colony numbers, confirming the success of this approach and indicating the loss of the major promoter activity within the short arm.

Visual counter selection and nucleus localisation sequence Another modification was introduced into the targeting vector to exclude events with random recombination of the vector. A visible counterselection marker was cloned into the backbone of the gene targeting constructs (compare [63]). It consists of a chicken beta-actin (CAGGS) promoter driven mCherry marker gene followed by a rabbit globin polyA. This cassette also integrates into the genome in case of random integration of the targeting construct and leads to an expression of the red fluorescent protein. Thus, these cell clones can then be excluded from further screening by microscopically identifying the visual marker gene.

Additionally, a SV40 nuclear localisation sequence (NLS) was isolated by PCR (primers NLSflank for/rev) and integrated into the vector backbone, to promote active DNA transport into the nucleus and thereby increase the frequency of HR. The NLS contains several transcription factor (TF) binding sites, that get recognised and occupied by TFs. Subsequently, the NLS is co-transported into the nucleus together with the TFs and thereby enriches the nuclear concentration of the targeting vectors.

These constructs represent the final versions of the p53 gene targeting vectors (p53TV-BS/neo), that are depicted in Figure 3.6.

3.1.2. Establishing PCR screening procedures

The initial screen of putatively gene targeted clones for a correct *TP53* modification was to be performed by PCR across the short homology arm. This "targeting PCR" successfully amplifies a fragment only if the LSL had integrated at the desired location. Therefore, the forward primer binding site had to be located outside of the homologous sequence used in the targeting vector. The reverse primer binding site had to be located in the exogenous modification – the LSL cassette. It also had to be possible to run the relatively large PCR of more than 3kb on small amounts of DNA. This was necessary, because the screening should be carried out as early as possible with a minimal amount of cells. Primary cells only show a limited number of cell divisions and early passage cells are less transformed by *in vitro* culture. This is important for the success of the somatic cell nuclear transfer (SCNT).

The PCR screening methods had to be stringently optimised to detect the positive HR events under these conditions. However, no actual positive targeting event was available to establish it on. Therefore, a PCR control plasmid was cloned for optimisation and as a positive control in subsequent experiments, containing the respective screening primer binding areas. In addition, its PCR product had to be distinguishable from an actual targeting event. Therefore, the product size was preemptively reduced by 485bp to allow the identification of a putative cross contamination with the plasmid. Figure 3.8 shows a schematic overview of the PCR control vector with respective primer binding sites.

Different forward primers were tested in combination with reverse primers binding in the splice acceptor or the respective resistance genes. The most specific combination of p53targF2 and p53SAR was optimised. For this step, PCR control vector was combined with porcine genomic DNA at a 1:1 ratio to mimick the later targeting situation. The PCR conditions could be optimised to detect down to approximately 10 copies of the PCR fragment, as shown in Figure 3.9. This detection limit was considered suitable for targeting screenings.

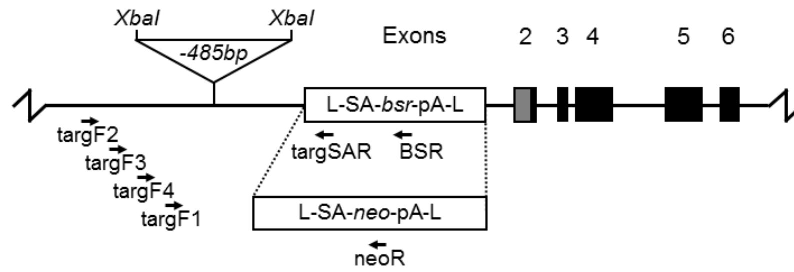

Figure 3.8.: Schmatic overview of the PCR control vector with the 485bp XbaI shortened fragment. The targeting primer binding sites are indicated by arrows.

3.1.3. Generating *TP53* gene targeted cell clones

Cell isolations used for gene targeting

Several mesenchymal stem cell (MSC) isolates were prepared from adipose tissue (adMSC) and bone marrow (bmMSC) of male Landrace/Pietrain cross bred or pure Landrace pigs. The isolates were assessed for sufficient *in vitro* proliferation, and transfectability using fluorescent marker genes. Both varied markedly between cell isolates, and the best isolates were used for the further experiments. The stem cell character of MSCs was confirmed by differentiation assays. Cell isolates capable of differentiation into osteogenic, adipogenic and chondrogenic lineages were used (data not shown).

Additionally, fibroblasts (KNDF) were isolated from the cortex of the kidneys of young Landrace/Pietrain cross piglets (2-3 months). These cells were kept on gelatin coated plates to promote surface attachment.

In this project, gene targeting experiments have been performed in KDNF, bmMSC and adMSCs.

The first successful gene targeting was achieved in bmMSCs. However, the efficiency was very low and many clones lacked proliferative potential. Therefore, the method of generating single cell clones was modified to allow a wider range of single cells to survive the process of antibiotic selection. It was proposed to select the transfectants in separate multiwell plates, so that on average one resistant clone was expected per well. The wells were microscopically assessed for cell clonality and the derived cells referred to as minipools, because a mixed resistant cell population could not be ruled out completely.

The gene targeting experiments in KDNF did not result in any positive minipools and the use of these cells was discontinued. However, the use of adMSCs and bmMSCs proved successful. For adMSCs the method of single cell cloning using cloning rings was taken up again, as these cells had better proliferatory abilities than bmMSCs.

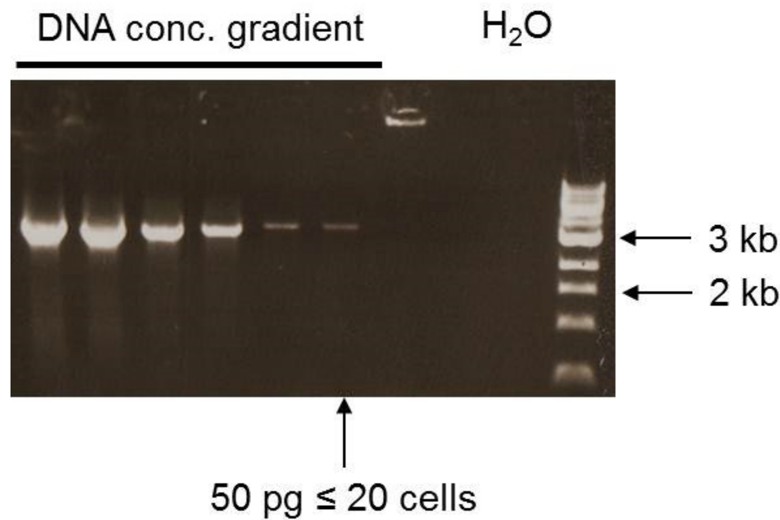

Figure 3.9.: PCR optimisation of PCR control vector diluted in genomic DNA at equal copy numbers. The DNA gradient amounts from left to right are 10ng, 5ng, 1ng, 500pg, 100pg, and 50pg. The lowest amount of 50pg corresponds to approximately 20 cells or 10 sequence copies. This would suffice for a targeting PCR from low amounts of material early in the screening process. (PCR by Margret Bahnweg)

Transfections

The different cell types were transfected separately with P53TV-BS or P53TV-neo vectors. These vectors differ only in the drug selectable marker used (bsr or neo). Transfections were optimised within the lab with varying DNA amounts and transfection methods: lipofection, nanofection, nucleofection and electroporation. The key factors were selection of the cells, while keeping the possibility to expand cell clones and finally the number of identified positive targeting events.

The best transfection conditions were identified to be electroporation of 10μg of plasmid into 1x10^6 cells.

Confirmation of gene targeting by PCR screening

Putative gene targeted cell clones were initially identified by lack of mCherry fluorescence. They were then expanded and screened by PCR. The latter amplified a 3.3kb DNA fragment from a point in the first intron outside the targeting vector, across the shorter arm of each targeting vector (BS/neo) into the splice acceptor in the LSL cassette. The DNA-quality of the sample was assessed by amplifying an endogenous 3.1kb fragment from the same forward primer to 17bp downstream of the LSL integration site (endogenous control PCR with p53Int1_5R reverse primer). All cells that carry the targeted modification of

the LSL in the *TP53* locus are referred to as "gene targeted". Figure 3.6 shows a schematic of the screening PCRs.

Cell clones carrying the R167H mutation were identified by PCR amplification across the mutation site in exon 5 (primers p53mut for/rev) and sequence analysis either by external sequencing or a diagnostic HaeII digest. In the presence of the mutation a HaeII recognition site is lost (compare figure 3.11). It was expected that the mutation site will be lost in some gene targeted clones, since the site of the mutation is more than 1kb downstream of the selectable marker. This could allow for a crossing over in between features.

Figure 3.10 and 3.11 show representative PCR screening and sequencing data, including targeted and untargeted cell clones, and cell clones that incorporated the R167H mutation hetero- and homozygously.

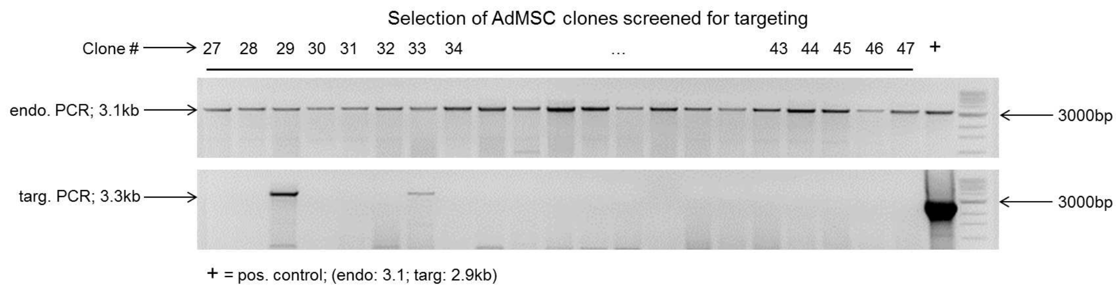

Figure 3.10.: targeting screening PCR: the expected size of the endogenous *TP53* control PCR for DNA quality is 3.1kb; in case of a positive targeting event the PCR results in a 2.9kb fragment; the screened clones all produce the endogenous PCR band, indicating sufficient DNA quality to support such a long range PCR; only clones adMSC29 and adMSC33 also show a band for positive targeting (PCR by Marlene Edlinger).

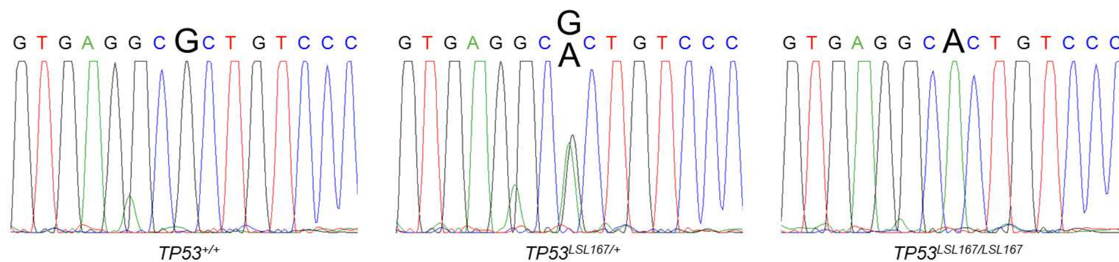

Figure 3.11.: Chromatograms of sequenced mutation sites from three *TP53* targeted cell clones; *TP53*$^{+/+}$ shows only the unmutated wildtype allele sequence: this clone lost the mutation during homologous recombination; *TP53*$^{LSL167/+}$ shows both the wildtype sequence and the mutated nucleotide as overlapping peaks, thus this clone carries a heterozygous mutation of *TP53*; *TP53*$^{LSL167/LSL167}$ shows only the mutated allele sequence: this clone carries two mutated *TP53* alleles. The HaeII restriction site is the central GGC**G**CT motif that changes to GGC**A**CT.

As expected, some positive minipools were comprised of too many subclones, or still

#	Cell type	Clones screened	Pos. events	Efficiency
1	bmMSC	24	4	16,6%
2	bmMSC	8	1	12,5%
3	bmMSC	10	2	20,0%
4	adMSC	57	10	17,5%
5	adMSC	78	7	9,0%
Total:	bmMSC	42	7	16,6%
	adMSC	135	17	12,6%

Table 3.1.: *TP53* targeting efficiencies confirmed by PCR: successful transfections with respective targeting efficiencies; all transfections are summarised per cell type at the bottom.

contained a few mCherry positive cells. Also, the growth characteristics of clones and minipools remained varied and sometimes too low. Unfortunately, some cells within minipools did not carry the $TP53^{R167H}$ mutation along with the LSL integration. This indicates homologous recombination of genome and targeting vector in the 1.2kb between the LSL and the mutation site downstream in exon 5. Therefore, not all cells that were confirmed positive for gene targeting could be used for further experiments.

Efficiency of *TP53* gene targeting

Only 3 tranfections into bmMSCs and 2 transfections into adMSCs lead to successful gene targeting. By far the most successful cell type in use were the adMSCs of the 110111 isolate with regard to proliferatory characteristics and SCNT behaviour. Transfections and efficiencies in relation to cell type and cell isolate are summarised in table 3.1. The proportion of drug selected cell clones with targeting events detected by PCR ranged between 9,0% to 20% in different experiments. It is important to note, that some of the positive clones did not show sufficient proliferatory abilities to warrant further experiments.

The outlier “neo314” – Homozygous targeting of *TP53*

One transfection into Landrace Pietrain hybrid cells from the 090210 isolation resulted in the homozygously targeted cell clone neo314. Surprisingly, the targeting PCR product was shorter than expected. Sequencing of the targeting PCR lead to identification of a 220bp deletion within intron 1. Moreover, the PCR amplifying the endogenous control fragment only gave rise to the 4.6kb fragment spanning the integrated LSL-cassette and no wildtype allele. The homozygous targeting was further confirmed by sequencing the mutation site in exon 5. The chromatogram only showed a singular adenine peak at the mutation site. This indicates the presence of only the mutation and no remaining guanine from the wildtype sequence.

These PCR and sequencing data were consistent with a loss of the wildtype allele. This can be explained either by a loss of heterozygosity (LOH) or a targeting of both *TP53* alleles and thus the generation of a potential $TP53^{LSL*/LSL*}$ homozygote.

Fluorescense in situ hybridisation A fluorescence in situ hybridisation (FISH) analysis was performed to further elucidate whether cell clone neo314 had undergone targeting at both *TP53* alleles or LOH occurred. A 41kb labeled FISH probe was designed to cover the *TP53* gene and 21kb and 7kb of flanking sequence. Hybridisation with this probe indicated no microdeletions of the locus. The expected signals at the ends of two small metacentric chromosomes were detectable on wildtype as well as neo314 metaphase spreads as shown in Figure 3.12. This is consistent with our PCR and sequencing findings of a gene targeting of both alleles. Smaller FISH probes without flanking regions were designed to get a more specific marker for the *TP53* gene or the integrated LSL-neo. These probes however could not bind to their target sites with sufficient specificity to detect the locus among background signals. This could be due to insufficient chromatin accessibility or probe specificity. In accordance with this, 2 SINE and 2 LINE elements were found by software analysis [273] in the part of intron 1 of *TP53*, which was used as targeting vector short arm.

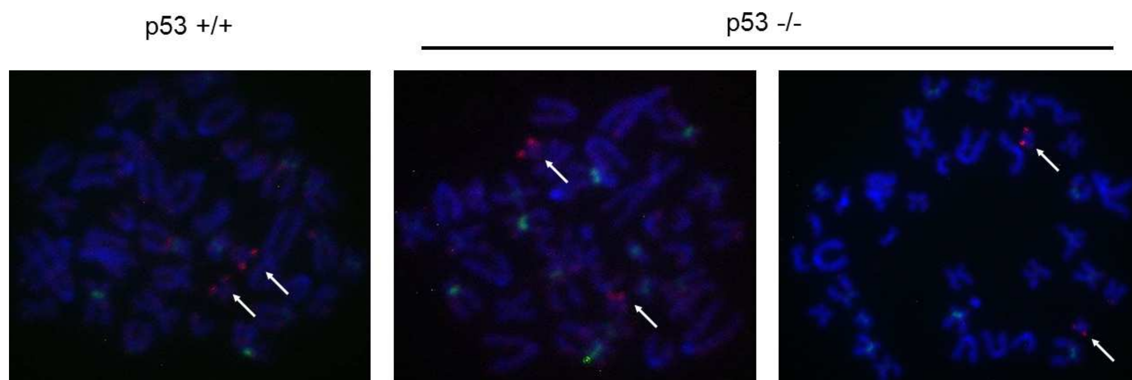

Figure 3.12.: FISH analysis of clone neo314 with homozygous loss of *TP53*: chromosomes are stained with dapi (blue) and the detected probe (red) covers the *TP53* locus with 21kb 5' and 7kb downstream of the gene; several prepared slides show the same signal pattern as the wildtype cells: two signals at the end of two small chromosomes (chromosome 12 in pig); this indicates no microdeletion within the region of the locus and thus supports homologous gene targeting over loss of heterozygosity. (FISH by Marlene Edlinger)

Proliferatory analysis The clone neo314 also changed its proliferatory behaviour after passage 5 in culture from doubling time of 72h to 24h. Moreover, it was able to undergo several single cell subcloning steps. Other cell clones have ceased proliferation after a

second round of subcloning. Due to that advantage, Cre-transduced single cell subclones of neo314 could be generated by Anja Saalfrank that carry only a heterozygous activation of the R167H mutated allele (compare [162]). This further confirms the homozygosity of a *TP53*^{LSL*} allele. The other allele still carries the characteristics of an integrated LSL.

Confirmation of gene targeting by Southern Blot

A Southern blot analysis of targeted cell clones was performed to confirm the structure of targeted alleles and the presence of the LSL cassette. Genomic DNA was digested with ScaI and hybridised to a endogenous probe spanning exon 2 to exon 4 (isolated with the primers p53probe for/rev). Figure 3.13 shows a schematic overview over the diagnostic ScaI fragments and probe binding site.

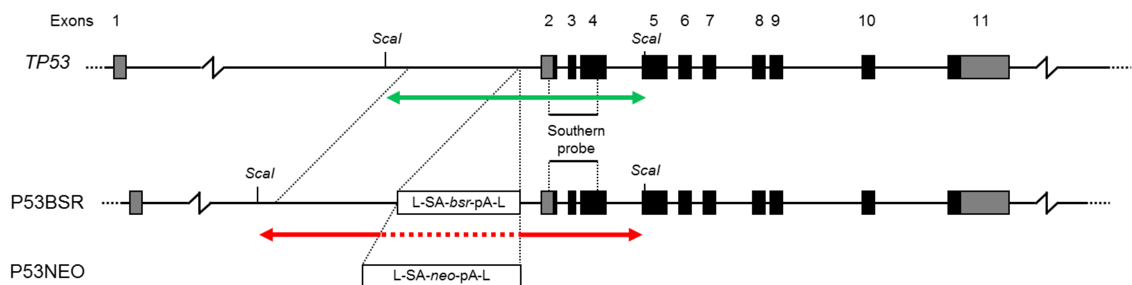

Figure 3.13.: *TP53* locus with diagnostic ScaI restriction sites used for Southern blot. Probe binding site is indicatied across exons 2-4. Green arrow: wildtype ScaI fragment of 3.2kb; Red arrow: gene targeted ScaI fragment of 4.7kb for LSL-BS and 4.9kb for LSL neo.

Predicted sizes for diagnostic ScaI fragments are: wild-type *TP53* 3.2kb, *TP53* with LSL-BS 4.7kb, *TP53* with LSL-neo 4.9kb (neo). Figure 3.14 shows samples from wildtype DNA and a selection of *TP53* targeted clones and piglets. The 3.2kb band indicative of a unmodified allele is detected in wildtype and heterozygous mutants. It is lost for the homozygously targeted clone and its derived piglets, in accordance with homozygous *TP53*^{LSL*} alleles. The larger bands appear at the expected sizes, indicating an allele carrying the LSL cassette. No additional random integrations of the constructs were detected by additional differently sized bands. In summary, the integrity of the modified and unmodified loci can be assumed.

Effective Cre mediated excision of the LSL

The *TP53* targeted cell clones were tested for the ability to excise the LSL by Cre-mediated recombination. This was important for the full functionality of the conditional targeting system. Therefore, cells were transduced with Cre protein and expanded to obtain sufficient material for subsequent analysis on DNA and RNA level.

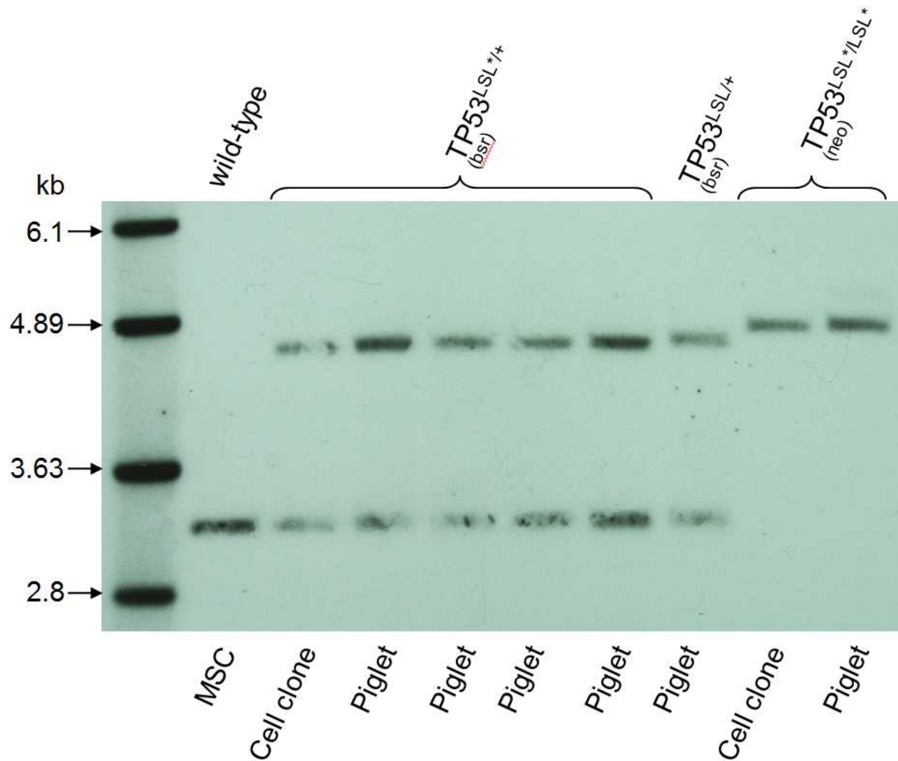

Figure 3.14.: Southern blot of *TP53* targeted cell clones and piglets; the probe detects exon 2 to 4 of *TP53*; the diagnostic ScaI digest results in a detectable 3.2kb fragment for a wildtype *TP53* allele and a bigger fragment of 4.7kb for an integrated LSL-BS and 4.9kb for an integrated LSL-neo cassette. Wildtype DNA shows only the expected fragment at 3.2kb; the cell clone and the piglets targeted with a LSL-BS cassette show the expected additional fragment at 4.7kb; the cell clone and piglet with homozygous integration of the LSL-neo cassette show only the diagnostic fragment of 4.9kb minus the intronic deletion of 220bp, and lacking a signal indicative of a wildtype *TP53* allele. (Southern blot by Margret Bahnweg)

Cre recombinase mediated excision of the LSL cassette was tested in targeted cell clones *in vitro*. Cre was introduced into cell clones by protein transduction.

On DNA level the LSL excision was confirmed by a PCR amplifying a fragment across the LSL integration site (Peggy Müller). Size differences are expected for the wildtype allele from the alleles with integrated LSL and the recombined alleles with remaining loxP site. Cre-mediated LSL excision results in a single 34bp loxP site and a 22bp cloning fragment remaining at the integration site. Therefore, the predicted fragment sizes are: wild-type *TP53* 198bp, integrated LSL (bsr) 1536bp, integrated LSL neo 1929bp, Cre-excised integration site 254bp.

Figure 3.15 shows results from non-targeted cells, heterozygous targeted ($TP53^{LSL*/+}$) cell clone adBS29 and homozygous $TP53^{LSL*/LSL*}$ cell clone neo314. In the heterozygous targeted clone both the wild-type integration locus and the loxP remnant are amplified. As expected, in the case of the homozygous targeting either the loxP remnant or the whole LSL cassette is amplified with no amplification of the wildtype 198bp fragment.

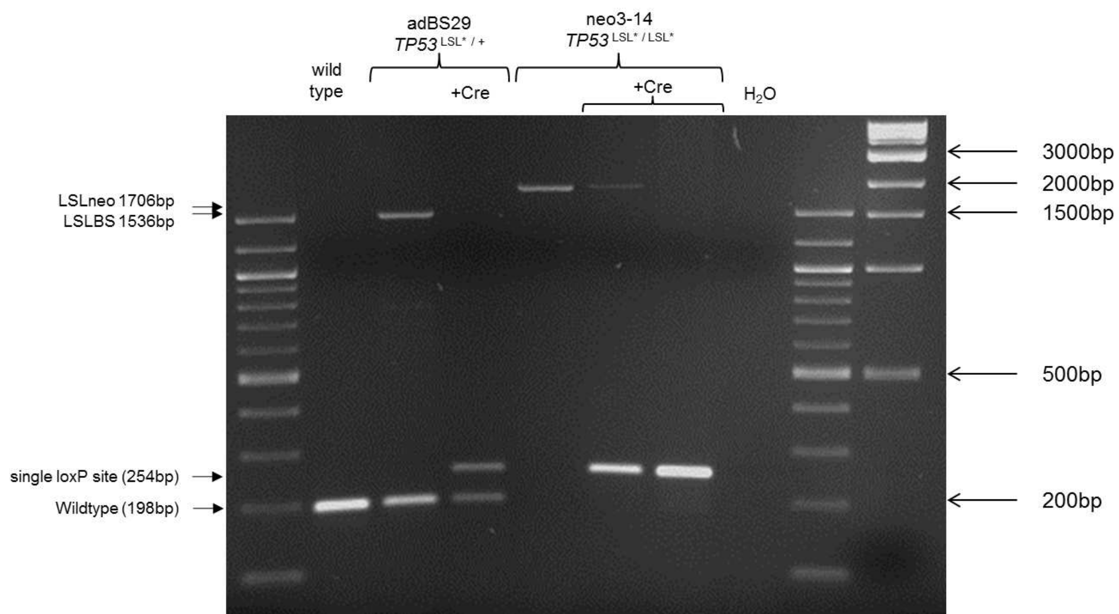

Figure 3.15.: loxP screening PCR covering the LSL integration site: the expected fragment amplified from a wildtype allele is 198bp, with a LSL cassette integrated it is 1.5kb in case of BS resistance, 1.9kb for neo resistance and after excision the fragment size is 254bp resulting from the residual loxP site and cloning remnants; as expected wildtype DNA only gives the unmodified sequence size; the heterozygous clone shows the presence of the LSL-BS and a wildtype allele and after activation the loxP remnant; from the homozygous clone no wildype sequence could be amplified, the partial and complete activated clone show the expected band sizes for integrated LSL-neo and the integrated loxP site (PCR by Peggy Müller).

Analyses of *TP53* targeting on RNA and protein level

RT-PCR analyses of *TP53* targeted cells Analyses on the RNA level produced further results confirming a functional integration and excision of the LSL. Cells with targeted integration of the LSL cassette in the first intron and the mutation in exon 5 were predicted to express mRNA from the first (non-coding) *TP53* exon spliced to the drug resistance gene. This eliminates the expression of the full length mRNA and after Cre-mediated LSL-excision leads to a mutated mRNA.

An RT-PCR screen was developed using one primer that hybridizes in exon 1 and one primer hybridising in exon 11. The resulting cDNA was digested with HaeII, to detect the restriction polymorphism caused by the R167H mutation (Peggy Müller). Predicted sizes for the digested 1313bp RT-PCR fragment are: 698bp, 310bp, 287bp and 18bp. Figure 3.16 shows RT-PCR exemplary data of a cell clone identified as heterozygously targeted and the homozygously targeted cell clone neo314. RNA from wildtype cells was used as positive control. Cell clone neo314 showed expression only of the mutated mRNA and only after partial or biallelic Cre-recombination.

Protein analyses of *TP53* targeted cells Targeted cell clones were also analysed on protein level by Western blot. The loss of p53 protein after gene targeting was confirmed. The p53 protein was detectable at approximately 46kDa in wild-type and heterozygous targeted cell clones. It was absent in the homozygous targeted neo314 and only reappeared after Cre mediated excision of the LSL cassette (see figure 3.17).

3.1.4. Phenotypic effects of the *TP53* knockout

The potency and functionality of the *TP53* gene knockout and the re-activation of the mutant allele should be assessed *in vitro*. For this, the following experiments were conducted mainly based on the p53 deficient cell clone neo314.

Enhanced proliferation

The proliferatory properties of a cell are a trait that is easy to assess yet still can give a good indication about existing abnormalities. With the loss of p53 a more rapid proliferation and even a transformation could be expected. Indeed, after a short time in culture (4-5 weeks post transfection), cell clone neo314 exhibited a noticeable change of phenotype. Cell proliferation increased compared to wild-type and heterozygous targeted cell clones, with cell doubling increasing to ~24h from ~72h. This was the first indication of an effect of p53 loss *in vitro* in porcine cells.

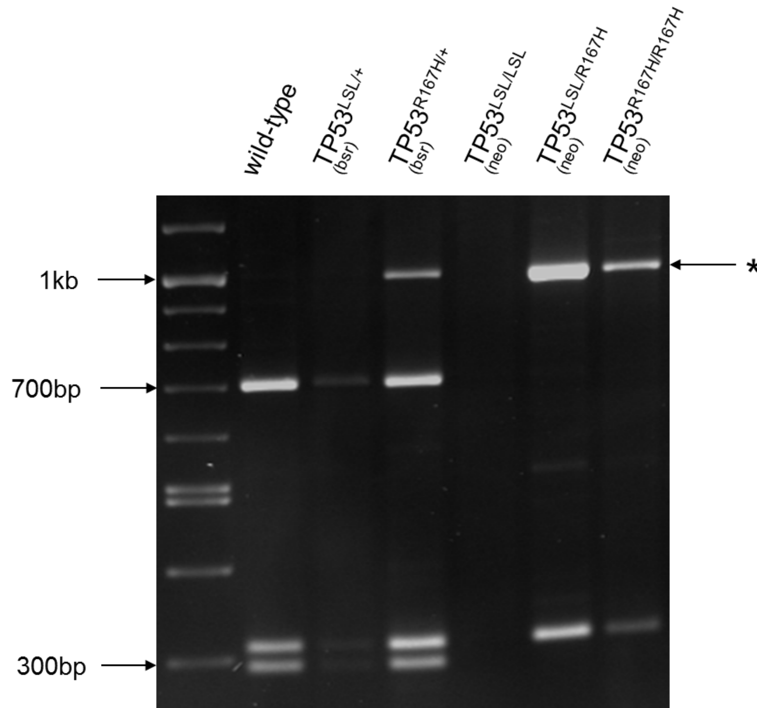

Figure 3.16.: RT-PCR confirming expression of wildtype and mutated mRNA from *TP53* and $TP53^{LSL/+}$: the amplified 1313bp fragment of *TP53* mRNA was digested by HaeII; one restriction site of this enzyme is lost through the mutation and a larger band remains undigestet (*); This results in the following fragment sizes: for wildtype *TP53* mRNA 698bp, 310bp, 287bp and 18bp; for mutated *TP53* mRNA 985bp, 310bp and 18bp. The 18bp fragment is undetectable on the gel. Wildtype and heterozygous clones show the expected fragment sizes of wildtype mRNA and only after Cre-activation the mutated mRNA is detected. For the homozygous $TP53^{LSL*/LSL*}$ no *TP53* mRNA was detectable; after Cre-activation the mutated mRNA is detected and as expected no trace of wildtype mRNA fragments. (RT-PCR by Peggy Müller)

Three dimensional growth

A standard test for transformed cell phenotypes is the soft agar assay for three dimensional growth. Cells from neo314, the heterozygous adBS29 and wildtype control cells were tested. For this 1 x 10^3 cells each were seeded in 0.4% noble agar. After 4 weeks, colonies with a diameter $>50\mu$m were counted (examplary colonies see figure 3.18). As expected, neither the wildtype bmMSCs nor the heterozygously targeted clone were able to form colonies. For neo314 it resulted in an average of 70 colonies (+/- 8). The homozygous Cre-activated p53 mutant of neo314 showed an even stronger transformation with an average of 100 formed colonies (+/- 20), summarised in figure 3.19. This demonstrates both the effect of p53 loss and a hint at the R167H mutation exerting the known gain of function and does not retain wildtype inhibition.

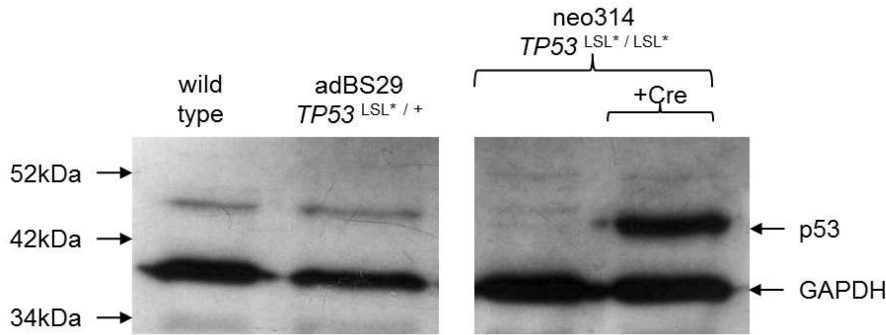

Figure 3.17.: Western blot of *TP53* targeted clones: the signal at 38kDa is the GAPDH protein detected as loading control; the signal at ~46kDa is the p53 protein; the latter is detectable weakly in wildtype and heterozygous clone adBS29, not at all for neo314 and strongly in the Cre-activated neo314. (Western blot by Daniela Fellner under the supervision of Anja Saalfrank)

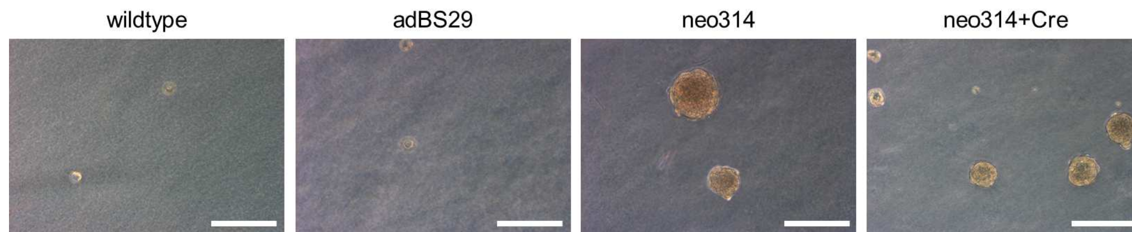

Figure 3.18.: Colonies grown in soft agar. Wildtype and heterozygous adBS29 cells are still embedded as singular cells. p53 deficient cells derived from neo314 grew as three dimensional colonies. White bars indicate 200μm

Chemotherapeutic resistance

It is known, that *TP53* deficiency can convey some forms of chemoresistance to cells. The intercalating drug doxorubicin for example triggers p53 dependent apoptosis, and is used for chemotherapy. The clone neo314 is completely p53 deficient and thus should be able to avoid cell death. Its Cre-activated mutant *TP53*R175H form is also expected to be doxorubicin resistant, as has been shown in human cells [301]. Wildtype cells, hetero- and homozygously targeted cell clones and their Cre-activated cell pools were challenged with this drug. After a 24h challenge the cells were kept under regular conditions for 14d, allowing potential colonies to outgrow. Wildtype and heterozygous targeted cells are killed completely by the treatment, also if the mutant allele is activated. As expected, only the p53 null clone neo314 and its Cre-activated form were able to form colonies, as can be seen in Figure 3.20. The *TP53*R167H expressing population might even show a slight increase in colony numbers, in accordance to the softagar assay results (see chapter 3.1.4).

In conclusion the acquired results were consistent with loss of p53 expression in alleles containing the LSL, and restoration of *TP53* after Cre recombination. As expected, a

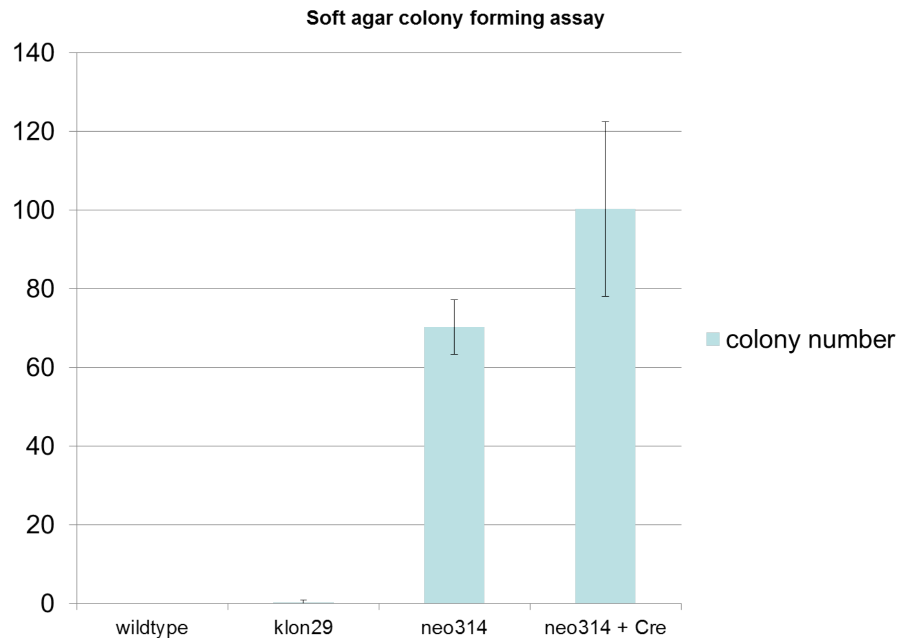

Figure 3.19.: Colonies of *TP53* targeted cell clones adBS29 and neo314 grown in soft agar. Wildtype cells serve as negative control. Only the *TP53* deficient, transformed cells of neo314 are able to form colonies with a diameter $>50\mu$m. The Cre-activated variant of neo314 shows even more prominent colonies, hinting at the functionality of the R167H mutation.

complete loss of *TP53* showed more prominent effects than a heterozygous loss. This *in vitro* data supports the validity of the knockout and inducible mutant p53 system. They are also indicators for a functional effect of p53 loss in homozygously bred animals.

3.1.5. Generation of *TP53* targeted pigs

TP53 gene targeted pigs can be derived from modified somatic cells via nuclear transfer and embryo transfer (NT/ET) into a foster mother. For this work, these experiments were conducted in collaboration with the group of Prof. Wolf (LMU).

Cells confirmed to be correctly gene targeted by the aforementioned methods were regarded as fit for nuclear transfer. Seven independently derived positive minipools and single cell clones from bmMSCs and adMSCs have been used for nuclear transfer and subsequent transfer of the *in vitro* reconstituted embryos into foster mothers. From our own experience and that of others we expected considerable variance of NT/ET success between cell isolates and even clones from the same isolate. Therefore, up to three different clones were mixed in one experiment. The clones were not tested individually for their suitability for NT/ET.

The initial 3 cell populations (BS22, BS51, neo314) that went to NT/ET resulted in two

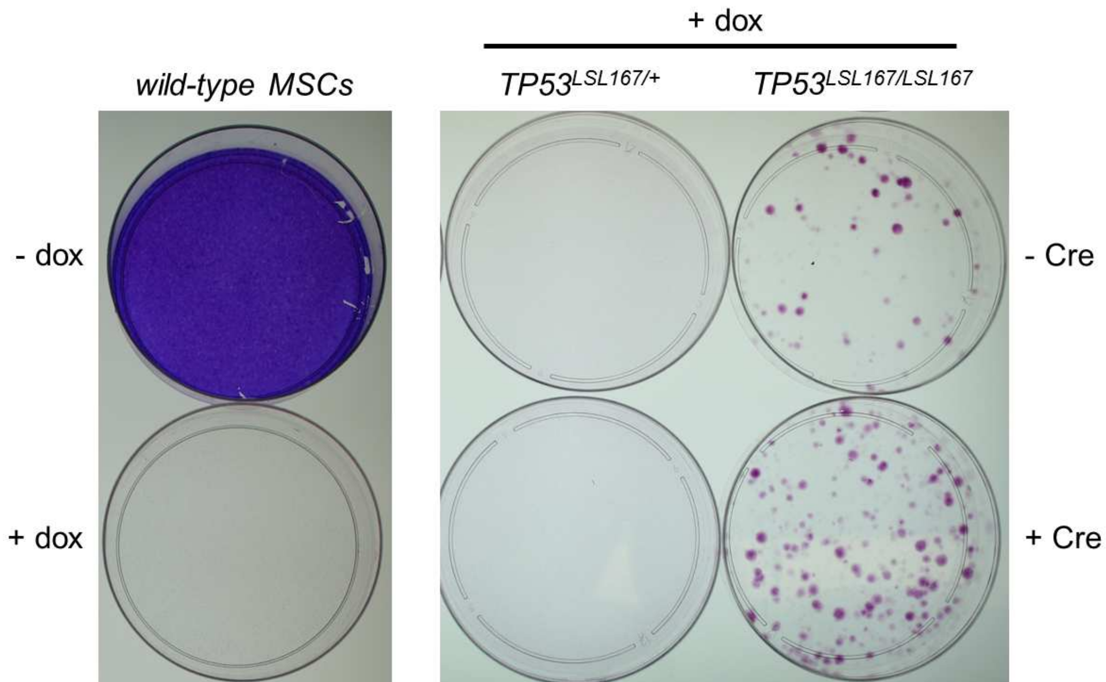

Figure 3.20.: Doxorubicin resistance assay of wildtype cells and *TP53* targeted cell clones; wild-type cells and the heterozygously targeted clone do not form colonies after doxorubicin treatment; the clone with homozygous loss of *TP53* forms colonies both with inactive and active R167H mutation. A slight tendency to more colonies is visible in the homozygously activated neo314 cells in the bottom right.

stillborn piglets. The stillborn piglets were derived from the homozygous *TP53* deficient cells of neo314. An early, untransformed passage of these cells was used for SCNT. These cells are originally derived from the 090210 Landrace-Pietrain hybrid isolation. These piglets showed a normal phenotype with a birth weight of 1.2 and 1.7 kg respectively. They did however exhibit macroglossia, which probably caused death by asphyxia at birth. This problem is not uncommon in nuclear transfer experiments and considered to be of epigenetic origin [115, 248]. Different NT/ET experiments of other projects with bmMSCs also showed this abnormality and in general a low rate of live birth and a high infant mortality. Therefore, the NT experiments with these isolates were discontinued.

To circumvent this problem, the cell type was exchanged. The follow up experiments were all conducted in adipose derived adMSCs, as KDNF did not generate positive events. Four adMSC derived, TP53 targeted cell populations (adBS27, adBS29, adBS33 and adBS310) were used for SCNT into 11 foster mothers. Table 3.2 (next page) summarises the cell clones used for nuclear transfer, the number of embryo transfer recipients, pregnancies obtained and piglets born.

Cell clone	ET recipients	Pregnancies	Born piglets
BS22			0
BS51	1	1	0
neo314			2
adBS27 adBS310	4	2	2
adBS29 adBS33	7	5	15

Table 3.2.: Summary of NT/ET experiments: clones are grouped in joint brackets according to their combined use in NT/ET

In total two stillborn and 15 liveborn founder piglets (G0) were obtained, that are confirmed as *TP53* gene targeted by PCR and some reconfirmed by Southern blot (see figure 3.14). The liveborn piglets are all derived from the pure Landrace adMSC isolate adMSC110101. The clones of origin of two p53 targeted piglets carry only the $TP53^{LSL}$ allele and lack the p53-R167H mutation. One of these piglets died at 58 days of age to a sepsis and meningitis. The other piglet has reached sexual maturity. No visible abnormal phenotype was observed in the boar as of today. In total, this boar was used to inseminate six sows, which resulted in 3 pregnancies and 19 liveborn offspring (F1) piglets of both genders with the $TP53^{LSL}$ allele.

All other *TP53* targeted pigs born carry both the LSL and the mutation and continue to grow and thrive.

These results are promising for future experiments with *TP53* gene targeted pigs: Firstly, the functionality of the *TP53* knockout was indicated *in vitro*. Secondly, fully *TP53* deficient pigs developed to birth comparable to mice [58] the only problem occurring due to the cell background. This supports the possibility of homozygous breeding because thirdly, the animals derived from adMSCs undergo normal fetal development, producing healthy piglets, which have now reached sexual maturity and produced offspring without apparent side effects of SCNT.

3.2. Indicator model

One of the cornerstones of the Cre-reversible *TP53* gene targeting was the possibility to activate the dominant negative gain of function mutation R167H, or to reactivate the wildtype allele with timing and tissue specificity. This necessitates ways to apply the Cre-recombinase *in vivo* into the desired tissue. Furthermore, detecting Cre-activity *in vivo* can be difficult.

3.2.1. Generating a Cre-reporter construct

To overcome these problems a double Cre-reporter construct was generated. It consists of a CAGGS promoter driving a loxP flanked beta-geo gene (a lacZ and neomycine resistance fusion gene) and quadruple polyA signal (schematic overview shown in figure 3.21). Downstream of the floxed cassette follows a mCherry reporter gene with its own polyA signal. This construct is referred to as CAGGS-geo-Cherry (CGC). The quadruple polyA of the beta geo gene was included to prevent a leaky readthrough into the mCherry RNA. In the case of Cre-activity the beta geo is excised and the fluorescent marker activated. Cloning details can be found in chapter A.1.4.

This construct now allows the visual identification of cells which underwent Cre-recombination by mCherry fluorescence. At the same time it allows for a counter-staining of unrecombined cells to ensure initial ubiquitous expression.

This can be extended to *in vivo* studies in transgenic animals. This would allow promoter studies as well as Cre-application studies.

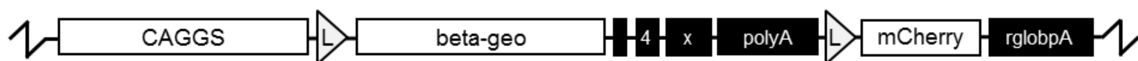

Figure 3.21.: Schematic overview of the CGC dual-reporter construct; the ubiquitous CAGGS promoter drives the expression of a floxed beta-geo fusion gene with quadruple polyA signals: SV40 polyA, BGH polyA, CMV polyA, *TP53* polyA; the 3' mCherry sequence and rabbit globin polyA (rglobpA) are only expressed after Cre-mediated excision of the floxed cassette.

3.2.2. Analysis of the Cre-reporter construct

Generating transgenic cell clones and pools

A cell pool carrying random integrations of the reporter construct was generated for initial testing. Porcine bmMSCs were tranfected with the dual-reporter constructs and selected for 14d. The functionality of the reporter cassette by lacZ staining and Cre-mediated mCherry activation was confirmed *in vitro* in the following ways: The cells were either

transfected with Cre-expression constructs with a phosphoglycerate kinase promoter (pgk-Cre) or treated with 5μM Cre-protein for 8h to excise the beta-geo cassette (Cornelia Brönner under the supervision of Anja Saalfrank). Figure 3.22 shows the results of the lacZ stain and fluorescence microscopy of the *in vitro* tests. These pools of selected cells were used for nuclear and embryo transfer to check for ubiquity of expression in fetuses.

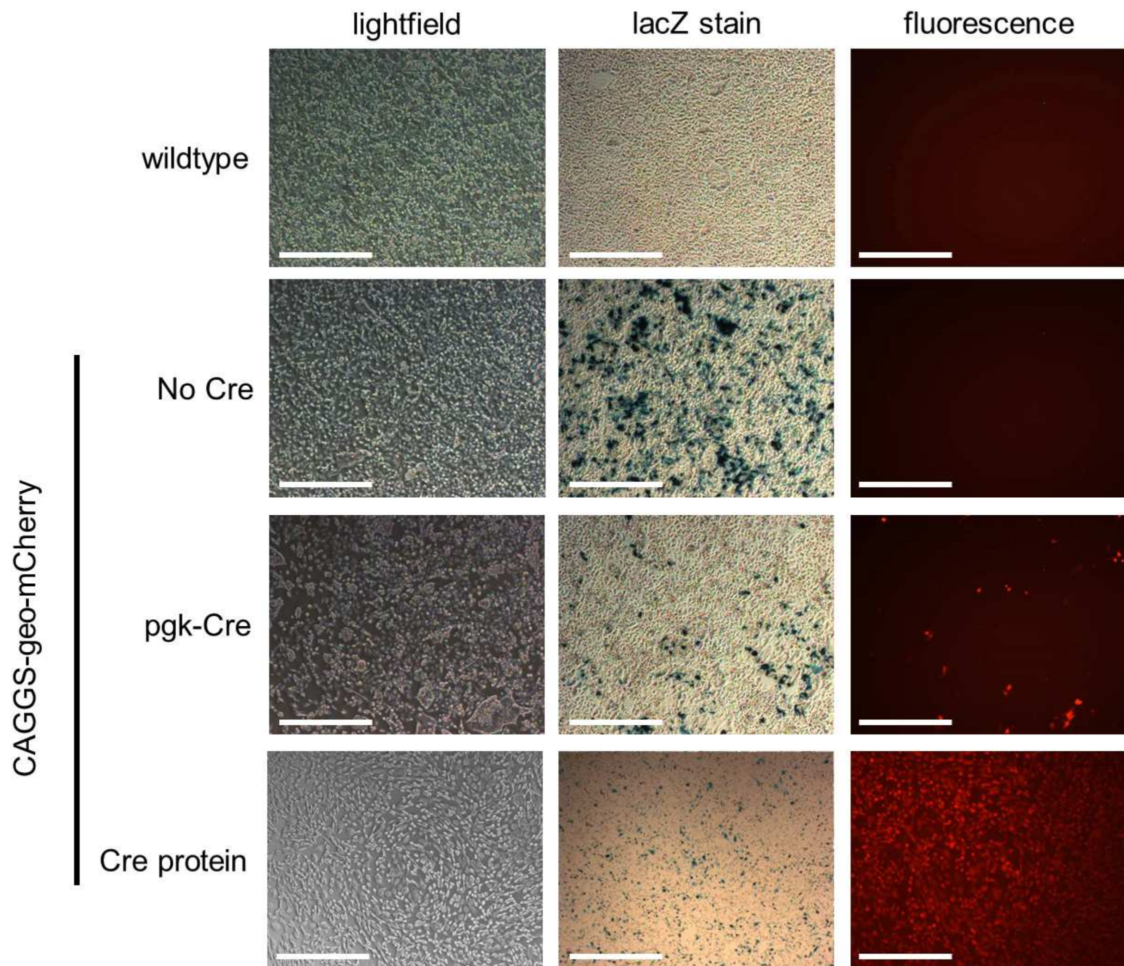

Figure 3.22.: lacZ stain and Cre-mediated fluorescence activation of reporter constructs: cells containing the CAGGS-geo-Cherry constructs are stainable for lacZ and after transient expression of Cre-recombinase show activated fluorescense; the lacZ staining is lost apart from some residual cells. Wildtype cells are used as negative control. The transfection of the Cre-expression plasmid pgk-Cre is less efficient than the direct application of the Cre-protein. White bars indicate 200μm. (Protein transduction images by Cornelia Brönner)

Generation and analysis of transgenic fetuses

The pregnancy was aborted at day 31 and four fetuses dissected from the uterus. Porcine fetal fibroblasts (poFF) were isolated from the front or hind legs and the remainder of the fetuses were dissected. The brain, lung, liver, kidney, stomach, heart and rest of the body were separately stained for lacZ. Similarly treated wildtype fetuses, aborted on day 28, served as negative controls.

Against expectations the endogenous beta-galactosidase activity in wildtype tissue was comparable to the transgenics. The poFFs derived from transgenic fetuses also showed no G418 resistance, lacZ staining and Cre-activatability. In contrast, a PCR analysis (primers LacZ intern for and neo intern rev) showed the presence of the beta-geo cassette (see figure 3.23). This indicates silencing of the integrated constructs during culture. Figure 3.24 on the next page shows the stained organs in comparison.

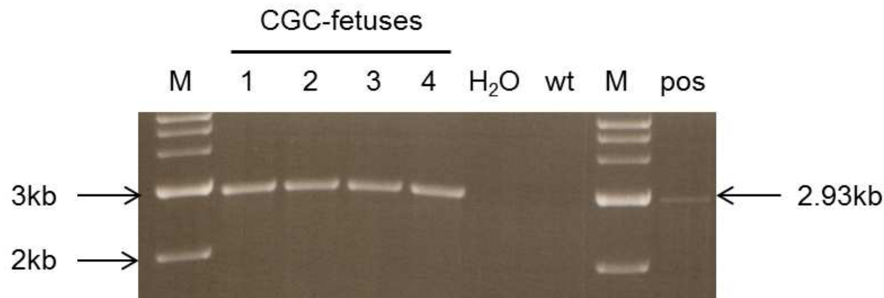

Figure 3.23.: A diagnostic PCR amplifying a 2.93kb fragment of the lacZ gene into the neo-resistance, which is present in the reporter construct but not in the wildtype genome (wt). It only is amplified from transgenic fetus DNA (1-4) and from the positive control – the reporter plasmid (pos); as expected the wildtype DNA (wt) does not produce an amplificate; this result is consistent with the silencing of the randomly integrated expression cassette in the transgenic fetuses. M = DNA length marker (PCR by Margret Bahnweg)

This is a known problem of integrated transgenes, especially with some extent of bacterial sequence like the lacZ. These sequences are prone to get epigenetically silenced in eukaryotic cells. This can potentially be circumvented by placing the expression cassette into more open chromatin [46, 195]. Furthermore, the actual activity of the cells used in nuclear transfer cannot be predicted with their origin in a cell pool.

Therefore, in a follow up experiment single cell clones were generated in bmMSCs and adMSCs and their individual activity screened. The adMSC derived clones with high expression of the reporter genes and functional Cre-mediated activation by Cre-expression plasmids were chosen for nuclear and embryo transfer. These traits were subjectively quantified by microscopy, and three clones used for a secondary nuclear and embryo transfer experiment. Again, the pregnancy was aborted and fibroblasts of the resulting four fetuses

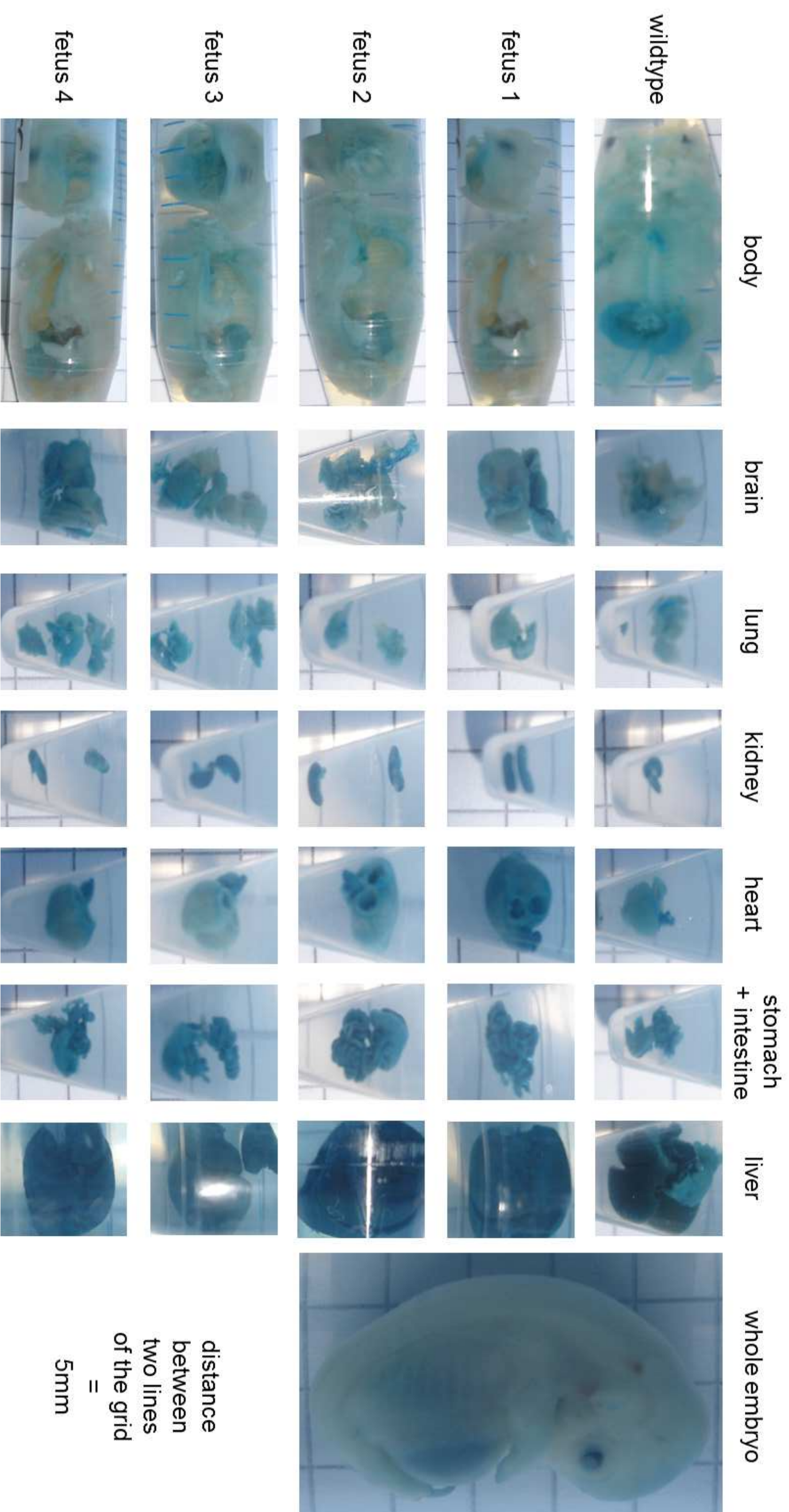

Figure 3.24.: Fixated and beta-gal stained organs of a wildtype fetus in comparison to four transgenic fetuses (1-4); all tissues show a slight blue lacZ stain, with no difference between wildtype and transgenics. The fetus on the right is an exemplary whole-mount stain of the 2.5cm long fetus.

analysed as before. All isolated poFFs now were positive in a lacZ staining and mCherry was activatable by Cre-transduction (5μM 4h) in all but fetus #2 (Tatiana Flisikowska). The latter showed a very high cell mortality after Cre-treatment. This was not observed in any of the initial clones but the difference in Cre-activation as well as cell type could account for this change.

Still a variation in lacZ stain was detectable in some poFFs. This could indicate a cell cycle dependent expression of the constructs even in clonal cells. A possible solution to this issue is currently getting addressed by Shun Li. He is targeting the reporter cassette into the putative porcine Rosa26 locus, known to be ubiquitously expressed and open for exogenous modification [124].

The dual-reporter system has also been used in one SCNT experiment to generate transgenic pigs through nuclear and embryo transfer. Recently, one live reporter piglet was born, confirmed by PCR to be transgenic and is in good health at the point of writing.

3.3. Tissue specific expression constructs

3.3.1. Tissue specific expression of Cre-recombinase

With the success of the conditional gene targeting and the dual reporter system, follow up experiments were designed. The conditional gene targeting of *TP53* done in this work necessitates a directed application of Cre-recombinase in order to activate the latent oncogenic mutation. The dual-reporter system facilitates the development of such techniques.

Cre-expression constructs were cloned by Vera Zywitza in order to provide a way of specific recombinase application. The Cre-recombinase was isolated by PCR (primers Cre +XbaI for and Cre +BglII rev) fused to a fluorescence marker gene (Cherry or EGFP) via a self cleaving T2A peptide, to further facilitate the evaluation of the Cre effectiveness. This coexpression system allows to visually confirm the presence of the protein and has been used for the generation of a multi-transgenic pig [55, 71].

In detail, the Cre-recombinase coding sequence without its stop codon was cloned upstream of a T2A peptide sequence with the EGFP and Cherry sequence located downstream. This Cre-T2A-EGFP cassette was cloned under the control of an ubiquitous CAGGS promoter and the epithelial keratin 19 (K19) promoter. The first can be used for *in vivo* transfections or simply for tissue culture work. For example, these constructs were used with their fluorescense counterparts on the dual-reporter constructs to establish the functionality. The latter was designed to lay the groundwork for a transgenic pig with Cre expression specifically in the gastrointestinal tract (see [228]).

3.3.2. Tissue specific expression of inflammatory factors

The development of gastric cancer often originates in a prolonged inflammation of the gastric mucosa. Oshima et al. successfully mimicked this process in mice by the directed expression of inflammatory as well as proliferatory genes in the gastric wall [228]. They expressed the microsomal prostaglandin E synthase (mPGES), cyclooxygenase 2 (COX2) and Wnt1 under the control of the epithelial keratin 19 (K19) promoter. These animals rapidly developed gastric cancer with similar phenotype to the human disease. This opens a second approach to generate a porcine cancer model for gastric cancer, next to modification of oncogenes or tumour suppressors.

Generating expression constructs

The cDNA of porcine mPGES and COX2 and the genomic sequence of the porcine Wnt1 gene were isolated. Subsequently they were subcloned under the control of the the human K19 promoter, followed by a rabbit globin polyA signal (details see chapter A.1.5).

Different promoter lengths were tested *in vitro* for activity and specificity by Thomas Briehl as part of his Masters thesis. The full length 3.7kb promoter was reduced in size by AccI and SacI restriction digests. These promoters were subcloned in front of Cre-T2A-EGFP/Cherry. Constructs were transfected into cells derived from different tissues: bone marrow, kidney, pancreatic cancer (Panc1) and gastric cancer (KatoIII and AGS).

The 2.1kb 3' promoter fragment showed the highest activity in gastric cell lines with the least activity in the other tissues tested. Therefore, the final expression constructs were cloned with this promoter length to drive the transgene expression.

Generating transgenic clones

The COX2, mPTGEs1 and Wnt1 expression constructs were linearised by a restriction digest while at the same time eliminating the bacterial vector backbone. The three linearised fragments were co-transfected with a G418 resistance expression vector, a neomycin gene driven by the ubiquitous phospho-glycerine kinase (PGK) promoter. Single cell clones were generated from bmMSCs and adMSCs. The clones were screened by PCR for the presence of the three constructs and one triple transgenic clone of adMSCs identified. This clone was cryopreserved at early passage and is ready for nuclear and embryo transfer.

4. Discussion

4.1. The pig as a model system

Medical progress in recent years has accelerated significantly with the rise of molecular biology and its revolutionary treatment options from monoclonal antibodies to somatic gene therapy. Even diseases as diverse as cancer nowadays are curable or can at least be reduced to a chronic state. However, in most cases this requires an established method of early diagnosis. This is only the case for a select few cancer types. Cancer is expected to turn into an even more severe health risk, with the increase in life expectancy. Modern molecular biology is now providing markers for early diagnosis that will have to be transferred into the clinic. This translation takes place with the help of model animals mimicking the human disease, notably rodents. Information drawn from these experiments have provided much insight into cancer biology and promoted therapeutic developments [5].

However, not all data is applicable for human treatment and small animals are simply physically unfit for the development of some therapies. Especially diagnostic endoscopy for early detection and surgical procedures demand an animal model more similar to human physiology. Pigs have begun to bridge this gap for medical applications. They are more similar to humans physiologically and ways to produce genetically modified and defined porcine models are emerging. It will still take a while to generate the multitude of mouse models and systems now available but initial experiments have generated valuable assets.

Currently wildtype pigs are used for surgical training, injury research and drug testing [87, 114, 234, 282]. Pig models based on genome modifications are now available in the fields of xenotransplantation [143], diabetes [257], cystic fibrosis research [138] and gastrointestinal cancer [73]. The field of cancer research was opened up by transplantation of transgenic cancerous cells into pigs where they indeed showed tumorous growth [1]. These experiments however are far from generating a defined model for human cancers. First genetically defined models have emerged by AAV assisted gene targeting of breast cancer type 1 susceptibility protein (BRCA1) and adenomatous polyposis coli gene (APC). The former should serve as a model breast cancer, however the generated animals did not survive longer than 18d [185]. The APC mutant animals are viable, and show the phenotype of familial adenomatous polyposis coli (FAP) in the colon [73].

4.2. Gene targeting of *TP53*

4.2.1. *TP53* as a target gene

A model with a mutated tumour suppressor gene would provide a platform suitable for a wider range of cancer research. The *TP53* gene is one of the central pillars of tumour suppression and its pathway mutated in the majority of human cancers. For example, a mutation is commonly found during pancreatic oncogenesis [109, 210, 238] or gastrointestinal cancers [252]. It is also one of the underlying defects in the human Li-Fraumeni disease, which is characterised by a heritable early onset of cancer. Consequently, it has been the center of extensive research and a well known candidate for genetically modified rodent animal models of cancer [58, 221, 335] (reviewed in [59]).

It is therefore logical when generating porcine cancer models, that one of the main target genes has to be *TP53*. Common defects of the *TP53* gene itself are allele losses or basepair mutations leading to amino acid changes. The latter frequently occur in so called hot spot locations predominantly in the DNA-binding domain [113, 314]. These mutations often not only inhibit wildtype function but also act as gain of function mutations. This has even prompted research to repair hot-spot mutants with zinc finger nuclease mediated targeted double strand breaks [106].

This work was also part of a larger research effort to generate a gastric cancer model in conjunction with pigs carrying an adenomatous polyposis coli (APC) gene mutation [73]. These two mutations should be combined by breeding to enhance carcinogenesis [258, 274, 316].

One of these is the mutation R175H in exon 5 (corresponds to R167H in pigs) leading to an arginine to histidine amino acid change. It is frequent in human cancers and correlates to a severe phenotype [210].

This hotspot mutation was chosen to generate a porcine model with high cancer penetrance and severe cancer phenotypes. It is known to promote metastasis [61], tumour vascularisation [134] and chemoresistance against the intercalating agent doxorubicin [301]. It has also been constructed into several mouse models [177, 221] (reviewed in [10]) and shown its effectiveness in conjunction with other oncogenic mutations e.g. K-ras [109, 238]. It is not subject to MDM2 independent degradation with its higher affinity to NADPH quinone oxidoreductase 1, that stabilises p53. However, it is still subject to MDM2 and E3 ligase mediated ubiquitinylation and degradation [9]. This leads to increased mutant protein levels, which in turn mitigates wildtype p53 function. It is also interesting to note that it is one of the main mutations promoting metastasis in pancreatic adenocarcinoma (PDAC) [210].

The technology of Cre-mediated DNA recombination allows for a combination of both the

gene knockout and point mutation. A Cre-excisable transcription stop cassette upstream of the R167H mutation acts as a gene knockout while turning the point mutation into a latent activatable variant. This has been used for comparable *TP53* gene targeting in the murine model [221].

The use of the Cre-recombinase for genome modification is widespread. It has been applied both for activating and inactivating mutations [128, 309] or in order to introduce site directed insertions [93]. The application techniques are also quite versatile, ranging from DNA-based expression to viral applications or the use of the actual protein [14, 241, 328, 347].

4.2.2. Design of the gene targeting approach

The targeting construct

The targeting construct was initially designed with a 3kb short arm and a 11kb long arm homology for homologous recombination. This is over 2 kb longer than the minimum length described in literature for murine ESCs [294, 54]. This excess sequence allowed the shortening of the 3kb fragment to 1.2kb after intrinsic promoter activity was identified.

The targeting vector was constructed from Duroc pig sequence available on a bacterial artificial chromosome (BAC) even though the cells in this work were isolated from Landrace X Pietrain or pure Landrace breed. The use of isogenic DNA for the homologous sequences can be advantageous for gene targeting [54, 291]. For pigs it does not appear a necessity though [50, 251]. This is also confirmed by the successful results of this work. The sequence could be isolated from the BAC clone by recombineering [48]. Recombineering is usually used to introduce modifications into a BAC but can also subclone fragments out of a BAC into a cloning vector, as was the case in this work.

Additional features were added into the vector backbone to enhance the gene targeting efficiency. Firstly, a CAGGS-mCherry fragment for the ubiquitous expression of the red fluorescent protein (compare [63]). This fluorescence can only be detected in transfected and selected cell clones, if the vector backbone integrated into the genome either by random integration or incomplete homologous recombination. Therefore, these clones were excluded from further screening procedures and the workload could be reduced. More importantly, it indicates whether identified clones are pure, or contain random integrants. This secures transgenic offspring through SCNT.

Secondly, a nuclear localisation sequence (NLS) was added, as it can improve the nuclear DNA uptake and thereby enhance the frequency of HR [6, 23, 53, 206].

Another possibility to enhance efficiency would have been the inclusion of a positive/negative selection (PNS), which is commonly used in mice gene targeting experiments.

For example by the counter selection against a diphtheria toxin A-chain gene in the vector backbone [196]. However, another addition to the already 21kb large vector was deemed unnecessary with the already successful PNS from the visual marker gene. Moreover, another selecting agent enhances the stress on the primary cells in culture, which is not the case with visual selection.

The floxed transcription stop cassette (LSL)

The transcription stop cassette design was comparable to the design used in other studies [108, 221, 304]. It was flanked by loxP sites for Cre-recombinase mediated excision. It was also designed to contain a splice acceptor 5' of a promoterless resistance gene to allow antibiotic selection and a simultaneous enrichment for positive gene targeting events by promoter trap. This common technique is known to be effective in genes that are actively expressed in a cells and therefore an obvious choice for targeting the "housekeeping" gene *TP53* (see review [78]). It is however not that common for porcine gene targeting. Both CFTR and BRCA1 gene targeting used phosphoglycerate kinase promoters driving the neo-resistance [185, 260], or cytomegalovirus promoter [200]. Another alternative is the use of an internal ribosomal binding site like in the APC targeting model [73] and alpha-1,3-galactosyltransferase knockout [153].

The stop of transcription within the LSL was ensured by three different subsequent polyA signals, as residual expression of the mutated allele had to be avoided in a defined tumour model. This is a novelty, compared to gene targeting vectors. Most only rely on the endogenous [200] or a singular polyA [73]. The functionality of the LSL was proven in *in vitro* assays for splicing and tightness. It was also confirmed by the Cre mediated reactivation of p53 expression *in vitro*.

Taken together these results comfirm the LSL to be suitable for a conditional gene knockout *in vivo*.

The cell type used for gene targeting

The choice of the cell type used for the experiments was made according to the following criteria.

First, the cells plasticity is an advantageous trait in cells used for homologous recombination (HR) and SCNT. High HR frequencies can be achieved in pluripotent embryonic stem cells (ESC) compared to somatic cells [79, 173, 293], as is reported in the literature. In ESCs shorter homology arms of 1-3kb can be used, in [54, 294]. Even genes with lower base expression level can be targeted [111]. Induced pluripotent stem cells (iPSC) have

been suggested to show similar susceptibility to reprogramming, and shown comparable efficiencies to ESCs [111].

Neither of these cell types are available for pigs as a stable and defined cell source. The reported iPSC are not yet fully independent of exogenous factors [67, 69] Therefore, in this work somatic cells were used. Mesenchymal stem cells from fat (adMSC) and bone marrow (bmMSC) were chosen, because they exhibit residual ability to differentiate into chondrogenic adipogenic and osteogenic cell linage. This has been shown in differentiation assays in the literature [8, 11, 25, 90, 189, 190] and in our laboratory. They have also been successfully used for genomic modifications (reviewed in [24]). The kidney fibroblasts were chosen because they also were utilised for the generation of transgenic pigs [139]. Also, the three cell types provide sufficient proliferative capacities to provide material for screening purposes. It is important to note, that a loss of p53 speeds up differentiation processes of murine MSCs, however it also promotes spontaneous transformation which could pose problems for SCNT [7].

The correct selection intensity for promoter trap gene targeting is also an important parameter [98, 266]. It was determined experimentally through selection tests on untransfected cells and adjusted during the actual targeting experiments during this work. The intensity was set so the untransfected controls are eliminated within the time the resistant clones need to proliferate to a manageable colony size of >100 cells. In this way, the untransfected cells still support the growth of the colonies and do not overflow the culture medium with cell death signalling molecules.

4.2.3. Efficiency of *TP53* gene targeting

The gene targeting efficiencies varied markedly between transfections and cell types used in this work between 0% and 20%. This was expected, as differences in efficiency between different cell isolations are described in the literature [260]. Here, the efficiencies between fetal fibroblast isolations of the same litter ranged from 0.07% to 10.93%. Our observed values of 16.6% (bmMSCs) and 12.6% (adMSCs) respectively varied within the range reported for porcine cells. Ramsoondar et al. report the disruption of the porcine immunoglobulin kappa light chain locus in fetal fibroblasts with overall 14 PCR positive clones out of 799 (1.7%) [250]. The knockout of a heavy chain locus was also ineffective with 0.63% targeted clones [200]. In contrast the highly efficient AAV-mediated targeting of CFTR reached up to 20% [260] and the BRCA1 locus a mean of 34.32% [185]. The relatively high targeting efficiency in this work can be explained through the more effective promoter trap of TP53, as it is ubiquitously expressed in contrast to the immune related genes. Another advan-

tage is the use of the visual negative selection with the CAGGS-mCherry cassette in the backbone as well as the nucleus localisation sequence.

The reasons for the overall variation of somatic gene targeting efficiency can be found in various aspects of the cellular background. There is a known influence of the cell cycle on transfection efficiencies [29, 209] and HR occurs in S late G2 phase [288]. This indicates an advantage in defined cell cycle control to achieve more efficient gene targeting. Cell cycle synchronisation has been shown to be effective in this context [105, 206].

Another reason could be the differing chromatin structure at the p53 locus between cell isolations. It is known that chromatin position effects can inhibit expression of integrated transgenes. Heterochromatin can form around genes with lower expression and this can silence the integrated construct. Also the homologous recombination between targeting construct and genome might be impaired (reviewed in [254]). It is however very unlikely, that the *TP53* locus with its role in cell cycle regulation is condensed in these primary cell isolates.

Yet, p53 expression levels could still vary in cells from different tissues of origin and between isolates. Lower expression of the locus would mitigate the efficiency of the promoter trap, and lead to a reduced antibiotic resistance and loss of targeted cell populations.

Furthermore, the method of single cell cloning can distort the gene targeting efficiencies. Some cell isolations ceased proliferating after subcloning. For example, the kidney derived fibroblasts rarely provided enough material for screening. The bmMSCs and adMSCs with their higher proliferatory potential were more suited for single cell cloning. In this work, the targeting of the TP53 locus in bmMSCs was slightly more efficient than in adMSCs (16.6% vs. 12.6%). However, for SCNT the bmMSCs did not show the viability of the adMSC isolation. This also distorts or masks the factual efficiency of *TP53* targeting.

4.2.4. Functionality of *TP53* knockout

The functionality of a porcine *TP53* knockout is an important cornerstone for the animal model to bring the cells closer to an oncogenic phenotype. While a mutation in *TP53* on its own does not directly cause cancer, it facilitates the acquisition of the oncogenic second hit. The first indications of this effect were shown in the course of this work. The behaviour of the homozygously targeted cell clone *in vitro* showed enhanced proliferation and chemoresistance opposing to heterozygous targeted and wild-type cells (compare [259, 263] reviewed in [31]). It also was able to promote anchorage-independent growth, comparable to transformed cancer cells and formed colonies in soft agar culture [2, 45, 281]. These are the first hallmarks of an oncogenic transformation facilitated by the loss of *TP53*, hinting at the validity of the system.

Indirect confirmation for this model are the already existing functional mouse models [58, 109, 335], even though gene functions can not be considered completely conserved across species.

4.2.5. Generation of *TP53* modified pigs by SCNT

With this *in vitro* knowledge, live pigs that carry a conditional *TP53* knockout on one allele were generated through somatic cell nuclear transfer (SCNT).

For SCNT the efficiencies do not differ as much in the literature, but still favor undifferentiated cells. For example, terminally differentiated neurons are unable to support fetal development in contrast to ESCs (reviewed in [218]). Therefore, gene targeting experiments were conducted in cell isolations able to still differentiate into cell types of the three germ layers (adMSC / bmMSC) or known to work in SCNT experiments (KDNF) [139] in this work.

Selection intensity and cloning procedure play an even more important role with the prospective SCNT of the cells. The use of impure cell pools often times results in wildtype SCNT animals [50, 197, 343].

Overall, the efficiency of SCNT derived and implanted embryos to develop into a live animal is low (reviewed in [218, 325]). Apparent bottlenecks are the sufficient reprogramming of the somatic nucleus and the ability of the fused and activated oocyte with mixed mitochondrial DNA and potentially shortened telomeres to develop into a viable organism. Therefore, widespread research on improving SCNT is found in the literature: several additives have been identified like valproic acid [207], dibutyryl cyclic adenosine monophosphate (dbcAMP) [280], latrunculin [107], anthocyanin [341], Cytochalasin B and trichostatin A [19], that all assist in the *in vitro* development and SCNT success. Also, the fusion timing [342] and osmolarity changes in the medium [208] influence the efficiency.

The success of the SCNT experiments in this work varied widely between cell isolations used. The adMSC isolation 110111 was the only isolation that reproducibly gave rise to healthy piglets.

The SCNT with the clones adBS27 and adBS310 from the adMSCn110111 isolation lead to the birth of two *TP53*^{LSL} targeted piglets without the R167H mutation. This indicates a mixed origin of the clones with cells carrying the targeted LSL integration but lacking the mutation and a subpopulation of random integrants carrying the mutation. This lead to screenings showing both the presence of the R167H mutation and correct LSL integration.

One heterozygous *TP53*^{LSL} piglet died to a meningitis and sepsis. It is important to note, that a cerebromeningitis in SCNT piglets has also been reported elsewhere [233]. This should warrant close observation in follow up experiments.

The heterozygous *TP53*^{LSL} phenotype offers interesting research possibilities as a potential tumour model allowing rescue by re-activation of wildtype p53. This has been shown to improve tumour behaviour, and lead to extensive research for example in the field of small molecules [28, 267].

A pig carrying only a heterozygous knockout of the *TP53* gene is not expected to show a severe cancer promoting phenotype yet. Heterozygous animals however are expected to have an increased cancer risk. In these animals, like in human Li-Fraumeni patients, only one additional mutation in the intact *TP53* allele directly leads to a complete p53 loss. In *TP53* heterozygous rats an early onset of sarcomas has been observed, together with a loss of heterozygosity of the wild type allele [306]. In mice one remaining wild-type allele exerts protective influence [335]. In addition, a p53 dosage dependent effect is possible on directly transactivated genes. This has been shown to be the case in human cells [339].

Still, the pigs with heterozygous *TP53* mutation could ultimately be bred to homozygosity. Indicated by our nuclear transfer experiments, cells with a complete loss of p53 will still support development to birth. This forecloses the second hit in regular cancer development and delivers a more severe animal model. The p53 deficient piglets were born with normal weight and appearance apart from macroglossia (enlarged tongue) that caused asphyxiation at birth. This resembles the human Beckwith Wiedemann syndrome, an overgrowth disorder. This particular pathological phenotype has also been noted by other nuclear transfer researchers and is understood to be a consequence of incomplete nuclear remodeling across mammalian species [115, 246].

It is known, that epigenetics play an important role for the reprogramming of the somatic cell in SCNT, yet the exact mechanisms are not fully understood [346]. The differences in epigenetics and reprogramming between wildtype and SCNT have been subject of research in bovine [215] and porcine background [321]. It has also been found, that the H3 acetylation levels in early embryo stages affect SCNT success in minipigs [336].

The gene targeting of *TP53* can also have an effect on the epigenetics of the cells. Both wildtype and mutant p53 does exert epigenetic influence on target promoters by activating histone acetylation [313]. Still, a connection between the macroglossia to the *TP53* mutation is unlikely: macroglossia also occurred in animals derived from the same MSC preparation in other projects and was not observed in SCNT animals derived from somatic cells of a different genomic origin.

In conclusion, the results in this work indicate that like in mice [335], p53 deficient pigs will be viable. It can be assumed, that the cancer development in these founder animals will be similar to the human disease. In the latter the loss of key tumour suppressor genes is also a common early event [240]. Pigs are close to humans physiologically immunologically and on a genomic level [21, 201]. Pigs can be kept on a diet similar to humans,

thereby setting a comparable background in gastrointestinal diseases [94] or diabetes research [149, 156]. Furthermore surveillance and treatment techniques like endoscopy or surgical instruments can directly be taken from human routines. In conclusion we postulate, that results generated in this porcine model are more easily and directly transferrable to the clinic.

The live homozygous animals derived from cells and breeding, carrying only the knockout and not the *TP53* R167H mutation could be used for research of tumour rescue by reactivation of wild-type p53 [40, 267, 309].

4.3. Dual reporter model

4.3.1. Design of the dual reporter cassette

Visual markers as means of developing proof of principle models are used extensively in molecular biology. In this work, it complements the conditional *TP53* gene targeting model. The tissue and timing specificity of the Cre-mediated R167H activation is important for the generation of a defined cancer model. Here visual markers can help to develop the means of specific Cre application and monitor the actual Cre activity.

A dual reporter approach was chosen comparable to the common mouse models [52, 213, 216]. In short, the construct consists of a CAGGS promoter controlling a floxed beta-geo fusion gene of lacZ and a neomycin resistance. A mCherry gene was positioned downstream and gets activated in case of Cre activity. LacZ expression can be visualised by the common beta-gal staining as a blue coloration of the cells. The mCherry gene expresses a fluorescent protein, that is visually detectable as a red glow at an emission wavelength of 610nm. A positive lacZ stain indicates no past or present Cre activity in direct contrast to detectable mCherry fluorescense.

Ideally, only one of the visual markers is detectable in a cell. The generation of the dual reporter cells however relies on the random integration of the construct. Therefore multiple copies can not be excluded completely. This can result in incomplete beta-geo excision and both a detectable lacZ and mCherry expression.

A chinese group reports the generation of porcine reporter poFFs and piglets, carrying a similar construct. [168, 169]. Their construct utilises an eukaryotic translation elongation factor 1 alpha 1 (EF1-alpha) promoter to drive a floxed neo-resistance gene with a singular polyA signal. EGFP is used as their activatable fluorescent reporter.

In contrast the construct used in this work employs a quadruple polyA signal, since the transcription termination in the floxed cassette is essential for its correct function. Initially a residual readthrough into the mCherry gene and thus visibly leaky expression

was detected in the earlier constructs with only a triple polyA signal. Secondly the Cre-activatable gene is visibly detectable even *in vivo*, comparable to mouse studies [216]. Also, the CAGGS promoter is a established ubiquitous promoter for example for miRNA [158] or fluorescent protein expression [84].

4.3.2. Functionality of the dual reporter cassette

In this work the constructs were first tested *in vitro* by transient transfections into porcine cells. Both the lacZ stain and Cre mediated mCherry activation were detectable. Fetuses from NT/ET of the reporter cells were also analysed, to get an indication of the functionality *in vivo*. In mice for example the ubiquitous lacZ expression can be seen in whole embryo stains [86].

The fetuses were isolated, dissected and analysed for lacZ staining. The analysis of wildtype fetuses showed a high endogenous activity of beta-galactosidase, comparable to the four fetuses derived from transgenic cells. Over the course of follow up experiments it became clear, that the transgene cassette was silenced. The cassette was still detectable by PCR but not by the lacZ stain or the the Cre-mediated mCherry activation. The cells also lacked G418 resistance. This was an unexpected result, since the initial cell pool showed acceptable lacZ stain and activation. But since a cell pool was used for nuclear transfer, the exact potential of the cells that generated the fetuses cannot be determined.

Single cell clones were used for the follow up experiment to avoid this initial variance. The fetuses analysed from this experiment and the *in vitro* characterisation of the isolated porcine fetal fibroblasts (poFF) showed complete functionality of the cassette as expected. This hints, that the initial problems were indeed part of the cell pool variance.

The remaining variance in lacZ expression however, is still not optimal for a live pig model with ubiquitous systemic expression. The reason for this probably lies in the still random positioning of the cassette in the genome. The cassette might be positioned in not constitutively open chromatin.

Silencing of exogenous constructs in cell culture is a known problem for example of a Tet ON/OFF system in pigs [148] or of a retroviral vector for iPS induction plasmid [116]. Also, the methylation of cytomegalovirus sequence has been observed in transgenic pigs [144]. A solution for this can be the directed positioning of the cassette into a suitable locus via gene targeting. A locus known for ubiquitous accessibility and stable expression is the Rosa26 locus in mice and human [344, 124]. Therefore as a follow up project by Shun Li the cassette is being targeted into the putative porcine Rosa26 locus, identified by a separate project in our department. The liveborn healthy piglet from SCNT can serve as an important founder and control for these experiments.

In general, animals carrying this transgene are of use in conjunction with the latent oncogenic $TP53^{R167H}$ mutation. They can serve to establish methods for tissue specific Cre application *in vivo*. For example, specialised endoscopes could directly detect the mCherry fluorescence in the gastrointestinal tract. Later stage autopsy of the animal followed by lacZ staining can indicate unwanted off target effects of the Cre delivery method. The beta-galactosidase shows a high stability in fixated tissue, therefore allowing these tests to be carried out with greater accuracy also over time.

In conclusion, these two animal models taken together synergise in the development of a tissue specific porcine cancer model.

4.3.3. Applicability of Cre recombinase *in vivo*

Another important aspect of the two aforementioned models is the *in vivo* recombination of the modified genomic loci. The porcine reporter model is effectively designed to establish the necessary application of Cre recombinase with the desireable specificity in a large animal. Recombinases can be used for site directed insertion of constructs [128], also in a porcine background (reviewed in [47]).

In mice the Cre recombinase is usually activated through in-crossing of e.g. a TET-on/off Cre background. The next generation can then be recombined with timing and tissue specificity by activation through tetracyclin (reviewed in [164]). The advantage of this is that both animals can be characterised thoroughly before mating. However, in pigs this approach is impractical. Most importantly, only a small number of pigs with trangenic Cre-expression are yet available for crossing [38]. Also pigs have a significantly longer generation interval, which would decrease the efficiency compared to mice.

Therefore, different approaches have to be used to fully utilise the potential of the models:

One approach can be *in vivo* transfections (compare [289]) or intravenous application. Cre expression constructs could be transfected into tissue. This would mean additional random mutations from the potentially integrated vectors, as well as potential collateral tissue damage. An advantage can be a cell type specific expression within a tissue if the expression plasmid contains a specific instead of a ubiquitous promoter. A more simplified version of this technology is just a *in vivo* injection of DNA solution into the tissue (compare [289]). This is accompanied by an ever lower efficiency of DNA uptake and therefore less expected Cre-activity.

A second kind of approach for Cre-application could be a virus based integration of Cre-expression constructs. This utilises the higher transduction efficiencies of viruses, both into dividing and non dividing cells. It also encompasses the advantages of the *in vivo* transfection of tissue specificity. Adeno-associated viruses are noteworthy in this context.

They couple cell type specificity through their serotype to a low copy DNA transfer per cell and are available for a range of tissues. They have been used to stably express genes in somatic cells [36, 333]. Their characteristics also made them an interesting asset in gene targeting experiments in somatic cells [105]. However, this method necessitates the design and construction of several tissue specific viruses, and has to cope with the lower basepair load available.

4.4. Transgene expression with gastrointestinal specificity

4.4.1. Assessing different promoters

A search of current literature was conducted, in order to identify promoters with gastric specificity, suitable for a porcine model with directed transgene expression. They should ideally meet several additional requirements: a sufficient level of expression for the transgene to be active, no expression in other tissues especially not during development. These restraints are especially true in case of Cre-expression. Too early and widespread Cre-expression would completely abolish the idea of tissue specificity. The transgene expression should also be reliable and not lost due to its own influence on the expression pattern.

The initial considered promoters were those of gastrin, carcinoembryonic antigen (CEA), Trefoil family factor (TFF), gastrokine 1 and 2, Pepsinogen A and C (PGA/PGC), gastric intrinsic factor (GIF) and the gastric H,K-ATPase beta-subunit (gATPase). Table 4.1 summarises the promoters.

Unfortunately, these promoters could not be put to use, as they did not meet the aformentioned requirements. Consequently, the idea of a strict gastric specific expression was discarded and the restrictions widened to cover the complete gastrointestinal system. In this context the promoter of cytokeratin 19 has been successfully used by Oshima et al. [228] and therefore was chosen in this work.

4.4.2. Overexpression of inflammatory factors

The transgenic model generated by Oshima in mice imitates the common trigger for gastrointestinal carcinogenesis - persistant inflammation [228]. The two overexpressed inflammatory factors are known to play a role in gastric carcinogenesis [76, 126] and the mice develop gastric cancers (GC) with high incidence with phenotypes resembling the human disease. Because of that, these mice offer a promising platform studying the early developmental stages of GC. However, the small size of the rodent model prevents the evaluation of

Gene	Expression pattern	Caveat	Source
Gastrin	G-Cells in gastric antrum	Also expression in embryonic pancreas	[88]
CEA	gastric mucosa & esophagus	CEA driven SV40 large T antigen gives rise to tumours also e.g. in lung, colon and bone	[295]
TFF	stomach & duodenum epithelia, pancreas, colon	also in sceletal muscle, in mice also in macrophages & lymphocytes	[151]
Gastrokine 1 and 2	native & metaplastic gastric epithelium / surface mucous cells	Low levels in placenta, uterus, liver, kidney, pancreas, adrenal & salivary glands	[220, 270]
PGA/PGC	PGA in pigs: specific to chief cells, PGC in stomach, esophagus, kidney	not yet used for transgene expression	
GIF	expression mostly restricted to parietal cells	In humans and rats also stomach margins and in salivary gland and pancreas	[117, 186]
gATPase	expression in parietal cells	parietal cells are lost early in gastric carcinogenesis	[77]

Table 4.1.: Gastric specific promoters

actual treatments designed for human patients like endoscopic resections. Another caveat is the differing dietary backgrounds of mouse and human, even though this is known to influence GC development (reviewed for example in [34]).

In the course of this work, the basis was laid to transfer the Oshima model onto pigs, complementing the p53 cancer model. This porcine model would specifically be suited for gastrointestinal cancer research, while the *TP53* mutations are common in a widespread array of cancers. It also opens up a second way of promoting carcinogenesis, should the *TP53* modification not yield satisfactory results. This triple transgenic approach with overexpression constructs could circumvent problems with inefficient gene targeting.

Design of the overexpression constructs

The constructs themselves followed the Oshima designs. The coding sequences of COX2 and mPTGES1 and the genomic Wnt1 locus were of porcine origin, while the cytokeratin 19 (KRT19) promoter was derived from human sequence. This was due to the testing procedures in human gastrointestinal cancer cell lines. The expression of the finished constructs was undetectable in the porcine primary cell culture, because non of them were of gastrointestinal/endothelial origin. Also, the three seperate constructs did not carry a selectable marker for antibiotic selection. Therefore, an additional fourth plasmid with a resistance gene was cotransfected.

Formation of the transgenic cell clones

Oshima et al utilised oocyte injections of expression constructs to form two transgenic mice - one with Wnt1 and one COX2 and mPTGES1 overexpression - and subsequent crossing of the mice. He also screened the singular or double transgenic mice for gastrointestinal abnormalities but only detected hyperplasia (Wnt1) [228] or more profound inflammation (COX2/mPTGES1) [126, 229] and no adenocarcinomas.

Therefore, to transfer these results into the porcine model all three factors were overexpressed. Even though with the porcine genomic background of relatively frequent development of gastritis, less extopic inflammatory factors might be needed. Regrettably, the oocyte injection technique in pigs is a lot less efficient than SCNT and therefore the constructs had to be introduced *in vitro* into primary cells. These cells were then screened by PCR for triple transgenics, as the stepwise selection for the three constructs would have taken too long for the established culture period of the primary cells. Especially, since these cells would still need to undergo complete nuclear reprogramming in SCNT.

However, this method of generating triple transgenics has several disadvantages. Firstly, not every transfected cell would incorporate all three constructs equally. This problem,

and the number of copies of the integrated plasmids were only adjusted beforehand by matching the plasmid copy numbers/concentrations prior to transfections.

Secondly, these constructs did not integrate into the genome in a directed way. The random integrations could potentially mitigate the cells potential for a successful SCNT. This could not be screened for initially, and neither could the expression of the transgenes be validated, due to the inactive KRT19 promoter. Only the presence of all three expression cassettes was confirmed by PCR.

Thirdly, the breeding of the triple transgenics will almost certainly lead to a divergence of the transgenes. The F1 generation might however show the initial phenotypes that Oshima et al. used for crossing. These might prove interesting to research potential differences in phenotype between mice and pigs.

In conclusion, the transfections proved to be successful and single cell clones could be selected. Among these was one clone with all three cassettes detectable by PCR. A SCNT with this subset could give rise to a founder generation of triple transgenic piglets with predisposition to gastrointestinal cancers. Oshima et al. found a very early and severe onset of these cancers in triple transgenic mice [228]. An intensive screening procedure should therefore be established for these piglets, as this phenotype probably also transfers over to pigs. Additionally, the directed expression of the constructs has to be confirmed in biopsies, preferably from diverse tissues.

Lastly the overexpression model should be put in context with the $TP53^{R167H}$/APC mutated models with regard to cancer development and similarity to human pathologies.

4.5. Outlook

Porcine cancer model The successful generation of a porcine model for cancerous diseases opens a wide field of new research. The *TP53* mutant founder animals now have to undergo regular screenings to allow the breeding of a initial population of the pigs and prevent premature complications from their heterozygous *TP53* loss. The F1 generation of animals would also be free from potentially harmful influences of the SCNT heritage.

The screenings could even serve as a first testrun of the directed research that will be conducted on these animals. Endoscopy or NOTES procedures, computer tomographies and even if need be the treatment of cancerous lesions with novel therapies.

In the further future the *TP53* model will be crossed into other genomic backgrounds like oncogenic mutants or Cre-models, to widen the applications. As mentioned before, this work is part of a larger research effort to generate gastrointestinal cancer models, especially through the combination of APC and *TP53* mutants. The APC mutant background is already established and with the *TP53* mutants reaching sexual maturity, breeding

experiments could commence soon. Moreover, the Cre-mediated *in vivo* activation of the latent oncogenic $TP53^{R167H}$ mutation can be established. While it will still take considerably longer to develop than in the common mouse models, a database of genetically modified pigs can already be imagined.

Alternative porcine cancer model The alternative cancer model based on the Oshima mouse model is not yet characterised. It can prove valuable to complement the tumour suppressor knockout as a comparison to tumour phenotypes and development. The stomach specificity however can still prove problematic as the keratin 19 promoter is widely expressed throughout gastrointestinal epithelia. In mouse models the inflammatory processes appeared either restricted to the stomach or to exert its strongest influence there. The porcine background with easy inflammatory processes in the stomach promotes the viability of the model.

Porcine reporter model The porcine reporter model will allow easy access to develop *in vivo* Cre-applications. The easy visual detection can be utilised to establish general *in vivo* protein application techniques. The primary use of this would be the targeted activation of the porcine cancer models. This might be usable for the administration of pharmaceuticals in protein form, given comparable stability and solubility to Cre-protein. The modified cells have already been used for SCNT, and viable animals will be probably be available within the upcoming months.

The role of the porcine disease model The role of pigs or large animal models in general will not replace but complement the rodent models. It can provide another perspective and serve as controls. This would strengthen the transfer of results from animal models into human medicine.

5. Bibliography

1. Adam, S., Rund, L., Kuzmuk, K., Zachary, J., Schook, L., and Counter, C. (2006). Genetic induction of tumorigenesis in Swine. *Oncogene*, 26(7):1038–1045.

2. Agrez, M. V., Kovach, J. S., and Lieber, M. M. (1982). Cell aggregates in the soft agar "human tumour stem-cell assay". *British journal of cancer*, 46(6):880–7.

3. Ajani, J. A., Barthel, J. S., Bekaii-Saab, T., Bentrem, D. J., D'Amico, T. A., Das, P., Denlinger, C., Fuchs, C. S., Gerdes, H., Hayman, J. A., Hazard, L., Hofstetter, W. L., Ilson, D. H., Keswani, R. N., Kleinberg, L. R., Korn, M., Meredith, K., Mulcahy, M. F., Orringer, M. B., Osarogiagbon, R. U., Posey, J. A., Sasson, A. R., Scott, W. J., Shibata, S., Strong, V. E. M., Washington, M. K., Willett, C., Wood, D. E., Wright, C. D., and Yang, G. (2010). Gastric cancer. *Journal of the National Comprehensive Cancer Network: JNCCN*, 8(4):378–409.

4. Anastassiadis, K., Rostovskaya, M., Lubitz, S., Weidlich, S., and Stewart, A. F. (2010). Precise conditional immortalization of mouse cells using tetracycline-regulated SV40 large T-antigen. *Genesis*, 48(4):220–32.

5. Anisimov, V. N., Ukraintseva, S. V., and Yashin, A. I. (2005). Cancer in rodents: does it tell us about cancer in humans? *Nature Reviews Cancer*, 5(10):807–819.

6. Arenal, A., Pimentel, R., García, C., Pimentel, E., and Aleström, P. (2004). The SV40 T antigen nuclear localization sequence enhances nuclear import of vector DNA in embryos of a crustacean (Litopenaeus schmitti). *Gene*, 337:71–77.

7. Armesilla-Diaz, A., Elvira, G., and Silva, A. (2009). P53 Regulates the Proliferation, Differentiation and Spontaneous Transformation of Mesenchymal Stem Cells. *Experimental cell research*, 315(20):3598–610.

8. Arrigoni, E., Lopa, S., de Girolamo, L., Stanco, D., and Brini, A. (2009). Isolation, characterization and osteogenic differentiation of adipose-derived stem cells: from small to large animal models. *Cell and Tissue Research*, 338(3):401–411.

9. Asher, G., Lotem, J., Tsvetkov, P., Reiss, V., Sachs, L., and Shaul, Y. (2003). P53 hot-spot mutants are resistant to ubiquitin-independent degradation by increased binding to NAD(P)H:quinone oxidoreductase 1. *Proceedings of the National Academy of Sciences*, 100(25):15065–15070.

10. Attardi, L. D. and Donehower, L. A. (2005). Probing p53 biological functions through the use of genetically engineered mouse models. *Mutation Research*, 576(1-2):4–21.

11. Augello, A. and De Bari, C. (2010). The Regulation of Differentiation in Mesenchymal Stem Cells. *Human Gene Therapy*, 21(10):1226–38.

12. Ayles, H., Friendship, R., and Ball, R. (1996). Effect of dietary particle size on gastric ulcers, assessed by endoscopic examination, and relationship between ulcer severity and growth performance of individually fed pigs. *Swine Health Prod*, 4(5):211–216.

13. Bachinski, L. L., Olufemi, S.-e., Zhou, X., Shete, S., Lozano, G., Amos, C. I., Strong, L. C., Krahe, R., Wu, C.-C., and Yip, L. (2005). Genetic mapping of a third Li-Fraumeni syndrome predisposition locus to human chromosome 1q23. *Cancer research*, 65(2):427–31.

14. Badorf, M., Edenhofer, F., Dries, V., Kochanek, S., and Schiedner, G. (2002). Efficient in vitro and in vivo excision of floxed sequences with a high-capacity adenoviral vector expressing Cre recombinase. *Genesis*, 33(3):119–124.

15. Baik, S.-c., Youn, H.-s., and Chung, M.-h. (1996). Increased Oxidative DNA Damage in Helicobacter pylori -infected Human Gastric Mucosa. *Cancer Research*, 56:1279–1282.

16. Baker, S. J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Jessup, J. M., VanTuinen, P., Ledbetter, D. H., Barker, D. F., Nakamura, Y., White, R., and Vogelstein, B. (1989). Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science*, 244(4901):217–21.

17. Bakhanashvili, M., Grinberg, S., Bonda, E., Simon, A. J., Moshitch-Moshkovitz, S., and Rahav, G. (2008). p53 in mitochondria enhances the accuracy of DNA synthesis. *Cell Death and Differentiation*, 15(12):1865–1874.

18. Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L.-V. F., Kolettas, E., Niforou, K., Zoumpourlis, V. C., Takaoka, M., Nakagawa, H., Tort, F., Fugger, K., Johansson, F., Sehested, M., Andersen, C. L., Dyrskjot, L., Ø rntoft, T., Lukas, J., Kittas, C., Helleday, T., Halazonetis, T. D., Bartek, J., and Gorgoulis, V. G. (2006). Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature*, 444(7119):633–7.

19. Beebe, L. F. S., McIlfatrick, S. J., and Nottle, M. B. (2009). Cytochalasin B and trichostatin a treatment postactivation improves in vitro development of porcine somatic cell nuclear transfer embryos. *Cloning and Stem Cells*, 11(4):477–482.

20. Belinsky, S. a., Swafford, D. S., Finch, G. L., Mitchell, C. E., Kelly, G., Hahn, F. F., Anderson, M. W., and Nikula, K. J. (1997). Alterations in the K-ras and p53 genes in rat lung tumors. *Environmental health perspectives*, 105 Suppl(June):901–6.

21. Bendixen, E. k., Danielsen, M., Larsen, K., and Bendixen, C. (2010). Advances in porcine genomics and proteomics–a toolbox for developing the pig as a model organism for molecular biomedical research. *Briefings in Functional Genomics*, 9(3):208–19.

22. Birch, J. M., Heighway, J., Teare, M. D., Kelsey, a. M., Hartley, a. L., Tricker, K. J., Crowther, D., Lane, D. P., and Santibáñez Koref, M. F. (1994). Linkage studies in a Li-Fraumeni family with increased expression of p53 protein but no germline mutation in p53. *British journal of cancer*, 70(6):1176–81.

23. Blomberg, P., Eskandarpour, M., Xia, S., Sylvén, C., and Islam, K. B. (2002). Electroporation in combination with a plasmid vector containing SV40 enhancer elements results in increased and persistent gene expression in mouse muscle. *Biochemical and Biophysical Research Communications*, 298(4):505–510.

24. Bosch, P., Pratt, S. L., and Stice, S. L. (2006). Isolation, characterization, gene modification, and nuclear reprogramming of porcine mesenchymal stem cells. *Biology of Reproduction*, 74(1):46–57.

25. Both, S. K., van Apeldoorn, A. A., Jukes, J. M., Englund, M. C. O., Hyllner, J., van Blitterswijk, C. A., and de Boer, J. (2011). Differential bone-forming capacity of osteogenic cells from either embryonic stem cells or bone marrow-derived mesenchymal stem cells. *Journal of Tissue Engineering and Regenerative Medicine*, 5(3):180–90.

26. Bourdon, J.-C. (2007). p53 and its isoforms in cancer. *British journal of cancer*, 97(3):277–82.

27. Brady, C. A., Jiang, D., Mello, S. S., Johnson, T. M., Jarvis, L. A., Kozak, M. M., Kenzelmann Broz, D., Basak, S., Park, E. J., McLaughlin, M. E., Karnezis, A. N., and Attardi, L. D. (2011). Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. *Cell*, 145(4):571–583.

28. Brown, C. J., Cheok, C. F., Verma, C. S., and Lane, D. P. (2011). Reactivation of p53: from peptides to small molecules. *Trends in pharmacological sciences*, 32(1):53–62.

29. Brunner, S., Sauer, T., Carotta, S., Cotten, M., Saltik, M., and Wagner, E. (2000). Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. *Gene therapy*, 7(5):401–7.

30. 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., and Prather, R. S. (2001). Transgenic pigs produced using in vitro matured oocytes infected with a retroviral vector. *Animal Biotechnology*, 12(2):205–214.

31. Cadwell, C. and Zambetti, G. P. (2001). The effects of wild-type p53 tumor suppressor activity and mutant p53 gain-of-function on cell growth. *Gene*, 277(1-2):15–30.

32. Cao, Y., Gao, Q., Wazer, D. E., and Band, V. (1997). Abrogation of wild-type p53-mediated transactivation is insufficient for mutant p53-induced immortalization of normal human mammary epithelial cells. *Cancer Research*, 57(24):5584–5589.

33. Capellá, G., Pera, G., Sala, N., Agudo, A., Rico, F., Del Giudicce, G., Plebani, M., Palli, D., Boeing, H., Bueno-de Mesquita, H. B., Carneiro, F., Berrino, F., Vineis, P., Tumino, R., Panico, S., Berglund, G., Simán, H., Nyrén, O., Hallmans, G., Martinez, C., Dorronsoro, M., Barricarte, A., Navarro, C., Quirós, J. R., Allen, N., Key, T., Bingham, S., Caldas, C., Linseisen, J., Nagel, G., Overvad, K., Tjonneland, A., Boshuizen, H. C., Peeters, P. H. M., Numans, M. E., Clavel-Chapelon, F., Trichopoulou, A., Lund, E., Jenab, M., Kaaks, R., Riboli, E., and González, C. a. (2008). DNA repair polymorphisms and the risk of stomach adenocarcinoma and severe chronic gastritis in the EPIC-EURGAST study. *International journal of epidemiology*, 37(6):1316–25.

34. Catalano, V., Labianca, R., Beretta, G. D., Gatta, G., de Braud, F., and Van Cutsem, E. (2009). Gastric cancer. *Critical Reviews in Oncology/Hematology*, 71(2):127–164.

35. Chandanos, E. and Lagergren, J. (2008). Oestrogen and the enigmatic male predominance of gastric cancer. *European journal of cancer*, 44(16):2397–403.

36. Chao, H., Liu, Y., Rabinowitz, J., Li, C., Samulski, R. J., and Walsh, C. E. (2000). Several log increase in therapeutic transgene delivery by distinct adeno-associated viral serotype vectors. *Molecular therapy*, 2(6):619–23.

37. Chen, H., Liu, Y.-q., Li, C.-h., Guo, X.-m., and Huang, L.-j. (2009). The susceptibility of three strains of Chinese minipigs to diet-induced type 2 diabetes mellitus. *Lab Animal*, 38(11):355–363.

38. Chen, L., Li, L., Pang, D., Li, Z., Wang, T., Zhang, M., Song, N., Yan, S., Lai, L. X., and Ouyang, H. (2010). Construction of transgenic swine with induced expression of Cre recombinase. *Animal*, 4(05):767.

39. Chen, M.-y., Tu, C.-f., Huang, S.-y., Lin, J.-h., Tzang, B.-s., Hseu, T.-h., Lee, W.-c., and Al, C. E. T. (2005). Augmentation of thermotolerance in primary skin fibroblasts from a transgenic pig overexpressing the porcine HSP70.2. *Asian-australasian journal of animal sciences*, 18(1):107–112.

40. Chen, P., Chen, Y., Bookstein, R., and Lee, W. (1990). Genetic mechanisms of tumor suppression by the human p53 gene. *Science*, 250(4987):1576.

41. Chen, V. K., Marks, J. M., Wong, R. C. K., McGee, M. F., Faulx, A. L., Isenberg, G. A., Schomisc, S. J., Deng, C. X., Ponsky, J. L., and Chak, A. (2008). Creation of an effective and reproducible nonsurvival porcine model that simulates actively bleeding peptic ulcers. *Gastrointestinal Endoscopy*, 68(3):548–553.

42. Chi, S.-W., Lee, S.-H., Kim, D.-H., Ahn, M.-J., Kim, J.-S., Woo, J.-Y., Torizawa, T., Kainosho, M., and Han, K.-H. (2005). Structural details on mdm2-p53 interaction. *The Journal of biological chemistry*, 280(46):38795–802.

43. Cho, Y., Gorina, S., Jeffrey, P. D., and Pavletich, N. P. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science*, 265(5170):346–55.

44. Christophorou, M. A., Ringshausen, I., Finch, A. J., Swigart, L. B., and Evan, G. I. (2006). The pathological response to DNA damage does not contribute to p53-mediated tumour suppression. *Nature*, 443(7108):214–7.

45. Cifone, M. A. and Fidler, I. J. (1980). Correlation of patterns of anchorage-independent growth with in vivo behavior of cells from a murine fibrosarcoma. *Proceedings of the National Academy of Sciences*, 77(2):1039–43.

46. Clark, A. J., Harold, G., and Yull, F. E. (1997). Mammalian cDNA and Prokaryotic Reporter Sequences Silence Adjacent Transgenes in Transgenic Mice. *Nucleic Acids Research*, 25(5):1009–1014.

47. Clark, K. J., Carlson, D. F., Foster, L. K., Kong, B.-W., Foster, D. N., and Fahrenkrug, S. C. (2007). Enzymatic engineering of the porcine genome with transposons and recombinases. *BMC Biotechnology*, 7(42).

48. Cotta-de Almeida, V., Schonhoff, S., Shibata, T., Leiter, A., and Snapper, S. B. (2003). A new method for rapidly generating gene-targeting vectors by engineering BACs through homologous recombination in bacteria. *Genome research*, 13(9):2190–4.

49. Crusius, J. B. a., Canzian, F., Capellá, G., Peña, a. S., Pera, G., Sala, N., Agudo, a., Rico, F., Del Giudice, G., Palli, D., Plebani, M., Boeing, H., Bueno-de Mesquita, H. B., Carneiro, F., Pala, V., Save, V. E., Vineis, P., Tumino, R., Panico, S., Berglund, G., Manjer, J., Stenling, R., Hallmans, G., Martínez, C., Dorronsoro, M., Barricarte, a., Navarro, C., Quirós, J. R., Allen, N., Key, T. J., Binghan, S., Caldas, C., Linseisen, J., Kaaks, R., Overvad, K., Tjø nneland, a., Büchner, F. C., Peeters, P. H. M., Numans, M. E., Clavel-Chapelon, F., Trichopoulou, a., Lund, E., Jenab, M., Rinaldi, S., Ferrari, P., Riboli, E., and González, C. a. (2008). Cytokine gene polymorphisms and the risk of adenocarcinoma of the stomach in the European prospective investigation into cancer and nutrition (EPIC-EURGAST). *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*, 19(11):1894–902.

50. 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., and Ayares, D. L. (2002). Targeted disruption of the alpha1,3-galactosyltransferase gene in cloned pigs. *Nature biotechnology*, 20(3):251–5.

51. Danilova, N., Sakamoto, K. M., and Lin, S. (2008). p53 family in development. *Mechanisms of development*, 125(11-12):919–31.

52. De Gasperi, R., Rocher, A. B., Sosa, M. A. G., Wearne, S. L., Perez, G. M., Friedrich Jr., V., Hof, P. R., Elder, G. A., Gasperi, R. D., Perez, M., and Jr, V. L. F. (2008). The IRG mouse: A two-color fluorescent reporter for assessing Cre-mediated recombination and imaging complex cellular relationships in situ. *Genesis*, 46(6):308–317.

53. Dean, D. A., Dean, B. S., Muller, S., and Smith, L. C. (1999). Sequence requirements for plasmid nuclear import. *Experimental Cell Research*, 253(2):713–722.

54. Deng, C. and Capecchi, M. R. (1992). Reexamination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. *Molecular and cellular biology*, 12(8):3365–71.

55. Deng, W., Yang, D., Zhao, B., Ouyang, Z., Song, J., Fan, N., Liu, Z., Zhao, Y., Wu, Q., Nashun, B., Tang, J., Wu, Z., Gu, W., and Lai, L. (2011). Use of the 2A peptide for generation of multi-transgenic pigs through a single round of nuclear transfer. *PloS One*, 6(5):e19986.

56. Dieckhoff, B., Kessler, B., Jobst, D., Kues, W., Petersen, B., Pfeifer, A., Kurth, R., Niemann, H., Wolf, E., and Denner, J. (2009). Distribution and expression of porcine endogenous retroviruses in multi-transgenic pigs generated for xenotransplantation. *Xenotransplantation*, 16(2):64–73.

57. Dockray, G., Dimaline, R., and Varro, A. (2005). Gastrin: old hormone, new functions. *Pflugers Arch*, 449(4):344–355.

58. Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A. J., Butel, J. S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*, 356(6366):215–221.

59. Donehower, L. A. and Lozano, G. (2009). 20 Years Studying P53 Functions in Genetically Engineered Mice. *Nature reviews. Cancer*, 9(11):831–41.

60. Dong, P., Tada, M., Hamada, J.-I., Nakamura, A., Moriuchi, T., and Sakuragi, N. (2007). p53 dominant-negative mutant R273H promotes invasion and migration of human endometrial cancer HHUA cells. *Clinical & experimental metastasis*, 24(6):471–83.

61. Dong, P., Xu, Z., Jia, N., Li, D., and Feng, Y. (2009). Elevated expression of p53 gain-of-function mutation R175H in endometrial cancer cells can increase the invasive phenotypes by activation of the EGFR/PI3K/AKT pathway. *Molecular Cancer*, 8:103.

62. Duran-Struuck, R., Cho, P. S., Teague, A. G. S., Fishman, B., Fishman, A. S., Hanekamp, J. S., Moran, S. G., Wikiel, K. J., Ferguson, K. K., Lo, D. P., Duggan, M., Arn, J. S., Billiter, B., Horner, B., Houser, S., Yeap, B. Y., Westmoreland, S. V., Spitzer, T. R., McMorrow, I. M., Sachs, D. H., Bronson, R. T., and Huang, C. A. (2010). Myelogenous leukemia in adult inbred MHC-defined miniature swine: a model for human myeloid leukemias. *Veterinary immunology and immunopathology*, 135(3-4):243–56.

63. Durkovic, M. (2012). *Immunodeficient pigs for biomedical research*. PhD thesis, Technische Universität München.

64. Eeles, R. A. (1995). Germline mutations in the TP53 gene. *Cancer surveys*, 25:101–24.

65. Efeyan, A., Garcia-Cao, I., Herranz, D., Velasco-Miguel, S., and Serrano, M. (2006). Tumour biology: Policing of oncogene activity by p53. *Nature*, 443(7108):159.

66. El-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., and Vogelstein, B. (1992). Definition of a consensus binding site for p53. *Nature genetics*, 1(1):45–9.

67. Esteban, M. A., Xu, J., Yang, J., Peng, M., Qin, D., Li, W., Jiang, Z., Chen, J., Deng, K., Zhong, M., and Others (2009). Generation of induced pluripotent stem cell lines from tibetan miniature pig. *Journal of Biological Chemistry*, 284(26):17634.

68. Evans, S. C., Liang, M., Amos, C., Gu, X., and Lozano, G. (2004). A novel genetic modifier of p53, mop1, results in embryonic lethality. *Mammalian genome*, 15(6):415–23.

69. Ezashi, T., Telugu, B. P., Alexenko, A. P., Sachdev, S., Sinha, S., and Roberts, R. M. (2009). Derivation of induced pluripotent stem cells from pig somatic cells. *Proceedings of the National Academy of Sciences*, 106(27):10993–10998.

70. Farinati, F., Cardin, R., Cassaro, M., Bortolami, M., Nitti, D., Tieppo, C., Zaninotto, G., and Rugge, M. (2008). Helicobacter pylori, inflammation, oxidative damage and gastric cancer: a morphological, biological and molecular pathway. *European journal of cancer prevention*, 17(3):195–200.

71. Fisicaro, N., Londrigan, S. L., Brady, J. L., Salvaris, E., Nottle, M. B., O'Connell, P. J., Robson, S. C., D'Apice, A. J. F., Lew, A. M., and Cowan, P. J. (2011). Versatile co-expression of graft-protective proteins using 2A-linked cassettes. *Xenotransplantation*, 18(2):121–130.

72. Fitzgerald, R. C. (2004). Clinical implications of E-cadherin associated hereditary diffuse gastric cancer. *Gut*, 53(6):775–778.

73. Flisikowska, T., Merkl, C., Landmann, M., Eser, S., Rezaei, N., Cui, X., Kurome, M., Zakhartchenko, V., Kessler, B., Wieland, H., Rottmann, O., Schmid, R. M., Schneider, G., Kind, A., Wolf, E., Saur, D., and Schnieke, A. (2012). A porcine model of familial adenomatous polyposis. *Gastroenterology*, 143(5):1173–1175.e7.

74. Flisikowska, T., Thorey, I. S., Offner, S., Ros, F., Lifke, V., Zeitler, B., Rottmann, O., Vincent, A., Zhang, L., Jenkins, S., Niersbach, H., Kind, A. J., Gregory, P. D., Schnieke, A. E., and Platzer, J. (2011). Efficient immunoglobulin gene disruption and targeted replacement in rabbit using zinc finger nucleases. *PloS one*, 6(6):e21045.

75. Foo, R. S.-Y., Nam, Y.-J., Ostreicher, M. J., Metzl, M. D., Whelan, R. S., Peng, C.-F., Ashton, A. W., Fu, W., Mani, K., Chin, S.-F., Provenzano, E., Ellis, I., Figg, N., Pinder, S., Bennett, M. R., Caldas, C., and Kitsis, R. N. (2007). Regulation of p53 tetramerization and nuclear export by ARC. *Proceedings of the National Academy of Sciences*, 104(52):20826–31.

76. Forones, N., Kawamura, K., Segreto, H., Neto, R., de Azevedo Focchi, G., and Oshima, C. (2008). Expression of COX-2 in Stomach Carcinogenesis. *Journal of Gastrointestinal Cancer*, 39(1):4–10.

77. Fox, J. G., Dangler, C. A., Taylor, N. S., King, A., Koh, T. J., and Wang, T. C. (1999). High-Salt Diet Induces Gastric Epithelial Hyperplasia and Parietal Cell Loss, and Enhances Helicobacter pylori Colonization in C57BL/6 Mice. *Cancer Research*, 59:4823–4828.

78. Friedel, R. H., Plump, A., Lu, X., Spilker, K., Jolicoeur, C., Wong, K., Venkatesh, T. R., Yaron, A., Hynes, M., Chen, B., Okada, A., McConnell, S. K., Rayburn, H., and Tessier-Lavigne, M. (2005). Gene targeting using a promoterless gene trap vector ("targeted trapping") is an efficient method to mutate a large fraction of genes. *Proceedings of the National Academy of Sciences*, 102(37):13188–93.

79. Friedrich, G. and Soriano, P. (1991). Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes & development*, 5(9):1513–23.

80. Fu, S., Ramanujam, K. S., Wong, A., Fantry, G. T., Drachenberg, C. B., James, S. P., Meltzer, S. J., and Wilson, K. T. (1999). Increased expression and cellular localization of inducible nitric oxide synthase and cyclooxygenase 2 in Helicobacter pylori gastritis. *Gastroenterology*, 116(6):1319–29.

81. Fuchs, S., Adler, V., Buschmann, T., Yin, Z., Wu, X., Jones, S., and Ronai, Z. (1998). JNK targets p53 ubiquitination and degradation in nonstressed cells. *Genes & development*, 12(17):2658.

82. Fujimura, T., Kurome, M., Murakami, H., Takahagi, Y., Matsunami, K., Shimanuki, S., Suzuki, K., Miyagawa, S., Shirakura, R., Shigehisa, T., and Nagashima, H. (2004). Cloning of the transgenic pigs expressing human decay accelerating factor and N-acetylglucosaminyltransferase III. *Cloning and stem cells*, 6(3):294–301.

83. Gao, L.-B., Rao, L., Wang, Y.-Y., Liang, W.-B., Li, C., Xue, H., Zhou, B., Sun, H., Li, Y., Lv, M.-L., Du, X.-J., and Zhang, L. (2009). The association of interleukin-16 polymorphisms with IL-16 serum levels and risk of colorectal and gastric cancer. *Carcinogenesis*, 30(2):295–9.

84. Garrels, W., Mátés, L., Holler, S., Dalda, A., Taylor, U., Petersen, B., Niemann, H., Izsvák, Z., Ivics, Z., and Kues, W. A. (2011). Germline transgenic pigs by sleeping beauty

transposition in porcine zygotes and targeted integration in the pig genome. *PloS One*, 6(8):e23573.

85. Gatza, C., Moore, L., Dumble, M., and Donehower, L. A. (2007). Tumor suppressor dosage regulates stem cell dynamics during aging. *Cell cycle*, 6(1):52–5.

86. Gaunt, S. J., Drage, D., and Cockley, A. (2003). Vertebrate caudal gene expression gradients investigated by use of chick cdx-A/lacZ and mouse cdx-1/lacZ reporters in transgenic mouse embryos: evidence for an intron enhancer. *Mechanisms of Development*, 120(5):573–586.

87. Giday, S. A., Dray, X., Magno, P., Buscaglia, J. M., Shin, E. J., Surti, V. C., Assumpcao, L., Marohn, M. R., Ruben, D., Zhigalin, A., Pipitone, L. J., and Kantsevoy, S. V. (2010). Infection during natural orifice transluminal endoscopic surgery: a randomized, controlled study in a live porcine model. *Gastrointestinal Endoscopy*, 71(4):812–816.

88. Gittes, G. K., Rutter, W. J., and Debas, H. T. (1993). Initiation of gastrin expression during the development of the mouse pancreas. *American journal of surgery*, 165(1):23–5; discussion 25–6.

89. Godar, S., Ince, T. A., Bell, G. W., Feldser, D., Donaher, J. L., Bergh, J., Liu, A., Miu, K., Watnick, R. S., Reinhardt, F., McAllister, S. S., Jacks, T., and Weinberg, R. A. (2008). Growth-inhibitory and tumor- suppressive functions of p53 depend on its repression of CD44 expression. *Cell*, 134(1):62–73.

90. Goldschlager, T., Rosenfeld, J. V., Jenkin, G., and Ghosh, P. (2009). Chondrogenic differentiation of adipose-derived stem cells. *ANZ Journal of Surgery*, 79(11):856–857.

91. Golovan, S. P., Meidinger, R. G., Ajakaiye, A., Cottrill, M., Wiederkehr, M. Z., Barney, D. J., Plante, C., Pollard, J. W., Fan, M. Z., Hayes, M. A., Laursen, J., Hjorth, J. P., Hacker, R. R., Phillips, J. P., and Forsberg, C. W. (2001). Pigs expressing salivary phytase produce low-phosphorus manure. *Nature biotechnology*, 19(8):741–5.

92. Götz, J. and Götz, N. N. (2009). Animal models for Alzheimer's disease and frontotemporal dementia: a perspective. *ASN neuro*, 1(4):251–264.

93. Graham, C., Cole, S., and Laible, G. (2009). Site-specific modification of the bovine genome using Cre recombinase-mediated gene targeting. *Biotechnology Journal*, 4(1):108–18.

94. Guilloteau, P., Zabielski, R., Hammon, H. M., and Metges, C. C. (2010). Nutritional programming of gastrointestinal tract development. Is the pig a good model for man? *Nutrition Research Reviews*, 23(1):4–22.

95. Halazonetis, T. D., Gorgoulis, V. G., and Bartek, J. (2008). An oncogene-induced DNA damage model for cancer development. *Science*, 319(5868):1352–5.

96. Hamilton, B., Feng, Q., Ye, M., and Welstead, G. G. (2009). Generation of induced pluripotent stem cells by reprogramming mouse embryonic fibroblasts with a four transcription factor, doxycycline inducible lentiviral transduction system. *Journal of Visualized Experiments: JoVE*, 33:e1447.

97. Hammer, R. E., Pursel, V. G., Rexroad, C. E., Wall, R. J., Bolt, D. J., Ebert, K. M., Palmiter, R. D., and Brinster, R. L. (1985). Production of transgenic rabbits, sheep and pigs by microinjection. *Nature*, 315(6021):680–683.

98. Hanson, K. D. and Sedivy, J. M. (1995). Analysis of biological selections for high-efficiency gene targeting. *Molecular and cellular biology*, 15(1):45–51.

99. Hao, Y., Zhang, J., Yi, C., and Qian, W. (1997). Abnormal change of p53 gene in gastric and precancerous lesions and APC gene deletion in gastric carcinoma and near tissues. *Journal of Tongji Medical University = Tong ji yi ke da xue xue bao*, 17(2):75–8.

100. Hao, Y. H., Yong, H. Y., Murphy, C. N., Wax, D., Samuel, M., Rieke, A., Lai, L., Liu, Z., Durtschi, D. C., Welbern, V. R., Price, E. M., McAllister, R. M., Turk, J. R., Laughlin, M. H., Prather, R. S., and Rucker, E. B. (2006). Production of endothelial nitric oxide synthase (eNOS) over-expressing piglets. *Transgenic research*, 15(6):739–50.

101. Hartgrink, H. H., Jansen, E. P., van Grieken, N. C., and van de Velde, C. J. (2009). Gastric cancer. *The Lancet*, 374(9688):477–490.

102. Hasty, P., Rivera-Perez, J., and Bradley, A. (1991). The length of homology required for gene targeting in embryonic stem cells. *Mol. Cell. Biol.*, 11(11):5586–5591.

103. Haupt, Y., Rowan, S., Shaulian, E., Vousden, K. H., and Oren, M. (1995). Induction of apoptosis in HeLa cells by trans-activation-deficient p53. *Genes & development*, 9(17):2170–83.

104. Hauschild, J., Petersen, B., Santiago, Y., Queisser, A.-L., Carnwath, J. W., Lucas-Hahn, A., Zhang, L., Meng, X., Gregory, P. D., Schwinzer, R., Cost, G. J., and Niemann, H. (2011). Efficient generation of a biallelic knockout in pigs using zinc-finger nucleases. *Proceedings of the National Academy of Sciences*, 108(29):12013–7.

105. Hendrie, P. C. and Russell, D. W. (2005). Gene targeting with viral vectors. *Molecular therapy*, 12(1):9–17.

106. Herrmann, F., Garriga-Canut, M., Baumstark, R., Fajardo-Sanchez, E., Cotterell, J., Minoche, A., Himmelbauer, H., and Isalan, M. (2011). p53 Gene Repair with Zinc Finger Nucleases Optimised by Yeast 1-Hybrid and Validated by Solexa Sequencing. *PloS One*, 6(6):e20913.

107. Himaki, T., Mori, H., Mizobe, Y., Miyoshi, K., Sato, M., Takao, S., and Yoshida, M. (2010). Latrunculin a dramatically improves the developmental capacity of nuclear transfer embryos derived from gene-modified clawn miniature pig cells. *Cellular Reprogramming*, 12(2):127–131.

108. Hingorani, S. R., Petricoin III, E. F., Maitra, A., Rajapakse, V., King, C., Jacobetz, M. A., Ross, S., Conrads, T. P., Veenstra, T. D., Hitt, B. A., Kawaguchi, Y., Johann, D., Liotta, L. A., Crawford, H. C., Putt, M. E., Jacks, T., Wright, C. V., Hruban, R. H., Lowy, A. M., and Tuveson, D. A. (2003). Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell*, 4(6):437–450.

109. Hingorani, S. R., Wang, L., Multani, A. S., Combs, C., Deramaudt, T. B., Hruban, R. H., Rustgi, A. K., Chang, S., and Tuveson, D. A. (2005). Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell*, 7(5):469–483.

110. Ho, W. C., Fitzgerald, M. X., and Marmorstein, R. (2006). Structure of the p53 core domain dimer bound to DNA. *The Journal of biological chemistry*, 281(29):20494–502.

111. 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., and Jaenisch, R. (2009). Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. *Nature biotechnology*, 27(9):851–7.

112. Hofmann, A., Kessler, B., Ewerling, S., Weppert, M., Vogg, B., Ludwig, H., Stojkovic, M., Boelhauve, M., Brem, G., Wolf, E., and Pfeifer, A. (2003). Efficient transgenesis in farm animals by lentiviral vectors. *EMBO reports*, 4(11):1054–1058.

113. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. (1991). p53 mutations in human cancers. *Science*, 253(5015):49–53.

114. Hon, S. S. F., Ng, S. S. M., Lee, J. F. Y., Li, J. C. M., and Lo, A. W. I. (2010). In vitro porcine training model for colonic endoscopic submucosal dissection: an inexpensive and safe way to acquire a complex endoscopic technique. *Surgical Endoscopy*, 24(10):2439–43.

115. Hong, I.-H., Jeong, Y.-W., Shin, T., Hyun, S.-H., Park, J.-K., Ki, M.-R., Han, S.-Y., Park, S.-I., Lee, J.-H., Lee, E.-M., Kim, A.-Y., You, S.-Y., Hwang, W.-S., and Jeong, K.-S. (2011). Morphological abnormalities, impaired fetal development and decrease in myostatin expression following somatic cell nuclear transfer in dogs. *Molecular reproduction and development*, 78(5):337–46.

116. Hotta, A. and Ellis, J. (2008). Retroviral vector silencing during iPS cell induction: An epigenetic beacon that signals distinct pluripotent states. *Journal of Cellular Biochemistry*, 105(4):940–948.

117. Howard, T. A., Misra, D. N., Grove, M., Becich, M. J., Shaoi, J., Gordon, M., and Alpers, D. H. (1996). Human gastric intrinsic factor expression is not restricted to parietal cells. *J Anat*, 189:303–313.

118. Howlader, N., Noone, A., Krapcho, M., Neyman, N., Aminou, R., Altekruse, S., Kosary, C., Ruhl, J., Tatalovich, Z., Cho, H., Mariotto, A., Eisner, M., Lewis, D., Chen, H., Feuer, E., and Cronin, K. (2011). SEER Cancer Statistics Review 1975-2009 (Vintage 2009 Populations). *National Cancer Institute. Bethesda, MD.*

119. Hu, W., Feng, Z., Teresky, A. K., and Levine, A. J. (2007). p53 regulates maternal reproduction through LIF. *Nature*, 450(7170):721–4.

120. Huang, L., Yan, Z., Liao, X., Li, Y., Yang, J., Wang, Z.-G., Zuo, Y., Kawai, H., Shadfan, M., Ganapathy, S., and Yuan, Z.-M. (2011). The p53 inhibitors MDM2/MDMX complex is required for control of p53 activity in vivo. *Proceedings of the National Academy of Sciences*, 108(29):12001–6.

121. Huang, L. C., Clarkin, K. C., and Wahl, G. M. (1996). Sensitivity and selectivity of the DNA damage sensor responsible for activating p53-dependent G1 arrest. *Proceedings of the National Academy of Sciences*, 93(10):4827–32.

122. Humar, B., Blair, V., Charlton, A., More, H., Martin, I., and Guilford, P. (2009). E-cadherin deficiency initiates gastric signet-ring cell carcinoma in mice and man. *Cancer Research*, 69(5):2050–2056.

123. Huntsman, D. G., Carneiro, F., Lewis, F. R., MacLeod, P. M., Hayashi, a., Monaghan, K. G., Maung, R., Seruca, R., Jackson, C. E., and Caldas, C. (2001). Early gastric cancer in young, asymptomatic carriers of germ-line E-cadherin mutations. *The New England journal of medicine*, 344(25):1904–9.

124. Irion, S., Luche, H., Gadue, P., Fehling, H. J., Kennedy, M., and Keller, G. (2007). Identification and targeting of the ROSA26 locus in human embryonic stem cells. *Nature Biotechnology*, 25(12):1477–1482.

125. Isomoto, H., Ohtsuru, A., Braiden, V., Iwamatsu, M., Miki, F., Kawashita, Y., Mizuta, Y., Kaneda, Y., Kohno, S., and Yamashita, S. (2006). Heat-directed suicide gene therapy mediated by heat shock protein promoter for gastric cancer. *Oncology Reports*, 15(3):629–635.

126. Itadani, H., Oshima, H., Oshima, M., and Kotani, H. (2009). Mouse gastric tumor models with prostaglandin E2 pathway activation show similar gene expression profiles to intestinal-type human gastric cancer. *BMC Genomics*, 10(1):615.

127. Jakobsen, J. E., Li, J., Kragh, P. M., Moldt, B., Lin, L., Liu, Y., Schmidt, M., Winther, K. D., Schyth, B. D., Holm, I. E., Vajta, G., Bolund, L., Callesen, H., Jø rgensen, A. L., Nielsen, A. L., and Mikkelsen, J. G. (2010a). Pig transgenesis by Sleeping Beauty DNA transposition. *Transgenic Research*, 20(3):533–45.

128. Jakobsen, J. E., Li, J., Moldt, B., Kragh, P. M., Callesen, H., Hertz, J. M., Bolund, L., Jø rgensen, A. L., Mikkelsen, J. G., and Nielsen, A. L. (2010b). Establishment of a pig fibroblast-derived cell line for locus-directed transgene expression in cell cultures and blastocysts. *Molecular Biology Reports*, 38(1):151–61.

129. Jeffers, J. R., Parganas, E., Lee, Y., Yang, C., Wang, J., Brennan, J., MacLean, K. H., Han, J., Chittenden, T., Ihle, J. N., McKinnon, P. J., Cleveland, J. L., and Zambetti, G. P. (2003). Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell*, 4(4):321–328.

130. Judd, L. M., Alderman, B. M., Howlett, M., Shulkes, A., Dow, C., Moverley, J., Grail, D., Jenkins, B. J., Ernst, M., and Giraud, A. S. (2004). Gastric cancer development in mice lacking the SHP2 binding site on the IL-6 family co-receptor gp130. *Gastroenterology*, 126(1):196–207.

131. Junttila, M. R. and Evan, G. I. (2009). p53 — a Jack of all trades but master of none. *Nature Reviews Cancer*, 9(11):821–9.

132. Kabir, S. (2009). Effect of Helicobacter pylori Eradication on Incidence of Gastric Cancer in Human and Animal Models: Underlying Biochemical and Molecular Events. *Helicobacter*, 14(3):159–171.

133. Kanfi, Y., Peshti, V., Gozlan, Y. M., Rathaus, M., Gil, R., and Cohen, H. Y. (2008). Regulation of SIRT1 protein levels by nutrient availability. *FEBS letters*, 582(16):2417–23.

134. Khromova, N. V., Kopnin, P. B., Stepanova, E. V., Agapova, L. S., and Kopnin, B. P. (2009). p53 hot-spot mutants increase tumor vascularization via ROS-mediated activation of the HIF1/VEGF-A pathway. *Cancer Letters*, 276(2):143–151.

135. Kido, T. and Lau, Y.-F. C. (2005). A Cre gene directed by a human TSPY promoter is specific for germ cells and neurons. *Genesis*, 42(4):263–275.

136. Kind, A. and Schnieke, A. E. (2008). Animal pharming, two decades on. *Transgenic Research*, 17(6):1025–1033.

137. Klymiuk, N., Aigner, B., Brem, G., and Wolf, E. (2010). Genetic modification of pigs as organ donors for xenotransplantation. *Molecular Reproduction and Development*, 77(3):209–221.

138. Klymiuk, N., Böcker, W., Schönitzer, V., Bähr, A., Radic, T., Fröhlich, T., Wünsch, A., Keß ler, B., Kurome, M., Schilling, E., and Others (2011). First inducible transgene expression in porcine large animal models. *The FASEB Journal*, 26(3):1086–99.

139. Klymiuk, N., Mundhenk, L., Kraehe, K., Wuensch, A., Plog, S., Emrich, D., Langenmayer, M., Stehr, M., Holzinger, A., Kröner, C., and Others (2012). Sequential targeting of CFTR by BAC vectors generates a novel pig model of cystic fibrosis. *Journal of Molecular Medicine*, 90(5):597–608.

140. Knudsen, S. (1999). Promoter 2.0: for the recognition of PolII promoter sequences. *Bioinformatics*, 15:356–361.

141. Kodama, M., Murakami, K., Sato, R., Okimoto, T., Nishizono, A., and Fujioka, T. (2005). Helicobacter pylori-infected animal models are extremely suitable for the investigation of gastric carcinogenesis. *World J Gastroenterol*, 11(45):7063–7071.

142. Kogan-Sakin, I., Tabach, Y., Buganim, Y., Molchadsky, A., Solomon, H., Madar, S., Kamer, I., Stambolsky, P., Shelly, A., Goldfinger, N., Valsesia-Wittmann, S., Puisieux, A., Zundelevich, A., Gal-Yam, E. N., Avivi, C., Barshack, I., Brait, M., Sidransky, D., Domany, E., and Rotter, V. (2011). Mutant p53(R175H) upregulates Twist1 expression and promotes epithelial-mesenchymal transition in immortalized prostate cells. *Cell Death and Differentiation*, 18(2):271–281.

143. Kolber-Simonds, D., Lai, L., Watt, S. R., Denaro, M., Arn, S., Augenstein, M. L., Betthauser, J., Carter, D. B., Greenstein, J. L., Hao, Y., Im, G.-S., Liu, Z., Mell, G. D., Murphy, C. N., Park, K.-W., Rieke, A., Ryan, D. J. J., Sachs, D. H., Forsberg, E. J., Prather, R. S., and Hawley, R. J. (2004). Production of alpha-1,3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations. *Proceedings of the National Academy of Sciences*, 101(19):7335–40.

144. Kong, Q., Wu, M., Huan, Y., Zhang, L., Liu, H., Bou, G., Luo, Y., Mu, Y., and Liu, Z. (2009). Transgene expression is associated with copy number and cytomegalovirus promoter methylation in transgenic pigs. *PloS one*, 4(8):e6679.

145. Kragh, P. M., Nielsen, A. L., Li, J., Du, Y., Lin, L., Schmidt, M., Bø gh, I. B., Holm, I. E., Jakobsen, J. E., Johansen, M. G., Purup, S., Bolund, L., Vajta, G., and Jø rgensen, A. L. (2009). Hemizygous minipigs produced by random gene insertion and handmade cloning express the Alzheimer's disease-causing dominant mutation APPsw. *Transgenic Research*, 18(4):545–558.

146. Kress, M., May, E., Cassingena, R., and May, P. (1979). Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum. *Journal of virology*, 31(2):472–83.

147. Krueger, C., Danke, C., Pfleiderer, K., Schuh, W., Jäck, H.-M., Lochner, S., Gmeiner, P., Hillen, W., and Berens, C. (2006). A gene regulation system with four distinct expression levels. *The Journal of Gene Medicine*, 8(8):1037–1047.

148. Kues, W. A., Schwinzer, R., Wirth, D., Verhoeyen, E., Lemme, E., Herrmann, D., Barg-Kues, B., Hauser, H., Wonigeit, K., and Niemann, H. (2006). Epigenetic silencing and tissue independent expression of a novel tetracycline inducible system in double-transgenic pigs. *FASEB Journal*, 20(8):1200–1202.

149. Kurome, M., Ishikawa, T., Tomii, R., Ueno, S., Shimada, A., Yazawa, H., and Nagashima, H. (2008). Production of transgenic and non-transgenic clones in miniature pigs by somatic cell nuclear transfer. *The Journal of Reproduction and Development*, 54(3):156–163.

150. Kurome, M., Ueda, H., Tomii, R., Naruse, K., and Nagashima, H. (2006). Production of transgenic-clone pigs by the combination of ICSI-mediated gene transfer with somatic cell nuclear transfer. *Transgenic research*, 15(2):229–240.

151. Kurt-Jones, E. A., Cao, L., Sandor, F., Rogers, A. B., Whary, M. T., Nambiar, P. R., Cerny, A., Bowen, G., Yan, J., Takaishi, S., Chi, A. L., Reed, G., Houghton, J., Fox,

J. G., and Wang, T. C. (2007). Trefoil family factor 2 is expressed in murine gastric and immune cells and controls both gastrointestinal inflammation and systemic immune responses. *Infect Immun*, 75(1):471–480.

152. Lai, L., Kang, J. X., Li, R., Wang, J., Witt, W. T., Yong, H. Y., Hao, Y., Wax, D. M., Murphy, C. N., Rieke, A., Samuel, M., Linville, M. L., Korte, S. W., Evans, R. W., Starzl, T. E., Prather, R. S., and Dai, Y. (2006). Generation of cloned transgenic pigs rich in omega-3 fatty acids. *Nature biotechnology*, 24(4):435–6.

153. 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., and Prather, R. S. (2002a). Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science*, 295(5557):1089–92.

154. Lai, L., Park, K.-W., Cheong, H.-T., Kühholzer, B., Samuel, M., Bonk, A., Im, G.-S., Rieke, A., Day, B. N., Murphy, C. N., Carter, D. B., and Prather, R. S. (2002b). Transgenic pig expressing the enhanced green fluorescent protein produced by nuclear transfer using colchicine-treated fibroblasts as donor cells. *Molecular reproduction and development*, 62(3):300–6.

155. Lane, D. P. and Crawford, L. V. (1979). T antigen is bound to a host protein in SY40-transformed cells. *Nature*, 278(5701):261–263.

156. Larsen, M. O. and Rolin, B. (2004). Use of the Göttingen minipig as a model of diabetes, with special focus on type 1 diabetes research. *ILAR Journal / National Research Council, Institute of Laboratory Animal Resources*, 45(3):303–313.

157. Lavitrano, M., Busnelli, M., Cerrito, M. G., Giovannoni, R., Manzini, S., and Vargiolu, A. (2006). Sperm-mediated gene transfer. *Reproduction, Fertility and Development*, 18(2):19.

158. Lebbink, R. J., Lowe, M., Chan, T., Khine, H., Wang, X., and McManus, M. T. (2011). Polymerase II promoter strength determines efficacy of microRNA adapted shRNAs. *PloS one*, 6(10):e26213.

159. Lee, H.-T. H.-G. H.-C., Kim, S. W., Lee, P., Chung, H.-J., Lee, Y.-K., Han, J.-H., Hwang, I.-S., Yoo, J.-I., Kim, Y.-K., Kim, H.-T., Chang, W.-K., and Park, J.-K. (2009). Production of recombinant human von Willebrand factor in the milk of transgenic pigs. *The Journal of reproduction and development*, 55(5):484–490.

160. Lefebvre, O., Chenard, M. P., Masson, R., Linares, J., Dierich, A., LeMeur, M., Wendling, C., Tomasetto, C., Chambon, P., and Rio, M. C. (1996). Gastric mucosa abnormalities and tumorigenesis in mice lacking the pS2 trefoil protein. *Science*, 274(5285):259–262.

161. Leontieva, O. V. and Blagosklonny, M. V. (2010). DNA damaging agents and p53 do not cause senescence in quiescent cells, while consecutive re-activation of mTOR is associated with conversion to senescence. *Aging*, 2(12):924–935.

162. Leuchs, S., Saalfrank, A., Merkl, C., Flisikowska, T., Edlinger, M., Durkovic, M., Rezaei, N., Kurome, M., Zakhartchenko, V., Kessler, B., Flisikowski, K., Kind, A., Wolf, E., and Schnieke, A. (2012). Inactivation and Inducible Oncogenic Mutation of p53 in Gene Targeted Pigs. *PLoS ONE*, 7(10):e43323.

163. Levine, A. J. and Oren, M. (2009). The first 30 years of p53: growing ever more complex. *Nature Reviews Cancer*, 9(10):749–758.

164. Lewandoski, M. (2001). Conditional control of gene expression in the mouse. *Nature Reviews Genetics*, 2(10):743–755.

165. Li, F. P. and Fraumeni, J. F. (1969a). Rhabdomyosarcoma in children: epidemiologic study and identification of a familial cancer syndrome. *Journal of the National Cancer Institute*, 43(6):1365–73.

166. Li, F. P. and Fraumeni, J. F. (1969b). Soft-tissue sarcomas, breast cancer, and other neoplasms. A familial syndrome? *Annals of internal medicine*, 71(4):747–52.

167. Li, F. P. and Fraumeni, J. F. (1982). Prospective study of a family cancer syndrome. *JAMA : the journal of the American Medical Association*, 247(19):2692–4.

168. Li, L., Pang, D., Chen, L., Wang, T., Nie, D., Yan, S., and Ouyang, H. (2009a). Establishment of a Transgenic Pig Fetal Fibroblast Reporter Cell Line for Monitoring Cre Recombinase Activity. *DNA and Cell Biology*, 28(6):303–308.

169. Li, L., Pang, D., Wang, T., Li, Z., Chen, L., Zhang, M., Song, N., Nie, D., Chen, Z., Lai, L., and Ouyang, H. (2009b). Production of a reporter transgenic pig for monitoring Cre recombinase activity. *Biochemical and Biophysical Research Communications*, 382(2):232–235.

170. Li, Q., Karam, S. M., and Gordon, J. I. (1996). Diphtheria toxin-mediated ablation of parietal cells in the stomach of transgenic mice. *J Biol Chem*, 271(7):3671–3676.

171. Li, S., MacLaughlin, F. C., Fewell, J. G., Gondo, M., Wang, J., Nicol, F., Dean, D. A., and Smith, L. C. (2001). Muscle-specific enhancement of gene expression by incorporation of SV40 enhancer in the expression plasmid. *Gene Therapy*, 8(6):494–497.

172. Li, X., Gutierrez, D. V., Hanson, M. G., Han, J., Mark, M. D., Chiel, H., Hegemann, P., Landmesser, L. T., and Herlitze, S. (2005). Fast noninvasive activation and inhibition of neural and network activity by vertebrate rhodopsin and green algae channelrhodopsin. *Proceedings of the National Academy of Sciences*, 102(49):17816–21.

173. Limaye, A., Hall, B., and Kulkarni, A. B. (2009). Manipulation of mouse embryonic stem cells for knockout mouse production. *Current Protocols in Cell Biology*, Chapter 19(Unit 19.13):19.13.1–24.

174. Lin, J., Chen, J., Elenbaas, B., and Levine, A. J. (1994). Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. *Genes & Development*, 8(10):1235–1246.

175. Lindor, N. M., McMaster, M. L., Lindor, C. J., and Greene, M. H. (2008). Concise handbook of familial cancer susceptibility syndromes - second edition. *Journal of the National Cancer Institute. Monographs*, 2008(38):1–93.

176. Linzer, D. I. and Levine, A. J. (1979). Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell*, 17(1):43–52.

177. Liu, G., McDonnell, T. J., Montes de Oca Luna, R., Kapoor, M., Mims, B., El-Naggar, A. K., and Lozano, G. (2000). High metastatic potential in mice inheriting a targeted p53 missense mutation. *Proceedings of the National Academy of Sciences*, 97(8):4174–4179.

178. Lobe, C. G., Koop, K. E., Kreppner, W., Lomeli, H., Gertsenstein, M., and Nagy, A. (1999). Z/AP, a double reporter for cre-mediated recombination. *Developmental biology*, 208(2):281–92.

179. Lombardo, A., Genovese, P., Beausejour, C. M., Colleoni, S., Lee, Y.-L., Kim, K. A., Ando, D., Urnov, F. D., Galli, C., Gregory, P. D., Holmes, M. C., and Naldini, L. (2007). Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nature biotechnology*, 25(11):1298–306.

180. López-Vidal, Y., Ponce-de León, S., Castillo-Rojas, G., Barreto-Zúñiga, R., Torre-Delgadillo, A., and Fox, D. (2008). High Diversity of vacA and cagA Helicobacter pylori Genotypes in Patients with and without Gastric Cancer. *PLoS ONE*, 3(12):e3849.

181. Lorson, M. A., Spate, L. D., Samuel, M. S., Murphy, C. N., Lorson, C. L., Prather, R. S., and Wells, K. D. (2011). Disruption of the Survival Motor Neuron (SMN) gene in pigs using ssDNA. *Transgenic research*, 20(6):1293–304.

182. Lozano, G. and Zambetti, G. P. (2005). What have animal models taught us about the p53 pathway? *The Journal of pathology*, 205(2):206–20.

183. Lu, H.-L. H.-b. H.-l., Zhou, J.-H., Ma, Y.-Y., Tang, Y.-l., Zhang, Q.-Y., and Zhao, C.-H. (2010). Five serum proteins identified using SELDI-TOF-MS as potential biomarkers of gastric cancer. *Japanese Journal of Clinical Oncology*, 40(4):336–342.

184. Luche, H., Weber, O., Nageswara Rao, T., Blum, C., and Fehling, H. J. (2007). Faithful activation of an extra-bright red fluorescent protein in "knock-in" Cre-reporter mice ideally suited for lineage tracing studies. *European Journal of Immunology*, 37(1):43–53.

185. Luo, Y., Li, J., Liu, Y., Lin, L., Du, Y., Li, S., Yang, H., Vajta, G., Callesen, H., Bolund, L., and Sø rensen, C. B. (2011). High efficiency of BRCA1 knockout using rAAV-mediated gene targeting: developing a pig model for breast cancer. *Transgenic Research*, 20(5):975–988.

186. Maeda, M., Asahara, S., Nishi, T., Mushiake, S., Oka, T., Shimada, S., Chiba, T., Tohyama, M., and Futai, M. (1995). The rat intrinsic factor gene: its 5'-upstream region and chief cell-specific transcription. *J Biochem*, 117(6):1305–1311.

187. Malkin, D. (2011). Li-fraumeni syndrome. *Genes & cancer*, 2(4):475–84.

188. Marchenko, N. D., Zaika, A., and Moll, U. M. (2000). Death Signal-induced Localization of p53 Protein to Mitochondria. *The Journal of biological chemistry*, 275(21):16202–12.

189. Markway, B., Tan, G.-K., Brooke, G., Hudson, J., Cooper-White, J., and Doran, M. (2010). Enhanced Chondrogenic Differentiation of Human Bone Marrow-Derived Mesenchymal Stem Cells in Low Oxygen Environment Micropellet Cultures. *Cell Transplantation*, 19(1):29–42.

190. Martínez-Lorenzo, M. J., Royo-Cañas, M., Alegre-Aguarón, E., Desportes, P., Castiella, T., García-Alvarez, F., and Larrad, L. (2009). Phenotype and chondrogenic

differentiation of mesenchymal cells from adipose tissue of different species. *Journal of Orthopaedic Research*, 27(11):1499–1507.

191. Masciari, S., Dewanwala, A., Stoffel, E. M., Lauwers, G. Y., Zheng, H., Achatz, M. I., Riegert-Johnson, D., Foretova, L., Silva, E. M., Digianni, L., Verselis, S. J., Schneider, K., Li, F. P., Fraumeni, J., Garber, J. E., and Syngal, S. (2011). Gastric cancer in individuals with Li-Fraumeni syndrome. *Genetics in medicine*, 13(7):651–7.

192. Matkar, S. S., Durham, A., Brice, A., Wang, T. C., Rustgi, A. K., and Hua, X. (2011). Systemic activation of K-ras rapidly induces gastric hyperplasia and metaplasia in mice. *American Journal of Cancer Research*, 1(4):432–445.

193. Matoba, S., Kang, J.-G., Patino, W. D., Wragg, A., Boehm, M., Gavrilova, O., Hurley, P. J., Bunz, F., and Hwang, P. M. (2006). p53 regulates mitochondrial respiration. *Science*, 312(5780):1650–3.

194. Matsunari, H., Onodera, M., Tada, N., Mochizuki, H., Karasawa, S., Haruyama, E., Nakayama, N., Saito, H., Ueno, S., Kurome, M., Miyawaki, A., and Nagashima, H. (2008). Transgenic-cloned pigs systemically expressing red fluorescent protein, Kusabira-Orange. *Cloning and stem cells*, 10(3):313–23.

195. Matzke, M. A., Mette, M. F., and Matzke, A. J. (2000). Transgene silencing by the host genome defense: implications for the evolution of epigenetic control mechanisms in plants and vertebrates. *Plant molecular biology*, 43(2-3):401–15.

196. McCarrick, J. W., Parnes, J. R., Seong, R. H., Solter, D., and Knowles, B. B. (1993). Positive-negative selection gene targeting with the diphtheria toxin A-chain gene in mouse embryonic stem cells. *Transgenic Research*, 2(4):183–190.

197. McCreath, K. J., Howcroft, J., Campbell, K. H. S., Colman, A., Schnieke, A. E., and Kind, A. (2000). Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. *Nature*, 405(6790):1066–1069.

198. Means, A. L., Xu, Y., Zhao, A., Ray, K. C., and Gu, G. (2008). A CK19(CreERT) knockin mouse line allows for conditional DNA recombination in epithelial cells in multiple endodermal organs. *Genesis*, 46(6):318–323.

199. Meek, D. W. (2009). Tumour suppression by p53: a role for the DNA damage response? *Nature Reviews Cancer*, 9(10):714–723.

200. Mendicino, M., Ramsoondar, J., Phelps, C., Vaught, T., Ball, S., Leroith, T., Monahan, J., Chen, S., Dandro, A., Boone, J., Jobst, P., Vance, A., Wertz, N., Bergman, Z.,

Sun, X.-Z., Polejaeva, I., Butler, J., Dai, Y., Ayares, D., and Wells, K. (2011). Generation of antibody- and B cell-deficient pigs by targeted disruption of the J-region gene segment of the heavy chain locus. *Transgenic Research*, 20(3):625–41.

201. Merrifield, C., Lewis, M., Claus, S., Beckonert, O., Dumas, M., Duncker, S., Kochhar, S., Rezzi, S., Lindon, J., Bailey, M., and Others (2011). A metabolic system-wide characterisation of the pig: a model for human physiology. *Mol. BioSyst.*, 7(9):2577–2588.

202. Meyerholz, D. K., Stoltz, D. A., Pezzulo, A. A., and Welsh, M. J. (2010). Pathology of gastrointestinal organs in a porcine model of cystic fibrosis. *The American Journal of Pathology*, 176(3):1377–1389.

203. Michalak, E. M., Villunger, A., Adams, J. M., and Strasser, A. (2008). In several cell types tumour suppressor p53 induces apoptosis largely via Puma but Noxa can contribute. *Cell death and differentiation*, 15(6):1019–29.

204. Milne, A. N., Carneiro, F., O'Morain, C., and Offerhaus, G. J. A. (2009). Nature meets nurture: molecular genetics of gastric cancer. *Human Genetics*, 126(5):615–628.

205. Mimata, A., Fukamachi, H., Eishi, Y., and Yuasa, Y. (2011). Loss of E-cadherin in mouse gastric epithelial cells induces signet ring-like cells, a possible precursor lesion of diffuse gastric cancer. *Cancer Science*, 102(5):942–950.

206. Mir, B. and Piedrahita, J. A. (2004). Nuclear localization signal and cell synchrony enhance gene targeting efficiency in primary fetal fibroblasts. *Nucleic Acids Research*, 32(3):e25.

207. Miyoshi, K., Mori, H., Mizobe, Y., Akasaka, E., Ozawa, A., Yoshida, M., and Sato, M. (2010). Valproic Acid enhances in vitro development and oct-3/4 expression of miniature pig somatic cell nuclear transfer embryos. *Cloning and Stem Cells*, 12(1):67–74.

208. Mizobe, Y., Kurino, S., Sata, Y., Mori, H., Yoshida, M., and Miyoshi, K. (2010). Stage-specific effects of osmolarity of a culture medium on development of pig oocytes and miniature pig somatic cell nuclear transfer embryos activated by ultrasound treatment. *Animal Science Journal = Nihon Chikusan Gakkaiho*, 81(4):453–460.

209. Mortimer, I., Tam, P., MacLachlan, I., Graham, R. W., Saravolac, E. G., and Joshi, P. B. (1999). Cationic lipid-mediated transfection of cells in culture requires mitotic activity. *Gene therapy*, 6(3):403–11.

210. Morton, J. P., Timpson, P., Karim, S. A., Ridgway, R. A., Athineos, D., Doyle, B., Jamieson, N. B., Oien, K. A., Lowy, A. M., Brunton, V. G., Frame, M. C., Evans, T.

R. J., and Sansom, O. J. (2010). Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer. *Proceedings of the National Academy of Sciences*, 107(1):246–251.

211. Münst, B., Patsch, C., and Edenhofer, F. (2009). Engineering cell-permeable protein. *Journal of visualized experiments : JoVE*, (34).

212. Murphy, D. J., Junttila, M. R., Pouyet, L., Karnezis, A., Shchors, K., Bui, D. A., Brown-Swigart, L., Johnson, L., and Evan, G. I. (2008). Distinct thresholds govern Myc's biological output in vivo. *Cancer cell*, 14(6):447–57.

213. Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L., and Luo, L. (2007). A global double-fluorescent Cre reporter mouse. *Genesis*, 45(9):593–605.

214. Nakano, K. and Vousden, K. H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. *Molecular cell*, 7(3):683–94.

215. Niemann, H., Carnwath, J. W., Herrmann, D., Wieczorek, G., Lemme, E., Lucas-Hahn, A., and Olek, S. (2010). DNA methylation patterns reflect epigenetic reprogramming in bovine embryos. *Cloning and Stem Cells*, 12(1):33–42.

216. Novak, A., Guo, C., Yang, W., Nagy, A., and Lobe, C. G. (2000). Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis*, 28(3-4):147–155.

217. Nowak-Imialek, M., Kues, W. A., Petersen, B., Lucas-Hahn, A., Herrmann, D., Meena Haridoss, S., Oropeza, M., Lemme, E., Schöler, H. R., Carnwath, J. W., and Niemann, H. (2011). Oct4-EGFP transgenic pigs - a new large animal model for reprogramming studies. *Stem Cells and Development*, 20(9):1563–75.

218. Oback, B. (2008). Climbing Mount Efficiency Small Steps, Not Giant Leaps Towards Higher Cloning Success in Farm Animals. *Reproduction in Domestic Animals*, 43(Suppl. 2):407–416.

219. O'Farrell, T., Ghosh, P., Dobashi, N., Sasaki, C., and Longo, D. (2004). Comparison of the effect of mutant and wild-type p53 on global gene expression. *Cancer research*, 64(22):8199.

220. Oien, K. A., McGregor, F., Butler, S., Ferrier, R. K., Downie, I., Bryce, S., Burns, S., and Keith, W. N. (2004). Gastrokine 1 is abundantly and specifically expressed in superficial gastric epithelium, down-regulated in gastric carcinoma, and shows high evolutionary conservation. *J Pathol*, 203(3):789–797.

221. Olive, K. P., Tuveson, D. A., Ruhe, Z. C., Yin, B., Willis, N. A., Bronson, R. T., Crowley, D., and Jacks, T. (2004). Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. *Cell*, 119(6):847–860.

222. Olivier, M., Goldgar, D. E., Sodha, N., Ohgaki, H., Kleihues, P., Hainaut, P., and Eeles, R. A. (2003). Li-Fraumeni and Related Syndromes: Correlation between Tumor Type, Family Structure, and TP53 Genotype. *Cancer Research*, 63(20):6643–6650.

223. Olivier, M., Hollstein, M., and Hainaut, P. (2010). TP53 mutations in human cancers: origins, consequences, and clinical use. *Cold Spring Harbor perspectives in biology*, 2(1):a001008.

224. Onishi, A. (2000). Pig Cloning by Microinjection of Fetal Fibroblast Nuclei. *Science*, 289(5482):1188–1190.

225. Oren, M. and Rotter, V. (2010). Mutant p53 gain-of-function in cancer. *Cold Spring Harbor Perspectives in Biology*, 2(2):a001107.

226. Orgad, S., Goldfinger, N., Cohen, G., Rotter, V., and Solomon, B. (2005). Single chain antibody against the common epitope of mutant p53 restores wild-type activity to mutant p53 protein. *FEBS letters*, 579(25):5609–5615.

227. Oshima, H., Itadani, H., Kotani, H., Taketo, M. M., and Oshima, M. (2009). Induction of Prostaglandin E2 Pathway Promotes Gastric Hamartoma Development with Suppression of Bone Morphogenetic Protein Signaling. *Cancer Research*, 69(7):2729.

228. Oshima, H., Matsunaga, A., Fujimura, T., Tsukamoto, T., Taketo, M. M., and Oshima, M. (2006). Carcinogenesis in mouse stomach by simultaneous activation of the Wnt signaling and prostaglandin E2 pathway. *Gastroenterology*, 131(4):1086–1095.

229. Oshima, H., Oshima, M., Inaba, K., and Taketo, M. M. (2004). Hyperplastic gastric tumors induced by activated macrophages in COX-2/mPGES-1 transgenic mice. *The EMBO Journal*, 23(7):1669.

230. Paleyanda, R. K., Velander, W. H., Lee, T. K., Scandella, D. H., Gwazdauskas, F. C., Knight, J. W., Hoyer, L. W., Drohan, W. N., and Lubon, H. (1997). Transgenic pigs produce functional human factor VIII in milk. *Nature Biotechnology*, 15(10):971–975.

231. Park, J.-K., Lee, Y.-K., Lee, P., Chung, H.-J., Kim, S., Lee, H.-G., Seo, M.-K., Han, J.-H., Park, C.-G., Kim, H.-T., Kim, Y.-K., Min, K.-S., Kim, J.-H., Lee, H.-T., and Chang, W.-K. (2006). Recombinant human erythropoietin produced in milk of transgenic pigs. *Journal of Biotechnology*, 122(3):362–371.

232. Park, K. W., Choi, K. M., Hong, S. P., Han, G. S., Yoo, J. Y., Jin, D. I., Seol, J. G., and Park, C. S. (2008). Production of transgenic recloned piglets harboring the human granulocyte-macrophage colony stimulating factor (hGM-CSF) gene from porcine fetal fibroblasts by nuclear transfer. *Theriogenology*, 70(9):1431–8.

233. Park, M.-R., Cho, S.-K., Lee, S.-Y., Choi, Y.-J., Park, J.-Y., Kwon, D.-N., Son, W.-J., Paik, S.-S., Kim, T., Han, Y.-M., and Kim, J.-H. (2005). A rare and often unrecognized cerebromeningitis and hemodynamic disorder: A major cause of sudden death in somatic cell cloned piglets. *Proteomics*, 5(7):1928–1939.

234. Parra-Blanco, A., Arnau, M. R., Nicolás-Pérez, D., Gimeno-García, A. Z., González, N., Díaz-Acosta, J. A., Jiménez, A., and Quintero, E. (2010). Endoscopic submucosal dissection training with pig models in a Western country. *World Journal of Gastroenterology: WJG*, 16(23):2895–2900.

235. Pathak, S., Multani, A. S., McConkey, D. J., Imam, A. S., and Amoss, M. S. (2000). Spontaneous regression of cutaneous melanoma in sinclair swine is associated with defective telomerase activity and extensive telomere erosion. *International journal of oncology*, 17(6):1219–24.

236. Patience, C., Switzer, W. M., Takeuchi, Y., Griffiths, D. J., Goward, M. E., Heneine, W., Stoye, J. P., and Weiss, R. A. (2001). Multiple groups of novel retroviral genomes in pigs and related species. *Journal of virology*, 75(6):2771–5.

237. Peitz, M., Pfannkuche, K., Rajewsky, K., and Edenhofer, F. (2002). Ability of the hydrophobic FGF and basic TAT peptides to promote cellular uptake of recombinant Cre recombinase: a tool for efficient genetic engineering of mammalian genomes. *Proceedings of the National Academy of Sciences*, 99(7):4489–94.

238. Pellegata, N., Sessa, F., Renault, B., Bonato, M., Leone, B., Solcia, E., and Ranzani, G. (1994). K-ras and p53 gene mutations in pancreatic cancer: ductal and nonductal tumors progress through different genetic lesions. *Cancer research*, 54(6):1556.

239. Petitjean, A., Achatz, M. I. W., Borresen-Dale, A. L., Hainaut, P., and Olivier, M. (2007a). TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. *Oncogene*, 26(15):2157–65.

240. Petitjean, A., Mathe, E., Kato, S., Ishioka, C., Tavtigian, S. V., Hainaut, P., and Olivier, M. (2007b). Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database (R15). *Hum Mutat.*, 28(6):622–9.

241. Pfeifer, A., Brandon, E. P., Kootstra, N., Gage, F. H., and Verma, I. M. (2001). Delivery of the Cre recombinase by a self-deleting lentiviral vector: efficient gene targeting in vivo. *Proceedings of the National Academy of Sciences*, 98(20):11450–11455.

242. Pietsch, E. C., Perchiniak, E., Canutescu, A. A., Wang, G., Dunbrack, R. L., and Murphy, M. E. (2008). Oligomerization of BAK by p53 utilizes conserved residues of the p53 DNA binding domain. *The Journal of biological chemistry*, 283(30):21294–304.

243. Plog, S., Mundhenk, L., Bothe, M. K., Klymiuk, N., and Gruber, A. D. (2010). Tissue and Cellular Expression Patterns of Porcine CFTR: Similarities and Differences to Human CFTR. *The Journal of Histochemistry and Cytochemistry*, 58(9):785–97.

244. Polejaeva, I. A., Chen, S.-H. H., Vaught, T. D., Page, R. L., Mullins, J., Ball, S., Dai, Y., Boone, J., Walker, S., Ayares, D. L., Colman, A., and Campbell, K. H. S. (2000). Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature*, 407(6800):86–90.

245. Pompei, F., Polkanov, M., and Wilson, R. (2001). Age distribution of cancer in mice: the incidence turnover at old age. *Toxicology and industrial health*, 17(1):7–16.

246. Prather, R. S., Shen, M., and Dai, Y. (2008). Genetically modified pigs for medicine and agriculture. *Biotechnology & genetic engineering reviews*, 25:245–65.

247. Prather, R. S., Sims, M. M., and First, N. L. (1989). Nuclear transplantation in early pig embryos. *Biology of reproduction*, 41(3):414–8.

248. Prather, R. S., Sutovsky, P., and Green, J. A. (2004). Nuclear remodeling and reprogramming in transgenic pig production. *Experimental biology and medicine*, 229(11):1120–6.

249. Qiu, F.-M., Yu, J.-K., Chen, Y.-D., Jin, Q.-F., Sui, M.-H., and Huang, J. (2009). Mining novel biomarkers for prognosis of gastric cancer with serum proteomics. *Journal of Experimental & Clinical Cancer Research*, 28(1):126.

250. Ramsoondar, J., Mendicino, M., Phelps, C., Vaught, T., Ball, S., Monahan, J., Chen, S., Dandro, A., Boone, J., Jobst, P., Vance, A., Wertz, N., Polejaeva, I., Butler, J., Dai, Y., Ayares, D., and Wells, K. (2011). Targeted disruption of the porcine immunoglobulin kappa light chain locus. *Transgenic Research*, 20(3):643–53.

251. Ramsoondar, J. J., Macháty, Z., Costa, C., Williams, B. L., Fodor, W. L., and Bondioli, K. R. (2003). Production of alpha 1,3-galactosyltransferase-knockout cloned pigs expressing human alpha 1,2-fucosylosyltransferase. *Biology of reproduction*, 69(2):437–45.

252. Ranzani, G. N., Luinetti, O., Padovan, L. S., Calistri, D., Renault, B., Burrel, M., Amadori, D., Fiocca, R., and Solcia, E. (1995). p53 gene mutations and protein nuclear accumulation are early events in intestinal type gastric cancer but late events in diffuse type. *Cancer Epidemiology, Biomarkers & Prevention*, 4(3):223–231.

253. Ray, K. C., Bell, K. M., Yan, J., Gu, G., Chung, C. H., Washington, M. K., and Means, A. L. (2011). Epithelial tissues have varying degrees of susceptibility to Kras(G12D)-initiated tumorigenesis in a mouse model. *PloS One*, 6(2):e16786.

254. Recillas-Targa, F. (2006). Multiple strategies for gene transfer, expression, knockdown, and chromatin influence in mammalian cell lines and transgenic animals. *Molecular Biotechnology*, 34(3):337–354.

255. Reed, S. D. and Li, S. (2009). Electroporation advances in large animals. *Current Gene Therapy*, 9(4):316–326.

256. Reisman, D., Greenberg, M., and Rotter, V. (1988). Human p53 oncogene contains one promoter upstream of exon 1 and a second, stronger promoter within intron 1. *Proceedings of the National Academy of Sciences*, 85(14):5146–5150.

257. Renner, S., Fehlings, C., Herbach, N., Hofmann, A., von Waldthausen, D. C., Kessler, B., Ulrichs, K., Chodnevskaja, I., Moskalenko, V., Amselgruber, W., Göke, B., Pfeifer, A., Wanke, R., and Wolf, E. (2010). Glucose intolerance and reduced proliferation of pancreatic beta-cells in transgenic pigs with impaired glucose-dependent insulinotropic polypeptide function. *Diabetes*, 59(5):1228–38.

258. Rhyu, M. G., Park, W. S., Jung, Y. J., Choi, S. W., and Meltzer, S. J. (1994). Allelic deletions of MCC/APC and p53 are frequent late events in human gastric carcinogenesis. *Gastroenterology*, 106(6):1584–8.

259. Rieber, M. and Rieber, M. S. (2009). DN-R175H p53 mutation is more effective than p53 interference in inducing epithelial disorganization and activation of proliferation signals in human carcinoma cells: Role of E-cadherin. *International Journal of Cancer*, 125(7):1604–12.

260. 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., and Prather, R. S. (2008a). Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. *The Journal of clinical investigation*, 118(4):1571–7.

261. Rogers, C. S., Stoltz, D. A., Meyerholz, D. K., Ostedgaard, L. S., Rokhlina, T., Taft, P. J., Rogan, M. P., Pezzulo, A. A., Karp, P. H., Itani, O. A., Kabel, A. C., Wohlford-Lenane, C. L., Davis, G. J., Hanfland, R. A., Smith, T. L., Samuel, M., Wax, D., Murphy, C. N., Rieke, A., Whitworth, K., Uc, A., Starner, T. D., Brogden, K. A., Shilyansky, J., McCray, P. B., Zabner, J., Prather, R. S., and Welsh, M. J. (2008b). Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. *Science*, 321(5897):1837–1841.

262. Sarkisian, C. J., Keister, B. A., Stairs, D. B., Boxer, R. B., Moody, S. E., and Chodosh, L. A. (2007). Dose-dependent oncogene-induced senescence in vivo and its evasion during mammary tumorigenesis. *Nature cell biology*, 9(5):493–505.

263. Schmitt, C. A., McCurrach, M. E., de Stanchina, E., Wallace-Brodeur, R. R., and Lowe, S. W. (1999). INK4a/ARF mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53. *Genes & development*, 13(20):2670–7.

264. Schnieke, A. E., Kind, A. J., Ritchie, W. A., Mycock, K., Scott, A. R., Ritchie, M., Wilmut, I., Colman, A., and Campbell, K. H. (1997). Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science*, 278(5346):2130–3.

265. Scian, M. J., Stagliano, K. E. R., Ellis, M. A., Hassan, S., Bowman, M., Miles, M. F., Deb, S. P., and Deb, S. (2004). Modulation of gene expression by tumor-derived p53 mutants. *Cancer Research*, 64(20):7447–7454.

266. Sedivy, J. M. and Dutriaux, A. (1999). Gene targeting and somatic cell genetics: a rebirth or a coming of age? *Trends in Genetics*, 15(3):88–90.

267. Selivanova, G. (2010). Therapeutic targeting of p53 by small molecules. *Seminars in Cancer Biology*, 20(1):46–56.

268. Shibue, T., Takeda, K., Oda, E., Tanaka, H., Murasawa, H., Takaoka, A., Morishita, Y., Akira, S., Taniguchi, T., and Tanaka, N. (2003). Integral role of Noxa in p53-mediated apoptotic response. *Genes & development*, 17(18):2233–8.

269. Shimada, S., Mimata, A., Sekine, M., Mogushi, K., Akiyama, Y., Fukamachi, H., Jonkers, J., Tanaka, H., Eishi, Y., and Yuasa, Y. (2012). Synergistic tumour suppressor activity of E-cadherin and p53 in a conditional mouse model for metastatic diffuse-type gastric cancer. *Gut*, 61(3):344–53.

270. Shiozaki, K., Nakamori, S., Tsujie, M., Okami, J., Yamamoto, H., Nagano, H., Dono, K., Umeshita, K., Sakon, M., Furukawa, H., Hiratsuka, M., Kasugai, T., Ishiguro, S., and

Monden, M. (2001). Human stomach-specific gene, CA11, is down-regulated in gastric cancer. *International journal of oncology*, 19(4):701–7.

271. Sigal, A. and Rotter, V. (2000). Oncogenic Mutations of the p53 Tumor Suppressor: The Demons of the Guardian of the Genome. *Cancer Research*, 60(24):6788–6793.

272. Silver, D. P. and Livingston, D. M. (2001). Self-excising retroviral vectors encoding the Cre recombinase overcome Cre-mediated cellular toxicity. *Molecular Cell*, 8(1):233–243.

273. Smit, A., Hubley, R., and Green, P. (2010). RepeatMasker Open-3.0. *http://www.repeatmasker.org*.

274. Smith, G. V., Feakins, R., Farthing, M. J., and Ballinger, A. (2005). Cyclooxygenase 2, p53, b-Catenin, and APC Protein Expression in Gastric Adenomatous Polyps. *American Journal of Clinical Pathology*, 123(3):415–420.

275. Sommer, J. R., Estrada, J. L., Collins, E. B., Bedell, M., Alexander, C. A., Yang, Z., Hughes, G., Mir, B., Gilger, B. C., Grob, S., Wei, X., Piedrahita, J. A., Shaw, P. X., Petters, R. M., and Zhang, K. (2011). Production of ELOVL4 transgenic pigs: a large animal model for Stargardt-like macular degeneration. *The British journal of ophthalmology*, 95(12):1749–54.

276. Soussi, T. and Béroud, C. (2001). Assessing TP53 status in human tumours to evaluate clinical outcome. *Nature reviews. Cancer*, 1(3):233–40.

277. Specke, V., Plesker, R., Wood, J., Coulibaly, C., Suling, K., Patience, C., Kurth, R., Schuurman, H.-J., and Denner, J. (2009). No in vivo infection of triple immunosuppressed non-human primates after inoculation with high titers of porcine endogenous retroviruses. *Xenotransplantation*, 16(1):34–44.

278. Stewart, S. A. and Weinberg, R. A. (2000). Telomerase and human tumorigenesis. *Seminars in cancer biology*, 10(6):399–406.

279. Suerbaum, S. and Michetti, P. (2002). Helicobacter pylori infection. *The New England journal of medicine*, 347(15):1175–86.

280. Sugimura, S., Yamanaka, K.-I., Kawahara, M., Wakai, T., Yokoo, M., and Sato, E. (2010). Early metaphase II oocytes treated with dibutyryl cyclic adenosine monophosphate provide suitable recipient cytoplasm for the production of miniature pig somatic cell nuclear transfer embryos. *Animal Science Journal = Nihon Chikusan Gakkaiho*, 81(1):48–57.

281. Sun, Y., Nakamura, K., Wendel, E., and Colburn, N. (1993). Progression toward tumor cell phenotype is enhanced by overexpression of a mutant p53 tumor-suppressor gene isolated from nasopharyngeal carcinoma. *Proceedings of the National Academy of Sciences*, 90(7):2827–2831.

282. Suter, M. J., Jillella, P. A., Vakoc, B. J., Halpern, E. F., Mino-Kenudson, M., Lauwers, G. Y., Bouma, B. E., Nishioka, N. S., and Tearney, G. J. (2010). Image-guided biopsy in the esophagus through comprehensive optical frequency domain imaging and laser marking: a study in living swine. *Gastrointestinal Endoscopy*, 71(2):346–353.

283. Suzuki, S., Iwamoto, M., Saito, Y., Fuchimoto, D., Sembon, S., Suzuki, M., Mikawa, S., Hashimoto, M., Aoki, Y., Najima, Y., Takagi, S., Suzuki, N., Suzuki, E., Kubo, M., Mimuro, J., Kashiwakura, Y., Madoiwa, S., Sakata, Y., Perry, A. C. F., Ishikawa, F., and Onishi, A. (2012). Il2rg gene-targeted severe combined immunodeficiency pigs. *Cell stem cell*, 10(6):753–8.

284. Swanson, K. S., Mazur, M. J., Vashisht, K., Rund, L. A., Beever, J. E., Counter, C. M., and Schook, L. B. (2004). Genomics and clinical medicine: rationale for creating and effectively evaluating animal models. *Experimental biology and medicine*, 229(9):866–75.

285. Swanson, M. E., Martin, M. J., O'Donnell, J. K., Hoover, K., Lago, W., Huntress, V., Parsons, C. T., Pinkert, C. A., Pilder, S., and Logan, J. S. (1992). Production of Functional Human Hemoglobin in Transgenic Swine. *Biotechnology*, 10(5):557–559.

286. Syder, A. J., Karam, S. M., Mills, J. C., Ippolito, J. E., Ansari, H. R., Farook, V., and Gordon, J. I. (2004). A transgenic mouse model of metastatic carcinoma involving transdifferentiation of a gastric epithelial lineage progenitor to a neuroendocrine phenotype. *Proceedings of the National Academy of Sciences*, 101(13):4471–4476.

287. Takahashi, K. and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, 126(4):663–76.

288. Takata, M., Sasaki, M. S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A., and Takeda, S. (1998). Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. *The EMBO journal*, 17(18):5497–508.

289. Takehara, T., Hayashi, N., Yamamoto, M., Miyamoto, Y., Fusamoto, H., and Kamada, T. (1996). In vivo gene transfer and expression in rat stomach by submucosal injection of plasmid DNA. *Human Gene Therapy*, 7(5):589–593.

290. Tasdemir, E., Maiuri, M. C., Galluzzi, L., Vitale, I., Djavaheri-Mergny, M., D'Amelio, M., Criollo, A., Morselli, E., Zhu, C., Harper, F., Nannmark, U., Samara, C., Pinton, P., Vicencio, J. M., Carnuccio, R., Moll, U. M., Madeo, F., Paterlini-Brechot, P., Rizzuto, R., Szabadkai, G., Pierron, G., Blomgren, K., Tavernarakis, N., Codogno, P., Cecconi, F., and Kroemer, G. (2008). Regulation of autophagy by cytoplasmic p53. *Nature cell biology*, 10(6):676–87.

291. te Riele, H., Maandag, E. R., and Berns, A. (1992). Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. *Proceedings of the National Academy of Sciences*, 89(11):5128–32.

292. Telugu, B. P. V. L., Ezashi, T., Sinha, S., Alexenko, A. P., Spate, L., Prather, R. S., and Roberts, R. M. (2011). LIF-dependent, pluripotent stem cells established from inner cell mass of porcine embryos. *The Journal of Biological Chemistry*, 286(33):28948–53.

293. Tenzen, T., Zembowicz, F., and Cowan, C. A. (2010). Genome modification in human embryonic stem cells. *Journal of Cellular Physiology*, 222(2):278–281.

294. Thomas, K. R., Deng, C., and Capecchi, M. R. (1992). High-fidelity gene targeting in embryonic stem cells by using sequence replacement vectors. *Molecular and cellular biology*, 12(7):2919–23.

295. Thompson, J., Epting, T., Schwarzkopf, G., Singhofen, A., Eades-Perner, A. M., van Der Putten, H., and Zimmermann, W. (2000). A transgenic mouse line that develops early-onset invasive gastric carcinoma provides a model for carcinoembryonic antigen-targeted tumor therapy. *International journal of cancer*, 86(6):863–9.

296. Toledo, F. and Wahl, G. M. (2006). Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. *Nature reviews. Cancer*, 6(12):909–23.

297. Tomioka, N., Morita, K., Kobayashi, N., Tada, M., Itoh, T., Saitoh, S., Kondo, M., Takahashi, N., Kataoka, A., Nakanishi, K., Takahashi, M., Kamiyama, T., Ozaki, M., Hirano, T., and Todo, S. (2010). Array comparative genomic hybridization analysis revealed four genomic prognostic biomarkers for primary gastric cancers. *Cancer Genetics and Cytogenetics*, 201(1):6–14.

298. Tomita, Y., Marchenko, N., Erster, S., Nemajerova, A., Dehner, A., Klein, C., Pan, H., Kessler, H., Pancoska, P., and Moll, U. M. (2006). WT p53, but not tumor-derived

mutants, bind to Bcl2 via the DNA binding domain and induce mitochondrial permeabilization. *The Journal of biological chemistry*, 281(13):8600–6.

299. Touati, E., Michel, V., Thiberge, J.-M., Wuscher, N., Huerre, M., and Labigne, A. (2003). Chronic Helicobacter pylori infections induce gastric mutations in mice. *Gastroenterology*, 124(5):1408–19.

300. Tripathi, S., Ghoshal, U., Ghoshal, U. C., Mittal, B., Krishnani, N., Chourasia, D., Agarwal, A. K., and Singh, K. (2008). Gastric carcinogenesis: Possible role of polymorphisms of GSTM1, GSTT1, and GSTP1 genes. *Scandinavian journal of gastroenterology*, 43(4):431–9.

301. Tsang, W.-p., Ho, F. Y. F., Fung, K.-p., Kong, S.-k., and Kwok, T.-t. (2005). p53-R175H mutant gains new function in regulation of doxorubicin-induced apoptosis. *International Journal of Cancer*, 114(3):331–336.

302. Tu, S., Bhagat, G., Cui, G., Takaishi, S., Kurt-Jones, E. A., Rickman, B., Betz, K. S., Penz-Oesterreicher, M., Bjorkdahl, O., Fox, J. G., and Wang, T. C. (2008). Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. *Cancer cell*, 14(5):408–19.

303. Turk, J. R. and Laughlin, M. H. (2004). Physical activity and atherosclerosis: which animal model? *Canadian journal of applied physiology*, 29(5):657–83.

304. Tuveson, D. A., Shaw, A. T., Willis, N. A., Silver, D. P., Jackson, E. L., Chang, S., Mercer, K. L., Grochow, R., Hock, H., Crowley, D., Hingorani, S. R., Zaks, T., King, C., Jacobetz, M. A., Wang, L., Bronson, R. T., Orkin, S. H., DePinho, R. A., and Jacks, T. (2004). Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. *Cancer cell*, 5(4):375–87.

305. Umeyama, K., Watanabe, M., Saito, H., Kurome, M., Tohi, S., Matsunari, H., Miki, K., and Nagashima, H. (2009). Dominant-negative mutant hepatocyte nuclear factor 1alpha induces diabetes in transgenic-cloned pigs. *Transgenic Research*, 18(5):697–706.

306. van Boxtel, R., Kuiper, R. V., Toonen, P. W., van Heesch, S., Hermsen, R., de Bruin, A., and Cuppen, E. (2011). Homozygous and heterozygous p53 knockout rats develop metastasizing sarcomas with high frequency. *The American journal of pathology*, 179(4):1616–22.

307. Van Cott, K. E., Lubon, H., Gwazdauskas, F., Knight, J., Drohan, W. N., and Velander, W. H. (2001). Recombinant Human Protein C Expression in the Milk of

Transgenic Pigs and the Effect on Endogenous Milk Immunoglobulin and Transferrin Levels. *Transgenic Research*, 10(1):43–51.

308. Van Cott, K. E., Monahan, P. E., Nichols, T. C., and Velander, W. H. (2004). Haemophilic factors produced by transgenic livestock: abundance that can enable alternative therapies worldwide. *Haemophilia*, 10(Suppl 4):70–6.

309. Ventura, A., Kirsch, D., McLaughlin, M., Tuveson, D., Grimm, J., Lintault, L., Newman, J., Reczek, E., Weissleder, R., and Jacks, T. (2007). Restoration of p53 function leads to tumour regression in vivo. *Nature*, 445(7128):661–665.

310. Vinall, R. L., Tepper, C. G., Shi, X.-B., Xue, L. A., Gandour-Edwards, R., and de Vere White, R. W. (2006). The R273H p53 mutation can facilitate the androgen-independent growth of LNCaP by a mechanism that involves H2 relaxin and its cognate receptor LGR7. *Oncogene*, 25(14):2082–93.

311. Vintersten, K., Monetti, C., Gertsenstein, M., Zhang, P., Laszlo, L., Biechele, S., and Nagy, A. (2004). Mouse in red: red fluorescent protein expression in mouse ES cells, embryos, and adult animals. *Genesis*, 40(4):241–246.

312. Vitale-Cross, L., Amornphimoltham, P., Fisher, G., Molinolo, A. A., and Gutkind, J. S. (2004). Conditional expression of K-ras in an epithelial compartment that includes the stem cells is sufficient to promote squamous cell carcinogenesis. *Cancer research*, 64(24):8804–7.

313. Vrba, L., Junk, D. J., Novak, P., and Futscher, B. W. (2008). p53 induces distinct epigenetic states at its direct target promoters. *BMC Genomics*, 9:486.

314. Walker, D. R., Bond, J. P., Tarone, R. E., Harris, C. C., Makalowski, W., Boguski, M. S., and Greenblatt, M. S. (1999). Evolutionary conservation and somatic mutation hotspot maps of p53: correlation with p53 protein structural and functional features. *Oncogene*, 18(1):211–8.

315. Walsh, D. M., Klyubin, I., Shankar, G. M., Townsend, M., Fadeeva, J. V., Betts, V., Podlisny, M. B., Cleary, J. P., Ashe, K. H., Rowan, M. J., and Selkoe, D. J. (2005). The role of cell-derived oligomers of Abeta in Alzheimer's disease and avenues for therapeutic intervention. *Biochemical Society transactions*, 33(Pt 5):1087–90.

316. Wang, J.-Y., Hsieh, J.-S., Chen, C.-C., Tzou, W.-S., Cheng, T.-L., Chen, F.-M., Huang, T.-J., Huang, Y.-S., Huang, S.-Y., Yang, T., and Lin, S.-R. (2004). Alterations of APC, c-met, and p53 genes in tumor tissue and serum of patients with gastric cancers. *The Journal of surgical research*, 120(2):242–8.

317. Wang, T. C., Dangler, C. A., Chen, D., Goldenring, J. R., Koh, T., Raychowdhury, R., Coffey, R. J., Ito, S., Varro, A., Dockray, G. J., and Others (2000). Synergistic interaction between hypergastrinemia and Helicobacter infection in a mouse model of gastric cancer. *Gastroenterology*, 118(1):36–47.

318. Wang, X., Chen, X., and Yang, Y. (2012). Spatiotemporal control of gene expression by a light-switchable transgene system. *Nature methods*, 9(3):266–9.

319. Watanabe, M., Umeyama, K., Matsunari, H., Takayanagi, S., Haruyama, E., Nakano, K., Fujiwara, T., Ikezawa, Y., Nakauchi, H., and Nagashima, H. (2010). Knockout of exogenous EGFP gene in porcine somatic cells using zinc-finger nucleases. *Biochemical and Biophysical Research Communications*, 402(1):14–8.

320. Webster, N. L., Forni, M., Bacci, M. L., Giovannoni, R., Razzini, R., Fantinati, P., Zannoni, A., Fusetti, L., Dalprà, L., Bianco, M. R., Papa, M., Seren, E., Sandrin, M. S., Mc Kenzie, I. F. C., and Lavitrano, M. (2005). Multi-transgenic pigs expressing three fluorescent proteins produced with high efficiency by sperm mediated gene transfer. *Molecular Reproduction and Development*, 72(1):68–76.

321. Wei, Y., Zhu, J., Huan, Y., Liu, Z., Yang, C., Zhang, X., Mu, Y., Xia, P., and Liu, Z. (2010). Aberrant expression and methylation status of putatively imprinted genes in placenta of cloned piglets. *Cellular Reprogramming*, 12(2):213–222.

322. Weinmann, L., Wischhusen, J., Demma, M. J., Naumann, U., Roth, P., Dasmahapatra, B., and Weller, M. (2008). A novel p53 rescue compound induces p53-dependent growth arrest and sensitises glioma cells to Apo2L/TRAIL-induced apoptosis. *Cell Death and Differentiation*, 15(4):718–729.

323. Wheeler, M. B., Bleck, G. T., and Donovan, S. M. (2001). Transgenic alteration of sow milk to improve piglet growth and health. *Reproduction Supplement*, 58:313–24.

324. Whibley, C., Pharoah, P. D. P., and Hollstein, M. (2009). p53 polymorphisms: cancer implications. *Nature Reviews. Cancer*, 9(2):95–107.

325. Whitworth, K. M. and Prather, R. S. (2010). Somatic cell nuclear transfer efficiency: How can it be improved through nuclear remodeling and reprogramming? *Molecular Reproduction and Development*, 77(12):1001–15.

326. Whyte, J. J. and Laughlin, M. H. (2010). Placentation in the pig visualized by eGFP fluorescence in eNOS over-expressing cloned transgenic swine. *Molecular Reproduction and Development*, 77(7):565.

327. Whyte, J. J., Samuel, M., Mahan, E., Padilla, J., Simmons, G. H., Arce-Esquivel, A. A., Bender, S. B., Whitworth, K. M., Hao, Y. H., Murphy, C. N., Walters, E. M., Prather, R. S., and Laughlin, M. H. (2011). Vascular endothelium-specific overexpression of human catalase in cloned pigs. *Transgenic research*, 20(5):989–1001.

328. Will, E. (2002). Unmodified Cre recombinase crosses the membrane. *Nucleic Acids Research*, 30(12):59e–59.

329. Willis, A., Jung, E. J., Wakefield, T., and Chen, X. (2004). Mutant p53 exerts a dominant negative effect by preventing wild-type p53 from binding to the promoter of its target genes. *Oncogene*, 23(13):2330–2338.

330. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J., and Campbell, K. H. S. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature*, 385(6619):810–813.

331. Wong, R. P. C., Tsang, W. P., Chau, P. Y., Co, N. N., Tsang, T. Y., and Kwok, T. T. (2007). p53-R273H gains new function in induction of drug resistance through down-regulation of procaspase-3. *Molecular cancer therapeutics*, 6(3):1054–61.

332. Wu, C.-C., Shete, S., Amos, C. I., and Strong, L. C. (2006). Joint effects of germ-line p53 mutation and sex on cancer risk in Li-Fraumeni syndrome. *Cancer research*, 66(16):8287–92.

333. Xiao, X., Li, J., and Samulski, R. J. (1996). Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *Journal of virology*, 70(11):8098–108.

334. Xie, W. and Evans, R. M. (2002). Pharmaceutical use of mouse models humanized for the xenobiotic receptor. *Drug Discovery Today*, 7(9):509–515.

335. Yamamoto, M., Tsukamoto, T., Sakai, H., Shirai, N., Ohgaki, H., Furihata, C., Donehower, L. A., Yoshida, K., and Tatematsu, M. (2000). p53 knockout mice (-/-) are more susceptible than (+/-) or (+/+) mice to N-methyl-N-nitrosourea stomach carcinogenesis. *Carcinogenesis*, 21(10):1891–1897.

336. Yamanaka, K.-I., Sugimura, S., Wakai, T., Kawahara, M., and Sato, E. (2009). Acetylation Level of Histone H3 in Early Embryonic Stages Affects Subsequent Development of Miniature Pig Somatic Cell Nuclear Transfer Embryos. *The Journal of Reproduction and Development*, 55(6):638–44.

337. Yamaoka, Y., Kato, M., and Asaka, M. (2008). Geographic Differences in Gastric Cancer Incidence Can be Explained by Differences between Helicobacter pylori Strains. *Internal Medicine*, 47(12):1077–1083.

338. Yasuda, K., Shiraishi, N., Suematsu, T., Yamaguchi, K., Adachi, Y., and Kitano, S. (1999). Rate of detection of lymph node metastasis is correlated with the depth of submucosal invasion in early stage gastric carcinoma. *Cancer*, 85(10):2119–23.

339. Yoon, H., Liyanarachchi, S., Wright, F. A., Davuluri, R., Lockman, J. C., de la Chapelle, A., and Pellegata, N. S. (2002). Gene expression profiling of isogenic cells with different TP53 gene dosage reveals numerous genes that are affected by TP53 dosage and identifies CSPG2 as a direct target of p53. *Proceedings of the National Academy of Sciences*, 99(24):15632–7.

340. Yoshizawa, N., Yamaguchi, H., Yamamoto, M., Shimizu, N., Furihata, C., Tatematsu, M., Seto, Y., and Kaminishi, M. (2009). Gastric carcinogenesis by N-Methyl-N-nitrosourea is enhanced in db/db diabetic mice. *Cancer Science*, 100(7):1180–1185.

341. You, J., Kim, J., Lim, J., and Lee, E. (2010a). Anthocyanin stimulates in vitro development of cloned pig embryos by increasing the intracellular glutathione level and inhibiting reactive oxygen species. *Theriogenology*, 74(5):777–85.

342. You, J., Song, K., and Lee, E. (2010b). Prolonged interval between fusion and activation impairs embryonic development by inducing chromosome scattering and nuclear aneuploidy in pig somatic cell nuclear transfer. *Reproduction, Fertility, and Development*, 22(6):977–986.

343. Yu, G., Chen, J., Yu, H., Liu, S., Chen, J., Xu, X., Sha, H., Zhang, X., Wu, G., Xu, S., and Cheng, G. (2006). Functional disruption of the prion protein gene in cloned goats. *The Journal of general virology*, 87(Pt 4):1019–27.

344. Zambrowicz, B. P., Imamoto, A., Fiering, S., Herzenberg, L. A., Kerr, W. G., and Soriano, P. (1997). Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proceedings of the National Academy of Sciences*, 94(8):3789–94.

345. Zhang, X.-P., Liu, F., and Wang, W. (2011). Two-phase dynamics of p53 in the DNA damage response. *Proceedings of the National Academy of Sciences*, 108(22):8990–8995.

346. Zhao, J., Whyte, J. J., and Prather, R. S. (2010a). Effect of epigenetic regulation during swine embryogenesis and on cloning by nuclear transfer. *Cell and tissue research*, 341(1):13–21.

347. Zhao, Z., Hou, N., Sun, Y., Teng, Y., and Yang, X. (2010b). Atp4b promoter directs the expression of Cre recombinase in gastric parietal cells of transgenic mice. *Journal of Genetics and Genomics = Yi Chuan Xue Bao*, 37(9):647–652.

348. Zhao, Z., Sun, Y., Hou, N., Teng, Y., Wang, Y., and Yang, X. (2009). Capn8 promoter directs the expression of Cre recombinase in gastric pit cells of transgenic mice. *Genesis*, 47(10):674–679.

349. Zhukova, E., Afshar, A., Ko, J., Popper, P., Pham, T., Sternini, C., and Walsh, J. H. (2001). Expression of the human insulin gene in the gastric G cells of transgenic mice. *Transgenic research*, 10(4):329–41.

6. Abbreviations

A	adenine
AA	amino acid
AAV	adeno associated virus
AB	antibody
AD	Alzheimer's disease
adMSC	adipose tissue derived MSC
AGS	cell line of a human adenocarcinoma of the stomach
APC	adenomatous polyposis coli gene
ATM	ataxia telangiectasia mutated protein
ATP4B	ATPase, H+/K+ exchanging, beta polypeptide
ATR	ataxia telangiectasia and Rad3-related protein
BAC	bacterial artificial chromosome
Beta-gal	beta-galactosidase
Beta-geo	fusion gene of beta-galactosidase and neomycine resistance
bm	bone marrow
bmMSC	bone marrow derived MSC
bp	base pair
Bcl-2	B-cell lymphoma 2
BH3	Bcl-2 homology domain 3
BS	Blasticidin S
BSA	bovine serum albumine
C	cytosine
CCN	CAGGS-Cherry-NLS
Cdh1	cadherin 1
cDNA	copy DNA
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance receptor
CMV	cytomegalovirus
c-myc	avian myelocytomatosis viral oncogene homolog
COX2	cyclooxygenase 2

Cre	causes recombination
Dapi	4',6-diamidino-2-phenylindole
DBD	DNA binding domain
DMEM	Dulbeccos modified Eagle medium
DMSO	dimethyl-sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide-triphosphate
DSB	(DNA) double strand break
dsDNA	double stranded DNA
DTT	dithio-threitole
E. coli	Escherichia coli
EDTA	ethylene diamine tetra-acedic acid
EGFP	enhanced green fluorescent protein
ESC	embryonic stem cell
ET	embryo transfer
FAP	familial adenomatous polyposis
FCS	fetal calf serum
FGF	fibroblast growth factor
FISH	fluorescense in situ hybridisation
FRT	Flp-recombinase-target
G	guanine
G418	geneticin
GC	gastric cancer
GGTA1	alpha 1,3-galactosyltransferase
GIP	glucose-dependent insulinotropic polypeptide
HBS	HEPES buffered saline
HBSS	Hanks buffered salt solution
HEK293	human embryonic kidney cell line 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNF1 alpha	hepatocyte nuclear factor 1-alpha
HPRT	hypoxanthine ribosyl transferrase
H. pylori	Helicobacter pylori
HR	homologous recombination
HRAS	Harvey rat sarcoma viral oncogene homolog
HSV-tk	herpes simplex virus - thymidin kinase
hTERT	human telomerase reverse transcriptase
ICSI	intracytoplasmatic sperm injection

iPSC	induced pluripotent stem cells
ITS	insuline transferrine selenite
Kato III	cell line of a human stomach carinoma
kDa	kilo Dalton
KDNF	kidney fibroblast
Klf4	Krueppel-like factor 4
KO	knockout
KRT19	cytokeratin 19
LFS	Li-Fraumeni syndrome
LINE	long interspersed element
LSL	loxP-stop-loxP transcription stop cassette
MDM2	murine double minute 2
MDM4	murine double minute 4
MNU	N-methyl-N-nitrosourea
mPTGES1	microsomal prostaglandin E synthase 1
mRNA	messenger RNA
MSC	mesenchymal stem cell
NaCl	sodium chloride
NaOH	sodium hydroxide
NEAA	non-essential amino acid
neo	neomycin
NHEJ	non-homologous end joining
NO	nitric oxide
NOXA	phorbol 12-myristate 13-acetate induced protein 1
NT	nuclear transfer
Oct4	Octamer binding transcription factor 4
P	passage
p53	tumour suppressor protein p53
Panc1	cell line of a human carcinoma of the exocrine pancreas
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pen/Strep	Penicillin / Streptomycine
PERV	porcine endogenous retrovirus
PGK	phosphor glycerate kinase
poFF	porcine fetal fibroblast
prD	proline rich domain
pS2	estrogen-responsive gene trefoil protein

Pu	purine
PUMA	p53 upregulated modulator of apoptosis
Pyr	pyrimidine
qPCR	quantitative PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RT	reverse transcription
RT-PCR	reverse transcriptase PCR
SCID	severe combined immunodeficiency
SCNT	somatic cell nuclear transfer
SDS	sodium dodecyl sulphate
SHP2	Src homology protein 2
SINE	short interspersed element
SMA	spinal muscular atrophy
SMN1	survival motor neuron
Sox2	sex determining region Y-box 2
SV40	simian virus 40
T	thymidin
TAD	transactivation domain
TAE	tris acetate EDTA
TALEN	transcription activator-like effector nuclease
TBE	tris borate EDTA
TP53	gene of the tumour suppressor protein *TP53*
tet	tetracyclin
Tris	tris(hydroxymethyl-)-aminomethane
Ub	ubiquitylation
X-gal	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ZFN	zinc finger nuclease

A. Appendix

A.1. Appendix

A.1.1. Cloning of *TP53* constructs

Construction of the targeting vectors

The main homology region was subcloned by recombineering of a 14kb fragment from a BAC (GenBank: AC127472.4) into a pGEM T easycloning vector by Tatiana Flisikowska. This sequence serves as a template for homologous recombination with the porcine genome in order to introduce a set of modifications. A shorter fragment covering exon 5 was amplified by PCR, subcloned and a G to A transition mutation introduced at the second position of codon 167 of exon 5 by a site directed mutagenesis kit. This leads to an amino acid change from arginin to histidin (R167H) and corresponds to the R175H hot spot mutation in human *TP53*. This construct is referred to as pTP53-ex5mut.

The SfoI/SphI-fragment carrying the mutation was exchanged from pTP53-ex5mut to pTP53-complete, resulting in the vector TP53-completemut. Subsequently, the LSL cassettes (both BS and neo) were initially cloned into the ClaI site in the intron 1 sequence of pGEM-p53completemut, finalising the homologous cassette of the *TP53* gene targeting vectors p53TV-BS and p53TV-neo.

The following trunctation of the short arm was conducted in two steps. First, the LSL was recloned into the PmlI site, 385bp downstream of the original ClaI site within intron 1. This allowed a sufficient short arm length after the second shortening step. The second step was done by excising the short arm fragment up to the AvrII site, and blunt religation of the vector.

Cloning of the PCR control vectors

A 3.8kb fragment spanning from 3.3kb upstream of exon 2 to 440bp downstream in exon 4 was amplified by PCR using the primers p53Int1 for and p53Ex4 rev and subcloned into a pGEM T-easy vector system resulting in the vector pTP53-int1/ex4. This sequence should incorporate the 3' primer binding site for the targeting PCR with the short arm and partial long arm targeting vector homology. The potential forward primer binding location

5' of the intended homology arms was isolated by PCR using the primers p53Int1_2 for and p53Int1 rev and cloned 5' of the aforementioned short homologous sequence. The LSL-BS/neo was cloned into the ClaI restriction site 3' of the short homology sequence. A final XbaI restriction digest and religation reduced the distance of the binding site and LSL by 485bp. This finished construct is referred to as PCR control vector.

Cloning of the splice acceptor test vector

A 678bp fragment of exon 1 and surrounding sequence ranging 229bp upstream and 336bp downstream of the exon was isolated by PCR using the primers p53Ex1 for and p53Int1 rev and subcloned into the pBluescript vector system. The *TP53* sequence surrounding exon 1 was isolated from this vector by NotI/BglII digest and cloned into the NotI/BamHI sites of the vector pBl-CAGGS 3' of the chicken beta-actin promoter and enhancer (CAGGS) promoter. The NotI/NotI fragment of pTP53-int1/ex4 was cloned into the NotI site of this vector to add *TP53* exons 2-4 as alternative splice acceptors. Then the two lox-stop-lox cassettes with the neo and BS resistance were cloned into the ClaI restriction site, to complete the splicetest vectors splice-LSLneo and splice-LSLBS.

A.1.2. *In silico* promoter predictions in the p53 short homology arm

The following sequence in figure A.1 shows the predicted promoter elements within the 3kb short homology arm of the initial *TP53* targeting vector up to the beginning of exon 2. Predicted promoter elements are marked in red, the TATA-box in yellow, the AvrII restriction site that was used for shortening in green and the LSL-cassette locations (ClaI and later PmlI) in blue. This prediction coincides with the luciferase assay in figure 3.7 and the later findings of reduced false positive colonies.

```
CATACAGATAATTCACTTCCCTAAACCACTGCAGTAAATTTCTCCTGTGAGTGAACACACCGTGTTTCCTTTATCTCCTCCCTACTG
ATATAAGGTTGTTTCTTGTCACCTGCCATCAGGAACAACGATGACATGAACATTTTGGGGCAAGGCTCCTGCTCCTGCCGCCTGCAA
CCATTTTTCCAGGGAGTCCATCTAAAAGTGGTTTTGCTAAGTAGTAAGATGTATGTGTTTTCAGTTTTACCAGAGGCTGCCTTGTTT
CTTCCCAGAATCAATTGTTTTTTCCCCTATCACATGGCTTTCAGCCAGATGTTTTTTTCCATCAGACTTCTGGGTGAAGCCAGGGGA
GCCGTTATTTAGAGCTTGGGTGTGGGAGTTCCCTGGTGGCCCAGCAGTTAAAGGACCTGGCCGTTGTCACTGCTGTGACGCAGCTTC
GATCCCCGGCCCGGGAACTTCTGCATGCCACAGGTGAGGGCAAAAATAAAAATGAAACGTAGGGCTTGGCTGTGGCCTCGGATAGCC
CAGGTTCATATTCCTGCCTCTGCCCCTTGGGAGCTTTCTGGGCCTGAGTAATTCCTTTAACCTCAGTTCCCTCCTCTGATGATACAG
CACCTGCCTCACTGGCTTTTTGAGAGGATTAATGAGATAAGCCAGCGAAAGCTGTGGGTCCAGATAGGGTCTGTTTTCGCAGCAGCC
TTGTTGTTCTGATAATGGGCAGGCCTGCCCTGCCTTTGAGTCTCAGCCGAGTTTATTGCCTTCCCACCTGGCTGCCGCCCCGATCAG
CTTAGCCGGAGAGTCTGGTTCCTCTTCTGACAGTTTAAGATCATGACCCCCTGATGTTCAGACCCATGGAAAGCACCTACTCTGAAG
AGGTTTCCTAGAGAAAAGAACATTGAAAATTTAGTTGCTACCAGCCACTGAGGGAGAGACACCTCCTATGTCCATGGGGCTGTCTCC
CCCTCAGTGGGCCTGAGACCCTGACCCACCATCCCCCGACAGCCCACATACCCACGGGTTTATTGAACACGGGGTTGCGGAAAGAA
GTGAGCTAGCACAGATGTGGGCAGAACCACAAAAACCCCACCCCCCTGCAATCACAGAGCTCTCGCTCTCAATAATAGAGAACCAGG
AGTTCCCACTGTGGTTCAGCAGTAACGAACCCGAATGGGCTCCATGAGGACGCAGGTTCGATCCCTGGCCTCACTCAGTGGGTTAAG
GATCCGGTGTCGCCATGAGCTGAGCTGTGGTGTAGGTTGCAGACATGGCTCGGATCCCGAGTTACTGTGGCTGTATCGTAGGCTGGC
GGCTCTAGCTCTCATTCAGCCCCTAGCCTGGGAACTTCCATATGCCACAGGTATGGCCCTAAAAACGTATATATATATCTGGCCCTT
TTCTCAACGCTACATTTCCCTTAATAACTGGAGTACTATCTTAGAGCTGAGGACACTTAAGGGACAGAGACGTTAAGCAACCGCCCA
AAGTTACACAGCTATTCCATGGTGAGCTTGGGATGCAGGCCTAGCGCATCTGCCTCTAGAGCCCGTGCTCTGCCATCTTTGCCCTGG
GAGCTTTGATGAGTGAAATCATGCAGTGAATTTAAGTGATGATACAACATCAAACCCAGGGCTTCCGACTTCTAAGCCCCATCTCTG
TTTACACAAGCCTCCCTTCCATAAAAACCTCAAAGTTATTTTTATCCTCAATTTAGAGAGGGGGACCCTGGTCCTTCCCCCCAAGGC
AAATATTTAGAGTCCCACTGCTGTCCCCTCTTGCCTTCCTTCGTGCACGTTCATGGCCTGTCCCCTGTCATCATTGGGAGCTATATA
AGTGGCCCTTTGCCGGCCTCTGACTTTCATCAGCTAAAATCTGCACCTCTAGACGTAACTGATGACAAAAGTTACGTTATGCAAAGT
CATCTTAATCCTTAATCCTAGGCATTCAAATGGTCAGTTTTTTGTAAAAGCTCCTTCACTAACCTGTGCATTGCAGGCTGCTCCCTT
AACCTCTCTGAGACTGTCAAGAATCAGGTGTGCTTAGCGAGACAGACAACCCATGTAGAAAGGGTTTGCACACTCTAAATAAAGCAG
          AvrII
AAAGCAGGCCCTAGGTCAACATAAAGGAGCGAGTGAATATTAGAGGAGATTAGAAAACAGCTGCTTGTTGGAGTTCCTGTCGTGGCA
CAGCGGAAACAAATCCAATTAAGAACCATGAGGTTGCAGGTTCGATCCCTGGCCTCGCTCCGTGGGTTAAGGATCGGGCATTGCTCT
GAGCTGTGGTGTACGTCGCAGATGTGGCTCGGATCTGGTGTGGCTGTGGCTGTGGTGTAGGCCGACAGCTGTACCTCCGATTGGACC
CCTAGCCTGGGAACCTCCATATGCCGTGGGTGCAGCCATTTTAAAAAAAAAAAAAAAAAAAAAAAAAGCTGCTTGCTGCGGGTGGGGC
GGTGGGGAGGTGTTTTCAGTGCCATTACATTAACACAGAAGGAGAAGAGTAATGGCAGGGATTTTTGCAGCGAGCATAATTCTGAGC
AAAGCTGATGACAGATTTGGCTTCGAGAAAGATGGCATGATGAAACCATTTGAAAGCTGAGCTGGGAGATGAGATGATAGAGGCATA
GCCTAGTGCGGGGCCCTGGCCCAGACCACACAGCTACCCCTCGGTCCTCCCTCGTACCCTTGACCAATGATAACCAGCTCACAGCCT
GGGCACAACAATTCATTCAGTCACCACGGTGACTGAGCCCCGAGCAGGTGCAGGGCAGTATGCTAATAGCTGGCGAAGGAGATGCTT
CCCTGCCCTGAGGAAGTTTATATCCCAGCAGCACACACAGAGCGAGCGAGAAAGGTCAGCAGGTAGTGTAAGCACGAGGAGCACTAA
                                                            ClaI
CAACAGGGTGACCTGAATTCGAATAAGGTGACAGAGTTTGGCTGGCTGTCCTTGATCGATCGAGCGGTCTGGGTCCCTGCTGAGGTG
TGACCCTCAGGATGGGGTCCCAGTGAGAAGGAGCTTGCCCTTCAGTGATGGCAGGACCACTGTTCCCAGGAGTGAAATGAACCAGCG
AAGGTCCGCCGTGCAGGGAAGGGCAGCCACTGCCGCTCGAGCATCGGGAAGGGAGAGGGTGCTAGAAGATGAGATCAGAGAGGTGGG
CAGGGGCTGGGTGGTGAGGAATTTGTATGCCAAGGTGGGGGGGGCCGGCCCCAAGGGAGCAGGGGGGAACGTGGGGGGGCCAGGGAG
GCATCTCCCCCTGCCCCGCCCTGACCGCGGTGGAGGGACAGGGCTGAGTTACTTCATCCTGATTCCCACAGAGGAGGGCAGACATGG
    PmlI
GTACACGTGGCGGAAGGCTTGTGGATTCAGTGGTGGAAGGGTGGACAATCCCTCTCTGGTTATCTTTTCCCCGCGGGGATGTGCGTG
GTGAGGGGTGGGGCAGGTGCAGCCTGGCATGTGAAGGGCAGGGCGCTGCCCCAGCAGCAGAGCCTCACCACGGGTGAGTGTGGACGG
AGGGGGTCCCTCTTCCCTCGGGGCTTGGGTGTGTCTCAGGCTGGATCCTGGCCTTTCTCCCCACAGCGGCCACACTCCCCTCCAGGG
AGCTGCAATGGAGGAGTCGCAGTCCGAGCTGGGCGTGGAGCCCCCTCTGAGTCAGGAGACATTTTCAGACTTGTGGAAACT
```

Figure A.1.: Sequence of *TP53* intron 1 from the 5' end of the short homology arm of the *TP53* targeting vector to the beginning of exon 2. red = Predicted promoter elements, yellow = TATA-box, green = AvrII restriction site that was used for shortening, blue = LSL-cassette locations (initial ClaI and final construct PmlI).

A.1.3. Detailed *TP53* pathway

Figure A.2 shows a more detailed overview of the *TP53* pathway. It displays the complex interplay that makes *TP53* one of the central regulators of the cell cycle.

A.1.4. Cloning of the reporter constructs

The pCAGGS plasmid containing a CAGGS and rabbit globin polyA was obtained from Tobias Richter. An mCherry reporter gene was isolated by PCR with the primers Cherry XbaI ATG for and Cherry EcoRI Stop rev and subcloned into the XbaI/EcoRI sites of the vector, generating pCAGGS-mCherry. A similar construct pCAGGS-EGFP was cloned with the EGFP gene isolated by PCR with the primers EGFP+ATG +XbaI and EGFP+Stop +EcoRI by Vera Zywitza. She also cloned the pCAGGS-Cre-T2A-EGFP/Cherry expression vectors as part of her Bachelor thesis.

The beta-geo reporter gene was isolated from pbgeo plasmid obtained from Angelika Schnieke. The XbaI fragment of beta-geo was blunt end subcloned in the blunt BamHI/EcoRI position of the LSL-neo, thereby providing it with flanking loxP sites and triple polyA signals and replacing the neo-resistance gene. This lox-geo-pA-lox cassette was cloned into the pCAGGS-mCherry vector, into the XbaI/NheI restriction site 5' of the mCherry gene, thereby completing pCAGGS-loxgeo-mCherry. The identical subcloning steps were used to clone pCAGGS-loxgeo-EGFP.

A.1.5. Cloning of the KRT19 expression vectors

The KRT19 promoter sequence was isolated by Thomas Briehl from human DNA with the primers KRTfor and KRTrev and subcloned into pJET1.2/blunt. As part of his Master thesis, he cloned the KRT19 promoter into the pCAGGS-Cre-T2A-EGFP/Cherry constructs, replacing the CAGGS promoter leading to KRT19-Cre-T2A-EGFP/Cherry. The KRT19 promoter was shortened to 2.1kb by an AccI/SacI digest to amplify specificity for gastric cell lines.

For the expression constructs of the inflammatory and proliferatory factors the sequence of COX2 cDNA was isolated from porcine kidney RNA by RT-PCR with the primers COX2 F and COX2 R. Also, the mPTGES1 cDNA was isolated from porcine kidney RNA by RT-PCR with the primers PTGE1 F and PTGE1 R and the genomic sequence of Wnt1 was isolated by PCR from porcine DNA with the primers Wnt1 F and Wnt1 R. All three were subcloned into pGEM-T-easy vectors. Subsequently, the sequences were cloned in the EcoRI sites between the KRT19 promoter and the rabbit globin polyA signal of the KRT19-Cre-T2A-EGFP plasmids, replacing the Cre-T2A-EGFP cassette. This lead to the expression constructs KRT19-COX2, KRT19-mPTGES1 and KRT19-Wnt1.

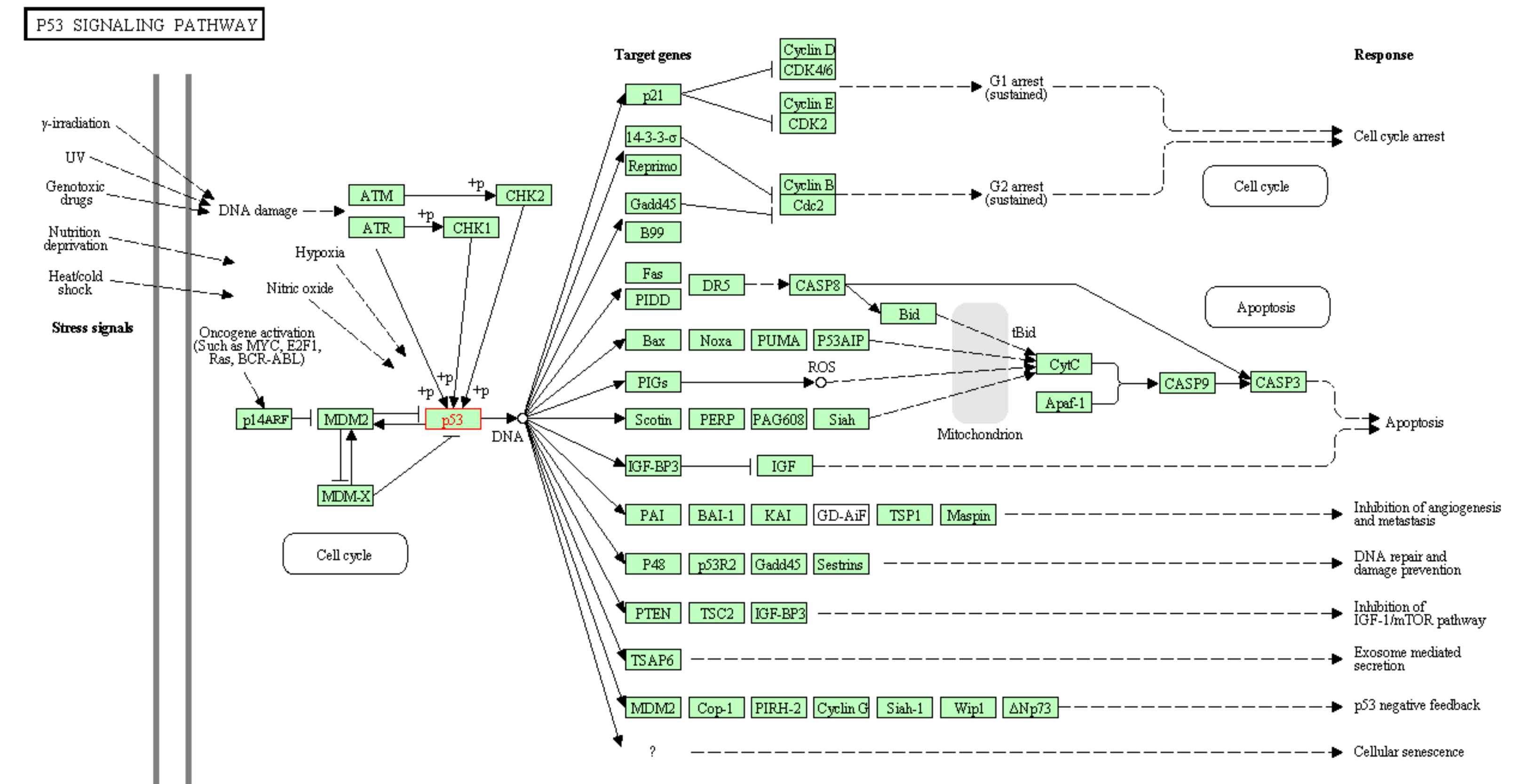

Figure A.2.: Detailed schematic of p53 pathway. Phosphorylations are noted together with "+p". p53 target genes and the resulting responses are shown on the right. Figure taken from Kyoto Encyclopedia of Genes and Genomes (http://www.kegg.jp/).

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"Fertsch da Lack" - [P.M.]

Declaration

I herewith declare that I have produced this work without the prohibited assistance of third parties and without making use of aids other than those specified; notions taken over directly or indirectly from other sources have been identified as such. This work has not previously been presented in identical or similar form to any other German or foreign examination board.

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Munich, June 4, 2013

Curriculum Vitae

Persönliche Daten

Name	Simon Leuchs
Geburtsdatum	04.09.1982
Geburtsort	München, Deutschland

Ausbildung

06/2008 - 03/2012	Promotionsstudium, Molekulare Biotechnologie Technische Universität München - WZW - Livestock Biotechnology Thesis: "Conditional gene targeting of TP53 in pig - a model for Li-Fraumeni disease and gastric cancer"
10/2006 - 05/2008	Master of Science, Molekulare Biotechnologie Technische Universität München Abschlussnote 1,2 (ECTS A) Thesis: "RNA Interferenz in Nutztieren - Knockdown des bovinen beta-Lactoglobulin Gens"
10/2003 - 09/2006	Bachelor of Science, Molekulare Biotechnologie Technische Universität München Abschlussnote 1,3 (ECTS A) Thesis: "Screening und Produktion monoklonaler Antikörper zur immunchromatografischen Isolierung von Proglycinin aus Soja-Extrakt"
09/1993 - 05/2002	Louise-Schröder-Gymnasium, München Allgemeine Hochschulreife Abschlussnote 1,2

Veröffentlichungen

Leuchs, Saalfrank, Kind, Wolf, Schnieke et al.: "Inactivation and Inducible Oncogenic Mutation of p53 in Gene Targeted Pigs." Public library of Science: PloS One (2012), Cambridge, UK; Vol. 7; Nr. 10; S. e43323

Merkl, **Leuchs**, Saalfrank, Kind, Schnieke: "RNA interference in pigs: comparison of RNAi test systems and expression vectors." Molecular Biotechnology (2011), Humana Press, New York NY, USA; Vol. 48 Nr. 1: S. 38-48.