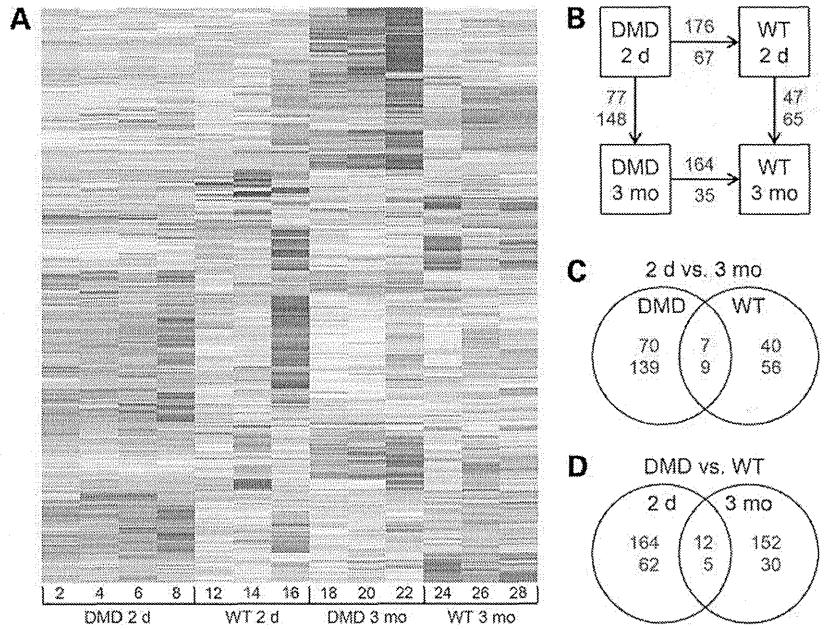

Figure 5. Differential gene expression in biceps femoris muscle of 2-day-old and 3-month-old DMD and WT pigs. (**A**) Heat-map of the DEGs among the four groups defined by genotype × age. Increased abundance of transcripts is indicated in red, decreased abundance in blue colours. (**B**) Number of DEGs between the four groups. Numbers in red and blue indicate the genes up- and down-regulated between the group at the end of an arrow versus the group at the arrow head. (**C**) Age-related transcriptome changes. The numbers in the overlap segment indicate DEGs commonly up- and down-regulated between 2-day-old and 3-month-old pigs of the DMD and WT groups. (**D**) Genotype-related transcriptome changes. The numbers in the overlap segment indicate DEGs commonly up- and down-regulated between DMD and WT pigs of both age classes.

structure organisation', 'collagen'). In WT pigs, the genes expressed at higher levels in 2-day-old animals were summarized by GO term 'extracellular matrix', while in 3-month-old animals transcripts belonging to GO terms 'muscle contraction', 'glucose metabolic process' and 'chloride ion binding' were more abundant (Supplementary Material, Table S4A and B).

Transcriptome changes in skeletal muscle of 3-month-old DMD pigs reflect human DMD and *mdx* mouse muscle

Next, we asked if the transcriptome changes observed in DMD pigs reflect the findings of gene expression studies in skeletal muscle of DMD patients and of the *mdx* mouse. Pescatori *et al.* (25) determined gene expression profiles in muscle biopsies of 19 DMD patients younger than 2 years, thus addressing the pre-symptomatic phase of the disease. Haslett *et al.* (26) investigated the transcriptome of skeletal muscle tissue in DMD children older than 5 years, when impaired muscle function and ambulation becomes clinically manifest. A systematic study of transcriptome changes in skeletal muscle of *mdx* mice between 7 and 112 days of age was performed by Porter *et al.* (27). To compare the changes in gene expression profiles of muscle samples from 2-day-old and 3-month-old DMD pigs versus age-matched controls with these published data sets, we performed gene set enrichment analyses (GSEAs) (28) providing a measure (normalized enrichment score, NES) of the concordance of DEG sets.

The transcripts with increased abundance in 3-month-old DMD pigs were in good concordance with the genes found to be up-regulated in DMD children younger than 2 years (25) (Fig. 7C), older than 5 years (26), and in *mdx* mouse muscles at 23 and 28 days of age (27) (Supplementary Material, Fig. S3A), as indicated by NES >2 with q-values of less than 0.001. A similarly good concordance was found for the down-regulated genes (Supplementary Material, Fig. S3B).

Transcriptomics of 2-day-old DMD pig muscle identifies a mechanical stress signature

The gene expression profiles of biceps femoris muscle samples from 2-day-old DMD piglets were rather different from the published sets of DEGs in DMD patients and in *mdx* mouse muscles (Supplementary Material, Fig. S3C). Specifically, samples from 2-day-old DMD pigs did not display the characteristic transcriptome signatures of ECM remodelling, inflammatory response and decreased energy metabolism which were observed even in DMD patients younger than 2 years, i.e. in the pre-symptomatic period. Instead, the set of transcripts with increased abundance in 2-day-old DMD piglets was similar to a set of genes up-regulated in muscle after acute exercise injury in humans (29) (Fig. 6C) and to transcripts induced by contractile overload (high-force eccentric contractions) in murine skeletal muscle (30) (NES = 1.7624; $q = 0.002$; data not shown).

DISCUSSION

In this study, we generated the first pig model for a genetic muscle disease by gene targeting. In mouse (31) and human

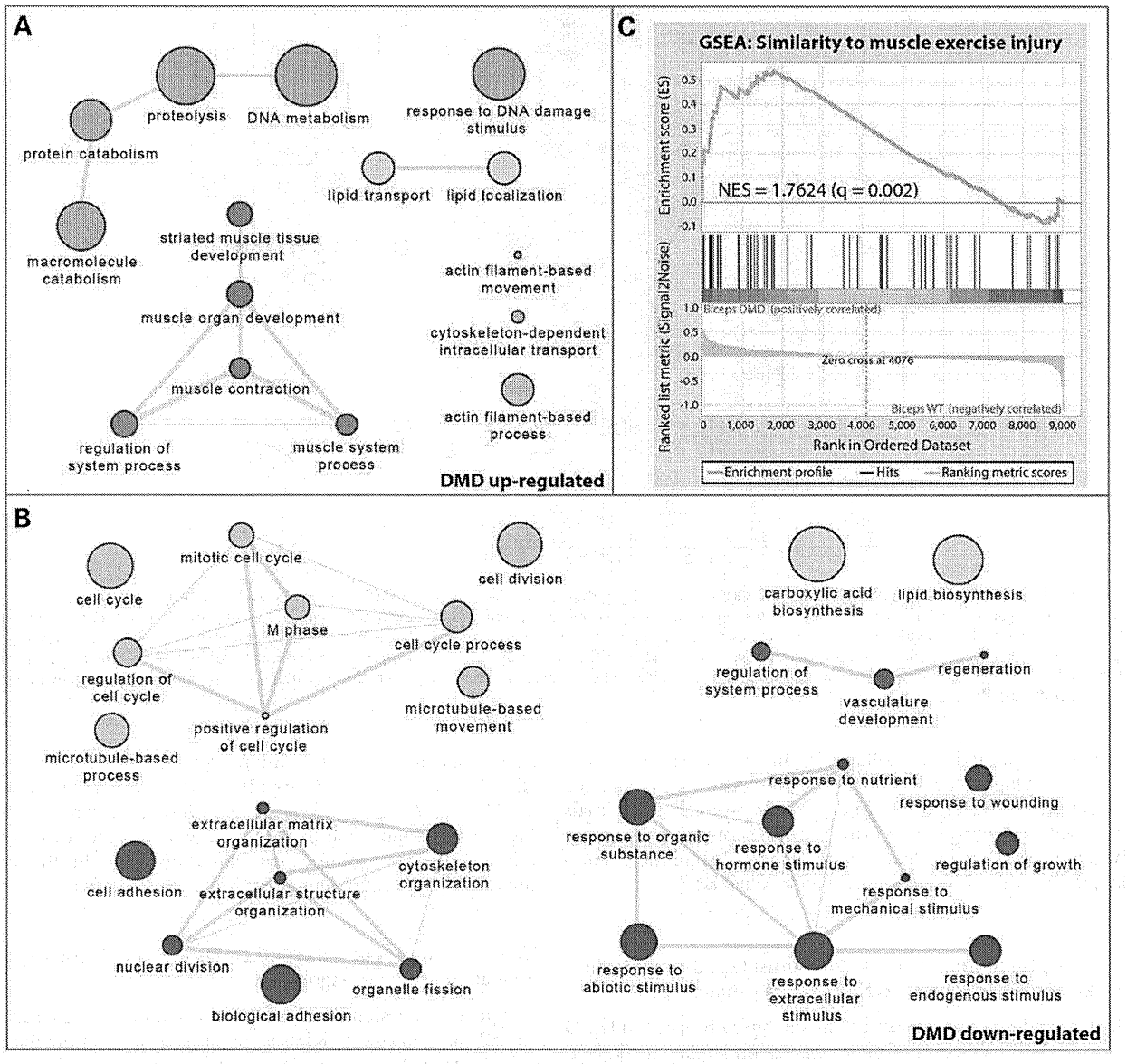

Figure 6. Graph-based visualization of enriched GO terms of genes differentially expressed between DMD and WT pigs at the age of 2 days. (**A**) GO terms of up-regulated and (**B**) GO terms of down-regulated genes in DMD versus WT pigs. The graphs were produced from all significantly enriched GO terms found by DAVID functional annotation clustering using the tools REVIGO (23) and Cytoscape (24). Each GO term is a node in the graph, and 3% of the strongest GO term pairwise similarities are designated as edges in the graph. The generality of GO terms is indicated by bubble size, with smaller bubbles implying more specific terms. (**C**) Gene set enrichment analysis of DEGs up-regulated in 2-day-old DMD pigs versus age-matched WT pigs in a set of genes up-regulated in human skeletal muscle exposed to acute endurance exercise (29). The enrichment profile (green curve) shows a non-random distribution indicated by a roughly triangular outline and a peak close to one of the extremes of the signal-to-noise ordered data set. This distribution means that a higher proportion than expected under the null hypothesis of the genes up-regulated in human skeletal muscle after acute endurance exercise is up-regulated in DMD pigs and fewer than expected are down-regulated or neutral. The leading edge subset containing the most up-regulated genes are the genes positioned left of the peak in the enrichment curve profile. The NES was calculated as outlined in the Materials and Methods section. The *q*-value is the false discovery rate (FDR) corrected *P*-value.

embryonic stem cells (32), a high rate of homologous recombination was observed with large targeting vectors based on BACs, avoiding the need for isogenetic DNA and negative selection markers (reviewed in 33). Therefore, we used a BAC containing the porcine *DMD* exon 52 and replaced it by recombineering (16). After nucleofection of the modified BAC into pre-tested nuclear donor cells, exon 52 replacement was observed in around 2% of the stable nucleofected cell clones, rendering BAC targeting an attractive method for introducing targeted mutations into large animal genomes. Importantly, the two targeted cell clones used for nuclear transfer produced pregnancies and offspring at a rate within the usual range of nuclear transfer in

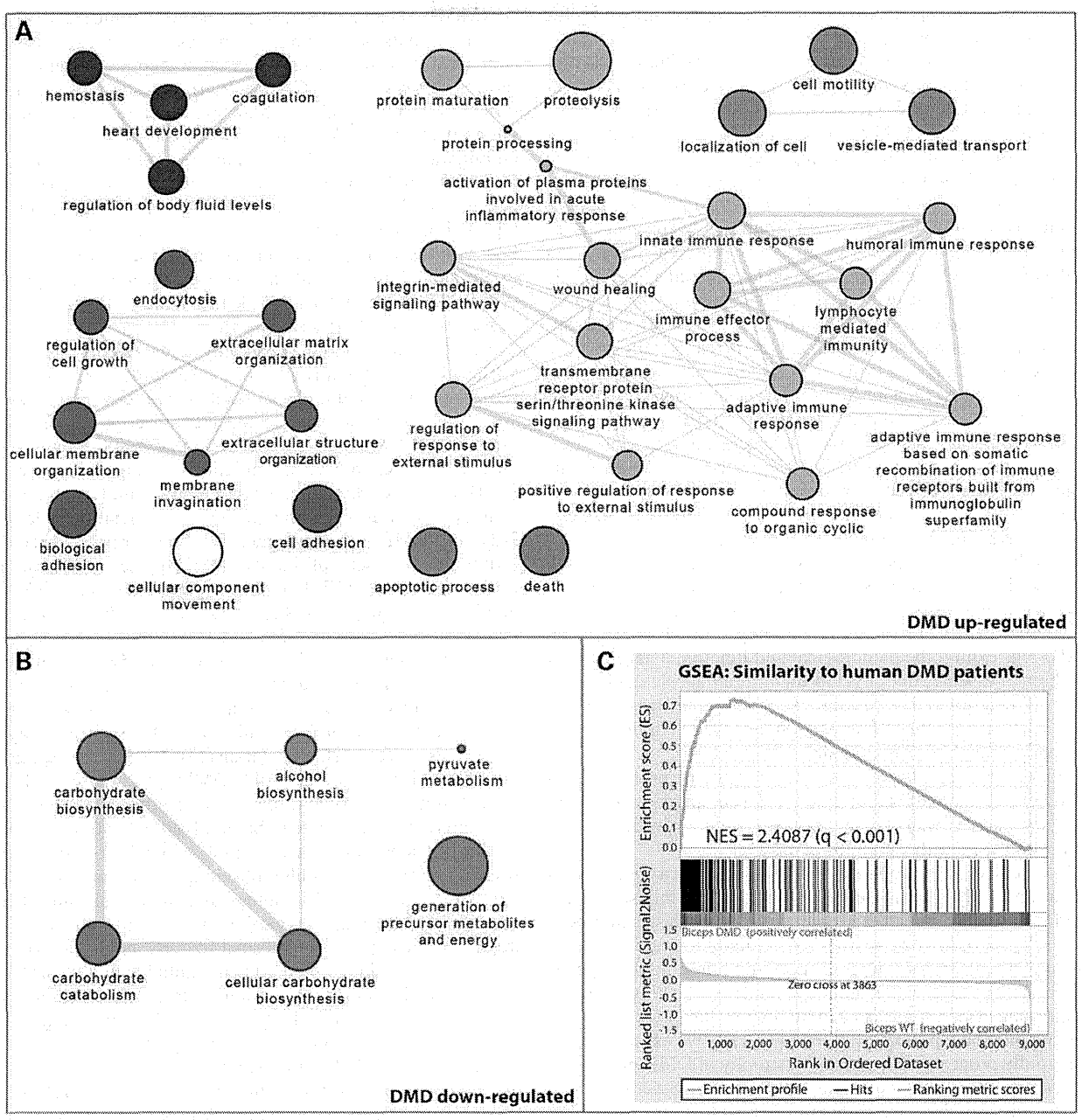

Figure 7. Graph-based visualization of enriched GO terms of genes differentially expressed between DMD and WT pigs at the age of 3 months, and comparison with a transcriptome study of DMD patients. (**A**) GO terms of up-regulated and (**B**) GO terms of down-regulated genes in DMD versus WT pigs. The graphs were produced from all significantly enriched GO terms found by DAVID functional annotation clustering using the tools REVIGO (23) and Cytoscape (24) as explained in legend of Figure 6. (**C**) Gene set enrichment analysis of DEGs up-regulated in 3-month-old DMD pigs versus age-matched WT pigs in a set of genes up-regulated in skeletal muscle of DMD boys younger than 2 years (25). The enrichment profile (green curve) shows a non-random distribution indicated by a roughly triangular outline and a peak close to one of the extremes of the signal-to-noise ordered data set. This distribution means that a higher proportion than expected under the null hypothesis of the genes up-regulated in DMD boys is up-regulated in DMD pigs and fewer than expected are down-regulated or neutral. The leading edge subset containing the most up-regulated genes are the genes positioned left of the peak in the enrichment curve profile. The NES was calculated as outlined in the Materials and Methods section. The *q*-value is the false discovery rate (FDR) corrected *P*-value.

the pig (34), demonstrating that BAC targeting and selection of single clones do not interfere with the potential of donor cells to yield cloned offspring.

Pigs lacking *DMD* exon 52 displayed a complete loss of dystrophin, as it is the case in the majority of DMD patients (17). In addition, the levels of dystrophin-associated glycoproteins

α-sarcoglycan and β-dystroglycan were reduced, mirroring the situation in DMD patients.

The DMD pig appears to be a bona fide model of the human dystrophy as ascertained by the absence of the dystrophin protein, elevated serum CK and progressive muscular dystrophy. The percentage of muscle fibre cross-sections with central nuclei (~10%) in 3-month-old DMD pigs appeared rather low when compared with *mdx* mice of the same age (~60%) (35). This might on the one hand reflect a lower regenerative potential of porcine DMD muscles, and might contribute to the accelerated severe muscle phenotype seen in DMD pigs when compared with *mdx* mice. On the other hand, the low percentage of muscle fibres with central nuclei might reflect the young age of the investigated pigs. Likewise, studies in *mdx* mice show that the percentage of muscle fibres with central nuclei increases with age (36). Another important point is that in *mdx* mouse muscle the myonuclei remain central for a long time after regeneration and can thus be used as a marker for regenerated muscle fibres, while in larger mammals the majority of nuclei in regenerating muscle fibres quickly become subsarcolemmal (reviewed in 37). Thus the higher percentage of central nuclei in the *mdx* mouse when compared with the DMD pig may reflect the difference in post-regenerative remodelling in the two species.

In addition to progressive muscle pathology, DMD pigs show characteristic disturbances of locomotion, including the inability in climbing a platform, which is comparable with the early difficulties of DMD patients in climbing stairs. It appears that DMD pigs exhibit the pathological and functional hallmarks of the human disease, but develop them in an accelerated mode. This offers improved opportunities for early and clear-cut read-outs in efficacy studies of new treatments when compared with currently available animal models.

Our findings raise the questions (i) why disease progression in DMD pigs is markedly accelerated when compared with human patients; and (ii) why the severity of muscular dystrophy was associated with birth weight of DMD pigs.

Up-regulation of utrophin at the sarcolemma is a common observation in dystrophin-deficient human and mouse muscle (reviewed in 20). Functional compensation of dystrophin deficiency by utrophin has been demonstrated by amelioration of the pathology in *mdx* mice expressing utrophin transgenes (38,39) and by the fact that double mutant mice lacking both dystrophin and utrophin present a more severe phenotype than mice lacking only one of these proteins (40). Thus we asked whether the rapidly progressing muscular dystrophy of DMD pigs is due to a lack of sarcolemmal utrophin up-regulation. Since increased levels of utrophin have been observed in regenerating, dystrophin-deficient, or inflamed muscle without a corresponding increase in *UTRN* mRNA (41), we compared utrophin expression between DMD and WT pigs on the protein level by western blot and immunohistochemistry. While in muscle specimens of 3-month-old pigs, the sarcolemma stained clearly positive for utrophin, we did not detect sarcolemmal utrophin expression in 2-day-old DMD pigs. The utrophin expression pattern in DMD pigs reflects the situation in humans where young cases of DMD show very little sarcolemmal utrophin, but as the disease progresses utrophin is detectable on many mature muscle fibres (reviewed in 42).

Consistent with these findings, sarcolemmal utrophin was markedly increased in canine X-linked muscular dystrophy (CXMD) in skeletal muscle of 30- and 60-day-old and adult dystrophic animals (43). Moreover, in these dogs, utrophin expression was more persistent when compared with controls and female carriers. In the Golden Retriever muscular dystrophy (GRMD) animal model, a high mortality rate was observed during the first 2 weeks of life (44). Notably, utrophin immunostaining was absent, faintly or variably positive in skeletal muscle from 2-day-old dystrophic CXMD animals which died spontaneously. Similarly absent or variable utrophin expression was reported in puppies which died after days 8, 9 and 11 (43). In line with the findings in neonates of the canine muscular dystrophy model, the lack of sarcolemmal utrophin up-regulation in the 2-day-old DMD pig muscle may contribute to the lethality of some animals shortly after birth.

To gain insight into the hierarchy of disease mechanisms of muscular dystrophy of DMD pigs, we performed a holistic transcriptome analysis of skeletal muscle specimens from 2-day-old and 3-month-old animals. The muscle transcriptome changes of 3-month-old DMD pigs were very similar to those reported for muscle samples of DMD patients, demonstrating that the DMD pig reflects the human disease on the molecular level. In contrast, the transcriptome profile of skeletal muscle from 2-day-old DMD pigs was rather different from published transcriptome data sets of human DMD muscle. Interestingly, *CCL2*—coding for CC chemokine ligand 2, a ligand of CC chemokine receptor 2 (CCR2)—was one of the most down-regulated transcripts in 2-day-old DMD pigs. Experiments in *Ccl2* knockout mice demonstrated that CCL2 deficiency results in reduced inflammation and impaired regeneration after acute muscle injury (45). Further, $Ccl2^{-/-}$ mice exhibited reduced expression of *Igf1*, which was also seen in 2-day-old DMD pigs. In contrast, *CCL2* transcript levels were elevated in 3-month-old versus 2-day-old DMD pigs associated with marked inflammatory and regenerative processes. Thus *CCL2* is an interesting candidate to be involved in the transition from the early lesions observed in 2-day-old DMD piglets to the severe muscular dystrophy observed in 3-month-old DMD pigs with all clinical and pathological hallmarks of human DMD.

A classical hypothesis regarding the pathophysiology of dystrophin deficiency is that abnormal fragility and leakiness of the muscle cell membrane represents the initial pathology of DMD, which is made worse by mechanical stress (reviewed in 20). A primary role of mechanical stress in early DMD pathology is supported by the fact that the set of transcripts with increased abundance in 2-day-old DMD piglets was similar to a set of genes up-regulated in muscle after acute exercise injury (29,30). Our observation that the survival time of DMD pigs was inversely associated with birth weight (Fig. 2A) points to an effect of intrauterine and postnatal growth rate on the severity of the disease. Human foetal growth reaches a maximum between 30 and 36 weeks and declines thereafter as the mother fails to meet the increasing energy demand of her growing foetus. In contrast, pig foetuses have not reached their peak growth rate at birth and can double their birth weight within 7–10 days due to high rates of protein deposition and lean tissue growth and a marked increase in body fat, while human babies need 5 months to double their birth weight (reviewed in 46). Thus, the pig is a model of accelerated growth, which may aggravate the phenotypic consequences of DMD deficiency via different routes. Foetal pig muscles grow by hypertrophy from around day 75 of pregnancy (47). Since

mechanical strains per unit surface area increase with the calibre of muscle fibres (48), rapid muscle growth may render muscle fibres of DMD pigs particularly susceptible to sarcolemmal damage. Recently, Grounds and Shavlakadze (49) proposed that the sarcolemma of an actively growing myofibre has different properties to the sarcolemma of a mature adult myofibre and that the timing and pattern of muscle fibre growth affects the severity of phenotype of DMD deficiency. This concept is fully supported by the pathology of the DMD pig. Parturition is another potential source of mechanical stress on the muscle fibres which is expected to be proportionate to body weight. This hypothesis is supported by the observation that DMD pigs with a very high birth weight were most severely affected and could not move at all (Fig. 2A and Supplementary Material, Video S2). A third source of mechanical stress on the muscle fibres of DMD pigs may result from the fact that they start to move on their own shortly after birth, resulting in greater mechanical strains on the muscle fibres when compared with human DMD newborns. As the mechanical stress due to movement is expected to be correlated with body weight, this would also explain the longer survival of DMD pigs with low birth weight when compared with pigs with normal or high birth weight. In the genetic background used for our DMD model, the birth weight of wild-type piglets generated by breeding usually ranges between 1.0 and 1.8 kg, depending on litter size. Only three DMD piglets had birth weights slightly higher than 1.8 kg (Fig. 2A), arguing against the notion that the cloning process may have indirectly—by increasing birth weight—aggravated the phenotypic consequences of the *DMD* mutation. Using a genetic background with reduced growth rate might decelerate the DMD pathology and facilitate longer survival. Interestingly, the DMD piglets surviving up to 3 months showed a low postnatal growth rate and a markedly reduced body weight at 3 months when compared with age-matched controls (15.49 ± 1.63 versus 42.60 ± 3.66 kg; $P < 0.001$).

In summary, pigs lacking *DMD* exon 52 display progressive and—compared with DMD patients—markedly accelerated muscular dystrophy. Our transcriptome studies of skeletal muscle samples from young (2 days) and older (3 months) DMD pigs provide new insights into early changes associated with dystrophin deficiency and secondary changes during postnatal development, and thus into the hierarchy of physiological derangements in a severe dystrophin-deficient animal model. Since loss of exon 52 is a frequent mutation in human DMD and can be treated by exon 51 skipping (50), our pig model has potential for testing and refinement of this therapeutic strategy. In order to provide sufficient numbers of DMD pigs for systematic treatment trials, we successfully generated $DMD^{+/\Delta exon52}$ female cells and are currently using them for nuclear transfer to produce heterozygous females, which are expected to have 50% male DMD offspring. These may prove helpful in developing new therapies for muscular dystrophy, including exon skipping, gene and stem cell therapies.

MATERIALS AND METHODS

BAC targeting

Exon 52 of the porcine *DMD* gene was identified on BAC clone CH242-9G11. We replaced the exon by a neomycin resistance cassette (*neo*®) using bacterial recombineering (16). The homologous arms for recombineering were amplified by the primer pairs 5′-atg agc tct taa tta agg tgt tct ctc ctc tat g-3′ and 5′-tgg atc ctc gcg act gca gcc tta gaa gca gtc tcc ttc-3′ as well as 5′-atg gat ccg cgg ccg caa act gga acc aca aga c-3′ and 5′-atg gta cct taa tta atc tgc tct ctg gtc act c-3′. After endotoxin-free preparation of a correctly modified BAC and its linearization with *Sfi*I, 5–10 μg of the modified BAC was nucleofected into primary kidney cells (51) from a 3-month-old male piglet. Cells were selected with 1.2 mg/ml G418 in Dulbecco modified Eagle medium supplemented with 15% fetal bovine serum (FBS), 293 mg/l L-glutamine, antibiotics and 0.1 mM 2-mercaptoethanol and neomycin-resistant clones were expanded for screening and nuclear transfer.

Screening of clones was performed by qPCR using the target site specific primer pair 5′-tgc aca atg ctg gag aac ctc a-3′ and 5′-gtt ctg gct tct tga ttg ctg g-3′ as well as the reference primer pairs 5′-tgt ctg cga ccc aca cca-3′ and 5′-gca tgc atc agt aag gaa ctg g-3′ and 5′-tca tca gtg gat tca ccc caa-3′ and 5′-cac cac ggg aat gcc ttc-3′.

Nuclear transfer and embryo transfer

All animal procedures in this study were approved by the local Animal Welfare Committee (Regierung von Oberbayern) and were performed according to the German Animal Welfare Act and the European Communities Council Directive of 24 November 1986 (86/609/EEC). Two *DMD* mutant cell clones were used for somatic cell nuclear transfer according to Kurome *et al.* (52) with minor modifications. HEPES-buffered medium 199 plus 10% FBS was used for all manipulations and post-activation culture. Fusion was done in Eppendorf fusion medium using the Multiporator® device (Eppendorf, Hamburg, Germany). Embryo transfer was performed as previously described (53). The second round of nuclear transfer experiments was done with the *DMD* targeted cell clone used to generate piglet #1263 or primary kidney cells from #1263 as nuclear donors.

Genotyping and clinical chemistry

Genotyping of the animals was done by PCR analysis of DNA isolated from fibroblast cell cultures established from ear biopsies. Target site specific primers 5′-cag cag cag tca aag ggc ata-3′ and 5′-agg caa gtc tgg gaa gca tca-3′ were applied for detection of exon 52 of the *DMD* gene. The primer pair 5′-cgc tcg tgg tcg aca acg-3′ and 5′-ctg gat ggc cac gta cat g-3′ designed on the sequence of the *ACTB* gene was used as a reference. PCR products were separated on a 2% agarose gel and stained with ethidium bromide. Serum CK levels were determined using the Hitachi 912E Automatic Analyser (Roche Diagnostics, Mannheim, Germany).

RNA sequencing and bioinformatics

Total RNA from two newborn *DMD* knockout piglets and two wild-type controls was isolated from cryopreserved tissue samples using Trizol® (Invitrogen, Carlsbad, CA, USA). Purity and integrity of RNA was assessed by spectrophotometry (nanodrop ND-100, Nanodrop, Wilmington, USA) and agarose gel electrophoresis. Fifty nanograms of total RNA was used for random primed cDNA synthesis and isothermal amplification

using the Ovation RNA-Seq kit (Nugen, San Carlos, CA, USA). The primers p50f7361 (5′-aga aag tta gaa gat ctg agc-3′) and p53r7879 (5′-ttg cct tct gtt ctg aag g-3′) were used for cDNA amplification. Amplified double-stranded cDNA was end-repaired and ligated to bar-coded adapters provided with the Encore Multiplex Kit (Nugen). Finally, the adapter ligated library was enriched and amplified by 10 PCR cycles according to the kit protocol. Concentration and size distribution of the library was determined with a DNA1000 chip on a 2100 Bioanalyser (Agilent, Santa Clara, CA, USA). The four bar-coded libraries were mixed at equimolar amounts and sequenced on one lane of Illumina's Genome Analyser IIx in single read mode and 75 nucleotides read length. Sequence reads were de-multiplexed according to a four-base leading bar-code and mapped to the pig genome (susScr2 from UCSC genome browser) with the spliced-read aligner Tophat v1.2.0 (54). Normalized read counts (FPKM, fragments per kilobase of transcript and per million mapped reads) based on the Ensembl annotation were calculated with Cuffdiff (v1.0.3) from the Cufflinks program package (55).

Microarray analysis

Total RNA from seven *DMD* knockout piglets and six wild-type controls was isolated from RNAlater preserved M. biceps femoris samples using Trizol® (Invitrogen). Purity and integrity of RNA was assessed by spectrophotometry (nanodrop ND-100, Nanodrop) and Agilent Bioanalyser 2100 (Agilent). Two hundred nanograms of total RNA was used for cDNA synthesis, amplification, fragmentation and labelling using the Nugen Applause WTA ST and Encore Biotin labelling kits (Nugen, San Antonio, TX, USA). Labelled probes were hybridized to Affymetrix PorGene 1.0 ST GeneChips, washed, stained in an Affymetrix FS450 station and scanned on an Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA, USA). The array CEL-files were RMA normalized by apt tool from Affymetrix and analysed for differential expression in R using the limma package. Gene annotations were extended by their orthologous human gene symbol. Porcine transcripts (susScr10.2) were aligned against the human transcripts (hg19) using BLAST and best matching hits with a bit score > 80 were accepted. Genes were regarded as differentially expressed when passing the thresholds of fdr < 0.05 and $\log_2$fold-change > 1. Functional analysis of DEGs was done by DAVID functional annotation clustering (21,22) with an enrichment score of ≥ 1.3, REVIGO (23) and Cytoscape (24) for summarization and visualization of GO annotations obtained by functional annotation clustering from DAVID, and GSEA (28). As a measure of concordance between sets of DEGs, the NES was used, which accounts for differences in gene set size and in correlations between gene sets. The normalization allows for comparison across gene sets.

Immunoblot analysis

Muscle tissue was homogenized in lysis buffer [125 mM Tris pH 8.8, 40% glycerol, 4% SDS, 0.5 mM PMSF, 100 mM DTT, Complete® protease inhibitor (Roche), bromophenol blue] (56), and protein concentration was determined by the Pierce 660 nm Protein Assay (Thermo Fisher Scientific, Rockford, IL, USA). Defined amounts of total muscle protein were separated by 5% SDS–PAGE and blotted to PVDF membrane. Dystrophin and dysferlin were detected using mouse monoclonal antibodies (NCL-DYS1 and NCL-DYS2, dilution 1:400, Novocastra, Newcastle upon Tyne, UK; and NCL-Hamlet, dilution 1:1000, Novocastra, respectively) and horseradish peroxidase-coupled polyclonal goat anti-mouse antibodies (115-035-146, dilution 1:10 000, Jackson ImmunoResearch, Suffolk, UK). Utrophin was detected using polyclonal rabbit anti-utrophin (sc-15377, dilution 1:800, Santa Cruz Biotechnology, Heidelberg, Germany) and horseradish peroxidase-coupled polyclonal goat anti-rabbit antibodies (dilution 1:2000, Cell Signaling). Bound antibodies were visualized using ECL reagent (RPN2106; GE Healthcare Amersham Biosciences, Freiburg, Germany).

Locomotion studies

A kinematic gait analysis was performed in a 9-week-old *DMD* mutant pig and a size-matched wild-type control using a digital video camera. The animals were trained to move in a linear test track (15 m long, 80 cm broad), which was built by spanning a wire fence in front of a wall. The animals were provoked to walk, trot or gallop by offering food as an incentive. The videos were evaluated in real time and time lapse, taking various kinematic parameters, such as stride length, stride speed, swing and stance phase, regularity and rhythm into account. In addition, the ability to jump up and down a small platform (height: 25 cm) was evaluated. The video is provided in full length as Supplementary Material, Video S1. A second set of kinematic movement analyses was performed on three 10-week-old DMD pigs and a size-matched control.

Necropsy and histopathology

DMD-deficient pigs which had to be euthanized due to progressive worsening were necropsied, and tissue samples of the left and right biceps femoris muscle, triceps brachii muscle, longissimus dorsi muscle, thyreohyoideus muscle, of the diaphragm (left pillar), intercostal muscles and the left heart ventricle were taken for histological examination. Control tissues were obtained from age-matched male wild-type pigs. In total, 22 DMD-deficient pigs [aged 1 day ($n = 6$), 2 days ($n = 4$), 3 days ($n = 3$), 4 days ($n = 1$), 1 week ($n = 1$), 2 weeks ($n = 1$), 7 weeks ($n = 1$) and 3 months ($n = 5$)], and six healthy male wild-type pigs [2 days ($n = 3$) and 12 weeks of age ($n = 3$)] were investigated. Samples were routinely fixed in neutrally buffered formaldehyde solution (4%) for 24 h. Formalin-fixed tissue specimens were embedded in paraffin or in plastic (glycol methacrylate and methyl methacrylate; GMA/MMA) (57). Cross and longitudinal muscle sections were stained with haematoxylin and eosin (H&E), or Masson's trichrome-stain, respectively.

Immunofluorescence and immunohistochemical studies

For immunofluorescence studies, samples of the biceps femoris muscle, triceps brachii muscle, longissimus dorsi muscle, diaphragm and heart from DMD pigs and age-matched wild-type control animals were immersed in 10% gum tragacanth (Sigma-Aldrich, Taufkirchen, Germany) and immediately frozen in liquid nitrogen-cooled isopentane (−150°C). Immunofluorescence studies were performed on 8 μm thick frozen sections. Primary antibodies used in this study were

anti-dystrophin (NCL-DYS1, 1:10, and NCL-DYS2, 1:50, Novocastra), anti-spectrin (MAB1622, Clone AA6, 1:200, Millipore, Schwalbach, Germany), anti-α-sarcoglycan (NCL-a-SARC, 1:50, Novocastra) and anti-β-dystroglycan (NCL-b-DG, 1:10, Novocastra). The isotype-specific secondary antibodies were anti-mouse IgG (H+L) coupled to Alexa Fluor 488 (A11029, 1:300, Invitrogen) and anti-mouse IgG coupled to TRITC (R0270, 1:300, Dako, Glostrup, Denmark). Utrophin immunohistochemistry was performed on sections of formalin-fixed and paraffin-embedded samples of the biceps femoris muscle of each two 2-day-old and 3-month-old DMD pigs and age-matched WT controls, using a polyclonal rabbit anti-utrophin antibody (sc15377, 1:100, Santa Cruz Inc., USA) according to the standard avidin–biotin peroxidase complex method (secondary antibody: biotinylated goat anti-rabbit immunoglobulins, E0432, Dako). Diaminobenzidine was used as the final chromogen and haemalum as nuclear counterstain. For negative controls, slides were incubated with an irrelevant primary antibody (polyclonal rabbit anti-*Escherichia coli*, B0357, Dako) instead of the anti-utrophin antibody.

Morphometric analyses

Morphometric analyses were performed on H&E-stained plastic (GMA/MMA) cross-sections of the left and right biceps femoris muscle of three 2-day-old and five 3-month-old DMD pigs, and of corresponding age-matched control pigs ($n = 3$ and 3, respectively). For quantification of muscle fibre sizes, at least 12 locations per case were taken by systematic random sampling in the sections at ×250 magnification, and superimposed with an unbiased counting frame (58). The minimal Feret's diameters of all muscle fibre cross-section profiles ($n = 697 \pm 166$) sampled with the unbiased counting frames were measured (59), using a Videoplan image analysis system (Zeiss-Kontron, Munich, Germany). To display the distribution of fibre diameters, the percentage deviation of each single measured muscle fibre diameter from the mean diameter of all muscle fibres was calculated separately for each investigated case. The respective single values were categorized into 23 classes of 10% deviation of the mean fibre diameter. The number of single values per class was counted, and their respective proportion (%) of the total number of evaluated fibre diameters was calculated. The volume density of muscle fibres in the biceps femoris muscle was determined by point counting (60) (294 ± 0.6 points per case) in six systematically randomly sampled locations at ×250 magnification, using an automated stereology system (VIS-Visiopharm Integrator System® Version 3.4.1.0 with newCAST® software, Visiopharm A/S, Hørsholm, Denmark). In the same locations, the proportion of muscle fibre cross-section profiles displaying at least one internalized centrally located nuclear section profile was determined, using an unbiased counting frame. Per case, 723 ± 250 muscle fibre cross-section profiles were evaluated.

Data analysis

Data are presented as means ± standard deviations. Unpaired two-sided *t*-tests were used for statistical analysis, assuming equal variances for the parameter body weight, and unequal variances for morphometric muscle parameters. The correlation between birth weight and life expectancy was evaluated using GraphPad Prism. *P*-values of < 0.05 were considered statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

AUTHOR CONTRIBUTIONS

N.K., C.T., H.L., M.C.W. and E.W. designed the study. N.K., A.B., S.K., K.B., A.W., St.K., A.G., B.K., V.Z., M.K., E.K., H.N., B.S., N.H., H.B., R.W., M.C.W. and E.W. performed experiments and analysed data. N.K., H.L., M.C.W. and E.W. wrote the paper with contributions from all authors. N.K, A.W. and K.B. did the gene targeting experiments. B.K., V.Z., M.K. and H.N. were involved in nuclear transfer experiments. St.K., A.G. and H.B. performed the RNA expression and bioinformatics studies. S.K. and B.S. did the immunofluorescence, A.B. the immunohistochemistry and E.K. the western blot analyses. A.B., K.B., N.H. and R.W. performed the pathological and stereological studies. B.K., M.C.W. and E.W. designed the locomotion studies. A.A.-R. contributed to the discussion. H.L., M.C.W. and E.W. gave conceptual advice and supervised the project.

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Conflict of Interest statement. None declared.

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Factors influencing the efficiency of generating genetically engineered pigs by nuclear transfer: multi-factorial analysis of a large data set

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Abstract

Background: Somatic cell nuclear transfer (SCNT) using genetically engineered donor cells is currently the most widely used strategy to generate tailored pig models for biomedical research. Although this approach facilitates a similar spectrum of genetic modifications as in rodent models, the outcome in terms of live cloned piglets is quite variable. In this study, we aimed at a comprehensive analysis of environmental and experimental factors that are substantially influencing the efficiency of generating genetically engineered pigs. Based on a considerably large data set from 274 SCNT experiments (in total 18,649 reconstructed embryos transferred into 193 recipients), performed over a period of three years, we assessed the relative contribution of season, type of genetic modification, donor cell source, number of cloning rounds, and pre-selection of cloned embryos for early development to the cloning efficiency.

Results: 109 (56%) recipients became pregnant and 85 (78%) of them gave birth to offspring. Out of 318 cloned piglets, 243 (76%) were alive, but only 97 (40%) were clinically healthy and showed normal development. The proportion of stillborn piglets was 24% (75/318), and another 31% (100/318) of the cloned piglets died soon after birth. The overall cloning efficiency, defined as the number of offspring born per SCNT embryos transferred, including only recipients that delivered, was 3.95%. SCNT experiments performed during winter using fetal fibroblasts or kidney cells after additive gene transfer resulted in the highest number of live and healthy offspring, while two or more rounds of cloning and nuclear transfer experiments performed during summer decreased the number of healthy offspring.

Conclusion: Although the effects of individual factors may be different between various laboratories, our results and analysis strategy will help to identify and optimize the factors, which are most critical to cloning success in programs aiming at the generation of genetically engineered pig models.

Keywords: Transgenic pig, Knockout pig, Somatic cell nuclear transfer, Multi-factorial analysis

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Background

Somatic cell nuclear transfer (SCNT) has become widely used for the generation of genetically engineered large animals, especially since germ line competent pluripotent stem cells – the key to sophisticated reverse genetics in rodents – are not available in these species [1-4]. Genetic modification of pigs by SCNT facilitated gene targeting [5-7], inducible transgene expression [8], and the first successful examples of zinc finger nuclease mediated targeted gene modifications [9,10] to generate tailored large animal models and donor animals for xenotransplantation.

During the last decade, transgenic pigs have gained importance in the field of biomedical research because of major anatomical and physiological similarities with humans [11] as well as the need for non-rodent based studies to investigate disease mechanisms, the efficacy and safety of new therapies, and to identify biomarkers for companion diagnostics. Genetically tailored pig models have already been developed to investigate cystic fibrosis [12], diabetes mellitus [13-16], and neurodegenerative diseases [17] (reviewed in [18]). Multiple lines of genetically modified pigs have also been generated for xenotransplantation (reviewed in [19]), most notably α1,3-galactosyl transferase knockout pigs lacking α1,3-Gal, the major xeno-antigen [5]. SCNT has facilitated the generation of donor pigs carrying multi-transgene combinations designed to overcome immune rejection and to ensure functional compatibility between xenograft and recipient, e.g. regulation of blood coagulation.

Although the first successful SCNT experiments using cultured porcine cells were performed more than a decade ago [20-22], the efficiency of cloning (live offspring per reconstructed embryos transferred to recipients) is still low, usually ranging from 1 to 5%, and cloned animals may suffer from various developmental defects.

Genetic modification of nuclear donor cells necessarily involves a series of procedures, such as transfection or transduction, drug selection and extended growth in culture, which could possibly affect their ability to support normal development.

To date, several studies have reported key factors in the production of cloned pigs and suggested a number of approaches to improve efficiency. However, the majority of these studies have addressed only single factors, e.g. SCNT procedure [23-26], oocyte and embryo culture systems [27,28], donor cell type [29,30], and the method of genetic modification [31,32]. Combined assessment of multiple factors and comparative analysis of their relative contribution to cloning efficiency have not yet been performed to our knowledge.

Here, we investigate the impact of five factors on the crucial stages of a cloning experiment and ultimately the impact on cloning efficiency. We used a large data set comprising three years of porcine SCNT experiments, during which more than 300 cloned pigs were generated using different genetically modified cell cultures. The data contains simultaneous variations in season, type of genetic modification (additive gene transfer vs. gene targeting), donor cell source (mesenchymal stem cells, postnatal fibroblasts, fetal fibroblasts, and kidney cells), number of cloning rounds, and pre-selection of cloned embryos for early development. We assessed the impact pattern of the variable factors on pregnancy and delivery rates as well as the numbers of born, live and healthy offspring. Cloning efficiency was calculated as the number of cloned piglets born relative to the number of SCNT embryos transferred to recipients that gave birth.

Results

General information

A total of 18,649 SCNT embryos were transferred into 193 recipients. The average number of embryos transferred per recipient was 97 (range: 43–216). 109 recipients (56%) became pregnant and 85 (78%) of those gave birth to offspring. The pregnancy rate was significantly increased when more than 100 NT embryos were transferred to a recipient. Experiments in which over 135 NT embryos were transferred resulted in the maximum overall pregnancy rate of 79.3% (Additional file 1). Recipients that became pregnant displayed no tendency for delivering live offspring in dependence on the number of embryos transferred (Additional file 2). Of the 318 cloned piglets born, 243 (76%) were alive, but only 97 (40%) were clinically healthy, defined as the absence of any visible anatomical or physiological disturbance, and showed normal development. The proportion of stillborn piglets was 24% (75/318), and another 31% (100/318) of the cloned piglets died soon after birth. The major reason for early neonatal death within 2 weeks was underweight (<1000 g) and/or weakness of unknown causes, which was observed in several transgenic litters. In addition, we observed malformations such as oversized tongue (30 cases, 9.4%), cleft palate (2 cases, 0.6%) or *atresia ani* (1 case, 0.3%), abnormalities of the legs (6 cases, 1.9%), patent *urachus* (1 case, 0.3%) and umbilical hernia (6 cases, 1.9%). In 3 cases (0.9%), piglets showed contracted tendons in the forelegs, which improved with increasing body weight and did not affect survival. 39 piglets (12%) were lost for other reasons (killed by the mother or died from infection). The health status of the remaining 7 cloned piglets could not be estimated as they have been used for experiments immediately after birth. The overall cloning efficiency, defined as the number of offspring born per SCNT embryos transferred, including only recipients that delivered, was 3.95%. A detailed description of the data set is shown in Table 1.

Table 1 Data summary

Total no. of transferred SCNT embryos	18,649
Average no. of transferred embryos per recipient	97
Range of transferred embryos per recipient	43-216
No. of different cell sources used for SCNT	4[1]
Type of genetic modification	
Additive gene transfer (no. of constructs)	14[2]
Homologous recombination (no. of target genes)	6[3]
Total no. of recipient pigs	193
Pregnant recipients	109 (56%)
Delivering recipients	85 (78%)
Total no. of cloned offspring	318
Live cloned pigs	243 (76%)
Healthy cloned pigs	97 (40%)

[1] Mesenchymal stem cells, postnatal fibroblasts, fetal fibroblasts, and kidney cells.
[2] See Table 8 for details.
[3] See Table 9 for details.

Impact of individual factors

We assessed the influence on the cloning outcome of five factors: the season the embryo transfer (ET) was performed in, the type of genetic modification, the donor cell source, the number of cloning rounds, and selection of SCNT embryos for development before transfer to the recipient. The stratification and distribution of each varied factor is summarized in Table 2 (more details can be found in Methods, Additional file 3 and Additional file 4).

Season

The seasonal influence on the assessed parameters is presented in Table 3. Spring was used as the reference category and statistically significant differences of results obtained in other seasons are indicated relative to the reference category. The oocyte maturation rate was highest in spring (77.1%), slightly lower in autumn (75.8%) and summer (74.4%), and significantly decreased in winter (71.3%; $p < 0.05$). Similarly, significantly fewer pregnancies were established in winter (1:2 chance) than in spring (2:1 chance). In contrast, the proportion of offspring per SCNT embryos transferred (cloning efficiency) was highest when ET was performed in winter (5.3%), as compared to spring (3.5%; $p < 0.05$). Similarly, the average number of live cloned offspring from ET performed during winter (4.3) was significantly higher than during spring (2.6; $p < 0.05$). The lowest number of healthy cloned piglets was observed if the ET was done in summer (0.8 vs. 2.2 when ET was performed in winter).

Type of genetic modification

Genetic modifications were categorized into three classes: additive gene transfer, homologous recombination, and replication of already existing transgenic pigs. The effects of these classes of genetic modification on outcome are summarized in Table 4. Homologous recombination was used as the reference category. No significant difference was apparent between these three classes of modification with regard to cloning efficiency, pregnancy and delivery rate. However, the numbers of live and healthy cloned offspring per litter, respectively, were significantly higher ($p < 0.05$) in the additive gene transfer group than in the homologous recombination group (3.5 vs. 2.3 and 1.5 vs. 0.6, respectively).

Nuclear donor cell source

Four different cell sources – mesenchymal stem cells, fetal fibroblasts, postnatal fibroblasts, and kidney cells –

Table 2 Stratification and data distribution of the investigated experimental factors

Factor	No. of embryo transfers (%)	
Season[1]		
- Spring	39	(20.2)
- Summer	59	(30.6)
- Autumn	58	(30.0)
- Winter	37	(19.2)
Type of genetic modification[2]		
- de novo - AGT	57	(29.5)
- HR	48	(24.9)
- replication of transgenic pig	88	(45.6)
Donor cell source[3]		
- MSC	36	(18.7)
- PF	24	(12.4)
- FF	51	(26.4)
- KC	82	(42.5)
Cloning rounds		
- 1 time	110	(57.0)
- 2 times	62	(32.1)
- 3 times	21	(10.9)
Selection of SCNT embryos for early development[4]		
- no selection	45	(23.3)
- selection after 1 day	13	(6.7)
- selection after 2 days	15	(7.8)
- mixed selection	120	(62.2)

Additional file 3 and Additional file 4 show in more detail the distribution in season and embryo selection of specific SCNT configurations with respect to genetic modification, donor cell source and number of cloning rounds.
[1] Embryo transfer date.
[2] AGT: additive gene transfer, HR: homologous recombination.
[3] Mesenchymal stem cells (MSC), postnatal fibroblasts (PF), fetal fibroblasts (FF), and kidney cells (KC).
[4] No selection: all SCNT embryos transferred, selection for 1 day: 1-cell stage SCNT embryos transferred, selection for 2 days: 2-cell to 4-cell stage SCNT embryos transferred, mixed selection: mixed SCNT embryos transferred (no selection/1 day and 1 day/2 days).

Table 3 Seasonal variation pattern of the cloning outcome

Season	Temperature (°C)[1]	Oocyte maturation (%)	Chance for pregnancy	Chance for delivery	Cloning efficiency (%)	No. of live cloned piglets	No. of healthy cloned piglets
Spring	9.6	77.1	2	4.2	3.5	2.6	1.4
Summer	18.1	74.4	1.2	3.6	3.8	3.0	**0.8***
Autumn	9.2	75.8	1.8	3.1	4.0	2.6	1.4
Winter	0.1	**71.3***	**0.6***	3.7	**5.3***	**4.3***	2.2

Spring was used as the reference category. For details on the statistical analysis and definition of the cloning benchmarks listed, please see Methods.
* Statistically significant differences ($p < 0.05$).
[1] Average temperature in Munich during the experiments.

were used and their effect on cloning success was determined (Table 5). Mesenchymal stem cells were used as the reference category. The fusion rate of mesenchymal stem cells (93%) was significantly ($p < 0.05$) higher, while that of postnatal fibroblasts (80%) was lower than those of other donor cells. The pregnancy rate was highest with fetal fibroblasts, and lowest with postnatal fibroblasts used as donor cells, but the differences between donor cell sources were not statistically significant. In contrast, the delivery rate was higher with mesenchymal stem cells than with fetal fibroblasts and kidney cells. The cloning efficiency was not affected by the source of donor cells. The proportion of live and healthy cloned offspring in the fetal fibroblast and kidney cell groups was higher than in the mesenchymal stem cell reference group.

Number of cloning rounds

In this data set, up to three rounds of nuclear transfer were performed. One cloning round was used as the reference category (Table 6). Although no statistically significant difference was apparent in pregnancy and delivery rates, cloning efficiency decreased significantly ($p < 0.05$) with cloning round (4.4%, 3.5% and 2.9% for one, two and three cloning rounds, respectively). The number of live and healthy offspring after two rounds was significantly ($p < 0.05$) lower than after the first cloning round (2.2 vs. 3.2 and 0.5 vs. 1.7, respectively). This effect was not seen after three rounds of SCNT.

Selection of cloned embryos for initiation of development

The effect of selection of SCNT embryos on the cloning outcome is shown in Table 7. As reference category, we used the cases where no selection was performed. Pregnancy and delivery rates were not significantly affected by *in vitro* culture of cloned embryos and selection for early development. However, transfer of *in vitro* cultured SCNT embryos, which had developed to 2-cell to 4-cell stage, resulted in the highest proportion of offspring per embryos transferred (6.8% vs. 4.5% in the group where no selection was performed; $p < 0.05$). The numbers of live and healthy offspring were not affected by the pre-selection of cloned embryos for early development.

Statistically significant effects on different phases of development

From in vitro oocyte maturation to cloned offspring

As shown in Figure 1, the maturation of oocytes was significantly impaired in winter (reduced by almost 6 percentage points as compared to spring). We found high fusion rates to be associated with the use of mesenchymal stem cells (up to 13 percentage points better than other cell sources). Cloning efficiency and, thus, the chance for full term development was improved when 2-cell to 4-cell embryos, selected after 2 days *in vitro* culture, were transferred to the recipient. In contrast, the cloning efficiency was negatively affected by repeated SCNT (two rounds of cloning).

Table 4 Variation of the cloning outcome depending on the type of genetic modification

Genetic modification[1]	Chance for pregnancy	Chance for delivery	Cloning efficiency (%)	No. of live cloned piglets	No. of healthy cloned piglets
HR	1	4	3.8	2.3	0.6
AGT	1.8	6.2	4.2	**3.5***	**1.5***
Replication of transgenic pigs	1	2.4	3.9	2.7	1.2

Homologous recombination (HR) was used as the reference category. For details on the statistical analysis and definition of the cloning benchmarks listed, please see Methods.
* Statistically significant differences ($p < 0.05$).
[1] *HR*: homologous recombination, *AGT*: additive gene transfer, Replication of transgenic pigs: replication of already existing transgenic pig lines.

Table 5 Variation of the cloning outcome depending on different nuclear donor cell sources

Cell source[1]	Fusion rate (%)	Chance for pregnancy	Chance for delivery	Cloning efficiency (%)	No. of live cloned piglets	No. of healthy cloned piglets
MSC	93.0	1.1	5.3	3.5	1.6	0.3
PF	**80.2***	0.7	4.0	4.1	2.0	0.5
FF	**89.1***	1.8	**3.7***	4.4	**3.4***	**1.9***
KC	**90.4***	1.3	**2.9***	3.8	**3.4***	**1.4***

Mesenchymal stem cells (MSC) were used as the reference category. For details on the statistical analysis and definition of the cloning benchmarks listed, please see Methods.
* Statistically significant differences ($p < 0.05$).
[1] Mesenchymal stem cells (*MSC*), postnatal fibroblasts (*PF*), fetal fibroblasts (*FF*), and kidney cells (*KC*).

Outcome stage of the recipients (pregnancy/delivery) and the offspring (live/healthy)

As illustrated in Figure 2, we frequently observed pregnant pigs in spring (chance for pregnancy 2:1, i.e. the probability P (pregnancy = YES) was twice as high as the probability P (pregnancy = NO)), whereas pigs hardly became pregnant in winter (1:2 chance). In addition, delivering recipients occurred mostly when mesenchymal stem cells were used (superior chance for delivery of 5:1). Most noteworthy, two cloning rounds significantly ($p < 0.05$) increased the risk of both, pre- and post-natal death of cloned fetuses and offspring, respectively. Postnatal death of offspring also frequently occurred when the embryo transfer was performed in summer and the piglets were therefore born in late autumn/early winter. On the other hand, statistically significant increases in the offspring outcome were observed for embryo transfer in winter, genetic modification by additive gene transfer, and the use of fetal fibroblasts or kidney cells as nuclear donors. These experimental settings yielded on average between one and two more live and healthy piglets than the corresponding reference category.

Interestingly, we could confirm beneficial effects of cloning in winter, using additive gene transfer, and fetal fibroblasts or kidney cells, in an additional analysis (data not shown), where we explicitly targeted the fraction of early neonatal death cases out of live piglets in experiments, which in principle could produce viable offspring (indicated by at least one live piglet). Cloning in winter, using additive gene transfer, and fetal fibroblasts or kidney cells, resulted in 12 percentage points, 32 percentage points, and >35 percentage points less early neonatal death cases, as compared to the reference categories cloning in spring, using homologous recombination, and mesenchymal stem cells, respectively.

Discussion

The outcome of somatic cell nuclear transfer is affected by complex interactions between multiple factors. While some of these are difficult to control, others – such as choice of nuclear donor cell source – may help increase the efficiency of cloning.

Over a period of three years, we generated more than 300 genetically modified pigs by SCNT using multiple donor cell sources. These cells were either *de novo* modified by additive gene transfer or gene targeting, or were derived from existing transgenic or knockout pig lines. All data were collected within our routine workflow for the production of genetically engineered pigs for biomedical research [18]. We used this large data set to identify factors that affect efficiency of cloning and at which stage they act.

We have employed robust linear models, requiring minimal distribution assumptions adjusted to the underlying empirical distribution of the cloning outcome, as a straightforward approach to determine the statistically significant part of the network of factors affecting pig cloning. As shown in Figures 1 and 2, network-based interpretation concepts were used to model and discriminate the major genetic, environmental and experimental factors.

The factors addressed by our study influenced the outcome of cloning for the production of genetically modified pigs on different levels.

The season affected *in vitro* maturation of oocytes, pregnancy rate, and survival of cloned piglets. Even if

Table 6 Influence of the number of cloning rounds on the cloning outcome

No. of cloning rounds	Chance for pregnancy	Chance for delivery	Cloning efficiency (%)	No. of live cloned piglets	No. of healthy cloned piglets
1	1.3	3.4	4.4	3.2	1.7
2	1.0	6.8	**3.5***	**2.2***	**0.5***
3	3.2	1.7	**2.9***	3.1	1.6

One cloning round was used as the reference category. For details on the statistical analysis and definition of the cloning benchmarks listed, please see Methods.
* Statistically significant differences ($p < 0.05$).

Table 7 Effect of SCNT embryo selection on the cloning outcome

Selection timing[1]	Chance for pregnancy	Chance for delivery	Cloning efficiency (%)	No. of live cloned piglets	No. of healthy cloned piglets
No selection	1.1	1.9	4.5	3.4	1.4
Selection for 1 day	0.9	-	4.9	4.3	1.5
Selection for 2 days	0.6	4.0	**6.8***	3.2	2.0
Mixed selection	1.8	4.0	3.5	2.6	1.3

No selection was used as the reference category. For details on the statistical analysis and definition of the cloning benchmarks listed, please see Methods.
* Statistically significant differences ($p < 0.05$).
[1] No selection: all SCNT embryos transferred; selection for 1 day: 1-cell stage SCNT embryos transferred; selection for 2 days: 2-cell to 4-cell stage SCNT embryos transferred; mixed selection: mixed SCNT embryos transferred (no selection/1 day and 1 day/2 days)

the domestic pig shows an estrus cycle with fertility throughout the year, the reproductive performance in commercial pig breeding is notably reduced in late summer and early autumn [33-35]. Bertoldo et al. [36] have documented reduced developmental competence of oocytes during this period. In our data set, the best maturation rate of oocytes *in vitro* was observed in spring and the worst in winter. The latter may be caused, at least in part, by accidental exposure of oocytes to low temperatures during collection and transport. Pig oocytes are very sensitive to low temperature due to high levels of cytoplasmic lipids [37]. During the time span between removal from the incubator and finished embryo transfer, maintenance of an optimal temperature cannot always been guaranteed. Therefore, low temperatures might affect the developmental capability of the embryos and could be responsible for the lower pregnancy rate after ETs in winter, compared to ETs in spring. Nevertheless, cloning efficiency was highest when SCNT experiments and ETs were performed in winter. This finding seems to be contradictory on a first view, but it has to be considered that the cloning efficiency was calculated only for cases in which the recipient delivered offspring. Cases of unsuccessful transfers were not included into the calculation. Therefore, if the embryos survived the negative environmental influences in winter and the recipients became pregnant, the natural high fertility period of the recipients might provide a favorable environment for embryos and fetuses to develop to term.

Unexpectedly, the method of genetic modification had little effect on cloning efficiency in our data set. Generally, genetic modification of donor cells requires prolonged *in vitro* culture for transfection and selection, which could induce cellular changes leading to a decrease in cloning efficiency. Gene targeting by homologous recombination takes a particularly long time and multiple cell divisions to establish single cell clones with sufficient cell numbers for genetic analysis and nuclear transfer [5,38-40]. In contrast, our protocol for additive gene transfer uses pools of mixed cell clones, which have been maintained under selection for 7 to 10 days

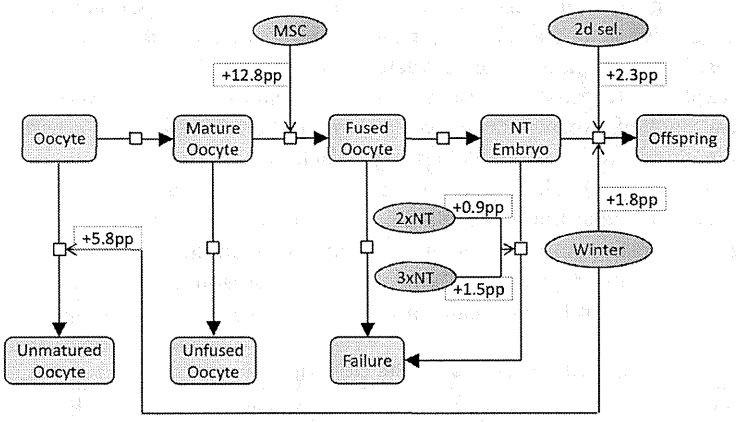

Figure 1 The major statistical effects of the investigated factors on the development from *in vitro* oocyte maturation to cloned offspring. According to the results listed in Tables 3, 4, 5, 6, and 7, transitions between developmental stages (green rectangles) are found to be affected by statistically significant impact categories (purple ellipses). For each impact, the gain during a certain transition is given in percentage points (pp) and as compared to the corresponding reference. For example, the cloning efficiency (offspring out of transferred embryos, including only recipients that delivered) is increased by 1.8 pp in winter (from 3.5% to 5.3%), as compared to spring, which was used as reference. MSC: mesenchymal stem cells, 2d sel.: selected embryos for initiation of development on day 2 (2-cell to 4-cell stage), NT: nuclear transfer.

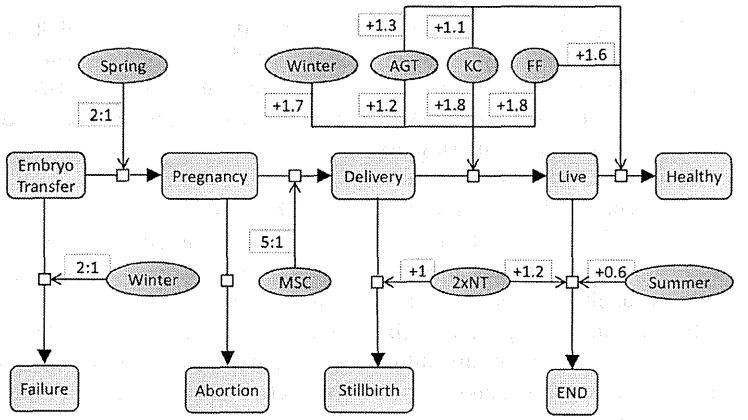

Figure 2 The major statistical effects of the investigated factors on the outcome stage of the recipients (pregnancy/delivery) and the offspring (live/healthy). For each impact, the gain during a certain transition is given in the respective outcome unit. For example, there are on average 1.7 more alive piglets for cloning in winter, as compared to the reference (cloning in spring). MSC: mesenchymal stem cells, AGT: additive gene transfer, KC: kidney cells, FF: fetal fibroblasts, NT: nuclear transfer, END: early neonatal death.

[8,18,41]. It might therefore be expected that extended *in vitro* culture of donor cells required for homologous recombination would negatively influence the cloning efficiency, compared to cells modified by additive gene transfer. However, our data showed no statistically significant difference in cloning efficiency between additive gene transfer, homologous recombination or replication of already existing transgenic pigs. It can be hypothesized that the conditions for transfection and selection did not adversely affect the developmental potential of donor cells, since we kept the passage numbers for SCNT donor cells as low as possible - less than 8 passages for additive gene transfer and less than 10 passages for gene targeting. Additionally, all wild-type primary cell lines used in this study were karyotyped and showed 68% to 90% normal karyotypes.

Interestingly, our analysis indicated that the number of live and healthy offspring was decreased when nuclear donor cells had undergone homologous recombination. However, this may – at least in part – be explained by the fact that 65% of nuclear transfers, designed to generate gene-targeted pigs, were carried out using only 4 particular mesenchymal stem cell lines, which later on turned out to be consistently poor in producing live cloned offspring.

Another important aspect to be considered in the context of genetic modification is the potential for lethal or toxic effects of modifications *per se*. For the experiments involving additive gene transfer this is unlikely, since live cloned piglets expressing the transgenes were obtained with all constructs. Nevertheless, we cannot rule out that cloned fetuses or offspring died due to a detrimental random integration of the construct. Of the gene targeting experiments, only mutation of the X-linked dystrophin (*DMD*) gene in male clones may cause a severe phenotype. In fact, *DMD* mutant male piglets showed severe muscular dystrophy already at birth, and a proportion died shortly later [42]. For all other target genes, the heterozygous knockout had either no specific phenotype or a phenotype that develops later in life.

A critical factor for the establishment of genetically engineered pig lines by SCNT is the viability of the cloned founder animals up to sexual maturity. In our data set, more than half of cloned pigs were stillborn (23.6%) or died soon after birth (31.4%). Associated pathological changes, such as underweight (average weight of the cloned piglets born under 1000 g: 686.4 +/− 181.0 g; range: 375 – 973 g), which is one of the major causes of early neonatal death, or cleft palate, contracted tendons, or enlarged tongues, have also been observed by other groups [43-47]. We have the impression that the percentage of underweight piglets (among normal weight littermates) is higher in cloned litters. However, we cannot prove this observation by statistical data, as the birth weights of naturally bred piglets are not routinely recorded in our facility. The average birth weight of healthy cloned pigs was higher than that of piglets that died in the neonatal period, or that of stillborn piglets (1409.2 +/− 343.1 g, 974.8 +/− 394.1 g, and 1065.5 +/− 479.0 g, respectively). These abnormalities could not be associated with any particular parameter, like donor cell source or genetic modification, and might be a general side effect in pig cloning. Previous studies reported that phenotypically abnormal cloned animals could produce normal offspring [48,49], suggesting that phenotypic abnormalities of the clones were more likely due to epigenetic rather than to genetic alterations.

In our data set, cloned piglets with enlarged tongues were mainly observed in offspring cloned from bone marrow derived mesenchymal stem cells, originating from 4 different animals (25 of 30 cases). However, this does not seem to be a general feature of mesenchymal stem cells, since in more recent cloning experiments with adipose tissue derived mesenchymal stem cells a high proportion of viable offspring without malformations was obtained (T. Flisikowska and A. Schnieke, unpublished data). Some groups reported that mesenchymal stem cells are superior to fibroblasts for SCNT in pigs [50-53], although this has not been generally observed [40,54,55]. Our results did not show any differences in the cloning efficiency among the different cell sources tested, although there was a tendency for a higher pregnancy rate when mesenchymal stem cells were used. The observation that the numbers of live and healthy cloned piglets were significantly lower in the mesenchymal stem cells group than other donor cell sources may be due to the fact that mesenchymal stem cells were only used for gene targeting. Thus, it cannot be distinguished at this stage, whether the low outcome of live and healthy piglets can be attributed to the cell source or type of genetic modification. In addition, different cell lines derived from the same cell source showed a considerable degree of variation in cloning efficiency (Additional file 5).

Re-cloning by using cells from a cloned animal for NT is a reasonable approach for the reproduction of specific transgenic animals, for example if animals of a defined genotype are required for an experiment or if the phenotype hinders natural breeding. However, the majority of studies on re-cloning have demonstrated that additional rounds of cloning lead to a decrease in cloning efficiency [49,56-58]. Our data also showed that repeated cloning rounds significantly decreased cloning efficiency (R1: 4.4%, R2: 3.5% and R3: 2.9%), and the number of live cloned offspring in the second round was in average one piglet less as compared to the initial cloning round. It should be mentioned that the lowest cloning efficiency for R3 may also be related to the high number of embryos transferred in these experiments. Xing et al. [59] recently demonstrated that reduced developmental potential of pig embryos generated by multiple rounds of cloning was associated with altered gene expression patterns, and a previous report stated that the reduction of cloning efficiency with additional rounds of cloning may be caused by accumulation of epigenetic errors [60].

The last factor addressed by our study was *in vitro* culture of cloned embryos and selection for normal development before transfer to recipients. This is possible since the *in vitro* culture systems for pig embryos have been markedly improved within the last decade [27,28]. Indeed, culture of embryos for two days and selection of 2-cell to 4-cell stage embryos for ET resulted in the highest proportion of offspring per SCNT embryos transferred. This suggests that SCNT embryos, which undergo normal cleavage *in vitro* within the expected time frame, have a greater chance of full term development *in vivo*.

Conclusion

We have investigated the influence of important experimental and environmental factors on the cloning outcome in a considerably large data set comprising over 270 porcine nuclear transfer experiments. Besides assessment of the cloning efficiency, we determined the respective steps of the cloning process from oocyte to offspring that are most critically influenced. We observed varying effects of individual factors, depending on the combination with other chosen factors and the parameters tested. Most importantly, more live and healthy offspring were obtained when fetal fibroblasts or kidney cells were modified by additive gene transfer and the resulting SCNT embryos were transferred in the winter period. Although our results cannot be simply extrapolated to other cloning labs, the approach used in this study may help to identify and optimize the specific factors most critical to cloning success in programs aiming to generate genetically engineered pigs.

Methods

Ethics statement

All animal procedures in this study were performed according to the German Animal Welfare Act and to a protocol approved by the Regierung von Oberbayern, under the reference numbers (55.2.1.54-2531-26-06; 55.2.1.54-2531-77-07; 55.2.1.54-2531-78-07; 55.2.1.54-2531-136-07; 55.2.1.54-2531-54-08; 55.2.1.54-2531-86-10; 55.2.1.54-2532-68-11).

Generation of genetically modified pigs

Genetically modified cells derived by transfection of primary cells or established from already existing transgenic pig lines were used as donors. The cells derived from transfection were genetically modified by additive gene transfer (Table 8) or by homologous recombination (Table 9). The latter group included bacterial artificial chromosome (BAC) targeting [7] and the use of classical targeting vectors [5]. The cells re-established from already existing transgenic pig lines were collected from 18 different transgenic pigs. Individual information on all cell lines used for these analyses is shown in the Additional file 6 and Additional file 7.

The following cell sources were used: mesenchymal stem cells, postnatal fibroblasts, fetal fibroblasts, and kidney cells. Mesenchymal stem cells, multi-potent tissue stem cells, as well as fibroblasts and kidney cells are

Table 8 Gene constructs for additive gene transfer

Gene	Promoter	Coding sequence	3'-UTR/pA
hTM	8.9 kb poTM	1.9 kb huTM gene	0.3 kb boGH
CAG-Case12	1.7 kb CAG [61][§]	1.2 kb Case12[a]	0.6 kb raHBB [61][§]
CAG-TA [8][§]	1.7 kb CAG [61][§]	1.0 kb rtTA[b]	0.3 kb boGH
CAG-LEA	1.7 kb CAG [61][§]	1.2 kb LEA29Y[c]	0.6 kb raHBB [61][§]
INS-LEA [62][§]	1.5 kb po INS	1.2 kb LEA29Y[c]	0.3 kb boGH
INS-C94Y [16][§]	2.5 kb po INS fragment including point mutation		
INS-C93S	2.5 kb po INS fragment including point mutation		
INS-TK	1.5 kb po INS	1.1 kb TK[c]	0.3 kb boGH
COL-TK	3.6 kb po COL1A1	1.1 kb TK[c]	0.3 kb boGH
CFTR-LacZ	CH242-248P18	3.5 kb lacZ[d]	0.3 kb boGH
GGTA-LacZ	CH242-21 F3	3.5 kb lacZ[d]	0.3 kb boGH
TRE-RANKL [8][§]	0.3 kb TRE[a]	0.9 kb po sRANKL	0.3 kb boGH
TRE-CTLA-4Ig [8][§]	0.3 kb TRE[a]	1.2 kb po CTLA4-Ig	0.3 kb boGH
HAC1 [63][§]	0.6 kb CMV	0.7 kb GFP	0.3 kb SV40

[§] See indicated references.
[a] Purchased from Evrogen, Moscow, Russia.
[b] Purchased from Clontech, Mountain View, CA.
[c] Custom-synthesized by Bio&Sell, Feucht, Germany.
[d] Purchased from Promega, Madison, WI.

already known to support full term development when used as donor cells in pig SCNT [21,23,41,53,64-67]. Briefly, mesenchymal stem cells from bone marrow were isolated from femurs and tibias of 6 to 7 months old Landrace x Pietrain pigs [64,68]. Fetal fibroblasts, postnatal fibroblasts and kidney cells originated from German Landrace, Swabian-Hall pig and crossbreeds of them [41]. Fetal fibroblasts were isolated from fetuses at day 27 and day 54, while postnatal fibroblast and kidney cells were from 1 day up to 3 months old piglets. The gender of all cell lines was male, except for one of the *GGTA1*$^{-/-}$ CD46 cell lines, which was female. Donor cells were isolated by standard methods mainly using collagenase II or trypsin/EDTA [41]. For details of transfection and characterization of *de novo* modified cells see references [7,8,41,64]. Cells were used for SCNT at passage 6–8 after additive gene transfer, passage 6–10 after homologous recombination, and passage 2–8 from re-established transgenic pig lines. 48 h prior to the SCNT experiment, donor cells were starved (0.5% FCS) for synchronization of donor cell cycle at G0/G1. All SCNT experiments included in this analysis were performed in the same laboratory by the same operators for micro-manipulation, using *in vitro* matured (IVM) oocytes, as previously reported [67].

Table 9 Target genes for homologous recombination

Target gene	Vector	Modification
CFTR [7][§]	CH242-248P18 (>100 kb)	ATG-STOP
DMD [42][§]	CH242-9G11 (>100 kb)	Δ exon 52
GGTA1	CH242-21 F3 (>100 kb)	ATG-STOP
APC [64][§]	12.5 kb	STOP
KRAS	13.5 kb	Point mutation
JAK3	13.8 kb	Δ exon 2-5

[§] See indicated references.

Up to three rounds of cloning (use of donor cells derived from an already cloned animal for a further round of SCNT) were performed for the generation and replication of multi-transgenic pigs. Specifically, one round of cloning was used for generating transgenic founder animals from transfected wild-type cells, or for replicating offspring of transgenic founder pigs. The second round of cloning involved donor cells from transgenic cloned pigs which were transfected with an additional construct or simply the replication of transgenic cloned pigs. In the third round, cloning was the re-cloning of transgenic pigs that had received an additional construct during the second round of cloning (for the individual information in each cell lines, see Additional file 6 and Additional file 7).

Fused reconstructed embryos were either directly transferred to recipients on the same day (no selection), or cultured *in vitro* and then selected for initiation of development on day 1 (1-cell stage) or day 2 (2-cell to 4-cell stage) after activation before embryo transfer.

Gilts of the breeds German Landrace, Swabian-Hall, and crossbreeds of them were used as recipients. Estrus was synchronized by oral administration of 4 ml Altrenogest (Regumate®) for 15 days, followed by intramuscular injection of 750 IU ECG (Intergonan®) and 750 IU HCG (Ovogest®) 24 h and 104 h later, respectively. ET was performed laparoscopically into one oviduct

[69]. Pregnancy was confirmed by ultrasonographic examination on day 21, repeated every 2 – 3 weeks.

Data description

The analysis is based on data from cloning experiments, performed in the period from April 2008 to February 2011, at the Chair for Molecular Animal Breeding and Biotechnology in Munich, Germany. The location is situated at an altitude of 444 m, and at latitude and longitude of 48°22'N and 11°49'E, respectively.

Changes in the experimental setup, described in the previous section, included variations of the season the ET was performed in, the type of genetic modification, the donor cell source, the number of cloning rounds, and selection of SCNT embryos for development before transfer to the recipient. The stratification and distribution of each varied factor is summarized in Table 2.

1. *Season:* Experiments were performed covering the whole year range, i.e. an approximately balanced sample size in each season – spring (March-May), summer (June-August), autumn (September-November) and winter (December-February) – was ensured. However, 10% more experiments were performed in summer and autumn. The average temperature in each season was 9.6°C, 18°C, 9.2°C, 0.1°C, respectively (http://www.dwd.de).
2. *Type of genetic modification:* Genetically modified cells were derived in roughly 30% of all experiments by additive gene transfer, in 25% of the experiments by homologous recombination, and in most cases (45%) established from transgenic pigs.
3. *Donor cell source:* Regarding the source of nuclear donor cells, most of the experiments were performed with kidney cells (43%), followed by fetal fibroblasts (26%), mesenchymal stem cells (19%), and postnatal fibroblasts (12%).
4. *Number of cloning rounds:* The vast majority of all cloning experiments were carried out with one round of cloning (57%), one third (32%) with two rounds, and the remaining experiments (11%) with three rounds of cloning.
5. *Selection of SCNT embryos for initiation of development:* In 23% of all experiments, all SCNT embryos were transferred to recipients on the same day on which the nuclear transfer was carried out (no selection for development). In other experiments, the SCNT embryos were cultured either 1 day (7%) or 2 days (8%) after activation and selected for initiation of normal development (1-cell stage on day 1, 2-cell to 4-cell stage on day 2). In most of the cases (62%) mixed populations of SCNT embryos (no selection, 1 day culture, 2 days culture) were transferred to the recipients. Those were not included in the analysis of this specific factor.

Cloning benchmarks

The success of each cloning experiment was progressively assessed based on the outcome of distinct evaluation stages. After the cloned embryos were transferred to the recipient, we first determined whether it became pregnant or not.

For a sample stratum under investigation, the *chance for pregnancy* is hence defined as the probability ratio

$$P(\text{pregnancy} = \text{YES}) \,/\, P(\text{pregnancy} = \text{NO}) \qquad (1)$$

The probabilities result from the relative frequencies of the corresponding event in the stratum.

Analogously, the *chance for delivery* is defined as

$$P(\text{delivery} = \text{YES}) \,/\, P(\text{delivery} = \text{NO}) \qquad (2)$$

For delivering recipients, we counted the number of offspring born, the number of live offspring among them, and if there were any, the number of healthy offspring.

In addition, we calculated for the experiments resulting in at least one delivered offspring the *cloning efficiency* as

$$\text{delivered cloned offspring/SCNT embryos transferred} \qquad (3)$$

As a benchmark for oocyte and donor cell quality, respectively, we also took the *oocyte maturation rate,* calculated as

$$\text{successfully matured oocytes/oocytes entering IVM,} \qquad (4)$$

and the *fusion rate,* calculated as

$$\text{successfully fused karyoplast} - \text{cytoplast complexes/complexes submitted to electrofusion,} \qquad (5)$$

into account.

Statistical analysis

Generalized linear models [70] were computed for each experimental factor (season, genetic modification, cell source, cloning rounds and SCNT embryo selection) in order to estimate its impact on each cloning outcome stage (pregnancy and delivery rate as well as numbers of total, live, and healthy offspring) and the cloning efficiency.

As all explaining variables, i.e. the experimental factors, are categorial, we designed the linear predictor of the regression models using indicator (dummy) variables

[71], yielding effects with respect to the correspondingly chosen reference category (*spring* for season, *additive gene transfer* for genetic modification, *mesenchymal stem cells* for cell source, *one round* for cloning rounds, and *no culture* for SCNT embryo selection). This design corresponds to an ANOVA model [72], where the sample mean of each stratum of the experimental factor under investigation is tested for deviation from the sample mean of the reference category assuming the sample means to be *t*-distributed. Consequently, all *p*-values reported here are *t*-test [73] derived, and should, thus, be interpreted as a statistical significance measure for equality of means, i.e. the lower the *p*-value, the more significant is the difference in the means. The link function of the regression models was selected according to the goodness of fit between the empirical distribution of the response (outcome) variable and the corresponding common distribution. Briefly, logistic regression was carried out for the binary factors (pregnancy and delivery), Poisson regression for the counts of live and healthy offspring, and Gaussian regression for the cloning efficiency (as well as for maturation and fusion rate).

Additional files

Additional file 1: Correlation of the number of embryos transferred with pregnancy rate. The absolute number of embryo transfers (left *y*-axis) that resulted in pregnancy of the recipient depending on the number of embryos transferred (*x*-axis) is shown in black over the number of all observations in grey. The red curve indicates the overall pregnancy rate (right *y*-axis) when more than *x* embryos have been transferred.

Additional file 2: Correlation of the number of embryos transferred with the number of live piglets. The number of transferred embryos is shown on the *x*-axis and the number of live piglets on the *y*-axis. No visible correlation can be detected (Pearson correlation 0.2).

Additional file 3: Seasonal distribution of specific SCNT configurations with respect to genetic modification, cell type and cloning round. For each season on the *x*-axis, the bar height denotes the total number of embryo transfers performed (as indicated on the *y*-axis). The three vertical slots in each of the bars correspond to the distribution of the respective categories of genetic modification (gen. mod), cell type (cell.type), and cloning rounds (clon.rds). The categories are alphanumerically encoded as denoted at the top: genetic modification = (1 = homologous recombination (HR), 2 = additive gene transfer (AGT), 3 = replication of transgenic pigs (replic. of tg pigs)), cell type = (1 = mesenchymal stem cells (MSC), 2 = postnatal fibroblasts (PF), 3 = fetal fibroblasts (FF), and 4 = kidney cells (KC)), cloning rounds = (1 = 1 round, 2 = 2rounds, 3 = 3rounds).

Additional file 4: Distribution of selected embryos derived from specific SCNT configurations with respect to genetic modification, cell type and cloning round. For a particular selection timing on the *x*-axis, the bar height denotes the total number of embryo transfers performed (as indicated on the *y*-axis). The three vertical slots in each of the bars correspond to the distribution of the respective categories of genetic modification (gen.mod), cell type (cell.type), and cloning rounds (clon.rds). The categories are alphanumerically encoded as denoted at the top: genetic modification = (1 = homologous recombination (HR), 2 = additive gene transfer (AGT), 3 = replication of transgenic pigs (replic. of tg pigs)), cell type = (1 = mesenchymal stem cells (MSC), 2 = postnatal fibroblasts (PF), 3 = fetal fibroblasts (FF), and 4 = kidney cells (KC)), cloning rounds = (1 = 1 round, 2 = 2 rounds, 3 = 3 rounds). Data for mixed selection timing not shown.

Additional file 5: Degree of variation in cloning efficiency within cell types. The variation in cloning efficiency on the *y*-axis is shown for the different cell lines within the four cell type categories (MSC: mesenchymal stem cells, FF: fetal fibroblasts, PF: postnatal fibroblasts, and KC: kidney cells). The numbers in brackets on the *x*-axis denote the number of embryo transfers (in total and for the corresponding fraction that delivered offspring, respectively). Details on the cell lines used can be found in Additional file 6 and Additional file 7.

Additional file 6: List of *de novo* modified cell lines by additive gene transfer or homologous recombination.

Additional file 7: List of transgenic cell lines from already existing transgenic pig.

Abbreviations

BAC: Bacterial artificial chromosome; ET: Embryo transfer; IVM: *In vitro* maturation; SCNT: Somatic cell nuclear transfer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MK, LG, RZ and EW conceived and designed the study. MK, BK, VZ, NK, AW, AR, AB, KK, KB, KF, TF, CM, TL, MD, AT, SK, DS, HN, AS, EW were involved in somatic cell nuclear transfer experiments. LG, MK, TP, RZ, EW analyzed the data and MK, LG, AK, RZ, EW drafted the manuscript. All authors read and approved the final manuscript.

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