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Investigating the role of angiogenin mutations in amyotrophic lateral sclerosis

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Titre / *Titel*

Investigating the role of novel genes that contribute to disease progression in amyotrophic lateral sclerosis

Description et Objectifs / *Beschreibung und Ziele*

In neurodegenerative conditions there is much evidence for defects in neuronal metabolism leading to activation of cell death pathways since mitochondrial dysfunction, aberrant protein aggregation, and oxidative stress are all pathological hallmarks of these conditions. Using gene silencing methods, the mechanism of neuron degeneration will therefore further be explored focussing on a selection of genes.

The objectives are to

- Generate gene-specific siRNA
- Examine the functional effects of siRNA in cell cultures
- Analyse the cell functions by Western Blot
- Generate a recombinant adeno-associated virus expressing gene-specific siRNA and examine the virus in cell culture.

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The Bachelor of Science thesis was carried out during the period of April - August 2009, under the supervision of Dr. Dairin Kieran and Prof. Patrick Aebischer, Neurodegenerative Studies Laboratory, EPFL and Dr. Bruno Schnyder, University of Applied Sciences (HES-SO), Sion.

ACKNOWLEDGEMENTS AND DEDICATION

I would like to express my gratitude to my supervisor at the EPFL, Dr. Dairin Kieran. Her scientific expertise, her skillful teaching (with patience when needed) in laboratory experiments contributed significantly to the success of the study. I thank Dr. Bruno Schnyder for discussions and general guiding. I would like to thank the entire group of Patrick Aebischer (LEN) for kindness and good mood daily life. Finally, I would like to dedicate this work in memory of my father.

CONTENTS

1. ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder where the selective loss of motor neurons and interneurons in the spinal cord, brainstem and cerebral cortex results in progressive paralysis and eventual death. ALS is the most common adult neuron disease, and it has no cure. Recent studies have identified mutations in the hypoxia inducible factor angiogenin (ANG) in patients with ALS. Based on structure analysis, the known angiogenin mutations are proposed to be loss-of-function mutations. Angiogenin is expressed in motor neurons, and has been identified as a neuroprotective factor suggesting that angiogenin or its signaling pathways may be considered as potential therapeutic target for ALS.

In this study the primary aim was to generate a recombinant adeno-associated virus (AAV) expressing wild-type or mutant (K40I) angiogenin. Here, we cloned wild type human angiogenin (*ANG* WT) and an ALS-associated angiogenin mutant (*ANG* K40I mut) into adeno-associated viral vectors. These constructions were confirmed by sequencing and will be used to examine the effect of *ANG* WT/K40I mut expression *in-vivo*.

Since the ALS-associated angiogenin mutations are associated with a loss-of-function, in this study we were also interested in developing an siRNA approach to silence the expression of angiogenin. Using motor neuron-like NSC34 cells, three different siRNA sequences against angiogenin were tested in order to identify the sequence with the most efficiency of knockdown.

Overall, the tools developed in this project will be used to further our understanding of the role of angiogenin in ALS pathogenesis.

RÉSUMÉ

La sclérose latérale amyotrophique (SLA) est une maladie neurodégénérative dans laquelle la perte sélective des motoneurones et des interneurones dans la moelle épinière, le tronc cérébral et le cortex cérébral provoque une paralysie progressive entrainant la mort. Chez l'adulte, la SLA est la maladie affectant les neurones la plus répandue, elle est incurable. Des études récentes ont identifiées, chez des patients atteints de SLA, des mutations dans l'angiogénine (ANG), un facteur de croissance vasculaire induit par l'hypoxie.

Basé sur l'analyse de structure, les mutations connues de l'angiogénine ont été présentées comme étant des modifications provoquant une perte de fonction de la protéine. L'angiogénine est exprimée dans les neurones moteurs et a été identifiée comme étant un facteur neuroprotectif. Ceci suggère que l'angiogénine ou l'une de ces voies de communication pourrait être considérée comme une cible thérapeutique potentielle pour la SLA.

Dans cette étude, le premier objectif était de générer un virus associé à l'adénovirus (AAV) recombinant exprimant l'angiogénine sauvage ou l'angiogénine mutante (K40I). Ici, le gène de l'angiogénine humaine sauvage (*ANG* WT) et celui de l'angiogénine mutante associé à la SLA (*ANG* K40I mut) ont été clonés dans des vecteurs viraux associés à l'adénovirus. Ces constructions furent confirmées par séquençage et seront utilisées, par la suite, afin d'examiner l'effet de l'expression de *ANG* WT/K40I *in-vivo*.

En sachant que les mutations de l'angiogénine, dans la SLA, sont associées à une perte de fonction ; Cette étude c'est également intéressée au développement d'une approche utilisant le siRNA, afin de réduire au silence l'expression de l'angiogénine. En utilisant les cellules NSC34 (possédant certaines caractéristiques des motoneurones) trois différentes séquences de siRNA ciblant l'angiogénine ont été testées. Ceci afin d'identifier la séquence ayant la plus grande efficacité de ' knockdown '.

D'une manière générale, les outils développés dans ce projet serviront pour de futures investigations afin de permettre une meilleure compréhension du rôle de l'angiogénine dans la pathogénicité de la SLA.

2.1 Amyotrophic Lateral Sclerosis

2.1.1 Definition of the disease

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the selective loss of upper and lower motor neurons and interneurons in the motor cortex, corticospinal tracts, brainstem and spinal cord. ALS is also termed Motor Neurone Disease in the UK, or Lou Gehrig's disease in the USA. The term "Amyotrophic" refers to the atrophy of muscle fibers, while "Lateral sclerosis" refers to the hardness to palpation of the lateral columns of the spinal cord in autopsy specimens, due to gliosis (reviewed by Rowland and Schneider, 2001). ALS is a rapidly progressing disease, with an average duration of 3 years. Moreover, there is no cure for ALS, and the only disease-modifing treatment is Riluzole (Rilutek®), which merely extends lifespan by 6 months in patients where it is effective (Bensimon et al., 1994). Therefore, there is an urgent need to understand the pathogenesis of ALS, and identify new therapeutics against the disease.

2.1.2 Clinical features

ALS was first described by the French clinician Jean-Martin Charcot in 1869. Despite the fact that this disease was identified over 150 years old ago, the precise causes of ALS remain unclear. ALS initially presents with limb weakness, and disease onset may first occur in bulbar regions (affecting muscles involved in speech and swallowing) or limb onset (affecting arms or legs) (Fig.1). Upper and lower motor neuron involvement is what defines ALS from other motor neuron diseases. Signs of upper motor neuron degeneration include exaggerated reflexes (hyperreflexia) and limbs spasticity, while signs of lower motor neuron degeneration include muscle weakness and fasciculation's. Because ALS involves predominantly motor neurons, it doesn't affect involuntary movement, such as eye movement, bladder and bowel control. ALS usually does not affect cognitive function. As ALS progresses, all voluntary muscles become progressively paralysed (reviewed by Wijesekera and Leigh, 2009).

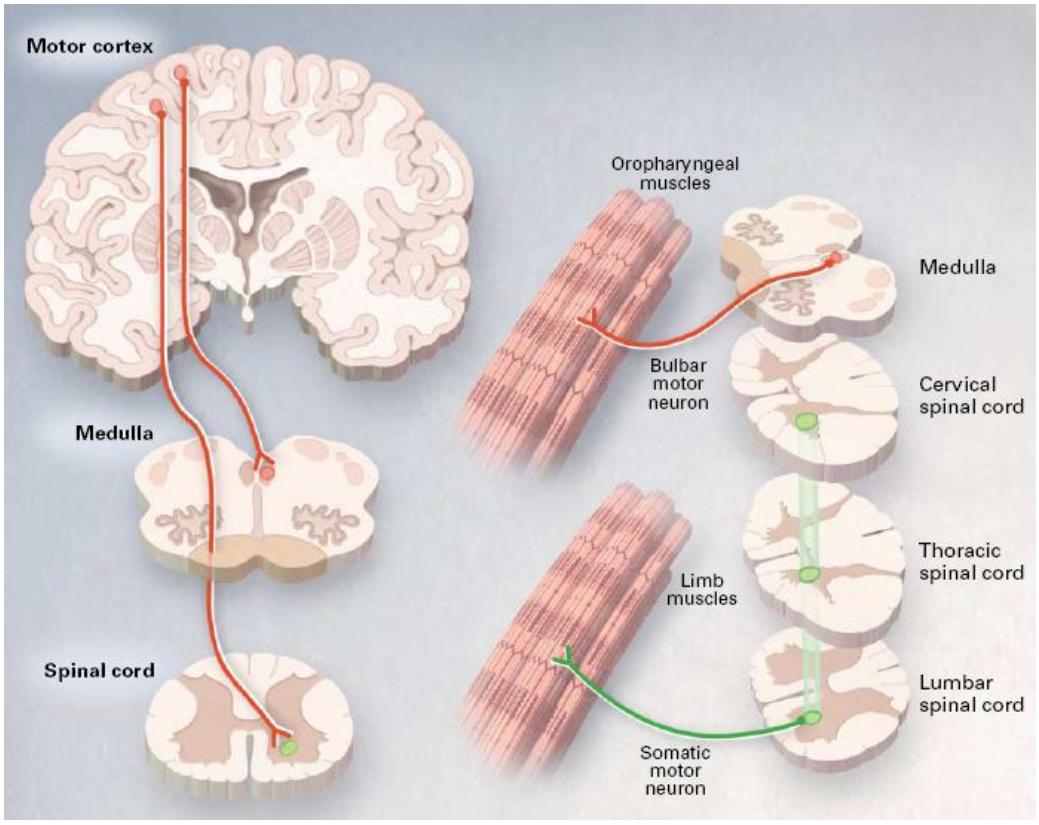

Figure 1. Motor neurons selectively affected in ALS. Degeneration of motor neurons in the motor cortex leads to clinically apparent signs of upper motor neuron abnormalities. Degeneration of motor neurons in the brain stem and spinal cord causes muscle atrophy, weakness, and fasciculation (from Rowland and Schneider, 2001)

2.1.3 Epidemiology and Etiology

ALS is an orphan disease, since its' incidence is 1-2 per 100,000 per annum. However, the incidence of ALS increases when a population is followed longitudinally. The prevalence is 4-6 cases per 100,000 people. The mean age of onset for sporadic ALS is 45-58 years, with the male to female ratio of 3:2. The greatest risk factors for developing ALS are age, gender and family history, while race, ethnicity, or socioeconomic boundaries appear to make no difference (reviewed by Passinelli and Brown, 2006).

Approximately 90% of ALS cases are sporadic (sALS), with no genetic or hereditary component, while 10% of cases are inherited (familial ALS, fALS). Both forms are clinically and histopathologically indistinguishable. Approximately 20% of fALS patients carry mutations in the Cu/Zn superoxide dismutase (SOD1) gene (Rosen et al., 1993). To date over 150 different SOD1 mutations have been identified in ALS patients, and transgenic mice

that over-express human mutant SOD1 develop an ALS-like phenotype of adult-onset motor neuron degeneration with a reduced lifespan (Gurney et al., 1994). Mutant SOD1 mice have become the standard laboratory model of ALS. A number of other gene mutations have been identified in sALS and fALS patients, these include TDP 43 (Sreedharan et al., 2008; Gitcho et al., 2008), FUS/TLS (Vance et al., 2009; Kwiatkowski et al., 2009) and angiogenin (Greenway et al., 2006). However, it remains unknown have these gene mutations are involved in ALS pathogenesis.

2.2 Angiogenin and ALS

Angiogenin (ANG) is an angiogenic and hypoxia-inducible factor, and as such had never previously been associated with ALS. This 14.4 kDa protein, containing 123 amino acids, is a member of the RNAse A superfamily. The function of angiogenin in the nervous system is relatively unknown, but in endothelial cells angiogenin has been shown to be a potent inducer of neovascularisation (Fett et al., 1985). Its angiogenic activity has been shown to involve nuclear translocation and stimulation of rRNA transcription (Xu et al., 2003), leading to the activation of signal transduction pathways important for angiogenesis and cell proliferation (Gao et al., 2008). Previous studies have shown a link between another angiogenic and hypoxic factor, vascular endothelial growth factor (VEGF), and ALS (Lambrechts et al., 2003). Mice carrying a disruption of the promoter element of the VEGF gene developed an ALS-like phenotype of adult onset motor neuron degeneration and hind limb muscle atrophy (Oosthuyse et al., 2001). Analysis of ALS patients identified that certain VEGF haplotypes, associated with a decreased circulating level of VEGF, are associated with increased incidence of the disease (Lambrechts et al., 2003). Thus, there appears to be a role for angiogenic and hypoxia-inducible factors in ALS pathogenesis.

Mutations in angiogenin were initially identified in 15 ALS patients, of whom 4 had familial ALS and 11 sporadic ALS. In the initial description seven missense mutations were described in *ANG* (Q12L, K17E, K17I, R31K, C39W, K40I and I46V). These were identified in five different populations, although 12 of the 15 individuals with *ANG* mutations were of Irish or Scottish descent (Greenway et al., 2006). The K40I mutation was identified at position 191 (A to T) in the coding region. This point mutation causes an amino acid substitution of lysine to isoleucine affecting a functionally important catalytic residue (Greenway et al., 2006). Analysis of the ANG crystal structure suggested that mutations including K40I mutation may disrupt the structure and result in a loss of function (Fig. 2A, B).

More recent studies have identified other *ANG* missense variants in ALS patients from different populations including North America (Wu et al., 2007), France (Paubel et al., 2008), Netherlands (Van Es et al., 2009), Germany (Fernandez-Santiago et al., 2009) and Italy (Gellera et al., 2007; Conforti et al., 2008).

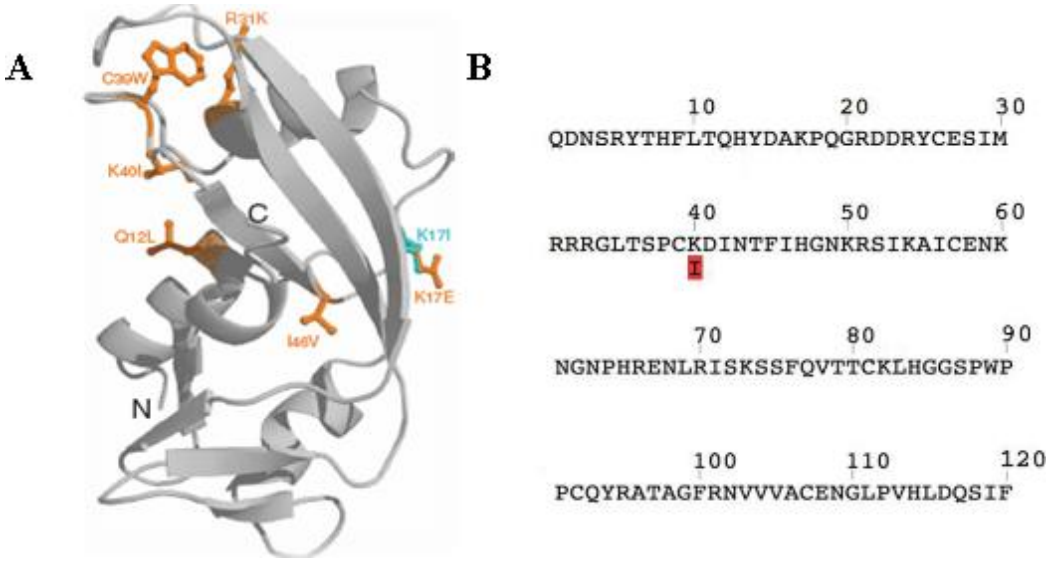

Figure 2. ALS-associated mutations in ANG. (A) 3-D structure of ANG. The majority of the mutations found are located within the catalytic core (Q12L, K17E, R31K, C39W, K40I and I46V). Only one of them is in the nuclear localization signal (K171) which is essential for angiogenin trafficking into the nucleus (from Greenway et al., 2006). **(B)** Amino acid sequence of ANG. The residue affected by ALS mutation K40I is highlighted (from Subramanian et al., 2008).

2.2.1 ANG is a neuroprotective factor

Since the identification of *ANG* mutations in ALS patients, the role of angiogenin in motor neurons has been examined in murine models. It has been shown that in neurons *in-vitro*, *ANG* plays an important role in neurite extension and pathfinding (Subramanian and Feng, 2007). *ANG* is also a hypoxia-inducible factor in motor neurons, and exposure of primary motor neuron cultures to hypoxic conditions reduces motor neuron viability (Sebastià et al., 2009). Treatment with recombinant ANG protein has been shown to protect primary motor neuron cultures from hypoxic injury *in-vitro* (Sebastià et al., 2009; Kieran et al., 2008). While *in-vivo*, in the mutant SOD1 mouse model of ALS, administration of recombinant ANG was shown to protect motor neurons and increase the lifespan of treated mice (Kieran et al., 2008). ALS-associated *ANG* mutations have also been studied *in-vitro*, and were found to

have no neuroprotective effect for primary motor neurons exposed to hypoxia. Interestingly, *ANG* K40I mutant has been associated with a loss of neuroprotective activity through a failure to appropriately activate the Akt cell survival-signaling pathway (Sebastià et al., 2009).

There are six angiogenin genes and three pseudogenes in mice whereas only one single angiogenin gene exists in humans (Cho et al., 2005). The most direct approach to define the function of *ANG* mutations in ALS pathogenesis would be to generate a transgenic mouse model expressing *ANG* mutant. However the expansion of *ANG* in mice makes the generation of such a transgenic mouse model difficult. In this study, we propose to generate recombinant adeno-associated viral vectors expressing either wild-type or ALS-associated *ANG* mutants *in-vivo* to overcome this problem.

2.3 Aim of this project

The aim of this study is to further understand the role of *ANG* mutations in ALS. This study has 3 overall aims:

1. **Generate *pAAV6-ANG K40I* mutant**

   ANG K40I mutant, marked with a polyhistidine tag, will be cloned into an AAV plasmid vector.

2. **Generate *pAAV6-ANG* wild type**

   ANG wild type, marked with a polyhistidine tag, will be cloned into an AAV plasmid vector.

3. **Identify *ANG* siRNA sequences**

   Three siRNA sequences against *ANG* will be tested on NSC34 cells, to identify which is the most efficient at knocking-down *ANG* expression.

3.1 Cloning of *ANG* WT and *ANG* mut into pAAV6 backbone

Minipreps of ANG. Plasmids pcDNA4/HisMaxAAngiogenin encoding *ANG* WT and pcDNA4/HisMaxAAngiogenin encoding *ANG* K40I mutant (Fig. 3A) (RCSI) were amplified into DH10B competent bacteria (Invitrogen Life Technologies) with an electroporation process. Briefly, DH10B competent bacteria were thawed on ice. The parameters of the Gene Pulser II Electroporation System (BioRad) were adjusted to 25 μF capacity, 200 ohms low resistance range, 500 ohms high resistance range, 1.7 kV ie. with an electrical shock of 12.5 kV/cm. Then, 2-3 μl of plasmid DNA (300 μg) was added to 50 μl of bacteria. This mix was placed into an electroporation cuvette and the electrical shock was performed. 1 ml of SOC medium (Sigma) was added immediately after the shock into the electroporation cuvette and the mix was incubated at 37°C, under agitation, for 1 h. Next, 200 μl of bacteria culture was plated on selective Zeocine LB agar (40 μg/ml) and incubated over-night at 37°C. The next day, 10 colonies of each plate were picked in order to perform minipreps. Each colony was grown over-night on 5 ml of LB + Zeocine (Invitrogen). Minipreps were performed with the QIAprep Spin Miniprep Kit (QIAGEN). These were digested with BamHI and EcoRI (Roche) at 37°C for 2 h. The presence of *ANG* was confirmed by band size on an agarose gel.

PCR. *ANG* WT and K40I mutant fragments were amplified by PCR, on minipreps positive candidates, with the following primers: forward, 5'-GGA GCC TGT GTT GGA AGA GA-3'; reverse, 5'-TGA ATG TTG CCA CCA CTG TT-3' (Microsynth). PCR was performed with the KOD Hot Start DNA Polymerase Kit (Merck) with 1 μl of template DNA in a total volume of 50 μl. Cycling conditions were the following; Polymerase activation: 95°C for 2 mins, followed by denaturing at 95°C for 20 s, annealing at 53.4°C for 10 s, followed by extension at 70°C for 5 s and repeated for 30 cycles.

Enzymatic digestions and ligations. Concentrations of PCR samples were determined with the Nanodrop. The amplification of the insert was confirmed on agarose gel. Next, PCR products of each positive candidate was extracted and purified from agarose gel, using the NucleoSpin Extract II Kit (Macherey-Nagel). PCR products purified were digested with XbaI and SacI (Roche) for 1.5 hrs at 37°C. *ANG* fragments were inserted into the backbone vector pAAV6:CMV:MCS (Fig. 3B) which had been previously digested by the same restriction enzymes.

Figure 3

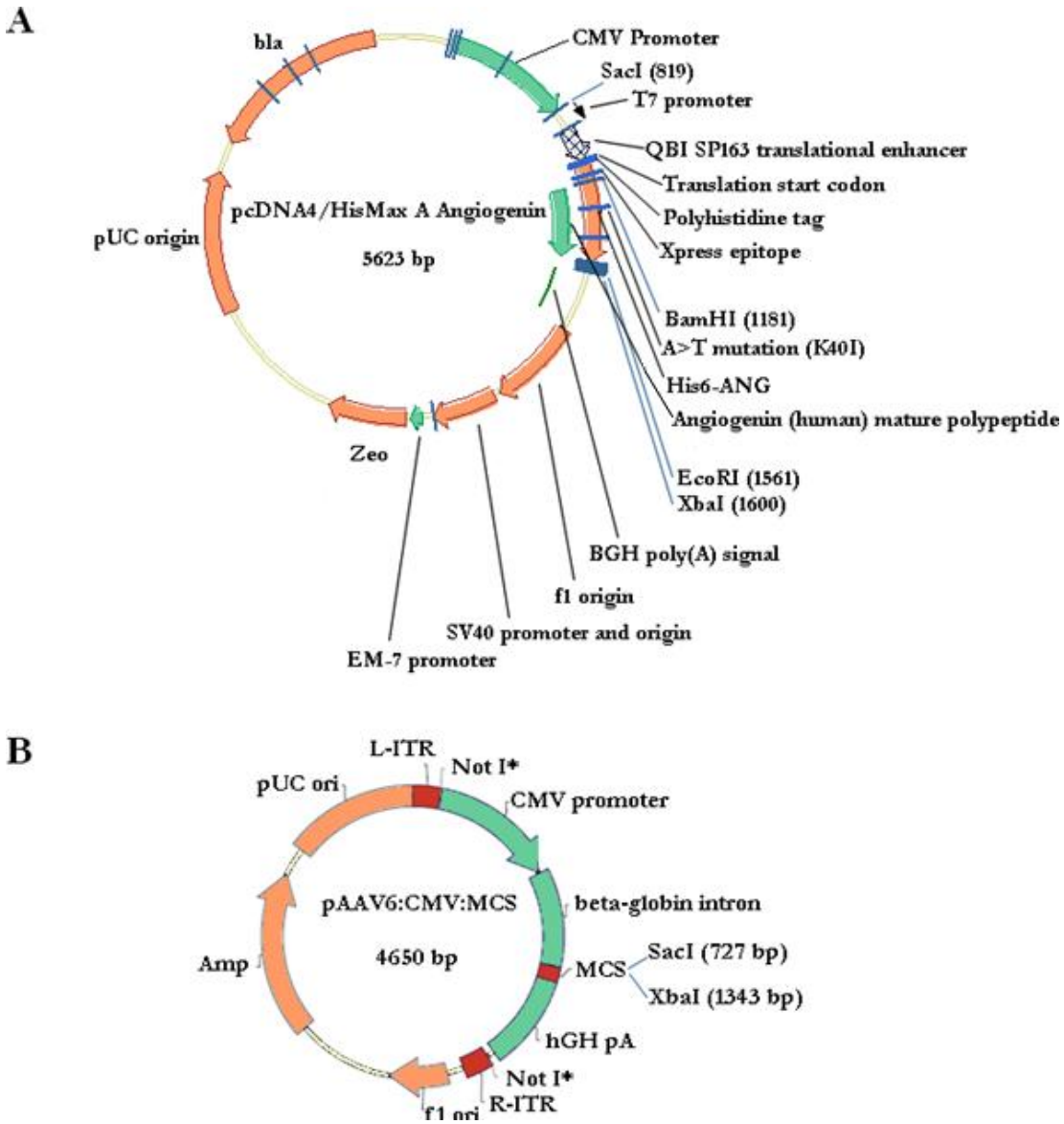

Figure 3. Map of plasmids using for the cloning. (A) Map of the pcDNA4/Mix A Angiogenin plasmid. Restriction sites of enzymes used for the cloning are located near the *ANG*. The polyhistidine tag used to marked angiogenin is located after the start codon. The substitution causing the K40I mutation is noted into the angiogenin mature polypeptide sequence (A>T mutation). **(B)** Map of the pAAV6:CMV:MCS. Restriction sites of the used enzymes are located into the multiple cloning site (MCS). This one is flanked by inverted terminal repeat (ITR) sequences (the number of nucleotides depending on the serotype). ITR sequences (*cis*-elements) are required for amplification of the viral genome within an infected cell and for encapsidation of the DNA (Grimm, 2002).

Transformations into DH10B competent bacteria, cultures on selective Amp LB agar (50 μg/ml) and minipreps were performed. Plasmids concentrations were analyzed with the Nanodrop. The inserted *ANG* WT and *ANG* K40I mutant were confirmed by enzymatic digestion with XbaI and SacI (Roche) and by sequencing. This one was performed by Microsynth: 0.8 ug of plasmid was added with 20 pmol of β-globin primer (Microsynth) were sent; in a total volume of 10 μl. Thereafter, sequences were analyzed to avoid the presence of point mutations not wished.

Transformations into DH10B competent bacteria with plasmids pAAV6 expressing respectively *ANG* WT and *ANG* K40I mutant and cultures on selective Amp LB agar were performed. The next day, 1 colony of each plate was picked and grown on 3 ml of LB + Amp (Sigma). Six hours later, this culture was used to inoculate 2 x 500 ml of LB + Amp during 16 hrs. From this culture, maxipreps were performed, using the Maxipreps Kit (Macherey-Nagel).

3.2 Transfection of NSC34 cells with *ANG* siRNA

Cell culture. NSC34 cells are an immortal motor-like cell line produced by fusion of motor neuron-enriched embryonic mouse spinal cord cells with mouse neuroblastoma cells (Cashman et al., 1992). For *ANG* knockdown, motor neuron-like NSC34 cells were plated on 6-well plates, grown to ~50% confluence in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Invitrogen) and 1% penicillin/streptomycin solution (Invitrogen).

siRNA transfection. Cells were transfected with 20 μM of predesigned siRNA to *ANG* (Invitrogen Stealth siRNA). Transfections were performed using Lipofectamine 2000 (Invitrogen) in OptiMEM medium (Invitrogen). A tube containing 9.6 μl siRNA at 20 μM and 40.4 μl of OptiMEM and a tube containing 9.6 μl of Lipofectamin and 40.4 μl of OptiMEM were prepared. These two mixes were incubated together at room temperature for 20 minutes. Cells were incubated in OptiMEM. Next, 100 μl of this mix was added to the cells. In this experiment three different siRNA sequences were tested in order to identify the most efficient sequence for knock-down. As a control, cells were treated with either scrambled siRNA or untreated (the plate medium was just changed for 900 μl of OptiMEM). Transfections were carried out for 48 hrs and 72 hrs at 37°C/ 5% CO_2, changing then the OptiMEM medium to DMEM medium after 24 hrs. After the different times of incubation, cultures were harvested immediately for protein extraction: The 6-well plate was placed on ice

and the DMEM was replaced with 150 µl of Lysis Buffer containing protease and phosphatase inhibitors (Roche). Lysate was left for 15 min on ice, then centrifuged for 10 mins at 4°C at 8000 g, after which the supernatant was recovered and conserved at -20°C.

Western blotting. The protein concentration of samples was determined using a modified Bradford Assay where 1 µl of sample is added to 1 ml of Bradford solution diluted (1x). After 2 mins, the sample optical density (OD) was measured at 600 nm. Standards were performed with 0, 4, 8, 16 and 20 ug/ul of Bovin Serum Albumin (BSA) (Pierce Biotech). A standard curve was generated, and from its' slope (y =mx + c), the protein concentrations of samples were calculated. Equal amounts of protein were separated by electrophoresis (25 µg). Samples are mixed with Loading Buffer and incubated for 15 mins at 95°C. Then separated onto a 12% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane (BioRad), followed by immunodectection using primary antibody to ANG (1:500, Abcam), overnight at 4°C, goat anti-rat secondary antibody (1:500, Invitrogen–Molecular Probes) at room temperature for 2 hrs, followed by detection the Odyssey® Infrared Imaging System (LI-COR Biosciences)

Localization of siRNA and cell survival. NSC34 cells were transfected with green fluorescent labeled *ANG* siRNA, mounted in mounting medium containing the nuclear stain DAPI (Vector Laboratories) and visualized with a fluorescent microscope.

4. RESULTS

4.1 Cloning of *ANG* WT and *ANG* mut into pAAV6 backbone

4.1.1 Digestion of plasmids and amplification of *ANG*

The two plasmids encoding for *ANG* WT and *ANG* K40I mut were amplified via transformation using competent bacteria. To confirm the presence of the angiogenin fragment, plasmids were digested with BamHI and EcoRI. Digestion products were separated by electrophoresis on agarose 1% TAE gel. All purified wild-type and mutant plasmids had the *ANG* fragment digested (Fig. 4A, B). PCR was used to amplify *ANG* WT and K40I mutant from the plasmid. The PCR was confirmed by analysis of PCR products**.** Initially, we used a set of primers that were designed *in-house*, without the use of a specific primer design software. The idea with these primers was to design primers where the forward primer encoded a Kozak sequence, a restriction site (EcoRI) and a start codon, and the reverse primer encoded a restriction site (HindIII) and stop codon (see Annex 8.2). This pair of primers did not work, as no fragment was visible after the analysis of PCR products (Fig. 5A). Having tried a number of different approaches (altering annealing temperature *etc*) we designed a second set of primers using Primer3 software. Here, the forward primer began at the translation start codon and the reverse primer began at the BGH-Poly(A) signal (see Annex 8.2), *ANG* WT and mut were amplified as shown the analysis of PCR products (Fig. 5B, C).

4.1.2 Ligations and sequencing

PCR products and pAAV6 backbone were digested by BamHI and SacI. Digestion products were ligated together using the T4 ligase. To confirm the correct insertion of the angiogenin inserts, products of ligation were digested by the same restriction enzyme and separated on agarose gel. And as can be seen in Figure 6A and B, all ligations products appeared to contain the *ANG* WT or mutant. These new plasmids were called respectively pAAV6-*ANG* WT and pAAV6-*ANG* K40I mut. Analysis of band size showed that they had approximately the theoretical length expected (Fig. 6C).

Figure 4

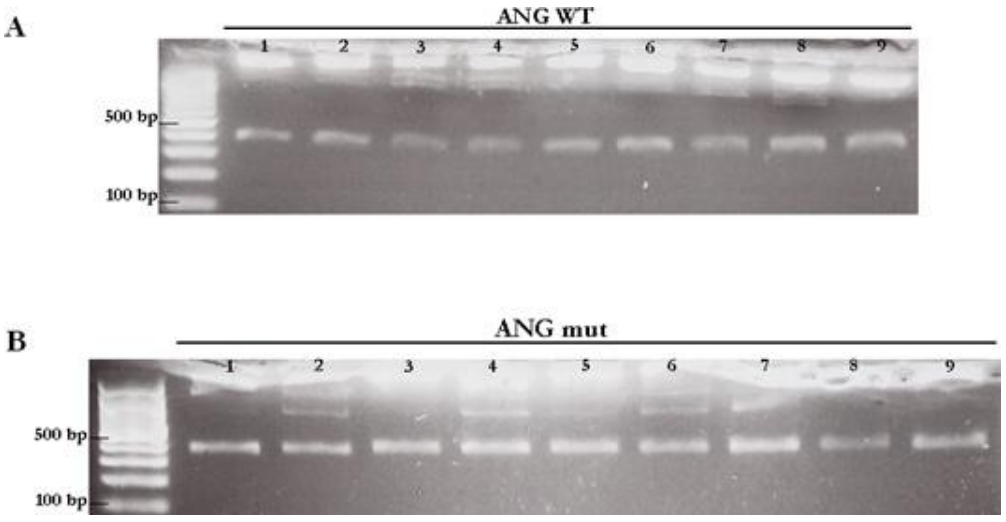

Figure 4. Verification of the presence of *ANG*. (A, B) Analysis of plasmids encoding for *ANG* WT (**A**) and *ANG* mut (**B**). All candidates have the presence of a band at 400 bp, after digestion by BamHI and EcoRI. This observation suggests that all plasmids have *ANG* fragments, because *ANG* has a length of 380 bp, after the digestion by the same enzymes.

Figure 5

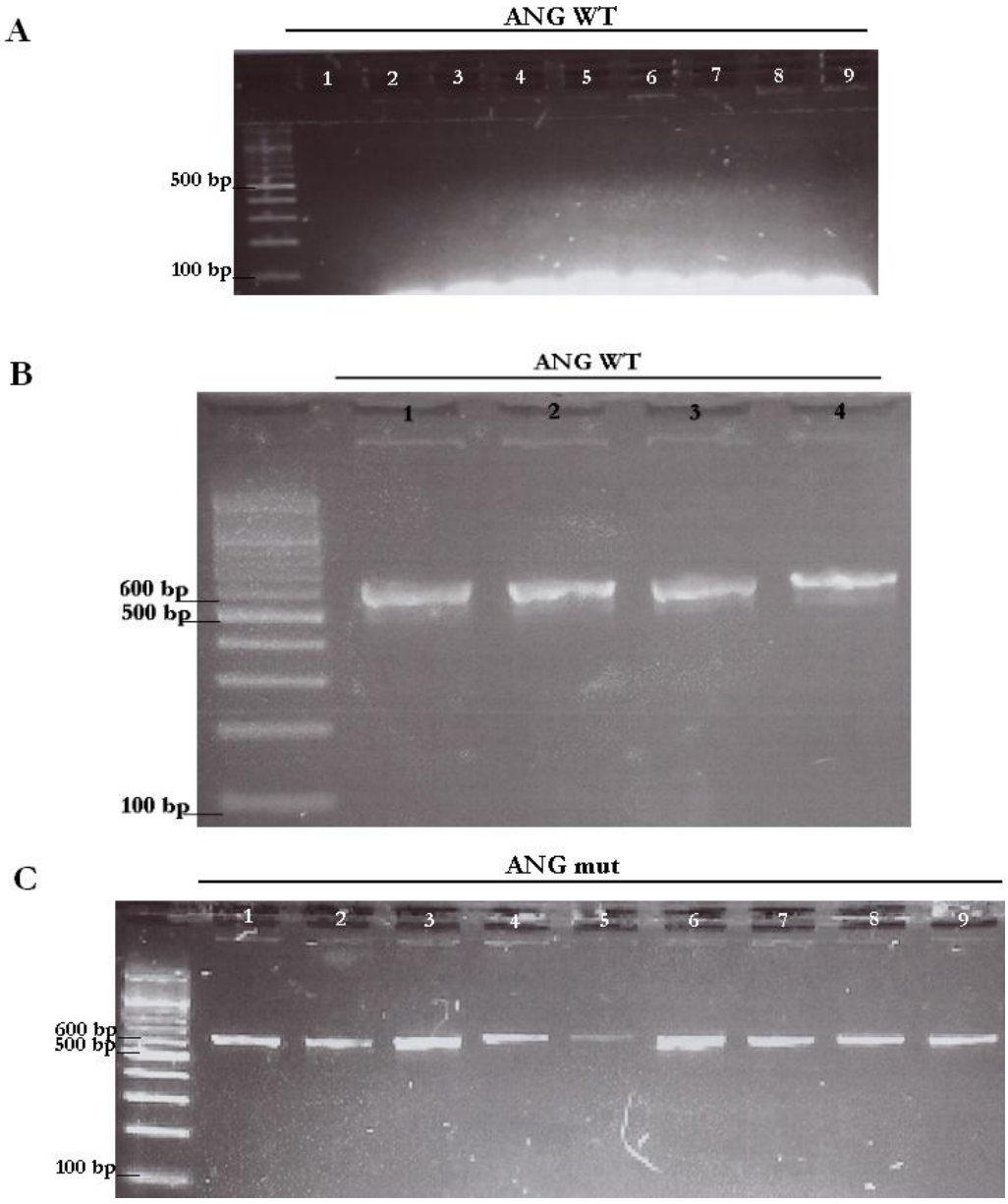

Figure 5. Amplification of *ANG* by PCR. **(A)** Analysis of PCR products, after the utilization of primers designed *in house*. No fragments are visible. **(B, C)** Analysis of PCR products after the utilization of primers designed via Primer3, from plasmids encoding for *ANG* WT (**B**) and *ANG* mut (**C**). Normally, the length of the amplified *ANG* fragment is 618 bp. For all candidates, the band at 600 bp attests that *ANG* fragments were amplified.

Figure 6

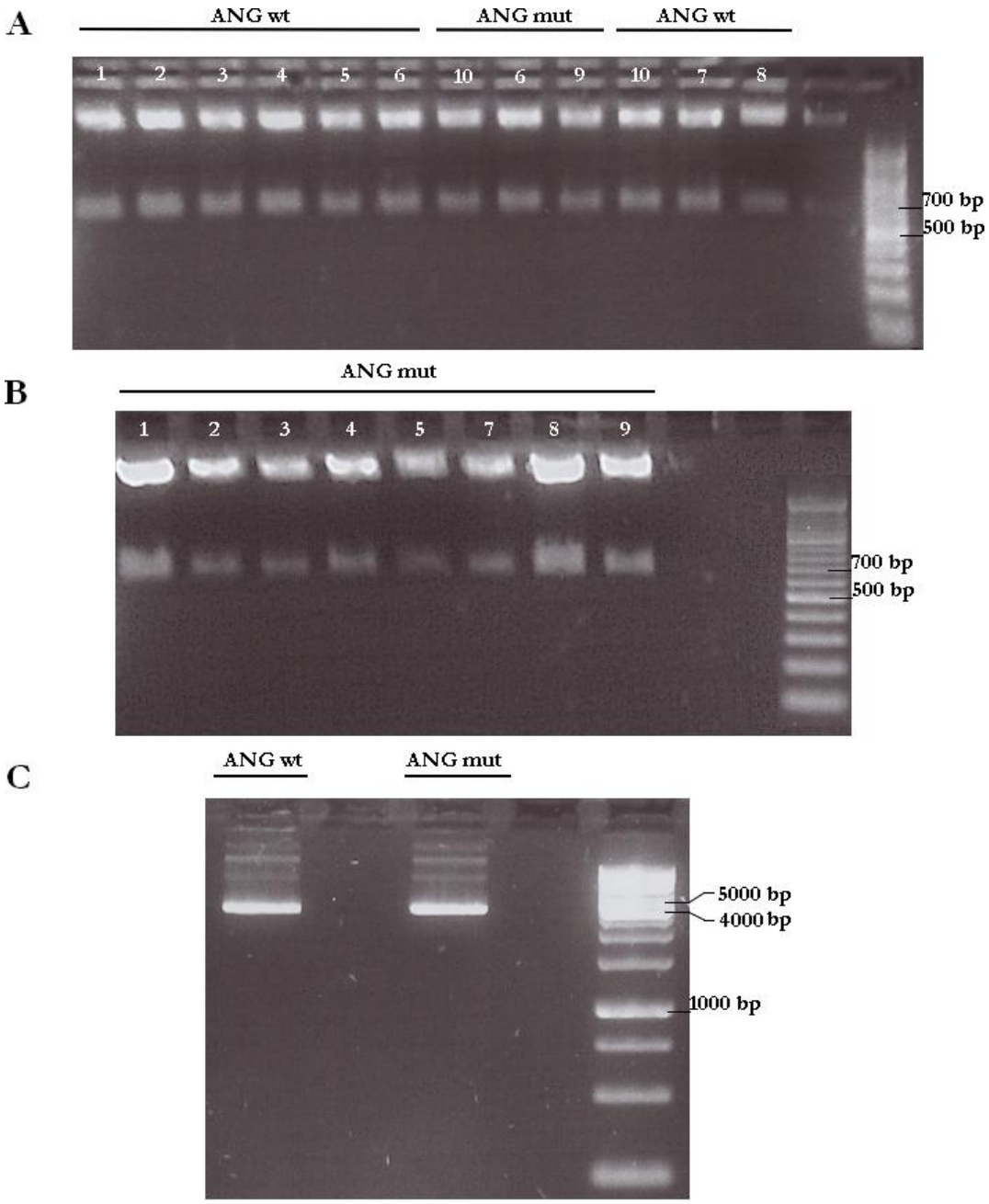

Figure 6. Verification of the ligations. **(A, B)** Analysis of ligation products encoding *ANG* WT (**A**) and *ANG* mut (**B**), digested by BamHI and SacI. All candidates have the presence of a band at 700 bp. This observation suggests that all plasmids have inserted *ANG* fragments, because *ANG* has a length of 781 bp, after the digestion by the same enzymes. **(C)** Gel analysis of ligation products without enzymatic digestion. Bands between 4000 bp and 5000 bp are in a range corresponding with the theoretical length (4815 bp).

To confirm the correct integration of *ANG* WT and *ANG* mut, two pAAV6-*ANG* WT and two pAAV6-*ANG* K40I mut plasmids were sequenced (see Annex 8.3). Analyze of these sequences demonstrated that one pAAV6-*ANG* WT plasmid had a point mutation in the coding sequence of angiogenin. This plasmid was not used for maxipreps. After maxipreps, concentrations of plasmids were respectively 453 ng/µl for pAAV6-*ANG* WT and 512 ng/µl for pAAV6-*ANG* K40I mut.

4.2 Transfection of NSC34 cells with *ANG* siRNA

4.2.1 Western blotting

Knockdown of exogenous *ANG* was successfully achieved in NSC34 cells using an siRNA approach. NSC34 cells were treated with three different siRNA constructs against *ANG*. At 72 hrs and 48 hrs after transfection, *ANG* siRNA sequence 1, reduced ANG expression more effectively than *ANG* siRNA sequence 2 or *ANG* siRNA sequence 3. Indeed, ANG is weakly expressed when cells are transfected with siRNA sequences and the weakest expression is visualized when *ANG* siRNA sequence 1 is used (Fig. 7A, B).

4.2.2 Localization of siRNA and cell survival

ANG siRNA sequence was directly associated with a green fluorescent label and the localization of siRNA was visualized with a microscope, showing expression in the cytoplasm. NSC34 cells transfected with *ANG* siRNA for 72 hrs, were visualized and as can be seen in Fig. 7C, there was cell death in *ANG* siRNA-treated cultures after 72hrs.

Figure 7

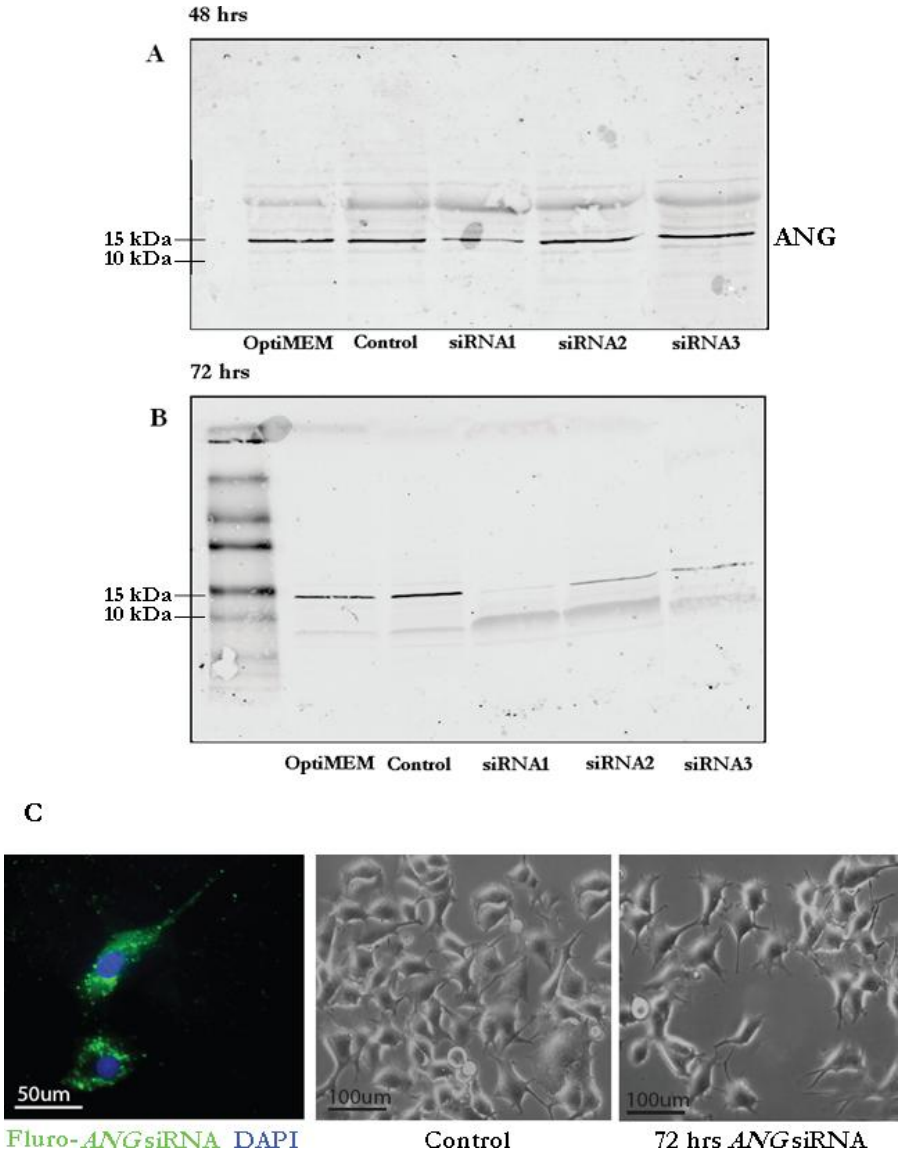

Figure 7. Knockdown of *ANG* with siRNA. **(A, B)** Western blot analysis showing ANG levels in NSC34 cells transfected with the three different *ANG* siRNA constructs, after 48 hrs (**A**) and 72 hrs (**B**). The lowest expression of ANG, characterized by the 15 kDa band, is visualized when *ANG* siRNA sequence 1 is used. **(C)** Fluorescent labeled *ANG* siRNA in NSC34 cells (green) with nuclear stain DAPI (blue). Phase contrast images showing fewer cells survived in 72hrs *ANG* siRNA vs control.

5. DISCUSSION

5.1 Cloning of *ANG* WT and *ANG* mut into pAAV6 backbone

5.1.1 Cloning strategy

In this study, *ANG* WT and *ANG* K40I mut were cloned from pcDNA4/HisMax plasmids into the pAAV6:CMV:MCS plasmid. The *ANG* K40I mutant was chosen as molecular modeling of the K40I substitution suggests that several key interactions are lost, including the ribonucleolytic activity of angiogenin (Greenway et al., 2006). Furthermore, recent *in-vitro* studies have shown that motor neurons expressing *ANG* K40I mut exerted no protective activity in *in-vitro* models of cell death (Kieran et al., 2008, Sebastià et al., 2009). Recent studies have shown identified the *ANG* K17I mutation, originally identified in ALS patients (Greenway et al., 2006), in non-ALS patients (Fernández-Santiago et al., 2009; Van Es et al., 2009). Functional assays have shown that the K17I mutation results in a loss-of-function (Wu et al., 2007). Therefore, in the future it will be interesting to compare the effects of *ANG* K40I and *ANG* K17I *in-vivo*.

The first attempt to clone *ANG* WT and *ANG* mut by PCR from the pcDNA4/HisMax plasmids using primers designed *in-house* did not work. A number of different factors can significantly impact on PCR sensitivity and specificity, most notably the annealing temperature and cycling parameters. In this case, melting points of primers were both close (66.5°C and 63.2°C). PCR cycling parameters such as cycle times and temperatures were verified. A number of 'trouble-shooting' experiments were made where the annealing temperature was modified, but no product was obtained. Finally we deduced that the problem was most likely to be the primers themselves, since they were designed *in-house* to force-fit the sequence of interest. Primer 3 software was used to generate a second set of primers to clone *ANG* WT and *ANG* mut, and these primers were successful. The cloning strategy was classical where *ANG* WT and *ANG* mut from pcDNA4/HisMax plamids were amplified by PCR and digested, before being ligated with the pAAV6:CMV:MCS plasmid.

5.1.2 Purpose of pAAV6 backbone

ANG WT and *ANG* mut were cloned into a pAAV6 backbone to generate adeno-associated viruses (AAV). These non-enveloped icosohedral viruses, that need the presence of a co-infecting helper virus to execute its complete cycle of replication, are very interesting for gene

transfer because they can infect large cells types in any state (dividing or non-dividing cells). And they will stably integrate into the host cell genome. The dependence between the adeno-associated virus and its auxiliary virus allows control of vector replication. The production of recombinant AAV viruses containing *ANG,* requires transfection of the following components into packaging cells: (i) AAV vector plasmid coding for *ANG*, as pAAV6-*ANG* WT or pAAV6-*ANG* mut, (ii) AAV helper plasmid, and (iii) Adenoviral helper plasmid. When the constructs are transfected into packaging cells together, *ANG* is replicated and incorporated in the single-stranded format into preformed empty capsids. The resulting infectious recombinant AAV virions are released from the cells and purified.

In this study the promoter of choice in the pAAV6 backbone was the cytomegalovirus (CMV) promoter. The cytomegalovirus promoter is a strong viral promoter used in many mammalian expression vectors, including the motor system (Towne et al., 2008). However, some studies have shown that the activity of this promoter decreases over-time (Smith et al., 2000). This could represent a problem for the proposed *in-vivo* studies to assess the effect of *ANG* mut over-expression in wild-type mice. Typically in this type of experiment mice are injected with virus and monitored for upto 24 months for changes in behaviour and/or lifespan. If the activity of the promoter driving the expression of the transgene of interest was to diminish, this could complicate the analysis of the experiment results. It will be important to assess the expression of the transgene over-time in injected mice, and perhaps prepare an AAV6 backbone with an alternative promoter such as the phosphoglycerate kinase (PGK) promoter.

5.1.3 Perspectives: To examine the effect of *ANG* WT/mut expression *in-vivo*.

Recombinant AAV serotype 6 (rAAV6) is a vector capable of particularly efficient muscle-to-motor neuron retrograde transport. Indeed, studies in mice have demonstrated that the intravenous administration of this rAAV serotype results in body-wide transduction of skeletal muscle (Gregorevic et al., 2004). Therefore, when it is delivered to skeletal muscle, rAAV6 is capable of retrograde transport along motor axons to motor neurons in the spinal cord (Kaspar et al., 2003). It has been also demonstrated that a single noninvasive injection of rAAV6:CMV:GFP can result in transduction of motor neurons at all levels of the spinal cord and brain stem. (Towne et al., 2008). Recombinant AAV will be injected into the muscles of wild-type or mutant SOD1 mice to stably integrate *ANG* WT and *ANG* mut into the genome of motor neurons. In this way, the neuroprotective effect of *ANG* WT in mutant

SOD1 mice, and the effect of ANG mut in wild-type mice (*do they develop and ALS-like phenotype*?) can be studied.

5.2 Transfection of NSC34 cells with *ANG* siRNA

5.2.1 *ANG* knockdown using siRNA

Small interfering RNA (siRNA) is the method of choice to silence a specific gene. Indeed, in most mammalian cells, long dsRNA (>30 bp) provokes the γ-interferon pathway (antiviral defense). This interferon response induces a global shutdown of protein synthesis, thus precluding the use of long duplex RNA for specific gene silencing. This obstacle can be overcome by the direct use of siRNA (19-23 nucleotides), which evades the radar of the mammalian interferon response. In this study, different commercially available *ANG* siRNA sequences were tested in NSC34 cells, and one of these sequences was found to be more efficient than the others. In this study we found that the viability of NSC34 cells transfected with *ANG* siRNA was reduced after 72 hrs of incubation. This can be explained by the absence of the protective effect of *ANG*. Indeed, a previous study has also demonstrated that the knock-down of endogenous angiogenin potentiates stress-induced cell death in motor neurons *in vitro* (Kieran et al., 2008).

5.2.3 Perspectives: *In-vivo ANG* siRNA delivery

The *ANG* siRNA sequences tested here are also commercially available for use *in-vivo*, and the next step will be to assess the identified sequence *in-vivo* in wild-type mice. *ANG* siRNA will be administered to the nervous system in these mice by intra-ventricular delivery, and the phenotype and behaviour of mice will be assessed in order to determine whether mice develop an ALS-like disease phenotype of muscle paralysis and motor neuron degeneration.

6. CONCLUSION

More than 150 years after its initial description, the ALS pathogenesis remains largely undetermined. The description of ALS-specific loss-of-function mutations in *ANG,* and the characterization of the neuroprotective functions of angiogenin have opened new prospects for ALS therapies. The therapeutic modulation of angiogenin expression and activity, such as motor neuron-targeted delivery of angiogenin, using viral-particles encoding *ANG* or manipulation of the pathways used by angiogenin could be a novel approach for ALS patients.

7. LITERATURE

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8. ANNEXES

8.1 Detailed material

8.1.1 Specific material for the cloning of *ANG* WT *and ANG* mut into pAAV6 backbone

pcDNA4/HisMaxAAngiogenin: Kind gift of Prof. Jochen Prehn; Royal College of Surgeon (RCSI); Ireland

pAAV6 backbone: Created from Towne et al., 2008

DNA primers ANG: (Microsynth AG; Switzerland)

Forward primer: 5'-GGA GCC TGT GTT GGA AGA GA-3'

Concentration: 100 µM / Melting point: 57.5°C

Reverse primer: 5'-TGA ATG TTG CCA CCA CTG TT-3'

Concentration: 100 µM / Melting point: 53.4°C

Human β-globin primer: (Microsynth AG; Switzerland)

Forward primer: 5'-ACCTGACTCCTGAGGAGAAG-3'

Concentration: 100 µM / Melting point: 54°C

8.1.2 Specific material for the transfection of NSC34 cells with *ANG* siRNA

NSC34 cells: Kind gift of Prof. Neil Cashman; University of Toronto; Canada

ANG siRNA; Invitrogen Stealth siRNA; Invitrogen AG; Switzerland

Primary antibody: Angiogenin antibody (ab10600); Mouse monoclonal; Abcam Inc.; USA

Secondary antibody: Alexa Fluor® 680 goat anti-rat IgG (H+L); 2 mg/ml; Highly cross-adsorbed; Invitrogen Molecular Probes; Invitrogen AG; Switzerland

8.1.3 Restriction enzymes and restriction enzymes buffers

BamHI; 1,000 U (10 U/µl); Roche AG; Switzerland

EcoRI; 1,000 U (10 U/µl); Roche AG; Switzerland

XbaI; 1,000 U (10 U/µl); Roche AG; Switzerland

SacI; 1,000 U (10 U/µl); Roche AG; Switzerland

SuRE/Cut Buffer A (10x); Roche AG; Switzerland

SuRE/Cut Buffer B (10x); Roche AG; Switzerland

T4 DNA Ligase; 500 U (1 U/µl); Invitrogen AG; Switzerland

T4 DNA Ligase Reaction Buffer (5x); Invitrogen AG; Switzerland

KOD Hot Start DNA Polymerase; 200 U (1 U/ul); Merck KGaA; Germany

Include: 10x PCR Buffer for KOD Hot Start DNA Polymerase; 25 mM $MgSO_4$; dNTPs (2 mM each)

8.1.4 Media, solutions and buffers

Quick Start Bradford Dye Reagent; BioRad Laboratories Inc.; USA

Bovine Serum Albumin (BSA); 2 mg/ml; Pierce Biotechnology Inc.; USA

Vectashield® Hard Set™ Mounting Medium with DAPI; Vector Laboratories Inc.; USA

Dulbecco's Modified Eagle Medium (DMEM); Invitrogen AG; Switzerland

Fetal Bovine Serum (FBS); Invitrogen AG; Switzerland

Penicillin-streptomycin solution (Pen/Strep); Invitrogen AG; Switzerland

OptiMEM® Reduced Serum Media; Invitrogen AG; Switzerland

SOC Medium; Sigma-Aldrich GmbH; Switzerland

Water; PCR Reagent; Sigma-Aldrich GmbH; Switzerland

TAE Buffer:

40 mM Tris (hydroxymethyl) aminomethane (Tris base); Molecular biology grade; BioSolve; ND

20 mM Acetic acid (glacial) 100%; Merck KGaA; Germany

1 mM EDTA disodium salt dihydrate; AppliChem GmbH; Germany

TBE Buffer:

45 mM Tris base (BioSolve)

88 mM Boric acid; Molecular biology grade; Sigma-Aldrich GmbH; Switzerland

1 mM EDTA (AppliChem)

LB Broth:

1.0% Trypton (Peptone from casein); AppliChem GmbH; Germany

0.5% Yeast Extract; Sigma-Aldrich GmbH; Switzerland

1.0% NaCl; Molecular biology grade; Sigma-Aldrich GmbH; Switzerland

SDS Gel-Loading Buffer:

50 mM Tris-base (pH 6.8) (BioSolve)

100 mM Dithiothreitol (DTT); Sigma-Aldrich GmbH; Switzerland

2% Sodium dodecyl sulfate (SDS); Molecular biology grade; Sigma-Aldrich GmbH; Switzerland

0.1% Bromophenol blue sodium salt; Molecular biology and electrophoresis grade; Sigma-Aldrich GmbH; Switzerland

2% 2-mercaptoethanol; Electrophoresis grade; Sigma-Aldrich GmbH; Switzerland

10% Glycerol; Electrophoresis grade, ≥99%; Sigma-Aldrich GmbH; Switzerland

Lysis Buffer:

50 mM Tris base (pH 6.8) (BioSolve)

2% SDS (Sigma-Aldrich GmbH)

5% Glycerol (Sigma-Aldrich GmbH)

1% 2-mercaptoethanol (Sigma-Aldrich GmbH)

\+ PhosSTOP Phosphatase Inhibitor Cocktail Tablets; 1 tablet for 1 liter total volume; Roche AG; Switzerland

Complete Protease Inhibitor Cocktail Tablets; 1 tablet for 1 liter total volume; Roche AG; Switzerland

Transfer Buffer:

25 mM TrisBase (BioSolve)

192 mM Glycine; Electrophoresis grade; Sigma-Aldrich GmbH; Switzerland

10% Methanol; Anhydrous, 99.8%; Sigma-Aldrich GmbH; Switzerland

Ponceau S Staining Solution:

0.1% (w/v) Ponceau S (sodium salt); Sigma-Aldrich GmbH; Switzerland

5% (v/v) Acetic acid (Merck KGaA)

Washing Solution:

Phosphate-Buffered Saline (PBS); Invitrogen AG; Switzerland

0.1% Tween® 20; Molecular biology grade; AppliChem GmbH; Germany

Blocking Solution:

1/3 Odyssey® Blocking Buffer; LI-COR Biosciences GmbH; Germany

2/3 PBS (Invitrogen)

8.1.5 Other chemical reagents

Zeocin™ Selection Reagent; 100 mg/ml (5 g); Invitrogen AG; Switzerland
Ampicilin sodium salt; Cell culture tested, powder; Sigma-Aldrich GmbH; Switzerland
Lipofectamine™ 2000 Transfection Reagent; Invitrogen AG; Switzerland
Gel Red; 10 000 x in Water; Biotium Inc.; USA
N,N,N',N'-Tetramethylethylenediamine (TEMED); Electrophoresis grade, ~99%; Sigma-Aldrich GmbH; Switzerland
Ammonium persulfate (APS); Electrophoresis grade, ≥98%; Sigma-Aldrich GmbH; Switzerland
Acrylamide : *N*,*N*'-Methylenebisacrylamide (32.5:1); Electrophoresis grade; Sigma-Aldrich GmbH; Switzerland

8.1.6 Practical

ElectroMAX™ DH10B™ Cells (*E.coli*); Invitrogen AG; Switzerland
Agarose tablets; Molecular biology grade (0.5 g /tablet); Serva GmbH; Germany
GeneRuler™ (1kb) and O'GeneRuler™ (100 bp) DNA Ladders; Fermentas Inc.; Canada
Precison Plus Protein™ Standards; BioRad Laboratories Inc.; USA
QIAprep Spin Miniprep Kit; QUIAGEN AG; Switzerland
NucleoBond® Xtra Maxi Kit; Macherey-Nagel GmbH; Germany
NucleoSpin Extract II Kit; Macherey-Nagel GmbH; Germany
GenElute™ HP Plasmid Miniprep Kit; Sigma-Aldrich GmbH; Switzerland
Electroporation cuvettes sterile and disposable; Thermo Electron Corp.; USA
Supported Nitrocellulose Membrane; 0.45µm; BioRad Laboratories Inc.; USA

8.1.7 Instruments and devices

NanoDrop; ND-1000 UV-Vis Spectrophotometer; NanoDrop Technologies; USA
Fluorescence Microscope Olympus AX70 + digital camera DP50 + mercury lamp device U-RFL-T; Olympus Suisse SA; Switzerland
Gene Pulser II Electroporation System; BioRad Laboratories Inc.; USA
PowerPac 200 + Sub-Cell GT Cell; BioRad Laboratories Inc.; USA
PowerPac 300; BioRad Laboratories Inc.; USA
Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell; BioRad Laboratories Inc.; USA
Shaker Vortex Mixer IKA 130 basic; IKA®-Werke GmbH & CO. KG; Germany

Multilmage® Light Cabinet Filter Positions + FluorChem Imager Software; Alpha Innotech Corp.; USA

Centrifuge Eppendorf 5415 D; Vaudaux-Eppendorf AG; Switzerland

Centrifuge Sorvall Evolution RC; Thermo Fisher Scientific Inc.; USA

Thermomixer Eppendorf compact; Vaudaux-Eppendorf AG; Switzerland

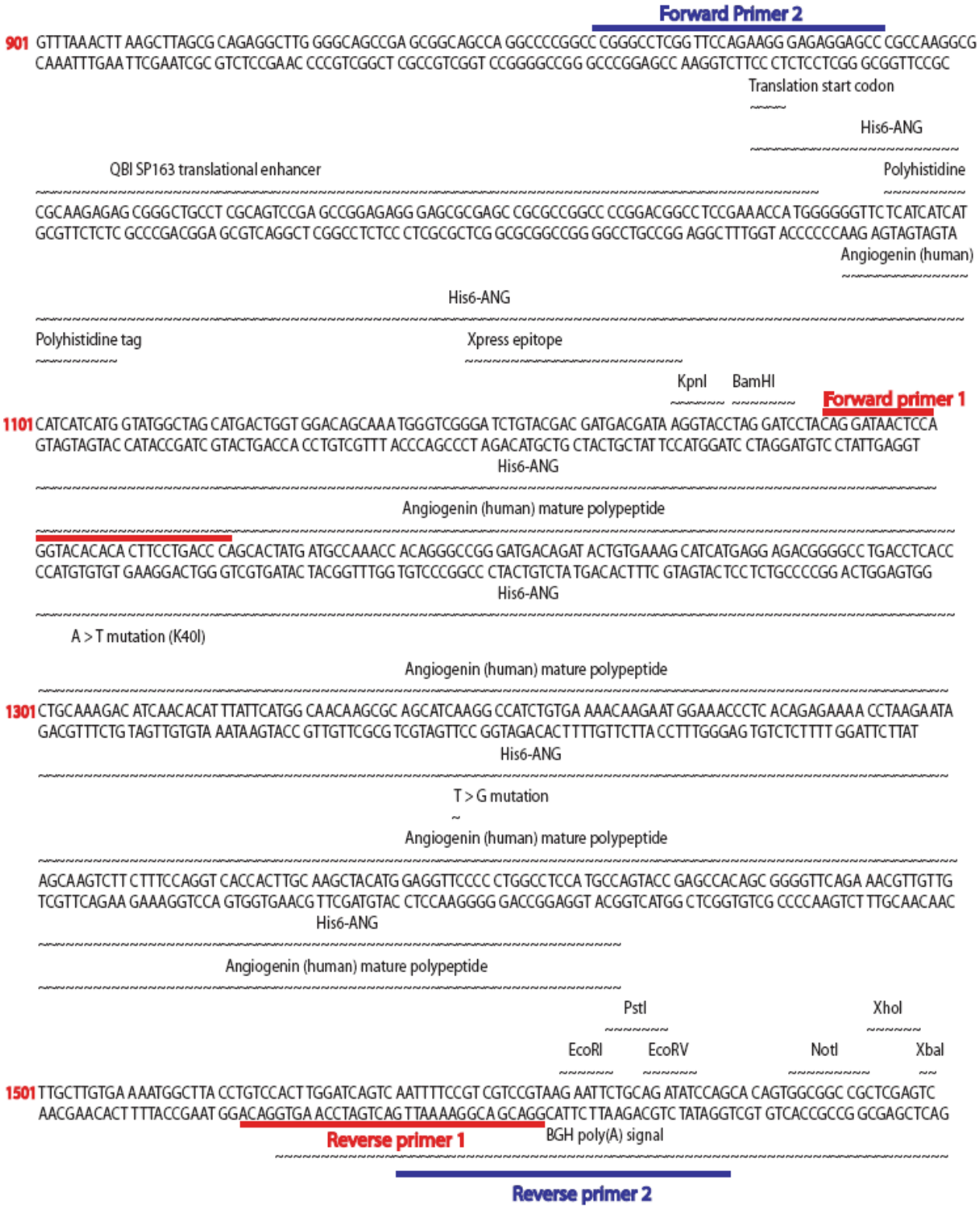
Biometra TGradient Thermal Cycler; Labrepco; USA

Odyssey® Infrared Imaging System; LI-COR Biosciences GmbH; Germany

Incubator Binder CB Series CO_2; G H Zeal Ltd; UK

Biological safety Cabinet; Labgard 430/435; Class II Type B2; NuAire, Inc.; USA

8.2 Localization of primers on the pcDNA4/HisMax A Angiogenin sequence



8.3 Sequence of *ANG* WT and *ANG* K40I mut

ANG WT

NNNCTGGTCTGTGTGCTGGCCNTNNTTTGGCAAANAATTGNGATTCGAACAT
CGATTGAATTCCCCGGGGATCCTCTAGAGTCGACCTGCAGAAGCTTGCCTCGA
GCAGCGCTGCTCGAGAGATCTACGGGTGGCATCCCTGTGACCCCTCCCCAGTG
CCTCTCCTGGCCCTGGAAGTTGCCACTCCAGTGCCCACCAGCCTTGTCCTAATA
AAATTAAGTTGCATCATTTTGTCTGACTAGGTGTCCTTCTATAATATTATGGGG
TGGAGGGGGGTGGTATGGAGCAAGGGGCAAGTTGGGAAGACAACCTGTAGG
GCCTGCGGGGTCTATTGGGAACCAAGCTGGAGTGCAGTGGCACAATCTTGGC
TCACTGCAATCTCCGCCTCCTGGGTTCAAGCGATTCTCCTGCCTCAGCCTCCCG
AGTTGTTGGGATTCCAGGCATGCATGACCAGGCTCAGCTAATTTTTGTTTTTTT
GGTAGAGACGGGGTTTCACCATATTGGCCAGGCTGGTCTCCAACTCCTAATCT
CAGGTGATCTACCCACCTTGGCCTCCCAAATTGCTGGGATTACAGGCGTGAAC
CACTGCTCCCTTCCCTGTCCTTCTGATTTTGTAGGTAACCACGTGCGGACCGAG
CGGCCGCAGGAACCCCTAGTGATGGAGTTG

ANG K40I mut

NNTNCTGGTCTGTGTGCTGGCCNTNCTTTGGCAAAGAATTGGGATTCGAACAT
CGATTGAATTCCCCGGGGATCCTCTAGAGTCGACCTGCAGAAGCTTGCCTCGA
GCAGCGCTGCTCGAGAGATCTACGGGTGGCATCCCTGTGACCCCTCCCCAGTG
CCTCTCCTGGCCCTGGAAGTTGCCACTCCAGTGCCCACCAGCCTTGTCCTAATA
AAATTAAGTTGCATCATTTTGTCTGACTAGGTGTCCTTCTATAATATTATGGGG
TGGAGGGGGGTGGTATGGAGCAAGGGGCAAGTTGGGAAGACAACCTGTAGG
GCCTGCGGGGTCTATTGGGAACCAAGCTGGAGTGCAGTGGCACAATCTTGGC
TCACTGCAATCTCCGCCTCCTGGGTTCAAGCGATTCTCCTGCCTCAGCCTCCCG
AGTTGTTGGGATTCCAGGCATGCATGACCAGGCTCAGCTAATTTTTGTTTTTTT
GGTAGAGACGGGGTTTCACCATATTGGCCAGGCTGGTCTCCAACTCCTAATCT
CAGGTGATCTACCCACCTTGGCCTCCCAAATTGCTGGGATTACAGGCGTGAAC
CACTGCTCCCTTCCCTGTCCTTCTGATTTTGTAGGTAACCACGTGCGGACCGAG
CGGCCGCAGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCGCT