Modeling Amyotrophic Lateral Sclerosis in hSOD1\textsuperscript{G93A} Transgenic Swine

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Key Words
Animal model · Swine · Amyotrophic lateral sclerosis · Somatic cell nuclear transfer · Cu/Zn superoxide dismutase 1 · SOD1\textsuperscript{G93A} · Pig

Abstract
Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that occurs in two clinically indistinguishable forms: sporadic (SALS) and familial (FALS), the latter linked to several gene mutations, mostly inheritable in a dominant manner. Nearly 20\% of FALS forms are linked to mutations in the Cu/Zn superoxide dismutase (SOD1) gene. Research on ALS relies on transgenic models and particularly on mice carrying a glycine-to-alanine conversion at the 93rd codon (G93A) of the hSOD1 gene. Although G93A transgenic mice have been widely employed in clinical trials and basic research, doubts have been recently raised from numerous reliable sources about their suitability to faithfully reproduce human disease. Besides, the scientific community has already foreseen swine as an attractive and alternative model to nonhuman primates for modeling human diseases due to closer anatomical, physiological and biochemical features of swine rather than rodents to humans. On this basis, we have produced the first swine ALS model by in vitro transfection of cultured somatic cells combined with somatic cell nuclear transfer (SCNT). To achieve this goal we developed a SOD1\textsuperscript{G93A} (superoxide dismutase 1 mutated in Gly93-Ala) vector, capable of promoting a high and stable transgene expression in primary porcine adult male fibroblasts (PAF). After transfection, clonal selection and transgene expression level assessment, selected SOD1\textsuperscript{G93A} PAF colonies were used as nuclei donors in SCNT procedures. SOD1\textsuperscript{G93A} embryos were transferred in recipient sows, and pregnancies developed to term. A total of 5 piglets survived artificial hand raising and weaning and developed normally, reaching adulthood. Preliminary analysis revealed transgene integration and hSOD1\textsuperscript{G93A} expression in swine tissues and 360° phenotypical characterization is ongoing. We believe that our SOD1\textsuperscript{G93A} swine would provide an essential bridge between the fundamental work done in rodent models and the reality of treating ALS.

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Introduction

Amyotrophic lateral sclerosis (ALS) is characterized by selective and progressive degeneration of upper and lower motor neurons, leading to muscle weakness and atrophy and evolving to complete paralysis. The disease impact on patients’ life quality is devastating and death occurs in 2–5 years [1]. ALS occurs in two forms: familial (FALS) and sporadic (SALS). FALS accounts for 5–10% of ALS cases and is related to several gene mutations, mostly inheritable in a dominant manner. Nearly 20% of FALS forms are linked to more than 100 mutations in the Cu/Zn superoxide dismutase (SOD1) gene, all showing dominant hereditary patterns [2, 3].

Research on ALS mainly relies on experimental animal models like transgenic rats and mice expressing mutant human SOD1 isoforms. Besides rodents, a variety of animal species such as zebrafish [4], Caenorhabditis elegans [5] and Drosophila melanogaster [6] has already been employed to unravel ALS pathogenetic mechanisms, although phylogenetic distance from human species prevents their employment in clinical research. Currently, the most widely employed model is a transgenic mouse with a glycine-to-alanine conversion at the 93rd codon (G93A) of the SOD1 gene. These mice reliably reproduce the ALS patient’s phenotype progression, developing a rapidly progressive motor neuron degeneration characterized by hind limb weakness that evolves to hyperreflexia, paralysis and premature death 4 months after symptom onset [7]. These ALS models have certainly facilitated investigations on the selective vulnerability of motor neurons. However, doubts have been recently raised by numerous reliable sources about the suitability of rodents to reliably reproduce the human disease [8, 9].

Since human and rodent species differ in life span, physiology, anatomy and biochemical aspects, data extrapolation may be difficult. As a matter of fact, encouraging results of drug tests in rodents have not been successfully translated to humans to date [10, 11]. While in some cases molecules delaying disease progression in transgenic mice, such as minocycline, have resulted in being even detrimental in ALS patients [12]. Given the limitations of the ALS rodents, a more homologous animal model should be created in an animal evolutionarily closer to the human species in order to provide a better tool to study the disease.

The scientific community has already accepted domestic and miniature swine as an attractive animal model for pharmacological and surgical testing as well as for research on a variety of diseases such as cystic fibrosis, type 1 diabetes, vascular disorders and multiple sclerosis [13, 14]. The emerging role of swine in biomedical research is based on its anatomical, physiological and biochemical features that are more closely related to the human species than the rodent ones. Furthermore, the prospect of obtaining genetically modified pigs further extended their biomedical potential, particularly that of mimicking inherited human diseases. Swine are ideal for this type of genetic research as the size and composition of the porcine genome is similar to that of humans [15].

Particularly with regard to CNS anatomy, the pig brain cortical surface resembles the human gyrencephalic neocortex and similarities with the human brain have also been demonstrated for the hippocampus, subcortical and diencephalic nuclei and brainstem structures. Adult pig brain weight ranges from 80 to 180 g, depending on adult body size, breed and duration of domestication, and it is comparable to the mass of several nonhuman primate species employed for experimental purposes.

As far as neurodegenerative diseases are concerned, the potential of murine models is limited because of their small-sized brain and difficulty in behavioral testing [16]. On the contrary, pig models exhibit physiological and anatomical characteristics similar to human ones and particular emphasis has been placed on swine potential for modeling symptoms and phenomena of neuropsychiatric diseases [17].

Furthermore, pig brain size permits an easier (compared with rodent models) identification of cortical and subcortical structures by conventional imaging techniques such as MRI and PET and offers invaluable opportunities for microsurgical techniques and intrathecal drug administration. Consistently, the large size and long lifespan of swine allow the selection of numerous and repetitive samplings from the same animal, thus enabling a higher amount of data to be collected to characterize in detail preclinical and clinical phases. The longer life span also makes swine a suitable animal model for long-term evaluation of safety and efficacy of innovative therapies.

Several swine models for neurodegenerative disease have already been produced. Bjarkam et al. [18] succeeded in producing an MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) Parkinson disease pig model, while other research groups developed transgenic pig models for Alzheimer’s disease [19], spinal muscular atrophy [20] and Huntington disease [21]. Here we report on the production of 5 healthy transgenic Yucatan minipigs expressing the hSOD1G93A transgene.

Modeling ALS in Swine

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Materials and Methods

Animal Experiments
All procedures involving animals and their care were conducted in conformity with national and international laws and policies (EEC Council Directive 86/609, OJL358, 1, 12 December 1987, Italian Legislative Decree 116/92, Gazzetta Ufficiale della Repubblica Italiana 10, 18 February 1992, and Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996) and after the approval of LTR-Avantea local ethical committee.

Vector Construction
We have previously developed a ubiquitous enhanced green fluorescent protein expression vector, driven by the pCAGGS hybrid promoter (CMV-IE enhancer + chicken β-actin promoter) [22], which is characterized by maintaining a high expression level throughout the next generation of pigs [23]. We created a destination vector pMGOrfA5′3′MARpuro5171, inserting the Multi-site Gateway system (Invitrogen) conversion cassette (OrfA) into the ubiquitous expression vector. The resulting vector carried the pCAGGS promoter inserted between two insulators (5′ MAR of chicken lysozyme gene) [24] to prevent various silencing effects (positional or copy number effects). The structure was also provided with a floxed, then removable recombination using the Cre recombinease, SV40-Puro cassette to select the transfected clones. We inserted the BamHI/Xhol fragment of hSOD1 G93A cDNA obtained by restriction of pcDNA3.0hSOD1 G93A plasmid into the pENTRL1L2OligoSacI site vector, obtaining pENTRL1L2-hSOD1 G93A.

The Sall-BamHI fragment was removed from the construct and the resulting pENTRL1L2-hSOD1 G93A delSB was used, after sequencing, in an LR exchange reaction with the destination vector pMGOrfA5′3′MARpuro5171. This exchange reaction, mediated by the LR Clonase, was used to transform chemically competent Escherichia coli cells (One Shot Mach1; Invitrogen). The resulting pMG5′3′MARpuro5171-hSOD1 G93A vector was purified with Plasmid Mini Kit (Qiagen, Hilden, Germany), analyzed by different restriction enzymes, confirmed by sequencing and finally linearized by ApaLI (Fermentas). After phenol/chloroform purification, the vector was precipitated and resuspended in Tris-EDTA buffer. Analog cloning procedures were performed to obtain the pMG5′3′MARpuro5171-hSOD1 wild-type vector.

Cell Isolation and Culture
Primary porcine fibroblast cultures were derived from adult Yucatan (miniature swine) male ear biopsies (pig adult fibroblasts, PAFs). Biopsy specimens were cut in small pieces by scalpel blade and resulting tissue pieces were distributed on the surface of gelatin-coated dishes containing 1.5 ml of DMEM/TCM199 with 20% of fetal bovine serum (FBS). The culture medium was changed every 3 days. Cells were allowed to grow until they reached 50% of confluence. Tissue pieces were then removed and the cells were subcultured until they reached confluence in DMEM/TCM199 with 10% FBS. Growth conditions consisted of a 38.5°C temperature and of an atmosphere composed of 90% N2, 5% O2 and 5% CO2. Exponentially growing cultures were cryopreserved in DMEM/TCM199 with 20% FBS and 10% DMSO and stored in liquid nitrogen. These batches of cells were used throughout the following experiments.

Transfection of hSOD1 G93A Vector into Fibroblasts
The day before transfection, passage 3 PAFs were trypsinized, counted and plated into 60-mm dishes in order to obtain about 1×106 cells at 80% confluence in 24 h. On the transfection day, cells were trypsinized, counted and resuspended in 100 μl of Nucleofector solution (Basic Nucleofector Kit, Primary Fibroblasts; Amaxa, Cologne, Germany), mixed with 5 μg of the linearized pMG5′3′MARpuro5171-hSOD1 G93A vector. PAF and linearized vector were then transfected into the nucleus of HIVontue vector by SCNT was performed (Nucleofector; Amaxa). The nucleofection cells were then plated in 60-mm culture dishes containing fresh culture medium. After 24 h, the drug (puromycin: 1 μg/ml) employed in colony selection was added. After 4 days, puromycin-resistant colonies were picked up using 5-mm cloning discs and transferred into 24-well dishes. Cells were then expanded in DMEM/TCM199 with 10% FBS and 5 ng/ml of bFGF at a temperature of 38.5°C and in a humidified atmosphere containing 5% CO2 and 5% O2. An aliquot was cryopreserved, as described above, to be employed in nuclear transfer and the remaining cells were subcultured to perform expression analysis.

Immunocytochemistry
Cells (transgenic hSOD1 G93A PAF cell clones and primary cultures from ear biopsy obtained from transgenic piglets generated by SCNT) were fixed with 4% paraformaldehyde and then stored in phosphate buffer until analysis.

We used a rabbit polyclonal antibody (07–403 Millipore, concentration 1:200), directed against the full-length wild-type hSOD1 (positions or copy number effects). The structure was also provided with a floxed, then removable recombination using the Cre recombinease, SV40-Puro cassette to select the transfected clones. We inserted the BamHI/Xhol fragment of hSOD1 G93A cDNA obtained by restriction of pcDNA3.0hSOD1 G93A plasmid into the pENTRL1L2OligoSacI SalI vector, obtaining pENTRL1L2-hSOD1 G93A.

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Preparation of Nuclear Donor Cells and SCNT
Twelve transgenic hSOD1 G93A fibroblasts clones were selected as nuclei donors, according to uniformity and intensity of their expression level. Cells were prepared and employed in SCNT procedures as described in Lagutina et al. [25] and Brunetti et al. [23].

Synchronization of Sows, Surgical Embryo Transfer, Pregnancy Diagnosis and Farrowing
In order to synchronize sow estrus, the animals were given 12 mg of alternogest (Regumate; Intervet, Peschiera Borromeo, Italy)
for 15 days. On the 15th day of Regumate administration the animals were treated with 0.15 mg of prostaglandin F2α (Dalmazin; Fatro, Ozzano Emilia, Italy) and finally, 96 h after the last Regumate administration, the sows were injected with 1,000 IU of human chorionic gonadotropin (Chorulon; Intervet).

On the 4th day after sow ovulation, SCNT blastocysts on day 5 of development were transplanted by midventral laparotomy to the uterus of the animals. The sows were then examined by ultrasound D-scanning on days 29, 36, 50 and 62 in order to assess pregnancy. A cesarean delivery was performed on day 114 of gestation. Because of their small size the piglets had to be hand-raised with reconstituted artificial milk.

Table 1. List of PAF clones transfected with pMG5′3′MARPuro5171-hSOD1G93A vector

<table>
<thead>
<tr>
<th>Clone</th>
<th>Score</th>
<th>Clone</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1</td>
<td>3</td>
<td>1D6</td>
<td>5</td>
</tr>
<tr>
<td>1A2</td>
<td>6</td>
<td>1E1</td>
<td>6</td>
</tr>
<tr>
<td>1A4</td>
<td>1</td>
<td>1E2</td>
<td>4</td>
</tr>
<tr>
<td>1A5</td>
<td>5</td>
<td>2A1</td>
<td>6</td>
</tr>
<tr>
<td>1B1</td>
<td>3</td>
<td>2A2</td>
<td>6</td>
</tr>
<tr>
<td>1B3</td>
<td>4</td>
<td>2A3</td>
<td>5</td>
</tr>
<tr>
<td>1B6</td>
<td>6</td>
<td>2A4</td>
<td>1</td>
</tr>
<tr>
<td>1C1</td>
<td>1</td>
<td>2A5</td>
<td>4</td>
</tr>
<tr>
<td>1C2</td>
<td>2</td>
<td>2A6</td>
<td>4</td>
</tr>
<tr>
<td>1C6</td>
<td>4</td>
<td>2B2</td>
<td>4</td>
</tr>
<tr>
<td>1D1</td>
<td>5</td>
<td>2C1</td>
<td>5</td>
</tr>
<tr>
<td>1D2</td>
<td>1</td>
<td>2C2</td>
<td>5</td>
</tr>
<tr>
<td>1D5</td>
<td>4</td>
<td>2C3</td>
<td>4</td>
</tr>
</tbody>
</table>

A score indicating the transgenic protein expression level, compared to the positive control one (score = 2), was assigned to each clone.

Western Blot

Cells (transgenic hSOD1G93A:PAF cell clones and primary cultures from ear biopsy obtained from transgenic piglets generated by SCNT) were lysed using Laemmli buffer 1× containing β-mercaptoethanol (5%) and boiled for 10 min. Total protein was quantified by Qubit fluorometer (Invitrogen) using the Quant-iT Protein quantification kit (Invitrogen).

Tissues obtained from stillborn piglets were homogenized (1:5 mg/ml) in lysis buffer [50 mM Tris HCl pH8, 150 mM NaCl, 5 mM EGTA pH8, 1.5 mM MgCl2, 10% anhydrous glycerol, 1% Triton, 100 μg/ml (= 0.57 mM) PMSF]. Protein quantification was performed by BCA Protein Assay kit (Pierce). 13 μg of each sample were loaded onto glycine-SDS-PAGE (4–12%) and electrophoretically separated for 45 min at 200 V using the Mini-Protean II...
chamber (Biorad). Blotting onto Immun-Blot PVDF membrane (Bio-Rad) was obtained after 1 h at 100 V using Mini-Protean II electroblotter (Biorad) according to the manufacturer’s protocol. The resulting PVDF membranes were subsequently processed following the technical specifications of the chemiluminescent detection system Lumi-Light PLUS Western Blotting Kit Mouse/Rabbit (Roche). Detection of transgenic target was achieved using a polyclonal antibody 07-403 (1:1,000; Millipore). Pig β-actin expression was detected using the mAb ab6276 (1:5,000; Abcam). The treated membranes were finally exposed, developed and fixed.

S o u t h e r n  B l o t
SOD1-DIG probes labeled with digoxigenin were obtained using the DIG DNA Labeling and Detection kit (Roche). Briefly, 25 μl of reaction mixture [1.25 mM MgCl2, 1 μM forward primer (ATGGACCAGTGAAGGTGTGGG), 1 μM reverse primer (AGTGTGCGGCCAATGATGC), 0.8 mM dNTPs-DIG, buffer 1×, Taq 1 unit] were added to 5 μl of DNA and then used as template for amplification cycles [once at 94 °C for 2 s; 35 × (denaturation at 94 °C for 30 s; annealing at 65 °C for 30 s; elongation at 72 °C for 20 s); once at 72 °C for 7 min].

Probes were then precipitated with absolute ethanol and finally resuspended in sterile ultrafiltered water. 10 μg of genomic DNA, extracted from hSOD1 G93A swine, were subjected to restriction analysis with SalI + BglII (10U/μgDNA) enzymes and incubated overnight at 37 °C. On the next day, samples were precipitated by centrifugation (10 min, 16,100 g) with 0.9 volumes of isopropanol and washed with 500 μl of 70% ethanol (5 min, 16,100 g). After ethanol removal, pellets were air dried, resuspended in Tris-EDTA buffer and eventually subjected to electrophoresis migration overnight at 15 V plus 2 h 30 min at 25 V in 0.7% agarose gel. DNA samples were then transferred by capillarity on modified nylon membrane (Hybond-N+, Amersham) under denaturing conditions (NaOH 0.4 N, 5 h). The membranes thus obtained were hybridized with the probe SOD-DIG (20 ng/ml) at 42°C overnight and processed the next day (blocking of nonspecific binding, incubation with antidigoxigenin secondary antibody conjugated with alkaline phosphatase and with chemiluminescent substrate and signal detection) following the technical specifications of DIG Luminescent Detection Kit (Roche). Finally, membranes were used to impress (3 h) photographic plates (BioMax; Kodak) that were subsequently developed (5 min) and fixed (10 min) in a darkroom using GBX (Kodak) chemical reagents.

Re s u l t s
Transfection of hSOD1G93A Vectors into Fibroblasts and Related Analysis
The transfection experiments conducted with the pMG5’3’3MARpuro-hSOD1 G93A vector have led to the isolation of 26 PAF clones, which were analyzed by Western blot and ICC. All clones, summarized in table 1, revealed transgene expression and were assigned a score: 4 clones showed a transgene expression level lower than the control one, and 3 clones achieved the same score as the control. The remaining 19 clones showed a higher expression level (scores ranging from 3 to 6).

Somatic Cell Nuclear Transfer
Six cloning experiments were conducted. In the first and second experiments, a pool of clones (1A1, 1C2, 1D2 and 1E2) was used as a nucleus donor. In the third and fourth experiments a pool composed of 1B1, 1D1, 2C1 and 2B2 clones was employed, while a pool composed of 1A2, 2A6 and 2C3 clones was employed in the fifth and sixth experiments. All the SCNT experiments conducted are listed in table 2 where preimplantation em-

<table>
<thead>
<tr>
<th>ID clones</th>
<th>SCNT, n</th>
<th>SCNT, N</th>
<th>Cl</th>
<th>%</th>
<th>MC/BL (day 6)</th>
<th>Tot embryo</th>
<th>%</th>
<th>Piglets vital/stillborn</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1, 1E2, 1C2, 1D2</td>
<td>1</td>
<td>195</td>
<td>181</td>
<td>94.66</td>
<td>8</td>
<td>61</td>
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<td>35.38</td>
</tr>
<tr>
<td>1A1, 1E2, 1C2, 1D2</td>
<td>2</td>
<td>204</td>
<td>185</td>
<td>92.82</td>
<td>11</td>
<td>63</td>
<td>74</td>
<td>36.27</td>
</tr>
<tr>
<td>1B1, 2B2, 2C1, 1D1</td>
<td>3</td>
<td>200</td>
<td>172</td>
<td>86.00</td>
<td>21</td>
<td>54</td>
<td>75</td>
<td>37.50</td>
</tr>
<tr>
<td>1B1, 2B2, 2C1, 1D1</td>
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<td>47</td>
<td>78</td>
<td>37.32</td>
</tr>
<tr>
<td>1A2, 2A6, 2A2, 2C3</td>
<td>5</td>
<td>205</td>
<td>179</td>
<td>87.32</td>
<td>96</td>
<td>96</td>
<td>46.83</td>
<td>3/1</td>
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<tr>
<td>1A2, 2A6, 2A2, 2C3</td>
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<td>202</td>
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<td>88.12</td>
<td>102</td>
<td>102</td>
<td>50.50</td>
<td>2/7</td>
</tr>
</tbody>
</table>

The ‘ID clones’ column shows the names of hSOD1 G93A PAF colonies that composed the cell pool used as nuclei donors. In the ‘SCNT’ columns, n is the number of SCNT experiments and N is the number of pairs of cytoplasts/PAFs formed after the fusion. ‘Cl’ is the number of segmented embryos and its value is compared to the total percentage of reconstructed embryos. ‘MC/BL’ indicates the number of compact morulae and blastocysts on the 6th day of in vitro culture. ‘Tot embryo’ indicates the number of viable embryos, with the relative percentage value obtained by comparison with the initial number of manipulated oocytes. ‘Piglets’ indicates the pregnancy outcome, with the number of vital and stillborn piglets obtained. MC = Compact morulae; BL = blastocysts.
bryo development is reported (ranging from 35.38 to 50.50%). These results are in line with those obtained from similar experiments conducted with other transgenes (data not shown).

**Development to Term of SCNT Embryos Derived from Adult hSOD1<sup>G93A</sup> Fibroblasts**

The transfer of 494 embryos to 6 recipient sows resulted in 4 pregnancies. The 4 pregnant sows received 75, 78, 96 and 109 embryos obtained from the third, fourth, fifth and sixth SCNT experiments, respectively. Pregnancies were developed to term and resulted in the birth of 10, 6, 4 and 9 piglets with a mean efficiency of blastocyst development to term of 8.78%. Among the produced piglets, 16 were viable and 13 were stillborn. 5 piglets, identified by an ear tag carrying the animal identification numbers 168, 173, 174, 204 and 205, survived and developed normally. The remaining 11 piglets died within 48–96 h from birth due to events commonly reported in commercial herds (i.e. some piglets did not survive hand-rearing, while others developed diarrhea, pneumonia, etc.).

**Detection of hSOD1<sup>G93A</sup> Expression in Tissues from Stillborn Piglets and Living Animals**

Fibroblasts obtained from ear biopsies of both alive and stillborn piglets were analyzed by ICC and revealed a transgene expression level comparable with that of PAFs used as nuclei donors. Figure 2 displays ICC on ear fibroblasts of the 5 pigs that reached adulthood.

Furthermore, Western and Southern blot analyses were performed on dead and stillborn piglets tissues. Snap-shot spinal cord samples were homogenized and analyzed by Western blot. Transgenic protein was revealed using the 07-403 Millipore antibody. In figure 3 it is possible to recognize two lanes, corresponding to the two SOD1 isoforms: the endogenous swine protein displaying a lower molecular weight (16 kDa) and the human transgenic one, with a higher molecular weight (20 kDa). In order to establish the integration pattern of the construct containing the ALS-related human SOD1<sup>G93A</sup>-mutated protein in the swine genome, Southern blot analysis was performed on DNA extracted from all piglets obtained during the project. In particular, figure 4 shows the results obtained on DNA from piglets born after the third and fourth embryo transfer experiments revealing 4 different integration patterns, corresponding to the 4 different PAF-SOD1<sup>G93A</sup> colonies amongst those employed as nuclei donors. Similarly figure 5 shows the results obtained on DNA from piglets born after the fifth and sixth embryo transfer experiments.

### Discussion

Anatomical and physiological swine features, so closely related to human ones, and the progress in pig genomics and proteomics attribute to this animal species an emerging role in biomedical research [26].

In vitro transfection of cultured somatic cells combined with SCNT has become the first-choice method to generate large transgenic animals. SCNT is advantageous since all founder animals are transgenic and the transgenic protein level is predictable to some extent during in vitro culture, which allows the expansion of sufficient numbers of cells for analysis aimed to select clones with the best transgene expression pattern.

The vectors that we used to produce hSOD1<sup>G93A</sup> transgenic pigs proved to be very effective due to the puromycin selection cassette (allowing us to select cells with the desired transgene expression level in a short time – 5 days), to the matrix attachments regions employment.
consenting to reduce transgene expression variability and to the pCAGGS promoter allowing to obtain an ubiquitous expression.

As this is the first hSOD1\textsuperscript{G93A} swine model produced so far, no data are available on the toxicity related to transgene expression levels in the early stage of porcine embryonic development and after embryo transfer in sows. For this reason we decided to use in SCNT experiments a pool of donor cells showing different transgene expression levels in order to minimize the risk of embryonic or fetal mortality of stillborn piglets. As a consequence, founder animals present variable integration patterns, ranging from 1 to 5 integration sites. However, site number does not determine hSOD1\textsuperscript{G93A} protein expression level, as integration randomly occurs in the genome and the vector could be integrated in rarely transcribed regions. Semen obtained from the boar displaying a single integration site (pig 168) has already been suc-
cessfully employed in artificial insemination procedures and we succeeded in inseminating a wild-type sow and in establishing a pregnancy, confirming the fertility of the founder animals.

Unlike rodent models that show an extremely high transgene expression level and a rapid disease course [7], our swine model presents an hSOD1\textsuperscript{G93A} expression level comparable to that of the endogenous swine protein. However, since an hSOD1\textsuperscript{G93A} swine model has never been produced before, no data are available on the correlation between the transgene expression level and the onset of disease, and we can only make rough estimates as to when the first neurological symptoms may occur. Since Yucatan swine live up to 13–15 years, pigs expressing a high hSOD1\textsuperscript{G93A} level are expected to show ALS symptoms in 3–4 years, while we cannot exclude that swine with a lower transgene expression could display clinical signs after a longer period.

On one hand, this could result in a longer preclinical phase and in an increase of animal-maintaining costs, on the other, our hSOD1\textsuperscript{G93A} swine could represent an invaluable opportunity to reproduce the human pathology since ALS is typically an adult-onset disease. As ALS has an insidious onset in human patients and we still do not know what the first symptoms would be in our swine, a wide and innovative approach has been developed to promptly recognize and characterize phenotypical alterations as well as to monitor disease course. As far as clinical characterization is concerned, specific neurological examination, objective gait analysis by means of a 3D motion capture system, neurophysiology and neuroimaging monitoring are ongoing. Besides, proteomic and metabolomics analyses are being performed on swine blood and tissues in order to detect novel biomarkers and hopefully unravel pathogenetic mechanisms underlying the disease.

An animal model recapitulating all the ALS crucial aspects has not yet been produced, although some transgenic mouse lines modulate a relatively reliable subset of disease features. However, since increasing difficulties are emerging in translating information gleaned from rodent models into therapeutic options for ALS patients, there is an urgent need for an intermediate research system. We do believe that a swine model could provide this essential bridge between insights gained from rodent models and the reality of treating a human disease.
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References