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Targeting angiogenin in therapy of amyotropic lateral sclerosis

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Abstract

Background—Missense heterozygous mutations in the coding region of angiogenin (*ANG*) gene, encoding a 14 kDa angiogenic RNase, were recently found in patients of amyotropic lateral sclerosis (ALS). Functional analyses have shown that these are loss-of-function mutations, implying that angiogenin deficiency is associated with ALS pathogenesis and that increasing *ANG* expression or angiogenin activity could be a novel approach for ALS therapy.

Objective—Review the evidence showing the involvement of angiogenin in motor neuron physiology and function, and provide a rationale for targeting angiogenin in ALS therapy.

Methods—Review the current understanding of the mechanism of angiogenin action in connection with ALS genetics, pathogenesis and therapy.

Conclusion—*ANG* is the first gene whose loss-of-function mutations are associated with ALS pathogenesis. Therapeutic modulation of angiogenin level and activity in the spinal cord, either by systemic delivery of angiogenin protein or through retrograde transport of *ANG*-encoding viral particles, may be beneficial for ALS patients.

Keywords

amyotropic lateral sclerosis; angiogenesis; angiogenin; endothelial cells; loss-of-function gene mutation; motor neurons; ribonuclease; rRNA transcription

1. Introduction

Amyotropic lateral sclerosis (ALS) is a progressive neurodegenerative disease with specific loss of motor neurons in the brain, brain stem and spinal cord [1]. The average age of onset is 55 years with upper and lower motor neuron signs, including distal muscle weakness and wasting, increased muscle tone with hyperreflexia and at times diaphragmatic and/or bulbar weakness. A significant percentage of ALS patients (up to 50%) have evidence of cognitive impairment, and 5 – 10% of them are demented [2–7]. Death occurs from respiratory failure at average 4 years after disease onset. It is a devastating disease without cure. At present, the

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Declaration of interest

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only recognized treatment for ALS is riluzole, which extends survival by about 3 months with no improvement in motor muscular functions [8].

The incidence of ALS is estimated at 0.6 – 2.4/100,000 population [9]. Approximately 90% of ALS cases are sporadic with no known family history, whereas the remaining 10% are familial cases inherited in either an autosomal dominant or recessive manner [1,10]. Mutations in the Cu/Zn superoxide dismutase gene 1 (ALS1; *SOD1*), have been identified in ~20% of familial [11–13] and in ~3% of sporadic [14–16] ALS patients.

Angiogenin (*ANG*) gene, encoding a 14 kDa angiogenic ribonuclease [17], seems to be the first loss-of-function gene identified in ALS. Since the original discovery of *ANG* as an ALS candidate gene [18], a total of 14 missense mutations in the coding region of *ANG* have been identified in 35 of the 3170 ALS patients of the Irish, Scottish, Swedish, North American and Italian populations [18–22]. Among the 14 mutations identified so far, 10 have been characterized in detail and shown to be loss-of-function mutations [22–24].

Mouse angiogenin is strongly expressed in the CNS during development [25]. Human angiogenin is strongly expressed in both endothelial cells and motor neurons of normal human fetal and adult spinal cords [22]. Wild type (WT) angiogenin has been shown to stimulate neurite outgrowth and pathfinding of motor neurons in culture and to protect hypoxia-induced motor neuron death, whereas the mutant angiogenin proteins not only lack these activities but also induce motor neuron degeneration [24]. Therefore, a role of angiogenin in motor neuron physiology and a therapeutic activity of angiogenin toward ALS can be foreseen.

2. ALS

2.1 ALS genetics

At present, *SOD1* is the only known autosomal dominant gene in which mutations have been functionally associated with ALS, although three other loci (ALS3, ALS6 and ALS7) have been identified for typical autosomal dominant ALS by linkage analysis [1,26]. Other dominantly inherited genetic loci, associated with an atypical ALS phenotype, have also been identified (ALS with dementia/parkinsonism, *MAPT*; progressive lower motor neuron disease, *DCTN1*; and ALS8, *VAPB*). In autosomal dominant ALS with frontotemporal dementia (FTD), genetic linkage has been reported to 9q21 – q22 [27]. Mutations in the *SETX* gene have been identified in juvenile onset autosomal dominant ALS. Genetic loci identified for juvenile onset autosomal recessive disease include *Alsin* (*ALS2*) and ALS5 [1,26]. It is notable that besides *SOD1*, the other genes and loci described above have only been found in very few ALS patients, and often in atypical ALS with slow progression.

Genetic association studies have also identified several risk factors in ALS, including deletions or insertions in the neurofilament heavy chain gene [28–30], polymorphisms in VEGF [31], hemochromatosis gene *HFE* [32] and paraoxonase-1 (*PON1*) [33]. A 5 bp deletion in mitocondrial cytochrome *c* oxidase subunit I (*COX1*) [34] and a T4272C mutation in isoleucine tRNA synthesase (*IARS2*) [35] have been linked to ALS, although only in a single case. A common mitochondrial DNA deletion mutation (mt DNA4977) is increased in the brain of ALS patients [36]. The apolipoprotein E epsilon4 allele has been associated with decreased survival of ALS patients [37]. Copy number variation in survival motor neuron (*SMN*) has also been shown to be a susceptibility factor [38]. More recently, whole genome association studies have identified genetic variations in dipeptidyl-peptidase 6 (*DPP6*), [39] and inositol 1,4,5-triphosphate receptor 2 (*ITPR2*) [40] genes in ALS patients. Whole genome association has also identified a minor association of a single nucleotide polymorphism near the *FLJ10986* gene to ALS [41]. Most recently, missense mutations in the coding region of *TARDBP* encoding the Tar DNA binding protein TDP-43 were found in both familial and sporadic ALS patients

[42–46] following the discovery that TDP-43 is a major constituent of the neuronal cytoplasmic inclusions [3,47,48]. Five studies have independently reported *TARDBP* mutations in ALS patients [42–46], whereas two studies failed to identify any mutations [49,50]. A total of 15 mutations have been identified in TARDBP among 1637 ALS patients [42–46,49,50], which places *TARDBP* as the third most frequently mutated gene so far identified in ALS (after *SOD1* and *ANG*).

Since 2004, *ANG* has emerged as an important gene in ALS [18,21]. A total of 14 different missense mutations in the coding region of *ANG* have been identified in 35 of 3170 patients from Irish, Scottish, Swedish, North American and Italian ALS populations [18–22]. Thus, *ANG* seems to be the second most frequently mutated gene in ALS (after *SOD1*). Importantly, although WT angiogenin induces angiogenesis, stimulates neurite outgrowth of motor neurons and protects them from hypoxia-induced death, mutant angiogenin proteins lack these activities [22,24]. Therefore, *ANG* seems to be the first loss-of-function gene so far identified in ALS [22]. Table 1 lists the genes and genetic factors whose alterations predispose to ALS.

2.2 ALS pathogenesis

Many theories including oxidative stress, excitotoxicity, mitochondrial dysfunction, defective axonal transport, abnormal protein aggregation, and loss of tropic and angiogenic factor support have been proposed as the underlying mechanism of ALS pathogenesis [1,51–53]. Each of these hypotheses is supported by some experimental evidence but at the same time is undermined by contradictory data. For example, motor neuron damage as a result of oxidative stress is a key hypothesis in ALS. It is supported by the findings of elevated oxidative metabolism in ALS, such as the detection of increased biochemical markers of oxidative injury in post mortem examinations of ALS patients [54]. It is also supported by the acquired capacity of some forms of mutant SOD1 to catalyze the production of reactive oxygen species such as superoxide anions (O_2^-) and peroxynitrite ($^-$ONOO) through either copper catalysis or improper copper and zinc binding [55–57]. However, this hypothesis is undermined by the report that oxidative markers are detected in *SOD1*^{G93A} mice but not in *SOD1*^{G37A} mice, although both developed ALS symptoms [58]. It is further undermined by the finding that deletion of copper chaperone for SOD1 diminished the copper load but did not affect the development of ALS [59], and that copper-binding-site-null *SOD1* still causes ALS in transgenic mice [60].

The etiology of ALS is likely to be multi-factorial, involving the interplay of several mechanisms to initiate disease and propagate the spread of motor neuron death. A generally accepted hypothesis at present is that several factors, both genetic and environmental, cause mitochondrial dysfunction and excitotoxicity, lead to abnormal protein precipitation and finally apoptosis of motor neurons [52]. Non-neuronal neighboring cells may also play a role in ALS pathogenesis. It has been shown that motor neurons, microglia [61] and astrocytes [62] affect the onset and progress of illness. Astrocytes and microglia harboring *SOD1* mutations secrete substances that kill motor neurons. Motor neurons with or without *SOD1* mutation showed neuro-degenerative properties when co-cultured with astrocytes that harbor *SOD1* mutation, whereas motor neurons with *SOD1* mutations showed less neuronal losses when they are surrounded by normal astrocytes [63–65].

Identification of *ANG* mutations in ALS patients [19–22] and demonstration that these mutations result in loss of angiogenin functions [22–24] provide an alternative viewpoint of ALS pathogenesis. Angiogenin was not the first angiogenic molecule reported to be associated with ALS. In 2001, Oosthuyse *et al.* have already reported that deletion of the hypoxia-response element in the *VEGF* promoter caused adult onset motor neuron degeneration similar to ALS [66]. Although mutations in the coding region of *VEGF* have not been found in ALS patients, a genetic variation in the *VEGF* promoter that lowers VEGF expression has been shown to be associated with an increased risk of ALS [31]. Reported involvement of both angiogenin and

VEGF in ALS suggests a link between angiogenesis and ALS pathogenesis. Further supporting this hypothesis are the findings of null mutations of progranulin (*PGRN*), another angiogenic protein, in patients with FTD [67,68]. Genetic variations of *PGRN* have also been recently reported in ALS patients [69]. Many patients with ALS develop dementia, and with FTD develop motor neuron diseases similar to ALS. FTD and ALS share some common neuropathological features such as the accumulation of ubiquitinated neuronal cytoplasmic inclusions containing TDP-43 [3]. Thus, a role of angiogenic factors in ALS pathogenesis has been proposed [53,70,71], and vascular abnormality has been recently demonstrated in *SOD1* transgenic mice [72–74]. The findings that the blood spinal cord barrier (BSCB) of *SOD1* transgenic mice is impaired and that the BSCB leakage occurs before motor neuron degeneration provide pathological data supporting an active role of blood vessels in ALS pathology [72–74].

2.3 ALS therapy, clinical trials and preclinical studies

There is presently no effective pharmacologic treatment for ALS to halt neuronal death or even slow it appreciably. Riluzole, the only drug approved for ALS since 1995, only extends survival by 2 – 3 months if it is taken for 18 months. Riluzole is thought to act in part by limiting glutamate release. It preferentially blocks tetrodotoxin-sensitive sodium channels, which are associated with damaged neurons [75]. This reduces influx of calcium ions and indirectly prevents stimulation of glutamate receptors. Together with direct glutamate receptor blockade, the effect of the neurotransmitter glutamate on motor neurons is greatly reduced. Riluzole was approved for use in ALS after two independent clinical trials showed a marginal increase in the survival time of ALS patients [8,76]. Unfortunately, patients taking riluzole do not experience any slowing in disease progression or improvement in muscle function. Therefore, riluzole does not present a cure, or even an effective treatment, and the search for better therapeutic agents continues.

Mutant *SOD1* transgenic mice have been widely used for ALS drug testing. *SOD1* mutations are the cause of ~20% of the familiar ALS and ~3% of the sporadic ALS, and, therefore, ~4% of all ALS cases. More than 100 mutations in *SOD1*, distributed throughout the gene, have been found in ALS patients [52]. Although it is still unknown why the mutant form of this abundant and ubiquitously expressed enzyme is specifically toxic to motor neurons and causes ALS, it is clear that mice overexpressing the mutant *SOD1* genes develop symptoms mimicking that of human ALS patients. Over 70 agents of various categories including tropic factors, antioxidant, antiviral, anti-inflammatory, immunomodulatory, antiapoptosis, antiglutamatergic, calcium regulators, proteasome inhibitors, metal ion regulators, structure proteins as well as energy metabolism-related compounds, have been tested in *SOD1*^{G93A} mice [51,52,77]. Many of these agents underwent clinical trials. However, the benefits in the mouse have not been translated into clinical efficacy except in the case of riluzole. All the others, including brain-derived neurotrophic factor [78], ciliary neurotrophic factor [79], IGF1 [80, 81], and glial-derived neurotrophic factor (GDNF) [82] have failed.

One of the main reasons for the disappointing clinical trials was that the beneficial effect of these agents observed in the *SOD1*^{G93A} mice was not significant and that the *SOD1*^{G93A} mice have high noise level in their survival. The ALS Therapy Development Institute (ALSTDI) has conducted a thorough retesting of > 70 drugs in 18,000 ALS mice across 221 studies and failed to reproduce the reported efficacy in *SOD1*^{G93A} mice [83]. ALSTDI has also reanalyzed the reported data from 5429 mice from 50 published papers and found that the reported beneficial effect in animal survival was actually the noise of the animals. ALSTDI has concluded that 24 ALS mice are needed in each group to reduce the noise and get conclusive results [83]. Moreover, all the *SOD1*^{G93A} mice in both control and experimental groups should be matched in age, gender, litter size and copy numbers of the transgene.

The reason for a relative minimal effect of exogenous tropic factors and other types of therapeutic proteins could be their failure to cross the blood–brain barrier (BBB) and BSCB. Gene therapy is, therefore, an alternative approach for ALS therapy. Many strategies are under investigation, including the delivery of genes encoding neurotrophic factors, antiapoptotic and antioxidants proteins using viral vectors administered directly into the affected areas of the CNS, or through retrograde transport to motor neurons from intramuscular injection, or through *ex vivo* gene transfer [84]. AAV (adeno-associated virus)-mediated delivery of *IGF-1* [85], *GDNF* [86] and *Bcl-2* [87] gene have been shown to be effective in the *SOD1*^{G93A} transgenic mice.

VEGF is a prominent angiogenic factor and a new neurotrophic factor linked to ALS. Since the demonstration that deletion of the hypoxia-response element in the promoter of *VEGF* causes motor neuron degeneration in mice [66] and that polymorphisms in the *VEGF* promoter that reduce VEGF expression are associated with ALS in the populations of Sweden, Belgium, UK [31] and New England [88], various attempts have been made to target VEGF as a therapeutic approach for ALS. VEGF delivered into *SOD1*^{G93A} rats intracerebroventricularly [89] or into *SOD1*^{G93A} mice with a retrogradely transported lentiviral vector [90] has been shown to improve motor neuron function marginally and prolong survival significantly. *VEGF* overexpression also improves motor muscular function and increases the survival in *SOD1*^{G93A} transgenic mice [91]. However, intraperitoneal injection of VEGF in *SOD1*^{G93A} mice had only a modest effect in delaying disease onset and in prolonging survival [92].

3. Angiogenin

3.1 Mechanism of action of angiogenin

Angiogenin was originally isolated from the conditioned medium of HT-29 human colon adenocarcinoma cells based solely on its angiogenic activity in the chicken embryo chorioallantoic membrane angiogenesis assay [17]. Subsequently, it has been found to have a wide tissue distribution with the highest expression in the liver [93]. It is a member of the pancreatic RNase superfamily with a 33% amino-acid identity and an overall homology of 56% to that of RNase A [94]. Angiogenin has a unique ribonucleolytic activity that is several orders of magnitude lower than that of RNase A but is important for its biological activity [95]. The amino-acid residues important for catalysis are conserved in all vertebrate angiogenin from fish to human [96]. Extensive studies on site-directed mutagenesis have shown that angiogenin variants with reduced enzymatic activity also have reduced angiogenic activity [95,97–104]. Structural work indicated that one of the reasons for angiogenin to have a reduced ribonucleolytic activity is that the side chain of Gln 117 occupies part of the enzymatic active site so that substrate binding is compromised [105,106].

Angiogenin is angiogenic, whereas the prototype family member RNase A is not. Two important structural differences between angiogenin and RNase A are responsible for this discrepancy. The first is the segment from amino-acid residues 59 – 68 that forms the receptor binding site in angiogenin [99,107]. Therefore, angiogenin binds to its target cells (including endothelial cells, cancer cells and motor neurons) but RNase A does not. Angiogenin binds to endothelial cells specifically [108] and induces second messenger responses including diacylglycerol and prostacyclin [109,110], and activates MAPK [111] and AKT [112]. Another structural difference between angiogenin and RNase A is that angiogenin has a nuclear localization signal consisting of ^{29}IMRRRGL^{35}, whereas RNase A does not [113]. Therefore, angiogenin undergoes nuclear translocation in endothelial cells where it accumulates in the nucleolus [114,115], binds to the promoter region of ribosomal DNA (rDNA) and stimulates rRNA transcription [116,117], an essential step for ribosome biogenesis, protein translation and cell proliferation.

An angiogenin binding protein has been identified from the surface of endothelial cells [107] and has been characterized to be a type of smooth muscle actin [118,119]. An ~170 kDa angiogenin receptor has also been identified from the endothelial cell surface to mediate nuclear translocation of angiogenin and cell proliferation [120]. Expression of the binding protein and the receptor on endothelial cells seems to be mutually exclusive. The binding protein is expressed on the surface of confluent cells. Binding of angiogenin to the binding protein activates tissue plasminogen activator [121] thereby inducing cell invasion and migration [122]. After the leading cells migrate away, the local cell density decreases, which triggers the expression of an angiogenin receptor. Binding of angiogenin to the receptor stimulates cell proliferation so that the gap created by the migrating cells is filled. Therefore, angiogenin is a multifunctional angiogenic molecule that plays a role in several steps in the angiogenesis process including cell invasion, proliferation and tube formation. Figure 1 shows the current understanding of the mechanism of angiogenin-induced angiogenesis.

3.2 Role of angiogenin in rRNA transcription

Angiogenin has been shown to undergo nuclear translocation in endothelial [114,115,123] and cancer [124,125] cells. Nuclear translocation of angiogenin in endothelial cells is under tight regulation and is cell density-dependent. It decreases as cell density increases and ceases when cells are confluent [115,120]. Nuclear translocation of angiogenin occurs through receptor-mediated endocytosis [114] and is independent of microtubule system and lysosomal processing [123]. Angiogenin seems to enter the nuclear pore by the classic nuclear pore input route [113]. Nuclear translocation of exogenous angiogenin is very fast. When exogenous angiogenin is added to the cell culture, nuclear angiogenin is detectable in 2 min and is saturated in 30 min [115]. On arriving at the nucleus, angiogenin accumulates in the nucleolus [114] where ribosome biogenesis takes place. Nuclear angiogenin has been shown to bind to the promoter region of rDNA [117] and stimulates rRNA transcription [116,126]. Cell growth requires the production of new ribosomes. Ribosome biogenesis is a process involving rRNA transcription, processing of the prerRNA precursor and assembly of the mature rRNA with ribosomal proteins [127–129]. The rate-limiting step in ribosome biogenesis is the synthesis of rRNA. Therefore, rRNA transcription is an important aspect of growth control. It is also important for maintaining a normal cell function as proteins are required for essentially all cellular activities. It may be particularly relevant for motor neurons to have a robust ribosome biogenesis because of long axonal transport of these cells.

Angiogenin-stimulated rRNA transcription has been demonstrated as a general requirement for angiogenesis [126]. In other words, angiogenin is a permissive factor for other angiogenic factors to induce angiogenesis. Experimental evidence for this contention includes: i) nuclear translocation of endogenous angiogenin in endothelial cells is stimulated by other angiogenic factors including aFGF, bFGF, VEGF and EGF [126]; ii) knocking down *ANG* expression in endothelial cells inhibits bFGF- and VEGF-induced cell proliferation, accompanied with a decrease in rRNA transcription. Addition of exogenous angiogenin can completely restore the proliferative activity of these angiogenic factors [126]; and iii) angiogenin-specific inhibitors have no effect on binding of VEGF and bFGF to their receptors but inhibit their angiogenic activity [130]. Figure 2 summarizes the function of angiogenin-stimulated rRNA transcription in cell proliferation.

As an angiogenic molecule, angiogenin may thus play a role in maintaining vasculature integrity in the spinal cord. Angiogenin insufficiency, caused by heterozygous missense mutations in the coding region, polymorphisms in the promoter region, or decreased expression owing to other genetic and environmental factors, may result in vascular abnormality and create an unhealthy environment for motor neurons. In addition, angiogenin may play a direct role in motor neuron physiology and its deficiency may accelerate or cause motor neuron

degeneration. To this end, angiogenin has been shown to be expressed in motor neurons of both human and mouse spinal cords during development and in adulthood [21,22,24]. Angiogenin deficiency may result in insufficient ribosomal biogenesis and improper mRNA translation either in the entire or in some parts of the cell. Local protein translation of asymmetrically localized mRNA within neurons has been shown to play an important role in neuronal polarity and synaptic plasticity [131]. For example, asymmetrical localization of tau protein and its abnormal metabolism has been linked in both ALS and FTD [132]. Aberrant mRNA formation and RNA processing errors of excitatory amino-acid transporter 2 were found only in neuropathologically affected areas of ALS patients but not in other brain regions [133]. Motor neurons are the longest cells in the body and asymmetrical protein expression may thus be crucial not only during development but also during repair and regeneration. Robust protein translation machinery is essential for motor neuron physiology and a potential role of angiogenin can be predicted from its function in ribosome biogenesis. It is noteworthy that angiogenin is a member of the pancreatic RNase family and that the RNase activity is essential for its biological activity [95]. A link between RNA processing and neurodegeneration has been established [134]. In fact, spinal muscular atrophy, another motor neuron degenerative disease, is caused by mutations in *SMN* gene that encodes SMN, a protein known to play roles in RNA splicing, ribosome assembly and gene transcription [135].

4. *ANG* mutations in ALS patients

Recently, linkage analysis in Irish ALS populations identified an association of the G allele of the single nucleotide polymorphism *rs11701* in the coding region of *ANG* (representing the amino-acid residue G86 in the mature protein) [18]. In the same study, a novel missense mutation at position 191 (A to T) in the coding region of *ANG*, which will result in a substitution of Lys 40 by Ile (K40I), was also found in 2 of the 169 Irish ALS patients but not in 171 control subjects [18]. Subsequently, seven heterozygous missense mutations in the coding region of *ANG* were identified in 15 patients by sequence screening of 1629 individuals with ALS [21], an overall frequency of ~1% with an overrepresentation of familial ALS (4/259, 1.5%) over sporadic ALS (11/1370, 0.8%). From sequencing 298 ALS patients of a Northern American cohort, four more mutations in *ANG* gene were identified (1.3% frequency) [22]. More recently, seven more mutations were identified in 9 of the 737 Italian ALS patients [20]. *ANG* mutations in Italian population also seem to segregate familial ALS (3/132, 2.3%) from sporadic ALS (6/605, 1%) with an overall frequency of 1.2% [20].

A total of 14 missense mutations (at 13-positions) in the coding region of *ANG* have been identified in 35 of the 3170 ALS patients of the Irish, Scottish, Swedish [21], North American [22] and Italian [20] populations. Among these mutations, 3 occurred in the signal peptide regions and 11 in the mature protein. In the seven sequencing efforts carried out so far, a total of 3003 healthy controls were included and two mutations in the *ANG* gene were found in non-ALS controls [20,21]. The first is a K17I mutation that was found in an apparent healthy 65-year-old male of European descent. The second is the I46V mutation that was found in 11 of the 1568 Italian healthy controls [19,20,136,137]. Therefore, I46V mutation does not seem to be associated with Italian ALS patients but does seem to be associated with the Scottish ALS patients in whom 3 of the 398 ALS patients but none of the 299 controls harbor this mutation [21]. Table 2 lists the frequencies of *ANG* mutations that occurred in 3170 ALS patients.

5. Properties of mutant angiogenin proteins

Except for the three mutations in the signal peptide region (M-24I, F-13S, P-4S) and the two most recently reported mutations in the mature protein region (V113I, H114R) [20], all the mutant angiogenin proteins have been prepared and characterized by ribonuclease [22,23], nuclear translocation [22] and angiogenesis [22,23] assays. Except for R31K, all of these ALS-

associated angiogenin mutant proteins have severely impaired ribonucleolytic activity ranging from < 1% (K40I) to 19% (K17E) of that of the WT angiogenin. R31K has 69% of the enzymatic activity of WT angiogenin [23]. Some of the mutant angiogenin proteins also have reduced thermal stability [23]. Among the three mutant angiogenin proteins (K17I, S28N, P112L) that have been tested in the nuclear translocation assay, S28N and P112L do not undergo nuclear translocation and K17I has a reduced capacity [22]. Two different angiogenesis assays have been used to examine the angiogenic activity of the mutant angiogenin proteins. The endothelial cell tube formation assay on fibrin gel was used to examine the mutants identified from the Northern American ALS patients and the results showed that all three mutants (K17I, S28N, P112L) are inactive [22]. The aorta ring assay was used to test three of the seven mutants identified from the Irish and Scottish ALS populations. All three mutants (Q12L, C39W, K40I) were inactive in the aorta ring angiogenesis assay [23]. Taken together, these results demonstrated that *ANG* mutations identified in ALS patients are associated with a functional loss of the angiogenic activity of the angiogenin protein.

WT angiogenin has been shown to stimulate neurite outgrowth and pathfinding of motor neurons derived from P19 embryonal carcinoma cells [25]. WT angiogenin also protects P19-derived motor neuron from hypoxia-induced cell death but the ALS-associated mutant angiogenin proteins (Q12L, C39W, K40I) lack this neuroprotective activity [24]. Moreover, these mutant angiogenin proteins are cytotoxic to the P19-derived motor neurons and induce their degeneration, suggesting that *ANG* mutations may even be causative to ALS [24].

6. Expression of angiogenin in the CNS

Mouse angiogenin is strongly expressed in the developing mouse nervous system both in the brain and in the spinal cord [25]. Immunohistochemistry and immunofluorescence have been used to show that angiogenin expression is the strongest in the brain and spinal cord at 9.5 days postcoitum (pc) [25]. At 11.5 days pc, angiogenin expression remains high in the telencephalon, mesen and mylencephalon as well as in the spinal cord, spinal ganglia and choroids plexus [25]. Until mid-gestation, angiogenin expression is stronger in the nervous system than in any other tissues. Co-staining with peripherin and Islet1 showed that angiogenin is expressed in mouse motor neurons.

Immunohistochemistry was also used to detect expression of human angiogenin in normal spinal cords obtained from fetal (ranging from 15 to 30 weeks gestation) and adult human autopsies. Strong angiogenin staining was observed in the ventral horn motor neurons of both fetal and adult cases [22]. Angiogenin was also detected in the extracellular matrix and interstitial tissues in all cases, consistent with it being a secreted protein. Angiogenin expression in the spinal cord seems to be downregulated as development proceeds but is still strongly expressed in the adulthood. Strong cytoplasmic and nuclear accumulation of angiogenin in motor neurons of both prenatal and adult spinal cords suggests a physiological role of angiogenin, both early in development and later in adulthood, and supports the hypothesis that *ANG* mutations are relevant to ALS pathology.

Double immunofluorescence with an antiangiogenin mAb 26-2F and antivon Willebrand factor polyclonal antibody showed that angiogenin is also localized in spinal cord endothelial cells, suggesting that angiogenin plays a role in maintaining the integrity of spinal cord vasculature that is important for physiological health of motor neurons [22]. Thus, angiogenin abnormalities may have a dual role in ALS, directly through motor neuron function and indirectly through endothelial cells and aberrant angiogenesis in the spinal cord.

7. Conclusion

Angiogenin is an angiogenic molecule known to play an essential role in angiogenesis by mediating rRNA transcription in endothelial cells [116,117]. The recent discovery of loss-of-function *ANG* mutations in ALS patients [19–22] and the findings that angiogenin is strongly expressed in the spinal cords both during fetal development and in adulthood [22,25] indicate an important role of angiogenin in motor neuron physiology and pathology. Angiogenin may have a dual role in motor neuron function by acting both on endothelial cells and on motor neurons. Thus, angiogenin may mediate angiogenesis thereby maintaining a normal vasculature, which is essential for motor neuron development, health and survival under various environmental and genetic insults. This is supported by previous findings that angiogenin-stimulated rRNA transcription is required for angiogenesis induced by VEGF [126] and that VEGF is a prominent angiogenic molecule known to be associated with ALS [66,88–92]. Involvement of angiogenin and VEGF, two angiogenic proteins that mediate angiogenesis by different mechanisms, in ALS suggests that angiogenesis insufficiency is linked to ALS pathogenesis [53]. Novel mutations in *PGRN* gene that encodes progranulin, another angiogenic protein, have recently been reported in ALS patients [69]; adding more evidence that abnormal angiogenesis is associated with ALS.

In addition to a role in angiogenesis, angiogenin may also act on motor neurons directly. This hypothesis is supported by the finding that angiogenin is strongly expressed in the motor neurons of fetal and adult spinal cords [22]. It is also supported by the results that angiogenin undergoes nuclear translocation in motor neurons and stimulates neurite outgrowth and pathfinding [24,25]. The mode of action of angiogenin in both endothelial cells and motor neurons could be related to its activity in mediating ribosome biogenesis. Nuclear angiogenin has been shown to bind to the promoter region of rDNA both in endothelial cells and in cancer cells thereby stimulating rRNA transcription [124,126]. It is conceivable that the role of angiogenin in motor neurons would also be related to rRNA transcription and that a defect in this pathway is likely to result in insufficient synthesis of ribosomes thereby affecting motor neuron viability. The dual role model suggests an essential role of angiogenin in motor neuron physiology. It is consistent with the results that all the angiogenin mutations so far found in ALS patients are heterozygous. Homozygous mutations may be lethal as a complete loss of angiogenin function would be detrimental. This model also implies that a decrease in angiogenin expression, as a result of various environmental and genetic insults, would have a profound effect on motor neuron function. It, at the same time, provides a therapeutic opportunity for ALS treatment by manipulating angiogenin expression levels and activities.

8. Expert opinion

Since the first report in 2004 that missense mutations in the cording region of *ANG* gene was found in ALS patients [18], 3170 ALS patients and 3003 non-ALS controls have been sequenced in six independent studies [18,19,21,22,136,137] and a total of 14 mutations in 13-positions have been found in 35 patients. Four functional studies have been carried out in which WT angiogenin has been shown to play a direct role in motor neuron physiology and the ALS-associated *ANG* mutations result in a complete loss of angiogenin activity [22–25]. Although mutations in *ANG* gene occurred in only 1.1% (ranging from 0.8 to 1% in sporadic ALS and 1.3 to 2.3% in familial ALS), *ANG* remains to be the second most frequently mutated gene in ALS and is the only loss-of-function gene so far identified in ALS patients. There is a sound rationale for exploring a novel ALS treatment opportunity by manipulating angiogenin levels and/or activities. For this purpose, the efficacy of angiogenin in improving motor muscular function and survival of *SOD1*^{G93A} mice should be tested. First, WT angiogenin protein, with the ALS-associated mutant angiogenin proteins as controls, could be administered systemically by i.v., i.p., i.m. or s.c. injection. A beneficial effect could be expected from these routes of

administration if angiogenin could cross the BBB and BSCB and reaches the CNS. A human angiogenin-specific mAb is available so that the distribution and stability of systemically administered angiogenin could be readily detected. It has been reported that ALS patients and *SOD1*^{G93A} mice have disrupted BSCB [72–74] so the pharmacokinetic findings of systemically administered angiogenin from the above experiments should be confirmed with WT mice. Even if angiogenin dose not cross the BBB and BSCB, these experiments are still worthy doing because of the possibility that the site of action of angiogenin may be peripheral. The role of axon in ALS has been recognized [138–140] so the possibility that angiogenin acts directly on the neuromuscular junctions or on motor axons directly should not be excluded although there is no direct evidence at present. These experiments are thus both clinically and scientifically significant as they will tell us whether angiogenin is effective and where the site of action might be. If the above routes of administration are ineffective, intrathecal or intracerebroventricular administration of angiogenin protein directly into the CNS may be considered. Alternatively, retrograde delivery of angiogenin-encoding AAV or lentiviral particles could also be used to enhance angiogenin expression in the motor neurons.

Another informative experiment would be to generate and characterize *ANG:SOD1*^{G93A} double transgenic mice. Both universal and cell-specific promoters should be considered for generating *ANG* transgenic mice. Characterization of these mice will reveal whether the effect of angiogenin is cell autonomous. Furthermore because the ALS-associated mutant angiogenin proteins are toxic to cultured motor neurons [24], it would be worthwhile to create and characterize transgenic mice overexpressing the mutant forms of *ANG*. If these mice develop ALS-like symptoms, they will be a valuable animal model, in addition to the *SOD1* transgenic mice, to be used for mechanistic study and for screening and testing potential drugs.

Another approach to reveal the role of angiogenin in development in general, and in motor neuron physiology in particular, would be to create and characterize *ANG* knockout mice. Although humans have only a single *ANG* gene, mice have six [141]. It is not possible to knockout all of them simultaneously because they are spread out over ~8 million bp. However, mouse *ANG1* is clearly the prominent form and the ortholog of the human gene [141,142]. Therefore, it is likely that knocking out mouse *ANG1* will suffice for investigating the function of human angiogenin. Because of the possibility that the loss of mouse angiogenin-1 function may be embryonic lethal, it would be advisable to create conditional knockout so that the role of angiogenin-1 in motor neuron can be studied at the different stages during development. If *ANG1* deletion results in motor neuron degeneration, *ANG1* knockout mice may also be useful for ALS drug screening.

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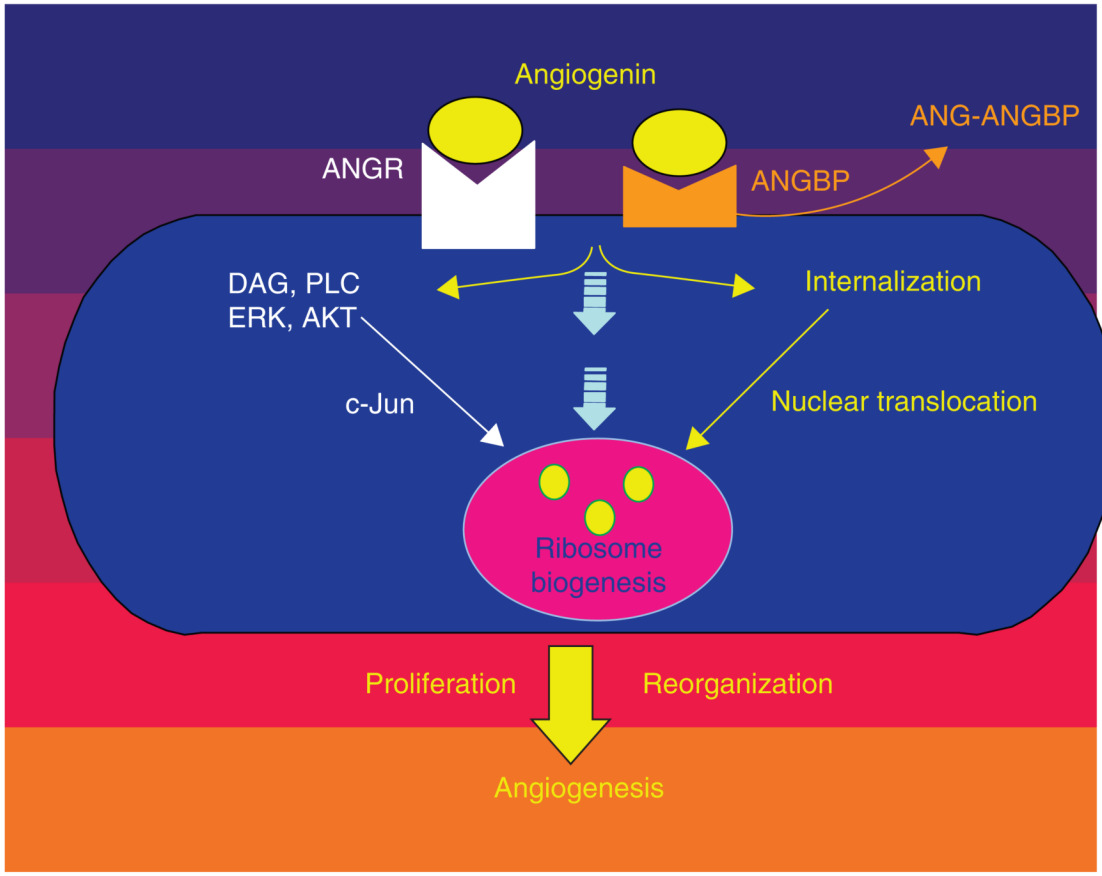

Figure 1. Conceptual framework of the interaction between angiogenin and its target cells

Angiogenin, shown in yellow, can bind to both the receptor and the binding protein, shown in white and orange, respectively. Most the angiogenin and its binding protein complex will dissociate from the cell surface and activates tissue plasminogen activator to produce plasmin, and induce cell invasion into the extracellular matrix. Binding to the 170 kDa receptor induces second messengers and triggers signal transduction. On binding, angiogenin is also internalized and translocated to the nucleus where it accumulates in the nucleolus. All these individual steps are necessary for angiogenesis.

ANG: Angiogenin.

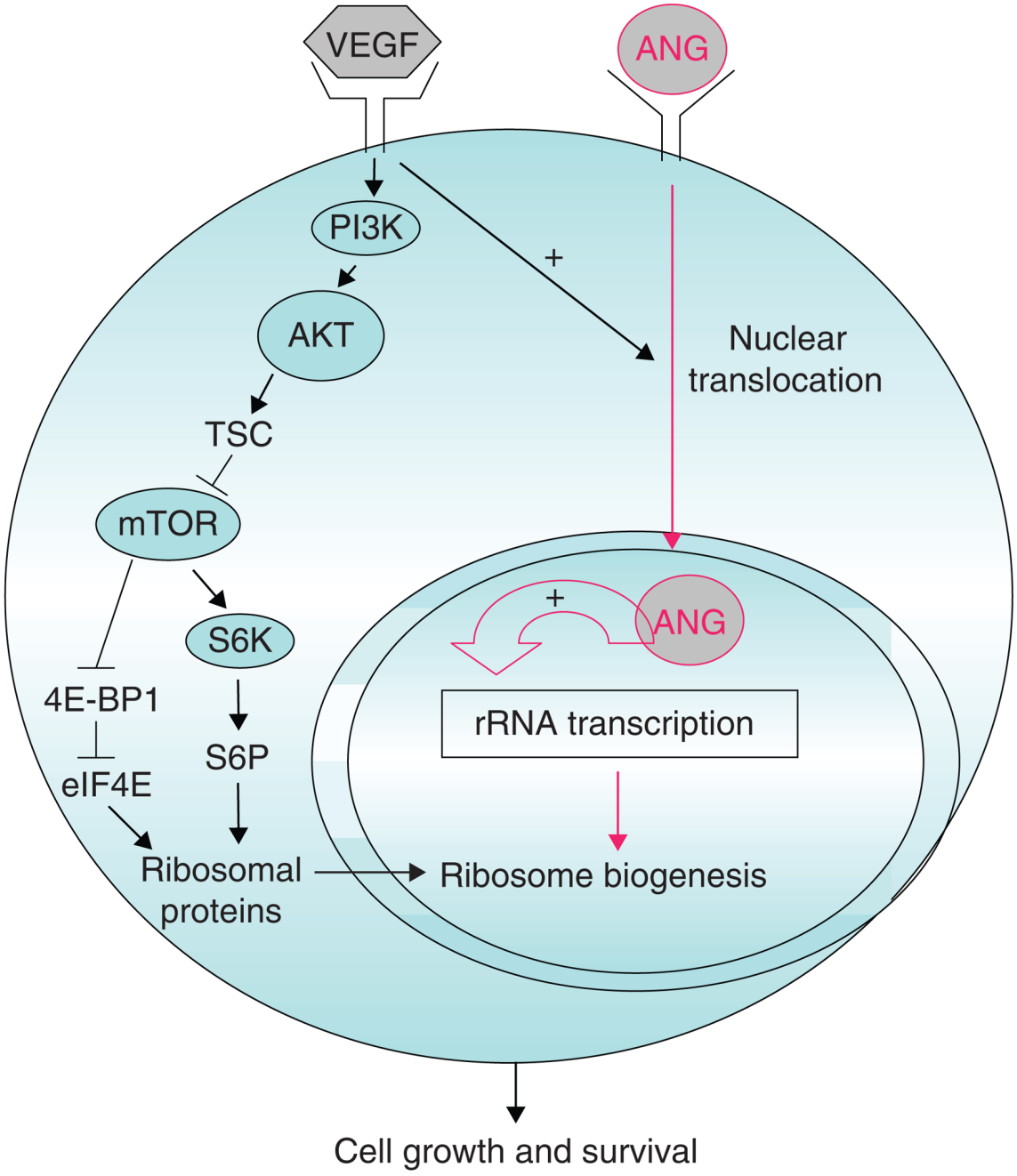

Figure 2. Angiogenin-stimulated rRNA transcription is a general requirement for angiogenesis

Angiogenin is a permissive factor for other angiogenic proteins to induce cell proliferation. Growth factors such as VEGF activate PI3K–AKT–mTOR pathway to enhance ribosomal protein production. Angiogenin is translocated to the nucleus where it enhances rRNA transcription so that ribosome biogenesis can occur. Angiogenin inhibitors have been shown to abolish cell proliferation stimulated by other angiogenic factors including bFGF and VEGF. ANG: Angiogenin.

Table 1

Genes and genetic factors associated with ALS.

Disease type	Locus	Gene	Inheritance	Clinic features	Ref.
ALS1	21q22.21	*SOD1*	AD	Adult onset, typical ALS	[143]
ALS2	2q33	*Alsin*	AR	Juvenile onset, atypical ALS and PLS, slow progression	[144]
ALS3	18q21	Unknown	AD	Adult onset, typical ALS	[145]
ALS4	9q34	*SETX*	AD	Juvenile onset, atypical ALS, slow progression	[146]
ALS5	15q15.1-21.1	Unknown	AR	Juvenile onset, atypical ALS, slow progression, no pseudobulbar signs	[147]
ALS6	16q12	Unknown	AD	Adult onset, typical ALS, short duration	[148]
ALS7	20ptel-13	Unknown	AD	Adult onset, typical ALS, short duration	[149]
ALS8	20q13.33	*VAPB*	AD	Adult onset, atypical ALS, slow progression	[150]
ALS-FTD	9q21-22	Unknown	AD	Adult onset, ALS with fronto-temporal dementia	[151]
ALS-PD	17q21	*MAPT*	AD	Adult onset, ALS with Parkinsonism and dementia	[152]
Progressive lower MND	2p13	*DCTN1*	AD	Adult onset, lower motor neuron disorder	[153]
ALS, sporadic	6q12	*VEGF*	Risk factors	Adult onset, typical ALS	[31]
	22q12.1-q13.1	*NFHC*			[28–30]
	6q21.3	*HFE*			[32]
	7q21.3	*PON1*			[33]
	5q13	*SMN*			[38]
	7q36	*DPP6*			[39]
	12p11.23	*ITPR2*			[40]
	1p32.1	*FLJ10986*			[41]
	17q21	*PGRN*			[69]
ALS, familiar and sporadic	14q11.2	*ANG*	AD	Adult onset, typical ALS	[20–22]
ALS, familiar and sporadic	1p36	*TARDBP*	AD	Adult onset, typical ALS	[42–46]

AD: Autosomal dominant; ALS: Amyotropic lateral sclerosis; ALS-FTD: Amyotropic lateral sclerosis–front-temporal dementia; ALS-PD: Amyotropic lateral sclerosis–Parkinsonism and dementia; *ANG*: Angiogenin; AR: Autosomal recessive; *DCTN1*: Dynactin p150 subunit; *DPP6*: Dipeptidyl peptidase 6; *FLJ10986*: A gene encoding a 48 kDa protein weakly similar to L-ribulokinase; *HFE*: A hemochromatosis gene involved in iron metabolism; *ITPR2*: Inositol 1,4,5-triphosphate receptor 2; *MAPT*: Microtubule-associated protein tau; MND: Motor neuron disease; *NFHC*: Neurofilament heavy chain; *PGRN*: Progranulin; PLS: Primary lateral sclerosis; *PON1*: Paraoxonase 1; *SETX*: Senataxin; *SMN*: Survival motor neuron; *SOD1*: Superoxide dismutase 1; *TARDBP*: Tar DNA binding protein 43; *VAPB*: Vesicle-associated membrane protein.

Table 2

ANG mutations identified in ALS patients.

Mutations	**M(−24)I**	**F(−13)S**	**P(−4)L**	**Q12L**	**K17I**	**K17E**	**S28N**
Number of cases	2	1	2	2	3	2	1
Ref.	[19,20]	[19]	[20,22]	[21]	[21,22]	[21]	[22]
Mutations	R31K	C39W	K40I	I46V	P112L	V113I	H114R
Number of cases	1	2	5	10	1	2	1
Ref.	[21]	[21]	[18,21]	[20,21,136]	[22]	[20]	[20]

ALS: Amyotropic lateral sclerosis; *ANG*: Angiogenin.