Angiogenin protects motoneurons against hypoxic injury

Running title: Angiogenin protects motoneurons

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Abstract

Cells can adapt to hypoxia through the activation of hypoxia-inducible factor-1 (*HIF-1*), which in turn regulates the expression of hypoxia-responsive genes. Defects in hypoxic signalling have been suggested to underlie the degeneration of motoneurons in amyotrophic lateral sclerosis (ALS). We have recently identified mutations in the hypoxia-responsive gene angiogenin (*ANG*) in ALS patients, and have demonstrated that *ANG* is constitutively expressed in motoneurons. Here we show that although *HIF-1α* is sufficient and required to activate *ANG* during hypoxia, *ANG* expression does not change in a transgenic ALS mouse model or in sporadic ALS patients. Administration of recombinant ANG, or expression of wild-type *ANG* protected motoneurons and against hypoxic injury, while gene silencing of *ANG* significantly increased hypoxia-induced cell death. The previously reported ALS-associated *ANG* mutations (*Q12L, K17I, R31K, C39W, K40I, I46V*) all demonstrated a reduced neuroprotective activity against hypoxic injury. Our data demonstrate that *ANG* plays an important role in endogenous protective pathways of motoneurons exposed to hypoxia, and suggest that loss-of-function rather than loss-of-expression of *ANG* is associated with ALS.

Keywords: ALS, angiogenin, HIF-1α, hypoxia, motoneuron.

Abbreviations: ALS, amyotrophic lateral sclerosis; ARE, adenylate/uridylate-rich element; ANG, angiogenin; BSA, bovine serum albumin; CSF, cerebrospinal fluid; DAPI, 4',6 diamidino-2-phenylindole; DFO, deferoxamine; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; *flk-1/vegfr2*, vascular endothelial growth factor receptor 2; HIF-1, hypoxia-

inducible factor-1; HRE, hypoxic response element; HRP, horseradish peroxidase; MAP-2, microtubule-associated protein 2; NeuN, neuronal nuclei; PBS, phosphate buffered saline; SMI-32, 200 kD neurofilament heavy antibody; *SOD1*, superoxide dismutase-1; TBS, tris buffered saline; VEGF, vascular endothelial growth factor.

Introduction

Hypoxic exposure of cells or organisms induces an adaptive response to compensate for the energy imbalance to maintain cell function (1). The most well studied adaptive mechanism is the expression of a cohort of hypoxia-responsive genes through the activation of the hypoxia-inducible factor-1 (HIF-1) (2, 3). Hypoxia leads to the accumulation of the α subunit of HIF-1 (HIF-1α). Heterodimerization of HIF-1α with its partner HIF-1β results in the formation of the HIF-1 complex. It binds to the hypoxic response element (HRE) that is present in all HIF target genes described to date (5'-RCGTG-3'), and enhances their transcription. While hypoxic exposure quickly stabilizes HIF-1α protein, normoxic exposure leads to immediate degradation of HIF-1α through hydroxylation of proline residues with subsequent ubiquitination and proteosomal degradation (4).

Angiogenin (ANG) is an evolutionary highly conserved 123-residue, 14.1 kDa member of the pancreatic ribonuclease A (RNaseA) superfamily. It is a potent inducer of neovascularization (5, 6, 7) and a hypoxia-inducible factor (8, 9, 10). We have recently reported 7 missense mutations in the *ANG* gene in 15 patients suffering from amyotrophic lateral sclerosis (ALS), 4 with familial and 11 with apparently 'sporadic' ALS, in 5 distinct populations (11). ALS is a progressive late-onset neurodegenerative disorder with a fatal outcome, characterised by relatively selective motoneuron loss in

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the spinal cord, brain stem and motor cortex, resulting in progressive paralysis and death (12).

A role for hypoxia-responsive genes in the pathogenesis of ALS was first suggested by the finding that mice with deletions in the HRE of the *vascular endothelial growth factor (vegf)* gene develop an ALS-like disease of adult-onset motoneuron degeneration and paralysis (13). Furthermore, VEGF delivery protected motoneurons from degeneration in both *in vitro* and *in vivo* models of ALS (14, 15). However, mutations in *VEGF* or indeed genes involved in HIF signalling have not been identified so far in ALS patients (16).

The present study was therefore conducted to explore whether ANG is capable of protecting motoneurons against hypoxic injury or is required for their survival under hypoxic conditions, and to determine whether changes in *ANG* expression can be detected during ALS-associated motoneuron degeneration. Furthermore, we wished to explore the effect of reported *ANG* mutations on hypoxic injury. Our results suggest that ANG activity greatly influences motoneuron survival in response to hypoxia, and that a loss-of-function, rather than loss-of-expression of *ANG* plays an important role in motoneuron degeneration seen in ALS.

Results

ANG is a hypoxia inducible factor in motoneurons

We could recently demonstrate that ANG is expressed in motoneurons *in vitro* and *in vivo* (11). To study the regulation of ANG in motoneurons under hypoxic conditions, primary mouse motoneuron cultures were exposed to atmospheric hypoxia of 10%,

3% and 1% O_2 for 24 hrs. Quantitative real-time PCR analysis of murine *ang1* mRNA expression, the murine ortholog of human *ANG* (17), showed strong induction of this gene under hypoxic conditions of 10%, 3% and 1% O_2 (**Fig. 1A**). In parallel we also examined *vegf* mRNA induction in these cultures. We noted a comparable *vegf* mrRNA induction in the motoneurons exposed to atmospheric hypoxia of 10%, 3% and 1% O_2 for 24 hrs (**Fig. 1B**). Moreover, a time course analysis of *ang1* mRNA induction in motoneurons under conditions of 1% O_2 over 24 hrs showed a time-dependent increase in expression (**Fig. 1C**), in a similar fashion to *vegf* mRNA induction (**Fig. 1D**). In addition, Western blotting analysis confirmed the hypoxia-induced increase in expression of ANG and VEGF proteins over time (**Fig. 1E**). NSC34 is a hybrid cell line obtained from embryonic mouse spinal cord and mouse neuroblastoma cells with characteristics of primary motoneurons, including generation of action potentials and acetylcholine synthesis, storage and release (18). In another set of experiments, we transfected NSC34 cells with luciferase reporter constructs containing two mouse *Ang1* promoters. Promoter 1 (Pr1) is universally active, while Promoter 2 (Pr2) is active only in hepatic cells in promoter assays *in vitro* (19) and which served as a negative control. Under hypoxic conditions of 1% O_2 for 24 hrs, activity from Pr1 was increased more than 6-fold over empty vector alone (**Fig. 1F**). We obtained similar results when cells were exposed to the HIF-1α stabilizer DFO, an iron chelator commonly used as a hypoxia-mimetic agent. In accordance with its hepatic specificity, Pr2 did not show any significant activity in NSC34 cells under hypoxia or DFO exposure (**Fig. 1F**).

Hypoxia-induced expression of ANG is associated with HIF-1α

We next investigated whether HIF-1α stabilisation was sufficient to activate murine *ang1*. There was strong HIF-1α stabilization in primary motoneurons under hypoxic conditions, already detectable after 4 hrs at 1% O_2 (**Fig. 2A**). Next, we expressed a constitutively active HIF-1α mutant in motoneurons and determined murine *Ang1* expression. This HIF-1α double mutant is constitutively active due to mutated residues P564A and P402A, the proline residues targeted for hydroxylation and degradation in normoxia). We found that in motoneurons expressing constitutively active HIF-1α there was a significant increase in *Ang1* mRNA fold induction even under normoxic conditions **(Fig. 2B)**.

We next examined the effect of endogenous *HIF-1α* knockdown on *ANG* expression. *HIF-1α* knockdown was successfully achieved in HeLa cells using siRNA transfection (**Fig. 2C**). In cells transfected with siRNA targeting *HIF-1α*, hypoxia-induced *ANG* mRNA transcription was significantly reduced when compared to cells transfected with a scrambled sequence **(Fig. 2D)**. Together, these findings supported the hypothesis that HIF-1α stabilisation is sufficient and required for hypoxia-induced *ANG* gene activation.

ANG levels are not altered in motoneurons from *SOD1^{G93A}* mice or ALS patients
In a previous study we could detect significant ANG expression in motoneurons (11), a finding subsequently confirmed in other studies (20, 21). Indeed, immunostaining with antibodies to ANG in adult murine spinal cord cross sections showed strong expression in motoneurons, in particular in the cytosolic compartment, and a less intense staining in non-neuronal cells (**Fig. 3A**). However, the staining showed no significant differences between *SOD1^{G93A}* and wild-type mice spinal cord samples

when examined at disease onset (90 days) and disease end stage. To further elucidate whether differences in *ang1* expression can be detected in this murine ALS model, we next compared the induction of murine *ang1* mRNA in lumbar spinal cord and motor cortex homogenates from $SOD1^{G93A}$ mice and their wild-type littermates **(Figs. 3B-C)**. Mice tissue samples were taken at 90 days of age (after symptom onset) or at disease end-stage (127 to 132 days). We did not observe any significant differences in *Ang1* mRNA expression between wild-type and $SOD1^{G93A}$ spinal cord **(Fig. 3B)** or motor cortex samples **(Fig. 3C)**. On the other hand, mRNA levels of both *vegf* and *flk-1/vegfr2* decreased significantly in spinal cord from $SOD1^{G93A}$ mice **(Fig. 3D,F)**, in agreement with previous findings (22).

Using *post mortem* spinal cord cross sections from ALS and non-ALS patients (see **Supplementary Table 1** for case details), we also detected ANG expression in the cytoplasm of human anterior horn motoneurons **(Fig. 4A)**. Semi-quantitative analysis of ANG staining intensity in the cell bodies of anterior horn motoneurons was assessed semi-quantitatively using a 4-point scale (23), and the results suggested no significant difference between ALS and non-ALS spinal cord samples **(Fig. 4B)**.

Exogenously added ANG rescues cultured motoneurons from hypoxia-induced cell death

We next examined whether ANG is active against hypoxia-induced motoneuron death *in vitro*. Neuronal and motoneuron survivals were analyzed in primary motoneuron cultures after exposure to hypoxic conditions of 1% O_2 for 24 hrs using the trypan blue exclusion method. Survivals of both motoneurons and the total neuronal pool decreased significantly following exposure to hypoxic conditions, such that only 45%

($\pm$ 3.0 S.E.M., p<0.05, n = 3) of MAP-2-immunopositive neurons and 36% ($\pm$ 4.6 S.E.M., p<0.05, n = 3) of peripherin-immunopositive motoneurons survived **(Fig. 5A)**, confirming the enhanced vulnerability of motoneurons in response to hypoxia (24, 25). To determine the neuroprotective effect of treatment with ANG, primary motoneuron cultures were exposed to hypoxic conditions of 1% O_2 for 24 hrs and treated with either 100 ng/ml of human recombinant ANG protein, vehicle (BSA 0.1%) or heat-denatured ANG. Following treatment, we examined motoneuron survival and found that addition of human recombinant ANG protein significantly increased motoneuron survival to 65% ($\pm$ 2.9 S.E.M., p<0.05, n = 12), compared to only 35% ($\pm$ 3.8 S.E.M., p<0.05, n = 12) in vehicle-treated cultures and 36% ($\pm$ 4.2 S.E.M., p<0.05, n = 12) in heat denatured ANG-treated cultures **(Figs. 5B-C)**.

Knockdown of *Ang1* enhances hypoxia-induced cell death

To explore the role of endogenous *ang1* in the response of motoneurons to hypoxia, we investigated the effect of gene silencing on the survival of NSC34 cells, which have an increased endogenous resistance to hypoxia. Gene silencing was successfully achieved in NSC34 cells using siRNA and tested by means of PCR **(Fig. 6A)**. In cells transfected with siRNA targeting *ang1*, hypoxia-induced injury significantly increased compared to mock-transfected cells or cells transfected with a scrambled sequence **(Figs. 6B-D)**, suggesting that *ang1* is required for maintaining motoneuron survival under hypoxic conditions.

Neuroprotective effect of *ANG* against hypoxic injury is lost in the ALS-associated mutants

We next investigated the effects of the ALS-related *ANG* mutants *K40I*, *R31K*, *K17I*, *Q12L*, *I46V* and *C39W* (11) on hypoxia-induced cell death. The *ANG* mutants were cloned into a mammalian expression vector also expressing the red fluorescent protein DsRed2 (pIRES2). Transfection of NSC34 cells with pIRES2-DsRed2 constructs containing any of these *ANG* mutations **(Fig. 7A)** did not reduce the levels of apoptosis in response to hypoxia when compared to empty vector-transfected cells, while wild-type *ANG*-overexpressing cells showed significantly reduced levels of apoptosis **(Fig. 7B-C)**.

Discussion

In the present study we provide evidence that murine *ang1* mRNA and protein is upregulated in motoneurons in response to hypoxia, and that HIF-1α is sufficient and required to upregulate *ANG* expression during hypoxia. An involvement of HIF-1α in *ANG* upregulation during hypoxia has so far not been directly demonstrated to our knowledge, but was expected owing to the existence of a consensus HRE (5'-RCGTG-3') in the murine *ang1* and human *ANG* promoter. The expression of murine *ang1* in response to hypoxia *in vitro* mirrored that of another HIF-1α target gene, *vegf*. We could also demonstrate a potent downregulation of *vegf* mRNA in the spinal cord of $SOD1^{G93A}$ transgenic mice *in vivo*, but interestingly we could not detect a concomitant decrease in murine *ang1* expression. We also detected a down-regulation of *flk-1* expression during disease progression. It has been suggested that an impairment to appropriately activate hypoxia-inducible factors like VEGF may be involved in disease progression in $SOD1^{G93A}$ mice (26) and sporadic ALS in humans (27). Significant reductions in expression of VEGF and its major receptor, Flk-1 have been seen on motoneurons in the spinal cord of patients with ALS (22). It is possible

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that the murine *ang1* gene is constitutively expressed at higher levels and/or subject to positive regulation by other transcription factors. In addition to HIF1-α, other hypoxia-responsive transcription factors may also contribute to a differentially regulated gene expression *in vivo* (28). It is also possible that the effects observed *in vivo* are more complex and subject to other regulatory events. It has been shown that mutant SOD1-linked ALS is associated with a destabilisation of *vegf* mRNA and with a downregulation of its expression (29). This negative effect was mediated through a specific interaction with the adenylate/uridylate-rich elements (AREs) of the 3'-untranslated region of this gene. It is possible that the downregulation of *vegf* mRNA expression in mutant SOD1-expressing cells is indeed mediated via a specific inactivation at the level of the 3-UTR of this particular gene, and not due to a general inability to activate HIF1-α target genes.

Interestingly, we also found no evidence for differences in ANG levels in motoneurons of patients who suffered from the sporadic form of this disease. This finding is also confirmed by a recent study performed in CSF ANG levels in ALS patients (30), and by our previous study demonstrating no downregulation of ANG serum levels in ALS patients at diagnosis (31), but rather a small, but statistically significant up-regulation of serum ANG levels in ALS patients. The latter study also demonstrated that there was no correlation between serum ANG and VEGF levels. Although further studies may be required to elucidate changes in ANG levels in the spinal cord or CSF in ALS patients, the data from the present study and from previous reports, argue against a major role for ANG down-regulation in mutant SOD1-induced motoneuron degeneration and in sporadic ALS.

Our study rather suggests that motoneuron degeneration may be triggered by a loss-of-function of the neuroprotective properties of the *ANG* gene. Motoneurons are particularly vulnerable to the inhibition of cellular bioenergetics that occurs during cellular hypoxia. In both *in vivo* and *in vitro* studies, motoneurons in particular have been shown to be more sensitive to short periods of oxygen deprivation than other spinal and central neurons (24, 25). In this study we provide evidence that ANG has significant neuroprotective activities on motoneurons exposed to hypoxic conditions *in vitro*. We also demonstrate that murine *ang1* was required for the survival of motoneuron-like NSC34 cells under hypoxic conditions. Our findings therefore support the hypothesis that hypoxia-inducible factors such as ANG and VEGF may have direct effects on motoneuron survival (32). Finally, we demonstrate that the *ANG* mutations reported in our previous study (11) (*K40I, Q12L, K17I, R31K* and *C39W*), including the *I46V* mutation (33) lack the neuroprotective activity against hypoxic exposure shown by wild-type *ANG*. These results are in accordance with a recent study, which showed that 3 of the identified *ANG*-ALS variants (*Q12L, C39W, K40I*) did not protect P19 embryonal carcinoma cells from hypoxic cell death (34). Based on the crystal structure of ANG, most of these point mutations affect functionally important residues, evolutionary highly conserved in ANG, Rnase A or both, and are involved in ANG nuclear import, nuclear localisation or ribonucleolytic activity (11, 35). Indeed, subsequent genetic and biochemical studies have identified further mutations in *ANG* in ALS patients (21, 33, 36), and have suggested that these may interfere with nuclear localisation, ribonucleolytic, and angiogenic activity in endothelial cells (20, 21).

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In summary, our results demonstrate that angiogenin plays a crucial role in the survival of motoneurons in response to hypoxia, and that loss-of-function rather than loss-of-expression of *ANG* may be involved in ALS.

Materials and methods

All experiments detailed here were carried out under licence from the Government of Ireland, Department of Health and Children and with ethical approval from the Royal College of Surgeons in Ireland Research Ethics Committee.

Cell culture. Primary motoneuron cultures were prepared from E13 mouse embryos. Donor animals were terminally anaesthetized and embryos removed by hysterectomy. Spinal cord ventral horns were dissected from individual embryos, and the tissue was cut into <1 mm pieces and incubated for 10 min in 0.025% trypsin in Ham F10 modified medium (Invitrogen, Paisley, Strathclyde, UK). The cells were then transferred into complete medium containing 0.4% BSA and 0.1 mg/ml DNAse1 (both from Sigma-Aldrich, Tallaght, Dublin, Ireland), and gently dissociated. The cell suspension was spun and re-suspended in complete neurobasal medium. Cells were seeded onto poly-$_{D,L}$-ornithine/laminin coated cell culture wells and maintained at 37 ºC and 5% CO_2.

Motoneuronal NSC34 cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin solution. HeLa cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin solution.

Hypoxic conditions. Cells were placed in one of three hypoxia chambers (Coy Laboratory Products, Grass Lake, MI, USA) allowing the establishment of graded,

humidified, ambient, atmospheric hypoxia of 10%, 3%, and 1% O_2, with 5% CO_2 and a balance of N_2 in all cases. Temperature was maintained at 37°C.

Immunological stainings. Human spinal cord sections were obtained from the MRC Brain Bank, Kings College London, UK. Sections were deparaffinised in xylene before antigen retrieval was performed using citrate buffer (pH 6). Human or mouse spinal cord sections and cell cultures were immunostained using similar protocols. Sections or cultures were blocked (5% milk solution with 3% normal serum) for 1 hr at room temperature, incubated with primary antibody (ANG 1:500, Abcam, Cambridge, UK; NeuN 1:500, Chemicon, Harrow, UK; Peripherin 1:2,000, Chemicon; MAP-2 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA; SMI-32 1:500, Abcam) overnight at 4°C, followed by secondary antibody (Rhodamine/ Fluorescein-conjugated 1:500, Jackson ImmunoResearch, Plymouth, PA, USA) at room temperature for 2 hrs, and then mounted in Vectastain containing 4',6 diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). Controls were prepared without either primary or secondary antibody, and no staining was observed.

Semi-quantitative analysis of immunohistochemistry. The intensity of ANG staining in the cell bodies of anterior horn motoneurons from human spinal cord cross sections was assessed semi-quantitatively using a 4-point scale (23): zero = staining absent, + = weak, ++ = moderate, +++ = strong. Immunoreactivity was considered weak (+) if it was poorly apparent at low-power magnification, but identifiable using the high-power objective. Moderate (++) and strong (+++) staining reactions were apparent at low power. Five fields (area = 0.125 mm^2) were assessed in each anterior horn. The number of motoneurons in each staining category was then expressed as a proportion of the total number of motoneurons counted in order to control for variation in the

absolute number of motoneurons examined. Statistical analysis was assessed using the Fisher's exact test.

Western blotting. Equal amounts of protein were separated by electrophoresis, and transferred to a nitrocellulose membrane followed by blocking for 1 hr (TBS containing 0.1% Tween, 3% skim milk). Membranes were then incubated in primary antibody to ANG (1:500, Abcam), VEGF (1:1,000, Abcam), murine HIF-1α (1:1,000, Biomol, Exeter, UK), human HIF-1α (1:500, BD Biosciences, Oxford, UK), DsRed protein (1:200, BD Biosciences Clontech) or ß-actin (1:2,500, Sigma-Aldrich) overnight at 4ºC. After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000, Jackson ImmunoResearch) at room temperature for 2 hrs, followed by detection using ECL detection reagent (Amersham Biosciences, Buckinghamshire, UK).

Analysis of mRNA expression. Total RNA was extracted from cell cultures using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and from tissue homogenates using the TRIZOL Reagent (Invitrogen). First strand cDNA synthesis was performed according to manufacturers' instruction using 2 μg Moloney murine Leukaemia virus reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using the LightCycler (Roche Diagnostics, Basel, Switzerland) and the QuantiTech SYBR Green PCR kit (Qiagen). Sense and antisense primers, respectively, were as follows: human *ANG*, 5'-GGGCGTTTTGTTGTTGGTCT-3' ; 5'-GCGCTTGTTGCCATGAATAA-3'. Human *β-actin,* 5'-TCACCCACACTGTGCCCATCTACGA-3' ; 5'-CAGCGGAACCGCTCATTGCCAATGG-3'. Murine *ang1*, 5'-TCCTGACTCAGCACCATGAC-3' ; 5'-TCTGTAAGGGCTTCCATTCG-3'. Murine *vegf*, 5'-GTACCTCCACCATGCCAAGT-3' ; 5'-

GCATTCACATCTGCTGTGCT-3'. Murine *flk-1*, 5'-CAGCTTCCAAGTGGCTAAGG-3' ; 5'-CAGAGCAACACACCGAAAGA-3'. Murine *β-actin*, 5'-AGGTGTGATGGTGGGAATGG-3' ; 5'-GGTTGGCCTTAGGGTTCAGG-3'. Each primer pair was tested with a logarithmic dilution of a cDNA mix to generate a linear standard curve, which was used to calculate the primer pair efficiency. The PCR reactions were performed in 20 µl volumes with the following parameters: 95°C for 15 min followed by 35 cycles of 94°C for 20 s, 59°C for 20 s, 72°C for 20 s. The generation of specific PCR products was confirmed by melting curve analysis and gel electrophoresis. The data was analysed using the Lightcycler Software 4.0 with all samples normalized to *β-actin*. All experiments were performed in triplicate.

Cloning of ANG 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). Point mutations were inserted in the *ANG* sequence using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Sense and antisense primers for the different mutations were: *K40I*: 5'-GGCCTGACCTCACCCTGCATAGACATCAACACATTTATTC-3' and 5'-GAATAAATGTGTTGATGTCTATGCAGGGTGAGGTCAGGCC-3'. *R31K*: 5'-GTGAAAGCATCATGAAGAGACGGGGCCTGAC-3' and 5'-GTCAGGCCCCGTCTCTTCATGATGCTTTCAC-3'. *K17I*: 5'-CAGCACTATGATGCCATACCACAGGGCCGGGATG-3' and 5'-CATCCCGGCCCTGTGGTATGGCATCATAGTGCTG-3'. *Q12L*: 5'-CACACACTTCCTGACCCTGCACTATGATGCCAAAC-3' and 5'-GTTTGGCATCATAGTGCAGGGTCAGGAAGTGTGTG-3'. *I46V*: 5'-

CAAAGACATCAACACATTTGTTCATGGCAACAAGCGCAG-3' and 5'-CTGCGCTTGTTGCCATGAACAAATGTGTTGATGTCTTTG-3'. *C39W*: 5'-CCTGACCTCACCCTGGAAAGACATCAACAC-3' and 5'-GTGTTGATGTCTTTCCAGGGTGAGGTCAGG-3'. Presence of the mutations was confirmed by sequencing. The *ANG* fragment was then subcloned into the pIRES2-DsRed2 vector (BD Biosciences Clontech).

Promoter reporter assays. NSC34 cells were plated at 75,000 cells per well in a 24-well plate 24 hrs prior to transfection. Cells were then transiently cotransfected with 0.3 g per well of the pGL3-derived constructs (pGL3 Basic, vector only; pGL3-Pr1, *Ang1* Pr1 promoter; pGL3-Pr2, *Ang1* Pr2 promoter) and 0.025 g per well of the phRL-TK control reporter (19). Transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation, and 24 hrs after transfection cells were exposed to hypoxia (1% O_2) or deferoxamine (DFO, Sigma-Aldrich). The Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA) was used to determine transcriptional activity of the reporter constructs according to manufacturer's suggestions. The firefly luciferase (experimental promoter construct) activities of each of the experimental constructs were normalized to the Renilla luciferase (co-transfectant control promoter construct) activity. All experiments were performed in triplicate.

HIF-1α overexpression. Primary motoneuron cultures were seeded in 6-well plates and transfected with either 2 µg per well of pcDNA3-*HIF-1α* wild-type, pcDNA3-*HIF-1α* double mutant or pcDNA3 empty vector, as previously described (37). The double mutant form of HIF-1α is constitutively active due to mutations at both Pro-564 and Pro-402 (the residues targeted for hydroxylation and degradation in normoxia). Cells were transfected using the Lipofectamine 2000 reagent in OptiMEM

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medium (Invitrogen) according to the manufacturer's instructions. Transfection was carried out for 24 hrs, changing then the medium to standard growth medium. After 24 hrs, cultures were harvested immediately for RNA extraction.

siRNA transfection. For *ang1* knockdown, NSC34 cells were plated on 24-well plates, grown to ~50% confluence and transfected with 100 nM of predesigned siRNA to murine *Ang1* (siGENOME-ON-TARGETplus, Dharmacon, Lafayette, CO, USA). For *HIF-1α* knockdown, HeLa cells were plated on 35mm plates, grown to ~50% confluence and transfected with 5 nM human specific *HIF-1α* siRNA (Dharmacon). Transfections were performed using Lipofectamine 2000 in antibiotic free media according to manufacturer's instructions. As a control, the same concentration of non target siRNA (Dharmacon) was used for each transfection. All transfections were repeated in triplicate.

NSC34 transient transfection and cell survival. Cells were transfected with pIRES2-DsRed2 plasmids using Lipofectamine 2000 and 24 hrs later exposed to hypoxia (1% O_2) for 24 or 48 hrs. Cell survival in transfected cells (red fluorescence) was assessed according to nuclear morphology after staining with Hoechst 33258 (1 g/ml, Sigma-Aldrich). Hoechst staining was used to quantify only strongly condensed and/or highly fragmented nuclei, late and key hallmarks of apoptosis. All experiments were repeated in triplicate.

Motoneuron viability. At the end of treatment cultures were incubated in trypan blue (Sigma-Aldrich) for 5 min, washed in PBS and fixed with 4% paraformaldehyde in 0.1 M PBS. Fixed cells on coverslips were then immunostained with antibodies to the motoneuron-specific markers peripherin (Chemicon) or SMI-32 (Abcam). Only motoneurons stained with the specific marker and containing no trypan blue were considered viable and counted.

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Acknowledgements

Figure legends

Figure 1. ANG expression is induced under hypoxia in motoneurons. **A-B,** Quantitative real-time PCR analysis showing murine *Ang1* **(A)** and *VEGF* **(B)** transcription levels in primary motoneurons exposed to atmospheric hypoxia of 10%, 3% and 1% O_2 for 24 hrs (*$P<0.05$, mean ± S.E.M. from 3 independent experiments). **C-D,** Time course analysis of *Ang1* **(C)** and *VEGF* **(D)** mRNA increase under exposure to 1% O_2 (*$P<0.05$, mean ± S.E.M. from 3 independent experiments). **E,** Representative Western blot analysis of ANG and VEGF protein levels after exposure of motoneurons to 1% O_2. **F,** Luciferase reporter assays showing *Ang1* promoter activity in NSC34 cells. Cell cultures were transfected with pGL3 reporter constructs containing Promoter 1 (Pr1) and Promoter 2 (Pr2) from mouse *Ang1* and then exposed to hypoxia (1% O_2) or deferoxamine (DFO, 100 M) for 24 hrs. Values are normalized to pGL3 basic, which lacks a promoter (*$P<0.01$ vs control Pr1, mean ± S.E.M. from 3 independent experiments).

Figure 2. Hypoxia induces ANG expression in a HIF-1α-dependent manner. A, Representative Western blot showing HIF-1α stabilization in motoneurons upon hypoxic exposure. **B,** *Ang1* mRNA levels are increased in motoneurons overexpressing wild-type HIF-1α or constitutively active (CA) HIF-1α (mutated at the oxygen-dependent degradation residues Pro-564 and Pro-402). Cells were transfected for 24 hrs, changing then the medium to standard growth medium. After 24 hrs, cultures were harvested immediately for RNA extraction and quantitative real-time PCR was carried out (*$P<0.05$ vs control untransfected cells, mean ± S.E.M. from 3 independent experiments). **C,** Representative Western blot showing effective silencing of HIF-1α following siRNA transfection in HeLa cells. **D,** Endogenous

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HIF-1α knockdown by siRNA in HeLa cells significantly reduced hypoxia-induced *ANG* mRNA transcription (*P<0.01 vs scramble, mean ± S.E.M. from 3 independent experiments).

Figure 3. ANG expression is not altered in motoneurons from *SOD1^{G93A}* mice. A, Immunostaining of adult spinal cord cross sections showing ANG expression in motoneurons from wild-type (left) and *SOD1^{G93A}* mice (right, scale bar = 50 µm). **B-G,** Quantitative real-time PCR analysis showing murine *Ang1* **(B-C)**, *VEGF* **(D-E)** and *Flk-1* **(F-G)** transcription levels in lumbar spinal cord **(B, D, F)** and motor cortex **(C, E, G)** homogenates from *SOD1^{G93A}* mice and wild-type littermates (mean ± S.E.M., n = 9).

Figure 4. ANG expression is not altered in *post mortem* spinal cord samples from ALS patients. A, Immunostaining of human spinal cord cross sections from ALS and non-ALS patients showing ANG expression (red) in anterior horn motoneurons. (Scale bar = 50 m). **B,** Immunohistochemistry quantitation. Intensity of ANG staining in the cell bodies of anterior horn motoneurons from human spinal cord cross sections was assessed using a 4-point scale: 0 = staining absent, + = weak, ++ = moderate, +++ = strong. P( ), proportion of anterior horn motoneurons showing 0, +, ++, or +++ staining intensity.

Figure 5. Hypoxia-induced cell death in motoneuron cultures can be alleviated by ANG. A, Direct counts of neuron survival (MAP-2-positive cells) and motoneuron survival (peripherin-positive cells) in primary ventral horn motoneuron cultures exposed to hypoxia (1% O_2) for 24 hrs (*P<0.05 vs sister cultures at atmospheric

oxygen, mean ± S.E.M. from 3 independent experiments). **B,** Direct counts of motoneuron survival (peripherin-positive cells) in primary motoneuron cultures exposed to hypoxia (1% O_2) for 24 hrs. Cultures were treated with ANG (100 ng/ml), heat denatured ANG (100 ng/ml) or vehicle (*P<0.05 vs vehicle or denatured ANG-treated cultures, mean ± S.E.M. from 3 independent experiments). **C,** Representative photomicrographs of SMI-32- (green, **a-c, g-i**), peripherin (red, **d-f, j-l**) and DAPI- (blue) immunostained primary motoneuron cultures. Cultures were treated with ANG (100 ng/ml), denatured ANG (100 ng/ml) or vehicle and exposed to hypoxia (1% O_2, **g-l**) for 24 hrs. (Scale bar = 20 m).

Figure 6. Hypoxia-induced cell death is potentiated by knockdown of *ang1*. A, Quantitative real-time PCR analysis showing knockdown of endogenous murine *ang1* by siRNA in NSC34 cells exposed to normoxia or 1% O_2 for 24 hrs (*P<0.01 vs scramble, mean ± S.E.M. from 3 independent experiments). **B-C,** Nuclear morphology in NSC34 motoneuron-like cells transfected with murine *ang1* siRNA and exposed to 1% O_2 for 24 **(B)** or 48 hrs **(C)** was assessed after Hoechst 33258 staining. (*P<0.01 vs hypoxia-treated mock/scrambled cultures, mean ± S.E.M. from 3 independent experiments). **D,** Nuclear morphology assessed by Hoechst 33258. Scale bar = 10 m.

Figure 7. Loss of neuroprotective activity against hypoxia of ALS-related *ANG* mutations. A, Western blot analysis showing DsRed2 and human ANG levels in NSC34 cells transiently transfected with pIRES-DsRed2 constructs alone or containing the different *ANG* mutants. **B,** NSC34 cells transiently transfected with pIRES2-DsRed2/*K40I, R31K, K17I, Q12L, I46V* or *C39W ANG* do not show a

significantly different response to hypoxia (1% O$_2$ for 48 hrs) than cells transfected with empty vector pIRES2-DsRed2. Nuclear morphology was assessed after Hoechst 33258 staining. (*P<0.01 vs hypoxia-treated empty vector, mean ± S.E.M. from 3 independent experiments). **C,** Representative photomicrographs of NSC34 cells transfected with empty vector pIRES2-DsRed2 or containing *ANG* or *K40I ANG*. Nuclear morphology was assessed after Hoechst 33258 staining (Scale bar = 20 m).
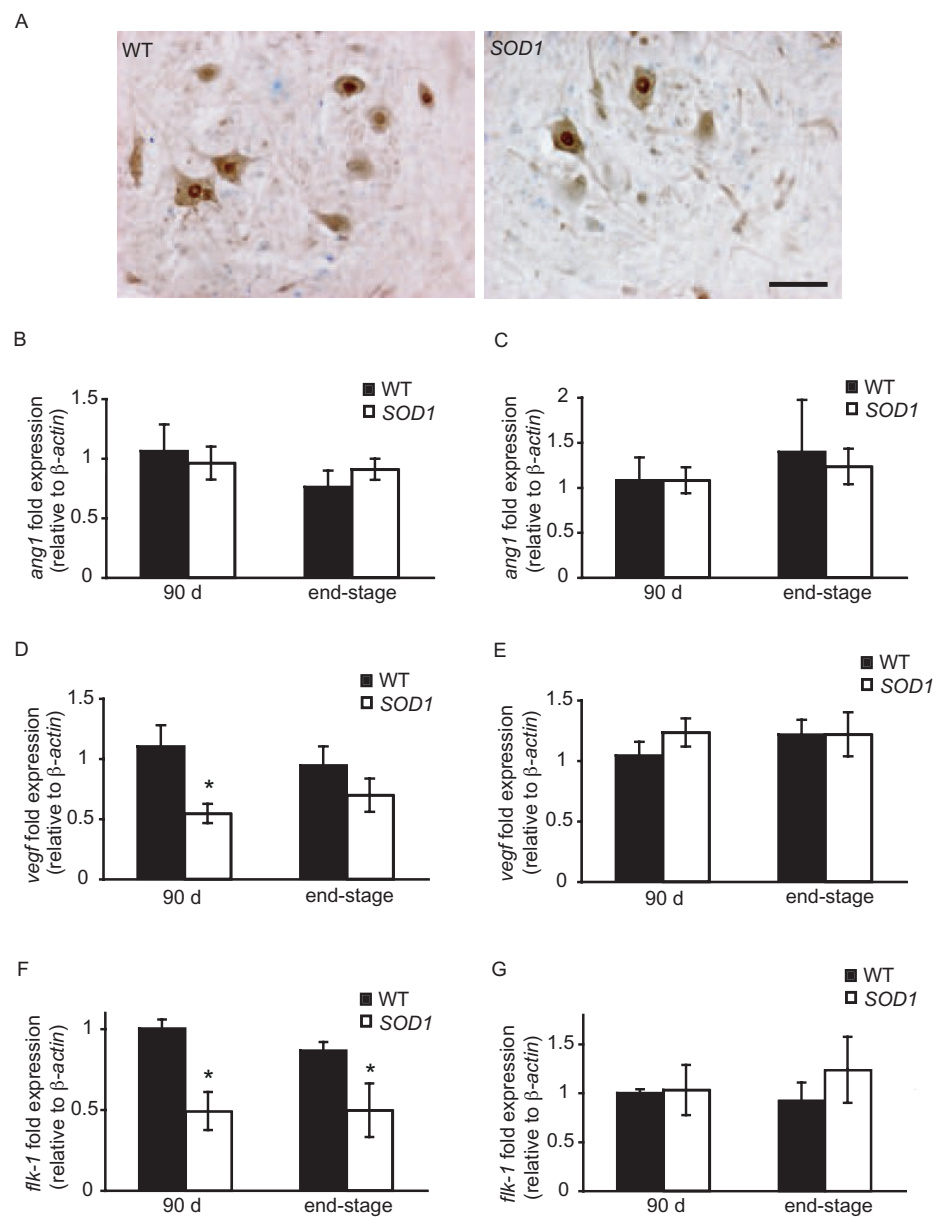
Figure 1

Figure 2

A

HIF-1α

β-Actin

| 4 | 8 | 24 | Ctrl |

1% Oxygen (hrs)

B

C

HIF-1α

β-Actin

| 0 | 0 | 30 | 30 | 1% Oxygen (hrs) |
| - | + | - | + | HIF-1α siRNA |

D

Figure 3

Figure 4

A

B

Group	Measure	P(0)	P(+)	P(++)	P(+++)
Controls	Median	0.06	0.17	0.44	0.32
	S.D.	0.09	0.08	0.13	0.17
ALS	Median	0.09	0.08	0.55	0.26
	S.D.	0.07	0.11	0.23	0.19
	p value	>0.5	>0.5	>0.5	>0.5

P( ), proportion of anterior horn motoneurons showing 0, +, ++, or +++
staining intensity.

Figure 5

SMI-32   Peripherin   DAPI

Figure 6

Figure 7

A

B

C