

## **Supplemental Experimental Procedures**

All experiments were carried out under licence from the Department of Health and Children (B100/3534), and following ethical approval by the Research Ethics Committee of RCSI.

### **Primary motoneuron cultures**

Primary motoneuron cultures were established from E13 C57 mouse embryos (Camu and Henderson, 1992). Spinal cord ventral horns were dissected, the ventral horn tissue cut into <1 mm pieces and incubated for 10 min in 0.025 % trypsin in Ham F10 medium (Gibco). The cells were then gently dissociated in complete medium containing 0.4 % BSA and 0.1 mg/ml DNase1 (Sigma) and seeded at  $5 \times 10^4$ /ml.

### **Immunohistochemistry**

Sections were blocked (5 % milk solution with 3 % normal serum) for 1 hr at room temperature, incubated with primary antibody (Akt-1, p-Akt-1 both 1:750, both Cell Signalling Technology), followed by secondary antibody (Rhodamine/ Fluorescein conjugated 1:500; biotinylated, 1:500, both Jackson ImmunoResearch) at room temperature for 2 hrs, and then mounted in Vectastain containing DAPI (Vector Labs). To detect serum protein leakage, sections were incubated with Alexa Fluor 594 rabbit anti-mouse IgG (1:200, Invitrogen) overnight at 4°C. Controls sections were prepared without either primary antibody or secondary antibody, and no staining was observed.

### **Real-time qPCR**

Total RNA was extracted using the RNeasy mini Kit (Qiagen). First strand cDNA synthesis was performed using 2 µg of total RNA as template and Moloney murine Leukemia virus reverse transcriptase (Invitrogen) primed with 50 pmol of random

hexamers. Quantitative real-time PCR was performed using the LightCycler (Roche Diagnostics) and the QuantiTech SYBR Green PCR kit (Qiagen). Specific primers were designed using Primer3 software ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)). Sense and antisense primers used were: CACAGATGGCCTTGATGTTG and CTCTGGCTCAGCATGACTCC for murine ANG1. The data was analysed using the LightCycler Software 4.0 with all samples normalised to  $\beta$ -actin.

### ***In vitro* models of motoneuron degeneration**

Primary motoneuron cultures at 7 days *in vitro* were treated with either AMPA (50  $\mu$ M, 24 hrs, Tocris Cookson) or tunicamycin (500 nM, Qbiogene) in complete Neurobasal medium for 24 hrs. Some cultures were co-treated with human recombinant angiogenin protein (100 ng/ml, R&D Systems), heat-denatured angiogenin, the PI-3-K inhibitor wortmannin (100 nM, Sigma) or angiogenin-conditioned medium, which was prepared from ventral horn cultures treated with angiogenin for 6 hrs, followed by extensive washing and a 18 hrs incubation in normal culture medium. All experiments were performed in cultures prepared from 12 separate donor females (n = 12). In each culture, all experimental conditions were repeated in triplicate (in at least 3 wells of plate). For analysis of Akt activation, cultures were treated with human recombinant angiogenin, IGF-1 (100 ng/ml, R&D Systems) or BSA (0.1 %) in the absence of GDNF and CNTF.

### **Cloning of angiogenin and site-directed mutagenesis**

The full length cDNA of human *ANG* was amplified by PCR (sense primer: 5'-GGAGCCTGTGTTGGAAGAGA-3'; antisense primer: 5'-TGAATGTTGCCACCACTGTT-3') and inserted into the PCR-Blunt II-TOPO vector

(Invitrogen). The *K40I* point mutation was inserted in the *ANG* sequence using QuikChange XL site-directed mutagenesis kit (Stratagene). Sense and antisense primers for the different mutations were; 5'-GGCCTGACCTCACCTGCATAGACATCAACACATTTATTC-3' and 5'-GAATAAATGTGTTGATGTCTATGCAGGGTGAGGTCAGGCC-3'. Presence of the mutation was confirmed by sequencing. The *ANG* fragment was then subcloned into pIRES2-DsRed2 vector (Clontech).

### **Expression and purification of recombinant angiogenin protein**

Two truncated variants of the human *ANG* gene, encoding either the wild type or *K40I* mutant of the mature angiogenin protein, were inserted as 0.4 kb *Bam*HI-*Eco*RI fragments into the pcDNA4-HisMaxA vector (Invitrogen). The integrity of the cloned sequences was confirmed by DNA sequencing and the resulting plasmids were designated as pcDNA4-HisMax-*ANG* and pcDNA4-HisMax-*K40I*. Purified plasmids were used to transfect subconfluent monolayers of HEK293 cells using Lipofectamine 2000 (Invitrogen), with cells being fixed for immunofluorescence or harvested at 24 hrs post-transfection. Protein expression was confirmed by immunofluorescence and immunoblotting using goat anti-angiogenin (data not shown). Polyhistidine-tagged angiogenin was purified using Ni<sup>2+</sup>-NTA resin (Qiagen) as suggested for mammalian cell lysates by the manufacturer.

### **NSC34 transient transfection and cell survival**

NSC34 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum and 1% penicillin/streptomycin solution. Cells were transiently transfected with pIRES-DsRed2 plasmids using Lipofectamine

2000 (Invitrogen) and 24 hrs later exposed to tunicamycin (500 nM, Qbiogene) for 24 hrs. Apoptosis in transfected cells (red fluorescence) was assessed according to nuclear morphology after staining with Hoechst 33258 (Sigma, 1 µg/ml). All experiments were performed in 7 to 10 independent transfection experiments (n=7-10). In each experiment all conditions were assayed in triplicate (3 wells of a plate).

### **MTT assay**

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was dissolved in PBS (5 mg/ml) and diluted 1:10 in culture medium, added to cells and incubated for 4 hrs at 37 °C. The media was then replaced with isopropanol containing 0.04 M hydrochloric acid. Emission values were then read at 570 nm. All experiments were repeated in triplicate.

### **Motoneuron survival *in vitro***

At the end of treatment, cultures were incubated in trypan blue (Sigma) for 5 min, washed in PBS and fixed with 4% paraformaldehyde. Cultures were then immunostained with antibodies to peripherin (1:2,000; Chemicon), a marker of motoneurons in cultures of ventral horn neurons from embryonic mice (Carriedo et al., 1996). Cultures were incubated in biotinylated secondary antibody (1:100; Vector Laboratories) and visualised using fast 3,3 diaminobenzadine tablets (Sigma). To quantify motoneuron death the number of peripherin and trypan blue co-stained neurons was counted, and expressed as a percentage of the total number of peripherin only-positive neurons. Experiments were carried out in cultures prepared from 12 separate donor females (n = 12). In each culture, all experimental conditions were repeated in triplicate (in at least 3 wells of plate).

### **Western blotting**

Equal amounts of protein were separated by electrophoresis and transferred to a nitrocellulose membrane followed by blocking for 1 hr (TBS, 0.1 % Tween, 3 % skim milk). Membranes were then incubated in primary antibody (Akt, phospho-Akt, ERK1/2, phospho-ERK1/2, all 1:1,000, all Cell Signalling Technology; ICAM-1, 1:500, R&D Systems),  $\beta$ -actin, 1:2,500,  $\beta$ -tubulin, 1:2,000, both Sigma). After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:500, Jackson ImmunoResearch) followed by detection using ECL.

### **Transfection with siRNA**

Primary motoneuron cultures at 7 DIV were transfected with predesigned siRNA's to murine *ANG1* (siGENOME-ON-TARGETplus, Dharmacon) using SMARTpool reagents (Dharmacon), according to manufacturer's instructions. The ability of the siRNA's to knock-down murine *ANG1* was determined in NSC34 cells using an identical transfection protocol and Western blot analysis. Controls included mock-transfected cells and cells transfected with scrambled sequences. All transfections were repeated in triplicate.

### **Treatment of *SOD1*<sup>G93A</sup> mice with ANG**

TgN[SOD1-G93A]1Gur mice were purchased from The Jackson Laboratory, and identified by genotyping (Gurney et al., 1994). Animals were injected daily with 1  $\mu$ g of human recombinant angiogenin protein (R&D systems, dissolved in sterile PBS) i.p. The dose was chosen based on a similar *in vivo* study (Lambrechts et al., 2003). For repeated administration we chose to administer human recombinant angiogenin protein by i.p. injection. Gene transfer of neurotrophic factors via retrogradely transported viral vectors has been shown to prolong the survival of *SOD1*<sup>G93A</sup> mice, however the clinical utility of such therapy remains to be determined. In addition, alternative routes, such as intrathecal

and intraventricular delivery, would involve the use of mini-osmotic pumps which require repeated surgical procedures for refilling. Delivery of human recombinant angiogenin protein offers flexible control of both the dose and the duration of treatment with the administered protein.

To examine the uptake of angiogenin protein following i.p. injection we labelled human recombinant angiogenin protein with fluorescein (EZ-label Fluorescein Protein Labelling, Pierce). Animals were sacrificed and perfused with saline followed by 4% paraformaldehyde fixative at time points after administration of labelled angiogenin. Following post-fixation, spinal cord cross sections were cut on a cryostat and sections were imaged using a Nikon Eclipse TE300 epifluorescence microscope. Fluorescently labeled angiogenin protein crossed the blood brain barrier, and was taken up in the spinal cord.

Treatment with angiogenin began from either i) 50 days of age, which is an early stage in the disease progression when motoneurons are beginning to degenerate but overt disease features have not yet manifested, or ii) 90 days of age, when disease features have manifested. For lifespan, body weight and stride length studies each experimental group contained angiogenin-treated (n = 12) and untreated SOD1<sup>G93A</sup> (n = 15) littermates, as well as untreated WT littermates (n = 15). In the group treated from 50 days, the sex of the mice in each group was as follows: angiogenin-treated SOD1 n = 12 (7 male, 5 female), untreated SOD1 n = 15 (9 male, 6 female) and untreated WT littermates n = 15 (8 males, 7 females). In the group treated from 90 days, the sex of the mice in each group was as follows: angiogenin-treated SOD1 n = 12 (8 male, 4 female), untreated SOD1 n = 15 (10 male, 5 female) and untreated WT littermates n = 15 (8 males, 7 females).

Administration of angiogenin to WT littermates for 75 days had no effect on mouse viability, breeding behaviour or motoneuron survival. Disease end stage was determined by the inability of the mouse to right itself when placed on its side, and a 20% reduction in body weight compared to body weight at 11 weeks.

### **Assessment of disease progression *in vivo***

The body weight (Kieran et al., 2004), PaGE test (Weydt et al., 2003) and stride length (Gurney et al., 1994) assessments were carried out blind, twice weekly. The PaGE test was repeated three times for each animal. Stride length was measured manually over an average of 20 steps. The tibialis anterior (TA) and extensor digitorum longus (EDL) muscles in the hindlimbs were dissected out and weighed. To measure motoneuron survival, mice were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. The lumbar region of the spinal cord (L2–L5) was removed, post-fixed and 20 µm sections cut serially on a cryostat. Sections were stained with haematoxylin and the number of surviving motoneurons in the sciatic motor pool was assessed by counting motoneurons in each ventral horn on every third section between the L2 and L5 levels of the spinal cord (Kieran et al., 2004).