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(54) Title: NOVEL TRANSGENIC ANIMAL MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

(57) Abstract: Transgenic swine suitable to develop Amyotrophic Lateral Sclerosis, being transgenic for expressing an exogenous gene in neuronal cells, wherein the exogenous gene is a human SOD1 gene carrying a mutation.

NOVEL TRANSGENIC ANIMAL MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

* * *

5 **FIELD OF THE INVENTION**

The present invention concerns a novel transgenic animal as model of Amyotrophic Lateral Sclerosis (ALS).

BACKGROUND ART

10 Amyotrophic lateral sclerosis (ALS) is characterized by selective and progressive degeneration of upper and lower motor neurons, leading to muscle weakness, atrophy and evolving to complete paralysis. The disease impact on patients life quality is
15 devastating and death occurs in 2 to 5 years. 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
20 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.

 A characteristic hallmark of FALS linked to mutant
25 SOD1 is the abnormal accumulation of ubiquitine-immunoreactive misfolded protein in the cytoplasm of degenerating motor neurons. It has been hypothesized that protein misfolding and aggregation may contribute to disease pathogenesis, although a causative role
30 remains controversial and pathogenic mechanisms underlying ALS still remain essentially undisclosed.

 Research on ALS mainly relies on experimental
 animal models like transgenic rats and mice overexpressing mutant human SOD1. Besides rodent
35 models, a variety of animal species such as zebrafish,

Caenorhabditis elegans and *Drosophila melanogaster* has already been employed to unravel ALS pathogenetic mechanisms, although phylogenetic distance from human species prevents their employment in clinical research.

5 On the contrary, rats certainly represent an interesting alternative for modelling neurodegenerative disorders as their relative larger size allows some experimental procedures which are not easily carried out in other laboratory animal species. The lack of
10 efficient tools for genetic manipulation however has dramatically limited rat employment as model for human diseases (Bugos et al., 2009).

A great number of murine models carrying a variety of SOD1 mutations has been hitherto produced.
15 Currently, the most widely employed animal model is a transgenic mouse with a glycine to alanin conversion at the 93rd codon (G93A) of the SOD1 gene. These mice reliably reproduce the ALS patients phenotype progression developing a rapidly progressive motor
20 neuron degeneration characterized by limb muscles weakness that evolves to paralysis and premature death four months after symptoms onset (Turner and Talbot, 2008), a too short interval compared to le lifespan of the mouse relative to the onset of the disease in
25 humans. These animal models have certainly facilitated investigations on the selective vulnerability of motor neurons, however doubts have been recently raised about rodents suitability to faithfully reproduce the human disease (Schnabel, 2008; Scott et al., 2008). As a
30 matter of fact, encouraging results of drug tests in rodents have never been so far successfully translated to humans (Benatar, 2007; Van de Bosch, 2011) and in some cases, molecules delaying disease progression in transgenic mice, such as minocycline, have resulted
35 even detrimental in ALS patients (Gordon et al., 2007).

Since human and rodent species differ in size, life-span, physiology, anatomy and biochemical aspects, data extrapolation is difficult.

In the patent literature the international patent application WO-A-2007/124751 concerning a transgenic pig showing ALS phenotype has been identified. Such a transgenic pig has been realized using the Sperm Mediated Gene Transfer (SMGT) technique. Such a technique is nevertheless not efficient. In fact, this technique allows the transgene integration in the host cell but not guarantees its expression as the transgene is rearranged thus preventing the protein expression. Animals obtained with SMGT are often mosaics, namely the integration takes place in different times after fertilization and therefore not all cells are transgenic. Moreover data about expression of the endogenous mutated gene (porcine SOD1 mutated protein) in pig's tissues are missing.

In view of the foregoing drawbacks, a more homologous animal model should be created in an animal evolutionarily closer to human species in order to provide a better tool to study the disease.

OBJECT AND SUMMARY OF THE INVENTION

Object of the represent invention is to provide a new animal model of ALS which may be used in clinical studies for identification of therapies for human species.

According to the invention, the above object is achieved thanks to the compositions specified in the ensuing claims, which are understood as forming an integral part of the present description.

In an embodiment, the present disclosure provides for a transgenic swine suitable to develop Amyotrophic Lateral Sclerosis, wherein the swine is transgenic for

expressing an exogenous gene preferably in neuronal cells, the exogenous gene being a human SOD1 gene carrying a mutation. The human SOD1 gene is preferably mutated at 93rd codon, with a glycine to alanine conversion.

In a further embodiment the transgenic swine has been obtained by means of i) *in vitro* transfection of somatic cells, preferably fibroblasts, with the exogenous gene and ii) somatic cell nuclear transfer technique, which allows the expression of the exogenous gene in all animal's cells, preferably animal's neuronal cells, thus representing more closely the human pathology.

A preferred embodiment of the instant description discloses the generation of live transgenic Yucatan minipigs expressing the mutated protein codified from the hSOD1-G93A gene and then suitable to develop ALS symptoms.

In a further embodiment, the present disclosure concerns a process for generation of the transgenic swine suitable to develop Amyotrophic Lateral Sclerosis, wherein the swine is transgenic for expressing an exogenous gene preferably in neuronal cells, the exogenous gene being a human SOD1 gene carrying a mutation (wherein the human SOD1 gene is preferably mutated at 93rd codon, with a glycine to alanine conversion). The process comprises the following steps:

- a. providing a vector encoding the exogenous gene;
- b. providing adult swine fibroblasts;
- c. nucleofecting the vector into the fibroblasts, obtaining transgenic fibroblasts;
- d. providing at least one enucleated swine oocyte by means of metaphase chromosomes removal from

an intact oocyte;

e. contacting the at least one enucleated oocyte with at least one transgenic fibroblast;

f. fusing the at least one enucleated oocyte with at least one transgenic fibroblast obtaining an embryo;

g. cultivating the embryo;

h. implanting at least one cultivated embryo, preferably 70, more preferably 120, to a sow uterus;

i. natural birth or cesarean section delivering of at least one newborn transgenic swine at the end of sow pregnancy.

In further embodiments, the transgenic swine herein described can be used for screening compounds pharmaceutically active in the treatment of human ALS and/or as a model for the study of human ALS.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described, by way of example only, with reference to the enclosed figures of drawing, wherein:

- **FIG. 1: the pMG5'3'MARPuro5171-hSOD1G93A structure in a graphical representation.**

- **FIG. 2: Complete sequence of the pMG5'3'MARPuro5171-hSOD1G93A vector.**

- **FIG. 3: ICC on PAF (pig adult fibroblasts) clones transfected with the pMG5'3'MARPuro5171-hSOD1-G93A vector.** [a] Human Umbilical Vein Endothelial Cells (Huvec) used as positive control, with score=2 of transgene expression level; [b] wild-type PAF used as negative control (score=0); [c] PAF clone 1D2 with score=1; [d] PAF clone 1A1 with score=3; [e] PAF clone 2B2 with scores=4 and [f] clone 2A1 with a score=6. DAPI is the filter used to highlight the Hoechst nuclear staining to identify the nucleus of the cells.

FITCH allows the detection of the fluorophore conjugated with the secondary anti-rabbit antibody and then the transgene expression.

- **FIG. 4: ICC on PAF biopsied from the 5**
5 **transgenic living pigs. ICC on PAF obtained from ear**
biopsy of the 5 transgenic living pigs. Picture shows FITCH signal obtained with Millipore 07-403 antibody staining. hSOD1-G93A expression is revealed in all animals analyzed. [A] and [B] display staining in wild-
10 type PAF and Huvec cells, respectively used as negative and positive controls. [C] Pig 168 is characterized by a moderate cytoplasmic and perinuclear staining. [D] Pig 173 displays an intense cytoplasmic labelling along with a perinuclear ring, noticeable in some cells. [E]
15 and [F] show nuclear labelling with faint cytoplasmic staining detected in pigs 204 and 205 respectively. [G] Pig 174 displays a faint cytoplasmic staining. Perinuclear rings and faint nuclei labelling can also be observed in some cells. [H] shows intense
20 cytoplasmic labelling detected in cells from stillborn piglets 169.

- **FIG. 5: WB on stillborn piglets spinal cord.** Lane [A] displays results obtained with Genetex GTX 100659 at the 1:800 dilution, while lane [B] shows
25 those obtained with Millipore 07-403 at the dilution 1:1000 on the same samples. Sample 162 corresponds to spinal cord homogenate from non-transgenic pig, used as negative control. In lane [C] it is possible to appreciate a WB analysis conducted with Millipore 07-403 at the
30 dilution 1:1000 on spinal cord from other piglets and on spinal cord homogenate from hSOD1-G93A mouse, used as positive control. In all hSOD1-G93A piglet samples it is possible to appreciate two lanes, corresponding to the two SOD1 isoforms: the endogenous swine protein
35 displaying a lower molecular weight (16 KDa) and the

human transgenic one, with a higher molecular weight (20 KDa).

- **FIG. 6: Genetex GTX 100659 IHC on FFPE piglet samples.** In figure A it is possible to appreciate hSOD1-G93A staining in the ileum with clusters of positive cells next to the *muscularis mucosa*. Figures B and C show deposition pattern at the level of area *Hypothalamica lateralis*. In the same area (figure D), it is also possible to appreciate isolated cells displaying hSOD1-G93A staining. Figure E shows a bundle of positive cells lining the optic tract. Figure F displays deposition pattern in the spinal cord: it is possible to appreciate granular aggregates along fibres.

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DETAILED DESCRIPTION OF THE INVENTION

The present invention created an animal model, consisting in a transgenic swine genetic line carrying the human SOD1 gene with mutation G93A responsible for the onset of ALS that is useful in studying such a disease and to develop novel diagnostic markers and therapeutic approaches for the human species.

Transgenic swine have been produced by means of many techniques such as pronuclear microinjection, sperm-mediated gene transfer and lentiviral transgene delivery.

These techniques present, nevertheless, some disadvantageous aspects. As a matter of fact, pronuclear microinjection is very inefficient (Clark and Whitelaw, 2003), sperm-mediated gene transfer is attractive due to its simplicity, but suffers from high variability and generates transgenic animals with a non uniform expression of the transgene (Lavitrano et al., 2002; WO-A-2007/124751) and although lentiviral transgene delivery offers an efficient method for the

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generation of transgenic pigs (Whitelaw et al., 2004), the use of lentivirus (HIV-1; EIAV) pose many safety and ethical concerns, due to the proven ability to activate oncogenes as well as the possibility of re-
5 acquiring their pathogenic characteristics.

Therefore, the present invention used a different technique with respect to the previous techniques used for generating ALS animal models: *in vitro* transfection of cultured somatic cells combined with Somatic Cell
10 Nuclear Transfer (SCNT).

The inventors constructed a vector capable of promoting in the swine species, preferably pigs, more preferably mini-pigs, the expression of the mutated SOD1 protein, in a manner similar to what happens in
15 the patient suffering from ALS.

To achieve this goal inventors were able to obtain a stable expression of exogenous gene (hSOD1-G93A) in a consistent, reproducible way and in the long term in living animals. In fact the inventors in a previous
20 study (Chieppa et al. "Creation of a ubiquitous vector for expression of hSOD1G93A in pigs" *Transgenic research* 2010; 19:326) - using a different vector (carrying neomycin as selectable marker, to express the mutated gene) - found that only 22% of positive clones
25 showed high and uniform transgene expression 25 days post-transfection, while the remaining positive clones showed variegated expression or transgene silencing.

In the present invention, using a puromycin as selectable marker, to express the mutated protein,
30 inventors solved this problem. In particular thanks to the ability of the drug (puromycin) to select in a more consistent and effective way and shorter time the transgenic clones four days after transfection, inventors have been capable to select, during *in vitro*
35 culture, suitable transgenic cell clones with the

desired protein expression level.

Genetic transformation of animals generally results in a large and random variation in transgene expression between individual transformants. This
5 variability has been attributed to the positioning effect that is the presence of different transgene integration sites and copy numbers.

Since integration of foreign DNA seems to occur randomly in the animal genome, it has been assumed that
10 some transgenes would be integrated into transcriptionally inactive regions, while others would be integrated into relatively transcriptionally active regions (Park et al., 2002).

To solve such a random insertion of the transgene
15 into the swine genome the inventors decided to introduce the chicken lysozyme an element (known as 5'MAR), which is located upstream of the chicken lysozyme gene and comprises a region of approximately 3 kb (Phi-Van and Stratling, 1996), in their pCAGGS
20 constructs.

The combination of puromycin selection cassette, with accurate screening of the cell clones for the desired characteristics in combination with SCNT turn out to be successful and the inventors managed to
25 produce a large number of piglets, five of which are alive and adult. All pigs generated in this way express hSOD1-G93A.

The vectors that were used to produce hSOD1-G93A transgenic pigs proved to be very effective to obtain
30 an ubiquitous expression in somatic cells and in the tissues of the animals generated by SCNT.

The inventors decided to use in SCNT experiments a pool of donor cells showing different transgene expression level in order to minimize the risk of using
35 cell clones unable to generate a viable animal. As a

matter of fact, since this is the first hSOD1-G93A swine model produced so far, no data are available about the toxicity related to transgene expression level in the early stage of porcine embryonic development and after embryo transfer in sows.

Since donors cells with different expression levels have been employed in SCNT, the cloned piglets present variable transgene expression levels depending on the particular cell from which they have been created.

However, transgenic swine could be re-cloned from the cells cryopreserved after birth from best expressing animal, in a successive SCNT experiment in order to further standardize the hSOD1-G93A pig model or could be bred to sows to produce F1 generation, of which accordingly Mendelian law 50% will inherit the disease.

The SOD1 protein is an enzyme with antioxidant function by reducing the superoxide ion (O_2^-) level, a toxic free radical product during the oxidative cellular metabolism. The superoxide ion is capable of altering proteins, membranes and DNA. Precisely the involvement of a mutated protein in its pathogenesis leads to include ALS in the proteinopathy family. Studies on transgenic rodent models was aimed at understanding the mechanisms by which mutated SOD1 gene leads to the onset of ALS: they have ruled out that the motor neuron degeneration is the result of loss of dismutase activity and allowed to detect the formation of aggregates of ubiquitinated proteins in tissues affected, among other containing the mutant SOD1: it is assumed that these inclusions protein play a role in the interruption of cell functions damaging mitochondria, proteasomes, mechanisms of protein folding or other proteins.

Unlike rodent models that show an extremely high expression transgene level and a rapid disease course (Bendotti and Carri, 2004), the instant swine model presents an expression level comparable to that of human patients, where a single allele mutation results in aforementioned toxic gain of function.

Piglets expressing the hSOD1-G93A protein, already express the mutated protein at birth, are expected to show the full degeneration of upper and lower motor neurons, leading to muscle weakness, atrophy and evolving to complete paralysis, with times and modalities similar to those that occur in ALS patients. On one hand this could result in a longer pre-clinical phase and in an increase of animal maintaining costs, on the other hand the present hSOD1-G93A swine represents an invaluable opportunity to find early biomarkers and a closer and more faithful model to reproduce human pathology since ALS is typically an adult-onset disease.

Currently, an animal model recapitulating all the ALS crucial aspects has not yet been produced.

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. The present inventors do believe that a swine model provides this essential bridge between insights gained from rodent models and the reality of treating a human disease.

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Materials and Methods

Animal experiments

All procedures involving animals and their care are conducted in conformity with national and international regulations (EEC Council Directive

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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, U.S. National
5 Research Council, 1996) and after the approval of LTR-Avantea Local Ethic Committee. Yucatan Black fibroblasts (PAF) were obtained from adult boar ear biopsy, coming from Italian pig farming. Subsequently PAF have been used in cloning procedures to obtain wild
10 type pigs.

Vector construction

The inventors have previously developed an ubiquitous EGFP expression vector, driven by the pCAGGS hybrid promoter (CMV-IE enhancer + chicken beta actin
15 promoter) (Niwa et al., 1991) which is characterized by maintaining high expression level through the F1 generation of pigs (Brunetti et al., 2008).

A Destination Vector pMGOrfA5'3'MARpuro5171 was created inserting the Multisite Gateway system
20 (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) (McKnight et al., 1992) to prevent various silencing effects
25 (positional or copy number effects). The structure was also provided with a floxed, then removable using the Cre recombinase, SV40-Puro cassette to select the transfected clones.

The *Bam*HI/*Xho*I fragment of hSOD1G93A cDNA obtained
30 by restriction of pcDNA3.0hSOD1-G93A plasmid was inserted into the pENTRL1L2oligoSacISalI vector, obtaining pENTRL1L2-hSOD1-G93A.

The *Sal*I-*Bam*HI fragment was removed from the construct and the resulting pENTRL1L2-hSOD1-G93A
35 was used, after sequencing, in a LR exchange reaction

with the Destination Vector pMGOrfA5'3'MARpuro5171. This exchange reaction, mediated by the LR Clonase, was used to transform chemically competent *E.coli* cells (One Shot Mach1-Invitrogen). The resulting
5 pMG5'3'MARPuro5171-hSOD1G93A vector (Figure 1 and 2 - SEQ ID No.:1) was purified with Plasmid Mini Kit (Qiagen, Hilden, Germany), analyzed by different restriction enzymes, confirmed by sequencing (Figure 2) and finally linearized by *Apa*LI (Fermentas). After
10 phenol/chlorophorm purification, the vector was precipitated and re-suspended in TE buffer.

Cell isolation and culture

Primary porcine fibroblasts cultures were recovered from adult male ear biopsy (pig adult
15 fibroblasts, PAF). Biopsy specimens were cut in small pieces with a scalpel blade and the 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). Culture medium was changed
20 every 3 days.

Cells were allowed to grow until they reached 50% of confluence. Tissue pieces were then removed and the cells sub-cultured until they reached confluence in DMEM/TCM199 with 10% of FBS and growth factors (bFGF).
25 Growth conditions consisted of a 38°C temperature and of an atmosphere composed by 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
30 throughout the following experiments.

Transfection of hSOD1-G93A vector into adult fibroblasts

The day before transfection, passage 3 PAF were trypsinized, counted, and plated into 60 mm dishes in
35 order to obtain about 1x10⁶ cells at 80% confluency in

24 hours. On the transfection day, cells were trypsinized, counted and resuspended in 100 μ l of nucleofector solution (Basic Nucleofector Kit, Prim. Fibroblasts; Amaxa, Cologne, Germany), mixed with 5 μ g of the linearized pMG5'3'MARPuro5171-hSOD1-G93A vector. PAF and linearized vector were then transferred into the nucleofection cuvettes and transfected with V-24 program (Nucleofector Amaxa).

After nucleofection cells were plated in 60-mm culture dishes containing fresh culture medium. After 24 hours, the drug (Puromycin: 1 μ g/ml) employed in colonies selection was added. After 4 days, puromycin-resistant colonies were picked up using 5-mm cloning discs and transferred into 24 mm 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 (ICC)

Cells (transgenic hSOD1-G93A PAF and primary culture cells from ear biopsy obtained from transgenic piglets) were fixed with 4% PFA and then stored in phosphate buffer (PB) until analysis.

In order to detect the expression of human SOD1 by immunocytochemistry (ICC) the inventors used a rabbit polyclonal antibody (07-403 Millipore, concentration 1:200), directed against the full-length wild-type hSOD1 plus an N-terminal methionine.

Unspecific binding blocking (10% goat serum) was followed by primary antibody incubation (RT, two hours). After 3 washes (PB with 0.2% BSA and 0.05% saponine, 3 minutes each) a secondary FITCH-conjugate antibody incubation (1 h at RT) was performed. Nuclei

were counterstained with Hoechst (RT, 15'). After two washes slides were finally mounted in mounting medium (Citifluor). We used Human Umbilical Vein Endothelial Cells (Huvec) and wild type PAFs respectively as positive and negative controls. Fluorescence was detected by exposing fibroblasts to an epifluorescent light mounted on an inverted microscope (Nikon TE-DH100W) equipped with a FITC (for green) and DAPI (for nuclei) filter and with a digital imaging system (Nikon DIGITAL SIGHT DS-L1). Subjective assessment of transgene expression level was based on the comparison of the fluorescence intensity revealed in PAF clones with that of the controls. The negative control was assigned a Score=0 while the positive a Score=2, as showed in Figure 3. It should be noted that this is as subjective scores attribution and values presented in this study are not objectively measured, neither absolute.

Preparation of nuclear donor cells and SCNT

Transgenic 1A1, 1A2, 1B1, 1C2, 1D1, 1D2, 1E2, 2A2, 2A6, 2B2, 2C1 and 2C3 hSOD1-G93A-fibroblasts clones were selected, as nuclei donors, according to uniformity and intensity of their expression level.

The day before nuclear transfer, donor cells were induced into quiescence by serum starvation (0.5% FBS). 30 minutes before nuclear transfer, cells were prepared by trypsinization, washed and resuspended in SOF (Tervit et al., 1972) supplemented with 25 mM HEPES (H-SOF). Ovaries with corpora lutea were collected at local slaughterhouse and carried to laboratory at 31-33°C. Oocytes were aspirated from follicles larger than 3 mm in diameter, washed, and transferred to maturation.

DMEM-F12 supplemented with 10% FBS, 110 µg/ml sodium pyruvate, 75 µg/ml ascorbic acid, 100 µg/ml

glutamine, 5 µg/ml myoinositol, 0.4 mM cystine, 0.6 mM cysteamine, ITS liquid media supplement (insulin, transferrin, selenite, Sigma, 1 µl/ml), gonadotropins (0.05 IU/ml FSH, and 0.05 IU/ml LH; Pergovet 75, 5 Serono), 100 ng/ml long IGF1 (recombinant insulin growth factor I analog), 50 ng/ml long EGF (recombinant epidermal growth factor analog), and 5 ng/ml bFGF (human recombinant) was used as maturation medium.

Oocytes were cultured at 38.5°C in 5% CO₂ in 10 humidified atmosphere. After 42 hours maturation, oocytes were denuded of cumulus cells by vortexing in the presence of hyaluronidase in HSOF and returned to maturation medium. Only oocytes displaying extruded polar body were selected. NT-embryos were reconstructed 15 following a zona-free method (Lagutina et al., 2005, 2006; Oback et al., 2003). The zona pellucida of oocytes with extruded polar body was digested with 0.5% pronase in PBS. The oocytes were washed in H-SOF with 10% FBS and returned to maturation medium. All the 20 following manipulations were performed in H-SOF with 10% FCS. Zona-free oocytes were exposed to cytochalasin B (5 µg/ml) and Hoechst (5 µg/ml) for 5 min prior to enucleation. Metaphase chromosomes were removed under very short exposure to UV light with a blunt 25 enucleation pipette.

After enucleation, zona-free cytoplasts were individually washed for few seconds in 300 µg/ml phytohemagglutinin P in PBS and then quickly dropped over a single donor cell (Vajta et al., 2003) settled 30 at the bottom of a microdrop of the diluted donor cell suspension.

Forty-six to forty-eight hours after maturation onset, formed cell couples were washed in 0.3 M mannitol (Ca⁺⁺-free, 100 µM Mg⁺⁺) solution, fused by 35 double DC-pulse of 1.2 kV/cm applied for 30 µsec and

returned to maturation medium. 2 hours later (after 48-50 hours of maturation), NT embryos were activated by double DC-pulses of 1.2 kV/cm for 30 μ sec applied in 0.3 M mannitol solution, containing 1 mM Ca^{++} (Cheong et al., 2002) and 100 μ M Mg. After activation, embryos were kept in culture maturation medium with 5 μ g/ml cytochalasin B for 4 hours.

NT embryos were cultured in SOF supplemented with essential and nonessential amino acids and with 4 mg/ml BSA (SOFaa) in a Well-of-the-Well system (WOW) modification (Vajta et al., 2000). During embryo culture half of the medium was renewed on day 3 (D+3) and on day 5 (D+5) with fresh SOFaa.

Synchronization of sows, surgical embryo transfer, pregnancy diagnosis, and parturition

Estrus was synchronized by feeding 12 mg of altrenogest (Regumate, Intervet, Peschiera Borromeo, Italy) per sow for 15 days and injecting 0.15 mg of PgF2a (Dalmazin, Fatro, Ozzano Emilia, Italy) on the 15th day of regumate treatment and 1000 IU of hCG (Chorulon, Intervet) 96 hours after the last altrenogest administration.

The SCNT embryos were transplanted to the sows uterus on D+5 of development. Embryo transplantation was performed 4 days after animals ovulation by midventral laparotomy and pregnancy was examined at day 29, 36, 50, and 62 by ultrasonography. A cesarean delivery was performed at D+114 of gestation.

Tissue banking

Ear biopsy was performed on all piglets, in order to obtain primary PAF cultures and subsequent cryopreservation to establish a cell bank from each individual piglet. Brain, spinal cord, peripheral nerves, muscles and organs were sampled from stillborn or euthanized animals. Tissue samples were either

formalin-fixed and paraffin-embedded (FFPE) or paraformaldehyde-fixed.

After 24 hours, paraformaldehyde samples were rinsed 2 times in PBS and then exposed to growing sucrose concentrations in order to achieve cryoprotection. Finally, samples were kept for 30 minutes in a 1:1 30% sucrose: OCT solution and then included in OCT, frozen in isopentane and stored at -80°C.

Finally, a part of every tissue was snap-shot freezed in isopentane and stored at -80°C.

Western Blot (WB)

Cells (transgenic hSOD1-G93A PAF and primary culture cells from ear biopsy obtained from transgenic piglets) were lysed using Laemmli buffer 1X containing β -mercaptoethanol (5%) and boiled for 10 minutes. Total protein was quantified by Qubit fluorometer (Invitrogen) using the QuantIT Protein quantification kit (Invitrogen).

Tissues obtained from stillborn piglets were homogenized (1:5 mg/ml) in lysis buffer (50mM Tris HCl pH8, 150mM NaCl, 5mM EGTA pH8, 1.5mM MgCl₂, 10% anhydrous glycerol, 1% Triton, 100 μ g/ml (=0.57mM) PMSF). Protein quantification was performed by BCA Protein Assay kit (Pierce).

Thirteen μ g of each sample were loaded onto Glicine-SDS-PAGE (4-12%) and electrophoretically separated for 45 minutes at 200V using the MiniproteanII chamber (Biorad). Blotting onto Immun-Blot PVDF membrane (Biorad) was obtained after 1h at 100V using MiniproteanII electroblotter (Biorad) according to manufacturer protocol. The resulting PVDF membranes were subsequently processed following the instructions of the chemiluminescent detection system Lumi-LightPLUS Western Blotting Kit Mouse/Rabbit

(Roche). Detection of transgenic target was achieved using either a polyclonal Antibody 07-403 (1:1000 Millipore) or a polyclonal Antibody GTX 100659 (1:800 Genetex) that is specifically directed against hSOD1 region within amino acids 75 and 138, which is different between human and swine. Pig β -actin expression was detected using the mAb ab6276 (1:5000 Abcam). The treated membranes were finally exposed, developed and fixed.

10 **Immunohistochemistry (IHC)**

All FFPE tissues were analysed by IHC. After antigen retrieval (95° bain-marie for 20 minutes), sections were treated with 3% hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase. Sections were incubated in Normal Goat Serum for 20 minutes and then overnight at 4°C in the primary antibody (GTX 100659; 1:250) dissolved in PBS.

Afterwards, sections were rinsed 3 times in PBS and incubated with relevant secondary antibody (1:200) for 1 hour. After 3 rinses in PBS, sections were incubated with Avidin Biotin Complex for 30 minutes. DAB was used as revealing agent. Sections were counterstained with haematoxylin.

Ubiquitine-immunoreactive misfolded protein was detected by the same IHC protocol, but the antigen retrieval step, with a rabbit polyclonal antibody (Dako Z0458, 1:100).

Results

30 **Transfection of hSOD1-G93A vectors into adult fibroblasts and related analysis.**

The transfection experiments conducted with the pMG5'3'MARPUro-hSOD1-G93A vector have led to the isolation of 26 PAF clones, which were analysed by WB and ICC. All clones, listed in Table 1, revealed

different degrees of transgene expression, to which a score was assigned. Four clones showed a transgene expression level lower than the control one (score=2), and three clones achieved the same score as the control. The remaining 19 clones showed a higher expression (Score ranging from 3 to 6).

Table 1

Clone	Score
1A1	3
1A2	6
1A4	1
1A5	5
1B1	3
1B3	4
1B6	6
1C1	1
1C2	2
1C6	4
1D1	5
1D2	1
1D5	4
1D6	5
1E1	6
1E2	4
2A1	6
2A2	6
2A3	5
2A4	1
2A5	4
2A6	4
2B2	4
2C1	5
2C2	5
2C3	4

10 **Somatic Cell Nuclear Transfer (SCNT)**

Six SCNT experiments were conducted. In the first and second experiment, a pool of clones (1A1, 1C2, 1D2 and 1E2) was used as a nucleus donor. In the third and fourth experiment a pool composed of 1B1, 1D1, 2C1 and 2B2 clones was employed, while a pool composed of 1A2, 2A2, 2A6 and 2C clones was employed in the fifth and sixth experiment. All the SCNT experiments conducted are listed in Table 2 where viable embryo percentages

(ranging from 35.38% to 50.50%) were obtained. Table 2 contains the following information: The "ID clones" column shows the names of hSOD1-G93A PAF clones that composed the cells pool, used as nuclei donors. In "N° SCNT" column the number of SCNT experiments is reported. The "average score" column indicates the transgene expression level owned by the pool of PAF clones. "N" is the number of pairs cytoplasts/PAF formed after the fusion. "Cl" is the number of segmented embryos and its value is compared to the total percentage of reconstructed embryos. "Mc/B1" indicates the number of compact morulae and blastocysts at the sixth 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 indicate the pregnancy outcome, with the number of vital and stillborn piglets obtained.

Table 2

<i>ID clones</i>	N° SCNT	Av. Sc.	N	Cl	%	Mc/B1 D6	TOT emb ryo	%	Piglets Vital/ Stillborn
A1,E2,C2,D2	1	2	195	181	94,66	8 mc, 61 bl	69	35,38	No pregnancy
A1,E2,C2,D2	2	2	204	185	92,82	11 mc, 63 bl	74	36,27	No pregnancy
B1, 2B2, 2C1,D1	3	3	200	172	86,00	21 mc, 54 bl	75	37,50	6/4
B1, 2B2, 2C1,D1	4	3	209	151	72,25	31 mc, 47 bl	78	37,32	5/2
1A2,2A6,2A2, 2C3	5	5	205	179	87,32	96 bl	96	46,83	3/1
1A2,2A6,2A2, 2C3	6	5	202	178	88,12	102 bl	102	50,50	2/7

Development to term of SCNT embryos derived from adult hSOD1-G93A fibroblasts

The transfer of 494 embryos to six recipient sows resulted in four pregnancies. The 4 pregnant sows received 75, 78, 96, 109 embryos obtained from, the third, fourth, fifth and, sixth SCNT experiment

respectively. Pregnancies were carried to term and resulted in the birth of 10, 7, 4 and 9 piglets with a mean efficiency of blastocyst development to term of 8,78%. Among produced piglets 16 were alive and 12 were
5 stillborn. Five piglets survived artificial hand raising, weaning 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 were not nursing, others developed diarrhoea,
10 pneumonia etc).

Detection of hSOD1-G93A expression in tissues from stillborn piglets and living animals

Fibroblasts obtained from ear biopsy of both living and stillborn piglets, were analyzed by ICC and
15 revealed a transgene expression level comparable with that of PAF used as nuclei donors (Figure 4).

Furthermore, IHC and WB analysis were performed on dead and stillborn piglets tissues. Snap shot spinal cord samples were homogenised and analysed by WB.
20 Transgenic protein was revealed using both antibodies (07-403 Millipore and GTX 100659 Genetex). In Figure 5 it is possible to appreciate two lanes, corresponding to the two SOD1 isoforms: the endogenous swine protein displaying a lower molecular weight (16 KDa) and the
25 human transgenic one, with a higher molecular weight (20 KDa).

Immunohistochemistry (Figure 6) was performed on coronary sections of all FFPE samples. Analysis performed by GTX 100659 revealed granular mutant
30 protein aggregates in neurites and perikarya in brain (from *area hypothalamica lateralis* to the third ventricle), spinal cord (especially at the thoracic level), peripheral nerves (brachial plexus and sciatic nerve) and in the Enteric Nervous System.

35 The same brain areas also revealed ubiquitine

immunoreactivity characterized by intracytoplasmatic aggregates.

Naturally, while the principle of the invention remains the same, the details of construction and the
5 embodiments may vary widely with respect to what has been described and illustrated by way of example, without departing from the scope of the present invention.

10

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CLAIMS

1. Transgenic swine suitable to develop Amyotrophic Lateral Sclerosis, being transgenic for
5 expressing an exogenous gene in all cells, preferably in neuronal cells, wherein the exogenous gene is a human SOD1 gene carrying a mutation.

2. Transgenic swine according to claim 1,
10 wherein the transgenic swine over-expresses the exogenous gene.

3. Transgenic swine according to claim 1 or claim 2, wherein the human SOD1 gene is mutated at 93rd
15 codon, wherein a glycine to alanine conversion is performed.

4. Transgenic swine according to any one of the preceding claims, wherein the transgenic swine carries
20 the exogenous gene in a single allele.

5. Transgenic swine according to any one of the preceding claims, wherein the transgenic swine express the exogenous gene in form of granular mutant SOD1
25 aggregates in brain, spinal cord, peripheral nerves and enteric nervous system.

6. Transgenic swine according to any one of the preceding claims, wherein the transgenic swine express
30 ubiquitine intracytoplasmatic aggregates in brain.

7. Transgenic swine according to any one of the preceding claims, wherein the transgenic swine is a pig, preferably a mini-pig.

8. Transgenic swine according to any one of the preceding claims, wherein the transgenic swine is obtained by means of i) *in vitro* transfection of somatic cells, preferably fibroblasts, with the exogenous gene and ii) somatic cell nuclear transfer technique.

9. A process for generating a transgenic swine according to any one of the previous claims comprising the steps of:

- a. providing a vector encoding the exogenous gene;
- b. providing adult swine fibroblasts;
- c. nucleofecting the vector into the fibroblasts, obtaining transgenic fibroblasts;
- d. providing at least one enucleated swine oocyte by means of metaphase chromosomes removal from an intact oocyte;
- e. contacting the at least one enucleated oocyte with at least one transgenic fibroblast;
- f. fusing the at least one enucleated oocyte with at least one transgenic fibroblast obtaining an embryo;
- g. cultivating the embryo;
- h. implanting at least one cultivated embryo, preferably 70, more preferably 120, to a sow uterus;
- i. natural birth or cesarean section delivering of at least one newborn transgenic swine at the end of sow pregnancy.

10. Process according to claim 9, wherein before step e. a pooling of more than two transgenic fibroblasts is carried out, and step e. is carried out by contacting the at least one enucleated oocyte with the pool of more than two transgenic fibroblasts.

11. Process according to claim 9 or claim 10, wherein after step c. analysis of vector expression in transgenic fibroblasts is carried out for selecting
5 transgenic fibroblasts having uniform and intensive expression of the vector, and wherein step e. is carried out using the selected transgenic fibroblasts.

12. Process according to any one of claims 9 to
10 11, wherein the vector encoding the exogenous gene is carrying the selectable marker Puromycin.

13. Process according to any one of claims 9 to
15 12, wherein the vector encoding the exogenous gene has the nucleotide sequence set forth in SEQ ID No.:1.

14. Use of the transgenic swine according to any
of claims 1 to 8 for screening compounds
pharmaceutically active in the treatment of ALS.

20

15. Use of the transgenic swine according to any
of claims 1 to 8 as a model for the study of ALS.

The pMG5'3'MARPuro5171-CXhSOD1G93A vector

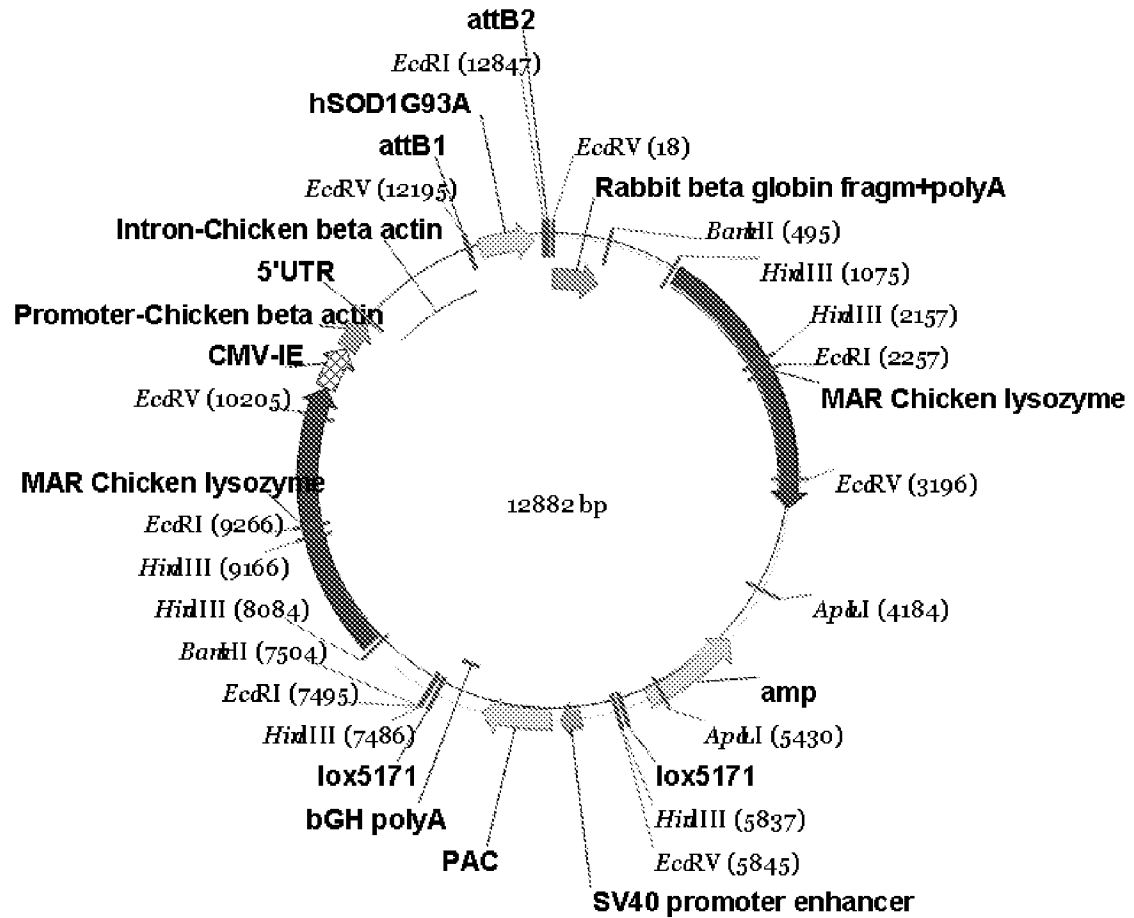


Figure 1

The pMG5' 3' MARPuro5171-hSOD1G93A sequence: 12882 bp

1 ttgtcaaaa tggatgatc ctogagtgt ctttttccct ctgcaaaaa ttatggggac atcatgaagc cctctgagca tctgacttct ggctaataaa ggaaatttat ttctattgca atagtgtgtt ggaatttttt gtgtctctca
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Figure 2

5401 tgagatccag ttogatgtaa cccactctg caccactctg atcttcagca tcttttact tcaccagcgt tctcgggtga gcaaaaacag gaaggcaaaa tgcgcgcaaaa aagggaataa gggcgacacg gaaatgttga atactcatic

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8401 ctgtgtgac aggtcaaca gactcactc ctattttat ttatagaatt ttatatgac tctgtctg gttctgtgt tga aggata cagcttaaa tttctagag ogatgctcag taaggggggt tgcacatgg gttcaaatg

8551 aaaaogggca cgtttctgc tgccttcca gatccagac actaaactgc tctgcaact gaagtaaaa togttcaga tccaggaag ttagatcca cgtcatatt cta aagaag aatgaatac ttttaaaa tgttgcata

8701 ggaagcaagc tgcaggtatt talttggac ttaaattatt ttgtaacog agtgcatagg ttttaaacac agttgcagca tgcataagac tcaagcatt tatgcagaag ttagtctgt tgcagctgt taogcacty ccttgcaag

8851 agcattgac atagggtgg ggtgtttgt gctgtttgg gacaogctgc cacacagcca cctcccagc atactcacc tctgtgtgac ttttcaacc atcttagcag tagtatgata gttactatg acaagagaag tttctcagtt

9001 gcatatttc atgggatgc ttttttcca tgttgggcaa agtatgataa agcactctca ttgttaatt atgcactgt tagttctga atctttcta tagcaccact tati gcagca ggttaggt ctggtgtgc ctgtctctg

9151 gcttcaact ttaagctc ttggaata cacogacty attgaagct ctgaaagata gtaaacagta ctaccttg atccccagca aatogagcat ttcagttgta aaagaattc gectattat acctgtaat gtaattttac

9301 accccagct ctgacactt ggaatattt caagatagc actttgctt caccctctg tgtactgtat ttgtaatag aaaatattt aaactgtgca tatgattat acattatgaa agagacatc tctgtatctt caaatgtaag

9451 aaaatgagga gtcogtgc ttttataat acaagtgatt gcaattagc gcaagtgcc ttaaaaaaaa aaaaaagta taaaaaagc accaggtgt ttacaagtga aata cttcc tatttggaaa acagttacat tttatgaa

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9751 atgcagagtt gcactgcaa atgtcaggaa atgatgtct ctgagaatgc ccaactcca agjatttata tgtgtatata gtaagcagtt tctgattcc agcaggccaa agagctctgt gaatgttgc ttgcoggaga cctgtattc

9901 tcaacaaggt aagatgtat ctagcaact gogatttita atacatttc agcagaagta cttagttat ctctacttt agggatgtt tcatcttt tagatgttat actt gaaata ctgcaact ttagcttcc atgggtctt

10051 tttttcagc ctttaggaga ctgttaagca atttgtgc caactttgt gttgtotta aactgcaata gtagtttacc ttgtattgaa gaaataaga ccattttat attaaaaat actttgtct gttctcatt tgaactgtct

10201 gatactctg cagtgctcat tagtcagtt ctgcagata ttcacacatc aaaaactaac gtagctcag tggagttaca gctcoggttt ttagctgttt attattctg aaactgaaa ttagttgtc ttcatctgt catcaaacac

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10501 taoggggtca ttagtctata gccatatal ggaagtcgc gttacataac ttaogttaa tggcccctt ggtcagccc ccaagacc cgcgccattg aogtcaataa tgaactatgt tcccatagta aogcaatag ggaacttcca

10651 ttgaogtcaa tgggtgact atttagctta aactgcccac ttgagctac atcaagtgta tcatatgca agtacgccc ctattgogt caatgoggt aaatggccc cctggcatta tgcacagatc atgacttatt ggaacttcc

Figure 2/cont.

10801 tacttggcag tacatctaog taltagtcat cgtattacc atgggtogag gtgagccca cgttctgctt cactctccc atctccccc cctcccacc cccaatttg tatt tathta tttttaatt attttgtca gogatgggg
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12301 cgaagcgcg gtgcgcgc aaggcgcgc gccagtcga gggatcctc aattgcgc agaaggaag taatgacca gtgaaggtg ggggaagc taaagcgc actg aagcgc tgcaggtc ccatgtctc gacttggag
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12751 ccaataaac attccttg atgtgtctg aggcctta actcctctg tctctgcg ccgcgcgc gctcagtc cgcgcgcg togaatc cggggctt tta tgcagc acccagctt cc

Figure 2/cont.

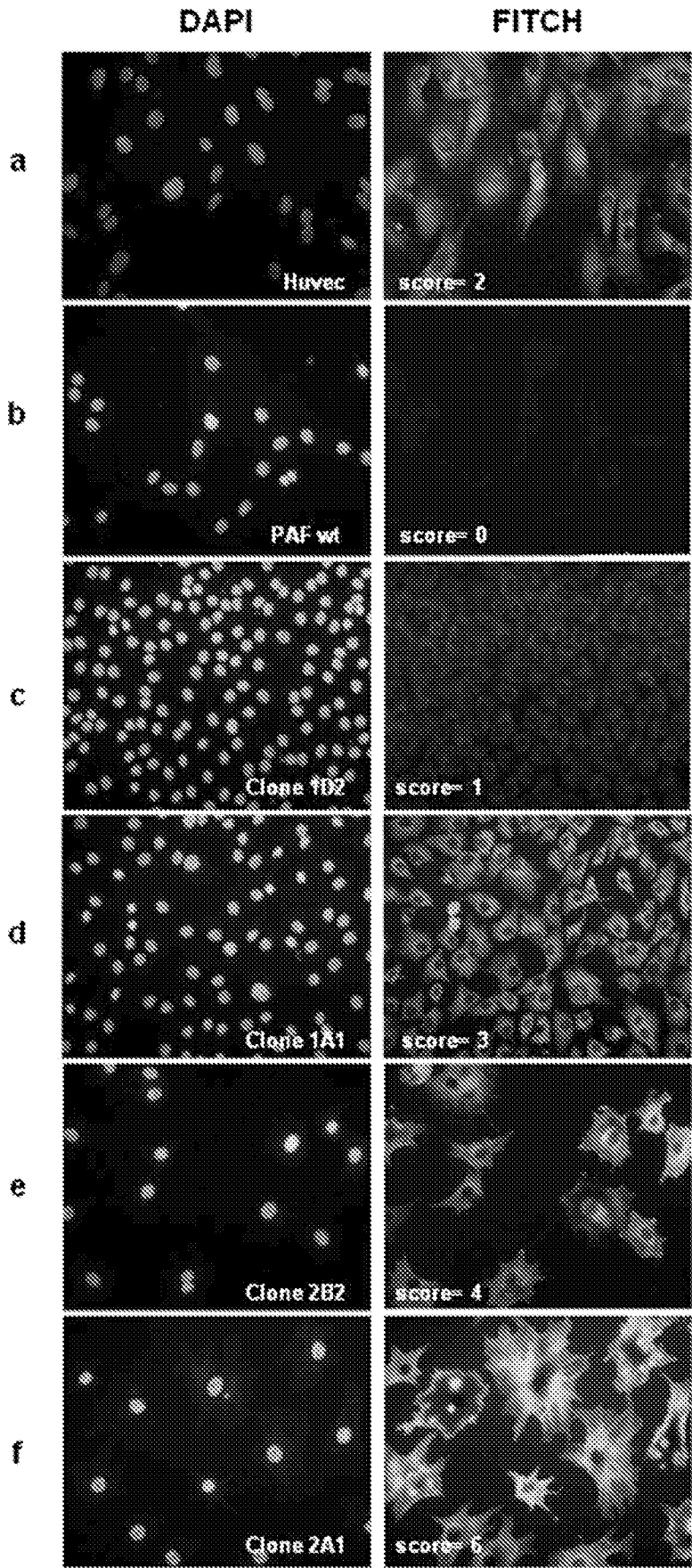


Figure 3

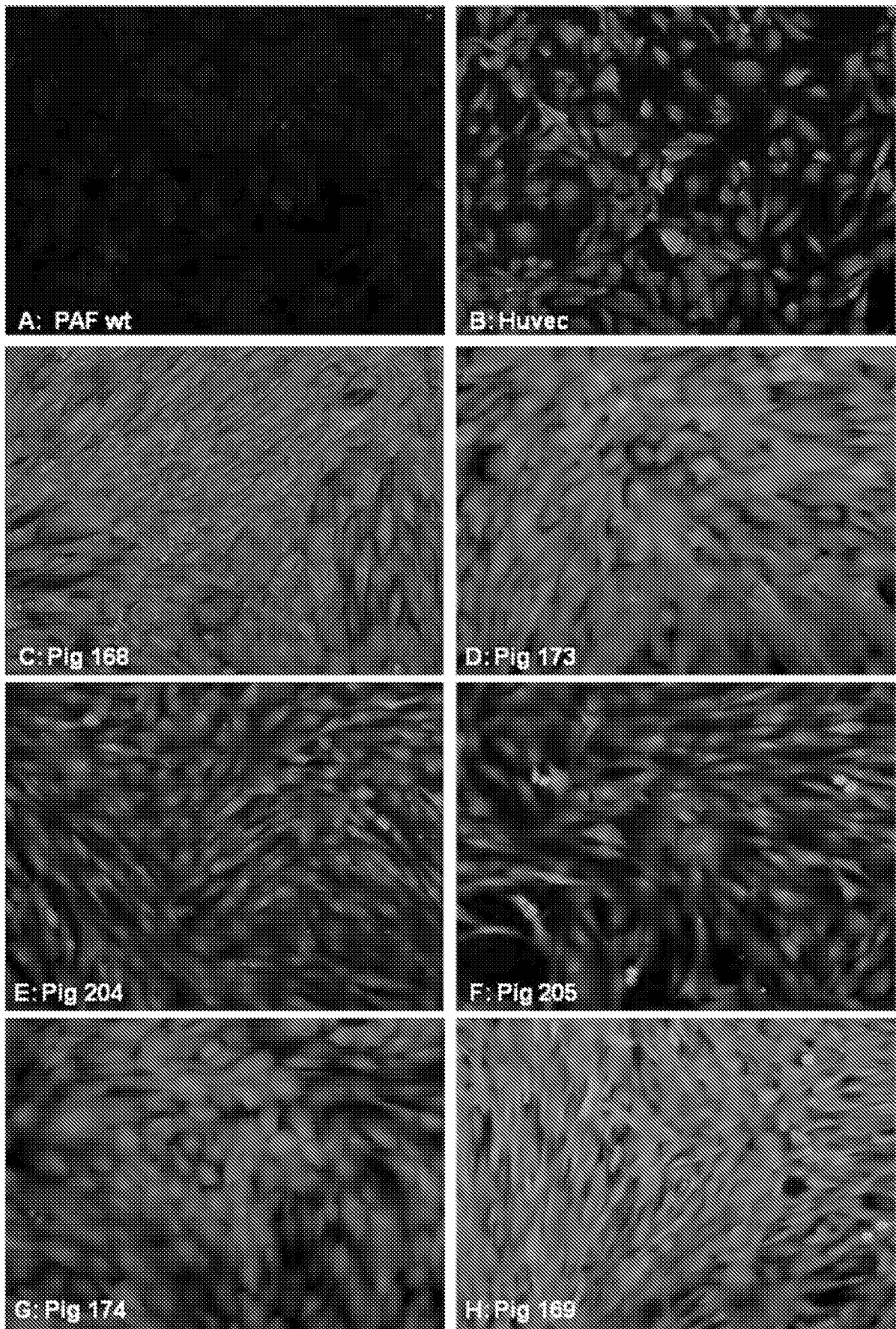


Figure 4

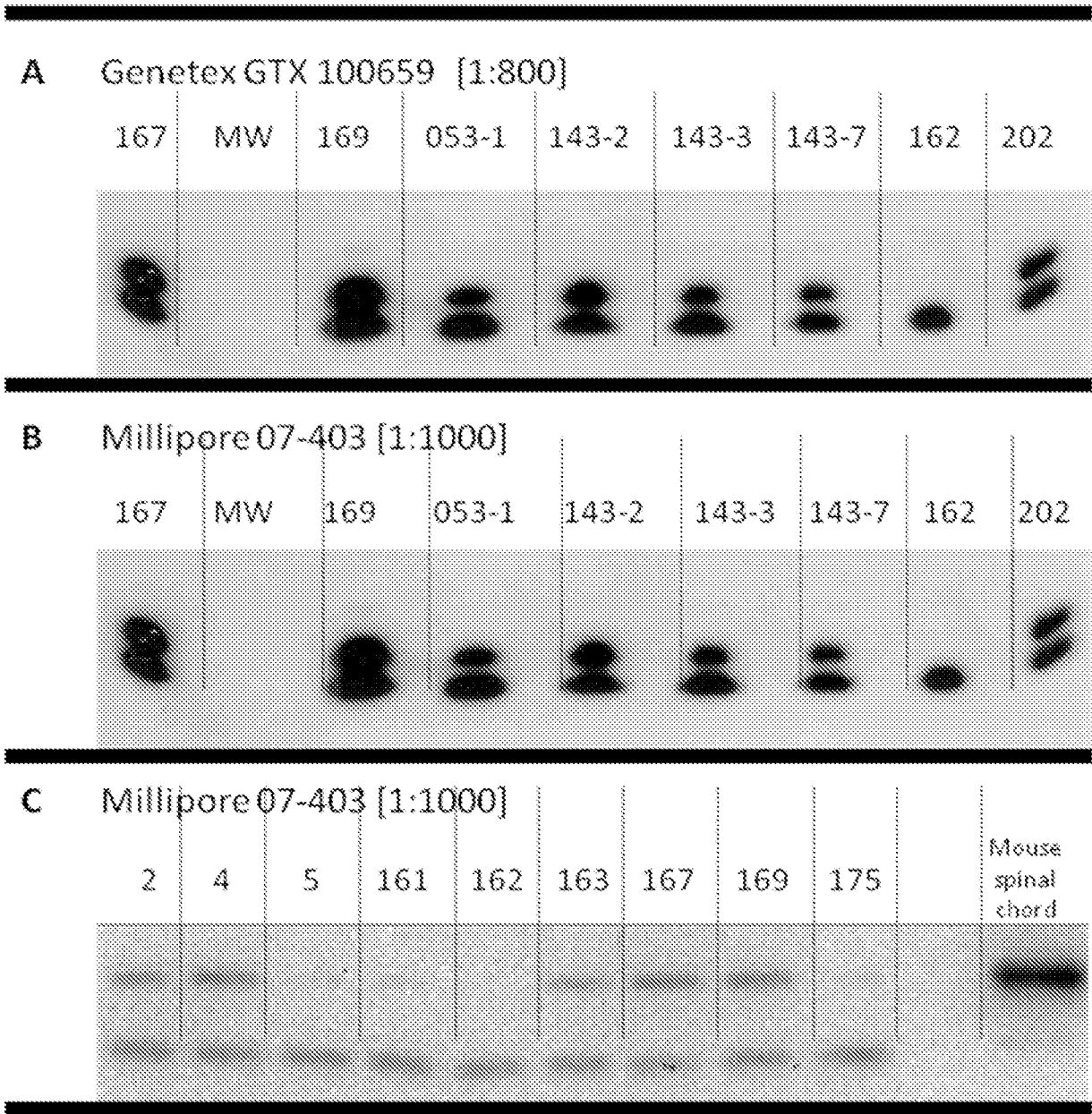


Figure 5

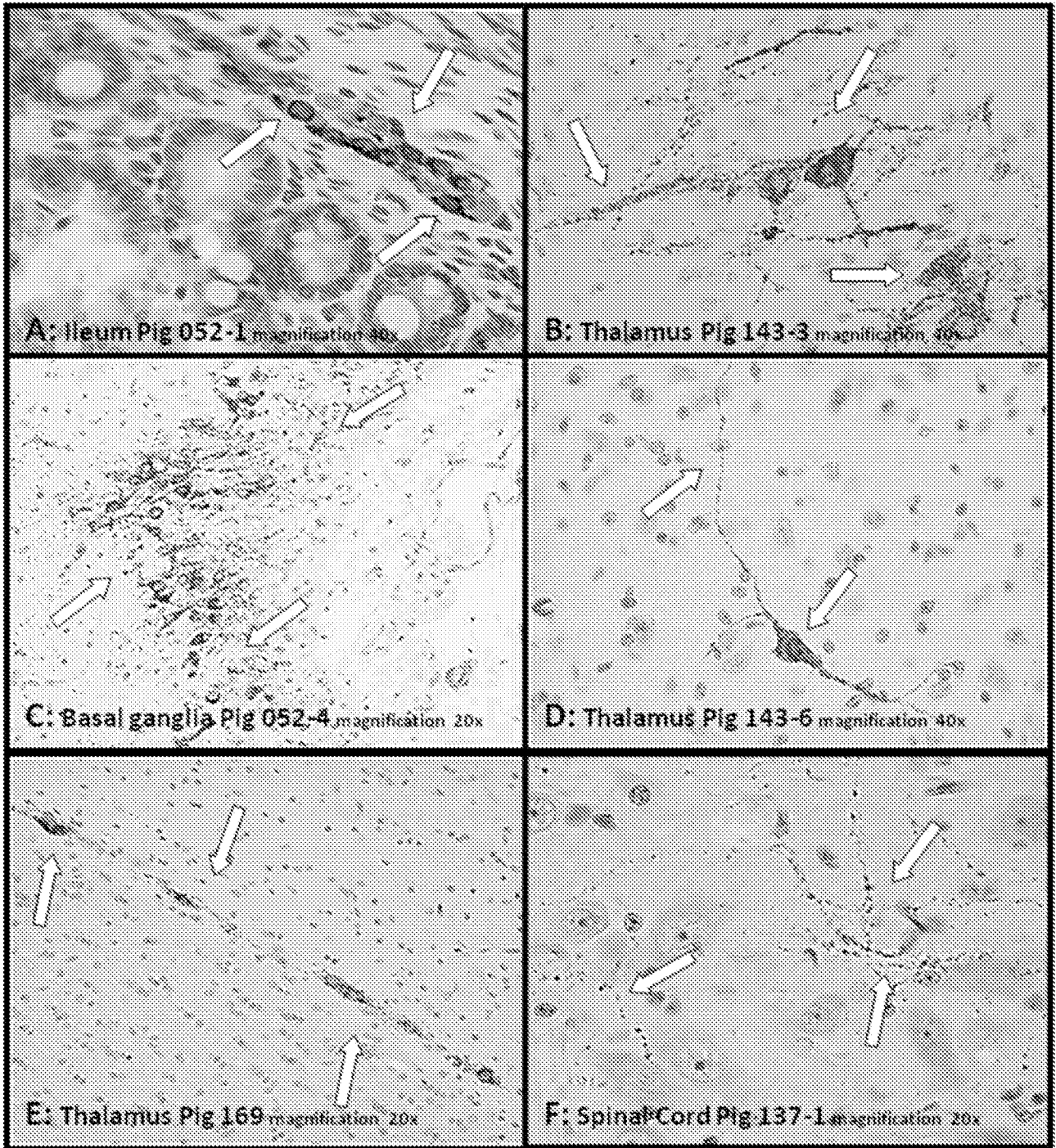


Figure 6

INTERNATIONAL SEARCH REPORT

International application No PCT/IB2013/053494

A. CLASSIFICATION OF SUBJECT MATTER INV. A01K67/027 C12N9/02 C12N15/877 C12N15/85 C12N9/00 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C12N A01K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data, BIOSIS, EMBASE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M N CHIEPPA ET AL: "Creation of a ubiquitous vector for expression of hSOD1G93A in pigs", TRANSGENIC RESEARCH, vol. 19, 1 April 2010 (2010-04-01), page 326, XP055050423, cited in the application	1-11,14,15
Y	abstract ----- -/--	12,13
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents :		
"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family	
Date of the actual completion of the international search	Date of mailing of the international search report	
25 July 2013	02/08/2013	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Brouns, Gaby	

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2013/053494

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>WATANABE S ET AL: "A Novel Method for the Production of Transgenic Cloned Pigs: Electroporation-Mediated Gene Transfer to Non-Cultured Cells and Subsequent Selection with Puromycin 1", BIOLOGY OF REPRODUCTION, NEW YORK, NY [U.A.] : ACADEM. PRESS, US, vol. 72, 1 January 2005 (2005-01-01), pages 309-315, XP003024055, ISSN: 0006-3363, DOI: 10.1095/BIOLREPROD.104.031591 page 313, paragraph 2 - page 315, paragraph 1</p>	12,13
A	<p>-----</p> <p>D. YANG ET AL: "Expression of Huntington's disease protein results in apoptotic neurons in the brains of cloned transgenic pigs", HUMAN MOLECULAR GENETICS, vol. 19, no. 20, 15 October 2010 (2010-10-15), pages 3983-3994, XP055006401, ISSN: 0964-6906, DOI: 10.1093/hmg/ddq313 page 3989 - page 3990</p>	1-15
A	<p>-----</p> <p>PETER M. KRAGH ET AL: "Hemizygous minipigs produced by random gene insertion and handmade cloning express the Alzheimer's disease-causing dominant mutation APP^{sw}", TRANSGENIC RESEARCH, vol. 18, no. 4, 1 August 2009 (2009-08-01), pages 545-558, XP055011723, ISSN: 0962-8819, DOI: 10.1007/s11248-009-9245-4 page 547, right-hand column, paragraph 2 - page 548, right-hand column, paragraph 1</p>	1-15
A	<p>-----</p> <p>CESARE GALLI ET AL: "Genetic engineering including superseding microinjection: new ways to make GM pigs", XENOTRANSPLANTATION, vol. 17, no. 6, 1 November 2010 (2010-11-01), pages 397-410, XP055007654, ISSN: 0908-665X, DOI: 10.1111/j.1399-3089.2010.00590.x page 402, left-hand column, last paragraph - page 403, right-hand column, paragraph 4</p> <p>-----</p> <p style="text-align: center;">-/--</p>	1-15

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2013/053494

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>YAQIONG HUANG ET AL: "Prospect of nuclear transfer technology in mammal animals", REMOTE SENSING, ENVIRONMENT AND TRANSPORTATION ENGINEERING (RSETE), 2011 INTERNATIONAL CONFERENCE ON, IEEE, 24 June 2011 (2011-06-24), pages 7735-7737, XP031906976, DOI: 10.1109/RSETE.2011.5966168 ISBN: 978-1-4244-9172-8 page 7735, left-hand column, last paragraph - right-hand column, paragraph 1</p> <p style="text-align: center;">-----</p>	1-15
A	<p>Randall S Prather ET AL: "Genetically Modified Pigs for Medicine and Agriculture" In: "Biotechnology and Genetic Engineering Reviews", 1 January 2008 (2008-01-01), Nottingham University Press, XP055050381, vol. 25, pages 245-266,</p> <p style="text-align: center;">-----</p>	1-15
A	<p>WO 2007/124751 A2 (UNIV AARHUS [DK]; MADSEN LONE BRUHN [DK]; BENDIXEN CHRISTIAN [DK]; LAR) 8 November 2007 (2007-11-08) example 1</p> <p style="text-align: center;">-----</p>	1-15
A	<p>E. BENDIXEN ET AL: "Advances in porcine genomics and proteomics--a toolbox for developing the pig as a model organism for molecular biomedical research", BRIEFINGS IN FUNCTIONAL GENOMICS, vol. 9, no. 3, 1 May 2010 (2010-05-01), pages 208-219, XP055050637, ISSN: 2041-2649, DOI: 10.1093/bfgp/elq004</p> <p style="text-align: center;">-----</p>	1-15
T	<p>MARIA N CHIEPPA ET AL: "Generation of pre-implantation pig SCNT embryos harboring the amyotrophic lateral sclerosis related hSOD1G93A gene", PRION, vol. 6, no. 5, 1 June 2012 (2012-06-01), page 84, XP055050413,</p> <p style="text-align: center;">-----</p>	

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Information on patent family members

International application No

PCT/IB2013/053494

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007124751 A2	08-11-2007	AU 2007245977 A1	08-11-2007
		CA 2682320 A1	08-11-2007
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