TECHNISCH UNIVERSITÄT MÜNCHEN

Lehrstuhl für Biotechnologie der Nutztiere

Gene-targeted pigs predisposed to colorectal cancer

Martina Landmann

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

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. M. Schemann

Prüfer der Dissertation:

1. Univ.-Prof. A. Schnieke, Ph.D.
2. Univ. Prof. Dr. Dr. H. H. D. Meyer

Die Dissertation wurde am 07.05.2010 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 29.09.2010 angenommen.

Abstract

Colorectal cancer (CRC) is the second most common cancer in Germany diagnosed in men and women. The Adenomatous Polyposis Coli (APC) protein plays a key role in colorectal tumor development. Germline mutations in the corresponding *adenomatous polyposis coli* (*apc*) gene cause the inherited human CRC syndrome familial adenomatous polyposis coli (FAP), which manifests itself by colorectal adeno-carcinoma with early onset. Mice with *apc* mutations fail to develop colorectal carcinoma and metastases characteristic for the human disease.

This work describes the generation of a potential porcine model predisposed to colorectal cancer. Pigs are supposed to be a better model for CRC due to their anatomy, physiology, size, genetics and diet. Targeting constructs for the two most common germline mutations in the human *apc* gene of FAP patients at codon 1061 and 1309 were generated. Both mutations introduce stop codons in exon 15 of the *apc* gene. The targeting vectors were based on a promotor-trap approach and designed with porcine *apc* sequences.

In contrast to mice, where embryonic stem cells are widely used for the development of transgenic animals, such cells are not available in pigs. Consequently, it was important to isolate proliferating and genetically stable primary cells, which are efficiently transfectable. These expectations were met by porcine mesenchymal stem cells. Accordingly, these cells were characterised and their genomic stability was analysed. Efficient transfection and selection methods were developed. Electroporation was chosen due to high transfection efficiency with low toxicity. Porcine mesenchymal stem cells with mutations at the codons corresponding to the human codons 1061 or 1309 were selected, screened and used as nuclear donors for somatic cell nuclear transfer.

Gene-targeted, heterozygous piglets with mutations at codon 1061 of the porcine *apc* gene were generated. These pigs represent the first porcine cancer model and the second porcine disease model. Additionally, the mutation in the *apc* gene is the third gene-targeted gene in the pig. Future work will reveal whether these pigs are predisposed to colorectal cancer and may therefore offer many advantages for research and clinic. The methods described herein should also enable the development of further livestock models of human diseases.

Zusammenfassung

Dickdarmkrebs ist die zweithäufigste Krebserkrankung in Deutschland. Eine zentrale Rolle in der Darmkrebsprogression nimmt das Adenomatous Polyposis Coli (APC) Protein ein. Keimbahnmutationen im humanen *adenomatous polyposis coli* (*apc*) Gen führen zu dem erblichen familiären adenomatösen Polyposis (FAP) Syndrom, bei dem die Patienten früh Dickdarmkarzinome entwickeln. In Mausmodellen mit *apc* Mutationen traten jedoch weder Dickdarmkarzinome noch Metastasen auf.

In der vorliegenden Arbeit wird die Entwicklung eines Schweinemodells mit einer genetischen Veranlagung für Darmkrebs beschrieben. Schweine eignen aufgrund ihrer Anatomie, Physiologie, Größe, Genetik und Ernährung sehr gut als Modelltiere für humane Erkrankungen. Für die Entwicklung des Schweinemodells wurden zwei Mutationsvektoren mit den beiden am häufigsten auftretenden Keimbahnmutationen bei FAP Patienten an Codon 1061 and 1309 des *apc* Gens erstellt. Beide Mutationen fügen ein Stopcodon in Exon 15 des *apc* Gens ein.

Im Gegensatz zu transgene Mausmodellen, die mit Hilfe von embryonalen Stammzellen erzeugt werden, stehen diese Zellen in Großtieren nicht zur Verfügung. Stattdessen mussten Zellen gefunden werden, die gute Wachstumseigenschaften aufweisen, genetisch stabil sind und sich effizient transfizieren lassen. Diese Anforderungen wurden von mesenchymalen Stammzellen des Schweins erfüllt. Die Zellen wurden charakterisiert, ihre genetische Stabilität untersucht und geeignete Transfektions- und Selektionsmethoden entwickelt. Dabei wies Elektroporation eine hohe Transfektionseffizenz und geringen Toxizität auf. Mittels homologer Rekombination wurden die *apc* Mutationen in die Zellen eingeführt, modifizierte Zellen mit den korrespondierenden Mutationen zu den humanen Codons 1061 oder 1309 ausgewählt und als Ausgangsmaterial für somatischen Kerntransfer verwendet.

Es wurden erfolgreich genetisch veränderte Ferkel mit Mutationen an Codon 1061 des *apc* Gen produziert. Diese Schweine stellen somit das erste Krebsmodell im Schwein dar und sind das zweite Modell einer Humanerkrankung im Schwein. Die Mutation im *apc* Gen ist erst die dritte gezielte Modifikation eines Gens im Schwein. Diese Ferkel haben eine Veranlagung für Dickdarmkrebs und bieten damit vielfältige Möglichkeiten für Forschung und Klinik. Die im Rahmen dieser Arbeit beschriebenen Methoden erlauben die Entwicklung weiterer Großtiermodelle menschlicher Erkrankungen.

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1 Introduction

1.1 Colorectal Cancer

Colorectal cancer (CRC) is the second most common cancer in men and women in Germany, and also the second leading cause for cancer-related deaths (in 2004) (Robert-Koch-Institut, 2008). Colorectal cancers include carcinomas of the colon, rectum and anus. 95% of colorectal carcinomas are adenocarcinomas, which arise from adenomatous polyps in the colon. Other types are lymphomas or squamous cell carcinomas. The life-time risk of developing CRC is 5% (Jemal *et al.,* 2006). The five year survival rate is 60% (Robert-Koch-Institut, 2008).

In men CRC is diagnosed at an average age of 69 years and in women at an average age of 75 years (Robert-Koch-Institut, 2008). An early onset before the age of 50 is usually connected to a familial history of colorectal cancer. Risk factors for colorectal cancer are excess weight, lack of exercise, chronic inflammatory bowel diseases and a low fibre / high fat diet. A contribution of a red meat rich diet, folate, nonsteroidal antiinflammatory drugs (NSAID), calcium and vitamin D is also suspected (Giovannucci *et al.,* 1994; Norat *et al.*, 2005; Rodriguez-Bigas *et al.*, 2003).

1.1.1 Molecular basis of colorectal cancer

Due to the high incidence rate of CRC and the accessibility of tumor samples by endoscopy, it has been possible to study the various stages in disease development from precancerous lesions to tumors. In 1990, Fearon and Vogelstein proposed a well-defined sequence in the progression of colorectal carcinomas, shown in Figure 1. According to this model, carcinoma development is a result of an accumulation of mutations, leading to activation of oncogenes and inactivation of tumor-suppressor genes. It is thought that at least four to five mutations are necessary for the development of CRC (Fearon and Vogelstein, 1990).

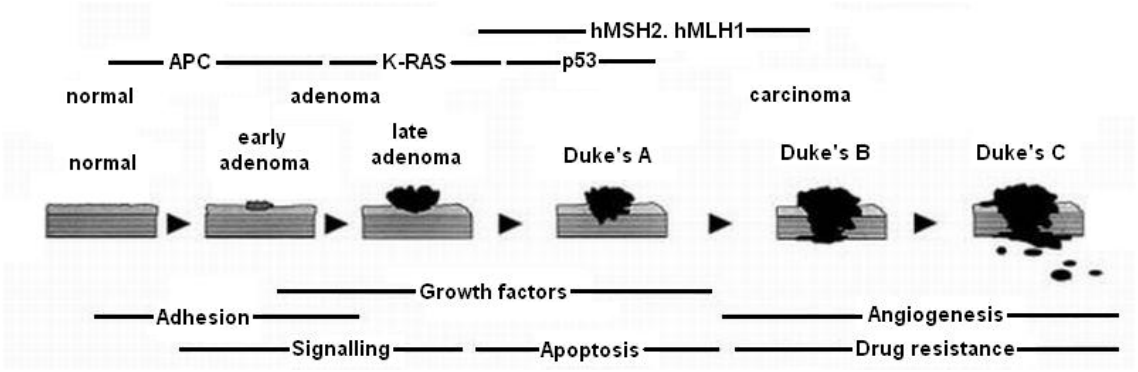

Figure 1: The colorectal cancer progression cascade. Mutations in the *apc* gene are the first mutations in tumor progression. Further mutations as in the K-*ras* or *Tp53* genes occur at later stages (adapted from Bodmer, 2006).

In about 70% of all sporadic colorectal cancers the earliest mutations occur in the *adenomatous polyposis coli* (*apc*) gene (Powell *et al.*, 1992). This leads to the first visible lesions, aberrant crypt foci (Figure 2).

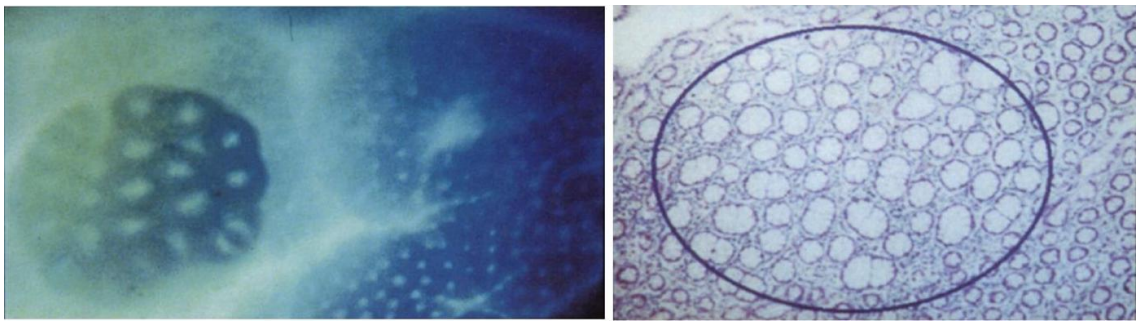

Figure 2: Aberrant crypt foci. Macroscopic (left) and microscopic (right) appearance of aberrant crypt foci (adapted from Ponz de Leon and Di Gregorio, 2001).

The progression of these aberrant crypt foci towards neoplasia is associated with an accumulation of further mutations. The chronological sequence of these mutations is less important for the biological properties of the carcinomas than the accumulation of mutations in key genes (Fearon and Vogelstein, 1990). In most cases the next steps are mutations in the genes K-*ras* and *Tp53* (Vogelstein *et al*., 1988), which occur in 50% of all adenomas (Bos *et al.,* 1987; Vogelstein *et al.*, 1988; Iacopetta, 2003). Later in the CRC progression deletions in the gene *DCC* (*Deleted in Colorectal Carcinoma*) appear in more than 70% of all carcinomas (Vogelstein *et al.*, 1988). Additional mutations are identified in genes responsible for mismatch repair (*hMLH1*, *hMSH2, hMSH6)*, cell cycle (*p16, p14*), growth factors and their signalling factors (*SMAD4*, *TGFβIIR*) and immune response (*beta2m*, *HLA Class I*) (Bodmer, 2006).

1.1.2 Intestinal homeostasis

An understanding of the mechanisms underlying neoplasia and progression to cancer in the intestine first requires a description of the normal intestinal physiology. The human gut can be divided into the small intestine, which is separated in duodenum, jejunum and ileum, and the large intestine consisting of caecum and colon. The epithelium of the small intestine consists of villi and crypts, whereas in the colon a flat surface epithelium only with crypts is present (Figure 3). At the bottom of each colonic crypt there are approximately six stem cells (Barker *et al*., 2007). In contrast to the colon, the stem cells of the small intestine do not reside at the bottom of the crypts, but above Paneth cells, which exist only in the small intestine. The intestinal stem cells give rise to the four different intestinal lineages: mucus secreting goblet cells, Paneth cells, absorptive and enteroendocrine cells. Cells differentiate along the crypt-villus-axis and shed off the tip of the villus. The cells are renewed every four to five days. The underlying molecular mechanism, which determines the balance between self-renewal and differentiation, is the Wnt pathway.

1.1.3 Wnt signalling

The Wnt pathway is a highly conserved pathway in multicellular organisms, which plays an important role in embryogenesis, but also in tissue maintenance and cancer. Wnt signalling is also relevant in the homeostasis of the intestine (Korinek *et al.*, 1998; Kuhnert *et al*., 2004). As reviewed by Sancho *et al*. (2003) Wnt signalling in the intestine has three main functions: It mediates the proliferation and differentiation of cells along the crypt-villus-axis, it regulates the migration of the precursor cells along the axis and it regulates the positioning of Paneth cells in the small intestine.

The intracellular mechanisms of Wnt signalling (Figure 4) are mediated through β-catenin. In general, Wnt ligands are absent and β-catenin is phosphorylated in the cytoplasm by serine/threonin kinases, casein kinase Iα (CKIα) and glycogen synthase kinase 3β (GSK-3β) (Logan and Nusse, 2004). The phosphorylation is initiated by a complex made up of Axin, APC and GSK-3β, which interacts with β-catenin through binding regions on the APC protein (Rubinfeld *et al.*, 1996). After phosphorylation, β-catenin is ubiquitinated by the E3 ubiquitin ligase β-TrCP and follows the ubiquitin-dependent degradation in the proteosome (Figure 4, left side) (Gilbert, 2000). In the activated pathway, which is responsible for the maintenance of the stem cell

compartment, Wnt ligands bind to the receptors Frizzled (Fzl) and co-receptor Low Density Lipoprotein Receptor-related Protein (LRP) (Logan and Nusse, 2004). The corresponding Wnt ligands in the developing murine colon are Wnt-5a and Wnt-11, which bind to Fz-5 / Fz-7 and LRP-5 / LRP-6 (Gregorieff *et al.,* 2005; Lickert *et al*., 2001).

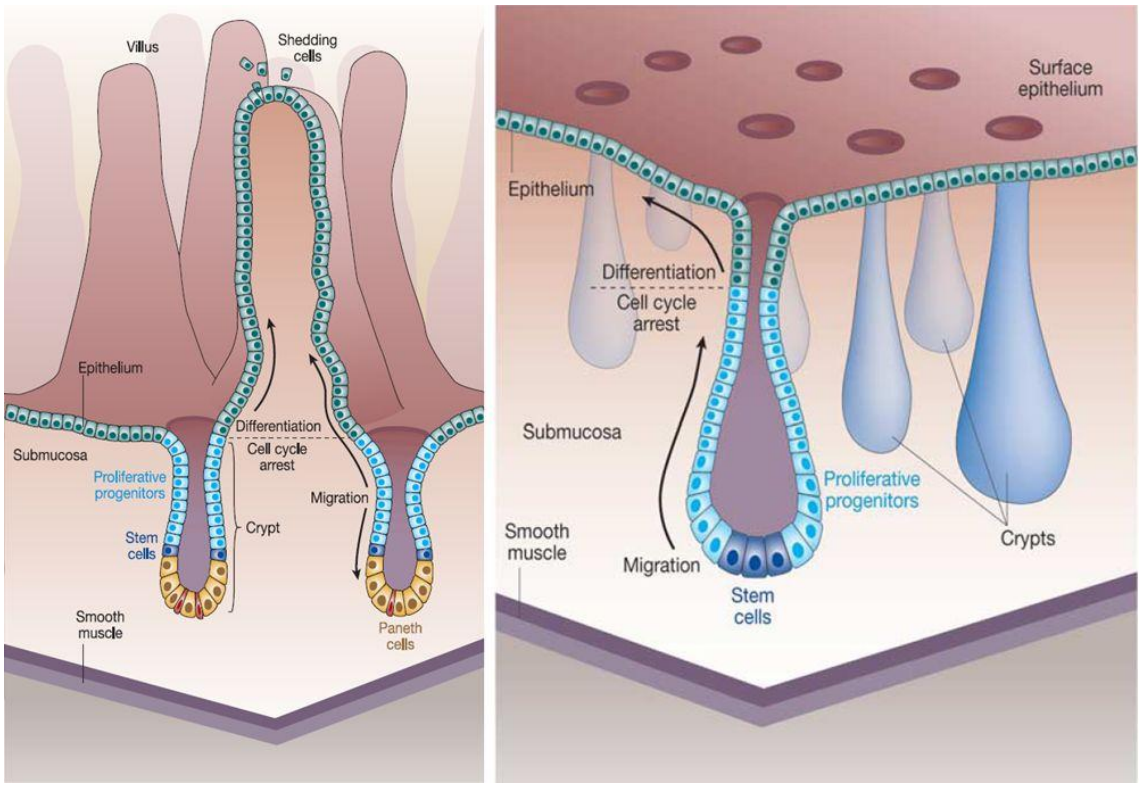

Figure 3: Normal physiology of small intestine and colon. Left: Epithelium of the small intestine with crypts and villi. Stem cells (dark blue) are within the crypts above the Paneth cells (red). Right: Colon epithelium with crypts. The stem cells rest (dark blue) on the bottom of the crypts. Cells migrate from the bottom of the crypt to the tip, where they shed off into the intestinal lumen (adapted from Reya and Clevers, 2005).

The molecular components of the Wnt pathway are shown in Figure 4. When both receptors are activated by a Wnt signal, Fzl receptor phosphorylates and activates Dishevelled (Dsh). The protein Dsh inactivates the destruction complex via an inhibition of GSK-3β and Axin (Logan and Nusse, 2004). Therefore β-catenin is not ubiquitinated, accumulates within the cytoplasm and is translocated into the nucleus. In the nucleus β-catenin forms heterodimers with transcription factors of the T-cell Factor (Tcf) and/or Lymphoid Enhancer Binding Protein Factor (Lef) family and activates Tcf/Lef-regulated genes, which promote proliferation and inhibit differentiation of stem cells (Figure 4) (Gilbert, 2000). Targets of Tcf-4 signalling are the receptors EphB2 and EphB3 and their ligand ephrin-B1, which maintain the boundaries between the proliferating and

differentiated cell compartment in the crypts (Batlle *et al.,* 2002). Important for the tumor development might be the effect of β-catenin/Tcf signalling on the expression of the tumor oncogene c-myc (He *et al*., 1998).

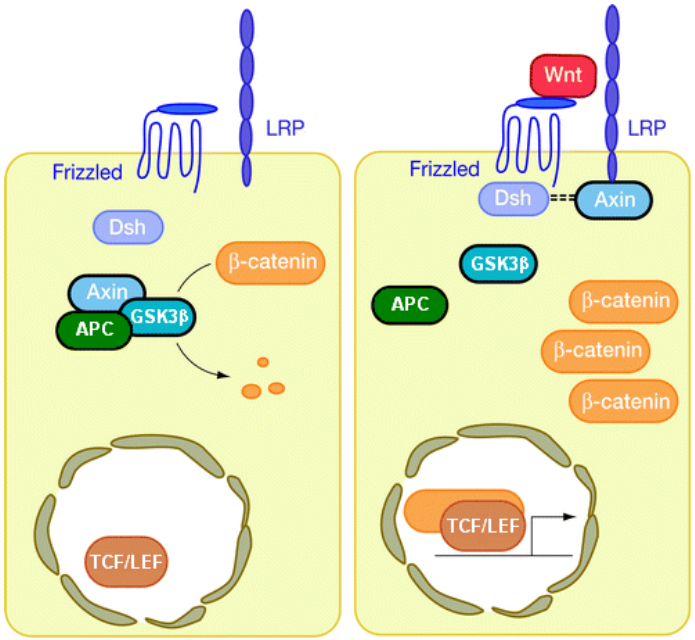

Figure 4: The Wnt pathway. Left side: Without a Wnt signal, GSK-3β is active and associates with APC, Axin and β-catenin, which leads to the degradation of β-catenin. Right side: Active Wnt binds to Frizzled and the LRP receptor, which leads to the activation of Dsh. Dsh inhibits GSK-3β. Inactive GSK-3β leads to free β-catenin, which translocates into the nucleus and – in combination with Tcf/Lef-transcription factors – activates Wnt-dependent gene transcription (adapted from Logan and Nusse, 2004).

Cell fate along the crypt-villus-axis is regulated through differential β-catenin expression and the resulting Tcf-4 regulation (van de Wetering *et al.,* 2002). In Tcf-4$^{-/-}$ mice the stem cells are absent from the small intestinal crypts and the intestinal epithelium consists entirely of differentiated, non-proliferating cells (Korinek *et al.,* 1998).

1.1.4 The Wnt pathway and colorectal cancer

In normal intestinal epithelium the intestinal stem cells receive Wnt-5a signals from the underlying mesenchymal cells (Gregorieff *et al.,* 2005; Lickert *et al.,* 2001). This activation of the Wnt pathway in the stem cells leads to β-catenin accumulation. The stem cells then proliferate and give rise to intestinal progenitor cells. These progenitor cells migrate upwards, where the Wnt signalling decreases and APC expression increases (Miyashiro *et al.,* 1995; Smith *et. al.*, 1993). This causes the cells to

differentiate and shed off into the intestinal lumen after three to four days. Without an APC signal, β-catenin is not degraded resulting in an accumulation of undifferentiated, proliferating cells. These cells migrate upwards, cluster and give rise to adenomatous polyps (Figure 5) (Midgley *et al.*, 1997; van de Wetering *et al.*, 2002).

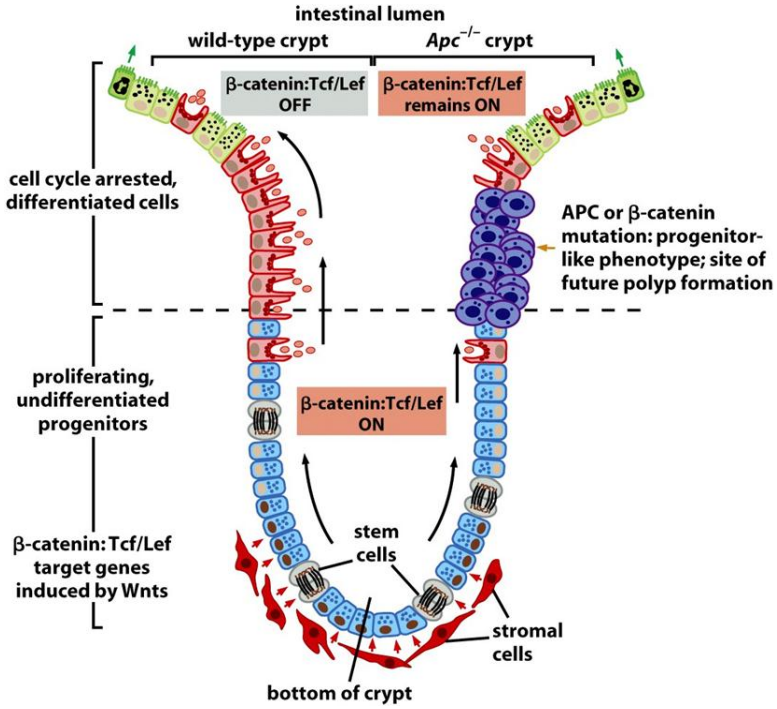

Figure 5: β–catenin expression in colon epithelium. Cells migrate from the bottom of the crypt, where the stem cells reside (red arrows), to the tip, where they shed off. Cells on the bottom of the crypt show high levels of β–catenin, as they receive Wnt signals from the underlying cells (red stroma cells). In normal intestinal epithelium, which is shown on the left side of the picture, the progenitor cells migrate upwards, where Wnt signalling decreases and therefore the cells differentiate and shed off into the lumen after 3 to 4 days (green arrow). In APC deficient crypts (shown on the right side), β–catenin is not degraded - even in the absence of Wnt signals - and undifferentiated, proliferating cells (purple cells) migrate upwards where they cluster and may lead to adenomatous polyps (from Weinberg, 2006).

Therefore *apc* mutations and the consequential β-catenin accumulation may lead to an overpopulation of the crypt with proliferating cells (Boman and Huang, 2008). Experiments with transgenic mice support this theory. APC^{580S} mice (see Table 2) have a conditional frameshift mutation at APC amino acid (aa) 580 that is activated with Cre recombinase. If the Cre recombinase is active within the amplification and differentiation compartment of the intestine no adenomas are found (Barker *et al.*, 2008). However, an activation of the *apc* mutation within the stem cell compartment leads to a rapid development of microadenomas in the small intestine and the colon, which develop into adenomas within three to five weeks (Barker *et al.*, 2008).

The APC protein regulates the Wnt pathway and therefore acts as a 'gatekeeper' of the intestinal equilibrium of proliferation and differentiation (Kinzler and Vogelstein, 1996). APC balances proliferation and apoptosis to maintain a certain cell number in the intestinal epithelium in normal physiology as well as after injuries. Because of its central role in tissue maintenance and cancer development, APC is here described in more detail.

1.2 The *apc* gene, protein and its function

The *adenomatous polyposis coli* (*apc*) gene is a tumor suppressor gene, which is located on human chromosome 5 (5q21-q22) (Herrera *et al.*, 1986; Bodmer *et al.*, 1987; Leppert *et al.*, 1987). The human *apc* locus spans 98 kb and consists of 15 exons (Groden *et al.*, 1991). It encodes a 10719 bp mRNA with an 8532 bp open reading frame. This mRNA is translated into a 2843 amino acid protein with a molecular weight of 312 kDa (Joslyn *et al.*, 1991; Kinzler *et al.,* 1991; van Es *et al.*, 2001). In the colon, APC localises in the basolateral cells of the crypt epithelial cells (Smith *et al.*, 1993).

The APC protein interacts with β-catenin, γ-catenin, GSK-3β, End-binding Protein 1 (EB1), homologues of the Drosophila Discs Large Tumor Suppressor Protein (DLG), microtubules, Axin and Conductin (Rubinfeld *et al.*, 1993; Su *et al.*, 1993b; Rubinfeld *et al.*, 1996; Su *et al.*, 1995; Matsumine *et al.*, 1996; Munemitsu *et al.*, 1994; Kishida *et al.*, 1998; Behrens *et al.*, 1998). Figure 6 shows the functional domains of APC.

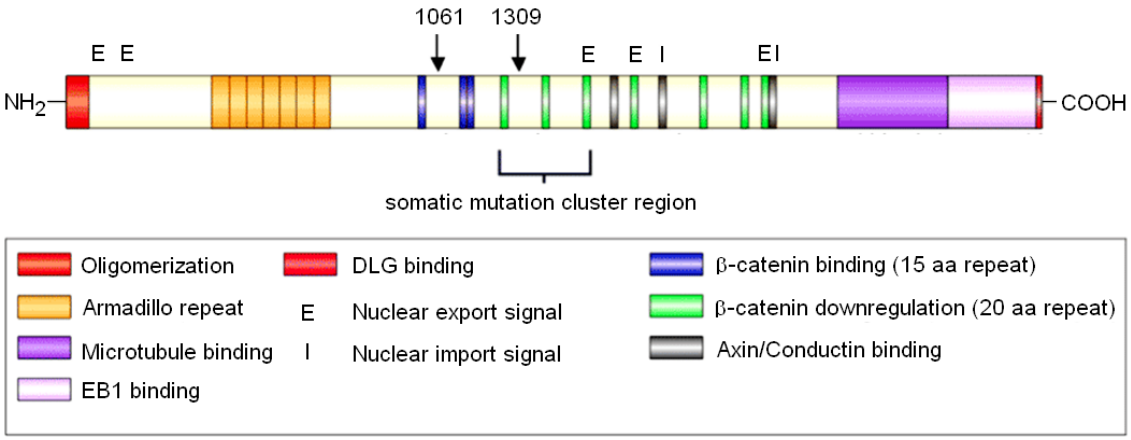

Figure 6: Structure, binding regions and mutations of APC. The structure of the APC protein: Dimerization of APC takes place between aa 1 and 171. The Armadillo motifs span from aa 453 to 767. β-catenin is bound at two sites (aa 1020 to 1169 and aa 1324 to 2075). The microtubules bind to APC within aa 2130 and 2843. EB1 interacts with APC between aa 2560 and 2843 and hDLG binds APC between aa 2711 and 2843 (adapted from Fodde *et al.,* 2001).

The APC protein dimerizes between amino acid (aa) 1 and 171 within heptad repeat motifs that form coiled-coil protein structures (Su *et al.*, 1993a). Between aa 453 and 767, there are seven armadillo repeats each consisting of a 42-amino-acid-motif (Groden *et al.*, 1991). These are named after the β-catenin like Armadillo protein of *Drosophila melanogaster.*

Two domains of APC can bind β-catenin: the first binding region consists of 15 aa repeats (aa 1020 to 1169), the second of 20 aa repeats (aa 1324 to 2075) (Su *et al.,* 1993b; Rubinfeld *et al.,* 1993). The second binding region also includes the GSK-3β phosphorylation site (Rubinfeld *et al.,* 1996). The interaction of APC with the cytoskeleton is mediated by microtubules, which are bound at a region located between aa 2130 to 2843, and also EB1, which interacts with APC between aa 2560 and 2843 (Munemitsu *et al.*, 1994; Groden *et al.*, 1991; Su *et al.*, 1995). The DLG protein is bound between aa 2771 and 2843 (Matsumine *et al.*, 1996). Additionally, there is some evidence that APC is also involved in double-strand break DNA repair via an interaction with histones between codon 1440 and 2077 of the APC protein (Kouzmenko *et al.*, 2008).

In addition to its role within the Wnt pathway there is evidence that APC plays a direct role in cell adhesion and cell migration (reviewed by van Es *et al.*, 2001). High expression can be identified in the outer layer of crypt cells, indicating that APC might be involved in detachment or apoptosis of cells at the crypt apex (Midgley *et al.,* 1997). Also, a role in chromosome segregation is considered for the APC protein (Kaplan *et al.*, 2001). APC binds the (+)-ends of microtubules via kinetochores and can interact with them through EB1 (Mimori-Kiyosue *et al.*, 2000).

1.3 Hereditary colorectal cancer

Various mutations in genes involved in the Wnt pathway are known to promote colorectal cancer formation. Inherited CRC syndromes such as familial adenomatous polyposis coli (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) account for 5 to 10% of all cases of CRC. A further 20 to 25% are thought to depend on otherwise inherited susceptibility (Sancho *et al.,* 2004; Bodmer, 2006). Study of these inherited cancer syndromes has revealed the role of the underlying signalling pathways – such as the Wnt pathway in FAP and the DNA mismatch repair system in HNPCC. FAP is caused by mutations in the *apc* gene, these mutations are an early

event in sporadic CRC as shown in Figure 1. Mutations in the *apc* gene have been identified in 60 to 80% of all spontaneous colorectal carcinomas and adenomas (Powell *et al.*, 1992; Miyoshi *et al*., 1992; Nagase and Nakamura, 1993). HPNCC is caused by germline mutations in DNA mismatch repair genes of the *mut*S or *mut*L family (Lynch *et al.*, 1996). These mutations lead to microsatellite instability, which is also observed in up to 15% of sporadic colon cancer (Samowitz *et al.*, 2001). In contrast to FAP, where patients have accelerated tumor initiation, but a normal progression rate, HNPCC patients show slow tumor initiation and accelerated tumor progression compared to patients with spontaneous CRC (Reitmair *et al.*, 1996). This suggests that tumor progression in FAP is more similar to spontaneous colorectal tumor progression than is HNPCC and therefore might provide deeper insight into the more prevalent spontaneous disease.

1.3.1 Familial adenomatous polyposis (FAP)

FAP is an inherited autosomal-dominant trait with a high penetrance caused by germline mutations in the *adenomatous polyposis coli* (*apc*) gene (Herrera *et al.,* 1986; Kinzler *et al.*, 1991; Bodmer *et al.*, 1987). Most of these mutations lead to truncated forms of APC protein lacking some or all of the β-catenin binding sites (Powell *et al.*, 1992; Miyoshi *et al.*, 1992; Nagase and Nakamura, 1993). This leads to β-catenin accumulation in the cytosol and activation of Tcf/Lef-mediated gene transcription. This shifts the balance from apoptosis to proliferation in the colorectal epithelium leading to the formation of numerous polyps and later adenomas in the large bowel.

Depending on the site of mutation, the average age of onset of colorectal cancer in these patients is 20 years (Caspari *et al*., 1994; Friedl *et al.,* 2001). Apart from the adenomas in the large bowel, other symptoms and diseases are linked with the FAP syndrome. Mutations in the *apc* gene are also associated with an increased risk of extra-colonic tumors. Here, a correlation with of the region of the germline mutation on the *apc* gene and the localization of cancer formation could be found (Table 1).

A very common manifestation in FAP patients is congenital hypertrophy of the retinal pigment epithelium (CHRPE). FAP patients also have an increased risk for upper gastrointestinal tract and desmoid tumors, Gardner's syndrome (epidermoid skin cysts), Turcot syndrome (cerebellar medulloblastoma), hepatoblastoma, adrenocortical tumors and papillary thyroid cancer (Fearnhead *et al.,* 2001). As described above,

mutations in the *apc* gene have also been detected in 60 to 80% of spontaneous colorectal carcinomas highlighting the importance of *apc* mutations in CRC initiation (Powell *et al.*, 1992; Miyoshi *et al.*, 1992; Nagase and Nakamura, 1993).

Table 1: Mutations in the *apc* gene and related extra-colonic tumor risk in FAP patients. In addition to a predisposition to colorectal cancer, FAP patients have an increased risk for tumors in other tissues. The tissues affected vary with the mutation site in the *apc* gene.

Codon	Phenotype	Reference
140 - 1309	increased risk for papillary thyroid cancer	Cetta *et al.,* 2000
543 - 1309	high risk for congenital hypertrophy of the retinal pigment epithelium (CHRPE)	Spirio *et al.,* 1993; Olschwang *et al.,* 1993
976 - 1067	increased risk for duodenal adenomas	Friedl *et al.,* 2001; Bertario *et al.,* 2003
1445 - 1580	increased risk for desmoid tumors	Caspari *et al*., 1995
from 1444	increased risk for osteomas	Davies *et al.,* 1995
141 - 1230	increased risk for hepatoblastoma	Giardiello *et al.,* 1996

In contrast to classical FAP, which has high penetrance, some *apc* mutations have weaker effects with incomplete penetrance. These mutations are very rare and due to their low penetrance, clustering of CRC cases does not occur in all affected families, but nevertheless there is increased risk for CRC (Frayling *et al*., 1998). For example, in the Ashkenazim Jew population a mutation in the *apc* gene (I1307K) occurs with a rate of 6 to 8%, whereas this mutation is observed in 28 to 38% of Ashkenazim CRC patients with a family history of CRC (Laken *et al.*, 1997), again emphasising the importance of mutations in the *apc* gene in colorectal cancer incidence.

1.3.2 Mutations of the *apc* gene in FAP

As described above, mutated APC proteins may lead to an accumulation of β-catenin in the cytoplasm and the transcriptional activation of Tcf/Lef-family dependent genes involved in proliferation, differentiation, migration and cellular apoptosis. Most *apc* mutations observed in polyps and carcinomas of FAP patients lead to truncated APC proteins, most commonly through frameshift mutations, but also through nonsense mutations or large deletions (Laurent-Puig *et al.*, 1998; Friedl *et al.*, 2001).

Mutations 5' to codon 168 and 3' to codon 1580 or at an alternatively spliced region of exon 9, lead to an attenuated phenotype of FAP with only a few polyps (< 100) and a late onset at an average age of 52.2 years (van der Luijt *et al*., 1995; Friedl *et al.,* 2001). APC proteins with mutations at the very 5' end are transcribed through an internal ribosomal entry site (IRES) from an AUG at codon 184 (Goss *et al*., 2002). So, the amino-terminus of the APC protein seems to play a minor role in tumor suppression. Mutations between codon 450 and 1600 lead to large numbers of polyps. The two most common germline mutations in FAP patients are truncating mutations at codons 1061 and 1309, which are both within the β-catenin binding region of exon 15 (Nagase and Nakamura, 1993; Aretz *et al.*, 2004). The most common mutation, a 5 base pair deletion at codon 1309, leads to the most severe phenotype with the occurrence of the highest number of polyps (> 5000) and the earliest malignant transformation with an onset of colorectal cancer at an average age of 20 years (Nugent *et al.*, 1994; Caspari *et al.*, 1994; Friedl *et al.*, 2001; Bertario *et al.*, 2003). Nevertheless there are great variations in phenotype and age of onset in patients with the same germline mutations (Friedl *et al.,* 2001).

The first step in the progression from neoplasia to colorectal adenoma in FAP patients is the somatic mutation of the second *apc* allele (Ichii *et al.,* 1992; Levy *et al.*, 1994). Most of the germline mutations in FAP patients are observed in the 5' half of the *apc* gene and only ~20% within the mutation cluster region from codon 1281 to 1554 (Figure 7) (Miyoshi *et al.*, 1992). In tumors of sporadic CRC cases, however, mutations preferably occur within the mutation cluster region, which is part of the β-catenin binding region (Powell *et al.,* 1992; Miyoshi *et al.*, 1992).

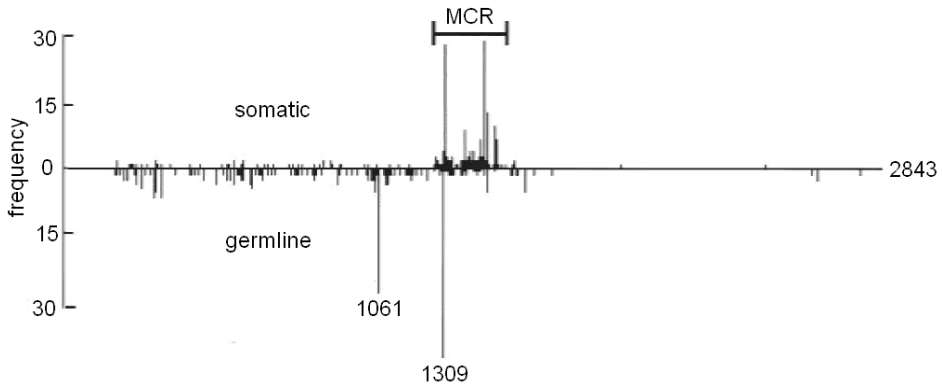

Figure 7: The frequency of germline and somatic *apc* mutations in FAP patients. Most of the somatic mutations observed in tumor tissues from FAP patients are clustered in the mutation cluster region (MCR). The germline mutations do not show such a clustering, but most are identified within the 5' half of the *apc* gene. The two most common mutations occur at codon 1061 and 1309 (adapted from Polakis, 1997).

According to Knudson's two-hit hypothesis, both *apc* mutation events should occur independently of each other (Knudson, 1971). But notably, there is an interdependence of the first and second hit in the *apc* genes in sporadic CRC and in FAP patients (Rowan *et al*., 2000; Cheadle *et al.,* 2002). A functional selection for the localization of the somatic mutations depending on the position of the germline mutation was noticed (Lamlum *et al.,* 1999) (Figure 8). Albuquerque *et al*. (2002) explained this with the "just-right" hypothesis: Tumorigenesis can only take place, if some of the regulatory functions of APC are conserved and lead to an optimal β-catenin level for tumor development. In humans excess β-catenin leads to apoptosis of the tumor cells. This hypothesis explains why germline mutations around codon 1300, which lead to APC proteins with one or two β-catenin regulation sites, are more likely to lead to loss of heterozygosity (LOH). Whereas germline mutations that cause complete deletion of the β-catenin regulation sites will force a selection for somatic mutations with two or three of the β-catenin regulation sites (Albuquerque *et al.*, 2002). So, in contrast to classical tumor suppressor genes, human colorectal tumors are not caused by the complete loss of APC protein and function. This interdependence of the first and second hit in *apc* mutations is not observed in mice (Giles *et al*., 2003).

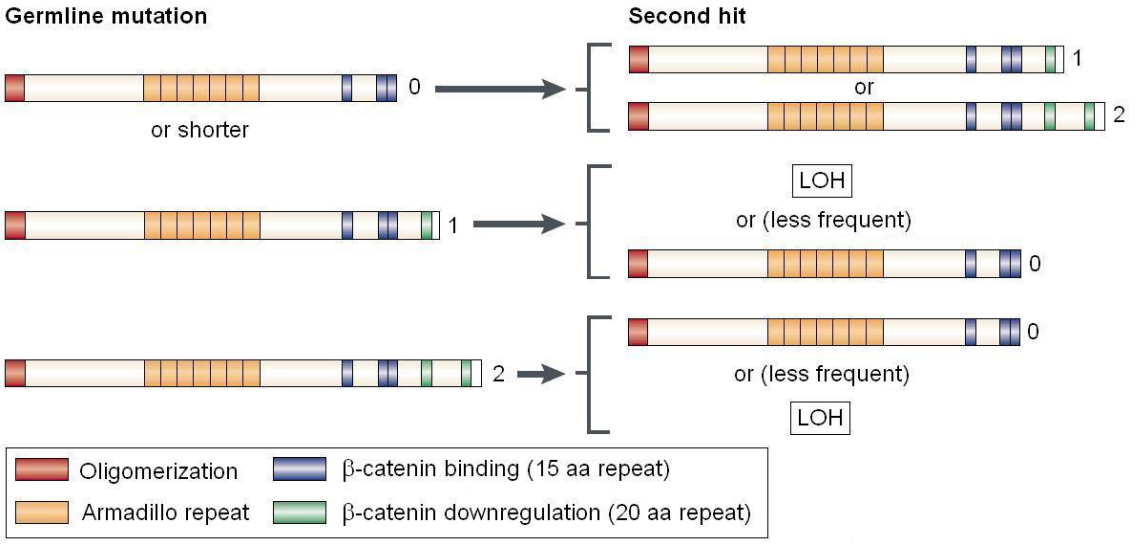

Figure 8: Correlation of germline and somatic *apc* mutations in FAP. The position and type of the somatic mutation in FAP depends on the localisation of the germline mutation. Germline mutations in the *apc* gene, which leave one or two β-catenin regulation sites, lead to the loss of all β-catenin regulation sites or loss of heterozygosity in the somatic mutations. In contrast the depletion of the regulation sites due to the germline mutation will force the selection of somatic mutations with some residual β-catenin regulation sites (adapted from Fodde *et al.,* 2001).

1.4 Animal models for colorectal cancer research

Animal models are widely used in research and medicine. The most common animal model in biomedicine is the mouse. Mice are small, easy to keep and reproduce quickly in laboratory conditions. Extensive knowledge of murine genetics, genomic sequence data and well established methods to induce carcinogenesis chemically are available. Most importantly, the discovery of murine embryonic stem cells (ES cells) and the genetic manipulation of these cells enabled the tailored genetic modification of mice and made them to an essential tool in biomedicine. It was possible to mimic human mutations in mice and create animal models with a defined genotype. These technologies were the basis for the development of various animal models for medical, developmental and regulatory studies in recent years.

1.4.1 Rodent colorectal cancer models

Although later studies revealed the limitation of *apc* mutation in rodents to mimic the human disease, these rodent models helped to understand the mechanism of tumor progression and to develop new screening methods and better therapies.

Many of the underlying signalling pathways in CRC research have been revealed through animal models. The importance of mutations within the *apc* gene on spontaneous and hereditary colorectal cancer progression has been described above. This has motivated attempts to generate various mouse and rat models reproducing the mutations observed in FAP patients. A summary of the existing rodent models with mutations in the *apc* gene is given in Table 2.

1.4.1.1 Non-transgenic CRC models

Chemically induced mouse and rat models are widely used for CRC research. The chemical induction of tumor formation is usually performed with 1,2-dimethylhydrazine (DMH) or azoxymethane (AMO) (reviewed by Rosenberg *et al.*, 2009). Often long latencies and incomplete penetrance is observed in these models and only certain tumor types are induced (Amos-Landgraf *et al*., 2007; Frese and Tuveson, 2007).

Table 2: Overview of mouse and rat models with mutations in the *apc* gene.

Model	Mutation	Phenotype		Reference
		heterozygous (number of adenomas)	homozygous	
APC^{min} (mouse)	truncation at aa 850	gastrointestinal adenomas (200 - 500)	embryonic lethal	Moser *et al.,* 1990; Su *et al.*,1992
APC^{580S} (mouse)	floxed exon 14, frame shift at aa 580 after viral infection with a Cre-recombinase	no tumors four weeks after infection	colorectal adenomas within four weeks after infection	Shibata *et al.*, 1997
$APC^{\Delta 716}$ (mouse)	truncation at aa 716	gastrointestinal adenomas (200 - 300)	embryonic lethal	Oshima *et al.*, 1995; Oshima *et al.*, 1997
APC^{1638T} (mouse)	hygromycin resistance cassette introduced at aa 1638	normal	viable, no tumors	Smits *et al.*, 1999
APC^{1638N} (mouse)	neomycin resistance cassette introduced at aa 1638	small number of gastrointestinal adenomas (< 15)	embryonic lethal	Fodde *et al.,* 1994
$APC^{\Delta 474}$ (mouse)	neomycin resistance cassette introduced at aa 474	gastrointestinal adenomas (100 - 150), mammary tumors	embryonic lethal	Sasai *et al.*, 2000
APC^{1309} (mouse)	nonsense mutation at codon 1309	small number of gastrointestinal adenomas	not published	Quesada *et al.*, 1998
APC^{am1137} (rat)	truncated APC at codon 1137	multiple neoplasms in the gastrointestinal tract, extensive polyposis after 4 months	embryonic lethal	Amos-Landgraf *et al.*, 2007

One of the main shortfalls of rodent models of CRC is the lack of an invasive and metastatic tumor phenotype as present in human CRC. Xenograft transplantation in immunodeficient mice or allograft transplantation in immunocompetent animals has been used to mimic human metastatic development (de Jong *et al.,* 2009). But in these models relevant aspects of the tumor microenvironment are absent and are therefore of limited clinical use (reviewed by Frese and Tuveson, 2007).

1.4.1.2 Mutant CRC models

The first mouse models for CRC were chemically induced mutant mice. The best characterised animal model for FAP is the multiple intestinal neoplasia (Min) mouse, which was the result of random mutagenesis with ethylnitrosourea (ENU) (Moser *et al.,* 1990; Su *et al.*, 1992). $APC^{min/+}$ mice carry a truncating mutation at codon 850 of one *apc* allele that leads to the development of adenomas in the small intestine and death within 150 days of birth. Microadenomas in the small intestine are also common in FAP patients and these are similar to those in the $APC^{min/+}$ mice (Preston *et al.*, 2008).

But importantly, in contrast to FAP patients, $APC^{min/+}$ mice do not exhibit adenomas in the colon. Polyps are formed preferably in the small intestine. There is no invasion into the submucosa and very little progression to adenocarcinomas (McCart *et al.*, 2008).

Another difference to the human phenotype is the origin of these polyps. In $APC^{min/+}$ and also in $APC^{\Delta 716}$ mice, polyps arise from beneath the normal epithelium, whereas in the human disease the polyps and adenomas originate from the epithelium itself as explained above (Oshima *et al.*, 1995). There are also great variations in tumor size and multiplicity between different mouse breeds with the same *apc* mutations, which limits their use as well-defined models for FAP. Although it led to the discovery of genetically modifying factors, the so called *Modifier of min* genes, no equivalent mutations have been found in humans so far (Dietrich *et al*., 1993; reviewed by McCart *et al.*, 2008).

1.4.1.3 Gene-targeted CRC models

For a better characterization and a human-like phenotype of CRC, various gene-targeted rodent models of CRC have been developed. These models take advantage of the efforts of the last 30 years, during which transgene technologies (see 1.5) have

been developed and enabled the addition, depletion and replacement of genes in animals.

Figure 9 summarizes the various *apc* mutations in the different *apc* models. Interestingly, from all the *apc* mutations of the rodent models, only the mutations at codon 850 and 1309 are described in the APC database of the Human Genome Variation Society (http://www.umd.be/APC). One case with a mutation at codon 850 and 263 cases with mutations at codon 1309 are described in the database. It is also surprising that no mouse model with the second most common germline mutations in FAP patients at codon 1061 exists.

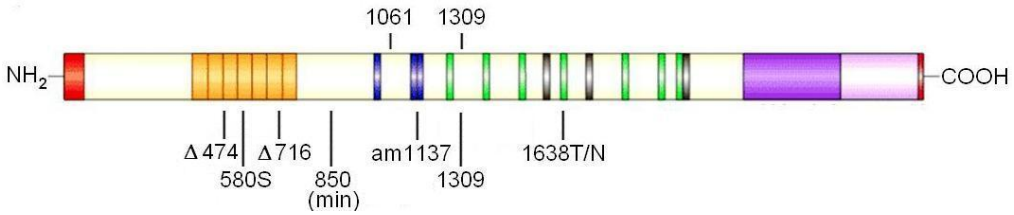

Figure 9: Mutations sites of the various mouse and rat models on the *apc* gene. The localisations of the various mutations of the discussed mouse models are indicated (adapted from Fodde *et al.,* 2001).

The examination of early gastrointestinal lesions in $APC^{\Delta 716/+}$ and the rapid tumor development after inactivation of both *apc* alleles in $APC^{580S/580S}$ mice confirmed *apc* mutations as initiating events in tumorigenesis (Figure 1). However, the severity of the symptoms in mice does not correlate with the human FAP phenotype with the same mutation (Sasai *et al.*, 2000). Furthermore the short life span of mice may not allow sufficient time for a human-like tumor progression (McCart *et al.*, 2008). Accelerated tumor formation was observed in mice with additional mutations in genes important for tumor progression such as the K-*ras* gene (Figure 1). Examples are APC^{580S}/K-ras^{LSLV12} or K-ras^{V12G}/$APC^{+/1638N}$ mice (Sansom *et al.,* 2006; Janssen *et al.*, 2006).

Other mouse models used for the investigation of CRC progression had mutations in the genes encoding for Smad3, Smad4, Cdx2, β-catenin, or in the DNA mismatch repair genes like in HPNCC (Romagnolo, *et al.,* 1999; Chawengsaksophak *et al.,* 1997; Takaku *et al.,* 1999; Zhu *et al.,* 1998; Taketo and Edelmann, 2009). But all these models share the same disadvantages of murine colorectal cancer models described above and do not resemble the human CRC phenotype.

In general, the physiology and size of mice and rats limit their application in screening and therapy development. Due to the considerable size difference endoscopy, surgical techniques and devices designed for the use in humans cannot easily be tested in mice. Radiation or hyperthermia treatments are difficult to scale down for their use in mouse studies (Adam *et al*., 2007). There are also differences between mice and humans regarding drug metabolism and immune system (Martignoni *et al*., 2006; Frese and Tuveson, 2007). Both are relevant factors for the development and evaluation of new drugs. Besides the development of new therapies and devices for human CRC, there is also a need for animal models to allow the investigation of dietary and environmental factors on CRC development. But the considerable differences in intestinal physiology and diet make rodent models unsuitable for these studies. For example, a comparison of the effect of aspirin, calcium and wheat bran on prevention of intestinal tumors showed no correlation between mice and men (Corpet and Pierre, 2005). Therefore well-defined animal models in other species are necessary to provide predictive information and allow the transition of research results from the animal to human medicine.

1.4.2 Pigs in biomedicine

Due to the limitations of rodent animal models, there is a clinical need for better animal models. An optimal model should closely resemble the human phenotype and allow clinical utilization. An alternative to mouse models are large species, especially those with a size and physiology more similar to humans and with a longer lifespan to allow extended observation periods. This is especially important for diseases with longer latency periods such as cancer, diabetes or neurodegenerative diseases.

Pigs reach sexual maturity after six months, have a gestation time of 114 to 115 days and have more than two litters per year with ten to twelve piglets per litter. This makes them more suitable for research than other large animals such as sheep or dog. Apart from their breeding behavior, they resemble humans in organ size, anatomy and physiology offering many possibilities for biomedicine. Pigs are already used as models for diabetes, arteriosclerosis and myocardial infarction (Turk and Laughlin, 2004; Larsen and Rolin, 2004; Bellinger *et al.*, 2006; Granada *et al.,* 2009). Most porcine models of human diseases either occur spontaneously or are induced chemically or mechanically. For example, in porcine models of diabetes the insulin-producing cells of the pancreas are destroyed with streptozotocin or alloxan (Larsen and Rolin, 2004;

Bellinger *et al.*, 2006). Another example is the subcutaneous injection of tumorigenic porcine cells to induce tumors in pigs (Adam *et al.,* 2007). But these models are genetically not defined and are of limited use as they do not resemble the progression of a disease in humans.

1.4.3 Genetically modified pigs

In recent years there has been increasing interest in genetically modified pigs especially due to the shortage of human organs for transplantation and genetically engineered animal organs are discussed as an alternative (xenotransplantation). To overcome obstacles such as rejection and zoonoses, gene-targeted pigs have been considered. Accordingly, the first gene-targeted animals for xenotransplantation were *α-1,3-galactosyltransferase* (*ggta1*) knock-out pigs to avoid hyperacute rejection in pig-to-human xenotransplantation (Lai *et al.*, 2002b; Dai *et al.*, 2002; Ramsoondar *et al.*, 2003). Shortly afterwards, a double-knockout with the disruption of both *ggta1* alleles was accomplished (Phelps *et al.,* 2003).

In 2008 the first porcine disease model was developed, which mimics cystic fibrosis. Previous mice models were used to study the underlying mechanism, but none showed a similar lung and pancreas disease as cystic fibrosis patients (Grubb and Boucher, 1999). Pigs with either a knock-out ($cftr^{+/-}$) of the *cystic fibrosis transmembrane conductance regulator* (*cftr*) gene or a knock-in ($cftr^{\Delta F508}$) with the most common mutation observed in humans were produced (Rogers *et al.*, 2008a). After reaching sexual maturity, heterogeneous *cftr*-null pigs were mated, leading to $cftr^{-/-}$ animals, which showed a phenotype similar to newborn children with cystic fibrosis (Rogers *et al.*, 2008b).

In contrast to classic genetic diseases such as cystic fibrosis, FAP shows a broad range of phenotypes (Galiatsatos and Foulkes, 2006). Patients with a germline mutation in one *apc* allele carry only a predisposition for colorectal cancer. Further somatic mutations are necessary for tumor development. Therefore the phenotypes can vary broadly depending on these mutations (Ichii *et al.,* 1992; Levy *et al.,* 1994). The somatic mutation rate is influenced by environmental, dietary or other effects and this is of considerable interest for research (Nyberg *et al*., 2003). In contrast to mouse models, pigs offer the possibility of studying dietary effects on CRC as they are omnivores. Moreover the human lifestyle (diet with high fat / low fibre and lack of

exercise) can be easily modelled (Jankord *et al.*, 2008). And, due to their size and gastrointestinal physiology pigs are already used for the evaluation of new colonoscopy devices (Pfeffer *et al.,* 2006).

1.5 Transgene technology and animal models

The methods used for producing the animal models described above are explained in the following chapters.

1.5.1 The generation of transgenic animals

The first transgenic mice were established by injecting simian virus 40 (SV40) viral DNA in murine blastocysts (Jaenisch and Mintz, 1974). This method was later modified and DNA was injected directly into the pronucleus of a fertilised egg. In 1981 this led to the production of transgenic mice overexpressing growth hormone (Gordon *et al*., 1980). When the same experiments were reproduced in pigs, rabbits and sheep the first transgenic livestock were obtained (Hammer *et al*., 1985). Besides microinjection, other methods are sperm-mediated transgenesis or retroviral gene transfer into zygote or oocytes (Lavitrano *et al.,* 1989; Hofmann *et al.,* 2003). All these methods lead to random integration of the transgene and the transfer of varying numbers of transgenes, which leads to varying expression levels and insertion mutagenesis with position-effects (Clark *et al.,* 1994). The main disadvantage of these methods is that they allow no genetic modification other than gene addition.

1.5.2 The generation of gene-targeted animals

In contrast to gene addition, the specific modification of endogenous genes has to be performed by gene targeting. The most convenient way to create mice from gene-targeted cells is the use of embryonic stem (ES) cells, which were first isolated from mice (Evans and Kaufman, 1981; Martin, 1981). The combination of gene targeting with embryonic stem cells was a breakthrough for the development of mouse models with specific mutations. In 2007, it was honoured with the Nobel Prize for Medicine to Mario Capecchi, Martin Evans and Oliver Smithies.

So far, ES cells have been isolated not only from mouse, but also from human, monkey and rat (Thomson *et al.,* 1995; Thomson *et al.,* 1998; Ueda *et al.,* 2008). After genetic

manipulation they can be injected into early embryos where they participate in embryo development and transferred into a recipient mouse. Chimeric mice are produced and those in which the ES cells contribute to the germline can be used for further breeding (Figure 10). The first gene-targeted mouse was achieved by the inactivation of the murine *hypoxanthine phosphoribosyl transferase* (*hprt*) gene in ES cells, followed by a second round of gene targeting to correct the disrupted *hprt* gene (Thomas and Capecchi, 1987; Doetschman *et al.,* 1987). In the following years these technologies were the basis for the development of various animal models for medical, developmental and regulatory studies.

Despite considerable efforts, definitive ES cells have not been isolated from livestock. Therefore other methods were necessary to generate transgenic livestock animals. A solution to this problem is somatic cell nuclear transfer (SCNT) (Campbell, *et al*., 1996; Wilmut *et al.*, 1997). That means a donor nucleus from a (genetically manipulated) cell replaces the nucleus of an enucleated oocyte. Initially, these cells were obtained from embryos, but Wilmut *et al.* (1997) showed that it is also possible to clone sheep from cultured somatic mammary epithelial cells. So far, various somatic cell types such as fetal fibroblasts, mammary epithelial cells, cumulus cells and muscle cells have been used as nuclear donor cells (Campbell *et al*., 1996; Wilmut *et al.*, 1997; Baguisi *et al*., 1999; Wakayama *et al*., 1998; Shiga *et al*., 1999; Zakhartchenko *et al.,* 2001). For the generation of cloned pigs only granulosa-derived cells, preadipocytes and fetal fibroblasts have been used for SCNT (Polejaeva *et al.*, 2000; Onishi *et al.*, 2000; Tomii *et al*., 2005). After nuclear transfer the reconstructed embryo can be transferred into a recipient animal (Figure 10).

Nuclear transfer has the advantage that founder animals are derived entirely from the donor cell nucleus and in contrast with blastocyst injection of ES cells, resulting animals are not chimeric. The whole animal has the genetic background of the donor nucleus. Additionally, appropriate choice of the donor cells, allows sex and other characteristics of the transgenic animals to be decided in advance.

Very quickly, SCNT was also applied to other livestock species such as goat, pig and cattle and cloned mice and pets such as cats were also established (Campbell *et al*., 1996; Wilmut *et al.*, 1997; Baguisi *et al*., 1999; Wells *et al.,* 1998; Polejaeva *et al.,* 2000; Wakayama *et al*., 1998; Shin *et al.,* 2002). In 1997, SCNT was used to produce gene-targeted sheep from modified fetal fibroblasts, expressing human factor IX (Schnieke *et al.*, 1997).

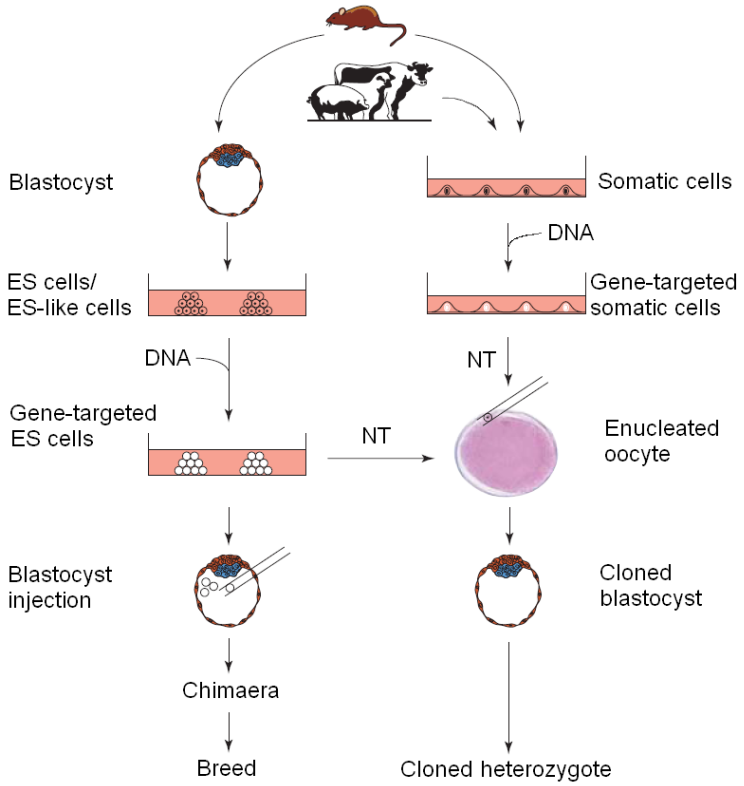

Figure 10: Comparison of blastocyst injection (left) and somatic cell nuclear transfer (right). For blastocyst injection ES cells are injected into a blastocyst, whereas for SCNT somatic cells are transferred to an enucleated oocyte. Afterwards they are transferred into recipients. Blastocyst injection leads to chimeric offspring, whereas with SCNT heterozygous offspring are obtained (adapted from Denning and Priddle, 2003).

But not all somatic cell types are suitable for the production of gene-targeted animals. Clark *et al.* (2000) proposed that roughly 45 population doublings are necessary for expansion, transfection, selection and screening of modified cells for SCNT. This requires the use of cell types capable of that many population doublings in culture. Additionally, efficient transfection methods have to be available for these cell types, as higher transfection efficiencies increase the possibility for homologous recombination (Arbonés *et al*., 1994). Most commonly used are fetal fibroblasts, but due to the limited time for genetic manipulation, there is a need to move to new cell types. A possible solution might be the use of cells that are not terminally differentiated, such as adult neural stem cell, skin stem cells or mesenchymal stem cells (Clarke *et al*., 2000; Zhu *et al*., 2004; Bosch *et al*., 2006). Mesenchymal stem cells (MSC) are easily isolated from bone and they proliferate well *in vitro*, which makes them an interesting cell type for genetic manipulation. They are multipotent progenitor cells with the capacity to differentiate into several distinct mesenchymal lineages *in vitro*, e.g. adipocytes, osteoblasts and chondrocytes (Jaiswal *et al.,* 1997; Johnstone *et al.,* 1998; Mackay *et al.,* 1998; Pittenger *et al.,* 1999; Ringe *et al.,* 2002). Accordingly MSCs are widely

discussed for tissue repair (Bianchi *et al.,* 2001). They might also be more suitable for SCNT than differentiated cell types. Human MSCs showed up to 42 population doublings without senescence (Bruder *et al.,* 1997), which would meet the demands for culturing and manipulation of the cells. With addition of basic fibroblast growth factor (FGF-2) the proliferation potential was enhanced to more than 70 population doublings (Martin *et al.,* 1997; Bianchi *et al.*, 2003). Moreover, using electroporation, transfection rates up to 67% have been achieved with porcine MSCs (Colleoni *et al.,* 2005). Bovine bone-marrow derived MSCs were successfully used for nuclear transfer after fusion with an enucleated oocyte (Kato *et al*., 2004). Additionally, nuclear transfer embryos reconstructed with porcine MSCs showed a higher proportion of development to blastocyst stage and lower numbers of apoptotic cells within the embryo compared to those made with porcine fetal skin fibroblasts (Jin *et al.*, 2007). A comparison of gene expression profiles from embryos reconstructed with porcine fetal fibroblasts (poFFs) or poMSCs showed that MSC-derived embryos resemble the transcript profile of *in vivo* produced embryos more closely (Kumar *et al*., 2007).

1.5.3 Gene-targeting and homologous recombination

Gene targeting allows endogenous genes to be modified in various ways - including replacement, depletion or insertion of DNA fragments. It is the specific modification of an endogenous gene by homologous recombination between exogenous DNA and the cognate genomic DNA sequence. It was first shown in mammalian cells by targeting the human *β-globin* gene (Smithies *et al.,* 1985).

In mouse ES cells homologous recombination occurs at a rate between 1 in 10^5 to 1 in 10^9 transfected cells (Schenkel, 1995). As non-homologous recombination or random integration is more frequent than homologous recombination, an enrichment and/or selection system is needed. For positive selection various antibiotic resistance genes, for example neomycin, blasticidin, puromycin or hygromycin, can be incorporated in the targeting vector. For negative selection thymidine kinase or diphtheria toxin A are being used, which enrich targeted cells 5- to 20-fold in murine ES cells (Koller and Smithies, 1992; Yagi *et al.*, 1993; McCarrick *et al.*, 1993). However, silencing of targeting constructs harbouring the diphtheria toxin A gene was described in mice (Palmiter *et al*., 1987; Behringer *et al*., 1988). More common is an antibiotic resistance cassette in combination with a negative selection, a so called positive negative selection (PNS) (Figure 11). In murine ES cells an enrichment from 0.1% targeted cell within all

resistant cells to 79% with PNS was obtained (2000-fold enrichment) (Mansour *et al.,* 1988) But in rat fibroblasts the enrichment did not exceed 2- to 10-fold (Hanson and Sedivy, 1995).

For transcriptionally active genes, a promoter-trap approach is the method of choice (Jasin and Berg, 1988; Sedivy and Sharp, 1989). Promotor-trap systems are based on a reporter gene or selection marker lacking a promoter. Therefore the reporter gene will only be expressed if it is placed under the control of an endogenous promoter. In NIH 3T3 cells one targeted cell out of 100 antibiotic resistant cells was identified using this approach and in rat fibroblasts targeting efficiencies between 9 and 32% were obtained (Sedivy and Sharp, 1989; Hanson and Sedivy, 1995).

Harrison *et al.* (2002) compared a PNS strategy with isogenic DNA with a promotor-trap approach with non-isogenic DNA in porcine fetal fibroblasts. With 2.3% targeted cell the promotorless vector was more effective compared to 0.1% for the PNS system. For the modification of transcriptionally inactive genes, an induction of gene expression or the choice of a somatic cell type in which the relevant gene is expressed might be considered (Clark *et al.*, 2000).

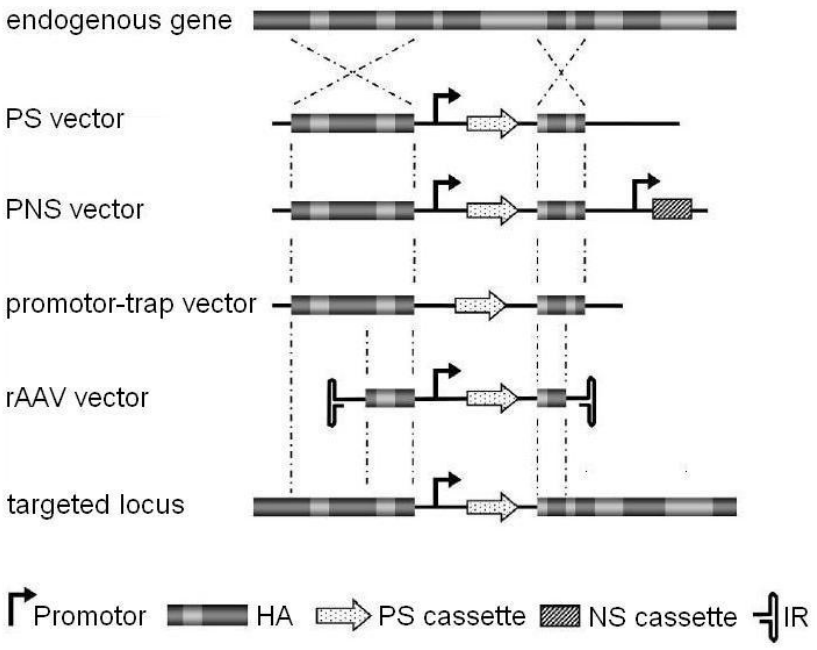

Figure 11: Designs of targeting vectors. The different designs of targeting vectors are explained in detail in the text. PS: positive selection; PNS: positive negative selection; NS: negative selection; HA: homology arms; IR: inverted repeats (adapted Laible and Alonso-Gonzáles, 2009).

Apart from the selection or enrichment strategy, the efficiency of homologous recombination is strongly dependent on the design of the targeting vector, the choice of appropriate cells and the transfection method. The most common methods of delivery are electroporation, lipofection and viral transduction (Clark *et al.,* 2000). The choice of the system influences the design of the targeting vector, as for example the use of recombinant adeno-associated virus (rAAV) limits the size of the targeting construct to 4.5 kb (Hendrie and Russell, 2005). Generally, only small amounts of naked DNA should be introduced into the cell to minimize cellular stress. The targeting vector can be constructed from isogenic or non-isogenic DNA. The construction of the targeting construct using isogenic DNA can raise the targeting efficiency 10 to 20-fold in mouse ES cells (te Riele *et al.*, 1992; Deng and Capecchi, 1992). In human somatic cells isogenic DNA did not show an advantage over non-isogenic DNA (Sedivy *et al.,* 1999). Therefore it is likely that the use of isogenic DNA is especially recommend in inbred species such as the mouse, in which large genetic differences are established between different strains. Another important factor is the length of the targeting construct. Deng and Capecchi (1992) compared targeting vectors of different lengths for their targeting efficiency in mouse ES cells and showed a strong correlation between the length of the homology and the targeting frequency. In total roughly 14 kb of homologous sequence are needed for efficient homologous recombination in mouse ES cells (Deng and Capecchi, 1992). The mutation sites should be surrounded by at least 1 kb of homologous sequence for efficient homologous recombination. Less than 1 kb of sequence homology will lead to a lower fidelity of the homologous recombination with rearrangements and integration of concatemerized targeting vectors (Deng and Capecchi, 1992; Thomas *et al.*, 1992).

1.6 Aims

The aim of this work was to generate a porcine animal model predisposed to colorectal cancer based on the human familial adenomatous polyposis coli (FAP) syndrome. The two most common germline mutations in the human *apc* gene of FAP patients at codon 1061 and 1309 were chosen. Both mutations are located within the β-catenin binding region in exon 15 and introduce a stop codon. The targeting vectors were based on a promotor-trap approach and designed with porcine *apc* sequences, which at the start of the project were unknown.

Porcine mesenchymal stem cells were chosen as cells for genetic manipulation. These cells had to be characterised and their genomic stability had to be analysed. The most efficient transfection and selection method had to be developed. Porcine mesenchymal stem cells with mutations at the codons corresponding to the human codons 1061 or 1309 had to be selected, screened and used as nuclear donors for somatic cell nuclear transfer. Additionally, methods for the analysis of the gene-targeted pigs had to be established.

2 Material and Methods

2.1 Material

2.1.1 Chemicals

Acetic acid	Fluka Laborchemikalien GmbH, Seelze, Germany
Alcian blue	Sigma, Steinheim, Germany
Ammonium persulfate (APS)	Carl Roth GmbH, Karlsruhe, Germany
Biozym LE Agarose	Biozym, Oldendorf, Germany
Boric Acid	Fluka Laborchemikalien GmbH, Seelzle, Germany
Bovine Serum Albumin (BSA), pH 7.0	PAA, Pasching, Austria
Bromphenol blue	Sigma, Steinheim, Germany
Chloroform	Sigma, Steinheim, Germany
4',6'-Diamidino-2'-phenylindole-dihydrochloride (DAPI)	Roche Diagnostics GmbH, Mannheim, Germany
Dithiotreitol (DTT)	Omnilab Life Science OLS, Bremen, Germany
Ethylene diamine tetracetic acid (EDTA)	Sigma, Steinheim, Germany
Ethanol absolute	Riedel-de-Haen, Seelze, Germany
Ethidiumbromid solution	Sigma, Steinheim, Germany
Formalin	Sigma, Steinheim, Germany
Formamide (deionized)	Sigma, Steinheim, Germany
GenAgarose L.E.	Genaxxon Bioscience GmbH, Biberach, Germany
Glacial acetic acid	Fluka Laborchemikalien GmbH, Seelze, Germany
Glutaraldehyde	Fluka Laborchemikalien GmbH, Seelzle, Germany
Glycerol	Carl Roth GmbH, Karlsruhe, Germany
Glycine	Carl Roth GmbH, Karlsruhe, Germany
Isopropanol (2-Propanol)	Carl Roth GmbH, Karlsruhe, Germany
Magnesium chloride-hexahydrate	Merck KGaA, Darmstadt, Germany
Methanol	Sigma, Steinheim, Germany
Milk powder	Carl Roth GmbH, Karlsruhe, Germany

N,N-Diemethylformamide	Sigma, Steinheim, Germany
Non fat dry milk powder	Carl Roth GmbH, Karlsruhe, Germany
Oil Red O	Sigma, Steinheim, Germany
Potassium hexacyanoferrat(II)-trihydrate	AppliChem, Darmstadt, Germany
Potassium hexacyanoferrat(III)	AppliChem, Darmstadt, Germany
Rotiphorese Gel 40 (29:1)	Roth, Karlsruhe, Germany
Saccharose	Sigma, Steinheim, Germany
Silver nitrate	Sigma, Steinheim, Germany
Sodium chloride	J.T. Baker, Deventer, Holland
Sodium citrate tribasic dihydrate	Sigma, Steinheim, Germany
Sodium thiosulfate	Sigma, Steinheim, Germany
Sodium acetate	Roth, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Sigma, Steinheim, Germany
Sodium hydroxide pellets	Riedel-de Haen Laborchemikalien GmbH, Seelze, Germany
Sucrose	Sigma, Steinheim, Germany
TEMED	Roth, Karlsruhe, Germany
Trizma base (Tris base)	Sigma, Steinheim, Germany
Tris hydrochloride (Tris HCl)	Sigma, Steinheim, Germany
Trizol	Invitrogen, Karlsruhe, Germany
Tween 20	Sigma, Steinheim, Germany

2.1.2 Plasticware and other consumables

0.5 ml, 1.5 ml and 2.0 ml reaction tubes	Brand, Wertheim, Germany
14 ml Polypropylene Round-Bottom Tube	Becton, Dickinson and Company, Sparks, USA
15 and 50 ml centrifugation Tubes	Corning Inc., New York, USA
Cell culture flasks (25, 75, 150 cm^2)	Corning Inc., New York, USA
Cell culture plates (6-, 12-, 24-, 48-well)	Corning Inc., New York, USA
CryoTubes	Nunc, Wiesbaden-Biebrich, Germany

Disposable inoculating tube (10 µl)	Cole-Parmer Instrument Company, Illinois, USA
Electroporation Cuvettes, 2 mm	peqlab, Erlangen, Germany
Electroporation Cuvettes, 4 mm	peqlab, Erlangen, Germany
Glass transfer pipettes	Brand, Wertheim, Germany
Glassware (bottles, flasks)	Marienfeld GmbH, Lauda-Königshofen, Germany
Hybond-N^+, positively chgd. nylon transfer membrane, 0.45 µm pore size	Amersham Bioscience, Freiburg, Germany
Immobilon-P Transfer Membrane, PVDF membrane, 0.45 µm pore size	Millipore Corporation, Billerica, USA
Petri dish (10 cm)	Brand, Wertheim, Germany
Photometer Cuvettes	Eppendorf, Hamburg, Germany
Pipette tips	Brand, Wertheim, Germany
Plugged pipette tips (2, 20, 200 and 1000 µl)	Mettler Toledo GmbH, Dreieich, Germany
Rainin pipette tips (2, 20, 200 and 1000 µl pipettes)	Mettler Toledo GmbH, Dreieich, Germany
Rotilabo® Blotting paper (1 mm)	Carl Roth GmbH, Karlsruhe, Germany
Sterile plastic pipettes 1 - 25 ml	Corning Inc., New York, USA

2.1.3 Cell Culture Material

2.1.3.1 Culture media

Advanced Dulbecco's Modified Eagle's Medium (Avanced DMEM)	Gibco BRL, Paisley, Scotland
Dulbecco's Modified Eagle's Medium (DMEM), high glucose (4.5 g/l)	PAA, Pasching, Austria
RPMI 1640 with L-Glutamine	PAA, Pasching, Austria

2.1.3.2 Supplements and solutions for cell culture

Accutase	PAA, Pasching, Austria
Ascorbic acid	Sigma, Steinheim, Germany
β-Glycerophosphate	Fluka Laborchemikalien GmbH, Seelze, Germany
Cell Culture Water, EP-grade	PAA, Pasching, Austria
Chicken serum	PAA, Pasching, Austria
Dexamethasone (water-soluble)	Sigma, Steinheim, Germany
Dulbecco's PBS, w/o Ca & Mg	PAA, Pasching, Austria
Fetal calf serum (FCS)	PAA, Pasching, Austria
GlutaMAX	Gibco BRL, Paisley, Scotland
Hank's Buffered Salt Solution (HBSS), w/o Phenol red, with Mg and Ca	PAA, Pasching, Austria
Human Fibroblast Growth Factor (FGF-2)	Genaxxon, Biberach, Germany
Hypoosmolar Buffer	Eppendorf, Hamburg, Germany
Isobutylmethylxanthine (IBMX)	Sigma, Steinheim, Germany
ITS+1	Sigma, Steinheim, Germany
Lipofectamine 2000	Invitrogen, Karlsruhe, Germany
Lymphocyte Separation Medium LSM 1077	PAA, Pasching, Austria
Non-essential amino acids (NEAA)	PAA, Pasching, Austria
Opti-MEM Reduced Serum Medium modification of MEM (Eagle's)	Gibco BRL, Paisley, Scotland
Sodium pyruvate	PAA, Pasching, Austria
TGF-β1	Serotec
Trypsin-EDTA	PAA, Pasching, Austria

2.1.3.3 Antibiotics and antimycotics

for selection:

Blasticidin	InvivoGen, San Diego, USA
G418	PAA, Pasching, Austria

for cell isolation:

Amphotericin B solution	PAA, Pasching, Austria
Gentamycin	PAA, Pasching, Austria
Penicillin/Streptomycin solution	PAA, Pasching, Austria

2.1.3.4 Chemicals for cell culture

Dimethyl sulfoxide (DMSO)	Sigma, Steinheim, Germany
Heparin sodium salt	Sigma, Steinheim, Germany

2.1.4 Media, supplements and cells for microbiology

2.1.4.1 Antibiotics for microbiology

Ampicillin	Sigma, Steinheim, Germany
Kanamycin	Sigma, Steinheim, Germany

2.1.4.2 Bacterial strains for microbiology

Bacterial strain	Genetic background	Supplier
DH5α	F^- Φ80lacZΔM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17 (r_{k-}, m_{k+}) phoA supE44 λ^- thi-1 gyrA96 relA1	Invitrogen, Karlsruhe, Germany
GM2163 / ER2925	ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210::Tn10)TetS endA1 rpsL136 dam13::Tn9 xylA-5 mtl-1 thi-1 mcrB1 hsdR2	New England Biolabs, Frankfurt, Germany
DH10B	F^- mcrAΔ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15, ΔlacX74 deoR recA1 araD139 Δ(ara, leu)7697 galU galK rpsL endA1 nupG	Invitrogen, Karlsruhe, Germany

2.1.4.3 Bacterial growth media

Difco Luria Bartani B Agar, Miller	Becton Dickinson and Company, Sparks, USA

Difco Luria Broth Base	Becton Dickinson and Company, Sparks, USA
S.O.C. medium	Invitrogen, Karlsruhe, Germany

2.1.5 RNA, DNA and Plasmids

2.1.5.1 Enzymes for cloning

All restriction enzymes were supplied by New England Biolabs (Frankfurt, Germany) besides BglII, which was supplied by Fermentas (St. Leon-Rot, Germany).

Calf Intestinal Alkaline Phosphatase (CIP)	New England Biolabs, Frankfurt, Germany
DNA Polymerase I, Large (Klenow) Fragment	New England Biolabs, Frankfurt, Germany
T4 DNA Ligase	New England Biolabs, Frankfurt, Germany
Proteinase K	Sigma, Steinheim, Germany
RNase A solution	Sigma, Steinheim, Germany

2.1.5.2 Polymerases

GoTaq Hot Start Polymerase	Promega, Mannheim, Germany
JumpStart AccuTaq LA DNA Polymerase	Sigma, Steinheim, Germany
MolTaq	Molzym GmbH & Co. KG, Bremen, Germany
OmniTaq Polymerase	Omnilab Life Science OLS, Bremen, Germany
PCR Extender System	5 Prime GmbH, Hamburg, Germany
Phusion High-Fidelity DNA Polymerase	New England Biolabs, Frankfurt, Germany
Platinum Taq DNA Polymerase	Invitrogen, Karlsruhe, Germany
SuperScriptIII RNase H^- Reverse Transcriptase	Invitrogen, Karlsruhe, Germany
SuperScriptIII One-Step RT-PCR with Platinum Taq	Invitrogen, Karlsruhe, Germany
BigDye Terminator v1.1 Cycle Sequencing Kit	Applied Biosystems, Carlsbad, USA

2.1.5.3 Cloning vectors and plasmids

pGEM-T Easy Vector System I	Promega, Mannheim, Germany
pBluescript SK+	Stratagene, La Jolla, USA

2.1.5.4 DNA Isolation

GenElute Mammalian Genomic DNA Miniprep Kit	Sigma, Steinheim, Germany
Nucleo Bond, Xtra Maxi	Macherey-Nagel GmbH & Co.KG, Düren, Germany
Nucleo Bond, Xtra Midi	Macherey-Nagel GmbH & Co.KG, Düren, Germany
Wizard SV Gel and PCR Clean-Up System	Promega, Mannheim, Germany

2.1.6 Chemicals for western blot analysis

CytoBuster	Merck KGaA, Darmstadt, Germany
Complete, Mini Protease Inhibitor Cocktail Tablets	Roche Diagnostics GmbH, Mannheim, Germany
Pierce ECL Western Blotting Substrate	Thermo Electron GmbH, Dreieich, Germany
Cytoskeleton Advanced Protein Assay Reagent (5x)	tebu-bio, Offenbach, Germany
APC antibody C-20	Santa Cruz Biotechnology Inc., Santa Cruz, USA
APC antibody #2504	Cell Signaling Technology Inc., Danvers, USA
Anti-APC N-15	Santa Cruz Biotechnology Inc., Santa Cruz, USA
SeeBlue Plus2 Pre-Stained Standard, 4 - 250 kDa	Invitrogen, Karlsruhe, Germany
Anti-Rabbit IgG, H&L (Donkey)	Rockland Immunochemicals Inc., Gilbertsville, USA
Anti-Rabbit IgG A0545 (Goat)	Sigma, Steinheim, Germany

2.1.7 Chemicals for Southern blot analysis

DNA molecular weight marker VII, Digoxigenin-labelled	Roche Diagnostics GmbH, Mannheim, Germany
Blocking Reagent	Roche Diagnostics GmbH, Mannheim, Germany
DIG Easy Hyb Granules	Roche Diagnostics GmbH, Mannheim, Germany
Anti-Digoxigenin-AP, Fab fragments	Roche Diagnostics GmbH, Mannheim, Germany
Digoxigenin-11-2'-deoxy-uridine-5'-triphosphate, alkaline-labile (DIG-labelled UTP)	Roche Diagnostics GmbH, Mannheim, Germany
CDP-Star	Roche Diagnostics GmbH, Mannheim, Germany

2.1.8 Miscellaneous

dNTPs	Biomers.net GmbH, Ulm, Germany
100 bp Ladder	New England Biolabs, Frankfurt, Germany
1 kb Ladder	New England Biolabs, Frankfurt, Germany
TURBO DNA-free	Ambion, Huntingdon, UK
RNase Away	Roth, Karlsruhe, Germany
TRIZOL Reagent	Invitrogen, Karlsruhe, Germany
Pig RNA (colon tissue)	BioChain Insitute Inc., Hayward, USA
QuantumRNA ß-Actin internal standard	Ambion, Huntingdon, UK

2.1.9 Equipment

Agarose Gel Electrophoresis, Classic CSSU78	Thermo Electron GmbH, Dreieich, Germany
Agarose Gel Electrophoresis, Classic CSSU1214	Thermo Electron GmbH, Dreieich, Germany
Analytical balance, 440-33N	Kern, Balingen, Germany
Axiovert 25	Zeiss AG, Oberkochen, Germany
AxioCAM MRc	Zeiss AG, Oberkochen, Germany
Bio Imaging System Gene Genius	Bio Imaging System Gene Genius, Syngene, Cambridge, UK

BioPhotometer 6131	Eppendorf, Hamburg, Germany
Centrifuge, Zentrifuge 5810	Eppendorf, Hamburg, Germany
Digital Graphic Printer UP-D895MD	Syngene, Cambridge, UK
+4°C fridge	Beko Technologies, Dresden, Germany
-20°C freezer	Liebherr International, Bulle, Switzerland
-80°C freezer (Therma Forma)	Thermo Electron GmbH , Dreieich, Germany
Handy Step multi pipette	Brand, Wertheim, Germany
HERAsafe Type HSP	Heraeus Instruments, Munich, Germany
Ice maker	Eurfrigor, Lainate, Italy
Incubator	Binder GmbH, Tuttlingen, Germany
Microwave MDA MW12M706	Haushaltswaren GmbH, Barsbuettel, Germany
Microwave NN-E202W	Panasonic
Minispin	Eppendorf, Hamburg, Germany
Multiporator	Eppendorf, Hamburg, Germany
Mr. Frosty	Nalgene, Rochester, U.S.A.
Laboratory centrifuge 4K15C	Sigma, Osterode, Germany
Laboratory centrifuge 1-15	Sigma, Osterode, Germany
Luminometer, Glomax 20/20	Turner Biosystems, Mannheim, Germany
Mini Trans-Blot Electrophoretic Transfer Cell	BioRad, Luton, UK
Mini-PROTEAN 3 Cell	BioRad, Luton, UK
PCR Unit	MJ Research PTC Thermal Cycler, GMI, Inc., Minnesota, USA
Pipetman Ultra U2 0.2 - 2 µl	Gilson, Bad Camberg, Germany
Pipetman P20, P200, P1000	Gilson, Bad Camberg, Germany
Pipettus reddot	Hirschmann Laborgeräte, Eberstadt, Germany
Power supply EC105	Thermo Electron GmbH, Dreieich, Germany
Electrophoresis Power Supply EPS 301	Amersham Bioscience, Buckinghamshire, UK
Rainin Pipet-Lite LTS (2, 20, 200 and 1000 µl)	Mettler Toledo GmbH, Giessen, Germany
Shaker, Forma orbital shaker	Thermo Electron Corporation, Dreieich, Germany
Steri-Cycle CO_2 incubator	Thermo Electron GmbH, Dreieich, Germany
Trans-Blot Module	BioRad, Luton, UK
Vortex Mixer	VELP Scientifica, Usmate, Milano, Italy

Waterbath Haake C10	Thermo Electron GmbH, Dreieich, Germany

2.1.10 Software

VectorNTI	Invitrogen, Karlsruhe, Germany
GLOMAX SIS	Turner Biosystems, Sunnyvale, USA
AxioVision	Zeiss AG, Oberkochen, Germany

2.2 Methods

2.2.1 Mammalian cell culture

2.2.1.1 General cell culture

All cells were cultivated at 37°C in a 5% CO_2 humidified atmosphere in a Steri-Cycle CO_2 incubator. Apart from the first steps of isolating cells from tissue and bones, all other steps were carried out in sterile class II laminar flow hoods. Only plugged pipette tips and autoclaved or sterile material were used. The medium was changed every 2 to 3 days after rinsing the cells once with PBS. Cells were split on a regular basis using Accutase to prevent the cells growing to more than 90% confluence. All cell lines were checked for mycoplasma infections on a regular basis. Where necessary, an improved Neubauer chamber was used to count cells.

2.2.1.2 Long-term storage of cell lines

Cells were detached with Accutase and centrifuged for 5 min at 430g. The supernatant was discarded and the pellet was dissolved in freezing medium, which consisted of the appropriate culture medium supplemented with 20% FCS and 10% DMSO. The cell solution was aliquoted in cryo vials and frozen in a freezing device (Mr. Frosty) at -80°C. After two days the cells were stored in liquid nitrogen. To thaw the cells the cryo vials were transferred to a 37°C water bath. The cell suspension was diluted in pre-warmed culture medium and centrifuged for 5 min at 430g. After discarding the supernatant the cells were dissolved in cell culture medium and plated.

2.2.1.3 Isolation and cultivation of porcine mesenchymal stem cells (poMSCs)

Multipotent mesenchymal stroma cells were isolated from femurs and tibia of 6 to 7 month old pigs. Before isolation, the bones and the equipment were cleaned with 80% ethanol. The epiphyses of the bones were opened with a saw and the bone marrow was flushed into a 100 mm petri dish with a HBSS heparin solution (1000 U/ml). A maximum of 20 ml of the cell suspension was loaded onto 25 ml of Lymphocyte Separation Medium LSM 1077 and centrifuged for 20 min at 1000g with a slow acceleration and deceleration. After centrifugation roughly 7 ml of the interphase - containing the mononuclear cells - were transferred into 20 ml HBSS for washing. After centrifugation (10 min, 600g), the pellet was resuspended in poMSC medium (Advanced DMEM, 2 mM GlutaMAX, 1x NEAA, 10% FCS, 5 ng/ml FGF-2) supplemented with 100 µg/ml Penicillin/Streptomycin and 100 µg/ml Amphotericin B. The medium containing the cells was transferred to a 75 cm² vented flask and incubated at 37°C with 5% CO. The medium was changed after 24 h to remove hematopoietic cells. After three days Penicillin/Streptomycin and Amphotericin B were removed from the medium. The poMSC medium was changed every 2 to 3 days after rinsing the cells once with PBS. Cells were split on a regular basis using Accutase to prevent the cells growing to more than 80% confluence.

2.2.1.4 Isolation and cultivation of porcine fetal fibroblasts (poFFs)

For the isolation of porcine fetal fibroblasts, pigs were slaughtered 30 days after fertilization and the uterus with the embryos was removed. The uterus was opened carefully with sterile scissors and the amniotic sacs with the embryos transferred to pre-warmed PBS. For every embryo a 100 mm² dish with pre-warmed PBS was prepared. The embryos were handled separately to avoid pooling of the male and female. They were then removed from the amniotic sacs. The liver and the head were excised. The head was stored in a 2 ml tube and frozen at -80°C to isolate genomic DNA afterwards to allow sex determination by PCR. The body was transferred to a 60 mm² dish with a pre-warmed trypsin-EDTA-solution with 2% chicken serum and cut into small pieces with a sharp cutter. After incubating for 10 min at 37°C the cell solution was pipetted up and down with a 10 ml pipette and 2 to 3 ml of fresh trypsin-EDTA-chicken serum-solution was added. After a second incubation at 37°C the solution was pipetted up and down with a 5 ml pipette and transferred into a 15 ml falcon tube. After roughly 2 min, undigested tissue sedimented at the bottom of the

tube. The supernatant (8 to 10 ml) was carefully transferred to a 50 ml tube and 20 ml poFF medium (DMEM with 4.5 g/l glucose, 2 mM GlutaMAX, 1x NEAA, 1x sodium pyruvate, 10% FCS, 5 ng/ml FGF-2) was added. The cells were centrifuged for 6 min at 430g and the supernatant was discarded. The pellet was dissolved in poFF medium supplemented with 100 µg/ml Penicillin/Streptomycin and 100 µg/ml Amphotericin B and transferred into a 75 cm² vented flask. After 24 h at 37°C with 5% CO_2 and without moving the flask, the medium was changed. After three days, Penicillin/Streptomycin and Amphotericin B were removed from the medium. The poFF medium was changed every 2 to 3 days after rinsing the cells once with PBS. Cells were split on a regular basis using Accutase to prevent the cells growing to more than 90% confluence.

2.2.1.5 Cultivation of porcine muscle fibroblasts

Muscle fibroblasts were isolated by C. Merkl (Livestock Biotechnology, TU München). The cultivation medium consisted of DMEM (4.5 g/l glucose), 2 mM GlutaMAX, 1x NEAA and 10% FCS.

2.2.1.6 Cultivation of colorectal carcinoma cells

As a control for the APC western blot the cell line SW480 was used. Cells were split in a ratio of 1:2 to 1:8 once a week. The cultivation medium consisted of DMEM (4.5 g/l glucose), 2 mM GlutaMAX and 10% FCS. The medium was changed every 2 to 3 days after rinsing the cells once with PBS.

2.2.1.7 Chromosome analysis of porcine mesenchymal stem cells

One day before the chromosome preparation the slides were prepared. They were stored in a slide chamber in 100% ethanol over night. On the next day they were flushed with dH_2O two to three times and stored under running dH_2O for 10 min. Afterwards they were put into a supersonic device three times for 3 min. After this step the chamber with the slides was filled with UltraPure Water, stored on ice and pre-cooled to 0°C.

Porcine multipotent mesenchymal stroma cells were grown to 75 to 80% confluence in a 75 cm² vented flask. After rinsing the cells with PBS, 10 ml of fresh poMSC medium supplemented with 0.6 to 0.8 ml colcemid was added to the cell culture flask and the

cells were incubated for 4 h at 37°C. After this incubation period the supernatant was transferred to a falcon tube to improve the yield of mitotic cells. The adhering cells were released with Accutase and added to the supernatant. The cells were centrifuged at 300g for 10 min and the supernatant discarded leaving 0.5 ml, in which the cells were resuspended. Cells were gently vortexed and 8 ml of pre-warmed (37°C) 0.8% sodium citrate solution was added drop wise to the resuspended cells. The cells were then incubated at 37°C for 30 min and afterwards centrifuged for 7 min at 300g (1100 rpm) at room temperature (RT). The supernatant was discarded leaving 0.5 ml, in which the cells were again resuspended. A precooled (-20°C) methanol / glacial acetic acid (3+1) solution was added to the cells - first 1 ml slowly and drop wise in which the cells were carefully resuspended, then additional 7 ml at once. Cells were centrifuged for 7 min at 300g at RT and the supernatant was discarded leaving 0.5 ml. This step was repeated three times. After the last centrifugation only 1 ml of precooled methanol / glacial acetic acid (3+1) was added and the cells were incubated at -20°C for at least 10 min. The cell suspension was spread onto the prepared glass slides and dried using a 40°C pre-warmed heat block. The slides with the chromosome spreads were dried for 7 days at RT in a dark box. After drying chromosomal spreads were incubated for 5 min in 4x SCC with 0.2% Tween 20 and stained for 2 min with 1 ml of a 10 ng/ml DAPI solution (4',6-Diamidino-2-phenylindole dihydrochloride). DAPI binds to AT-rich regions of DNA, with double-stranded DNA its absorption maximum is at 358 nm and its emission maximum is at 461 nm. Each glass slide was covered with antifade and a coverslip.

2.2.1.8 Fixation of cells

For the fixation of cells, the cells were rinsed with PBS and 10% formalin in 90% methanol was added for 5 min.

2.2.1.9 Differentiation of porcine multipotent mesenchymal stroma cells

Porcine mesenchymal stem cells were characterised by differentiation assays.

Osteogenic differentiation: For osteogenic differentiation, cells were cultivated in a 6-well plate with poMSC medium to a confluence of 60 to 70%. Then the cells were rinsed with PBS and osteogenic differentiation medium added. This medium consists of poMSC medium supplemented with 100 mM dexamethasone, 10 mM β-glycero-phosphate and 0.28 mM ascorbic acid and was changed every 2 to 3 days. The osteo-

genic differentiation medium was prepared fresh each time, as ascorbic acid is not stable in storage. After 14 days cells were fixed and stained for calcium salts within the bone matrix. For the staining the fixed cells were covered with 1 ml of a 5% silver nitrate solution and incubated for 30 min in the dark. After incubation they were rinsed once with dH_2O. The cells were covered with dH_2O and irradiated for 30 min with UV-light. After a second washing step, 2 ml of a 5% sodium thiosulfate solution were added for 2 min. After a final washing step with distilled water (dH_2O), calcium salts appeared brown / black.

Adipogenic differentiation: For adipogenic differentiation, cells were cultivated in a 6-well plate with poMSC medium to 80% confluence. The cells were then rinsed with PBS and adipogenic differentiation medium was added, which consists of poMSC medium supplemented with 0.5 µM isobutylmethylxanthine (IBMX), 10 µg/ml ITS+1, 100 µM indomethacine and 1 µM dexamethasone. The differentiation medium was changed every 2 to 3 days. After 14 to 21 days cells were fixed and stained with an Oil Red O Solution (1.5 parts of Oil Red O Stock solution + 1 part ddH_2O, filtrated). Oil Red O Solution was made from a stock solution with 0.5% oil red o in isopropanol. For staining, fixed cells were covered with 2 ml of an Oil red O working solution for 1 h at RT. After a washing step with dH_2O, accumulations of lipids were stained red.

Chondrogenic differentiation: For chondrogenic differentiation, cells were detached with Accutase, mixed with the chondrogenic differentiation medium and transferred to a 50 ml falcon tube. For chondrogenic differentiation poMSC medium without FGF-2 was supplemented with 10 ng/ml TGF-β1. The differentiation medium was carefully changed every 2 to 3 days without disturbing the pellets. The fixed cells were stained after 14 to 21 days. For staining, the fixed cells were covered with 2 ml Alcian Blue Solution (1% w/v alcian blue in 3% acetic acid (pH 2.5)) for 30 min at RT. After a washing step with dH_2O mycopolysaccharids appeared blue.

2.2.1.10 Transfection of mammalian cells

Transfection by electroporation: For electroporation of porcine MSCs, cells were rinsed with PBS, detached with Accutase and centrifuged at 430g for 5 min. The supernatant was discarded and the pellet resuspended in pre-warmed, hypoosmolar buffer to a concentration of 5×10^5 to 1×10^6 cells/ml. For each electroporation experiment 400 µl (2 mm cuvette) or 800 µl (4 mm cuvette) of cell suspension were mixed with the

corresponding DNA and incubated for 5 to 10 min at RT. The suspension was pipetted into the cuvette and electroporated with one pulse at 1200 V for 85 µs. After electroporation cells were left in the cuvette for 5 to 10 min at RT before being transferred into pre-warmed medium. The cells were split into selection medium supplemented with 8 µg/ml blasticidin in 150 cm² dishes 24 h after transfection. The medium was changed every 2 to 3 days until colonies with roughly 100 cells were visible. For picking colonies, cell culture dishes were rinsed with PBS. Small, autoclaved filter papers saturated with Accutase were placed over each colony for 2 min. The filter papers were then transferred to a 12- or 24-well plate with fresh selection medium. The filter papers were removed after 24 to 48 h.

Transfection with Lipofectamine 2000: Porcine mesenchymal stem cells were split into a 6-well-plate one day before transfection, in order that they reach 80 to 90% confluence the following day when the transfection was carried out. For each well 3 µg DNA were mixed with 250 µl Opti-MEM and in a second tube 10 µl Lipofectamine 2000 were added to 250 µl Opti-MEM. Both solutions were incubated for 5 min at RT, before the diluted DNA was combined with the diluted Lipofectamine 2000. The mixture was incubated at RT for 20 min. Meanwhile the cells were rinsed once with PBS and 2 ml culture medium was added to each well. Then the combined solutions were added to the well. After 2 to 4 h the medium was changed.

Transfection with Nanofectin: Porcine MSCs were split into a 6-well-plate one day before transfection, in order that they reach 80 to 90% confluence the following day, when the transfection was carried out. For each well 3 µg DNA were mixed with 100 µl Diluent and in a second tube 9.6 µl Nanofectin were added to 100 µl Diluent. Then the Nanofectin solution was added to the DNA solution and the mixture was incubated at RT for 20 min. Meanwhile the cells were rinsed once with PBS and 2 ml culture medium was added to each well. The combined solution was added drop wise to the well. After 2 to 4 h the medium was changed.

2.2.1.11 Selection of cells and picking of colonies

Two days after transfection, cells were removed from the 75 cm² culture flasks with Accutase and replated into three 150 cm² plates with MSC medium supplemented with 8 µg/ml blasticidin. The medium was changed every 2 to 3 days until single colonies were visible. The plates were then rinsed with PBS and small filter papers soaked in

Accutase were placed carefully on the colonies and incubated for 2 min. Then the filter papers were removed and put into a 48-well plate with MSC medium supplemented with 8 µg/ml blasticidin.

2.2.1.12 PCR from cell culture

For the screening of transfected cells, each single-cell colony was transferred to a single well of a 12-well-plate. Once confluent, cells were rinsed once with PBS and detached with 150 µl Accutase. 50 µl of this cell suspension were aliquoted in aliquots of 6 µl and frozen at -20°C for further screening. Each PCR was carried out with one 6 µl aliquot of the cell suspension.

2.2.1.13 Preparation of cells for Somatic Cell Nuclear Transfer (SCNT)

Gene-targeted clones were induced to cell cycle synchronisation by serum starvation before cells were used for SCNT. Two days before the nuclear transfer, MSC medium was exchanged with Starvation medium, which consisted of Advanced DMEM, 2 mM GlutaMAX, 1x NEAA and 0.5% FCS.

2.2.2 Microbiological methods

Bacteria culture: *Escherichia coli* (*E. coli*) were grown overnight at 37°C on agar plates or in LB medium in an orbital shaker. Agar plates and LB medium were supplemented with the appropriate antibiotic; either 100 µg/ml ampicillin or 30 µg/ml kanamycin. For liquid culture, 5 to 300 ml LB medium were inoculated using autoclaved toothpicks.

Storage of *E. coli*: For short-time storage agar plates with *E. coli* colonies were stored at +4°C. For long-term storage 750 µl of an overnight culture were carefully mixed with 250 µl sterile 99% glycerol and stored at -80°C.

Transformation of *E. coli*: For the electroporation of electro-competent *E. coli*, 40 to 50 µl of the bacteria suspension were thawed on ice and carefully mixed with 1 to 3 µl DNA from a ligation reaction or with diluted plasmid. The cell suspension was immediately pipetted into an electroporation cuvette with a 2 mm gap. Bubbles were carefully removed. The cells were electroporated with one pulse at 2500 V for 5 ms. Directly afterwards 600 µl pre-warmed S.O.C. medium were added and the cells

incubated in an orbital shaker at 37°C and 230 rpm for 20 to 30 min. Cells were then plated on LB plates with the corresponding antibiotic supplement and incubated at 37°C over night.

2.2.3 Molecular biological methods

2.2.3.1 DNA isolation

Plasmid DNA preparation from *E. coli*: Minipreps were carried out to obtain small amounts of DNA. For minipreps 5 ml of LB medium with the appropriate antibiotic were inoculated with bacteria and incubated over night at 37°C in an orbital shaker at 230 rpm. From the bacterial suspension 4 ml were centrifuged for 1 min at 14000 rpm and the supernatant was discarded. 100 µl Lysis Buffer (5 mM Sucrose, 10 mM EDTA, 25 mM Tris (pH 8.0)) were added to the pellet and the mixture was vortexed. 200 µl Lysis Solution (0.2 M NaOH, 1% (w/v) SDS) were then added, the solution was inverted to avoid sheering of the DNA and incubated for 3 min at RT. 150 µl Neutralization Solution (3.0 M Sodium acetate (pH 5.3)) were then added, again inverted and stored on ice for 30 min (or 10 min at -20°C). To remove proteins the solution was centrifuged for 5 min at 14000 rpm. The supernatant was poured into a new Eppendorf vial and 1 ml of 95% ethanol added. After mixing the solution thoroughly, it was centrifuged for 15 min at 14000 rpm and the ethanol discarded. The pellet was first washed with 0.5 ml of 80% ethanol followed by 0.5 ml of 95% Ethanol. The pellet was then dried at RT. The pellet with the DNA was dissolved in 40 to 50 µl ddH_2O. RNase was added to the ddH_2O and incubated for 15 min at RT to degrade the residual RNA.

For larger amounts of DNA, midi- or maxi preps were carried out. For midi preps 50 to 100 ml and for maxi preps 200 to 300 ml of LB medium supplemented with the appropriate antibiotic were inoculated with bacteria. Purification of DNA was carried out with NucleoBond Xtra kits according to the manufacturer's manual.

Isolation of genomic DNA: Genomic DNA was isolated with the GenElute Mammalian Genomic DNA Miniprep Kit. Genomic DNA was eluted in 150 µl of the supplied elution buffer.

2.2.3.2 DNA manipulation

Restriction digestion: For restriction digestion 2 to 3 units of enzyme were used per µg DNA. To check restriction patterns, 1 to 3 µg DNA were digested. For preparation of fragments of plasmid DNA up to 100 µg DNA were digested. All digests were carried out with the appropriate buffer and at the specific temperature according to the supplier's manual.

Klenow reaction: For some ligation reactions overhangs of DNA fragments had to be removed or filled in with DNA Polymerase I, Large (Klenow) Fragment. The enzyme forms blunt ends by removing 3' overhangs and filling in 5' overhangs. Usually the removal of the overhangs was followed by a ligation reaction.

Klenow reaction mix:

Reagent	Final concentration
DNA	up to 5 µg
T4 Ligase Buffer	1x
dNTPs	0.1 mM
Klenow	1 µl
ddH_2O	up to 25 µl
T4 Ligase	1 µl

In this case the Klenow reaction mix was prepared without T4 ligase, incubated at RT for 15 min and then the T4 ligase was added to the mixture.

Dephosphorylation and ligation of DNA fragments: Ligation was carried out with T4 ligase for 1 h at RT. If higher ligation efficiency was needed, the reaction was stored at +4°C overnight and transformed in *E. coli* the following day. The reaction was set up according to the supplier's recommendation. If it was necessary to prevent self-ligation of the plasmid backbone, calf intestine alkaline phosphatase was used according to the supplier's manual.

Agarose gel electrophoresis of DNA: Agarose gel electrophoresis was used to separate DNA fragments according to size. It was used to analyse the restriction pattern of plasmids and to isolate DNA fragments from the gel. Gels with 0.8 to 3% agarose supplemented with ethidium bromide were used depending on the fragment size. For

fragments up to 1 kb TBE gels were prepared and TBE buffer (0.9 M Tris-HCl, 0.2 M EDTA, 0.9 M boric acid) was used. For longer fragments and for isolating DNA fragments from the gel, TAE gels and buffer (1.0 M Trisma-HCl, 50 mM EDTA, 2.0 M acetic acid) were used. For size estimation 100 bp and 1 kb DNA ladders from NEB were used. All samples were mixed with Gel Loading Buffer (6.8 M glycerol, 0.2 M EDTA, spatula bromphenol blue in 10 ml ddH_2O) before loading onto the gel. Gels were run at 80 to 100 V for 30 min to 2 h according to the size of the DNA fragments. To visualize DNA fragments with UV light, the gels were examined with the Bio Imaging System Gene Genius.

Isolation of DNA fragments from agarose gels: For isolation of DNA fragments from the gel, the gels were put on a UV table. Fragments were cut from the gel with a clean scalpel. The DNA was purified from the gel pieces with the Wizard SV Gel and PCR Clean-Up System according to the supplier's manual.

Precipitation of DNA with sodium chloride and ethanol: Whenever sterile DNA was required, it was precipitated before use. 0.1 volumes of 3 M NaCl were added to the DNA solution. 2 volumes of 100% ethanol (precooled to -20°C) were added and the solution vortexed. It was then incubated over night at -20°C or for 2 h at -80°C. After the incubation step, the solution was centrifuged for 10 min at 14000 rpm (or 30 min at 5000 rpm). The following steps were carried out under a laminar flow hood. The supernatant was discarded, 1 ml of 70% ethanol was added (for maxi preps 2 ml) and centrifuged at 14000 rpm for 5 min (or 10 min at 5000 rpm). The supernatant was discarded and the pellet dried under a laminar flow hood. The DNA was then dissolved in 50 to 100 µl Tris-low EDTA (10 mM Tris, 0.1 mM EDTA).

Rapid plasmid size screening: Bacterial colonies were picked with a sterile toothpick and resuspended in 20 µl ddH_2O. 20 µl Cracking Buffer (0.2 M NaOH, 0.5% SDS, 0.6 M Sucrose in ddH_2O) and bromphenol blue solution were added. The mixture was centrifuged for 30 sec at maximum speed. From the supernatant 20 to 40 µl were loaded onto a 0.8 to 1% gel. As a control an uncut, empty vector was loaded on the gel.

2.2.3.3 Oligonucleotide annealing

For the introduction of the mutation sites into the targeting vector, oligonucleotides with the corresponding mutations were used. To obtain double-stranded oligonucleotide

DNA an annealing reaction was set up with 1 µg forward and reverse oligonucleotide each, 1x Annealing Buffer (0.1 M Tris (pH 7.5), 1.0 M NaCl, 10 mM EDTA) and ddH_2O up to 20 µl. The mixture was incubated at 95°C for 10 min in a PCR thermocycler. The heating unit was then turned off and the annealing mixture slowly cooled down to RT. A 3% TAE gel was used to check the annealing reaction.

Oligonucleotides for the introduction of the mutation sites at codon 1061 and 1311 are shown below:

Name	Oligonucleotide sequence	size of annealed oligonucleotides
oligo1060s	5'- ccg gaa ggc aaa gtc ctt cac aga atg aaa ggt ggg caa gac cca aac ata taa tag aag atg aaa tat aat aag gat ccg cgg ccg ctt aag tta acg aat tca cca ggt atc gat c -3'	126 bp
oligo1061c	5'- aat tga tcg ata cct ggt gaa ttc gtt aac tta agc ggc cgc gga tcc tta tta tat ttc atc ttc tat tat atg ttt ggg tct tgc cca cct ttc att ctg tga agg act ttg cct t -3'	
oligo1311c	5'- aat tga tcg ata cct ggt cca gaa gcc tgg gaa ttc gtt aac tta agc ggc cgc gga tcc tat tat ttg att tcg gct att tgt aga gta tta gca gaa tct gtt agc atg -3'	118 bp
oligo1311s	5'- cta aca gat tct gct aat act cta caa ata gcc gaa atc aaa taa tag gat ccg cgg ccg ctt aag tta acg aat tcc cag gct tct gga cca ggt atc gat c -3'	

2.2.3.4 Southern blot analysis

Southern blot hybridisation was used to analyse the structure of the targeted *apc* gene and to distinguish the wildtype and targeted alleles.

Preparation of DIG-labelled probes: For the detection of DNA on the membrane, DIG-labelled probes were used. The oligonucleotides APC_S_XhoI_BglII_f and APC_S_XhoI_BglII_r amplified a fragment with a size of 1206 bp, the oligonucleotides IRES-BS-pA-for and IRES-BS-pA-rev a fragment with a size of 915 bp. Additionally a

PCR reaction without Digoxigenin-11-2'-deoxy-uridine-5'-triphosphate was set up to compare the labelled and unlabelled probe by gel electrophoresis.

Oligonucleotides for the amplification of the probe for the Southern blot are shown below:

Name	Oligonucleotide sequence	expected fragment size
IRES-BS-probe-for	5'- tta ctg gcc gaa gcc gct tg -3'	915 bp
IRES-BS-probe-rev	5'- atg gac agc cga cgg cag tt -3'	
APC_S_XhoI_BglII_f	5'- aag tca ggc ggc tac cac tt -3'	1206 bp
APC_S_XhoI_BglII_r	5'- gga cag tcc tcg att ctc ac -3'	

PCR setting for Southern blot probes are shown below:

Reagent	Final concentration
Moltaq Buffer	1x
APC_S_XhoI_BglII_f or IRES-BS-pA-for	400 nM
APC_S_XhoI_BglII_r or IRES-BS-pA-rev	400 nM
MolTaq	1 U
dNTPs	160 µM
Digoxigenin-11-2'-deoxy-uridine-5'-triphosphate	20 µM
poAPC9.2	20 pg
ddH_2O	up to 50 µl

Pre-denaturation		94°C	2 min
40 cycles	Denaturation	94°C	15 sec
	Annealing	55°C	30 sec
	Extension	68°C	90 sec
Final Extension		68°C	5 min
		8°C	forever

Analysis of PCR-labelled probes by gel electrophoresis: For the analysis the non-labelled and DIG-labelled samples were loaded onto a 0.8% TAE gel. The labelled probe was expected to be slightly larger than the unlabelled PCR product.

Dot blot: As gel electrophoresis did not reveal anything about the binding efficiency of the probe to the target DNA, a dot blot was carried out afterwards. Different amounts of the plasmid poAPC9.2 were pipetted dropwise onto a Hybond-N^+ membrane. The examined amounts were 0.1, 1, 5, 10, 50, 150 and 500 pg of plasmid DNA. The DNA was then linked to the membrane by baking at 120°C for 30 min. Afterwards all steps followed the Southern blot protocol described below, but with 1 and 2 µl probe per milliliter of Hybridization Solution.

Southern blot: The first step for the Southern blot was the digestion of DNA. For each lane on the gel 10 µg genomic DNA were digested with 40 U BglII for 3 h at 37°C. The digested DNA was separated by gel electrophoresis on a 1% TAE gel without ethidium bromide to avoid a high background. For size control 4 µl DIG-labelled molecular weight marker VII were used. Additionally 6 µl of the 1 kb size marker were loaded onto the gel for subsequent staining with ethidium bromide. All samples were mixed with 5x Gel Loading Buffer. The gel was run at 40 V for 16 h followed by an additional 12 h at 80 V. The gel lane with the 1 kb marker was then cut off the gel, stained with ethidium bromide for 30 min and the DNA marker visualized with UV light by the Bio Imaging System Gene Genius. According to the separation of the marker, all parts of the gel with DNA fragments shorter than 4 kb and longer than 8 kb were cut off the gel to minimize the gel area for blotting. The gel was submerged in 250 mM HCl for 10 min to depurinate the DNA. Afterwards the gel was rinsed with dH_2O. To denature the DNA the gel was incubated twice in Denaturation Solution (0.5 M sodium hydroxide, 1.5 M sodium chloride) for 15 min at RT. The gel was then rinsed with dH_2O. To neutralize the gel, it was incubated twice in Neutralization Solution (0.5 M Tris-HCl (pH 7.5), 1.5 M sodium chloride) for 15 min at RT. The gel was then equilibrated in 20x SSC (3.0 M sodium chloride, 0.3 M sodium citrate, adjust with 1.0 M HCl to pH 7.0) for 10 min. All steps were carried out with gentle shaking. The capillary blot was assembled according to the manual. After blotting the DNA onto the membrane, the membrane was washed in 2x SSC and the DNA then linked to the membrane by baking for 30 min at 120°C. For the hybridization of the probe to the DNA the membrane was incubated with DIG Easy Hyb for 30 min to 1 h at RT in a rotating 50 ml falcon tube (pre-hybridization step). Meanwhile 2.5 ml of DIG Easy Hyb were pre-warmed to 37°C; 2.5 µl Southern blot probe were diluted in 50 µl ddH_2O and denatured for 5 min at 95°C. The probe was then immediately pipetted into the pre-warmed DIG Easy Hyb and mixed thoroughly. The Pre-Hybridization Solution was discarded and the Hybridization Solution was added to the 50 ml falcon with the membrane. To improve the sensitivity, hybridization took place over night at 37°C. After the hybridization step, the Hybridization Solution

was discarded or stored for further use and the membrane incubated twice in Low Stringency Buffer (2x SSC, 0.1% SDS) for 15 min at RT with gentle shaking. Meanwhile the High Stringency Buffer (0.5x SSC, 0.1% SDS) was preheated to 54°C and the membrane incubated twice in High Stringency Buffer for 15 min at 54°C with gentle shaking. Following this step the membrane was washed with Washing Buffer (0.1 M maleic acid, 0.15 M sodium chloride (adjusted with sodium hydroxide to pH 7.5), 0.3% Tween 20) for 2 min and afterwards Blocking Solution (0.1 M maleic acid, 0.15 M sodium chloride (adjusted with sodium hydroxide to pH 7.5), 1x blocking solution) was added to the tray. After 1 h at RT under gentle shaking, the Antibody Solution (Blocking solution, anti-Digoxigenin-AP, Fab fragments 1:1000) was added to the tray and the membrane incubated for 30 min at RT while shaking. The membrane was then washed twice with Washing Buffer for 15 min at RT and equilibrated in Detection Buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 3 min at RT. The liquid was discarded and the membrane sealed into plastic wrap. The chemiluminescent substrate was diluted 1:100 in detection buffer and added to the membrane drop wise. After 5 min incubation at RT the liquid was carefully squeezed off from the membrane and the plastic wrap carefully sealed around the membrane. The sealed membrane was exposed to X-ray film for 5 to 15 min.

Stripping of the membrane: The membrane was rinsed with dH_2O and afterwards incubated twice in Stripping Buffer (0.2 M sodium hydroxide, 0.1% SDS) for 15 min at RT. After incubation in 2x SSC for 5 min at RT, the membrane was used for the hybridisation with the second Southern blot probe.

2.2.3.5 Sequencing of DNA

Sequencing of DNA was either performed by Entelechon GmbH (Regensburg, Germany), 4BaseLabs (Ulm, Germany) or the BigDye Terminator v1.1 Cycle Sequencing Kit was used.

Oligonucleotides for general sequencing are shown below:

Name	Oligonucleotide sequence	
T7	5'- taa tac gac tca cta tag g -3'	T7 promotor region
SP6	5'- att tag gtg aca cta tag -3'	SP6 promotor region

PCR settings for the sequencing of DNA are shown below:

Reagent	Final concentration
BigDye Reaction Mix	1x
BigDye Sequencing Buffer	1x
Oligonucleotide	2.5 pmol
DNA	20 - 100 ng
ddH_2O	up to 10 µl

Pre-denaturation		96°C	20 sec
30 cycles	Denaturation	96°C	10 sec
	Annealing	51°C	8 sec
	Extension	60°C	4 min
		8°C	forever

Further processing of the sequencing samples was performed by Dr. K. Flisikowska (Chair of Animal Breeding, TU München).

2.2.3.6 Polymerase Chain Reaction (PCR)

All oligonucleotides were supplied by biomers.net GmbH (Ulm, Germany).

Screening for Malignant Hyperthermia sensitive pigs: Isolated MSCs were examined for mutations in the ryanodine receptor 1 (ryr1) with the oligonucleotides RYR1_for and RYR1_rev according to Binder (2004).

For the examination of the ryanodine receptor the following oligonucleotides were used:

Name	Oligonucleotide sequence	expected fragment size
RYR1_forward	5'- gtt ccc tgt gtg tgt gtg tgt gca at-3'	118 bp
RYR1_reverse	5'- ctg gtg aca tag ttg atg agg ttt g -3'	

PCR cycling conditions for malignant hyperthermia screening are shown below:

Reagent	Final concentration
Green GoTaq Buffer	1x
RYR1_for	500 nM
RYR1_rev	500 nM
GoTaq Polymerase	0.25 µl
dNTPs	800 µM
genomic DNA	200 ng
ddH_2O	up to 50 µl

Pre-denaturation		95°C	5 min
35 cycles	Denaturation	95°C	30 sec
	Annealing	56°C	30 sec
	Extension	72°C	30 sec
Final Extension		72°C	5 min
		8°C	forever

Sexing PCR: Genomic DNA was isolated from fetus heads and used as a template for the PCR. The four oligonucleotides SRYB5', SRYB3', 5'ZFX+Y and 3'ZFX+Y were mixed. The oligonucleotide set SRYB5'/SRYB3' amplifies a 163 bp fragment of the *sex-determining region Y* gene (*Sry*) (Pomp *et al.*, 1995). The second oligonucleotide set 5'ZFX+Y/3'ZFX+Y amplifies a 447 / 445 bp fragment of the *Zfy-Zfx* genes (Aasen and Medrano, 1990). Female fetuses showed only one PCR fragment at 445 bp, whereas male fetuses showed an additional PCR fragment at 163 bp.

Oligonucleotides for the porcine sexing PCR:

Name	Oligonucleotide sequence	expected fragment size
SRYB5'	5'- tga acg ctt tca ttg tgt ggt c -3'	163 bp
SRYB3'	5'- gcc agt cgt ctc tgt gcc tcc t -3'	
5'ZFX+Y	5'- ata atc aca tgg aga gcc aca agc -3'	445 bp
3'ZFX+Y	5'- gca ctt ctt tgg tat ctg aga aag -3'	

PCR cycling conditions for sexing screening of porcine fetal:

Reagent	Final concentration
Moltaq Buffer	1x
SRYB5’	400 nM
SRYB3’	400 nM
5’ZFX+Y	400 nM
3’ZFX+Y	400 nM
MolTaq	1.25 U
dNTPs	800 µM
genomic DNA	200 ng
ddH_2O	up to 50 µl

Pre-denaturation		95°C	5 min
35 cycles	Denaturation	95°C	30 sec
	Annealing	58°C	30 sec
	Extension	72°C	45 sec
Final Extension		72°C	5 min
		8°C	forever

Confirmation of blasticidin-resistant poMSC clones: As a primary screening for targeted cell clones, cells were screened for the presence of the *blasticidin deaminase* (*bsd*) gene. The expected fragment size was 331 bp.

Oligonucleotides for the screening for the existence of the *bsd* gene:

Name	Oligonucleotide sequence	expected fragment size
BSf	5'- gag caa cgg cta caa tca -3'	331 bp
BSr	5'- ggc agc aat tca cga atc -3'	

PCR cycling conditions for *bsd* screening:

Reagent	Final concentration
Green GoTaq Flexi Buffer	1x
BSf	500 nM
BSr	500 nM
GoTaq	1.25 U
dNTPs	800 µM
genomic DNA	200 ng
ddH_2O	up to 50 µl

Pre-denaturation		95°C	5 min
35 cycles	Denaturation	95°C	30 sec
	Annealing	58°C	30 sec
	Extension	72°C	40 sec
Final Extension		72°C	5 min
		8°C	forever

Screening for targeting-positive poMSC clones: The PCR Extender System was used for the screening of the poMSC clones. The expected fragment sizes were 3747 bp for APC1061-targeted poMSCs, 3333 bp for APC1311-targeted poMSCs and 3995 bp for the control vector.

Oligonucleotides for the PCR-Screening after gene targeting:

Name	Oligonucleotide sequence
TVR1	5'- tcc gaa ctc ctg gaa tgt ga -3'
BSf	5'- gag caa cgg cta caa tca -3'

PCR cycling conditions for targeting screening:

Reagent	Final concentration
High Fidelity Buffer	1x
BSf	400 nM
TVR1	400 nM
Polymerase Mix	0.7 µl
dNTPs	800 µM
genomic DNA	120 - 200 ng
ddH_2O	up to 50 µl

Pre-denaturation		94°C	2 min
40 cycles	Denaturation	94°C	30 sec
	Annealing	60°C	30 sec
	Extension	72°C	4 min
Final Extension		72°C	7 min
		8°C	forever

2.2.3.7 RNA isolation

For RNA isolation and handling, only RNase-free materials were used. Pipettes and the work bench were treated with RNase Away. Only plugged pipette tips were used. Glass ware was incubated at 180°C for 4 h before being used for RNA isolation.

The cells were cultivated in a 6-well plate, rinsed once with PBS and the supernatant discarded. In each well 1 ml of Trizol was added and incubated for 5 min at RT. Cell lysis was checked under a light microscope. The supernatant was transferred to a 2 ml Eppendorf tube and stored at -80°C. For the isolation of RNA, the supernatant was thawed, 200 µl chloroform added and the tube shaken for 15 sec. It was then incubated at RT for 2 to 3 min and centrifuged for 15 min at 12000g at 4°C. The clear, aqueous, upper phase with the RNA was transferred to a fresh 1.5 ml tube. 500 µl ice-cold isopropanol (stored at -20°C) were added, the liquids were mixed well and incubated for 10 min at RT. After centrifugation for 10 min at 12000g at 4°C, the supernatant was discarded and the pellet with the RNA washed with 1 ml 75% ethanol and vortexed. It was centrifuged for 5 min at 7500g and 4°C, the supernatant was discarded and the pellet dried under a laminar flow at RT or at 55°C for 5 min. The pellet was dissolved in 50 µl UltraPure water. The Turbo DNA-free kit was used according to the manufacturer's protocol to remove DNA from the RNA preparation. RNA was then stored at -80°C.

2.2.3.8 Assessing RNA integrity on agarose gels

For the determination of the RNA integrity the protocol of Masek *et al.* (2005) was used. 5 µl of the RNA sample were mixed with 5 µl 5x buffer and 15 µl deionised formamid. The solution was incubated for 5 min at 65°C followed by 5 min incubation on ice and then loaded onto a 1.2% TAE agarose gel. For high RNA quality two bands with the 18 S and 28 S ribosomal RNA (rRNA) were expected, of which the 28 S rRNA band should contain the double amount of RNA.

2.2.3.9 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR reactions were carried out with the SuperScriptIII One-Step RT-PCR with Platinum Taq Kit, according to the supplier's manual. To exclude contamination with genomic DNA, PCR controls were carried out with Platinum Taq analogous, but without the step for the cDNA synthesis. RNA transcribed from the APC targeted locus was analysed by RT-PCR with the oligonucleotides EST15f and BSr. As a control the wildtype allele was amplified with the oligonucleotides EST15f and Seq7r.

Oligonucleotides for the RT-PCR after gene targeting are shown below:

Name	Oligonucleotide sequence
EST15f	5'- gac tac agg cca ttg cag aa -3'
BSr	5'- ggc agc aat tca cga atc -3'
Seq7r	5'- ggg gct tat aat gcc act ca -3'

Expected fragment sizes for RT-PCR are shown below:

oligonucleotides	fragment size for APC1061 targeting	fragement size for APC1311 targeting
EST15f / BSr	2752 bp	3501 bp
EST15f / Seq7r	3703 bp	4038 bp

RT-PCR cycling conditions for screening for targeting events are shown below:

Reagent	Final concentration
Reaction Mix	1x
EST15f	200 nM
BSr / Seq7r	200 nM
SuperScript RT mix	1.0 µl
RNA	200 ng to 1 µg
ddH_2O	up to 50 µl

cDNA synthesis		55°C	30 min
Pre-denaturation		94°C	2 min
40 cycles	Denaturation	94°C	15 sec
	Annealing	55°C	30 sec
	Extension	68°C	3 min
Final Extension		68°C	7 min
		8°C	forever

If sequencing of the amplified products was necessary, the fragments were excised from the TAE agarose gel, purified and used as a template for a PCR with Phusion Taq. The fragments were then purified again from the gel and used for a sequencing PCR.

2.2.3.10 Isolation of proteins

Protein was isolated either from frozen tissue or from cells. For the isolation from tissue, 85 µl CytoBuster and 15 µl Mini Protease Inhibitor Cocktail Solution was added to the tissue and the tissue was homogenised with a pestle. Cells were detached from the cell culture flask with Accutase and centrifuged for 5 min at 330g. The supernatant was removed carefully and the pellet was resuspended in 85 µl CytoBuster and 15 µl 7x Mini Protease Inhibitor Cocktail. After incubating for 5 min at RT the solution was centrifuged for 5 min at 14000 rpm to pellet remaining cells and tissue. The supernatant was put into a new tube and stored at -80°C. Protein concentration was determined with Advanced Protein Assay Reagent at 590 nm.

2.2.3.11 Western blot analysis

SDS-PAGE: For the detection of truncated APC protein SDS-PAGE based on a tris-glycine system was carried out. A 6% resolving gel with a 3% stacking gel was used.

Compositions of resolving and stacking gel for SDS-PAGE:

Reagent	6% resolving gel	3% stacking gel
40% Polyacrylamide	0.75 ml	0.5 ml
1 M Tris (pH 8.8)	1.88 ml	-
0.5 M Tris (ph 6.8)	-	1 ml
10% SDS	50 µl	40 µl
10% APS	50 µl	40 µl
TEMED	4 µl	4 µ
ddH_2O	2.28 ml	2.42 ml

For each lane 50 µg total protein were prepared. The samples were mixed with 4x Lämmli buffer, heated to 95°C for 5 min and then centrifuged. The Mini-PROTEAN 3 Cell was assembled according to the manufacturer's instructions. The gel chamber was filled with Electrophoresis Buffer for SDS-PAGE (0.25 M Tris base, 1.9 M glycine, 35 mM SDS) and the samples were loaded onto the gel. Additionally 10 µl SeeBlue Plus2 Pre-Stained Standard was used as a size control and all empty gel pockets were filled with 4x Lämmli Buffer (0.25 M Tris-Cl (ph 6.8), 0.1 M SDS, 1.2 M sucrose, 26 mM dithiotreitol, 1 % saturated bromphenol blue) to prevent uneven running of the gel. The gel ran for 60 min at 200 V and 400 mA.

Western blot: Before blotting the gel, filter papers and fibre pads were equilibrated in Wet-Blotting Buffer for 30 min. A PDVF membrane was used for the transfer of proteins onto the membrane. The membrane was activated in 100% methanol for 1 min and equilibrated in Wet-Blotting Buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.025% SDS) for 2 min. The blotting unit was then assembled according to the manual. Blotting occurred over night at 4°C at 30 V and 90 mA.

After blotting the membrane was submerged in 100% methanol for 2 min and then washed with TBS Buffer (20 mM Tris base, 140 mM NaCl) for 5 min at RT. To decrease background signals, the membrane was incubated with Blocking Buffer (5% BSA, 20 mM Tris base, 140 mM NaCl, 0.1% Tween20) for 1 h at RT. Afterwards it was washed with TBS-T Buffer (20 mM Tris base, 140 mM NaCl, 0.1% Tween20) three times for 15 min at RT. The membrane was then incubated with the primary antibody in 10 ml Blocking Buffer over night at 4°C. After additional washing steps with TBS-T

Buffer three times for 15 min at RT, the membrane was incubated with a peroxidase-labelled secondary antibody diluted in Blocking Buffer for 1 h at RT. The membrane was then washed three times with TBS-T Buffer for three times for 15 min at RT and sealed into plastic wrap. The Pierce ECL Western Blotting Substrate was prepared according the manufacturer's protocol and added to the membrane drop wise. After 2 min incubation at RT the liquid was carefully squeezed off from the membrane and the plastic wrap carefully sealed around the membrane. The sealed membrane was exposed to X-ray film for 5 to 15 min.

3 Results

The aim of this work was to generate gene-targeted pigs with a predisposition to colorectal cancer (CRC) modelling the inherited human disease familial adenomatous polyposis coli (FAP). FAP is caused by germline mutations in the *adenomatous polyposis coli* (*apc*) gene. The generation of gene-targeted pigs was to be facilitated by means of somatic nuclear transfer (SCNT) with genetically modified porcine mesenchymal stem cells (poMSCs) as nuclear donors.

3.1 Isolation and characterisation of porcine mesenchymal stem cells

In pigs granulosa-derived cells, preadipocytes and fetal fibroblasts have been used as nuclear donors for SCNT (Polejaeva *et al.,* 2000; Onishi *et al.,* 2000; Tomii *et al*., 2005). Jin *et al.* (2007) used porcine mesenchymal stem cells (poMSCs) as nuclear donors for SCNT and the reconstructed embryos showed a higher blastocysts rate than skin fibroblasts, but these embryos have not been used for embryo transfer. Therefore major focus of this work lay on the evaluation of the suitability of poMSCs for gene targeting and SCNT. To this end, a protocol for the isolation of competent poMSCs has been established. Porcine MSCs, cultured under these conditions, have thereupon been characterised and different transfection methods have been compared.

3.1.1 Isolation of porcine mesenchymal stem cells

Mesenchymal stem cells are part of the stromal bone marrow compartment, which comprises the haematopoietic and the mesenchymal stem cell niche (Yin and Li, 2006). In culture, isolated human MSCs are characterised by adherence to plastic, expression of certain surface antigens and *in vitro* differentiation into osteogenic, adipogenic and chondrogenic cells (Dominici *et al*., 2006). The porcine MSCs in this work were characterised by plastic adherence and their differentiation potential.

Animals produced by SCNT are genetically identical to the cells used as donors for nuclear transfer. Therefore, an advantage of SCNT is the ability to predetermine the offsprings' sex by choosing corresponding donor cells. As male offspring facilitate the

rapid expansion of a herd, only cells isolated from male animals were used for the following experiments.

Porcine MSCs were isolated by flushing the cells of the bone marrow with buffered heparin solution from pig bones. Mononuclear cells were then separated with a Ficoll gradient. After washing, the cells were transferred to standard cell culture dishes and flasks. Within 24 h, single poMSCs were observed adhering to the plastic surface. The medium was exchanged daily for the first three days in order to remove non-adherent cells. Five to six days after the isolation the first colonies were visible. After nine to eleven days the cells covered 80% of the surface of the flasks and the cells were cryopreserved.

Human MSCs have also been isolated from tissues such as adipose tissues, muscle or umbilical cord (Zuk *et al.*, 2001; Bosch *et al.,* 2000; Bieback *et al.*, 2004). Thus, poMSCs were also isolated from other tissues than bone marrow at our institute. However, the isolation of poMSCs from umbilical cord was not successful due contamination of the umbilical cords with bacteria und fungi during the birth process. Porcine MSCs isolated from adipose tissue were also used in this work. These cells were isolated and contributed by C. Merkl (Livestock Biotechnology, TU München).

3.1.2 Effect of FGF-2 on porcine mesenchymal stem cells

As described above, the project aimed to use poMSCs as nuclear donors for SCNT after genetic modification of the *apc* gene. Genetic manipulation of cells is a very time consuming procedure. Cells have to be expanded, transfected, selected, genetically modified cells have to be identified and prepared for nuclear transfer. Clark *et al.* (2000) proposed that roughly 45 population doublings are necessary before cells can be used for SCNT. It has been shown that the growth factor FGF-2 increases the growth rate of human and rabbit MSCs and prolongs the proliferation capacities of MSCs up to 70 population doublings (Martin *et al*., 1997; Tsutsumi *et al.*, 2001; Bianchi *et al.,* 2003). Since nothing is yet known about the effect of FGF-2 on poMSCs, the growth characteristics, stability of chromosomal number and differentiation potential of poMSCs cultivated with FGF-2 were examined.

Freshly isolated cells were cultured in poMSC medium with and without (w/o) 5 ng/ml FGF-2. Morphological differences were observed after six days. Porcine MSCs cultured without FGF-2 showed a fibroblast-like morphology similar to that described by Ringe

et al. (2002). Porcine MSCs cultured with 5 ng/ml FGF-2 show a different morphology (Figure 12), they were smaller, rounder and grew in less tightly packed colonies. They also detached more easily from the plastic surface and proliferated faster. Addition of FGF-2 allowed the long-term culture of poMSCs for 147 days. The cells were capable of 73 population doublings *in vitro* when cultured with FGF-2.

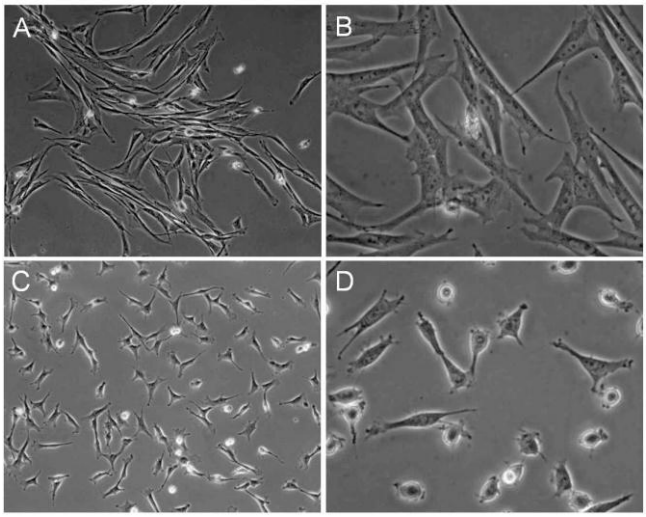

Figure 12: Multipotent mesenchymal stromal cells cultivated with and without FGF-2 (six days after isolation). A/B: poMSCs cultivated in poMSC medium without FGF-2. C/D: poMSCs cultivated in poMSC medium with 5 ng/ml FGF-2. Cells were exposed to FGF-2 directly after isolation.

3.1.3 Chromosome analysis of porcine mesenchymal stem cells

Successful nuclear transfer requires euploid nuclear donor cells. Chromosomal abnormalities in donor cells often prevent normal embryonic development. In MSCs from rhesus monkey, chromosomal abnormalities were observed around passage 20 (Izadpanah *et al.*, 2008). Thus, poMSCs cultivated with and without FGF-2 were examined for the stability of their chromosome number. As transfection, selection and screening of clones were expected to require a minimum of ten passages, metaphase spreads were prepared around passage 15 (Figure 13). For a comparison with long-term cultured poMSCs, additional chromosome spreads of the cells cultured with FGF-2 were carried out at passage 39. The results are summarized in Table 3.

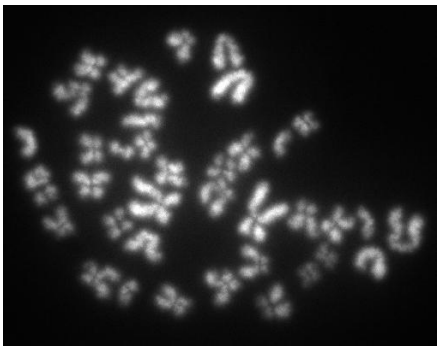

Figure 13: Metaphase spread of chromosomes from porcine mesenchymal stem cells (passage 15). The porcine karyotype consists of 12 metacentric and 6 acrocentric pairs of autosomes and two sex chromosomes. Cells were cultured in poMSC medium with 5 ng/ml FGF-2.

At passage 16, 89.8% of poMSCs cultivated with FGF-2 showed a normal diploid karyotype and in 8.3% of these cells a tetraploid karyotype was observed. Aneuploidy was only seen in one case (2.1%). In contrast, 24.2% of the poMSCs cultivated without FGF-2 showed aneuploidy at passage 15. This suggests that poMSCs cultivated with 5 ng/ml FGF-2 show a more stable karyotype and are therefore a better choice for nuclear transfer.

Table 3: Karyotyping of porcine mesenchymal stem cells. The diploid porcine karyotype consists of 38 chromosomes.

	diploid	aneuploid	tetraploid
poMSCs w/o FGF-2, passage 16, n = 33	75.8%	24.2%	0%
poMSCs with 5 ng/ml FGF-2, passage 15, n = 48	89.8%	2.1%	8.3%
poMSCs with 5 ng/ml FGF-2, passage 39, n = 46	39.1%	60.9%	0%

After long-term culture, poMSCs cultured in medium supplemented with FGF-2 displayed aneuploidy in 60.9% of the cells. Only 39.1% of the cells showed a normal karyotype. Izadpanah *et al.* (2008) showed that long-term *in vitro* culture of human and rhesus monkey MSCs caused cell cycle dysregulation - even in the absence of FGF-2. This might explain why embryos reconstructed with long-term cultured MSCs show higher rates of apoptotic blastomeres (Jang *et al.*, 2004). Therefore, prolonged *in vitro* culture of gene-targeted poMSCs was avoided and cells were stored in liquid nitrogen as soon as possible to minimize time in culture.

3.1.4 Differentiation of porcine mesenchymal stem cells

One criterion for the characterisation of human MSCs is their *in vitro* differentiation potential in osteogenic, adipogenic and chondrogenic cells (Dominici *et al*., 2006). This differentiation capacity was also shown for poMSCs (Ringe *et al.,* 2002), but these cells were not cultivated with FGF-2. Thus, differentiation was carried out to reassure that the isolated cells cultured with FGF-2 were poMSCs. All poMSC preparations were used for differentiation to ensure that the preparations were consistent in their differentiation potential. The cells were differentiated into adipocytes, osteoblasts and chondrocytes. As a control, cells were also cultured in MSC medium without the differentiation supplements.

3.1.4.1 Osteogenic differentiation

For osteogenic differentiation, MSC medium was supplemented with 0.28 mM ascorbic acid, 100 nM dexamethasone and 10 nM β-glycerophosphate according to Bosch *et al*. (2006), but using Advanced DMEM instead of MEM alpha. For the evaluation von Kossa staining was carried out, which indicates the mineralisation of bone matrix by staining calcium deposits. Extensive osteogenic differentiation of poMSCs was observed in the poMSCs cultured with osteogenic medium (Figure 14).

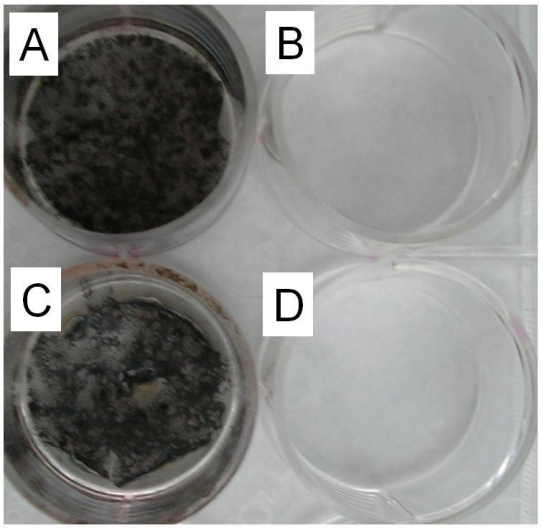

Figure 14: Von Kossa staining after osteogenic differentiation of poMSCs. A: poMSCs differentiated into osteoblasts and stained with von Kossa staining. Osteogenic medium consists of poMSC medium, 0.28 mM ascorbic acid, 100 nM dexamethasone, 10 mM β-glycero-phosphate and 5 ng/ml FGF-2. C: Cells are differentiated in the same medium as in A but w/o FGF-2. FGF-2 has no effect on the osteogenic differentiation potential of poMSCs. B: Control poMSCs in poMSCs medium with FGF-2. D: Control poMSCs in poMSCs medium w/o FGF-2. No calcium deposits were visible after von Kossa staining of the control cells.

The osteogenic differentiation medium was also prepared with and without 5 ng/ml FGF-2, but no differences were observed in osteogenic differentiation potential (Figure 14, A/C). The control cells cultured in poMSC medium without supplements did not show any staining (Figure 14, B/D).

3.1.4.2 Adipogenic differentiation

Six different media were tested for differentiation of poMSCs into adipocytes (Table 4). The first four media consisted of combinations of isobutylmethylxanthine (IBMX), dexamethasone and insulin in poMSC medium with and without FGF-2. No adipogenic differentiation was observed with any of these media. Therefore indomethacine was added and insulin replaced by ITS+1, which is a mixture of insulin, transferrin, sodium selenite, bovine serum albumin and linoleic acid.

Table 4: Overview of adipogenic differentiation media tested.

Medium no.	Medium composition	Adipogenic differentiation
AD1	poMSC medium, 500 µM IBMX, 100 nM dexamethasone	-
AD2	poMSC medium w/o FGF-2, 500 µM IBMX, 100 nM dexamethasone	-
AD3	poMSC medium, 500 µM IBMX, 100 nM dexamethasone, 5 µg/ml insulin	-
AD4	poMSC medium w/o FGF-2, 500 µM IBMX, 100 nM dexamethasone, 5 µg/ml insulin	-
AD5	poMSC medium, 250 µM IBMX, 10 µl/ml ITS+1, 250 nM dexamethasone	some cells differentiated into adipocytes (Figure 15, A)
AD6	poMSC medium, 50 µM IBMX, 10 µl/ml ITS+1, 1 µM dexamethasone, 100 µM indomethacine	extensive differentiation into adipocytes (Figure 15, C)

As shown in Figure 15A some cells showed adipogenic differentiation when insulin was replaced with ITS+1 (medium AD5). The effect was increased when indomethacine was added to the medium and the dexamethasone concentration was raised to 1 µM (medium AD6). This led to extensive lipid vacuolisation (Figure 15C). Lipid deposits were then stained with Oil Red O solution. No staining was visible in the control poMSCs (Figure 15, B/D). As shown in Figure 15C, adipogenic differentiation took

place in more than 50% of the cells. Bosch *et al*. (2006) used a different differentiation scheme and observed adipogenic differentiation in only 1 to 15% of the poMSCs.

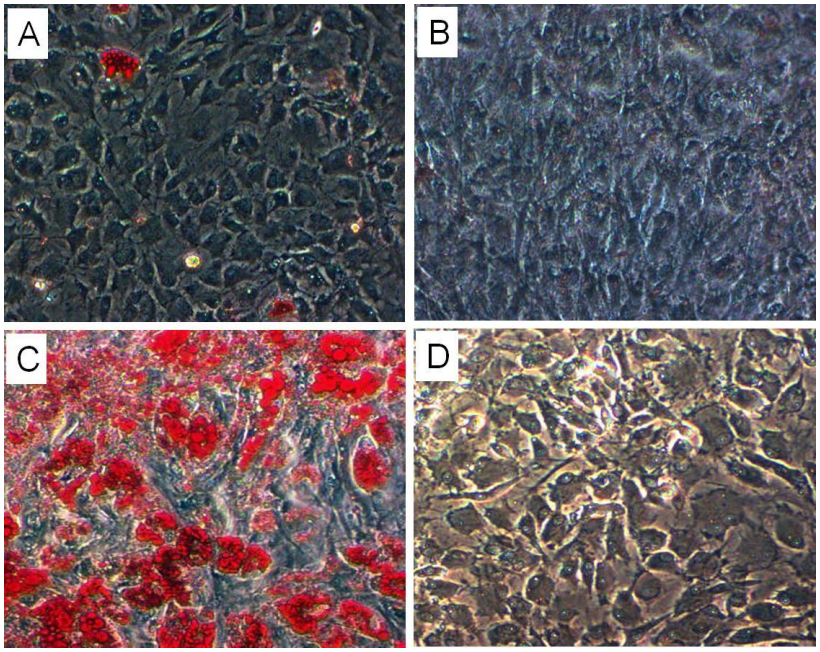

Figure 15: Oil Red O staining of poMSCs differentiated to adipocytes. Cells were cultured for 21 days in the adipogenic differentiation media before lipid vacuoles were stained with Oil Red O. A: poMSC medium supplemented with 250 µM IBMX, 250 nM dexamethasone and 10 µl/ml ITS+1. C: poMSCs medium supplemented with 50 µM IBMX, 1µM dexamethasone, 10 µl/ml ITS+1 and 100 µM indomethacine. B/D: control with poMSC medium.

3.1.4.3 Chondrogenic differentiation

The chondrogenic differentiation of poMSCs was first initiated in culture dishes with MSC medium supplemented with 10 ng/ml TGF-β (Alhadlaq and Mao, 2004). As shown in Figure 16 the formation of 3D spheres was visible, when poMSCs were cultured in chondrogenic differentiation medium. No 3D spheres were observed in the control. But when cells reached 100% confluence, cell layers started detaching from the plastic surface in differentiation and control wells, which complicated the analysis. Therefore differentiation was then carried out in 15 ml falcon tubes. Cells cultured in falcon tubes with chondrogenic differentiation medium started the formation of 3D pellets within seven days of culture. Within the next 14 days these pellets grew to diameters of 2 to 4 mm. In contrast, the control cells adhered to the plastic surface of the falcon tubes in single layers, without the formation of 3D spheres.

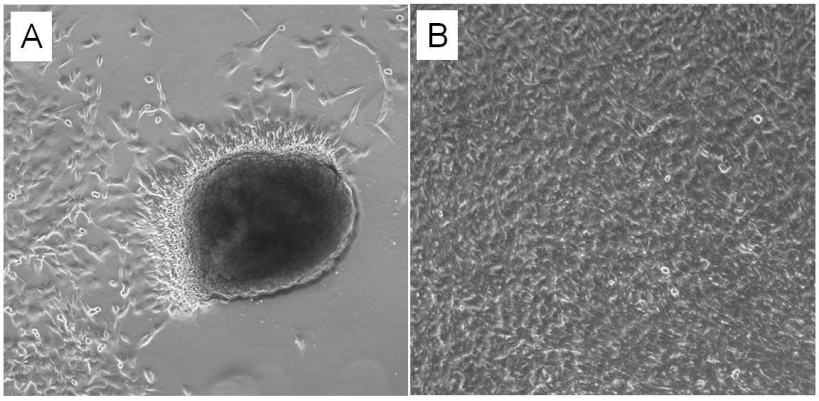

Figure 16: Chondrogenic differentiation of poMSCs in cell culture dishes. A: poMSCs were cultivated with poMSC medium supplemented with 10 ng/ml TGF-β. The formation of 3D spheres was visible. B: Control poMSCs were cultured with poMSC medium.

Summarizing the differentiation experiments, it was shown that the isolated porcine cells were capable of differentiation in cells of the adipogenic, osteogenic and chondrogenic lineage. This suggested that supplementation with FGF-2 did not change the differentiation potential and that the isolated cells were poMSCs.

3.1.5 Transfection efficiency in porcine mesenchymal stem cells

The modification of the endogenous porcine *apc* gene was accomplished by homologous recombination with a transfected plasmid coding for the mutated version. Unfortunately, homologous recombination is a very rare event. Higher transfection rates will increase the number of cells in which homologous recombination can take place. Therefore high transfection rates are necessary to compensate for the low recombination rates and to obtain a few gene-targeted cell clones.

Different methods were tested for their ability to transfect poMSCs effectively. Vasquez *et al*. (2001) proposed that the DNA delivery method used for gene targeting should introduce only few copies of the targeting construct in as many cells as possible. Introducing high amounts of DNA into cells might be toxic and reduce the number of cells able to undergo homologous recombination. Electroporation is supposed to be a suitable method. Regarding MSCs, in human, bovine and porcine MSCs high transfection rates were achieved by electroporation (Peister *et al*., 2004; Colleoni *et al*., 2005). Furthermore the commercial transfection reagents Lipofectamine 2000 and Nanofectin were compared to electroporation.

3.1.5.1 Nanofectin and Lipofectamine 2000

The transfection efficiencies obtained using Nanofectin and Lipofectamine 2000 were evaluated first. Both reagents were mixed as described in 2.2.1.10 with three different DNA combinations (only pPGK-EGFP1, a mixture of pPGK-EGFP1 and the targeting vector pAPC1311 and a control without DNA). The transfection mix was added to the cells in either normal poMSC medium or in serum-free poMSC medium. The medium was exchanged four hours after transfection. The ratio of green cells to non-green cells was estimated. As shown in Table 5, high transfection efficiencies of up to 50% were obtained. With increasing transfection efficiencies, cell viability decreased. Both transfection reagents led to very strong fluorescence signals, suggesting high copy numbers of the pPGK-EGFP1 plasmid within the transfected cells. No difference was observed between cells transfected with pPGK-EGFP1 alone or in combination with the targeting vector pAPC1311. The cell viability decreased in serum-free conditions, but the number of green fluorescent cells increased.

Table 5: Transfection efficiencies and survival rates after transfection of poMSCs with Nanofectin and Lipofectamine 2000 (72 h after transfection).

	Confluence of transfected cells [%]	green cells [%]
control	90	-
Nanofectin + 10% serum	85	10
Nanofectin w/o serum	30	20
Lipofectamine 2000 + 10% serum	80 - 90	40
Lipofectamine 2000 w/o serum	10 - 20	50

Very intense fluorescence was observed with both transfection reagents. Both reagents resulted in a morphological change in the cells. After a few days cells stopped proliferating and they appeared senescent. This might be due to toxic effects of high doses of DNA or EGFP protein on poMSCs. Such treatment seemed likely to damage the competence of poMSCs for nuclear transfer. Therefore electroporation was examined.

3.1.5.2 Electroporation of porcine mesenchymal stem cells

This method takes advantage of a reversible cellular membrane breakdown to allow DNA entry due to short duration and high intense electric fields (Zimmermann, 1996). Electroporation has been used for the transfection of human, bovine and porcine MSCs (Peister *et al*., 2004; Colleoni *et al*., 2005). Based on their results it is supposed that only low copy numbers of DNA enter the cell, which results in less cytotoxic effects compared to chemical transfection methods. The determination of suitable conditions for cell electroporation involves the optimisation of five key parameters: Field strength, pulse length, number of pulses, DNA concentration and cell density. Generally, electroporation with one pulse at room temperature (RT) with a cell density of 1×10^6 cells/ml is recommended in the manual of the electroporation device (Multiporator). This cell number should not be exceeded to avoid an inhomogeneous electric field (Zimmermann, 1996). The electroporation parameters were optimised by examining transient expression of EGFP protein 24 h after transfection (Figure 17). Cells with and without green fluorescence were counted and the ratio of green cells to non-green cells was calculated (Table 6).

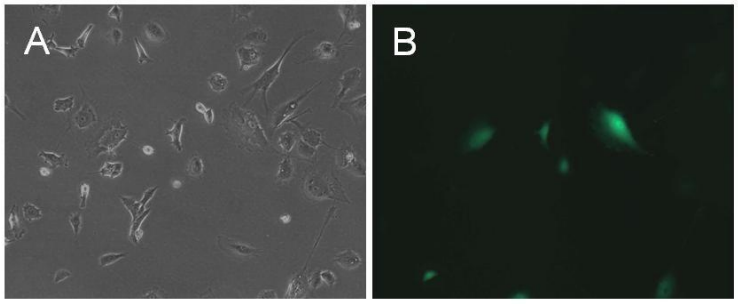

Figure 17: Porcine MSCs transfected with 6 µg pPGK-EGFP1. Electroporation parameters were 1200 V, one pulse, 85 µs, 2 mm electrode distance. Pictures were taken 24 h after electroporation. A: Phase-contrast. B: UV light.

The appropriate voltage for electroporation depends on the diameter of the cells. As poMSCs are very small, voltage numbers up to 1200 V are recommended by the supplier. In a preliminary experiment, the targeting efficiencies in poMSCs with voltages between 600 and 1000 V were determined with a pulse duration of 70 µs. For comparison porcine fetal fibroblasts (poFFs) were used. As poFFs have a greater diameter than poMSCs, lower voltages (400 to 1000 V) and a higher pulse duration of 100 µs were chosen as it was recommended by the supplier. The cells showed a high viability and no differences in cell morphology compared to an untransfected control.

Table 6: Effect of voltage on electroporation efficiencies. For each electroporation, 6 µg of the plasmid pPGK-EGFP1 were used. Electroporation was carried out with 0.4 x 10^6 cells in 400 µl hypoosmolar buffer with 2 mm cuvettes.

	voltage [V]	duration [µs]	$EGFP^+$ cells [%]
poMSC	600	70	0%
	800	70	8.9%
	1000	70	10.2%
poFF	400	100	0%
	600	100	0%
	1000	100	0%

No fluorescence was observed in poFFs with any of the chosen parameters (Table 6). As poFFs were not the subject of this work and only chosen as control cells for the poMSCs transfections, no further experiments were performed with these cells. In contrast to poFFs up to 10.2% of the poMSCs showed green fluorescence.

To increase the number of transfected cells a higher pulse duration of 85 µs was used and 1000 and 1200 V were compared in the next experiments. Additionally, the electrode distance was varied. The field strength depends, amongst other factors, on the distance between the electrodes. In the following experiments cuvettes with 2 and 4 mm electrode distances were compared. For 2 mm cuvettes a maximum of 0.4 x 10^6 cells is recommended for electroporation; therefore 4 mm cuvettes (0.8 x 10^6 cells) were preferred. The increase of the pulse duration to 85 µs had no noticeable influence on cell viability or morphology. As shown in Table 7 larger variations in electroporation efficiency, ranging from 7.1 to 43.3%, were observed with 2 mm cuvettes. An electrode distance of 4 mm led to more stable electroporation efficiencies from 26.0 to 51.6%. The highest efficiency was achieved with 4 mm electrode distance and 1000 V for 85 µs.

Table 7: Effect of cuvette diameter on electroporation efficiency. For each electroporation, 6 µg of the plasmid pPGK-EGFP1 were used. Electroporation was carried out with 400 µl hypoosmolar buffer in 2 mm cuvettes and with 800 µl in 4 mm cuvettes. Pulse duration was 85 µs for all electroporations.

Cuvette gap [mm]	voltage [V]	Cell number	$EGFP^+$ cells [%]
2	1000	1.0×10^6	43.3
	1000	0.4×10^6	7.1
	1200	0.2×10^6	28.3
	1200	1.0×10^6	16.3
	1200	1.0×10^6	47.7
4	1000	0.4×10^6	51.6
	1200	0.8×10^6	43.1
	1200	1.0×10^6	26.0
	1200	0.8×10^6	37.8

The previous experiments only examined the transient expression of EGFP. As the plasmid pPGK-EGFP1 contains also a neomycin resistance gene, viable transfected cells can be selected with the drug G418. To investigate stable expression of EGFP, selection with 250 µg/ml G418 was started two days after electroporation. As expected the number of green fluorescing cells decreased quickly in most of the preparations. Only the electroporation experiment with 1200 V showed an increase in the number of green fluorescing cells. This suggests that the transient transfection efficiency did not reflect the efficiency of stable integration of the *egfp* gene into the genome. Although a difference in the efficiency of colony formation due to homologous recombination and random stable integration has been assumed, these conditions will reflect the requirements better than the transient transfection. Therefore the electroporations with the targeting vectors were carried out with 4 mm electrode distance, one pulse for 85 µs with 1200 V.

3.2 Construction of the targeting vectors

Besides the cell type and the transfection method, the efficiency of gene targeting strongly depends on the design of the targeting vector. In mouse ES cells a total of 14 kb homologous sequence are recommended for efficient homologous recombination

(Deng and Capecchi, 1992). For the detection with PCR these homologous sequences are most commonly split in a short and a long arm when the targeting vectors are constructed. A minimum of 1 kb of homologous sequence is necessary for the short arm of the targeting vector (Thomas *et al.*, 1992; Deng and Capecchi, 1992).

Two targeting constructs were created for the targeting of the *apc* gene in pigs. When the project was started the sequence of the porcine *apc* gene was unknown. Thus, sequencing of the gene was necessary. A total of 16.8 kb of the porcine *apc* sequence from exon 11 of the *apc* gene to 0.78 kb 3' of the translational stop signal in exon 15 were sequenced by Dr. T. Flisikowska (Livestock Biotechnology, TU München). It was shown by sequence analysis that porcine APC protein has two additional amino acids compared to human APC protein. In the pig codon 1242 is followed by glutamine and threonine, which are not present in the human APC protein. Accordingly, mutations were introduced in the porcine *apc* gene at codon 1061 and 1311, which corresponds to the mutations at codon 1061 and 1309 of the human *apc* gene.

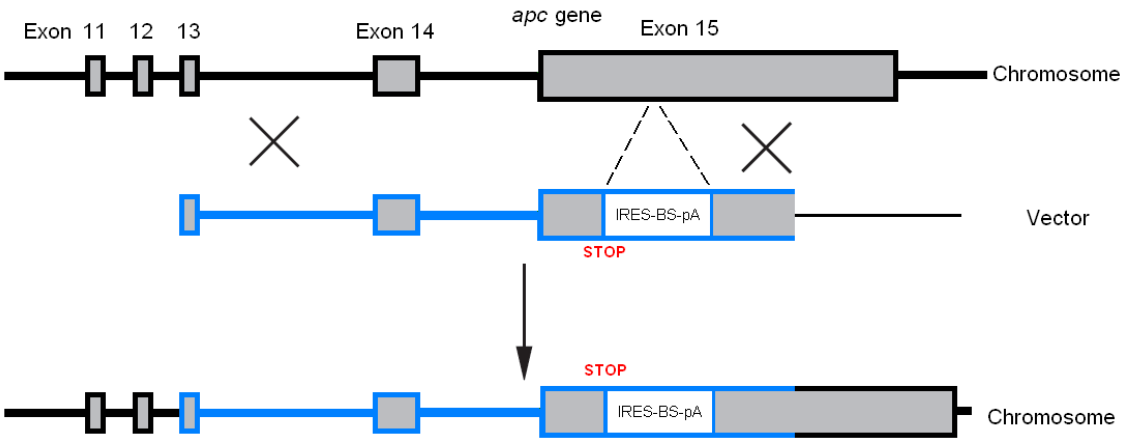

Figure 18: A schematic overview of the targeting event and localization of the targeting vectors' homology arms within the *apc* gene. Both targeting vectors cover sequences from exon 13 to 15 (blue). The chromosomal sequence (black) is replaced after homologous recombination and the mutation cassette is introduced. The mutation cassette includes a stop codon (red), an IRES, a blasticidin resistance gene and a poly(A) sequence.

Both targeting vectors covered sequences from exon 13 to 15 of the porcine *apc* gene and were designed to introduce stop codons at either codon 1061 or 1311 of the porcine *apc* gene. The mutations are based on the two most common mutations of the *apc* gene observed in the human FAP syndrome and lead to truncated APC proteins. A schematic figure of the targeting vector and the recombination event is shown in Figure 18. After the homologous recombination event a stop codon is introduced at either codon 1061 or 1311 of the porcine *apc* gene. The stop codon is followed by an internal

ribosomal binding site (IRES), a *blasticidin deaminase* (*bsd*) gene from *Aspergillus terreus* and a SV40 polyA (polyA) sequence. As both targeting vectors are based on a promotor-trap system the *bsd* gene will only be active if the cassette is inserted downstream of an exogenous promotor (see Figure 11).

In mouse ES cells the targeting efficiency increases with the use of isogenic DNA for the construction of the targeting vector (te Riele *et al.*, 1992; Deng and Capecchi, 1992). One reason might be the large genetic differences, which are established between different murine inbred strains, because in human somatic cells isogenic DNA did not show an advantage over non-isogenic DNA (Sedivy *et al.,* 1999). Additionally, in porcine fetal fibroblasts non-isogenic DNA was successfully used for homologous recombination (Harrison *et al*., 2002). As the cells for genetic manipulation were isolated from different animals, the use of isogenic DNA for the construction of the targeting vectors was not feasible. But as long non-homologous sequences can decrease the efficiency of the homologous recombination (te Riele *et al.*, 1992), the sequences of the porcine *apc* gene were amplified from porcine genomic DNA of a German landrace (DL) x Pietrain pig and the cells used for electroporation were either DL x DL or DL x Pietrain pigs.

3.2.1 Construction of the targeting vector for the mutation at codon 1061

In the targeting vector pAPC1061 the mutation cassette is surrounded by a total of 12454 bp of the porcine *apc* sequence. The targeting vector pAPC1061 has a total size of 16682 bp (Figure 19). The targeting construct pAPC1061 comprises 9357 bp homologous sequence 5’ to the mutation cassette and 3097 bp of homologous sequence 3’ to the mutation cassette. The mutation cassette includes a stop codon, which replaces codon 1061 of the *apc* sequence, an IRES, a blasticidin resistance gene (*bsd*) and a poly(A) sequence. The cloning steps for the construction of pAPC1061 are shown in the appendix (see Appendix 9.1).

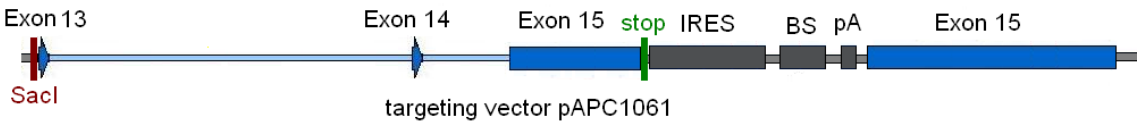

Figure 19: Targeting construct pAPC1061. The targeting construct pAP1061 contains parts of exon 13 to 15 (dark blue) of the porcine *apc* sequence. Introns are shown in light blue. The stop codon (green) is followed by a selection cassette (dark grey).

3.2.2 Construction of the targeting vector for the mutation at codon 1311

In the targeting vector pAPC1311 the mutation cassette is surrounded by a total of 12816 bp of the porcine *apc* sequence. The targeting vector pAPC1311 has a total size of 17017 bp (Figure 20). The targeting vector pAPC1311 consists of 10143 bp homologous sequence 5' to the mutation cassette and 2673 bp of homologous sequence 3' of the mutation cassette. The mutation cassette includes a stop codon, which replaces codon 1311 of the *apc* sequence, an IRES, a blasticidin resistance gene and a poly(A) sequence. The cloning steps for the construction of pAPC1311 are shown in the appendix (see Appendix 9.2).

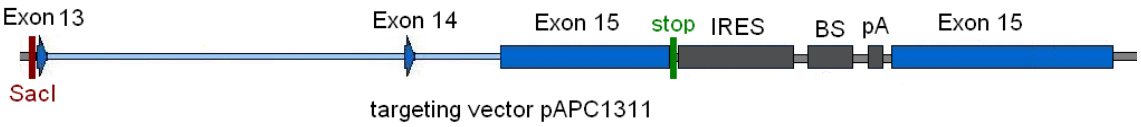

Figure 20: Targeting construct pAPC1311. The targeting construct pAP1311 contains parts of exon 13 to 15 (dark blue) of the porcine *apc* sequence. Introns are shown in light blue. The stop codon (green) is followed by a selection cassette (dark grey).

3.2.3 Construction of a positive control construct

To establish a robust PCR screening assay for gene-targeted colonies after transfection, a positive control construct (control vector) was designed that modelled the structure of the targeted locus after the homologous recombination event. It also served as a positive control for the screening PCR reactions. It contains the selection cassette and *apc* sequences downstream of the 3' flanking homology of the targeting vectors. To distinguish this vector from positive targeting events and to avoid possible contamination of the DNA templates for the screening PCRs, the control vector incorporated a duplication of 248 bp (Figure 21). The cloning steps for the construction of the positive control construct are shown in the appendix (see Appendix 9.3).

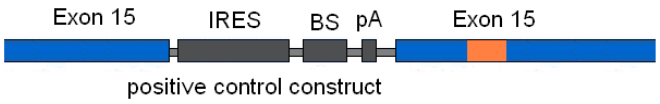

Figure 21: Positive control construct (control vector). To distinguish this vector from positive targeting events and to avoid a possible contamination of the DNA templates for the screening PCRs, the control vector incorporates a duplicated sequence of the *apc* gene (orange).

3.2.4 Linearization of both targeting vectors

Both targeting vectors were linearized with the restriction enzyme SacI before they were used for transfection. It has been shown for mammalian cells that linearized targeting constructs increase the homologous recombination frequency about 10-fold (Kucherlapati *et al*., 1984). The linearization and the concentration of the targeting vectors were checked by agarose gel electrophoresis.

3.3 APC targeting in porcine mesenchymal stem cells

3.3.1 Transfection of mesenchymal stem cells with *apc* targeting vectors

As described in 3.1.5.2, poMSCs were transfected by electroporation in cuvettes with 4 mm electrode distance and one pulse for 85 µs with 1200 V. For each electroporation 13 µg DNA were used due to the size of the targeting vectors. For each transfection two control reactions with pPGK-EGFP1 and without any DNA were carried out. Due to the smaller size of the pPGK-EGFP1 plasmid (4658 bp) only 5 µg DNA were used for electroporation.

Five isolations of poMSCs from different animals were used for transfections (Table 8). Cells were isolated from the bone marrow of four animals as described in 2.2.1.3 and from one animal poMSCs were isolated from adipose tissue by C. Merkl (Livestock Biotechnology, TU München). To estimate the electroporation efficiency, an electroporation with pPGK-EGFP1 was carried out in parallel in all 15 targeting experiments and green fluorescent cells were counted after 48 h. The transient transfection efficiency obtained was between 10 and 29%.

3.3.2 Selection for blasticidin-resistant clones

Two days after transfection the cells were split 1:4 into 150 cm² cell culture plates with poMSC medium supplemented with blasticidin. Blasticidin concentrations of 6, 8 and 10 µg/ml were compared. With a supplementation of 6 µg/ml blasticidin the control cells did not die within 10 days. With a blasticidin concentration of 8 µg/ml the control cells died within seven days and the cells electroporated with the targeting constructs formed single colonies within 10 to 14 days after electroporation. A concentration of

10 µg/ml blasticidin led to the formation of very few colonies after electroporation. However, these colonies grew very slowly and the cells did not attach to the surface after transfer to 24-well-plates. Thus, a concentration of 8 µg/ml blasticidin was chosen for the selection of electroporated cells.

For picking of the colonies different methods were compared: Filter papers soaked with Accutase were put on colonies and transferred to 24-well plates after 2 min. Alternatively, colonies were removed from the plastic surface either by scratching with glass or plastic pipettes or by the use of cloning rings. Best results were achieved with filter papers soaked in Accutase. After 10 to 14 days, when the colonies consisted of ~100 cells, clones were picked with filter papers and transferred to 24-well plates. Cells were detached from the 24-well plates when they reached 80% confluence. One third of the cell suspension was used for the screening PCRs, the remaining cells were transferred to 6-well plates. When the poMSCs reached 80% confluence in their 6-well, a part of the cell solution was used to prepare frozen stocks and the rest was expanded for further characterisation.

3.3.3 Screening procedures for gene-targeted clones

Published data on gene targeting in somatic cells show a considerable range in recombination frequency. Targeting of the *c-myc* gene in rat fetal fibroblasts with a promotor-trap approach led to targeting efficiencies from 2 to 32% depending on the antibiotic resistance genes and the selection procedure (Hanson and Sedivy, 1995). Using a promotor-trap vector in porcine fetal fibroblasts, a targeting efficiency of 2.3% was observed for the *α(1,3)galactosyltransferase* gene (Harrison *et al*., 2002). Therefore it was assumed that a large number of cell clones would have to be screened to identify correctly targeted clones. Several methods were established to screen for homologous recombination events in single cell colonies and afterwards in animals. Initial screening was performed using a PCR designed to detect the presence of the *bsd* gene (see 3.3.4). Screening for the presence of the *bsd* gene was performed as false-positive blasticidin-resistant clones of transfected poMSCs might arise due to insufficient blasticidin concentration or mixed clones. Porcine mesenchymal stem cells are heterogeneous primary cells and the blasticidin concentration for selection was determined empirically. Therefore the stringency of selection might vary. Additionally, it is possible that non-resistant cells are also present due to locally reduced blasticidin concentrations. The next screening was the amplification of a fragment from the *bsd*

gene to an *apc* sequence downstream of the 3' flanking homology of the targeting vectors (see 3.3.5 and Figure 23). Positive results were only expected for colonies in which homologous recombination took place. The PCR screening was established using a control vector. The flanking region 5' of the homology arms was checked by RT-PCR (see 3.3.7 and Figure 29). The amplified fragment extended from the *bsd* gene to the *apc* sequence upstream of the 5' flanking homology of the targeting vectors into exon 11. Additionally, Southern blot hybridisation was established for the examination of single clones (see 3.3.6.2, Figure 26). Southern blot analysis confirmed that no random integration of the targeting constructs occurred. Additionally, it was attempted to set up a Western blot hybridisation to examine the expression of truncated APC proteins (see 3.3.8).

3.3.4 PCR screening for the *blasticidin deaminase* gene

For this PCR the oligonucleotides BSf and BSr were used, which amplify a small fragment with a size of 331 bp of the *bsd* gene (Figure 23). The fragment size was the same for pAPC1061, pAPC1311 and the control plasmid. In total 316 clones were screened by PCR for the presence of the *bsd* gene. 191 clones transfected with pAPC1061 were examined and 119 clones were positive for the *bsd* gene. 125 clones transfected with pAPC1311 were examined and 71 of those clones were positive for the *bsd* gene. The screening revealed that 62.3% of the screened colonies transfected with pAPC1061 were positive for the *bsd* gene. For pAPC1311 in 56.8% of the colonies the 331 bp fragment of the *bsd* gene was amplified. The targeting efficiencies are summarized in Table 8 and Table 9.

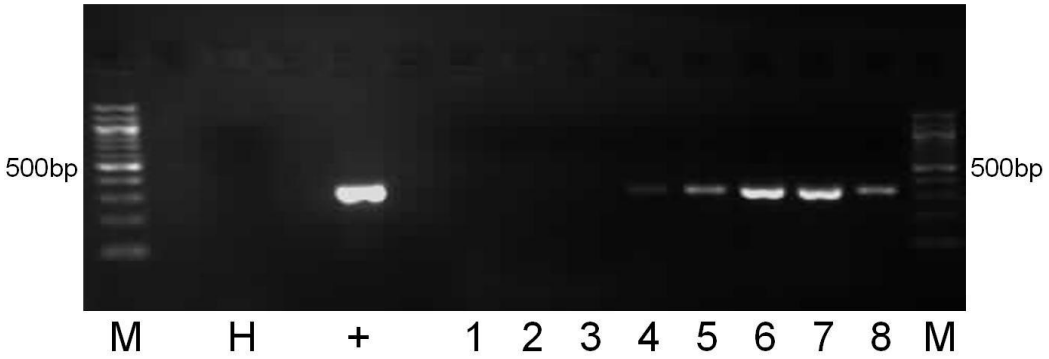

Figure 22: Screening for the *bsd* gene. Cells were transfected with targeting vector pAPC1061. A PCR reaction with the oligonucleotides BSf and BSr was carried out, which amplifies a fragment of 331 bp of the *bsd* gene. H: H_2O control. +: control vector. 1: clone 234. 2-7: clones 227-232. 8: clone 312. A 100 bp marker (M) was used.

An illustrative figure of the screening for the *bsd* gene is shown in Figure 22. In cell lysates of clones 229 to 232 the 331 bp fragment of the *bsd* gene was amplified. Every colony with a positive signal in the *bsd* screening was then used for the targeting PCR.

3.3.5 PCR screening for a targeting event

Clones positive for the *bsd* gene were further analysed for the targeting event by long-range PCR with the oligonucleotides BSf and TVR1 (Figure 23). The amplified fragments for both targeting vectors and the control plasmid were 3.7 kb for homologous recombination with pAPC1061, 3.3 kb for homologous recombination with pAPC1311 and 4.0 kb for the control vector.

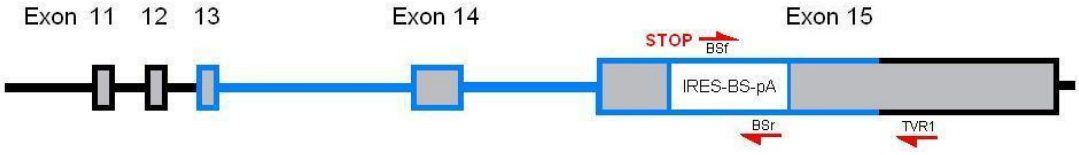

Figure 23: A schematic overview of the targeting event and the binding sites of the oligonucleotides for targeting PCR. The targeting vector (blue) replaces sequences of the *apc* gene. For the screening of targeted clones a PCR with the oligonucleotides BSf (within the *bsd* gene) and TVR1 (3' of the targeting vector's sequence) was performed.

3.3.5.1 Establishment of the PCR for targeting event

To establish the screening for targeting events, four different Taq polymerases were compared: MolTaq, omnitaq, TaKaRa Prime Star HS DNA polymerase and the TripleMaster PCR System (now: PCR Extender System). To achieve conditions comparable to the screening, the control vector was diluted in porcine genomic DNA. As shown in Figure 24, only with the TripleMaster PCR System an amplification of the 4.0 kb fragment of the positive control construct diluted in genomic DNA was possible. Hence, this system was used for the screening of the clones for targeting events.

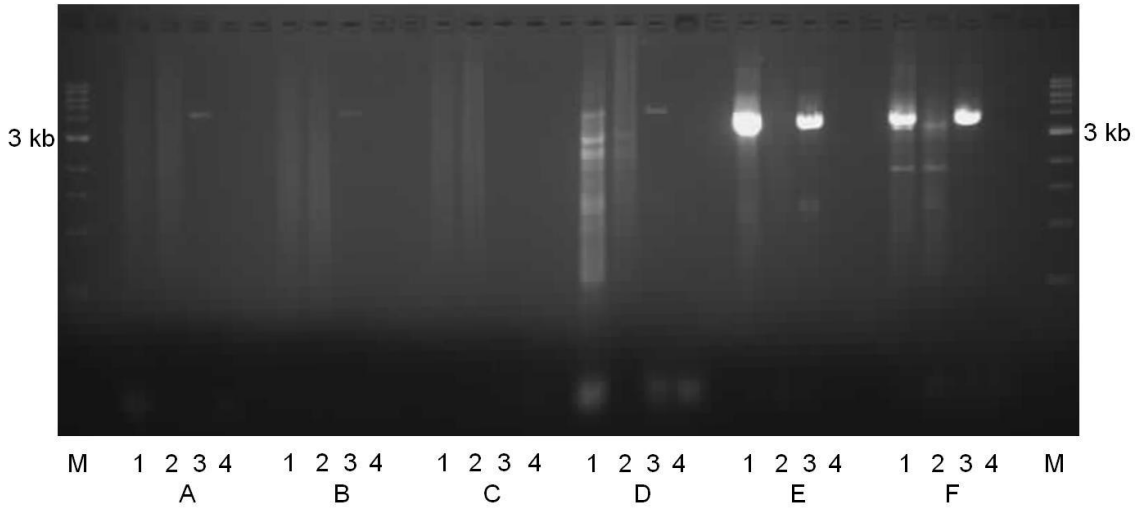

Figure 24: Comparison of Taq polymerases for the targeting PCR. The oligonucleotide pair BSf and TVR1 was used. The expected fragment size was 4.0 kb. A: MolTaq. B: omnitaq with buffer A. C: omnitaq with buffer C. D: TaKaRa Prime STAR HS DNA Polymerase. E: TripleMaster PCR System (now: PCR Extender System) with Tuning Buffer. F: TripleMaster PCR System with High Fidelity Buffer. 1: 0.2 µg genomic DNA with 4.5 x 10^{-6} µg control plasmid. 2: 0.2 µg genomic DNA. 3: 4.5 x 10^{-6} µg control plasmid. 4: water control.

3.3.5.2 Screening for targeting events

The screening for targeting events by PCR was carried out in 190 clones. In 35.3% of the colonies transfected with pAPC1061 targeted events were detected. For transfection with pAPC1311 the targeting PCR amplified the expected fragment in 22.5% of the cells.

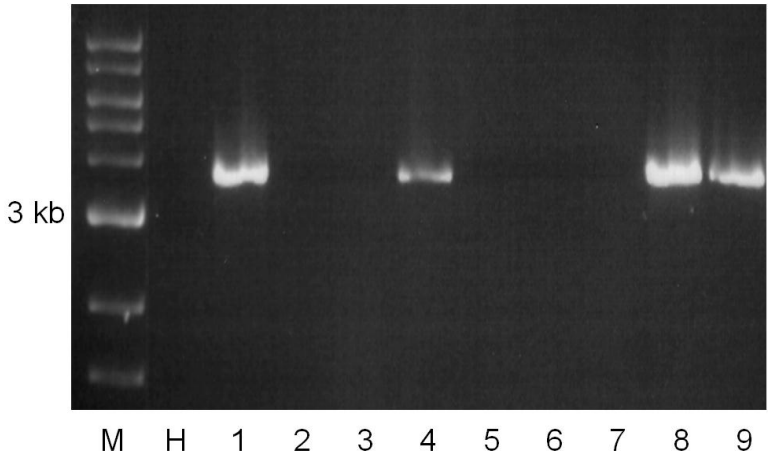

Figure 25: Screening for targeted clones transfected with targeting vector pAPC1061. A PCR reaction with the oligonucleotides BSf and TVR1 was carried out, which amplifies a 3.7 kb fragment for targeted clones. H: H_2O control. 1-9: clones 22, 711, 302, 214, 303, 332, 333, 301, 325. A 1 kb marker (M) was used.

Figure 25 shows the targeting PCR of nine clones. Clones 22, 214, 301 and 325 amplified the 3.7 kb fragment expected for the targeting PCR with APC1061-targeted clones. Clones with a positive targeting PCR were then used for RT-PCR to analyse the correct insertion of the mutation cassette in the 5' direction of the *apc* gene.

3.3.6 Southern blot analysis for detection of single copies

3.3.6.1 Establishment of Southern blot hybridisation

The structure of the APC targeted locus was analysed by Southern blot hybridisation (see 2.2.3.4). 10 µg of genomic DNA was digested with BglII to distinguish wildtype and gene-targeted alleles. As shown in Figure 26, the targeted locus should include the selection cassette between two endogenous BglII restriction sites. Therefore the fragment size varies depending on the wildtype or the targeted allele. The wildtype allele has a fragment size of 5.2 kb, while the gene-targeted allele has 6.0 kb.

Two different probes were used. The APC probe binds to flanking sequences 3' of the short homology arm of both targeting vectors. It detects the wildtype and the gene-targeted allele. To investigate whether additional random integration of the selection cassette had occurred, an IRES-BS probe was used as a second probe. This probe binds within the IRES-BS-polyA-cassette and detects only this cassette.

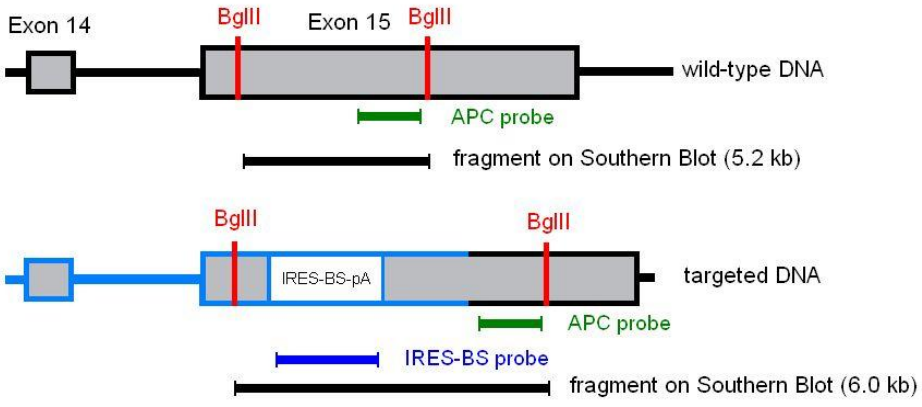

Figure 26: Schematic picture of the binding sites of both Southern blot probes. The APC probe(green) binds flanking regions 3' of the homology sequence from both targeting vectors (light blue). The IRES-BS probe (dark blue) detects the IRES-BS-polyA-cassette. BglII restriction sites are marked in red.

Analysis of PCR-labelled probes by gel electrophoresis: As recommended in the DIG Application Manual (Roche Diagnostics GmbH, 2000) gel electrophoresis with both Southern blot probes was carried out to check the labelling of the probes. Due to the integration of digoxigenin-labelled uridine into the PCR fragments, the labelled probe is larger than the control without labelling and moves slower through the agarose gel (data not shown).

Dot blot: To estimate the sensitivity of the probes for the Southern blot, dot blots were carried out. Concentrations of 1 and 2 µl probe per milliliter hybridization solution were compared for both Southern blot probes (Figure 27). For the APC probe 1 µl probe per milliliter hybridization solution showed a lower background and a higher sensitivity. As little as 5 pg plasmid was detectable. The IRES-BS probe also showed a very light signal at 5 pg plasmid for 1 µl probe per milliliter hybridization solution, but the signal was better detectable for 2 µl probe per milliliter hybridization solution. A minimum of 5 pg plasmid was detectable.

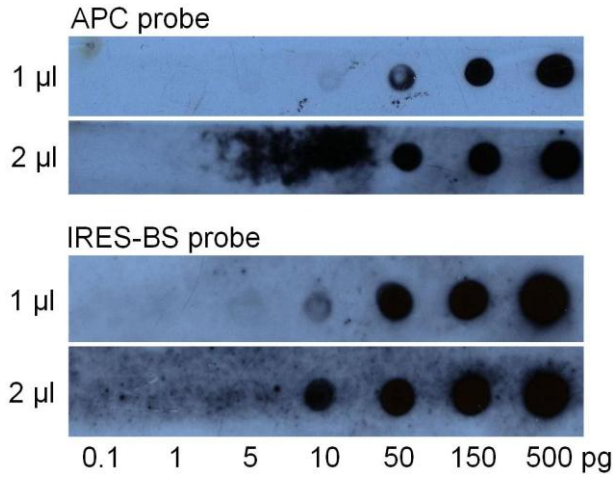

Figure 27: Dot blot for both Southern blot probes. The upper two blots show the APC probe with 1 and 2 µl probe per milliliter hybridisation solution. The lower two blots shows the IRES-BS probe also with 1 and 2 µl probe per milliliter hybridisation solution.

For Southern blot 1 µl APC probe and 2 µl IRES-BS probe per milliliter hybridization solution were used.

Reduction of background signal: To reduce the background staining of the Southern blot, incubation with the high stringency buffer at 54 and 60°C were compared as well as purified and non-purified probes. Hybridization was carried out at 37°C and 1 µl probe per milliliter hybridization solution was used. The probe was purified with the “Gel Wizard System”, but no improvement of the signal was observed. Incubation with the

high stringency buffer at 54°C showed a lower background signal than incubation at 60°C. Thus, unpurified probes were used and hybridisation was carried out at 54°C.

3.3.6.2 Analysis of clones by of Southern blot hybridisation

Due to the restricted number of cells generated from a transfected colony it was not possible to analyse all clones by Southern blot hybridisation. Clones 133, 214, 301 and 325 were expanded, genomic DNA was isolated from each clone and the genomic DNA was digested with BglII. As shown in Figure 28 the targeted and the wildtype fragments were detected in genomic DNA from clones 214, 301 and 325. No signal is visible for clone 133. This was expected as only 2 µg of genomic DNA could be isolated from clone 133 and 10 µg of DNA are necessary for Southern blot.

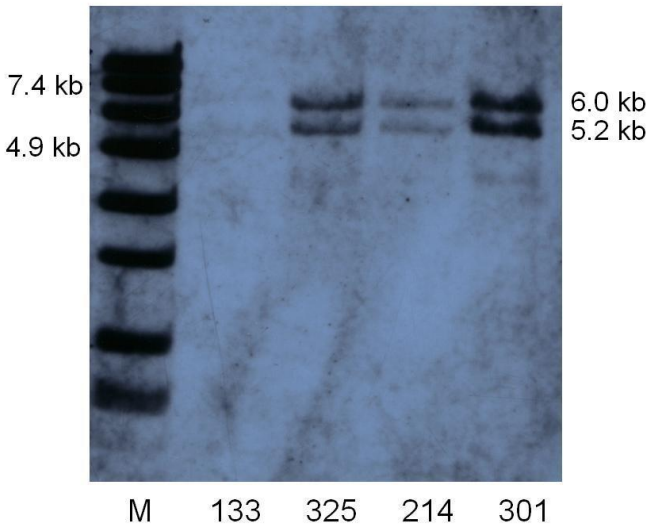

Figure 28: Southern blot of the clones 133, 214, 301 and 325 with the APC probe. No signals are visible for clone 133, but only 2 µg of genomic DNA were used from this clone. Clones 214, 301 and 325 show the wildtype (5.2 kb) and the gene-targeted (6.0 kb) alleles on the Southern blot. A digoxigenin-labelled marker (M) was used.

3.3.7 Analysis of colonies by RT-PCR

Clones which were positive in the screening for the targeting event were examined by RT-PCR to detect RNA transcription from the targeted allele and to analyse the insertion of the long arm of the targeting vector. The oligonucleotides EST15f and BSr were used. As a control, the endogenous *apc* allele was amplified with EST15f and Seq7r (Figure 29). Targeted clones show fragments in both RT-PCR reactions, whereas negative clones will only show the endogenous control.

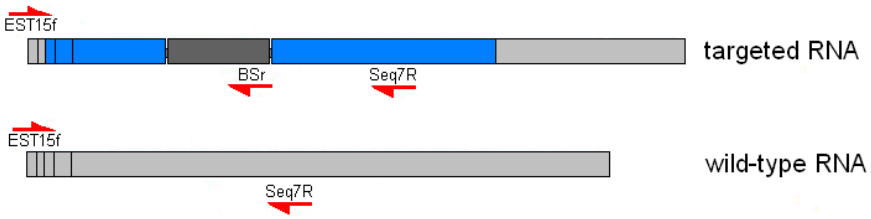

Figure 29: Binding sites of the oligonucleotides for RT-PCR of targeted clones. The oligonucleotides EST15F and BSr are used for the confirmation of targeting events within the clones. As an endogenous control, a second RT-PCR was set up with the oligonucleotides EST15F and Seq7r to amplify the second allele.

RT-PCR was not possible with all the clones, as some of them stopped proliferating and no RNA isolation was possible. A 2.8 kb fragment was amplified from the RNA of the clones with the primers EST15f and BSr. In addition an endogenous control reaction was set up with the primer set EST15f and Seq7r, which amplifies a 2.9 kb fragment for the endogenous allele. The gene-targeted allele would have a fragment size of 3.7 kb, which was not detected as the RT-PCR preferentially amplified the smaller wildtype fragment. From the RNA of APC1311-targeted clones a 3.5 kb fragment was amplified with the primers EST15f and BSr.

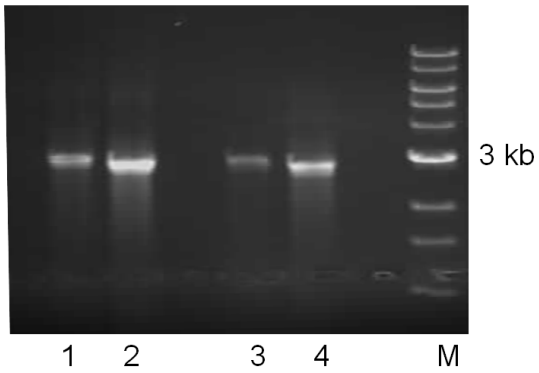

Figure 30: RT-PCR of clones transfected with targeting vector pAPC1061. 1+2: clone 325. 3+4: clone 230. 1+3: RT-PCR reaction with the oligonucleotides EST15f and Seq7r. A 2.9 kb fragment was expected. 2+4: RT-PCR reaction with the oligonucleotides EST15f and BSr. A 2.8 kb fragment was expected. A 1 kb marker (M) was used.

Sufficient amounts of RNA were isolated from eleven APC1061- and two APC-1311-targeted clones. For all 13 clones the targeting event was confirmed by RT-PCR. Figure 30 shows an exemplary figure of the RT-PCR of APC1061-targeted clones. Both clones 325 and 230 amplified the 2.8 kb fragment for the targeted allele and the 2.9 kb fragment for the wildtype allele.

3.3.8 Analysis by western blot hybridisation

It was attempted to set up a western blot hybridisation to analyse the expression of truncated APC protein (see 2.2.3.11). The human colorectal carcinoma line SW480 was used as a positive control. The cell line SW480 was isolated from a human colorectal adenocarcinoma and expresses APC proteins truncated at codon 1338 with a molecular weight of 150 kDa (Nishisho *et al.*, 1991; Midgley *et al.*, 1997). The molecular weight of porcine wildtype APC is 312 kDa. Porcine APC proteins truncated at codon 1061 have a molecular weight of 116 kDa and with a truncation at codon 1311 of 144 kDa. Protein was isolated from SW480 cells, APC1061-targeted poMSCs and wildtype poMSCs.

5% tris-glycine gels were used for the separation of proteins. Afterwards the proteins were blotted on a PDVF-membrane either by semi-dry or wet blotting. In preliminary experiments proteins larger than 250 kDa did not transfer to the membrane with semi-dry blotting, therefore wet blotting was chosen. Proteins were transferred to the membrane for 16 h at 4°C. For the detection of the proteins three different primary antibodies (APC #2504, C-20, N-15) were used. The antibodies APC #2504 and N-15 detect the N-terminus of APC proteins, whereas C-20 detects the C-terminus. For blocking of unspecific signals 5% BSA and 5% non fat dry milk in TBS-T were compared (Figure 31).

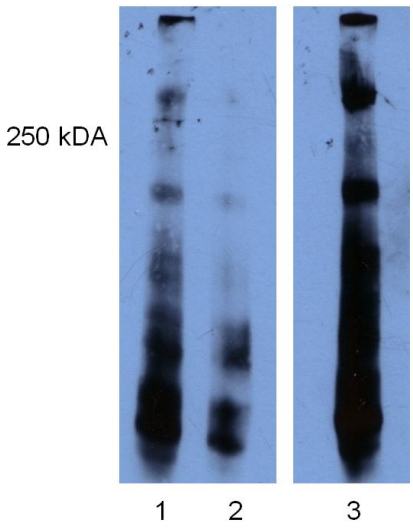

Figure 31: Comparison of 5% non fat dry milk and 5% BSA in TBS-T. No primary antibody was applied to the membrane. Secondary anti-Rabbit (donkey) antibody was diluted 1:10000. 1: 50 µg SW480, 1 h blocking with 5% non fat dry milk in TBS-T. 2: 30 µg SW480, 1 h blocking with 5% non fat dry milk in TBS-T. 3: 50 µg SW480, 1 h blocking with 5% BSA in TBS-T.

The signal at the very top in Figure 31 is protein which did not migrate out of the gel slot. Therefore it is unclear if 5% gels are suitable for the separation of large proteins up to 312 kDa. Due to the high unspecific signals from the donkey anti-Rabbit antibody, another secondary antibody was used in dilutions ranging from 1:30000 to 1:100000 (donkey anti-Rabbit). Protein amounts ranging from 50 to 100 µg and APC #2504 antibody was used. Nevertheless no signal was detected. On a 4 to 15% tris-glycine gradient gel the detection of β-actin was successful. It is likely that the blotting itself was functional, but the detection of the proteins was unsuccessful.

3.3.9 Differentiation of gene-targeted clones

Before cell clones were used for SCNT, further characterisation of gene-targeted clones was carried out. To investigate if the clones kept their differentiation potential during transfection and selection procedures, single clones were differentiated in cells of the chondrogenic, osteogenic and adipogenic lineage.

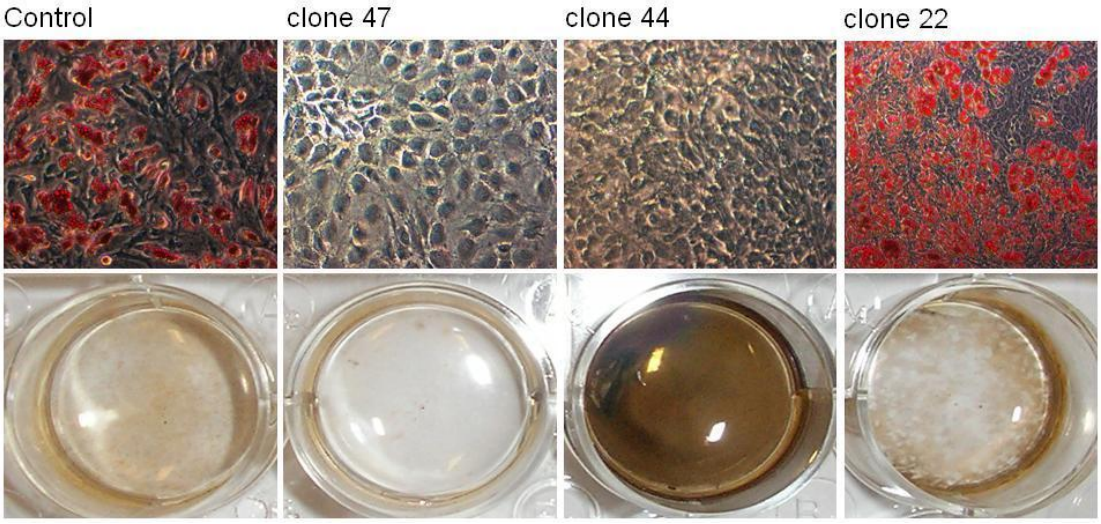

Figure 32: Adipogenic and osteogenic differentiation of single clones 22, 44 and 47. Adipogenic differentiation medium consisted of poMSC medium supplemented with 50 µM IBMX, 1µM dexamethasone, 10 µl/ml ITS+1 and 100 µM indomethacine. Lipid vacuoles were stained with Oil Red O. Osteogenic medium consisted of poMSC medium, 0.28 mM ascorbic acid, 100 nM dexamethasone and 10 mM β-glycerophosphate. Calcium deposits were stained with von Kossa staining.

As shown in Figure 32 and Figure 33, not all clones behaved in the same way. Clones 22, 26, 214, 301 and 325 differentiated in osteogenic and adipogenic lineage, whereas clone 47 did not differentiate at all. Clone 44 preferentially differentiated to the osteogenic lineage. Clones 22 and 301 did show extensive differentiation into the

adipogenic lineage. Pittenger *et al.* (1999) also observed that single cell clones of untransfected human MSCs displayed differences in their differentiation potential. They suggested that either certain cell clones lost their multilineage differentiation potential during *in vitro* culture or that some cells are progenitors with restricted differentiation potential.

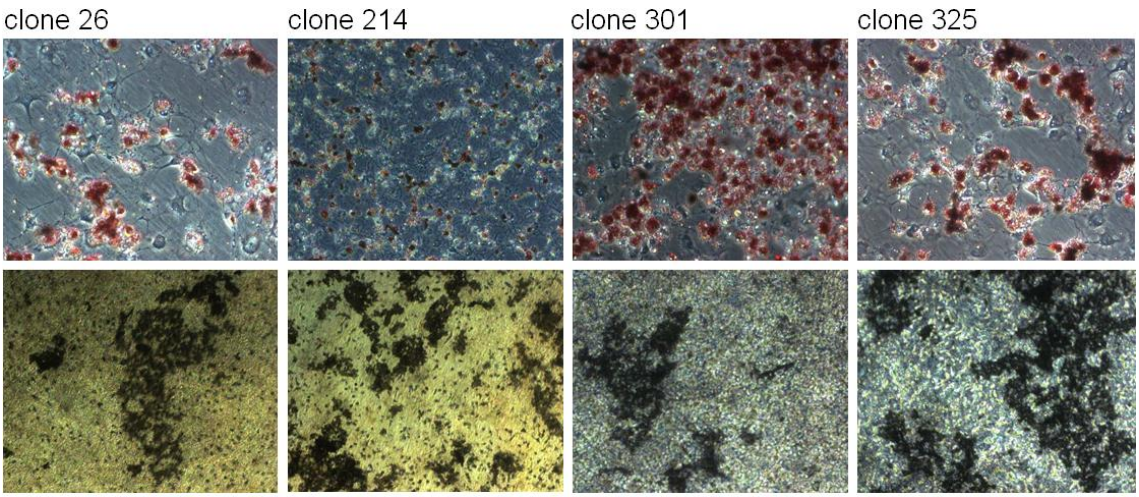

Figure 33: Adipogenic and osteogenic differentiation of clones 26, 214, 301 and 325. Adipogenic differentiation medium consisted of poMSC medium supplemented with 50 µM IBMX, 1µM dexamethasone, 10 µl/ml ITS+1 and 100 µM indomethacine. Lipid vacuoles were stained with Oil Red O. Osteogenic medium consisted of poMSC medium, 0.28 mM ascorbic acid, 100 nM dexamethasone and 10 mM β-glycerophosphate. Calcium deposits were stained with von Kossa staining.

3.4 Targeting efficiencies with targeting vectors pAPC1061 and pAPC1311

3.4.1 Homologous recombination with targeting vector pAPC1061

Nine electroporations with targeting vector pAPC1061 were performed in poMSCs from four different animals. The poMSCs electroporated with pAPC1061 showed normal morphology and growth characteristics compared to untransfected poMSCs (clone 301, Figure 34). The exception was poMSC-170209, which were poMSCs derived from adipose tissue (poADMSC). These cells grew much faster than bone-marrow derived poMSCs. Usually cells were split 48 h after electroporation into selection medium with 8 µg/ml blasticidin and control cells without the *bsd* gene died within seven days. However, poADMSC-170209 had to be split during selection, which was never the case with bone-marrow derived poMSCs. Even though most of the control cells died, some colonies were visible on the plate after seven days of selection. A possible explanation might be that the higher number of cells led to locally reduced drug

concentrations. However, the rate of *bsd* positive clones was very high for the poADMSC-170209 (85.7%) (Table 8).

On average 62.3% of the colonies integrated the *bsd* gene in their genome. As the transfection, selection and screening procedure was established with poMSC-300707, lower numbers of *bsd*$^+$ colonies could be recovered. At the time, nothing was known about the strength of the porcine *apc* promoter, its ability to direct blasticidin resistance and the strength of the IRES in porcine cells. Therefore the selection protocol had to be adapted to the growth characteristics of the electroporated cells. When cells were split after 24 h into selection medium, most cells died quickly. When cells were split in normal poMSC medium and blasticidin was added after adherence of the cells to the plastic surface, control cells did not die within 10 days. Therefore, for further transfections the cells were split directly in selection medium 48 h after transfection. As mentioned in 3.3.2, different methods were compared for picking of the colonies. Some of the methods might have led to low numbers of detached cells or damaged the cells and as a consequence reduced the number of cells which attached to the plastic surface after transfer. Nevertheless 39.5% of these cells integrated the *bsd* gene. From the other poMSC isolations 70.7 to 85.7% of the cells were *bsd*$^+$.

Table 8: Targeting efficiency with targeting vector pAPC1061.

poMSC preparation	no. of transfected cells	*bsd*$^+$ clones / total no. of analysed clones	APC$^{1061/+}$ / *bsd*$^+$ clones	targeting frequency†
poMSC-300707‡	3.7 x 10^6	39.5% (32/81)	43.8% (14/32)	3.8 x 10^{-6} (1 in 2.6 x 10^5)
poMSC-030309	0.4 x 10^6	82.9% (34/41)	41.2% (14/34)	35 x 10^{-6} (1 in 2.8 x 10^4)
poMSC-100209	0.7 x 10^6	70.7% (29/41)	48.3% (14/29)	20 x 10^{-6} (1 in 5.0 x 10^4)
poADMSC-170209	0.8 x 10^6	85.7% (24/28)	0% (0/24)	-
total	5.6 x 10^6	62.3% (119/191)	35.3% (42/119)	7.5 x 10^{-6} (1 in 1.3 x 10^5)

†Targeting frequency is expressed in gene-targeted colonies per total number of cells used for transfection.
‡The cell isolation poMSC-300707 was used for six transfections.

42 APC1061-targeted colonies were identified with the targeting PCR from the transfection experiments with pAPC1061. An overview of gene-targeted cell clones is given in the appendix (see 9.4). All of these colonies were derived from bone-marrow derived poMSCs. The average targeting efficiency of bone-marrow derived poMSCs alone was 44.2%. No APC gene-targeted colonies were identified when using the poADMSC, which was unexpected as these cells had the highest rate of *bsd*$^+$ colonies (85.7%).

3.4.2 Homologous recombination with targeting vector pAPC1311

Porcine MSCs from four different animals were used for six electroporations with targeting vector pAPC1311. In contrast to APC1061-targeted poMSCs, cells electroporated with pAPC1311 changed morphology and growth characteristics compared to untransfected and APC1061-targeted poMSCs. The cells were enlarged, more flattened and grew very slowly after picking of the colonies (Figure 34). The cell morphology was similar to the morphology of senescent human MSCs described by DiGirolamo *et al*. (1999). The senescent morphology occurred in all cell clones transfected with targeting vector pAPC1311. Both targeting vectors are constructed from the same portion of the porcine *apc* gene and three transfections were carried out in parallel with pAPC1061 with the same cell isolates. Thus, the differences in growth characteristics and morphology could be a direct cause of the mutation at codon 1311 or a toxic effect of the DNA due to a contamination in the DNA preparation. Different preparations of plasmid DNA were used for the transfections. Dihlmann *et al.* (1999) described the suppression of wildtype APC by APC1309, which might be an explanation for the decreased growth of APC1311-targeted clones (see 4.3.3.1). However, Thanisch (2009) did not observe morphological changes when the pAPC1311 was used for gene targeting in poMSCs derived from Göttingen minipigs.

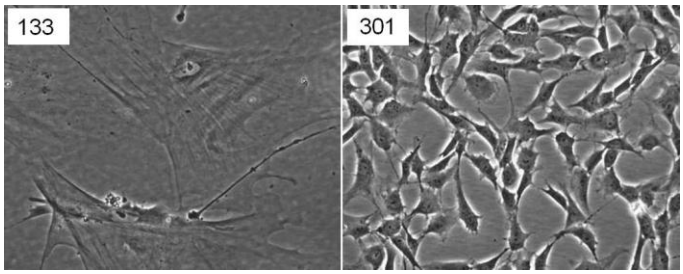

Figure 34: Morphology of APC1061- and APC1311-targeted clones. Left: The APC1311-targeted clone 133. The morphology of this clone is representative of gene targeting with pAPC1311. Right: The APC1061-targeted clone 301.

In addition to the poMSCs mentioned in Table 9, electroporations were also carried out with poMSC-141008 and poMSC-300707, but no colonies were obtained. In total, 125 colonies were examined for the integration of the *bsd* gene. Out of 71 *bsd*$^+$ clones 16 APC1311-targeted colonies were detected. An overview of gene-targeted cell clones is given in the appendix (see 9.4). For poMSC-030309 and poADMSC-170409 100% of the examined colonies were positive for the *bsd* gene (Table 9).

Table 9: Targeting efficiency with targeting vector pAPC1311.

poMSC preparation	no. of transfected cells	*bsd*$^+$ clones / total no. of analysed clones	APC$^{1311/+}$ / *bsd*$^+$ clones	targeting frequency†
poMSC-240707‡	1.5 x 10^6	19.7% (13/66)	76.9% (10/13)	6.7 x 10^{-6} (1 in 1.5 x 10^5)
poMSC-030309	0.4 x 10^6	100% (6/6)	16.7% (1/6)	2.5 x 10^{-6} (1 in 4.0 x 10^5)
poMSC-100209	0.7 x 10^6	90.1% (10/11)	30% (3/10)	4.3 x 10^{-6} (1 in 2.3 x 10^5)
poADMSC-170409	0.8 x 10^6	100% (42/42)	4.8% (2/42)	2.5 x 10^{-6} (1 in 4.0 x 10^5)
total	3.4 x 10^6	56.8% (71/125)	22.5% (16/71)	4.7 x 10^{-6} (1 in 2.1 x 10^5)

† Targeting frequency is expressed in gene-targeted colonies per total number of cells used for transfection.
‡The cell isolation poMSC-240707 was used for three transfections.

3.5 Somatic nuclear transfer with gene-targeted clones

Nuclear transfer with APC1061- and APC1311-targeted cells was carried out at the Institute of Molecular Animal Breeding and Biotechnology (Prof. Dr. Eckhard Wolf, LMU Munich). 48 h before nuclear transfer, poMSC medium was exchanged with starvation medium, which is poMSC medium with reduced serum content of only 0.5%. For all nuclear transfers, different gene-targeted clones were mixed as properties beneficial for SCNT are not yet known. Nuclear transfer was carried out on nine dates between March 2008 and July 2009 and a total of 1372 embryos were transferred (Table 10 and Table 11). On each date 80 to 209 reconstructed embryos were obtained by nuclear transfer and transferred into the recipient sows. Embryo transfer was carried out by

Dr. B. Kessler (Institute of Molecular Animal Breeding and Biotechnology, LMU Munich) at the pig facility in Thalhausen (TU München).

Table 10: Overview of somatic cell nuclear transfer.

Date of embryo transfer	Clones used for transfer	poMSC	no. of transferred embryos	no. of piglets
28.03.08	22, 44, 47	300707	80	-
11.04.08	22, 44, 47	300707	93	-
06.06.08	22, 26, 58	300707	120	4 (4 stillborn)◊
13.06.08†	22, 26, 58	300707	104 & 104	3 (2 died after 1 d, 1 died after 126 d)▲
21.11.08	22, 26, 58	300707	120	-
28.11.08†	22, 26, 58 and poMSC-071008♦	300707	83 & 85	4 (1 stillborn, 3 died within 3 d)▲ 2 (1 stillborn, 1 died after 2 d)▲
15.05.09†	133‡, 214, 301, 325	240707 100209 030309	102 & 102	5 (1 alive, 1 died within 1 h, 3 stillborn)▲
03.07.09†	214, 301, 325	100209 030309	105 & 104	6 (1 alive, 1 died after 1 d, 1 died after 5 d, 3 stillborn)
10.07.09†	168‡, 214, 301, 325	240707 100209 030309	85 & 85	-

◊Cesarean was performed at day 115 after embryo transfer.
†Two recipients were used for embryo transfer.
▲Birth was induced at day 114 after embryo transfer.
♦The cell isolation poMSC-071008 was isolated from one of the dead piglets from the embryo transfer at the 13.06.08.
‡Clones 133 and 168 were APC1311-targeted.

Due to changes in the animal facilities, one of the recipients from the last embryo transfer at the 10.07.2009 had a full bladder, which complicated the transfer of the embryos by endoscopy. Therefore, the second recipient was exposed to movement before the embryo transfer was carried out to ensure an empty bladder.

The first litter was born at the 29.09.2008 (embryo transfer 06.06.2008) comprising four stillborn piglets. Usually pigs give birth after a gestation of 112 to 115 days. Experience at the Institute of Molecular Animal Breeding and Biotechnology (LMU Munich) showed that embryo transfers lead to slightly prolonged gestation times. Therefore birth was expected on day 115, but no signs of parturition were observed at this date. So, it was decided to perform a caesarean section and four dead piglets were recovered from the uterus. One piglet proximal to the vagina showed signs of advanced decay and might have been the reason for the non-initiation of the birth process. Three piglets were dead very recently. The piglets had normal weights between 1.0 to 1.5 kg, only one piglet was slightly underweight with 890 g. For the following births, it was decided to activate the birth process with the administration of Estromate (2.0 ml at d 113) and Depotocin (2.5 ml at d 114). Birth usually started within a few hours after the administration of Depotocin.

The second litter was born on the 06.10.2008 (embryo transfer 13.06.2008) and consisted of three liveborn piglets. All 3 piglets, derived from poMSC-300707, showed a thickened tongue and upturned hoof tips. Otherwise no phenotypical abnormalities were observed. The piglets had great variations in their birth weight. One piglet had a normal weight, but two weighted only 670 g. Quite expectedly both underweight piglets died within 24 h, as these piglets moved very slowly and the sow hit them when she was turning. Porcine MSCs and muscle fibroblasts were isolated from both dead piglets. One poMSC population had to be discarded due to contamination with bacteria. The other poMSCs were used for SCNT at the 28.11.08. The third piglet died on day 126 due to thereupon diagnosed porcine malignant hyperthermia. Further analysis of this piglet (Adam) is described in detail in 3.6.1 and 3.6.2.

Two litters were born on the 22.03.2009 (embryo transfer 28.11.2008). One litter consisted of a liveborn and a stillborn piglet, the other one of four liveborn and one stillborn piglet. All piglets had normal birth weights, between 1.0 and 1.5 kg and made a healthy impression after birth. 12 h later, the first piglet started cramping and died very quickly within 2 h. Four hours later the second piglet started to suffer cramps and also died short after. The last two piglets died roughly 39 and 63 h after birth with the same symptoms and a rapid decline in symptoms. Dissections at the Institute of Veterinary Pathology (Prof. Dr. W. Hermanns, LMU Munich) did reveal an infection with corona virus in the small intestine, but this was not judged to be pathologically relevant. No inflammation of the brain was detected, which could have explained the observed symptoms.

The fifth litter was born on the 06.09.2009 (embryo transfer 15.05.2009). Two piglets were born alive, but one died within the first hour. Three stillborn piglets were born after these two piglets. The last stillborn piglet was born 20 h after the birth of the first alive born piglet. Such long time periods of the birth process were not observed in the first four litters. The liveborn piglet had a birth weight of 620 g and flexured tendon at both hind legs. Similar pathology has already been described by Lai *et al*. (2002b) for SCNT derived piglets. The four dead piglets had normal birth weights between 1.0 and 1.4 kg, but thickened tongues and upturned hoof tips as observed before in the other litters. One piglet is still alive and gives a healthy impression.

The sixth litter was born on the 26.10.2009 (embryo transfer 03.07.2009) and consisted of three liveborn and three stillborn piglets. The birth weights of the liveborn piglets were 0.8, 1.0 and 1.6 kg. All stillborn piglets had a birth weight around 1.6 kg. All liveborn piglets showed a slightly thickened tongue and no sign of upturned hoof tips. One of the liveborn piglets was made a slightly underdeveloped and weak impression and died after one day. The heaviest liveborn piglet died after five days from a combination of gastritis, enteritis and peritonitis after infection with *Escherichia coli* and *Clostridium*. The smallest liveborn piglet is still alive.

3.6 Analysis of gene-targeted piglets

3.6.1 Tissue analysis

Piglet Adam (embryo transfer 13.06.08) died at an age of 126 days after being exposed to stress, i.e. before an examination of the upturned hoof tips by a veterinary. According to the dissection at the Institute of Veterinary Pathology (Prof. Dr. W. Hermanns, LMU Munich) the piglet died of malignant hyperthermia. Part of the dissection was the further analysis of tissues from eye, stomach, small intestine and colon, but no pathological changes were observed (Figure 35). This result was unsurprising as familial adenomatous polyposis coli occurs with an onset at an average age of 20 years in humans (Caspari *et al*., 1994; Friedl *et al.,* 2001). Therefore no malignant transformations in the intestine of the pigs were expected at such a young age.

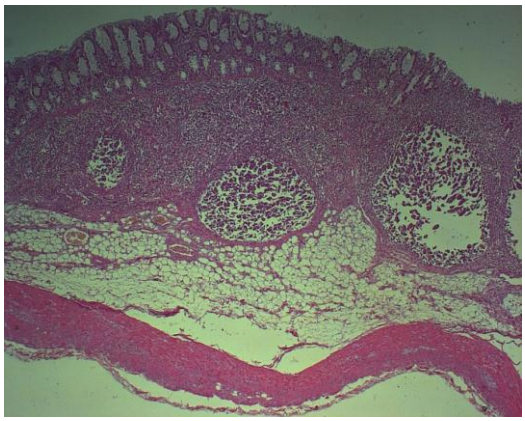

Figure 35: Histological sections of the colon. Hematoxylin and eosin staining was carried out at the laboratory of the II. Medizinische Klinik, TU München.

3.6.2 Malignant hyperthermia syndrome

The results from the dissection of piglet Adam suggested that malignant hyperthermia syndrome (MHS) was the reason for the unexpected death of the piglet. The malignant hyperthermia syndrome is an inherited myopathy that occurs in many species, which leads to hypercatabolism and muscle rigor triggered by inhalational anaesthetics and skeletal muscle relaxants (Fujii *et al*., 1991). In pigs the syndrome can also be caused by stress and therefore is also called porcine stress syndrome. The molecular cause is a mutation in the *ryanodine receptor 1* (*ryr1*) gene, which leads to the replacement of an arginine on codon 615 with a cysteine (MacLennan *et al*., 1990). The ryanodine receptor 1 encodes a Ca^{2+}-release channel within the skeletal muscle sarcoplasmic reticulum. This mutation causes the opening and closing of Ca^{2+} channels in the skeletal muscle to be altered, opening of the channel is facilitated and closing is restricted. After a trigger this leads to massive stimulation of the skeletal muscle metabolism, which causes a rapid increase in body temperature, and metabolic changes such as acidosis and hyperkalaemia.

As MHS was clearly diagnosed for the dead piglet, the genotype of the *ryr1* gene in this animal was examined. Additionally, all poMSCs were tested for the mutation in the *ryr1* gene to exclude those cells for further SCNTs. For the genetic test a PCR for the *ryr1* gene was performed (see 2.2.3.6). The PCR fragment was then used for a diagnostic restriction enzyme digestion with HhaI according to Binder (2004). After restriction digestion with HhaI two fragments with 33 and 85 bp were expected for homozygous animals without the *ryr1* mutation. In heterozygous animals the restriction site for HhaI is lost and three fragments with the sizes 33, 85 and 118 bp were expected.

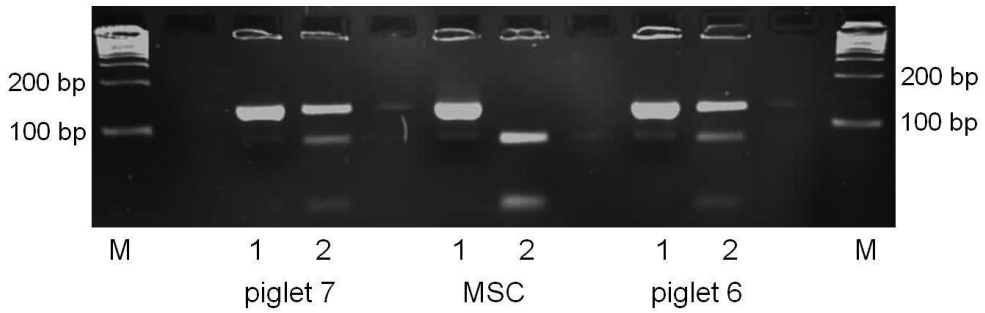

Figure 36: Analysis of the ryanodine receptor 1 in piglet 6, piglet Adam and poMSC-240707. 1: PCR fragment of oligonucleotides Ryr1_for and Ryr1_rev. A 118 bp fragment was expected. 2: PCR fragment digested with HhaI. Homozygous animals without a mutation show two fragments after HhaI digest. Heterozygous animals show three fragments. A 100 bp (M) length standard was used.

As shown in Figure 36, piglet 6 and piglet Adam were heterozygous for the mutation in the *ryr1* gene. These animals were created from poMSC-300707. From May 2009 on only poMSCs negatively tested for the mutation in the *ryr1* gene were used for SCNT. One example is shown in Figure 36, as genomic DNA from poMSC-240707 showed a homozygous genotype for the wildtype allele.

3.6.3 Genetic analysis of gene-targeted piglets

The first analysis of these piglets was carried out by PCR analysis for targeting events to distinguish APC1061- and APC1311-targeted piglets. So far, no APC1311-targeted piglet was born. The genomic DNA was also used for Southern blot analysis to show the insertion of the targeting cassette in the porcine genome and to exclude additional integrations of the selection cassette.

3.6.4 Analysis of gene-targeted piglets by PCR

PCR analysis as described in 3.3.5 was used to detect the correct insertion of the selection cassette in relation to *apc* sequences downstream of the 3' flanking homology of the targeting vectors. The oligonucleotides BSf and TVR1 were used (see Figure 23). The predicted fragment sizes were 3.7 kb for homologous recombination with pAPC1061, 3.3 kb for pAPC1311 and 4.0 kb for the control vector. PCR analysis for targeting events of the piglets from the embryo transfer at the 15.05.09 is shown in Figure 37. In four of the analysed piglets a fragment of 3.7 kb could be amplified by PCR analysis, which proofs the successful targeting of codon 1061 in the endogenous

porcine *apc* gene. Isolation of genomic DNA from piglet 2 was not successful due to decay of the tissue. Control PCRs on SacI treated targeting vectors were negative as expected, as the 3' primer binds only within the genomic DNA.

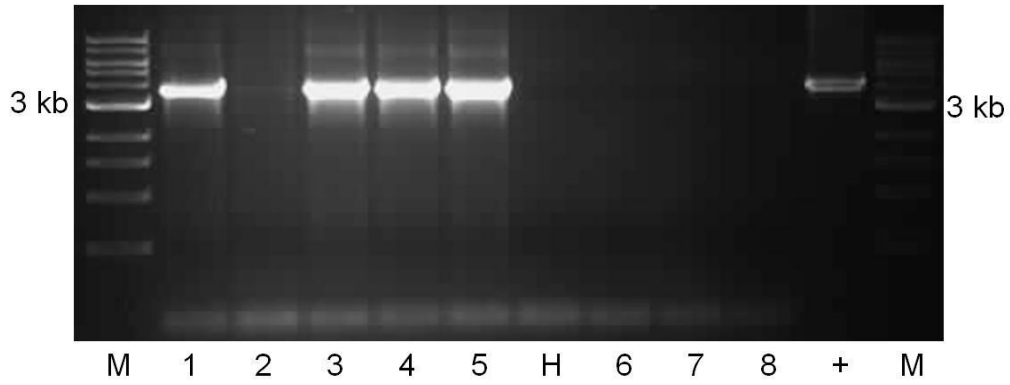

Figure 37: Targeting PCR with APC1061-targeted piglets. A PCR reaction with the oligonucleotides BSf and TVR1 was performed, which amplifies a 3.7 kb fragment. 1-4: Dead piglets 1 to 4 from the ET at the 15.05.09. DNA isolation from dead piglet 2 was not successful due to decay of the tissue. 5: Alive piglet from the ET at the 15.05.09. H: H_2O control. 6: genomic control. 7: Targeting vector 1061, SacI treated. 8: Targeting vector pAPC1311, SacI treated. +: control vector, predicted fragment size is 4.0 kb. A 1 kb marker (M) was used.

Further analysis of the gene-targeted piglets was carried out by Southern blot hybridisation.

3.6.5 Analysis of gene-targeted piglets by Southern blot hybridisation

The targeted insertion of the stop codon at 1061 and the antibiotic resistance cassette was analysed by Southern blot hybridisation as described in 3.3.6.1. As shown in Figure 26, the targeting cassette integrates between two endogenous BglII restriction sites. Therefore the wildtype allele has a fragment size of 5.2 kb, whereas the gene-targeted allele has a size of 6.0 kb. In addition to the APC probe, which indicates the integration of the targeting vectors in the *apc* gene, Southern blot analysis with the IRES-BS probe was carried out to detect random integrations in other parts of the genome. Figure 38 shows the results for the gene-targeted piglets from the embryo transfer at the 15.05.09 and a wildtype piglet. Four of the five piglets show the expected fragments at 5.2 and 6.0 kb with the APC probe. DNA isolation from piglet 2 was not successful due to decay of the tissue. Additionally, with the IRES-BS probe no random integration was detected in these piglets.

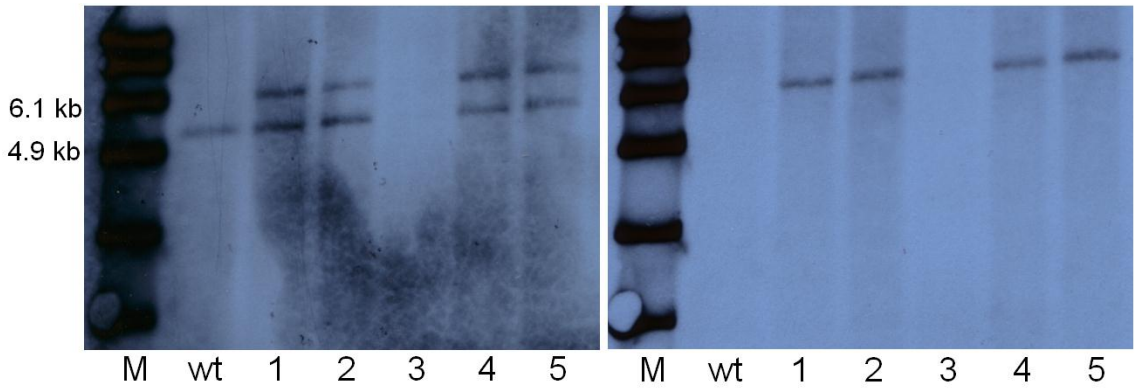

Figure 38: Analysis of gene-targeted piglets by Southern blot hybridisation. For each lane 10 µg of genomic DNA were used for restriction digestion with BglII. After hybridisation of the IRES-BS probe, the membrane was stripped and the APC probe was used for a second hybridisation. Left: Hybridisation with the APC probe. Expected fragments were 5.2 kb for the wildtype allele and 6.0 kb for the gene-targeted allele. Right: Hybridisation with the IRES-BS probe. No random integrations were detected. A digoxigenin-labelled marker (M) was used. Genomic DNA from a wildtype piglet was used as a control. 1: Alive piglet from the ET at the 15.05.09. 2-5: Genomic DNA from the dead piglets from the ET at 15.05.09. DNA isolation from dead piglet 2 was not successful due to decay of the tissue.

3.7 Summary of results

Gene-targeted, heterozygous piglets with mutations at codon 1061 of the porcine *apc* gene were generated. Two healthy piglets were born in September and October 2009 (Figure 39). These piglets represent the first porcine cancer model. When this work was started in 2006, no porcine disease models were described. However, in 2008, Rogers *et al*. (2008a) published a porcine model of cystic fibrosis. Thus, these piglets represent the second disease model described in pigs.

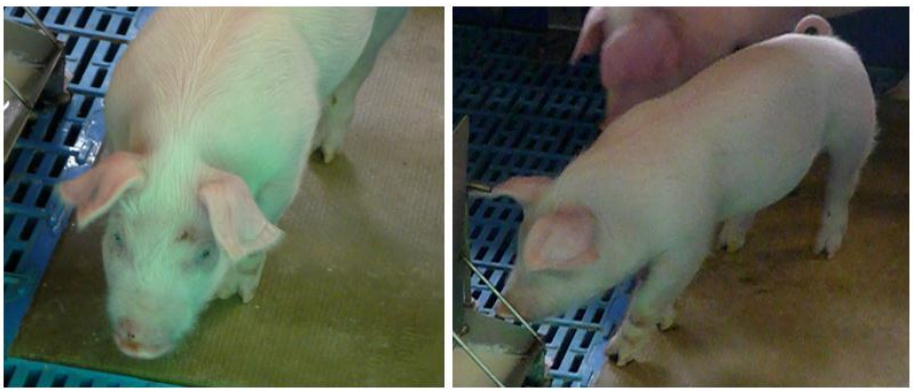

Figure 39: Gene-targeted, heterozygous piglet with mutation at codon 1061 of the porcine *apc* gene. Piglet Adam at an age of 43 days.

In this work an efficient method for the transfection of porcine MSCs was established. Gene targeting was more efficient with targeting vectors pAPC1061. A total of 42 APC1061-targeted clones were identified from nine transfections. The vector pAPC1311 was less efficient. However, 16 APC1311-targeted clones were detected in six transfections. Interestingly, higher number of *bsd* positive clones did not increase the number of gene-targeted clones. This variation in random integration to homologous recombination was also observed in targeting of the *cftr* gene (Rogers *et al*., 2008a). High variations in homologous recombination rate were observed either in different poMSCs targeted with the same vector or targeting the same poMSCs with both targeting vectors. The high variation within poMSCs isolated from different animals was also described by Rogers *et al*. (2008a), when porcine fetal fibroblasts were used for gene targeting of the porcine *cftr* gene.

Table 11: Summary of somatic nuclear transfer.

no. of recipients	no. of early pregnancies	no. of pregnancies to term	no. of piglets	no. still-born	no. alive born	death after	alive
14	50% (7/14)	43% (6/14)	24	50% (12/24)	50% (12/24)	1 h to 4 d, 126 d	2

APC1061- and APC1311-targeted poMSCs were used for SCNT (Table 10). In total 14 embryo transfers were performed (Table 11). In seven animals early pregnancies were detected around day 30 after transfer by ultrasound examination. Six of these pregnancies went to term and 24 piglets in total were born. Twelve of these piglets were stillborn. The litter size was between two and five piglets. One of the pregnancies that was detected around day 30 after embryo transfer by ultrasound did not go to term. The abortion of established pregnancies in pigs after embryo transfer was also described in other SCNT experiments in pigs (Dai *et al*., 2002; Lai *et al*., 2002b; Takahagi *et al*., 2005; Phelps *et al*., 2003). The rate of pregnancies to term after SCNT in this work was similar to other published experiments in pigs (Dai *et al*., 2002; Ramsoondar *et al.*, 2003).

Two piglets with mutations at codon 1061 of the porcine *apc* gene are alive and will be used for further analysis and breeding.

4 Discussion

Animal models for medical and developmental studies are still indispensable to research. Due to established transgene technologies a broad variety of rodent models is available, which model human diseases and enable the development of new drugs and therapies.

Colorectal cancer (CRC) is the second most common cancer in Germany diagnosed in men and women (Robert-Koch-Institut, 2008). It accounts for over 600000 deaths per year worldwide (World Health Organization, 2009). Animal models could increase the knowledge about this disease and help to develop treatment and prevention strategies. Rodent CRC models have been useful for addressing basic questions about the mechanisms of tumor progression and developing new screening methods and therapies (McCart *et al.*, 2008). However, rodents have limitations such as age, size, physiology and the symptoms of rodent models did not resemble human CRC (see 1.4.1.2 and 1.4.1.3). These problems could be overcome by livestock models. Thus, the aim of this work was the generation of a porcine model predisposed to colorectal cancer.

The mutations in the gene-targeted pigs are based on the inherited human CRC syndrome familial adenomatous polyposis (FAP) which manifests itself by colorectal adenocarcinoma with early onset (Caspari *et al.*, 1994; Friedl *et al.*, 2001). FAP is caused by germline mutations in the *adenomatous polyposis coli* (*apc*) gene and these mutations disturb the normal function of the APC protein within the Wnt pathway (see 1.1.4). However, rodent models with mutations in the *apc* gene equivalent to those observed in human FAP patients fail to develop colorectal carcinoma and metastases characteristic for the human disease (McCart *et al.*, 2008).

In contrast to mice, where embryonic stem cells are widely used for the development of transgenic animals such cells are not available in pigs. Thus, in this work porcine mesenchymal stem cells were chosen for the genetic manipulation. Accordingly, these cells were characterised, their genomic stability analysed and transfection and selection methods compared. Afterwards, gene-targeted clones with stop codons introduced at either codon 1061 or 1311 of the porcine *apc* gene were used as nuclear donors in somatic nuclear cell transfer (SCNT) and gene-targeted pigs were generated.

4.1 Isolation of cells for genetic manipulation

For SCNT in pigs, fetal fibroblasts, adipocytes and granulosa-derived cells have been used as nuclear donor cells (Polejaeva *et al.*, 2000; Onishi *et al.*, 2000; Tomii *et al.*, 2005). However, due to the limited life span of these cells and an estimated number of 45 population doublings necessary for expansion, transfection, selection and screening of gene-targeted cells (Clark *et al.*, 2000), a need for new cell types exists. Additionally, for the isolation of porcine fetal fibroblasts (poFFs) fertilization of sows is necessary, the animals need to be slaughtered to obtain the fetuses for the cell isolation and a sex determination is only possible after the isolation of the poFFs. Thus, in this work, porcine bone-marrow derived MSCs (poMSCs) were chosen for genetic manipulation. The methods described in this work were performed with slaughterhouse material and did not require experimental animals for cell isolation. Bones for the isolation of poMSCs and ovaries for the isolation of oocytes for somatic cell nuclear transfer (SCNT) were both obtained from the slaughterhouse.

4.1.1 Porcine mesenchymal stem cells

Multipotent mesenchymal stem cells (MSCs) were first isolated from bone marrow by Friedenstein (1961). They share the stem cell niche in the bone marrow compartment with progenitors of haematopoiesis and provide them with signalling and adhesion factors (Figure 40) (Yin and Li, 2006). In recent years the use of MSCs for tissue engineering and regenerative medicine has been proposed. Especially in the field of degenerative bone and cartilage diseases such as osteoporosis or osteoarthritis autologous and allogenic transplantations of modified MSCs are considered as a therapy (Tuan *et al*., 2003). The advantages of MSCs are their growth characteristics, their differentiation potential into cells of the mesenchymal lineage and the simple isolation and manipulation methods for these cells (Tuan *et al*., 2003). Human MSCs have been isolated not only from bone marrow, but also from other tissues such as adipose tissue, muscle, umbilical cord and many more (Zuk *et al*., 2001; Bosch *et al*., 2000; Bieback *et al*., 2004; reviewed by Tuan *et al*., 2003).

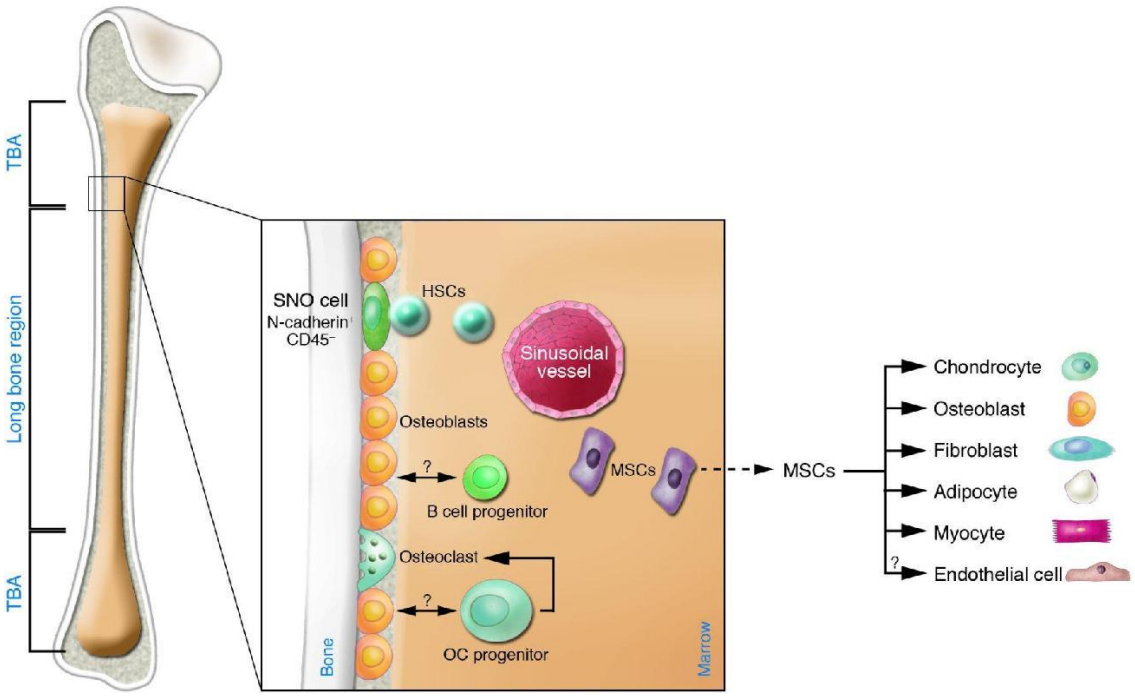

Figure 40: Stem cell niche in bone. Hematopoietic stem cells (HSCs) are surrounded by MSCs and their derivatives (adapted from Yin and Li, 2006).

In this work an efficient procedure for the isolation of poMSCs from bones is described. The material for the isolation of porcine MSCs was obtained from a slaughterhouse and the sex of the cells was determined in advance by choosing male pigs as bone donors. The cells were flushed from porcine bones, separated by gradient centrifugation and plated with MSC medium. Additionally, the isolation of porcine MSCs from adipose tissue was carried out at our institute and adipose-derived poMSCs were also used for gene targeting. The isolation from porcine umbilical cord was not successful.

In addition to the pre-determination of the donor's sex, a selection for other factors might also be considered. For example, poMSCs might be isolated from male pigs with superior breeding and health properties. These characteristics might increase the number of healthy piglets afterwards and ease the propagation of the herd.

4.1.2 Effects of basic fibroblast growth factor

The porcine MSCs used in this work were cultured with supplementation of basic fibroblast growth factor (FGF-2). Martin *et al*. (1997) showed that growth factors changed the growth characteristics of human MSCs. In these cells the growth rate and osteogenic differentiation were both significantly increased by supplementation of the cultivation medium with FGF-2. Additional publications confirmed the stimulatory effect

of FGF-2 on proliferation and differentiation of human MSCs (Mastrogiacomo *et al*., 2001; Tsutsumi *et al*., 2001; Bianchi *et al*., 2003; Solchaga *et al*., 2005). Bianchi *et al.* (2003) proposed, that the addition of FGF-2 to the culture medium selects for a subpopulation of MSCs with longer telomeres, increased life span and a higher differentiation potential in higher passages. This theory is supported by the fact that the total number of MSC colonies plated with FGF-2 is decreased compared to the number of colonies without a FGF-2 supplementation (Bianchi *et al.,* 2003; Solchaga *et al.,* 2005). The effect of FGF-2 was also observed in rabbit MSCs (Tsutsumi *et al.*, 2001). However, the cultivation of porcine MSCs with FGF-2 has not been described so far. Thus, in this work, the effects of FGF-2 on poMSCs were examined. It was shown in this work that supplementation with FGF-2 allows the cultivation of poMSCs for at least 73 population doublings. This is far beyond the “Hayflick limit” of 50 population doublings for somatic cells (Hayflick and Moorhead, 1961) and consistent with the observations in human MSCs, which were also capable of more than 70 population doublings (Bianchi *et al.,* 2003). The supplementation of the culture medium with FGF-2 is a convenient way to support the proliferation of porcine MSCs. The longevity of the cells shown in this work was one of the reasons for choosing porcine mesenchymal stem cells for genetic modification instead of porcine fetal fibroblasts.

FGF-2 had an effect on the morphology of the porcine MSCs. Porcine MSCs cultivated without FGF-2 showed a fibroblast-like morphology consistent with the poMSCs described by Ringe *et al.* (2002). When cells were cultivated with FGF-2, they had a more round phenotype and the cells were smaller. Martin *et al*. (1997) and Solchaga *et al*. (2005) also described an effect of FGF-2 on the morphology of human MSCs. In the presence of FGF-2 the human MSCs had a fibroblast like appearance, but without FGF-2 the cells were enlarged, with a flattened phenotype. The intracellular effects of FGF-2 on MSCs are not revealed yet. MSCs derived from telomerase knock-out ($mTR^{-/-}$) mice lost their multipotency and capacity to differentiate, which led Liu *et al*. (2004b) to the conclusion that the effect of FGF-2 might be mediated through telomerase activity. However, Zimmermann *et al.* (2003) could not detect telomerase activity in human MSCs. A possible explanation is that telomerase might only be expressed at specific time points or that a small subpopulation of MSCs exists with active telomerase, which is under the detection limit of the TRAP assay performed by Zimmermann *et al*. (2003).

So far, there are no reports on cultivation of porcine MSCs in FGF-2 containing medium and the effects on nuclear transfer are unknown. In the experiments described

in this work, FGF-2 was removed from the culture medium two days before the cells were used for nuclear transfer, but effects on the reconstructed embryos might not be excluded. Thus, other methods to enhance the proliferation potential of porcine MSCs might be considered such as the cultivation in a low oxygen (5%) environment or retroviral transduction with human telomerase reverse transcriptase (hTERT) or human papillomavirus type 16 E6 or E7 genes (Bosch *et al*., 2006; Takeda *et al*., 2004). It is suggested that a low oxygen environment resembles the *in vivo* conditions of mesenchymal stem cells more closely (Bosch *et al*., 2006). However, for practical reasons cultivation of poMSCs under low oxygen was not performed in this work. Still, it might be interesting to examine the effect of low oxygen on the porcine MSCs used in this work to replace the supplementation with FGF-2.

4.1.3 Characterisation of porcine mesenchymal stem cells

According to the International Society for Cellular Therapy, human MSCs are characterised by their adherence to plastic, the expression of certain surface antigens and the *in vitro* differentiation potential in osteogenic, adipogenic and chondrogenic cells (Dominici *et al*., 2006). Due to the fact that porcine MSCs cultivated with FGF-2 were not described so far, the cells isolated in this work were characterised in the same way as human MSCs. As described in 3.1.1, the adherence of the poMSCs to the plastic surface took place within 24 h and after five to six days the first colonies were visible. The differentiation of the isolated poMSCs was shown for the adipogenic, osteogenic and chondrogenic lineage, demonstrating that the isolated cells were truly mesenchymal stem cells. Walsh *et al*. (2000) propose that FGF-2 directs human MSCs in the osteogenic lineage, whereas Tsutsumi *et al*. (2001) did not observe such a determination of the cells. Therefore in this work, the differentiation assays were performed with and without FGF-2. However, no differences were observed and successful differentiation of poMSCs into adipogenic cells was shown in the presence of FGF-2 (Figure 15). The third criterion for the classification of MSCs is the expression of certain surface markers. Human MSCs are classified by a $CD105^+$, $CD73^+$, $CD90^+$ and $CD45^-$, $CD34^-$, CD14/$CD11b^-$, CD79α/$CD19^-$ and HLA-DR^- phenotype. However the surface markers for the characterisation of porcine MSCs are unknown. In preliminary experiments at our laboratory by Wiskow (2006), the expression of surface markers on poMSCs cultured without FGF-2 was examined and no expression of CD45 and CD11b was observed. Vacanti *et al*. (2005) and Liu *et al*. (2004a) were able to demonstrate that their poMSCs had a $CD105^+$, $CD90^+$, $CD45^-$ and/or HLA-DR^-

phenotype. In bovine MSCs a $CD105^-$, $CD90^-$ and $CD140a^-$ phenotype was observed and the gene expression profiles were significantly different from human MSCs (Kato *et al*., 2004; Colleoni *et al*., 2005). It is therefore unclear if all the criteria regarding human MSCs also apply for porcine MSCs. Moreover the examination of surface antigens of porcine cells is difficult as few anti-porcine antibodies are available.

4.1.4 Stability of chromosome number

Chromosomal count of the poMSCs in this work showed a normal karyotype of 38 chromosomes for up to 15 passages, when cultivation medium was supplemented with FGF-2. At higher passages aneuploidy was identified in 60% of the cells (Table 4). Izadpanah *et al.* (2008) also observed a loss of stable chromosome numbers at higher passage numbers in bone-marrow and adipose tissue derived MSCs from rhesus monkey. They compared human and rhesus monkey MSCs and a normal euploid chromosome number was noticed in 100% of the cells up to passage 10. At passage 30 the human cells were still diploid, whereas the rhesus monkey MSCs lost their chromosomal stability and only 20% of these cells were still diploid. A possible reason for the loss of stable chromosome numbers might be the need for different growth factors in MSC cultures of different species.

4.1.5 Induced pluripotency

Although it was shown in this work that poMSCs are a good cell source for genetic manipulation and SCNT, the use of embryonic stem (ES) cells would reduce the effort necessary to produce gene-targeted animals. Moreover, it is hypothesized that the homologous recombination machinery is less active in somatic cells than in embryonic stem cells. This hypothesis is based on the observation of Arbonés *et al*. (1994) that gene targeting of the interferon-γ receptor was 10-fold more effective in murine ES cells than in murine myoblasts. Additionally, the question whether non-terminally differentiated cells as nuclear donors for SCNT increase the rate of liveborn and surviving animals after SCNT is still open (see 4.4.2). But due to the unavailability of livestock ES cells, nothing is known about these effects in livestock. However, in 2006 Takahashi and Yamanaka described reprogramming of murine fibroblasts into induced pluripotent stem (iPS) cells. Pluripotency was induced by retroviral transduction with the transgenes *c-myc*, *klf4*, *sox2* and *oct3/4*. These iPS cells had stem-cell like features such as teratoma formation and contribution to chimeras after injection into blastocysts.

Okima *et al.* (2007) observed the formation of tumors in ~20% of the offspring generated from these cells due to the reactivation of the *c-myc* transgene. In 2009 porcine iPS cells were described (Wu *et al.*, 2009; Ezashi *et al.*, 2009, Esteban *et al.*, 2009), but it is still elusive if iPS cells can replace embryonic stem cells. Due to the advanced stage of the targeting project of the porcine *apc* locus porcine iPS cells were not used as an alternative cell source. Nevertheless, gene targeting of the *apc* locus in porcine MSCs and iPS cells derived from porcine MSCs cells could help to answer the question whether the rate of homologous recombination is increased in stem cells and which mechanisms are responsible.

Porcine MSCs could be used for the generation of iPS cells. Shi *et al*. (2008) generated iPS cells from murine neural progenitors, which already expressed Sox2. Thus, a reduced number of transgenes was necessary to induce pluripotency. In human and rabbit MSCs the expression of Oct4 and Sox2 has been described (Izadpanah *et al*., 2006). However, in the porcine MSCs isolated in this work the expression of c-Myc and Klf4 was observed by Dr. T. Flisikowska (Livestock Biotechnology, TU München), but no expression of Oct4 was detected. The expression of Sox2 was not examined. Porcine MSCs might be an interesting source of cells for the generation of iPS cells, as two of the necessary factors are already expressed. Additionally, due to the high targeting efficiencies of the *apc* locus with pAPC1061, APC targeting would offer a good model for the comparison of targeting efficiencies in somatic and stem cells.

4.2 Construction of the targeting vectors

At the onset of this work, the DNA sequence of the porcine *apc* gene was unknown. Thus, sequencing of the porcine *apc* gene was necessary. The strategy for the cloning of both targeting vectors was based on the amplification of the porcine *apc* gene from porcine genomic DNA. The mutation site, a selection cassette and restriction sites for further cloning steps were introduced with oligonucleotides. The selection cassette consisted of an internal ribosomal entry site (IRES), a *blasticidin deaminase* (*bsd*) gene from *Aspergillus terreus* and a SV40 poly(A) signal. Both targeting vectors were based on a promotor-trap approach for the enrichment of targeted cells, therefore no promoter sequences were introduced in the targeting vectors. The targeting vector pAPC1061 covered 12454 bp of the porcine *apc* sequence and pAPC1311 12816 bp. The short homology arms of both targeting vectors consisted of at least 2.7 kb of the *apc*

sequence. Therefore the sequences were longer than the minimal requirement of 1 kb for the short arm of a targeting vector (Thomas *et al.*, 1992; Deng and Capecchi, 1992).

An important factor for successful gene targeting in inbred species such as the mouse is the use of isogenic DNA for the construction of targeting constructs (te Riele *et al*., 1992; Deng and Capecchi, 1992). However, Dai *et al*. (2002) and Ramsoondar *et al*. (2003) did not observe that the construction of the targeting vector from isogenic DNA was necessary for homologous recombination in pig. In this work, the porcine *apc* sequence for the construction of the targeting vectors was derived from a German landrace (DL) x Pietrain pig and the cells for gene targeting were either isolated from DL x DL or DL x Pietrain pigs. Successful gene targeting was shown for the *apc* locus in these cells. Thus, it is likely that the use of isogenic DNA is only necessary in highly inbred strains with great differences in the genetic background due to a high inbred level such as the laboratory mouse. However, little is know about the frequency and extent of heterologous sequences that may affect the targeting efficiencies.

Recombineering: The construction of the targeting vectors involved considerable time and effort. Long-range PCR reactions were necessary to cover the porcine *apc* sequence and many cloning steps were necessary to construct the final targeting vectors. An alternative to conventional cloning for the construction of targeting constructs is bacterial artificial chromosome (BAC) recombineering (Cotta-de-Almeida *et al*., 2003). With this approach the targeting constructs are generated in *Escherichia coli* (*E. coli*) by homologous recombination between a linear donor plasmid and circular BAC DNA that includes the sequence of the target gene. The advantage is that only short homology arms of 50 to 500 bp are needed for efficient recombination in bacteria and these homology arms can easily be amplified by PCR. Homologous recombination is then mediated by the means of the bacteriophage systems λ Red or RecET in *E. coli* (Sharan *et al*., 2009). After recombination modified BACs can be isolated from the bacteria and used for the transfection of mammalian cells.

In mouse ES cells, targeting efficiencies between 6 and 28% were observed with BAC vectors constructed by recombineering (Testa *et al.,* 2003; Yang and Seed, 2003). This advantageous effect of long homology arms was unexpected, as Deng and Capecchi (1992) proposed that the recombination system is saturated at a homology length of roughly 14 kb and Lu *et al.* (2003) did not achieve an improvement in targeting efficiency when targeting the *β-globin* locus in murine ES cells with a 110 kb construct. It was shown in our laboratory by Böttcher (2008) that rapid generation of BACs

containing a mutated *apc* gene is possible with recombineering. However, no gene-targeted clones were obtained after transfections with these mutated BACs. The main problem was, that in contrast to the rapid generation of the targeting construct, the screening for positive clones is more complex than with shorter targeting constructs. Conventional targeting vectors are designed with short and long arms of homology to facilitate the screening by PCR as described in this work (Figure 23). Due to the size of the BACs (100 to 300 kb) standard PCR analysis to detect targeting events is not possible and more complex methods such as fluorescent *in-situ* hybridisation (FISH) or qPCR have to be used to detect and quantify the integration of the mutation cassette (Yang and Seed, 2003). Alternatively, conventional targeting vectors can be isolated from genetically modified BACs either by restriction digestion, long-range PCR or recombineering in targeting vectors. All of these methods have certain limitations such as the availability of restriction sites, the introduction of mutations by PCR or the need for additional recombineering steps.

4.3 Gene targeting of the *apc* gene

4.3.1 Electroporation

In this work, a protocol for the transfection of porcine MSCs by electroporation was successfully established. With this method, the poMSCs showed a high viability and transient expression of EGFP ranged from 10 to 29% of total cells (see 3.1.5.2). The same DNA preparation was used for all control reactions. Variations in handling of the cells were avoided but slightly different buffer temperatures or varying incubation time before and after electroporation could not be excluded. As primary cells isolated from different animals were used for transfections, the transfection capacity of each isolate might be different. Another explanation might be an influence of the cell cycle phase on electroporation efficiencies of porcine mesenchymal stem cells. Yorifuji *et al.* (1989) observed that the cell cycle phase of mouse fibroblasts influenced their electroporation efficiencies. Additionally, the variation in transfection efficiency may be due to the differences in transfected cell numbers leading to varying field strengths within the cuvette (see Table 8 and Table 9).

4.3.2 Enrichment, selection and homologous recombination

A promotor-trap strategy was used to enrich gene-targeted clones. Important issues with promoter-trap approaches are the matching of the expression of the selection marker and the target gene and the appropriate concentration of selection reagents (Hanson and Sedivy, 1995; Sedivy and Dutriaux, 1999). Hanson and Sedivy (1995) showed that it was possible to increase the targeting efficiency from 9 to 32% by increasing the G418 concentration from 600 to 3000 µg/ml, but meanwhile the absolute number of clones decreased from 16 to 6. Thus, it is necessary to find a balance between stringent selection and a sufficient number of clones. As the promotor strength of the *apc* promoter was unknown and the manufacturer's recommendation for selection with blasticidin ranged from 3 to 50 µg/ml, the concentration of 8 µg/ml was determined experimentally in this work. Marques *et al*. (2006) used a similar concentration of 7 µg/ml blasticidin for targeting the *collagen type1 α1* (*COL1A1*) gene in ovine fetal fibroblasts. With an increase of the concentration to 10 µg/ml blasticidin, no colonies were obtained.

In many gene targeting experiments non-transgenic offspring were obtained when selected pools or even selected clones were used for nuclear transfer (McCreath *et al*., 2000; Yu *et al*., 2006; Dai *et al*., 2002). Usually it is explained by mixed colonies consisting of resistant and non-resistant cells. These might arise because of locally reduced antibiotic concentration due to fast-growing resistant colonies or improper antibiotic concentrations. Therefore the first screening PCR in this work was performed to detect the *bsd* gene. In roughly 60% of the colonies the *bsd* gene was detected after selection. This number might be underestimated as PCR was carried out with cell lysates and the DNA concentration was not determined before the PCR reaction.

The aim in this work was gene targeting by homologous recombination between the porcine genomic DNA and the targeting constructs. Takata *et al*. (1998) proposed that homologous recombination preferentially occurs during late S- to G_2-phase. This was confirmed by gene targeting of the *hprt* gene in cattle and the *hTERT* gene in rhesus macaque (Mir and Piedrahita, 2004; Meehan *et al*., 2008). Moreover, gene targeting was achieved in 4.4% of resistant colonies when bovine fetal fibroblasts were synchronised in S-phase by thymidine treatment and transfected with the targeting construct after 24 h (Mir and Piedrahita, 2004). In future work, the influence of thymidine treatment on porcine MSCs should be examined. This might be a promising approach to increase the number of gene-targeted colonies. Mir and Piedrahita (2004)

also investigated the effect of nuclear localisation signals on targeting efficiencies. When this sequence was added to the *hprt* targeting construct and cells were synchronised, 32.7% of the resistant colonies were gene-targeted. The direct delivery of targeting construct DNA into the nucleus is also the basic principle of a newly developed electroporation method, called nucleofection. The method is based on specialized buffers and electrical pulses and DNA is directly delivered to the nucleus. In human MSCs 74% of the cells transiently expressed EGFP after nucleofection (Aluigi *et al*., 2006). In preliminary nucleofection experiments in our laboratory by Thanisch (2009), EGFP expression was detectable in the control reaction within hours after the transfection. However, nucleofection of the targeting vectors pAPC1061 and pAPC1311 did not result in gene-targeted colonies (Thanisch, 2009).

4.3.3 Targeting efficiency in porcine mesenchymal stem cells

Porcine MSCs isolated from five different animals were used for gene targeting of the *apc* gene. A sixth poMSC isolation was used for transfections with pAPC1311, but no colonies were obtained. After selection with blasticidin, the colonies were screened by PCR amplification directly from cell lysates. The primers for the PCR analysis were homologous to the *bsd* gene and to sequences outside the 3' arm of the targeting vector.

4.3.3.1 Variations in targeting efficiencies

A comparison of transfections with both constructs revealed large differences in targeting efficiencies. In all bone-marrow derived poMSCs the number of gene-targeted to blasticidin-positive colonies was roughly 42% with pAPC1061, whereas pAPC1311 led to targeting efficiencies ranging from 17 to 67% depending on the cell preparation used (see Table 8 and Table 9). With adipose-tissue derived MSCs no APC1061-targeted and only 4.8% APC1311-targeted clones were obtained from bsd^+ clones. Thanisch (2009) used both targeting vectors for gene targeting of the *apc* gene in poMSCs derived from bone marrow and adipose tissue of Göttingen minipigs. No gene-targeted cells were obtained from adipose-derived poMSCs, but 14 APC1061- and 15 APC1311-targeted clones were identified after transfecting 6×10^6 cells with each targeting vector. Rogers *et al*. (2008a) also described variations in homologous recombination rate between different cell preparations. Additionally, targeting frequencies, which are expressed in gene-targeted colonies per total number of cells used for transfection, differed between both targeting vectors. They were almost consistent for transfection with pAPC1311

ranging from 2.5 to 6.7 x 10^{-6}. For transfections with pAPC1061 they were in the range of 3.8 to 35 x 10^{-6}. In absolute numbers almost three times more gene-targeted clones were achieved with pAPC1061. This variation is striking as both vector sequences are based on the same section of the *apc* gene and pAPC1311 was constructed by exchanging the mutation cassette of pAPC1061. The short homology region of pAPC1311 was shorter than in pAPC1061 and had a length of 2673 bp, but in mouse ES cells as little as 500 bp of homology were successfully used for the formation of crossing over events (Hasty *et al*., 1991).

Mutations at codon 1311 might have had an effect on viability of the porcine MSCs. This would also explain the morphological differences between APC1061- and APC1311-targeted colonies. An explanation might be a dominant-negative effect of APC1311. Dihlmann *et al.* (1999) observed such an effect of APC1309 when a colorectal cancer cell line expressing only mutant APC truncated at codon 1114 was transfected with wildtype and three different mutated *apc* genes. A 5-fold excess of APC1309 over wildtype APC almost completely suppressed the function of the wildtype protein in a β-catenin reporter assay. The reporter assay comprised of a luciferase gene under the control of four Tcf/Lef binding sites and increased β-catenin levels activate luciferase activity. However, an excess of APC386 or APC1465 interfered only weakly with the function of wildtype APC. Additionally, Green and Kaplan (2003) investigated the effect of truncated APC1450 protein in 293 cells which express wildtype APC. They observed that truncated APC1450 dominantly interferes with microtubule function and chromosome segregation during mitosis. In addition to a direct effect of truncated APC on β-catenin levels, other effects on mitosis and cell integrity are possible and might explain the differences between APC1061- and APC1311-targeted cells observed in this work. However, Thanisch (2009) did not observe morphological changes when the pAPC1311 was used for gene targeting in poMSCs derived from Göttingen minipigs. This suggests that the differences in growth characteristics and morphology are mainly due to contaminations of the DNA preparations used for the transfections.

In future work, the effects of both *apc* mutations need to be investigated further. For example the level of β-catenin in APC1061- and APC1311-targeted cells could be examined by an improved β-catenin reporter assay described by Veeman *et al*. (2003) which comprises of seven Tcf/Lef binding sites. Future experiments could show if one mutated *apc* allele already alters the β-catenin levels and if differences between APC1061- and APC1311-targeted cells are observed. Also interesting is the expression of APC in bone-marrow and adipose-tissue derived poMSCs. Transfections of adipose-

tissue derived poMSCs were less successful. No gene-targeted colonies were achieved with pAPC1061 and only two colonies with pAPC1311. One possible explanation might be a lower or non-existent expression of APC in adipose-derived poMSCs. Thus, in further experiments the expression of APC should be quantified.

4.3.3.2 Comparison of targeting efficiencies

Porcine MSCs are a heterogeneous primary cell population and therefore the calculation of the targeting efficiency based on blasticidin resistant clones was necessary. As shown in the targeting experiments with both targeting vectors, great variations were observed in the same cell isolation (Table 8 and Table 9). This was also described by Sedivy *et al.* (1999) in human somatic cells. They compared 23 gene targeting experiments in human somatic cells and the targeting efficiencies were in the range between 0.1 and 37%. In this work high targeting efficiencies for gene targeting of the *apc* gene were achieved - 35.3% for APC1061 and 22.5% for APC1311 when blasticidin resistant clones and gene-targeted clones were compared. The ratio of homologous recombination to random integration was exceptional for both targeting vectors with 1:3 for pAPC1061 and 1:5 for pAPC1311. The ratio of homologous recombination to random integration in this work is even lower than the ratio of 1:10 estimated for gene targeting with viral vectors (Russell and Hirata, 1998). Zijlstra et al. (1989) and Mouellic et al. (1990) obtained ratios of 1:25 and 1:40 when targeting the *β2-m* or the *Hox-3.1* gene. They explained these ratios with an increased recombination rate within these genes due to recombination hotspots, but it remains unclear how these DNA sequences are characterised. Of total transfected cells 1 cell out of 1.3×10^5 transfected cells was targeted at codon 1061 and 1 cell out of 2.1×10^5 was targeted at codon 1311. However, a comparison based on the ratio of gene-targeted cells to electroporated cells in total is imprecise. Standardized electroporation and selection procedures led to variations in the transient expression of EGFP and no correlation to homologous recombination events in simultaneous transfections with the targeting vectors was observed. Moreover, differences in cell death due to variations in electroporation and selection procedures have to be taken in consideration. Generally, high targeting efficiencies of the *apc* gene were achieved in this work compared to gene targeting of the *apc* gene in murine ES, in which efficiencies ranged from 0.4 to 9.8% (6.2%, Smits *et al.*, 1999; 0.4%, Shibata *et al*., 1997; 4.2%, Oshima *et al*., 1995; 1.8%, Sasai *et al*., 2000; 9.8%, Fodde *et al*., 1994). No promotor-trap approaches were applied in murine ES cells; only for the generation of APC^{1638N} mice a negative

selection cassette was used. In this work, a promotor-trap strategy was applied effectively and led to the low rate of clones with random integration as mentioned above. Gene targeting in porcine cells was most commonly done with porcine fetal fibroblasts. Targeting of the *α-1,3-galactosyltransferase* (*ggta1*) locus with a promoter-trap approach was obtained in 0.9 to 8.7% of the antibiotic resistant porcine cells (Harrison *et al.*, 2002; Ramsoondar *et al*., 2003). Rogers *et al.* (2008a) achieved targeting efficiencies of 0.1 to 10.9% for the *cftr* gene after viral transduction with rAAV.

Summarizing the above, the promotor-trap approach applied in this work in combination with the established transfection and selection procedure for porcine mesenchymal stem cells led to highly efficient gene targeting of the *apc* locus.

4.3.4 Alternative methods for gene targeting

4.3.4.1 Zinc-finger nucleases

A new method for the establishment of gene targeted animals is the application of zinc-finger nucleases (ZFN), which can introduce sequence specific double-strand breaks in genomic DNA (Figure 41). They consist of DNA-binding domains and an attached DNA cleavage domain, the endonuclease FokI. Each binding domain contains three to four zinc-finger repeats in each monomer and each repeat recognises a specific 3 bp DNA sequence. With different combinations of these repeats the binding to specific DNA sequences can be facilitated. Dimerised forms of FokI introduce double-strand breaks in DNA (Bitinaite *et al*., 1998). Repair mechanisms for double-strand breaks include homology-directed repair and non-homologous end-joining (NHEJ), whereof NHEJ is most common and leads to the loss or gain of single base pairs (Valerie and Povirk, 2003).

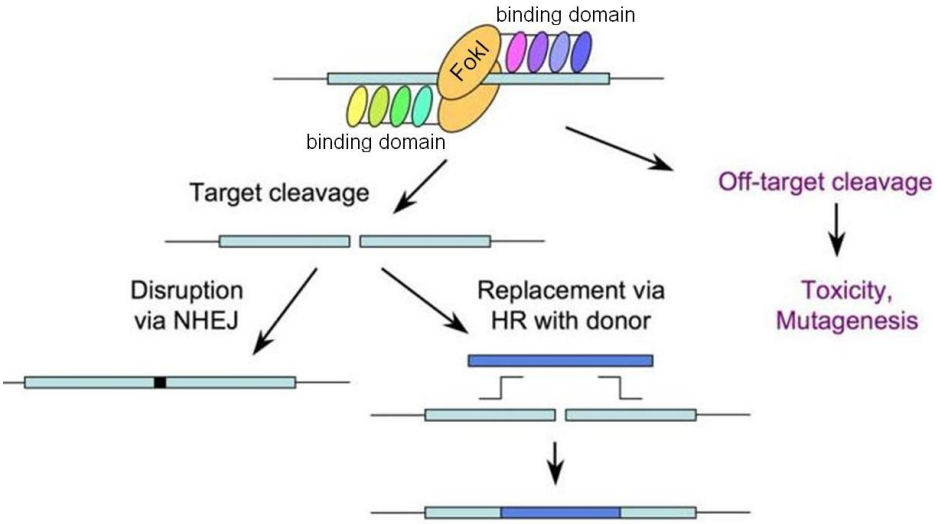

Figure 41: Application of zinc-finger nucleases. Each zinc-finger nuclease (ZFN) consists of DNA binding domains and an attached DNA cleavage domain, the endonuclease FokI. The DNA binding domains contain three to four zinc-finger repeats in each monomer and each repeat recognises a specific 3 bp DNA sequence. After dimerisation DNA is cleaved and double-strand breaks are introduced. Repair mechanisms include homology-directed repair and non-homologous end-joining (adapted from Carroll, 2008).

The ZFN approach has been used for the production of knockout animals. ZFN-encoding mRNA was injected in zebrafish embryos and 30 to 60% of the obtained animals carried the introduced mutations (Doyon *et al*., 2008; Meng *et al*., 2008). In rat, 12 to 24% of the founder animals had a mutation within the target sequences (Geurts *et al.*, 2009; Mashimo *et al*., 2009). In total numbers Geurts *et al.* (2009) found 35 knockout animals within 295 founders and Mashimo *et al*. (2009) 13 within of 54 founders. Although the injection of ZFN-mRNA into rat embryos leads to a considerably high percentage of transgenic animals, it is less attractive for large animals with smaller litter sizes and longer gestation times. Instead of direct injection in embryos, ZFN can also be used for the manipulation of single cells, which can then serve as nuclear donors for SCNT. ZFN were successfully used for the disruption of the *dihydrofolate reductase* (*dhfr*) gene in mammalian cells (Santiago *et al*., 2008). Mutations in the *dhfr* gene were observed in 2 to 3% of the analysed colonies and around 1% of these colonies showed mutations in both *dhfr* alleles. With conventional gene targeting mutations in both alleles are more complicated due to the rare recombination events, making ZFN an interesting alternative for double knockouts. Moreover it is possible to examine the introduced mutations more closely before animals are generated. In ZFN-mutated rats a variety of deletions ranging from 3 to 187 bp was observed (Geurts *et al*., 2009). Not all mutations might have an influence on the gene or protein function, especially those with very short, in frame deletions.

A promising application of ZNF for the generation of gene-targeted large animals is a supportive role in homologous recombination. Choulika *et al*. (1995) observed that double-strand breaks induced by rare cutting restriction enzymes stimulate homologous recombination in yeast. Therefore homology-directed repair of double-strand breaks can be utilized for gene targeting. Transfection with a targeting vector in parallel with the introduction of unique double-strand breaks at the target gene by ZFN might increase the number of homologous recombination events. The vector will serve as a template for the repair of the double-strand break. An additional benefit is that ZFN can be used for the modification of transcriptionally inactive genes. This is one of the main restrictions of promoter-trap approaches used in this work. A limitation of the ZFN method is the possible introduction of double-strand breaks in off-target sequences. These breaks might lead to gene rearrangements, chromosomal instability, random integration of transgenes or cell death. In knock-out rats generated with ZFN no off-targeted mutations were detected, but only 20 loci were analysed (Geurts *et al*., 2009).

Zinc-finger nucleases are not unique for the introduction of double-strand breaks. Alternative methods utilize conjugates of orthophenanthroline and triplex-forming oligonucleotides or homing endonucleases (Cannata *et al*., 2008; Grizot *et al.*, 2009). The triplex-forming oligonucleotides are conjugated with locked nucleic acids to obtain a strong, sequence-specific binding to oligopyrimidine-oligopurine sequences. In combination with orthophenanthroline, which is a DNA cleavage molecule, sequence specific double-strand breaks can be introduced in genomic DNA. Engineered homing endonucleases also introduce double-strand breaks at specific sites of the genome. By co-transfection with targeting constructs, which serve as a template for the repair of these double-strand breaks, mutations can be introduced in the genome. Cannata *et al*. (2008) achieved gene targeting in 1.5% of the cells with triplex-forming oligonucleotides and Grizot *et al.* (2009) used homing endonucleases and detected recombination in 6% of the screened cells.

However, these methods were not considered for gene targeting of the porcine *apc* gene as the project was started in 2006 and APC-targeted MSCs were already used for SCNT before effective production of knockout animals was shown.

4.3.4.2 Viral methods

Another alternative method is viral transduction. The most commonly used viral vectors for gene targeting are recombinant adeno-associated viruses (rAAV), which are single-stranded DNA viruses. They were successfully used for the transduction of human mesenchymal stem cells and the subsequent repair of mutations within the *COL1A1* gene of patients with osteogenesis imperfecta (Chamberlain *et al*., 2004). Rogers *et al*. (2008a) used the rAAV system for targeting of the *cftr* gene in porcine fetal fibroblasts, which do not express *cftr*. Therefore the application of a promotor-trap approach was impossible and electroporation and nuclear microinjection were not successful. In addition to targeting of transcriptionally inactive genes, the transduction with rAAV led to high homologous recombination efficiencies ranging from 0.1 to 1% and low numbers of random integrations (Russell and Hirata, 1998). The ratio of homologous recombination to random integration has been estimated to be around 1:10 (Russell and Hirata, 1998). It is hypothesized that the high targeting efficiencies by rAAV vectors are achieved by the delivery of the DNA directly to the nucleus of the transduced cells (Russell and Hirata, 1998). This is interesting as the other methods mentioned in 4.3.2 such as the addition of nuclear localisation signals or nucleofection are also based on an improved nuclear entry. One limitation of rAAV vectors is the restricted DNA capacity of 4.5 kb (Hendrie and Russell, 2005), but short sequence homologies of 2.7 to 3.8 kb were sufficient for homologous recombination with rAAV (Russell and Hirata, 1998; Rogers *et al*., 2008a). However, for the insertion of mutation cassettes with more than 1.5 kb the size limitation can be an obstacle. Moreover, high effective titers are necessary for effective gene targeting (Laible and Alonso-Gonzáles, 2009).

4.3.5 Further characterisation of APC-targeted clones

Southern blot analysis: For further characterisation of the APC-targeted clones, Southern blot analysis was carried out (see 3.3.6). With Southern Blot analysis (additional) random integrations of the selection cassette can be excluded and mixed colonies consisting of targeted and non-targeted clones might be detected. Due to the growth characteristics of poMSCs it was possible to expand enough APC1061-targed cells for Southern blot analysis before SCNT was performed. However, due to the different growth characteristics of APC1311-targeted clones, the isolation of 10 µg genomic DNA was not successful. Ramsoondar *et al*. (2003) also analysed single fetal fibroblast clones with Southern blot hybridisation, but due to the lower amount of

isolated DNA radioactive labelling was necessary for the detection of the DNA fragments. Thus, radioactive labelling might offer a possible solution for Southern blot analysis of APC1311-targeted clones. However, Southern blot analysis of APC1061-targeted clones clearly showed that no random integration of the targeting construct occurred in the examined clones.

Differentiation: As described in section 4.1.3, differentiation was used as a method for the characterisation of porcine MSCs. It will be discussed in more detail in 4.4.2 that multipotent cells as donors for nuclear transfer might increase the rate of liveborn and surviving animals after SCNT. Differentiation was also performed with gene-targeted clones, which were derived from single colonies. Interestingly, the differentiation potential of these clones varied from clone to clone. In some clones the differentiation in all three cell types was observed, whereas others did not differentiate at all. Generally, it is proposed that only one third of the cells of a bone-marrow derived MSCs population are multipotent (Pittenger *et al*., 1999). The variations in the differentiation potential of clonally derived MSCs in this work support this hypothesis.

4.4 Somatic cell nuclear transfer

4.4.1 Pregnancy rate after somatic cell nuclear transfer in pigs

Generally, the overall efficiency of SCNT is very low with only 0 to 4% of the transferred embryos developing to live animals (summarized by Wilmut *et al.,* 2002). The efficiency of SCNT in pigs is low with only 0.4 to 2% of the transferred embryos going through gestation and giving rise to liveborn animals (see Table 12 and Table 13). In this work early pregnancies were established in 50% (7 out of 14) of the recipient sows and six of these pregnancies went to term. These numbers are high compared to other embryo transfer experiments in pigs (see Table 12 and Table 13). It has been proposed that the low rates of liveborn and surviving animals are consequences of epigenetic alterations in the transferred embryo and problems with establishment and maintenance of the pregnancy in the recipient sow.

Table 12: Overview of non gene-targeted SCNTs in pigs.

	nuclear donor cell type	no. of transferred embryos	no. of recipients	no. of early pregnancies	no. of pregnancies to term	no. of piglets (alive / stillborn)	no. of transgenic animals	gestation time, way of delivery	Reference
Omega-3 fatty acid	poFF	1633	14	x[c]	5 (35.7%)	12 (10 / 2)	6	Caesarean / natural	Lai *et al*., 2006
H-trans-ferase$^{+/-}$	adult skin fibroblasts	217	5	x[c]	2[a] (40.0%)	2[a] (1 / 1)		116 d, Caesarean	Bondioli *et al*., 2001
-	poFF	902	7	7[b]	2[b] (28.6%)	5 (4 / 1)	-	x[c]	Betthauser *et al*., 2000
double NT	granulosa cells	401	7	2	1 (14.3%)	5	-	116 d, Caesarean	Polejaeva *et al*., 2000
-	poFF	110	4	4	1 (25.0%)	1 (1 / 0)	-	x[c]	Onishi *et al*., 2000
eGFP	poFF	308	3	3	1 (33.3%)	5		113 d, Caesarean	Park *et al.,* 2001

[a]One pregnancy was aborted at day 90 due to health issues. One normal and one mummified piglet were recovered.

[b]Three pregnancies were ongoing, two aborted at day 33 and 40.

[c]Not mentioned in publication.

Table 13: Overview of SCNT for the production of gene-targeted pigs.

	nuclear donor cell type	no. of transferred embryos	no. of recipients	no. of early pregnancies	no. of preg-nancies to term	no. of piglets (alive / stillborn)	no. of transgenic animals	gestation time, way of delivery	Reference
$CFTR^{+/-}$	poFF	> 1100	8	x[c]	5 (62.5%)	10	9	116-118 d, Caesarean	Rogers *et al*., 2008a
$CFTR^{\Delta F508}$			4	x[c]	3 (75.0%)	5	4		
$GGTA1^{+/-}$ $hHT^{+/-}$	poFF and HT^{+} poFF	2918	20	7[d]	3[d] (15.0%)	12 (8 / 4)		116 d, naturally; 119 d,	Ramsoondar *et al.*, 2003
$GGTA1^{+/-}$	poFF	2513	16	11	6 (37.5%)	6	5	x[c]	Dai *et al*., 2002
$GGTA1^{+/-}$	poFF	338	28	12	3 (10.7%)	13 (13 / 0)	7	Caesarean	Lai *et al.*, 2002b
$GGTA1^{+/-}$ $hDAF^{+}$ $GnT\text{-}III^{+}$	poFF ($hDAF^{+}$ $GnT\text{-}II^{+}$)	1197	12	x[c]	2 (16.7%)	-	(13)[a]	-	Takahagi *et al*., 2005
		633	6	2	1 (16.7%)	2 (2 / 0)	2	116 d, naturally	
$GGTA1^{-/-}$	poFF ($GGTA1^{+/-}$)	x[c]	16	10	2 (12.5%)	5	5	x[c]	Phleps *et al*., 2003
APC^{1061}	poMSC	1372	14	7	5 (35.7%)[b]	24 (12 / 12)	9 / 9[e]	114 d, naturally	

[a]Pregnancies were aborted at day 29 of gestation and used for a second round of SCNT. Only eight of the embryos showed normal ploidity.
[b]One pregnancy is ongoing. [c]Not mentioned in publication. [d]One terminated for the collection of poFF. [e]Not all animals were tested.
Genes: HT: α1,2-Fucosylosyltransferase. hDAF: human decay-accelerating factor. GnT-III: *N*-acetylglucosaminyltransferase III

Aspects of epigenetic alterations which are discussed are incomplete reprogramming of the donor cell nuclei, asynchronous cell cycle status of the recipient oocyte and the donor cell, shortened telomere length in cloned embryos, mitochondrial DNA incompatibility, incomplete X-chromosome inactivation and aberrant DNA methylation or imprinting. Imprinted genes are to be expressed according to their parental origin based on epigenetic modifications of the maternal or paternal genome. Imprinting is mainly mediated by methylation of cytosines of CpG dinucleotides. Amongst others imprinted genes regulate growth, development and function of fetus and placenta. Differential gene expression of imprinted genes in fetus and placenta was also shown in pig (Bischoff *et al*., 2009). In mice the paternal genome from the sperm is actively demethylated after fertilization, whereas demethylation of the maternal genome is based on a passive mechanism depending on DNA replication (Oswald *et al.*, 2000; Santos *et al.,* 2002). At implantation, re-methylation takes place in the murine embryo (Monk *et al.*, 1987). The demethylation process after fertilization was also shown for pigs (Fulka *et al.*, 2006). However, Dean *et al*. (2001) observed aberrant reprogramming in cloned, bovine embryos. Additionally, Shi and Haaf (2002) described an influence of culture conditions on the methylation status of the embryo and aberrant foetal growth and development due to abnormal methylation patterns in the early embryo. Besides the loss of the embryo, deformation and/or weak physiology of the offspring are described as consequences of aberrant or incomplete reprogramming. Other reasons for embryonic loss are alterations in X chromosome inactivation, which is a dosage compensation process during the development of female offspring. Xue *et al.* (2002) described altered X chromosome inactivation in aborted bovine SCNT fetuses and stillborn calves. However, only male cells were used for gene targeting of the *apc* gene and only male offspring were generated. Another important aspect during reprogramming is telomere length. In the first cloned sheep telomeres were significantly shortened when compared to age-matched controls (Shiels *et al.*, 1999). Betts *et al*., 2005 described differences in telomere length in cloned goats compared to age-matched control goats when clones were derived from adult granulose cells but not when they were derived from fetal fibroblasts. However, Jiang *et al.* (2005) and Jeon *et al.* (2005) did not find significant differences in telomere length in cloned transgenic pigs. Generally, the results on telomere length of cloned animals are conflicting and might depend on the cell type used for nuclear transfer.

In addition to reprogramming the cell cycle phase of the nuclear donor cell and the recipient oocyte have to be synchronised to obtain normal ploidity. Campell *et al.* (1996) successfully used serum starvation to arrest nuclear donor cells in G0 phase

before nuclear transfer was carried out. Thus, serum starvation was also used to induce quiescence in poMSCs before SCNT.

As mentioned above, establishment and maintenance of pregnancies after embryo transfer is also a critical aspect. To increase the numbers of established pregnancies after embryo transfer, two methods are being discussed: First the administration of hormones for the maintenance of an established pregnancy and second the use of helper embryos for the establishment of a pregnancy. It has been reported that in pigs at least four viable embryos are necessary to provide sufficient levels of signals for the recognition and establishment of a pregnancy (Polge *et al.*, 1966). Generally, porcine embryos undergo pre-implantational steps which include the elongation of the extraembryonic tissues. During this process embryos provide estrogen to the uterus, which is responsible for the maternal recognition of the pregnancy (Pusateri *et al.*, 1990). For the implantation of the embryos and the following maintenance of the pregnancy the production of progesterone by the corpus luteum is crucial. The maintenance of the corpus luteum is mediated through the luteotrophic (maintaining luteal function) and antiluteolytic (preventing release of prostaglandin) acting prostaglandin E2 (PGE_2) (Akinlosotu *et al*., 1986). Thereby prostaglandin E2 counteracts the luteolytic activity of prostaglandin F2α ($PGF_{2\alpha}$) (Ford and Christenson, 1991). Ziecik (2002) suggested that estradiol injections might increase the PGE_2 level, decrease the level of $PGF_{2\alpha}$ and therefore maintain the corpus luteum. In pigs estradiol injections were successfully used to induce the maintenance of the corpora lutea and liveborn piglets were achieved (Kolbe and Holtz, 2000; Lai *et al*., 2002a). In future embryo transfer experiments, this might offer a possibility to increase the number of pregnancies.

As an alternative to administration of hormones, untreated or parthenogenic helper embryos can increase the number of embryos and therefore increase the chance for the establishment of a pregnancy. This method was successfully used for the establishment of pregnancies with one to three piglets (King *et al*., 2002; Kawarasaki *et al*., 2009). But Betthauser *et al*. (2000) showed that blastocysts derived from nuclear transfer, parthenogenesis and *in vitro* fertilisation have a significantly reduced cell number compared to *in vivo* controls. They concluded that due to the lower cell numbers, decreased amounts of pregnancy recognition signals are expressed and therefore larger numbers of NT embryos are necessary to initiate a pregnancy. This might also explain why the use of untreated or parthenogenetic helper embryos does

not improve embryo implantation in all cases (Onishi *et al*., 2000; Lai *et al*., 2002a and 2002b).

Moreover, only a small number of the transferred embryos is viable and therefore many SCNTs in pigs resulted in small litters with a size of two to six piglets (Polejaeva *et al.,* 2000; Park *et al.,* 2001; Dai *et al.*, 2002; Lai *et al.,* 2002b; Ramsoondar *et al.*, 2003; Rogers *et al*., 2008a). In comparison, after normal fertilisation pigs have an average litter size of ten piglets (Weiss *et al*., 2008).

One of the pregnancies confirmed by ultrasound examination was lost after day 30 of gestation. In sheep and cattle one third of the pregnancies are lost during gestation after SCNT (reviewed by Wilmut *et al.,* 2002). In cattle placental abnormalities or abnormal embryo-maternal communication are thought to be responsible for early miscarriages (Hill *et al*., 2000; Bauersachs *et al*., 2009). In pigs placental abnormalities are not common, but abnormalities in the donor cells for nuclear transfer might have occurred that led to the termination of the pregnancy. Additionally, it was observed that some subpopulations of poFFs isolated from the same animal are more suitable as nuclear donors than others (Kühholzer *et al*., 2001). To minimize the effects of a clone with poor nuclear donor characteristics, three to four gene-targeted clones were mixed for each SCNT.

4.4.2 Cloning efficiency and differentiation

As a consequence of the arguments discussed in 4.4.1 it was assumed that undifferentiated or less terminally differentiated cells might be better nuclear donor cells as these cells are epigenetically less restricted and therefore need less extensive nuclear changes to gain totipotency. However, the question whether these cells give rise to offspring at a higher rate and with less side effects than differentiated cells is still open. The problems with the production of cloned livestock are high costs and long gestation times, which makes it difficult to carry out large-scale evaluation of donor cells and SCNT procedures that would provide statistically significant results. Cheong *et al.* (1993) showed a decrease in SCNT efficiency, which correlates with the stages of development. Zhu *et al*. (2004) compared fetal porcine skin stem cells with fetal fibroblasts and observed a higher development rate for the stem cells. In cattle, Kato *et al*. (2004) did show that bovine MSCs can be used as nuclear donors, as they were capable of nuclear reprogramming and gave rise to live birth. Additionally, somatic

bovine adult donor cells led to higher loss in late phases of gestation, whereas embryonic donor nuclei led to 34% SCNT efficiency (Heyman *et al*., 2002). In mice blastocyst rates were higher for MSCs than for fetal fibroblasts (Jin *et al*., 2007). For porcine and bovine MSCs preliminary experiments showed no differences in cleavage rate compared to fibroblasts or osteocytes, but more apoptotic cells were seen in fetal-fibroblast-derived blastocysts and less inner cell mass developed (Colleoni *et al*., 2005). Bosch *et al*. (2006) showed that porcine MSCs can be used as nuclear donors in SCNT, as they undergo nuclear reprogramming and generated cloned blastocysts after fusion with enucleated oocytes. Contrary to these findings, no effects on live birth number and survival were observed in cloned red deer, when either multipotent antler stem cells or differentiated progeny cells were used as nuclear donors (Berg *et al*., 2007). However, it appears that undifferentiated or less terminally differentiated cells such as mesenchymal stem cells lead to a higher rate of liveborn and surviving animals after SCNT.

Generally, the generation of sufficient numbers of gene-targeted cells for nuclear transfer is a problem. As somatic cells undergo only a limited number of cell divisions (Hayflick and Moorhead, 1961), the expansion of targeted cells might be difficult. To overcome this limitation, “recloning” is used. The method was established in pigs for double-knockout of the *ggta1* alleles (Phelps *et al.*, 2003). Heterozygous *ggta1* knockout fetal fibroblasts were used for a second round of gene targeting. One pregnancy went beyond day 35 of gestation and was terminated for the isolation of fetal fibroblasts. These cells were further characterised and then used for a third SCNT from which *ggta1*$^{-/-}$ piglets were obtained. However, the *ggta1*$^{-/-}$ fetal fibroblasts were not produced by homologous recombination with the targeting vector, but a random mutation in the second *ggta1* allele.

Successful targeting of two alleles was shown by Kuroiwa *et al.* (2004) with sequential targeting of the genes encoding for the immunoglobulin-μ and the prion protein in cattle. In pigs this method was also applied to ‘rejuvenate’ the somatic donor cells for nuclear transfer. Fujimura *et al.* (2008) compared recloning with conventional SCNT and obtained pregnancies only from those experiments where recovered porcine fetal fibroblasts were used as nuclear donors. Eight piglets were delivered from four recipients with this recloning method. Thus, recloning could be an option to increase the number of liveborn piglets and might therefore be considered for further SCNT experiments.

4.4.3 Parturition and birth process

Six litters with nine liveborn APC1061-targeted piglets were born between October 2008 and October 2009. As described in 3.5, no parturition signs were observed at the expected birth date of the first litter, which caused the loss of the litter. The absence of parturition signals was also described by Lai *et al*. (2002b) and Park *et al*. (2001). Additionally, the high rate of Caesarean delivery and the long gestation times (summarized in Table 13) indicate that this also occurred in other porcine SCNT experiments. As shown in Table 13 most of the piglets were delivered between day 116 and 118. This is longer than the average gestation time of 114 to 115 days in pigs (Weiss *et al*., 2008). It is described that piglets delivered by Caesarean show altered immune system function and gain less weight within the first weeks (Daniel *et al*., 2008). Therefore natural birth was intended for the delivery of the APC-targeted piglets. In the following pregnancies birth was induced with $PGF_{2\alpha}$ and oxytocin. On day 113 after embryo transfer $PGF_{2\alpha}$ was administered followed by oxytocin 24 hours later. With this scheme the birth of the first piglet should occur within 3 hours after the oxytocin injection (Gall and Day, 1987). For the second to fourth litter, the birth process started as expected. The cells used as nuclear donors for these first four pregnancies were clones from a single porcine MSC isolation. For these APC-targeted clones the gestation time was 114 days. The APC-targeted clones used for the embryo transfer of the fifth and sixth pregnancy were derived from different poMSCs isolations. The gestation time for these clones seems to be longer as the fifth birth started more than 12 hours after the oxytocin administration and the time interval between the birth of the first and the fifth piglet was 20 hours. It is therefore likely that parturition was induced too early and the gestation time for these clones is longer than 114 days. This effect of the MSC preparation on the gestation time should be considered for further SCNT experiments and induction of parturition has to be adjusted to the cell clones used as nuclear donors.

A contrary effect of the parturition induction with $PGF_{2\alpha}$ is an inhibitory effect on lactation (Farmer and Quesnel, 2009). This effect was reported in sows giving birth to cloned piglets (Park *et al*., 2001; Ramsoondar *et al*., 2003) and it was also observed in the pigs delivering the APC-piglets described in this work. Therefore lactation has to be monitored carefully. The collection of colostrum from other lactating pigs might be considered.

4.4.4 Physical appearance of APC-targeted piglets

Two piglets, one born at the 06.09.2009 and one born at the 26.10.2009, are still alive. Another piglet survived for 126 days before it died of the malignant hyperthermia syndrome (HMS). The other nine liveborn piglets died within the first four days after birth. Some of these piglets showed physical abnormalities. Most frequently observed was a thickened tongue and upturned hoof tips. This phenotype was observed in piglets from all litters with exception of the sixth litter born at the 26.10.2009, therefore a hereditary effect of the poMSCs might be excluded as cells from different poMSC preparations were used for the SCNT experiments. Phelps *et al*. (2003) also described piglets with an enlarged tongue and additionally an enlarged kidney. Therefore the thickened tongue in the piglets seems to be not caused by the genetic modification of the *apc* gene of the nuclear donor cells or the addition of FGF-2 but by the SCNT procedure or an incomplete reprogramming process. One of the APC1061-targeted piglets from the fifth litter showed flexure tendon deformations. This was previously described by Park *et al*. (2001) and Lai *et al*. (2002b). Moreover, a reduced fetal size, occasional respiratory and cardiac dysfunctions and organ dysplasia are commonly observed in SCNT pigs and the perinatal mortality is high (reviewed by Wilmut *et al.,* 2002). In contrast to cloned offspring from other large animals, no large offspring syndrome was observed in the described APC1061-targeted pigs. As such SCNT-derived side-effects are limited to the founder generation (Tamashiro *et al.*, 2002), further breeding of the APC-targeted pigs is essential for the establishment of a porcine disease model.

4.4.5 Malignant hyperthermia syndrome

As described in 3.6.4, one of the gene-targeted piglets died at day 126. According to the Institute of Veterinary Pathology (Prof. Dr. W. Hermanns, LMU Munich) malignant hyperthermia syndrome (MHS) was diagnosed post-mortally in the dead piglet. Until the clinical manifestation it was unknown that this mutation was within the pig population used for the isolation of the poMSCs. The genetic examination showed that the dead piglet was heterozygous for the arginine to cystein mutation at codon 615 of the *ryanodine receptor 1* (*ryr1*). This mutation was found to be the reason for MHS in five different pig breeds (Fujii *et al*., 1991). It is suggested that either the mutation originates from one founder animal or that this mutation was favoured by breeding for leanness and muscularity as it is found especially in pig breeds known for these

characteristics. In humans, dogs and rabbits MHS is inherited autosomal-dominant (Roberts *et al*., 2001). The inheritance pattern in pigs is controversial discussed. It seemed that only homozygous animals were susceptible to the standard MHS test, the halothane test. But with genetic testing for *ryr1* mutations it became obvious that some heterozygous animals are also susceptible and phenotypically not distinguishable from homozygous animals with the mutation (Fujii *et al*., 1991; O'Brien *et al*., 1990; Basic *et al*., 1997). As the piglet in this work was heterozygous for the *ryr1* mutation, it either adds evidence to the hypothesis that the syndrome is not inherited in a recessive manner or another, less frequent mutation occurred in the second *ryr1* allele or in another gene relevant for stress response. In retrospect, malignant hyperthermia might have also contributed to the unexpected and quick deaths of the piglets from the third and fourth litter. At the dissection no clear cause for the deaths was detected, but these piglets also carried the mutation at codon 615 of the *ryr1* gene. It is described that piglets susceptible to MHS die when they are exposed to heat (39-40°C) for 100 min, which was tolerated by non-susceptible piglets (Denborough *et al*., 1996). The APC-targeted piglets were raised in an environment with elevated temperature, as it is common practice for newborn piglets. Maybe they did not tolerate these temperatures due to the *ryr1* mutation. To avoid the loss of piglets due to MHS, only porcine MSCs negatively tested for the mutation in the *ryr1* gene were used for SCNT from May 2009 onwards.

4.5 Outlook

APC-targeted pigs may be used in many applications. One interesting application would be the examination of drug treatment against CRC. Due to the similarity of porcine pharmacokinetics, pigs are already used for the evaluation of cancer treatment. For example 5-fluorouracil, one of the standard drugs used in CRC treatment, is metabolised similar to humans (Manzano *et al*., 2007). Pigs are also useful for the development of new screening methods. Screening for CRC is (cost)effective in the reduction of incidence rate and mortality, but improved colonoscopy methods are needed to detect lesions or cancer also at the right side of the colon (Baxter *et al*., 2009). Gene-targeted pigs with mutations in the *apc* gene are not limited to CRC research. In mice$^{min/+}$ spontaneously developing gastric tumors are observed and dysregulation of β-catenin in human gastric tumorigenesis is described (Tomita *et al.,* 2007; Dar *et al*., 2009). Mouse models with mutations in the *apc* gene were also used to study tumorigenesis in kidney and liver (Sansom *et al*., 2005; Colnot *et al*., 2004).

Therefore pig models might reveal additional roles of *apc* mutations in carcinogenesis in other tissues. Also pigs with additional mutations are possible. Mice$^{min/+}$ with an additional knockout of p53 showed pancreatic neoplasia in addition to the intestinal tumors (Clarke *et al*., 1995). Also interesting would be a porcine model with mutations in the *apc* and the K-*ras* gene. In applications, where homozygous animals or animals with mutations in more than one gene are necessary, recloning might be considered. In contrast to mouse, where double mutations or homozygous animals are achieved quite easily through conventional breeding, longer generation times and smaller litter sizes inhibit this approach in large animals. Recloning was already successfully shown in pigs for double-knockout of the *ggta1* gene (Phelps *et al.*, 2003). When knockout mutations in both alleles of one gene are intended, zinc-finger nucleases might be an alternative (see 4.3.4.1). But this is no option for gene targeting of the *apc* gene as homozygous mutations are embryonic lethal (see Table 2).

The use of minipigs might also be considered. German landrace pigs reach a weight of 150 to 180 kg after 12 month and can gain up to 250 kg when they are fully grown, whereas minipigs gain a maximum of 30 to 60 kg (Weiss *et al*., 2008). For clinical applications smaller pigs with a weight more similar to the human one might be preferred.

4.6 Concluding remarks

Animal models for human diseases are of major importance for molecular understanding and drug development. Although, big steps have been made in the last years regarding transgenic methods, most of them have been optimized for small rodent models. This allowed the generation of several small animal models reflecting the *apc* mutation found in human CRC. Nevertheless, mice and rat models of *apc* mutation in CRC do not exhibit the symptoms observed in men and most importantly they do not form invading carcinomas. As CRC has a very high incidence and a high mortality, it is very important to improve the existing diagnosis and therapy. A large animal model, as pig, might resemble the conditions for disease outbreak more closely and might provide helpful insights in the tumor formation.

In this work, genetically manipulated porcine mesenchymal stem cells were used as nuclear donor cells in SCNT. Cultivation of poMSCs in FGF-2 containing medium and their use as nuclear donor cells in SCNT has not been previously published. It was

shown that these cells are capable of long-term *in vitro* culture, which is necessary to expand enough cells for selection, screening and nuclear transfer. The criteria for cell culturing, transfection and establishment of APC-targeted clones and the successful cloning of transgenic pigs have been addressed. Under these established conditions, piglets carrying one of the most common *apc* mutations have been generated. The animals described in this work provide the first gene-targeted pigs with a predisposition to colorectal cancer and the second disease model in pigs besides cystic fibrosis. It was first shown in this work, that poMSCs can be used as nuclear donor cells in SCNT to produce healthy piglets. Analysis of the piglets after SCNT confirmed the gene targeting of one of the porcine *apc* alleles. The *apc* gene is the third targeted gene in pigs after gene targeting of the porcine *cftr* and *ggta1* genes. If these pigs will suffer from CRC or related symptoms will be analysed in the adult animals.

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6 Abbreviations

%	Percent
°C	Degree Celsius
µg	Microgram
µl	Microliter
µM	Micromolar
µm	Micrometer
3D	Three dimensional
aa	Amino acid
APC	Adenomatous polyposis coli
APS	Ammonium persulfate
BAC	Bacterial artificial chromosome
bp	Base pairs
bsd	Blasticidin deaminase
BSA	Bovine serum albumin
CFTR	Cystic fibrosis transmembrane conductance regulator
CHRPE	Congenital hypertrophy of the retinal pigment epithelium
cm^2	Square centimeter
CRC	Colorectal cancer
COL1A1	Collagen type1 α1
CO_2	Carbone dioxide
d	Day
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DHFR	Dihydrofolate reductase
ddH_2O	Double-distilled water
dH_2O	Distilled water
DL	German landrace
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
Dsh	Dishevelled
EB1	End-binding Protein 1
EDTA	Ethylenediamine tetraacetic acid

EGFP	Enhanced green fluorescent protein
ES cells	Embryonic stem cells
ET	Embryo transfer
FAP	Familial adenomatous polyposis
FCS	Fetal calf serum
FF	Fetal fibroblast
FGF-2	Human fibroblast growth factor
Fzl	Frizzled
g	Gravitational acceleration
GGTA1	α-1,3-galactosyltransferase
GSK-3β	Glycogen synthase kinase 3β
h	Hour
HBSS	Hank's Buffered Salt Solution
HNPCC	Hereditary nonpolyposis colorectal cancer
HPRT	Hypoxanthine phosphoribosyl transferase
hTERT	Human telomerase reverse transcriptase
IBMX	Isobutylmethylxanthine
iPS cells	Induced pluripotent stem cells
IRES	Internal ribosomal entry site
kb	Kilobase
kDA	Kilo Dalton
Lef	Lymphoid Enhancer Binding Protein Factor
LRP	Low Density Lipoprotein Receptor-related Protein
M	Molar
min	Minute
ml	Milliliter
mM	Millimolar
mm^2	Square millimeter
MSC	Bone marrow mesenchymal stem cell
NEAA	Non-essential amino acids
ng	Nanogram
nm	Nanometer
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
pg	Picogram
PGE_2	Prostaglandin E2

$PGF_{2\alpha}$	Prostaglandin F2α
PGK	Phosphoglycerate kinase
PNS	Positive negative selection
poADMSC	Porcine adipose-tissue derived mesenchymal stem cells
poFF	Porcine fetal fibroblasts
poMSC	Porcine mesenchymal stem cell
polyA	Polyadenylation site
rAAV	Recombinant adeno-associated virus
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
RYR1	Ryanodine receptor 1
sec	Second
SCNT	Somatic cell nuclear transfer
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SV40	Simian virus 40
Tcf	T-cell Factor
U	Unit
UV	Ultraviolet
V	Volt
ZFN	Zink-finger nucleases

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9 Appendix

9.1 Construction of targeting vector pAPC1061

For the construction of the targeting vectors pAPC1061 and pAPC1311 porcine *apc* sequences from exon 13 to 15 were amplified by PCR from the porcine genome (Dr. T. Flisikowska, Livestock Biotechnology, TU München). The targeting vectors were constructed from four fragments of the porcine *apc* sequence which were amplified with the oligonucleotides described in Table 14.

Table 14: Oligonucleotides for the amplification of the porcine *apc* gene.

Fragment	Primer	Fragment size
APC2.9	APColap4f: 5'- gga gga tgg gca gta gtc ag -3'	2847 bp
	APColapr: 5'- gct ccc cat agc aat cat tt -3'	
APC4.5	EST3f: 5'- acc tcc acc tgt ggc aag aa -3'	4494 bp
	EST6r: 5'- gag cag cca tct cac ctc aa -3'	
APC6.4	EST12f: 5'- tgc gtg aag ttg gca gtg tg -3'	6414 bp
	EST11r: 5'- caa gct gga cac att ccg ta -3'	
APC7.6	EST8f: 5'- acg gaa tgt gtc cag ctt ga -3'	7569 bp
	T2r: 5'- gtt ctt ctc caa gtg ctt ac -3'	

The fragment APC2.9 was necessary to connect the fragments APC6.4 and APC7.6 (Figure 42).

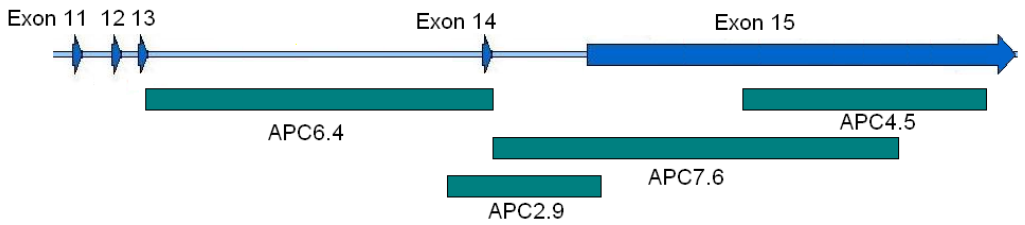

Figure 42: Localisation of the amplified fragments within the porcine *apc* gene.

After amplification by PCR the fragments APC7.6 and APC4.5 were subcloned in pGEM3ZL(+). Both fragments shared a 2901 bp overlapping region. A BamHI

restriction site within this region and a NotI restriction site in the backbone of the pGEM3ZL(+) plasmid were used to combine both fragments and to create poAPC9.2 (Figure 43).

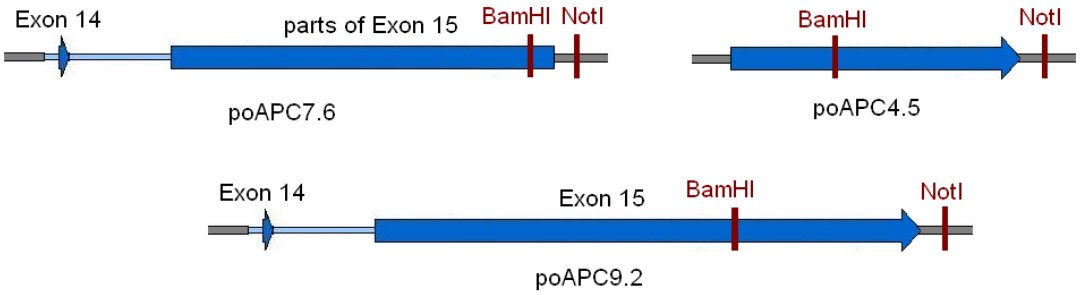

Figure 43: Construction of poAPC9.2. The plasmid poAPC7.6 and poAPC4.5 were used for restriction digestions with BamHI and NotI. The fragments were ligated and poAPC9.2 was created. poAPC9.2 contains 9180 bp of the porcine *apc* sequence.

For the introduction of the mutation cassette an annealed oligonucleotide containing the stop codon and further restriction sites for the following cloning steps was used (see 2.2.3.3). To allow the transfer of this oligonucleotide into poAPC9.2, a 1479 bp SphI-EcoRI-fragment of poAPC9.2 was subcloned in pGEM-3ZfP. From this plasmid 485 bp were removed with BspEI and EcoRI and the annealed oligonucleotide was introduced with the same restriction sites (not shown). Afterwards the selection cassette containing an internal ribosomal entry site (IRES), *blasticidin deaminase* (*bsd*) gene and a poly(a) sequence was inserted in poAPC1.5-oligo1061. The selection cassette was removed from pPGK-EGFP-IRES-BS and ligated to poAPC1.5-oligo1061 by a restriction digest with AflII and BamHI. With this step the IRES-BS-poly(A) cassette was introduced 3' of the introduced stop codon at position 1061 (Figure 44).

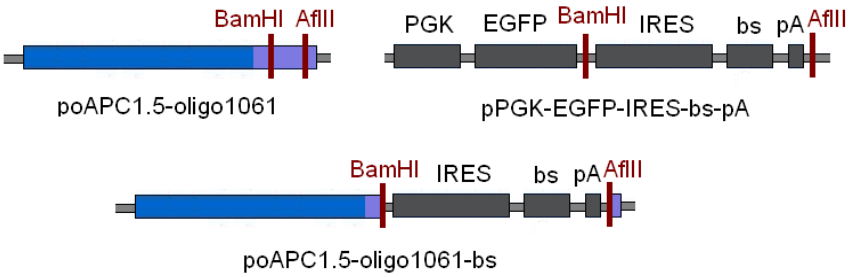

Figure 44: Construction of poAPC1.5-oligo1061-bs. The IRES-bs-poly(A) fragment was introduced in the *apc* sequence by BamHI and AflII restriction sites.

The mutation cassette was transferred to poAPC7.6 by SphI and EcoRI restriction sites. As poAPC7.6 contained more than one EcoRI restriction site, two restriction digestions with SphI/SexAI and EcoRI/SexAI were carried out (Figure 45).

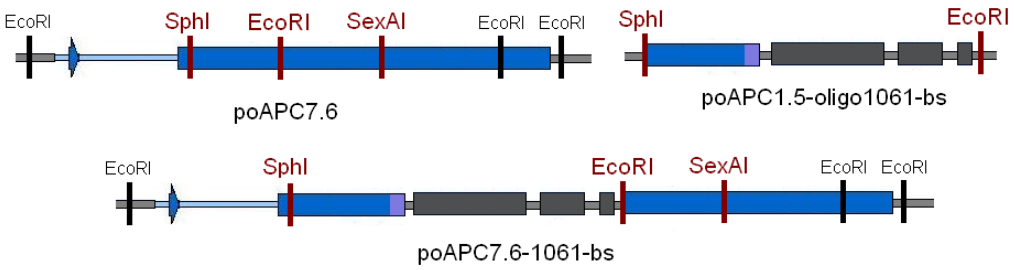

Figure 45: Construction of poAPC7.6-1061-bs. The SexAI-SphI- and EcoRI-SexAI-fragments of poAPC7.6 were ligated with the SphI-EcoRI-fragment of poAPC1.5-oligo1061-bs.

The apc sequence containing the mutation site and the selection cassette was then transferred to a pBluescript backbone by an XhoI restriction digestion (Figure 46). Due to the restriction digestion with XhoI, 1026 bp of the *apc* sequence were removed from the plasmid.

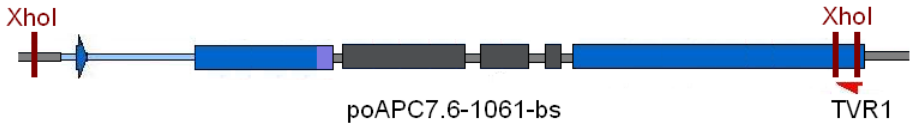

Figure 46: XhoI restriction digestion of poAPC7.6-1061-bs. An XhoI-XhoI-fragment was removed from poAPC7.6-1061-bs. The binding of the oligonucleotide TVR1 which was used for the screening of targeted clones is also marked.

To enlarge the long targeting arm, the 6.5 kb fragment of poAPC6.4 was added to the plasmid. As shown in Figure 42, the fragments APC6.4 and APC7.6 do not share overlapping sequences of the *apc* gene. Thus, it was necessary to first add APC2.9 by a SacII restriction digestion to APC6.4. The created plasmid was then used for two restriction digestions with NheI/SphI and NheI/SalI. Additionally, a restriction digestion with SphI and XhoI was carried out with pBS-poAPC7.6-1061-bs (Figure 47).

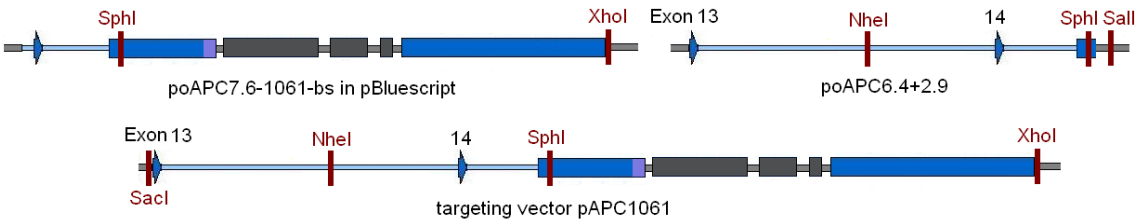

Figure 47: Construction of the targeting vector pAPC1061. The SphI-XhoI-fragment of poAPC7.6-1061-bs in pBluescript was ligated to the NheI-SalI- and NheI-SphI-fragments of poAPC6.4+2.9. XhoI and SalI share compatible ends for ligation. For electroporation the targeting vector was linearized with the restriction enzyme SacI.

The sequence was confirmed by sequencing. The targeting vector pAPC1061 covers porcine *apc* sequences from exon 13 to 15. The long arm of the targeting vector has

9357 bp of homologous region and the short arm 3097 bp. A unique SacI restriction site was used for linearisation of the plasmid (Figure 47).

9.2 Construction of targeting vector pAPC1311

The targeting vector pAPC1311 is based on the same porcine *apc* sequences as pAPC1061 (Figure 42). For the insertion of the mutation cassette other restriction enzymes were used and thus, a different approach was necessary for the construction of pAPC1311. A plasmid with the *apc* sequence from exon 14 to 15 was created, but in contrast to poAPC9.2 the sequence was introduced in reverse orientation into pGEM3ZL(+). The plasmids poAPC7.6rev and poAPC4.5rev were used for restriction digestions with EcoRV and ApaI, the fragments were ligated and poAPC9.2rev was created.

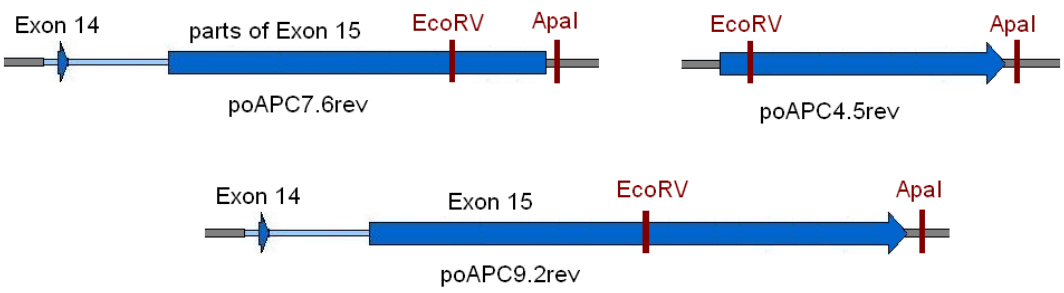

Figure 48: Construction of poAPC9.2rev. The EcoRV-ApaI-fragment of poAPC4.5rev was introduced in the plasmid poAPC7.6rev.

The next step was the annealing of the oligonucleotides containing the stop codon at codon 1311 and restriction sites for further cloning steps (see 2.2.3.3). The oligonucleotide was introduced in pGEM-3ZfP by a restriction digestion with EcoRI and SphI. Then 1229 bp of poAPC9.2rev were transferred to pGEM-3-ZfP-oligo1311 by a restriction digestion with BstXI and SexAI (Figure 49).

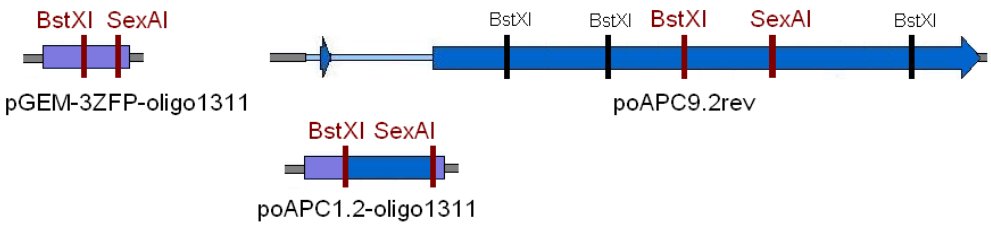

Figure 49: Construction of poAPC1.2-oligo1311. A BstXI-SexAI-fragment of poAPC9.2rev was introduced in pGEM-3ZfP-oligo1311.

Two restriction digestions were carried out with the plasmid poAPC1.2-oligo1311 with SphI/BseRI and BseRI/AlwNI/SphI. The 4093 bp BseRI-SphI- and 377 bp BseRI-AlwNI-SphI-fragments were ligated with the 1780 bp AlwNI-SphI-fragment of poAPC9.2rev (Figure 50).

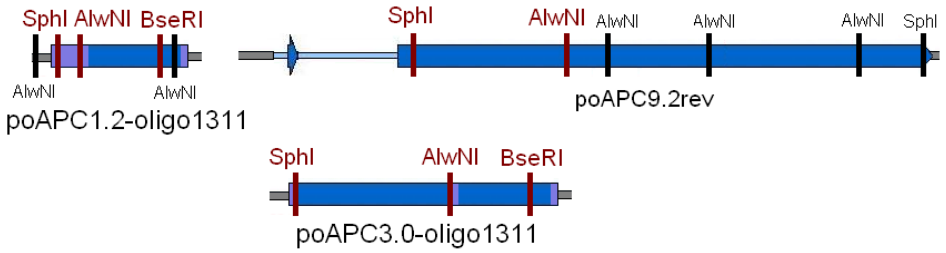

Figure 50: Construction of poAPC3.0-oligo1311. The BseRI-SphI- and BseRI-AlwNI-SphI-fragments of poAPC1.2-oligo1311 were ligated with the AlwNI-SphI-fragment of poAPC9.2rev.

The selection cassette was obtained from the plasmid poAPC1.5-oligo1061-bs by a BamHI and AflII restriction digestion and introduced in poAPC3.0-oligo1311 with the same restriction sites (Figure 51).

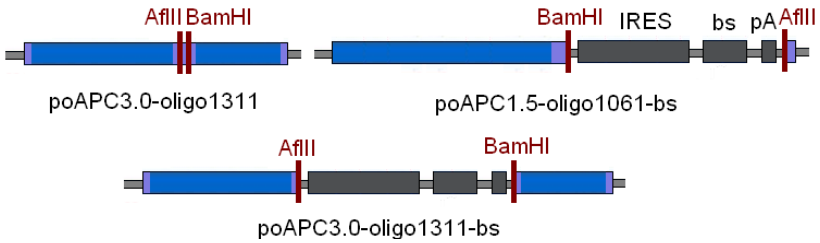

Figure 51: Construction of poAPC3.0-oligo1311-bs. The BamHI-AflII-fragment of poAPC1.5-oligo1061-bs containing the selection cassette was introduced in poAPC3.0-oligo1311 with the same restriction sites.

To enlarge the *apc* sequence in the final vector, the mutation site at codon 1311 and the selection cassette surrounded by *apc* sequence were removed from the plasmid poAPC3.0-oligo1311-bs by a restriction digestion with SphI and SexAI and subcloned in pAPC1061. This final step replaced the pGEM-T easy backbone with the pBluescript SK+ backbone of pAPC1061 (Figure 52).

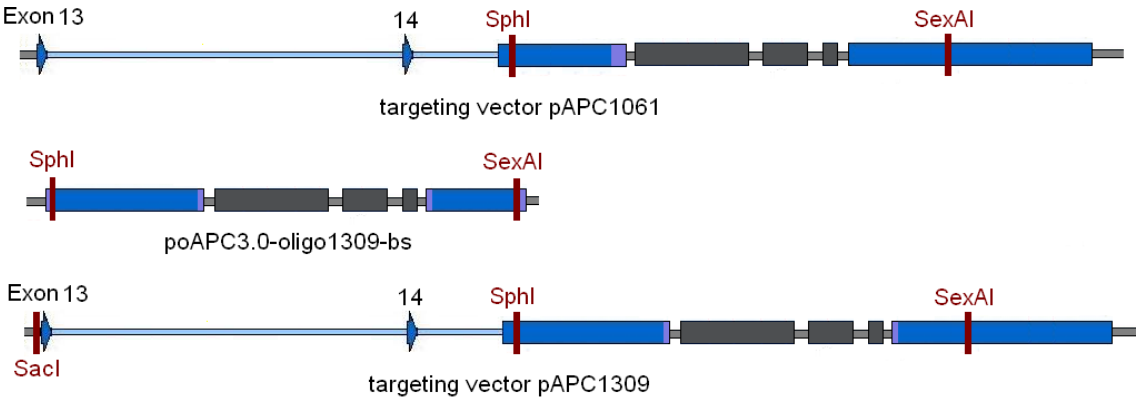

Figure 52: Construction of targeting vector pAPC1311. The SphI-SexAI-fragment of poAPC3.0-oligo1311-bs was ligated with the SexAI-SphI-fragment of pAPC1061.

The sequence was confirmed by sequencing. The targeting vector pAPC1311 covers porcine *apc* sequences from exon 13 to 15. The long arm of the targeting vector has 10143 bp of homologous region and the short arm 2673 bp. A unique SacI restriction site was used for linearisation of the plasmid (Figure 52).

9.3 Construction of a control vector

As a positive control for the targeting PCRs, a control vector was designed. For the construction of the control vector, the 3902 bp SphI-EcoRV-fragment of the plasmid poAPC7.6-1061-bs was ligated with the 8223 bp PmlI-SphI-fragment of the plasmid poAPC7.6rev (Figure 53). Both EcoRV and PmlI create blunt ends and therefore leave compatible ends for ligation. The positive control vector has a size of 12125 bp. To distinguish this vector from positive targeting events and to avoid a possible contamination of the DNA templates for the screening PCRs, the control vector incorporates a duplication of 248 bp.

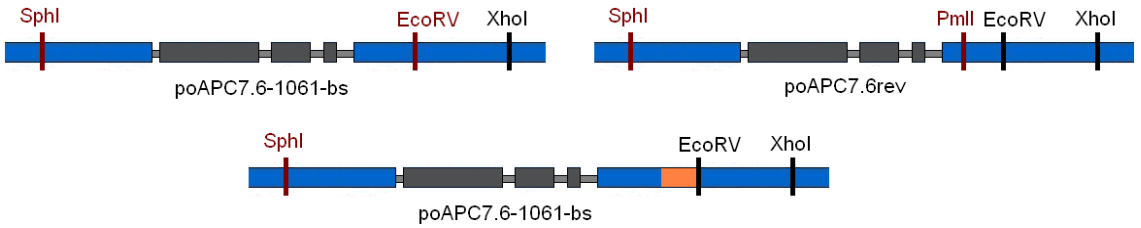

Figure 53: Construction of the positive control construct (control vector). The SphI-EcoRV-fragment of the plasmid poAPC7.6-1061-bs was ligated with the PmlI-SphI-fragment of the plasmid poAPC7.6rev The duplicated sequence is shown in orange.

9.4 Overview cell clones

poMSC-300707: APC1061 clones

Clone number	RT-PCR	Southern blot	Transfer
22	+		+
26	+		+
42			
44	+		+
47	+		+
56	+		
58	+		+
59			
63			
65			
67	+		
68	+		
91			
92			

poMSC-030309: APC1061 clones

Clone number	RT-PCR	Southern blot	Transfer
301		+	+
304	+		
305			
307			
318	+		
320	+		
322			
324			
325	+	+	+
329	+		
331	+		
335			
336			
339			

poMSC-090210: APC1061 clones

Clone number	RT-PCR	Southern blot	Transfer
201			
203	+		
208			
211	+		
212	+		
214	+	+	+
216			
217			
218			
230	+		
231			
232			
236			
241			

poMSC-030309: APC1311 clones

Clone number	RT-PCR	Southern blot	Transfer
716			

poMSC-170209: APC1311 clones

Clone number	RT-PCR	Southern blot	Transfer
835			
838			

poMSC-240307: APC1311 clones

Clone number	RT-PCR	Southern blot	Transfer
133			+
155			
157			
162			
165			
168	+		+
169			
171			
172			
175			

MSC-090210: APC1311 clones

Clone number	RT-PCR	Southern blot	Transfer
701	+		
714			
715			

10 Acknowledgement

This project was funded by the Dr. Mildred Scheel Stiftung für Krebsforschung, Bonn.

I would like to thank my supervisor Prof. Dr. Angelika Schnieke for the opportunity to work on this exciting project and for her supervision, the guidance and the support throughout my work.

I would like to thank Dr. Alex Kind for the discussion and his support during my work and the helpful suggestions on my thesis. Furthermore I would like to thank Prof. Dr. Oswald Rottmann and everyone else at the animal research station at Thalhausen for taking care of the animals. I also would like to thank Prof. Dr. Eckhard Wolf, Dr. Barbara Keßler and everyone involved in the nuclear and embryo transfers at the Institute for Molecular Animal Breeding and Biotechnology in Oberschleißheim. In addition, I would like to thank Prof. Dr. med. Roland Schmid and PD Dr. med. Dieter Saur for the outstanding support with materials and suggestions and the possibility to carry out the tissue stainings.

I would like to express my deepest gratitude to all members of the Institute for Livestock Biotechnology. I would like to thank Dr. Nooshin Rezaei, Dr. Tatiana Flisikowska and Dr. Hagen Wieland for their suggestions and their scientific and non-scientific support. I would like to thank Peggy Müller, Angela Zaruba, Marco Nörenberg, Kristina Mosandl, Dr. Simone Kraner, Marlene Edlinger, Sulith Christan, Tobias Kranz and Barbara Bauer for all their help and the fun during the past years. Thanks to my fellow Ph.D. students Simon Leuchs, Marina Durkovic, Tobias Richter, Xinxin Cui and Alexander Tschukes for their input and the exchange of ideas. I also want to thank my students Christin Ruoff, Sandra Grässle, Martina Mayer, Lisa Böttcher and Katharina Thanisch. I want to say a special thank you to Margret Bahnweg, Andreas Braun and Steffen and Viola Löbnitz for their outstanding support and their patience in the lab and in the stable. Huge thanks to Claudia Merkl for the great time, the professional and moral support and correction of homework during lunch break. It was great to work with you!

Finally, I want to thank Chris and my family for all their love and unlimited support during these years.

11 Curriculum Vitae

Persönliche Daten

Name: Martina Landmann

Geburtsdatum: 28.02.1981

Geburtsort: Dachau, Deutschland

Ausbildung

06 / 2006 – 04 / 2010	Promotion Technische Universität München, Deutschland Gene-targeted pigs predisposed to colorectal cancer
10 / 2003 – 11 / 2005	Master of Science Molekulare Biotechnologie Technische Universität München, Deutschland Abschlussnote: 1.2 Master's Thesis: Die Wirkung des Phytopharmakons STW5-NII auf die Motilität des Meerschweinchenmagens
10 / 2000 – 09 / 2003	Bachelor of Science Molekulare Biotechnologie Technische Universität München, Deutschland Abschlussnote: 1.8 Bachelor's Thesis: Aufreinigung und Lagerungsversuche des monoklonalen Antikörpers AD4G2
08 / 1991 – 06 / 2000	Abitur Ignaz-Taschner-Gymnasium, Dachau, Deutschland Abschlussnote: 1.5