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Angiogenin protects motoneurons against hypoxic injury

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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 signaling 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 shown that *ANG* is constitutively expressed in motoneurons. Here, we show that *HIF-1α* is sufficient and required to activate *ANG* in cultured motoneurons exposed to hypoxia, although *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 against hypoxic injury, whereas gene silencing of *ang1* significantly increased hypoxia-induced cell death. The previously reported ALS-associated *ANG* mutations (*Q12L*, *K17I*, *R31K*, *C39W*, *K40I*, *I46V*) all showed a reduced neuroprotective activity against hypoxic injury. Our data show 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.

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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. Although 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 evolutionarily highly conserved 123-residue, 14.1 kDa member of the pancreatic ribonuclease A (RNase) superfamily. It is a potent inducer of neovascularization[5–7] and induced by hyopxia.[8–10] 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, characterized by relatively selective motoneuron loss in 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, vascular endothelial growth factor (VEGF) delivery protected motoneurons from degeneration in both *in vitro* and *in vivo* models of ALS.[14,15] However, mutations in *VEGF* or other genes involved in HIF signaling have not been identified so far in ALS patients.[16]

This 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

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Abbreviations: ALS, amyotrophic lateral sclerosis; ANG, angiogenin; ARE, adenylate/uridylate-rich element; 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; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NeuN, neuronal nuclei; PBS, phosphate-buffered saline; SMI-32, 200 kDa neurofilament heavy antibody; *SOD1*, superoxide dismutase-1; TBS, Tris-buffered saline; VEGF, vascular endothelial growth factor

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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 show 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 h. 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 (Figure 1a). In parallel, we also examined *vegf* mRNA induction in these cultures. We noted a comparable *vegf* mRNA induction in the motoneurons exposed to atmospheric hypoxia of 10, 3 and 1% O_2 for 24 h (Figure 1b). A time course analysis of *ang1* mRNA induction in motoneurons under conditions of 1% O_2 over 24 h showed a time-dependent increase in expression (Figure 1c), in a manner similar to *vegf* mRNA induction (Figure 1d). In addition, western blotting analysis confirmed the hypoxia-induced increase in the expression of ANG and VEGF proteins over time (Figure 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, whereas 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 h, activity from Pr1 was increased more than six-fold over empty vector alone (Figure 1f). We obtained similar results when cells were

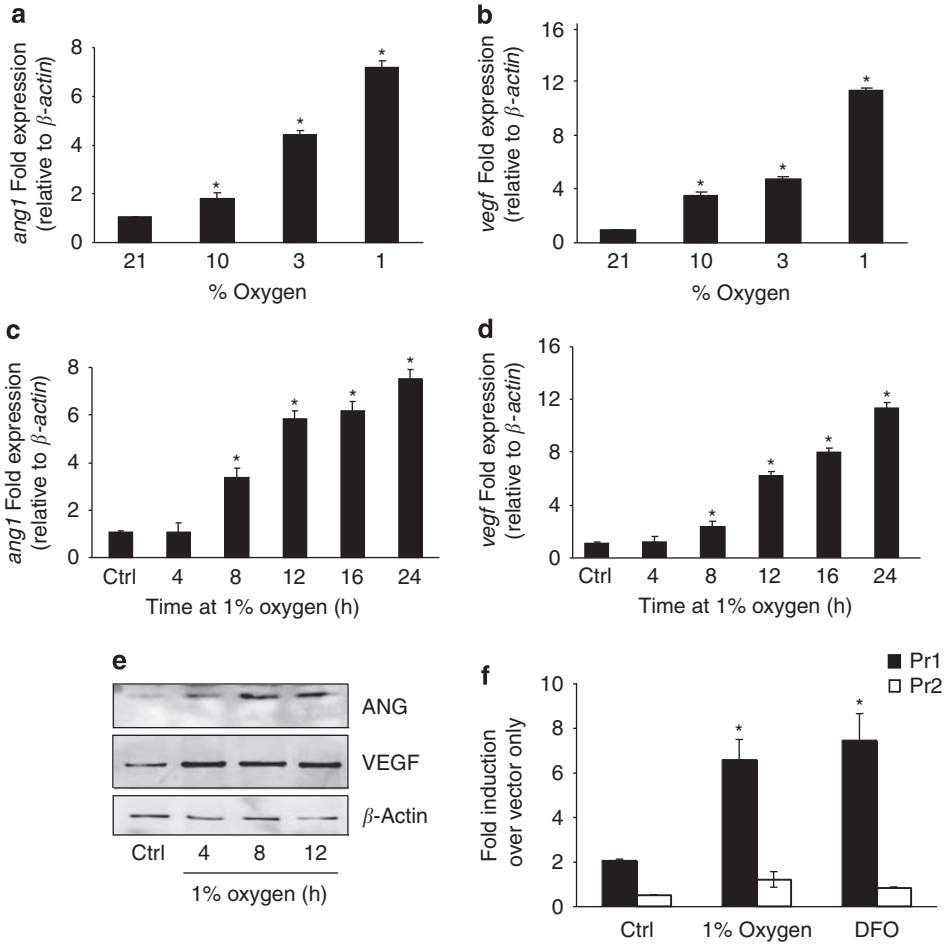


Figure 1 ANG expression is induced under hypoxia in motoneurons. (**a** and **b**) Quantitative real-time PCR analysis showing murine *ang1* (**a**) and *vegf* (**b**) transcription levels in primary motoneurons exposed to atmospheric hypoxia of 10, 3 and 1% O_2 for 24 h (*$P<0.05$, mean ± S.E.M. from three independent experiments). (**c** and **d**) Time course analysis of *ang1* (**c**) and *vegf* (**d**) mRNA increases under exposure to 1% O_2 (*$P<0.05$, mean ± S.E.M. from three 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 μM) for 24 h. Values are normalized to pGL3 basic, which lacks a promoter (*$P<0.01$ *versus* control Pr1, mean ± S.E.M. from three independent experiments)

exposed to the HIF-1α stabilizer deferoxamine (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 (Figure 1f).

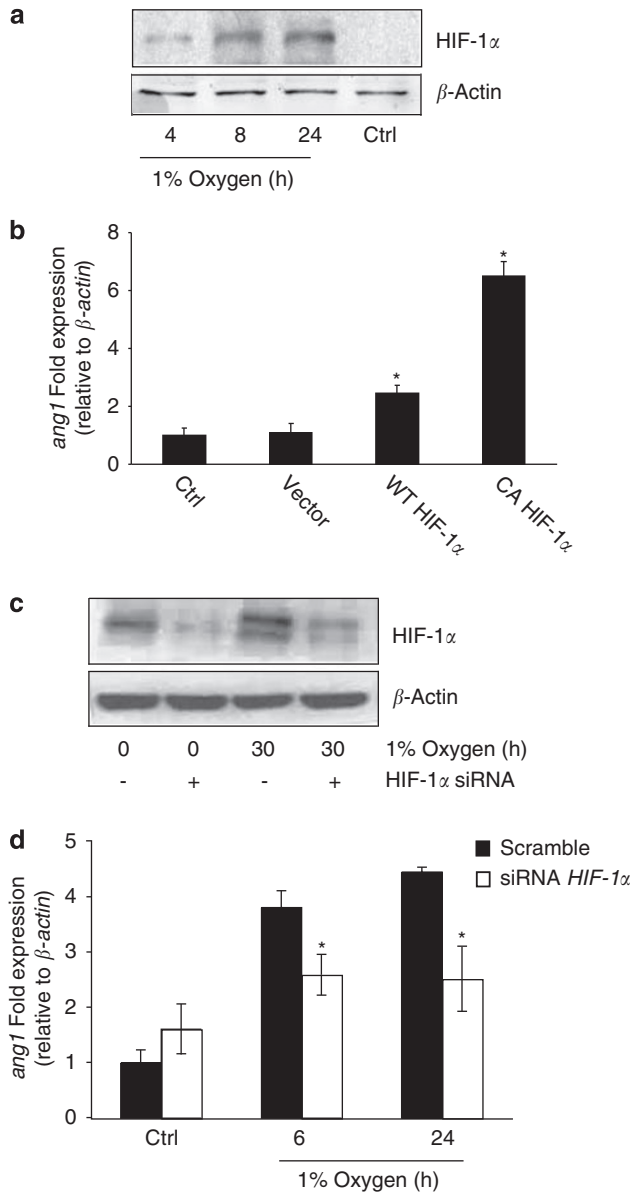


Figure 2 Hypoxia induces ANG expression in a HIF-1α-dependent manner. (**a**) Representative western blot showing HIF-1α stabilization in motoneurons upon hypoxic exposure. (**b**) *ang1* mRNA levels are increased in motoneurons overexpressing wild-type HIF-1α or constitutively active (CA) HIF-1α (mutated at the oxygen-dependent degradation residues Pro-564 and Pro-402). Cells were transfected for 24 h and subsequently cultured in standard growth medium. After 24 h, the cultures were harvested immediately for RNA extraction, and quantitative real-time PCR was carried out (*$P<0.05$ *versus* control untransfected cells, mean ± S.E.M. from three independent experiments). (**c**) Representative western blot showing effective silencing of HIF-1α after siRNA transfection in HeLa cells. (**d**) Endogenous *HIF-1α* knockdown by siRNA in HeLa cells significantly reduced hypoxia-induced *ang1* mRNA transcription (*$P<0.01$ *versus* scramble, mean ± S.E.M. from three independent experiments)

Hypoxia-induced expression of ANG is associated with HIF-1α. We next investigated whether HIF-1α stabilization was sufficient to activate murine *ang1*. There was strong HIF-1α stabilization in primary motoneurons under hypoxic conditions, already detectable after 4 h at 1% O_2 (Figure 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 induction even under normoxic conditions (Figure 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 (Figure 2c). In cells transfected with siRNA targeting *HIF-1α*, hypoxia-induced *Ang* mRNA transcription was significantly reduced when compared with those transfected with a scrambled sequence (Figure 2d). Together, these findings supported the hypothesis that HIF-1α stabilization is sufficient and required for hypoxia-induced *ANG* gene activation.

ANG levels are not altered in motoneurons from *SOD1^{G93A}* mice or ALS patients. Next, we were interested to determine whether the HIF1-α-dependent upregulation of ANG under hypoxic conditions was impaired in motoneuron cultures derived from ALS-transgenic mice. We carried out a quantitative real-time PCR analysis of *ang1* and *vegf* mRNA expression in primary motoneuron cultures derived from *SOD1^{G93A}* mouse embryos under hypoxic conditions of 10, 3 and 1% O_2. After 24 h of hypoxia, no differences in *ang1* and *vegf* upregulation were detected between wild-type and *SOD1^{G93A}* motoneuron cultures (Figure 3).

In an earlier 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 (Figure 4a). 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 an *in vivo* 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 (Figure 4b and c). Mouse tissue samples were taken at 90 days of age (after symptom onset) or at disease end stage (127–132 days). We did not observe any significant differences in *ang1* mRNA expression between wild-type and *SOD1^{G93A}* spinal cord (Figure 4b) or motor cortex samples (Figure 4c). On the other hand, mRNA levels of both *vegf* and *flk-1/vegfr2* decreased significantly in the spinal cord from *SOD1^{G93A}* mice (Figure 4d and f), in agreement with earlier findings.[22]

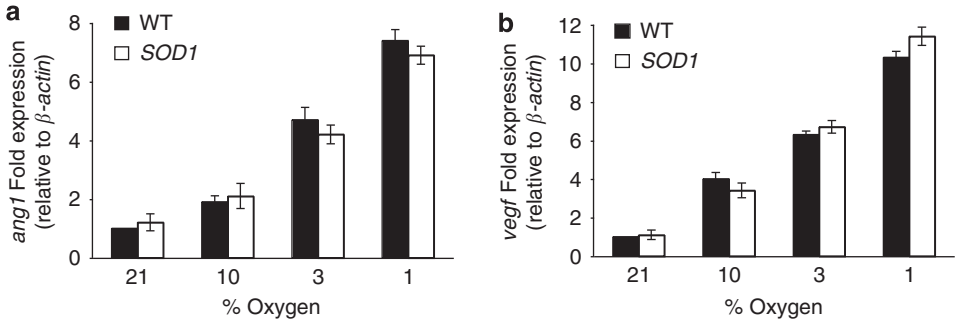


Figure 3 Wild-type and *SOD1*^{G93A} motoneurons show comparable levels of *ang1* and *vegf* upregulation under hypoxic conditions. Quantitative real-time PCR analysis showing murine *ang1* (**a**) and *vegf* (**b**) transcription levels in primary motoneuron cultures derived from wild-type and *SOD1*^{G93A} mice embryos exposed to atmospheric hypoxia of 10, 3 and 1 O_2 for 24 h (mean ± S.E.M. from three independent experiments)

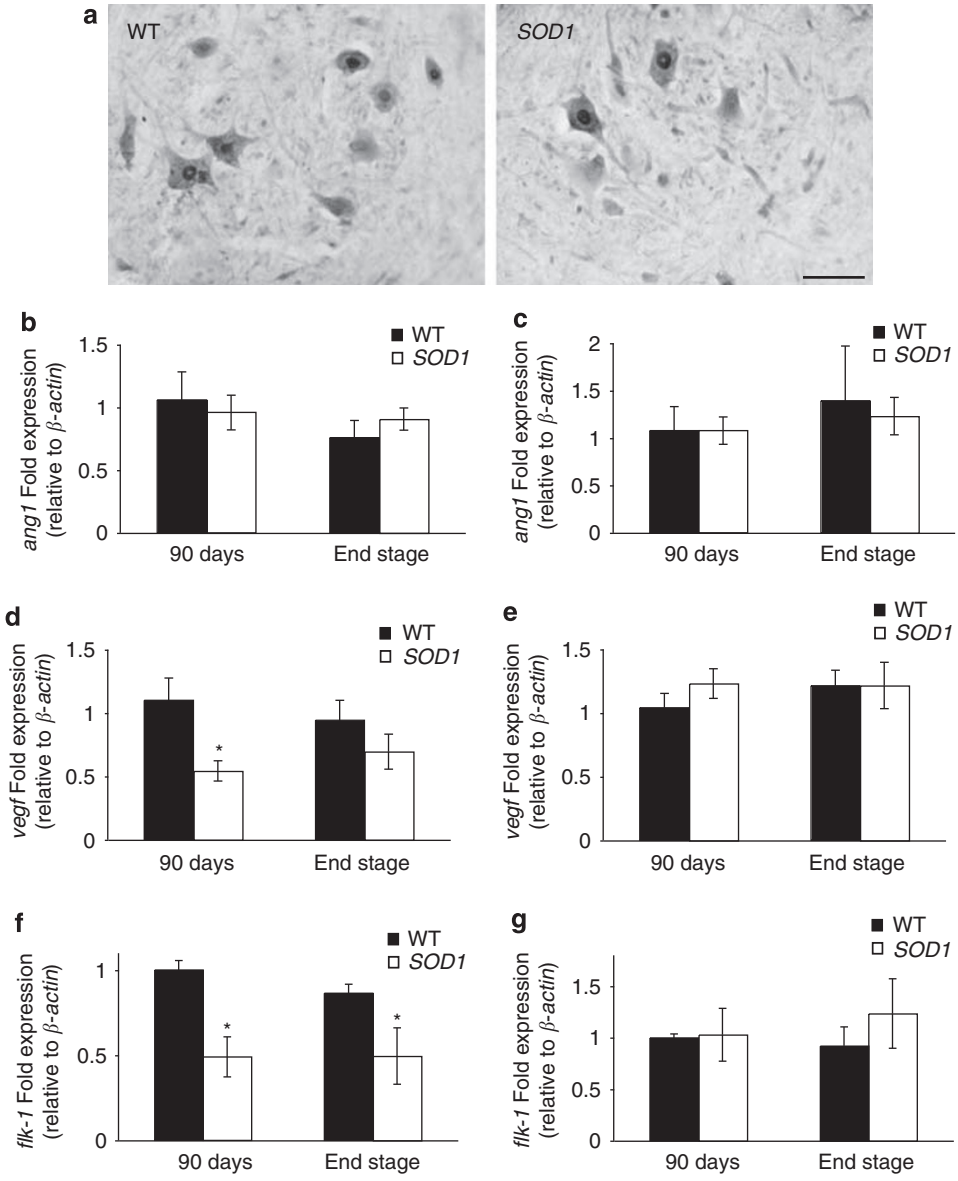
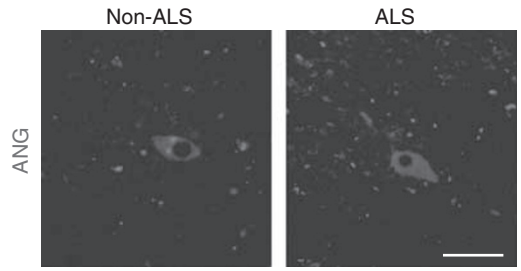


Figure 4 ANG expression is not altered in motoneurons from *SOD1*^{G93A} mice. (**a**) Immunostaining of adult spinal cord cross-sections showing ANG expression in motoneurons from wild-type (left panel) and *SOD1*^{G93A} mice (right panel, scale bar = 50 μm). (**b**–**g**) Quantitative real-time PCR analysis showing murine *ang1* (**b**) and (**c**), *vegf* (**d** and **e**) and *flk-1* (**f** and **g**) transcription levels in lumbar spinal cord (**b**, **d** and **f**) and motor cortex (**c**, **e** and **g**) homogenates from *SOD1*^{G93A} mice and wild-type littermates (*$P<0.05$ *versus* wild-type, mean ± S.E.M., $n=9$)

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 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 = 0.5 mm). (**b**) Immunohistochemistry quantitation. Intensity of ANG staining in the cell bodies of anterior horn motoneurons from the human spinal cord cross-sections was assessed using a 4-point scale: 0 = staining absent; + = weak; + + = moderate; and + + + = strong. P( ), proportion of anterior horn motoneurons showing 0, +, + + or + + + staining intensity (sections from $n = 11$ ALS patients; $n = 10$ non-ALS patients)

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 (Figure 5a). Semiquantitative analysis of ANG staining intensity in the cell bodies of anterior horn motoneurons was assessed semiquantitatively using a 4-point scale,[23] and the results suggested no significant difference between ALS and non-ALS spinal cord samples (Figure 5b).

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*. Primary motoneuron cultures exposed to 1% O_2 showed a decrease in viability over time, as seen with the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) reduction assay (Figure 6A). Neuronal and motoneuron survivals were then analyzed in primary motoneuron cultures after exposure to hypoxic conditions of 1% O_2 for 24 h using the trypan blue exclusion method. Survivals of both motoneurons and the total neuronal pool decreased significantly after exposure to hypoxic conditions, such that only 45% (±3.0 S.E.M., $P<0.05$, $n=3$) of microtubule-associated protein 2 (MAP-2)-immunopositive neurons and 36% (±4.6 S.E.M., $P<0.05$, $n=3$) of peripherin-immunopositive motoneurons survived (Figure 6B), 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 h and treated with 100 ng/ml of human recombinant ANG protein, vehicle (bovine serum albumin (BSA) 0.1%) or heat-denatured ANG. After treatment, we examined motoneuron survival and found that the addition of human recombinant ANG protein significantly increased motoneuron survival to 65% (±2.9 S.E.M., $P<0.05$, $n=12$), compared with only 35% (±3.8 S.E.M., $P<0.05$, $n=12$) in vehicle-treated cultures and 36% (±4.2 S.E.M., $P<0.05$, $n=12$) in heat-denatured ANG-treated cultures (Figure 6C and D).

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 (Figure 7a). In cells transfected with siRNA targeting *ang1*, hypoxia-induced injury significantly increased compared with mock-transfected cells or cells transfected with a scrambled sequence (Figure 7b–f), 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 (Figure 8a) did not reduce the levels of apoptosis in response to hypoxia when compared with empty vector-transfected cells, whereas wild-type *ANG*-overexpressing cells showed significantly reduced levels of apoptosis (Figure 8b and c). In order to test whether this effect was restricted to motoneuron-like cells, we performed similar experiments in rat pheochromocytoma cells (PC12). Interestingly, this cell type also showed significantly reduced levels of hypoxia-induced apoptosis after transfection with pIRES2-DsRed2 constructs containing wild-type *ANG*, whereas the *K40I* or *R31K* mutant forms did not reduce the levels of apoptosis in response to hypoxia when compared with empty vector-transfected cells (Supplementary Figure 1).

Discussion

In this study, we provide evidence that murine *ang1* mRNA and protein are upregulated in motoneurons in response to hypoxia, and that HIF-1α is sufficient and required to upregulate *ANG* expression during hypoxia. To our knowledge, an involvement of HIF-1α in *ANG* upregulation during hypoxia has so far not been directly shown, but was expected owing to the existence of a consensus HRE (5′-RCGTG-3′) in the murine *ang1* and human *ANG* promoter. The expression

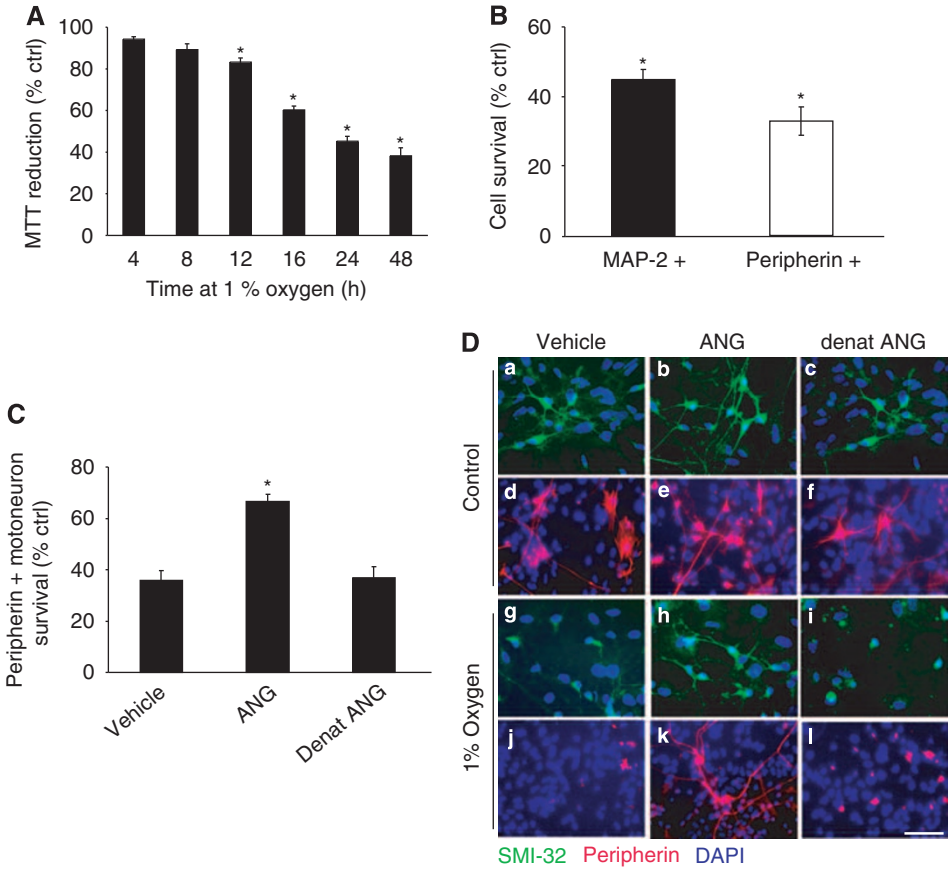


Figure 6 Hypoxia-induced cell death in motoneuron cultures can be alleviated by ANG. (**A**) Time course analysis showing MTT reduction in primary motoneuron cultures under exposure to 1% O_2 (*$P<0.05$ *versus* cultures at atmospheric oxygen, mean ± S.E.M. from three independent experiments). (**B**) 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 h (*$P<0.05$ *versus* sister cultures at atmospheric oxygen, mean ± S.E.M. from three independent experiments). (**C**) Direct counts of motoneuron survival (peripherin-positive cells) in primary motoneuron cultures exposed to hypoxia (1% O_2) for 24 h. Cultures were treated with ANG (100 ng/ml), heat-denatured ANG (100 ng/ml) or vehicle (*$P<0.05$ *versus* vehicle or denatured ANG-treated cultures, mean ± S.E.M. from three independent experiments). (**D**) 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 h. (scale bar = 20 μm)

of murine *ang1* in response to hypoxia *in vitro* mirrored that of another HIF-1α target gene, *vegf*. We also detected no significant difference in *ang1* and *vegf* upregulation under hypoxic conditions between wild-type and $SOD1^{G93A}$ motoneuron cultures. This finding suggests that, in this *in vitro* system, expression of $SOD1^{G93A}$ does not impair hypoxia signaling. *In vivo*, we could show a potent downregulation of *vegf* mRNA in the spinal cord of $SOD1^{G93A}$ transgenic mice, but we could not detect a concomitant decrease in murine *ang1* expression. We also detected a downregulation of *flk-1* expression during disease progression. It has been suggested that an impairment to appropriately activate HIFs such as 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 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] However, the effects observed *in vivo* may be more complex and subject to other regulatory events. It has been shown that mutant *SOD1*-linked ALS is associated with a destabilization 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 through 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.

It is interesting that 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 carried out in cerebrospinal fluid (CSF) ANG levels in ALS patients,[30] and by our earlier study showing no downregulation of ANG serum levels in ALS patients at diagnosis,[31] but rather a small, but statistically significant upregulation of serum ANG levels in ALS patients. The latter study also showed that there was no correlation

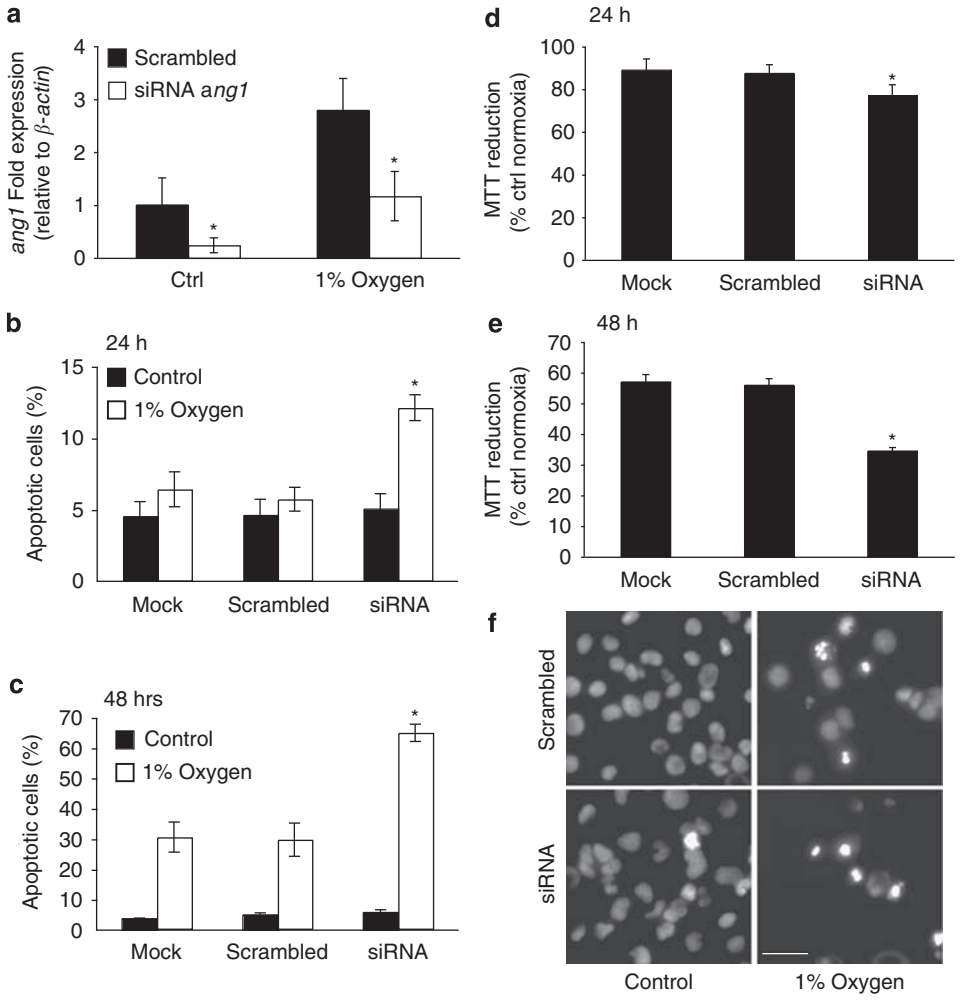


Figure 7 Hypoxia-induced cell death is potentiated by knockdown of *ang1*. (**a**) Quantitative real-time PCR analysis showing knockdown of endogenous murine *ang1* by siRNA in NSC34 motoneuron-like cells exposed to normoxia or 1% O_2 for 24 h (*$P<0.01$ *versus* scramble, mean ± S.E.M. from three independent experiments). (**b** and **c**) Nuclear morphology in NSC34 cells transfected with murine *ang1* siRNA and exposed to 1% O_2 for 24 (**b**) or 48 h (**c**) was assessed after Hoechst 33258 staining (*$P<0.01$ *versus* hypoxia-treated mock/scrambled cultures, mean ± S.E.M. from three independent experiments). (**d** and **e**) Cell viability in NSC34 cells transfected with murine *ang1* siRNA and exposed to 1% O_2 for 24 (**d**) or 48 h (**e**) was assessed with the MTT assay (*$P<0.01$ *versus* hypoxia-treated mock/scrambled cultures, mean ± S.E.M. from three independent experiments). (**f**) Nuclear morphology assessed by Hoechst 33258. Scale bar = 10 μm

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 this study and from earlier reports argue against a major role for ANG downregulation 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, and express particularly high levels of *ANG*.[11] 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 show that murine *ang1* was required for the survival of motoneuron-like NSC34 cells under hypoxic conditions. Our findings therefore support the hypothesis that HIF targets such as Ang and VEGF may have direct effects on motoneuron survival.[32] Finally, we show that the *ANG* mutations reported in our earlier 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 three of the identified *ANG*-ALS variants (*Q12L*, *C39W*, *K40I*) did not protect P19 embryonal carcinoma cells from hypoxic cell death.[34] On the basis of the crystal structure of ANG, most of these point mutations affect functionally important residues, evolutionarily highly conserved in ANG, Rnase A or both, and are involved in ANG nuclear import, nuclear localization 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

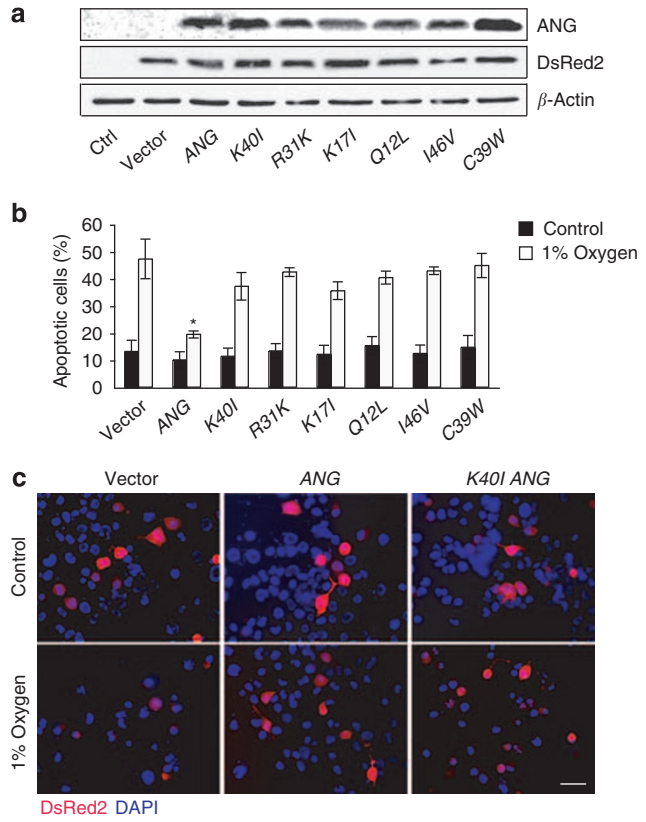


Figure 8 Loss of neuroprotective activity against hypoxia of ALS-related ANG mutations. (**a**) Western blot analysis showing DsRed2 and human ANG levels in NSC34 cells transiently transfected with pIRES-DsRed2 constructs alone or containing different *ANG* mutants. (**b**) NSC34 cells transiently transfected 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 h) than cells transfected with empty vector pIRES2-DsRed2. Nuclear morphology was assessed after Hoechst 33258 staining (*$P<0.01$ *versus* hypoxia-treated empty vector, mean ± S.E.M. from three 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 μm)

with nuclear localization, ribonucleolytic and angiogenic activity in endothelial cells.[20,21]

In summary, our results show that ANG 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 license 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 anesthetized and embryos removed by hysterectomy. Spinal cord ventral horns were dissected from individual embryos, and the tissue was cut into <1 mm slices 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 DNAse 1 (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°C 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. Rat pheochromocytoma PC12 cells were grown in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% horse serum (Sigma-Aldrich), 5% FBS, 1% glutamine (2 mM) 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). The sections were deparaffinized 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 h at room temperature, incubated with primary antibodies (ANG 1 : 500, Abcam, Cambridge, UK; NeuN 1 : 500, Chemicon, Harrow, UK; Peripherin 1 : 2000, Chemicon; MAP-2 1 : 500, Santa Cruz Biotechnology, Santa Cruz, CA, USA; SMI-32 1 : 500, Abcam) overnight at 4°C, followed by a secondary antibody (rhodamine/fluorescein-conjugated 1 : 500, Jackson ImmunoResearch, Plymouth, PA, USA) at room temperature for 2 h, and then mounted in Vectastain containing 4′,6 diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). Controls were prepared without either a primary or a secondary antibody, and no staining was observed.

Semiquantitative analysis of immunohistochemistry. The intensity of ANG staining in the cell bodies of anterior horn motoneurons from human spinal cord cross-sections was assessed semiquantitatively using a 4-point scale:[23] zero = staining absent, + = weak, ++ = moderate and +++ = 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. Anterior horn motoneurons were identified based on morphology and location within the spinal cord anterior horn, only motoneurons with an evident nucleus and a diameter >0.25 mm were included in counts. 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 Fisher's exact test (sections from $n=11$ ALS patients and $n=10$ non-ALS patients, see Supplementary Table 1).

Western blotting. Equal amounts of protein were separated by electrophoresis and transferred to a nitrocellulose membrane followed by blocking for 1 h (TBS (Tris-buffered saline) containing 0.1% Tween, 3% skim milk). The membranes were then incubated in the following primary antibodies ANG (1 : 500, Abcam), VEGF (1 : 1000, Abcam), murine HIF-1α (1 : 1000, Biomol, Exeter, UK), human HIF-1α (1 : 500, BD Biosciences, Oxford, UK), DsRed protein (1 : 200, BD Biosciences Clontech) or β-actin (1 : 2500, Sigma-Aldrich) overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1 : 5000, Jackson ImmunoResearch) at room temperature for 2 h, followed by detection using enhanced chemiluminescence (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 carried out according to the manufacturer's instruction using 2 μg Moloney murine leukemia 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′ and 5′-GCGCTTGTTGC CATGAATAA-3′; human *β-actin*, 5′-TCACCCACACTGTGCCCATCTACGA-3′ and

5′-CAGCGGAACCGCTCATTGCCAATGG-3′; murine *ang1*, 5′-TCCTGACTCAGC ACCATGAC-3′ and 5′-TCTGTAAGGGCTTCCATTCG-3′; murine *vegf*, 5′-GTACC TCCACCATGCCAAGT-3′ and 5′-GCATTCACATCTGCTGTGCT-3′; murine *flk-1*, 5′-CAGCTTCCAAGTGGCTAAGG-3′ and 5′-CAGAGCAACACACCGAAAGA-3′; murine *β-actin*, 5′-AGGTGTGATGGTGGGAATGG-3′; and 5′-GGTTGGCCTTAGG GTTCAGG-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 PCRs were performed in 20 μl volumes with the following parameters: 95 °C for 15 min followed by 35 cycles at 94 °C for 20 s, at 59 °C for 20 s and at 72 °C for 20 s. The generation of specific PCR products was confirmed by melting curve analysis and gel electrophoresis. The data were analyzed 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′-GGAGCCTGTGT TGGAAGAGA-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′-GGCCTGACCTCACCCTGCATAGACATCAACACATTTA TTC-3′ and 5′-GAATAAATGTGTTGATGTCTATGCAGGGTGAGGTCAGGCC-3′; *R31K*: 5′-GTGAAAGCATCATGAAGAGACGGGGCCTGAC-3′ and 5′-GTCAGGC CCCGTCTCTTCATGATGCTTTCAC-3′; *K17I*: 5′-CAGCACTATGATGCCATACCA CAGGGCCGGGATG-3′ and 5′-CATCCCGGCCCTGTGGTATGGCATCATAGTGC TG-3′; *Q12L*: 5′-CACACACTTCCTGACCCTGCACTATGATGCCAAAC-3′ and 5′-GTTTGGCATCATAGTGCAGGGTCAGGAAGTGTGTG-3′; *I46V*: 5′-CAAAGAC ATCAACACATTTGTTCATGGCAACAAGCGCAG-3′ and 5′-CTGCGCTTGTTGCC ATGAACAAATGTGTTGATGTCTTTG-3′; and *C39W*: 5′-CCTGACCTCACCCTGG AAAGACATCAACAC-3′ and 5′-GTGTTGATGTCTTTCCAGGGTGAGGTCAGG-3′. The 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 h before transfection. The cells were then transiently co-transfected with 0.3 μg per well of the pGL3-derived constructs (pGL3 Basic, vector only; pGL3-Pr1, *ang1* Pr1 promoter; and pGL3-Pr2, *ang1* Pr2 promoter) and with 0.025 μg 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 h after transfection, the cells were exposed to hypoxia (1% O_2) or DFO (Sigma-Aldrich). The Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA) was used to determine transcriptional activity of the reporter constructs according to the 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 six-well plates and transfected after 5 days *in vitro* with 2 μg per well of pcDNA3-*HIF-1α* wild-type, pcDNA3-*HIF-1α* double mutant or pcDNA3 empty vector, as described earlier.[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 medium (Invitrogen; 2 μl of Lipofectamine in 250 μl OptiMEM). Once all DNA were added, the cultures were incubated for 4 h, when the medium was replaced with standard growth medium. This reduced incubation time decreased Lipofectamine-induced cell toxicity (Supplementary Figure 2). After 24 h, 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 35 mm 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 the manufacturer's instructions. As a control, the same concentration of nontarget siRNA (Dharmacon) was used for each transfection. All transfections were repeated in triplicate.

Transient transfection and cell survival. NSC34 and PC12 cells were transfected with pIRES2-DsRed2 plasmids using Lipofectamine 2000 and 24 h later exposed to hypoxia (1% O_2). Cell survival in transfected cells (red fluorescence) was assessed according to nuclear morphology after staining with Hoechst 33258 (1 μg/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 phosphate-buffered saline (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.

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

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)