Supplementary information, Data S1 Methods

Antibodies. The following primary antibodies were used in this study: rabbit anti-SOD1 (HPA001401; Sigma), rabbit monoclonal anti-human specific SOD1 (2582-1; Epitomics), rabbit anti-ChAT (AB143; Millipore), rabbit anti-GFAP (Z0334; Dako), mouse monoclonal anti-Iba1 (MABN92; Millipore), mouse monoclonal anti-NeuN (MAB377; Millipore), mouse monoclonal anti-Ubiquitin (3936; Cell signaling), rabbit anti-Ubiquitin (UG9510; Enzo), mouse monoclonal anti-Parvalbumin (MAB1572; Millipore), rabbit anti-Calretinin (12278-1-AP, Proteintech), rabbit anti-Nogo-A (NBP1-51208; Novus), rabbit anti-NCAM (MS-204; Lab Vision or GTX101626; GeneTex), mouse monoclonal Anti-MyHC-II (M4276; Sigma), rabbit anti-ACTA1 (17521-1-AP; Proteintech), rabbit anti-Murf1 (55456-1-AP; Proteintech), rabbit anti-MAFbx (AP2041; ECM Biosciences), mouse monoclonal anti-β-actin (sc-47778; Santa Cruz), goat anti-PCBP1 (Abcam; ab133421), and mouse monoclonal anti-GAPDH (G9295; Sigma).

Protein extraction and western blot analysis. The tissues of transgenic pigs were lysed in

RIPA lysis buffer (50 mM Tris-HCl, pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml each of leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride). For analyzing the insoluble SOD1 oligomers or aggregates, 2% SDS was used to lyse transgenic pig tissues, followed by heating for 1 h at 95°C. Equal amounts of total proteins were resolved in a 10% polyacrylamide gel and transferred to PVDF membrane (Millipore, Bedford, MA, USA). The membrane was reacted with primary antibodies overnight at 4°C. Protein expression detection was done with horseradish peroxidase-conjugated goat anti-mouse or rabbit IgG secondary antibody and ECL Plus detection system (Amersham Pharmacia Biotech, Arlington Heights, IL, USA).

Estimating the copy numbers of the transgene by real-time PCR. The numbers of the transgene integrations in transgenic pigs were measured based on the method described by Mason et al (1). Standard curves of transgenic hSOD1 and endogenous control gene GAPDH were obtained by conducting real-time PCR reactions using a StepOne real-time PCR system (Applied Biosystems, Foster City, CA, USA) and three-fold serial dilutions of DNA extracted from transgenic pig ear tissues. Standard curve is a linear relationship between the log of the starting quantity (SQ) of a template and its threshold cycle (Ct) during real-time PCR. The primers and reaction system were optimized in advance to confirm the reaction efficiencies for hSOD1 and the same level for endogenous GAPDH. PCR reactions consisted of 1 × Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA), 0.4 μM of primers of hSOD1 or GAPDH, 1 μL of ROX, 0.5μL of DNA sample and water to a 20 μL final volume. For each transgenic pig, a single DNA extract was used in all experiments, and each reaction was run in triplicate wells. The cycling parameters used were as follows: one cycle at 50°C for 3 min for activation of UNG, one cycle a 95 °C for 2 min for DNA polymerase

activation, 40 cycles of 95 °C for 15 sec (denaturation), 65 °C for 30 sec (annealing), and 72 °C for 30 sec (extension). hSOD1 primer pairs used in real-time PCR are: hSOD1-qF: 5' - CCAGTGCAGGGCATCATCAAT - 3'; hSOD1-qR: 5' - TCCATGCAGGCCTTCAGTCAG - 3'; GAPDH primer pairs are: GAPDH-qF: 5' - CTTTGCCCCGCGATCTAATG - 3'; GAPDH-qR: 5' - CTCACCCGTTCACTCCGACC - 3'.

Based on standard curves and Ct value got in real-time PCR, we calculated SQ values of hSOD1 and GAPDH of each sample. From the SQ, we obtained the ratio: $r_{line} = SQ_{hSOD1}/SQ_{GAPDH}$. Since the endogenous gene GAPDH is present in two copies, we multiplied this ratios 2 times for assessing the copy number of the transgene. Virtual calibrator r_1 was used to determine the copy number for each transgenic pig. For each possible value of r_1, we defined the quantity: $F(r_1) = \Sigma lines\ [r_{line}/r_1 - N(r_{line}/r_1)]^2 / (\delta r_{line})^2$ where $N(r_{line}/r_1)$ is the nearest integer to r_{line}/r_1, δr_{line} is the uncertainty calculated from the value of triplicates for each sample. Value of r_1 is the one for which the quantity $F(r_1)$ reaches a minimum. The value of r_1 was 0.26 in this study. Once r_1 had been determined, the copy number of the transgene in each pig was determined as r_{line}/r_1.

TUNEL assay. The terminal deoxynucleotidyl transferase-mediated UTP end labeling (TUNEL) assay was performed on spinal cord and brain sections to detect apoptosis using DeadEnd™ Colorimetric Apoptosis Detection System (G7360; Promega, Madison, WI, USA). Tissue sections were deparaffinized and rehydrated in xylene and graded ethanol washes. Rehydrated tissues were permeablized using proteinase K, and the fragmented DNA of apoptotic cells was labled with biotinylated nucleotide using the terminal deoxynucleotidyl transferase (rTdT) enzyme. Horseradish peroxidase-labeled streptavidin was then bound to these biotinylated nucleotides, which were detected using DAB. DNase I (M6101; Promega, Madison, WI, USA) treatment of the fixed

tissues resulted in fragmentation of the chromosomal DNA and was included in experiment as the positive control.

Reference

(1) Mason G, Provero P, Vaira AM, Accotto GP. Estimating the number of integrations in transformed plants by quantitative real-time PCR. BMC Biotechnol 2002; 24; 2:20.