The secretion of the angiogenic and neurotrophic factor Angiogenin is COPII and microtubule dependent

Ross Ferguson and Vasanta Subramanian*

Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK

*Correspondence: Vasanta Subramanian, Department of Biology & Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK; Fax: +44 1225-386779, Tel: +44 1225-386315
Abstract

The RNaseA superfamily member Angiogenin (ANG) is a secreted protein involved in cell proliferation and stress response. Dysregulation of ANG expression is found in many cancers with poor prognosis and mutations in ANG are associated with neurodegenerative diseases. While the uptake and nuclear translocation of ANG is relatively well characterised, little is known about how it reaches the plasma membrane and its mode of secretion. We generated SH-SY5Y neuroblastoma cell lines constitutively expressing wild type (WT) Hemagglutinin (HA) epitope tagged mouse Ang1 (mAng1), and two amyotrophic lateral sclerosis associated ANG variants (C39W and K40I). Herein, we show that these cell lines secrete mAng1 into the culture media. Using small molecule inhibitors we probed the route taken between the endoplasmic reticulum and trans-Golgi network during secretion and have characterised it as COPII and microtubule dependent. In addition, we show that disruption by the PI3-kinase inhibitor wortmannin of the later stages of transit to the plasma membrane leads to mAng1 trafficking to lysosomal compartments. This suggests an autophagy dependent regulation of secretion.

Keywords: Angiogenin, secretion, small molecule inhibitors. Microtubule, SH-SY5Y, epitope tag.
Introduction

Human ANG is a member of the RNase A superfamily. It is a small single chain polypeptide of 123 residues with a relative molecular mass of 14.1kDa [1] and is translated as a pre-protein with a signal sequence. Other characteristic features of ANG include the nuclear localisation signal, a cell binding motif and the catalytic domain [2,3]. Secreted ANG is taken up and translocated to the nucleus in a variety of cell types [3,4]. Although the 3D structure of ANG is very similar to RNase A, the active site in ANG is blocked and this leads to the characteristic weak ribonucleolytic activity [5].

ANG is pleiotropic in function and its roles range from vascular remodelling during angiogenesis to protection from stress [5, 6]. Besides its role in neovascularization, ANG has been shown to be both neurotrophic and neuroprotective [8–11]. Variants in ANG have been reported in amyotrophic lateral sclerosis (ALS) [12–15]. ANG mutations are a risk factor for ALS in combination with those found in other neurodegeneration associated genes [12]. In addition, ANG has recently been shown to play a role in stem cell homeostasis in the haematopoietic system [16]. These functions of ANG are mediated intracellularly after endocytosis. Under differing cellular contexts ANG has been shown to activate the phosphoinositide 3-kinase pathway, cleave transfer RNA (tRNA) in the cytoplasm and upregulate ribosomal RNA (rRNA) synthesis by binding to the ANG binding element (ABE) of rRNA genes [17].

ANG is a secreted protein so its export is key to its post-uptake role in target cells/tissue. Elevated levels of ANG in the serum have been associated with progression in many types of cancers such as colorectal cancer [18,19]. It has also been associated with poor prognosis in chronic heart failure [20,21], and has been proposed as a biomarker for both these conditions [22].
ANG ALS variants can be broadly categorised by their phenotypic effect. While some variants alter gross structure (e.g. C39W, P112L) others predominantly affect ribonucleolytic activity (e.g. K40I) or the properties of the nuclear/nucleolar localisation signal (e.g. S28N) [8]. In addition mutations in the signal sequence such as P(-4)S are also common ALS variants but it is unclear how signal sequence mutations affect the function of ANG. As the signal peptide is cleaved during secretion from the source tissue, mutations must therefore affect secretion after translation and release into the extracellular space.

We have recently characterised the mechanism of uptake and intracellular trafficking to the nucleus [4]. We have shown that impaired intracellular trafficking occurs during uptake of gross structural mutants [8]. However, nothing is known about the pathway taken by ANG after translation in order to reach the extracellular space, or the mechanisms regulating secretion. We therefore investigated the post-translational trafficking and secretion of ANG and its ALS associated variants. ANG is expressed in the nervous system and is present in motor-neurons of the spinal cord and dorsal root ganglia [9]. Therefore we have used the neuroblastoma cell line SH-SY5Y to investigate the secretion of ANG. The trafficking and secretion of epitope tagged wild type mAng1 and ALS mAng1 variants were perturbed using small molecule inhibitors of intracellular transport.
Materials and Methods

Cell lines
Stable SH-SY5Y cell lines carrying constructs constitutively expressing HA tagged, full length wild type mAng1 or the mAng1 ALS-variant K40I and C39W were generated previously by transfection of the following constructs - pN1mAngHAWT, pN1mAngHAK40I and pN1mAngHAC39W using Fugene6 [18]. The resulting stable cell lines are referred to as SH-SY5Y WT-, K40I- and C39W mAng1HA respectively [8]. Cell lines were maintained in SH-SY5Y culture media comprised of DMEM:F12 with Glutamax (Life) and 15% fetal bovine serum (FBS) (Labtech), and passaged prior to confluence at least once between revival and experimental set-up. Cells were passaged using Trypsin EDTA (Invitrogen) following a phosphate buffered saline (PBS) wash.

Treatment with pharmacological agents
Exponentially growing untransfected and stable SH-SY5Y cell lines expressing wild type mAng1HA or the ALS variants were disassociated using Trypsin-EDTA (Lonza) and seeded at a density of $10^5$ cells/cm$^2$ onto acid-washed coverslips (SLS) in wells of 24-well plates (BD Falcon) in complete medium. Medium was exchanged after 24h with complete medium containing the following pharmacological agents at the concentrations indicated: Brefeldin A (18mM); Chloroquine (0.1mM); Wortmannin (0.1µM); Lovastatin (1mM); Nocodazole (17mM). After treatment with inhibitors for 2.5h, the cells were fixed for 15min in ice-cold 4% paraformaldehyde (PFA) (Sigma) in PBS, washed in PBS and then dehydrated to 70% ethanol and stored at -20°C.

Co-culture
Untransfected SH-SY5Y cells (5000 cells/cm²) were seeded onto acid-washed coverslips (SLS) in wells of 24-well plates (BD Falcon) in complete medium. Five thousand cells/cm² of WT-, C39W- and K40I-mAng1HA expressing SH-SY5Y clones were seeded onto 33.6mm² tissue culture inserts (Greiner, 0.1µm² pore size, 2x10⁶ cm⁻² pore density). After 24h the tissue culture inserts were transferred to wells containing the untransfected SH-SY5Y cells on coverslips and co-cultured for four days before the cells on coverslips were fixed in PFA as above.

**Cell Growth Assay**

Exponentially growing untransfected, WT or ALS variant mAng1HA expressing SH-SY5Y cells were trypsinised and resuspended in culture medium. After counting by haemocytometer, four wells on 24 well plates (BD Falcon) per cell line per time point were seeded in a final culture volume of 0.5ml/well of SH-SY5Y complete medium. Four wells per time point of media without cells were also plated as controls.

The Promega 96-well non-radioactive cell titre kit (G4000) was used to assay for cell growth, using the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Four replicate wells of each cell line were assayed at 24h intervals post-plating following the manufacturer’s protocols. Cells were incubated with the substrate for 4h at 37°C, 5% CO₂, the reactions were stopped and the insoluble products dissolved by the addition of solubilising reagent (G4000 kit) with vigorous rocking for 2h. Contents of each replicate was transferred to a flat bottom 96well plate (BD Falcon). Absorbances were read at 570nm with a 650nm reference wavelength. Three independent experiments were performed and the mean of the reference corrected absorbances were plotted against the experiment time course

**Differentiation of SH-SY5Y cells on PA6 stromal feeder cells**
Sub-confluent PA6 stromal cells were passaged 1:3 onto gelatin-coated acid-washed coverslips on 24 well plates (BD Falcon) in 0.5ml complete PA6 media (alpha MEM supplemented with ribonucleosides (Invitrogen), 1% Glutamax (Invitrogen), 10% FBS (Labtech). Once the PA6 cells had formed a confluent monolayer, SH-SY5Y cells were seeded onto the monolayer as single cells at a density of 5000 cells/well in 0.5ml differentiation media. Differentiation medium consists of DMEM:F12 supplemented with 5mM Glutamax (Invitrogen), 1% NEAA (Invitrogen), 0.5% Knock-out serum replacement KOSR; Invitrogen) and 10µm all-Trans Retinoic Acid (RA; Sigma). Differentiation media was changed every 48h. See [23] for details.

Collection of conditioned media

Untransfected, WT and ALS variant mAng1HA expressing SH-SY5Y cells were grown to confluence in normal medium on 15cm dishes (Becton Dickinson). Media was exchanged for serum free DMEM:F12 with additional 5mM L-Glutamine (Invitrogen). The conditioned media was collected after four days and passed through 0.22µm PES filters (Millipore) and concentrated 100x using Amicon Ultra centrifugal filters with a 3kDa cut-off. The concentrates were subsequently diafiltered with buffer containing 10mM Tris HCl pH 8.0 and 150mM NaCl. Protein concentration was estimated by A280 and 30µg of protein was used for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS- PAGE)

Western blotting

Proteins were resolved on a discontinuous tris-tricine SDS-PAGE (5% stacking gel, 10% resolving gel) using Biorad Miniprotean III apparatus. Resolved proteins were blotted onto 0.22µm PVDF (Pierce) using a Novex semi-dry blotter and buffer containing 48mM Tris (Sigma), 39mM glycine (Sigma), 0.037% SDS (Sigma) and 20% Methanol (HPLC grade, Fisher).
Blotted membranes were blocked in 5% Marvel milk powder, 0.1% Tween20 (Sigma) in PBS (Oxoid) for one hour at room temperature followed by incubation with mouse anti-HA.11 (1:5000 , Covance, Clone 16B12) overnight at 4°C in heat-sealed bags. Blots were washed (5X5min) in PBS containing 0.1% Tween20 followed by incubation with HRP conjugated goat anti-mouse (1:10,000 in block, Abcam, ab6789) secondary antibody for 2h at room temperature in heat-sealed bags. Blots were washed (5X5 min) in PBS containing 0.1% Tween20. Blots were incubated with electro-chemiluminescent reagents (100mM glycine, 0.4mM luminal, 8mM 4-iodophenol and 10% dimethyl sulphoxide mixed 1:1 with 0.12% hydrogen peroxide) for one minute and exposed to Amersham Hyperfilm MP film (GE Healthcare).

**Immunocytochemistry**

Fixed cells on coverslips stored in 70% ethanol were rehydrated to PBS and blocked for 1h in blocking buffer (1% Gelatin (Sigma), 0.5% Triton (Sigma) and 0.5% FBS (Labtech). Overnight incubation at 4⁰C was performed in block containing primary antibodies against the appropriate antigen. Cells on coverslips were then washed with 0.1% PBS-TritonX-100 (BDH) and PBS alone each 3X5min followed by incubation with fluorophore-conjugated secondary antibodies against appropriate primary species for 2h at room temperature. Five further washes with PBS were performed then nuclei were counter-stained with DAPI (4′,6-diamidino-2-phenylindole;Sigma). Two further PBS washes were performed prior to mounting in Mowiol with antifade DABCO (1,4-diazabicyclo[2.2.2]octane; BDH) on glass slides (Fisher).

**Quantification and statistics**

Images were acquired on a Leica DMRB microscope using Leica DFC490 camera and processed using Leica Application Suite (LAS) software. The lengths of neurofilament positive
neurites were measured in six (660x490µm) randomly selected fields each from three independent experiments. Cells positive for cleaved caspase 3 were counted in six (660x490µm) randomly selected fields each from three independent experiments. Total cell numbers were determined by counting DAPI positive nuclei. Fields of undifferentiated and differentiated cells contained 400-500 cells each. Data were compared by ANOVA with Dunnet’s post-hoc comparison to untransfected SH-SY5Y cells using Minitab 15. Due to their skewed distribution, neurite lengths were Log10 transformed before statistical testing.
Results

Wild type mAng1HA over-expression reduces post-passage recovery period

There was little observable difference in the growth rate of untransfected SH-SY5Y cells, SH-SY5Y clones expressing WT-mAng1HA, and C39W- mAng1HA or K40I-mAng1HA ALS variants between 24h and 48h after seeding (Fig. 1A). However, between 48h and 72h the growth rate of SH-SY5Y WT-mAng1HA cells nearly doubled while the growth rate of untransfected SH-SY5Y cells, SH-SY5Y C39W-mAng1HA and K40I-mAng1HA cell lines increased only marginally. SH-SY5Y WT-mAng1HA cell growth continued at this higher rate until the final time point. After 96h, growth rate of untransfected SH-SY5Y and C39W-mAng1HA SH-SY5Y increased to a similar level as WT-mAng1HA SH-SY5Y cells. The growth rate of SH-SY5Y K40I-mAng1HA cell line appears to remain lower than that of the other cell lines between 120h and 144h.

SH-SY5Y cells transfected with mAng1HA process, secrete and endocytose mAng1

It is unclear whether mAng1 exerts its effects upon proliferation through intra or autocrine signalling therefore first we investigated if it was secreted into the culture medium from our stable cell lines. In addition to robust uptake and nuclear translocation seen upon addition of purified hANG to the media, we observed a distinct speckled distribution of ANG coverslip surface outside the cell body (Fig. 1B). This suggests that ANG may be binding to an extracellular component. We observed a similar speckled distribution on the coverslips when immunostaining for the HA tagged mAng1 and mAng1 ALS variants. This suggested that the SH-SY5Y mAng1HA cell lines are secreting ANG into the media. Secretion of the mAng1HA variants from the SH-SY5Y cell lines was assessed by Western blot of conditioned media collected from each of the cell lines (Fig. 1C). The smallest band picked up by staining with
the anti-HA antibody consistently runs above the 15kDa marker and was calculated as 17kDa. This must represent HA tagged mAng1. The small discrepancy in mass may be due to partial refolding or differences in SDS binding in comparison to the marker band, exacerbated by the small sizes involved. The 21kDa band is uncharacterised. It is unlikely to be unprocessed ANG as it is concentrated from culture medium and therefore must have been secreted by the cells. The 24aa signal sequence would only account for an extra 2.48kDa. It is unlikely to be a multimer of mAng1HA as this would be a minimum of 34kDa and the SDS-PAGE used denaturing and reducing conditions. Therefore, the 21kDa band most likely represents an unknown post-translationally modified species of mAng1HA. Co-culture experiments were performed to then determine whether SH-SY5Y cells were capable of taking up the secreted Ang. Untransfected SH-SY5Y cells were grown on coverslips in co-culture with WT-, C39W- or K40I-mAng1HA expressing SH-SY5Y grown on transwell filters (Fig. 1D). Untransfected SH-SY5Y cells showed uptake of secreted WT-, C39W- and K40I- mAng1HA variants, all are seen at low levels in the cytoplasm (Fig. 1E). The two ALS variants were frequently seen to accumulate as large aggregates adjacent to the nucleus. WT-mAng1HA signal appears less intense than that seen for C39W- or K40I- mAng1HA. Whether this represents reduced uptake, faster turnover or is an artefact due to the aggregation of the mutant variants is unclear.

**Wild type mAng1 over-expression enhances neurite outgrowth of SH-SY5Y cells during differentiation**

We have previously shown that exogenously added ALS-associated ANG variant proteins to P19 pluripotent cell derived motor-neurons in culture led to impaired axon extension and cell death [8,10]. We therefore, investigated the effects of over-expression of WT- and ALS-variant mAng1HA on SH-SY5Y cells differentiated to a more mature neuronal identity. Length and
morphology of neurites expressing neurofilament, the mature axonal marker were assessed after differentiation (Fig. 2A, B). SH-SY5Y cells expressing WT mAng1HA possessed longer NF positive neurites (median length 79.6 µm, Q1-Q3 45.1-133.8 µm) than untransfected SH-SH5Y cells (median length 63.5 µm, Q1-Q3 41.9-97.6 µm) or cells expressing either the K40I (median length 61.7 µm Q1-Q3 41.4-94.1 µm) or C39W variants (median length 63.6 µm Q1-Q3 39.7-94.2 µm). No significant differences from untransfected SH-SY5Y neurite length were seen in SH-SY5Y expressing either mutant variant.

**mAng1 ALS-variants are mis-localised in differentiated SH-SY5Y cells**

TAR DNA-binding protein 43 (TDP-43) is a nuclear RNA-binding protein involved in post-transcriptional RNA processing. Mutations in TDP43 are also associated with ALS-FTD [24,25]. Export of TDP43 from the nucleus to the cytoplasm, and its subsequent cleavage and hyper-phosphorylation is associated with stress response and altered stress granule dynamics [26]. This is also seen in ALS-FTD pathology where it is found in inclusion bodies [27,28]. We have used TDP43 localisation during differentiation to monitor stress in SH-SY5Y cells over-expressing WT and ALS-mAng1HA variants.

The distribution of the WT-mAng1HA, mAng1HA ALS variants and the ALS associated protein TDP43 were analysed upon differentiation of the stably transfected SH-SY5Y cell lines (Fig. 2C). TDP43 was seen in the nuclei of both PA6 and SH-SY5Y cells. Staining with an anti-HA tag antibody showed that the HA tagged WT mAng1 appeared as small punctate spots distributed throughout the cytoplasm. No nuclear staining for the HA tag was observed. HA tag positive punctae appeared evenly distributed throughout the soma and neurites with an increase in the number of HA+ punctae seen at the distal tip of the neurites. The mAng1HA C39W clones showed increased numbers of HA+ punctae in the soma rather than the distal tip of neurites. Branching of neurites appears to occur more frequently in shorter
neurites in C39W mAng1HA expressing cells compared to those in the WT mAng1HA expressing clone (Fig. 2A). TDP43 was seen to partially relocate to the cytoplasm in these cells (Fig. 2C, asterisks). In K40I-mAng1HA SH-SY5Y cells, HA+ punctae appear throughout the soma and neurite but were found more frequently in the soma in comparison to the WT mAng1HA expressing clone. Unlike in the mAng1HA C39W clone, some HA staining was seen in the tips of neurites. As in the case C39W mAng1HA clones, TDP43 was observed to partially relocate to the cytoplasm in some K40I mAng1HA cells (Fig. 2C, asterisks).

**Mutant mAng1HA expression increases apoptosis in differentiated SH-SY5Y cells**

DAPI stained nuclei of SH-SY5Y mAng1HA ALS variant clones upon differentiation showed highly condensed or fragmented nuclear material and strongly staining for cleaved caspase 3 in both nuclei and apoptotic bodies. (Fig. 3A). Increased frequencies of cleaved caspase 3 positive cells were seen in differentiated cells on day ten when compared to undifferentiated populations. After differentiation, significantly more cells positive for cleaved caspase 3 were observed in differentiated SH-SY5Y cells expressing C39W mAng1HA in comparison with differentiated SH-SY5Y expressing WT- and K40I-mAng1HA or untransfected SH-SY5Y (Fig. 3C). In contrast, we found no significant differences in cleaved caspase 3 levels in the undifferentiated mAng1HA ALS variant over-expressing SH-SY5Y cell lines.(Fig 3B).

**Subcellular distribution of recombinant mAng1HA and mAng1HA ALS variants in SH-SY5Y cells**

Immunostaining for the HA tag in the stably transfected SH-SY5Y cells showed small punctuate granules of mAng1HA clustered adjacent to the nucleus with more diffuse organisation within the cytosol and along neurites. No signal was seen in the nucleus. SH-SY5Y cells expressing K40I mAng1HA and C39W mAng1HA showed a similar distribution,
however the dense perinuclear staining in the two ALS variant expressing cell lines appears larger and more intense when compared to WT mAng1HA (Fig. 4A, arrows). This was more prominent in the C39W mAng1HA clone, which also appears to show reduced staining in the neurites. We see no effect on organelle distribution due to overexpression of any of the mAng1HA variants. Immunostaining showing organelle distribution in untransfected SH-SY5Y can be found in Supp Fig 1.

We then sought to identify the intracellular route through which ANG transits during secretion. This was done using well characterised markers associated with distinct subcellular compartments: Protein disulphide isomerase (PDI) – a marker for the endoplasmic reticulum (ER) [29]; Trans-Golgi network protein 46 (TGN46) – a marker for the trans-Golgi network (TGN) [30], early endosome-associated protein (EEA1) – a marker for the early endosome [31], the G-protein RAB7 – a marker for the late endosome- [32] and the lysosome marker-lysosomal membrane protein 1 (LAMP1) [33].

Diffuse HA staining was associated with the endoplasmic reticulum marker PDI in all three cell lines (Supp Fig 2). The large perinuclear accumulation partially co-localised with the large TGN46 compartment of the Golgi stacks for both WT-mAng1HA and the two ALS variants (Fig. 4A, closed arrows). In the cells expressing the mAng1 ALS variants, a proportion of the perinuclear accumulation did not co-localise with TGN46, and this was more prominent in the case of C39W mAng1HA (Fig. 4A, open arrows). No co-localisation was observed between mAng1HA ALS variants and the early or late endosome markers EEA1 and RAB7 respectively (Fig. 6A and Supp Fig. 4). This strongly suggests that the vast majority of observable tagged mAng1HA is secretory rather than endocytic. Some limited co-localisation was seen between LAMP1 and the HA tagged mAng1 variants in neurites. (Fig. 7A, arrows).
Effect of intracellular trafficking inhibitors on mAng1HA and mAng1ALS variant distribution

We used small molecule inhibitors of intracellular trafficking and observed their effects upon the distribution of the HA tagged mAng1 and mAng1ALS variants, in order to identify the mechanisms and compartments through which mAng1 passes after synthesis. We used a broad range of inhibitors to perturb (1) ER to TGN transit (brefeldin A, [34,35]); (2) intracellular trafficking mediated by microtubules (nocodazole, [36]); (3) vesicular dynamics and lipidation of proteins (lovastatin, [37,38]); (4) pH dependent sorting (chloroquine, [39,40]), and (5) compartment dynamics and regulated or autophagy dependent secretion mechanism (wortmannin, [41,42]).

Treatment of cells with brefeldin A resulted in the loss of the main bulk of perinuclear TGN46, redistributing into the ER (confirmed by wheat germ agglutinin staining [43], (Supp Fig. 3). The perinuclear accumulations of the mAng1HA variants were absent, presumably having been redistributed throughout the cell (Fig. 4B). Despite this, WTmAng1HA still appeared to be in small punctae within the cell body and throughout the neurites with very few instances of co-localisation with TGN46. A small proportion of C39WmAng1HA remained in a large cluster adjacent to the nucleus (Fig. 4B, arrow).

Treatment with the microtubule disrupting agent nocodazole resulted in the loss of the thin well-defined neurites seen in untreated cells (Fig. 5A). Neurites, where present after nocodazole treatment, taperered from a wide origin at the cell body and had little WT-mAng1HA. In addition, the TGN46 positive trans-Golgi network redistributed into the endoplasmic reticulum. Within the cell bodies large irregularly sized areas of WT and ALS-variant mAng1HA partially colocalised with TGN46. In SH-SY5Y cells expressing the C39W and K40I mAng1HA variants, the TGN46 positive compartments were seen to co-localise with some of the large perinuclear accumulations of mAng1HA protein in nocodazole treated cells.
(Fig. 5A, closed arrows). No further co-localisation with TGN46 is seen. The frequency and size of the perinuclear accumulation of mAng1HA was greater in nocodazole treated cell lines when compared to untreated mAng1HA expressing SH-SY5Y (Fig. 5A, open arrows vs. Fig. 4A).

The large perinuclear pool of TGN46 seen in untreated mAng1HA and mAng1HA ALS variant expressing SH-SY5Y cell lines appeared to be more dispersed when treated with lovastatin (Fig. 5B, arrows) but remained adjacent to nucleus. HA tagged mAng1 ALS variants appear distributed throughout the cell in small vesicles however, co-localisation with TGN46 was substantially reduced after exposure to lovastatin.

Treatment with chloroquine causes larger EEA1 positive bodies to accumulate in the cell body adjacent to nucleus (Fig. 6B). The large perinuclear accumulations of the HA tagged mAng1 and mAng1ALS variants seen in untreated cells become dispersed throughout the cell body into smaller, more numerous accumulations.

Upon treatment with wortmannin, LAMP1 positive compartments appeared slightly larger in size. This was particularly noticeable in the LAMP1+ compartments present above the nucleus (Fig. 7B). Co-localisation of HA tagged mAng1HA and LAMP1 was observed in the discrete puncta found within the cell body and neurites but not the LAMP1+ bodies above the nucleus after wortmannin treatment (Fig. 7B, insets). Wortmannin appear to increase the frequency and size of the perinuclear accumulations of mAng1HA protein similar to nocodazole treatment but with less severity.
Discussion

We have demonstrated that SH-SY5Y cells can secrete constitutively expressed HA-tagged wild type and ALS-variant mAng protein which is in the processed form and is uptake ready. These cell lines were then used to investigate the effects of mAng1 and its ALS variants on cell proliferation and differentiation. We also used these overexpressing cell lines to investigate the intracellular secretory route taken by mAng1. We show that the neuronal cell line SH-SY5Y is competent to express the epitope tagged mAng1 and mAng1 ALS variant proteins from the transfected constructs and secrete them into the culture media for subsequent uptake by neuronal cells.

Under normal physiological conditions, ANG is present in human serum, plasma and cerebrospinal fluid. ANG is also known to be constitutively secreted by multiple cancer cell lines, such as the adenocarcinoma cell line HT29 from which it was first identified [1]. Extracellular levels of ANG can be regulated either transcriptionally or by intracellular sequestration and release post-translationally. A well studied mechanism of ANG upregulation occurs during hypoxia and involves the hypoxia inducible factor 1a (HIF-1a) [44], while HIF-1a independent upregulation occurs in response to ER stress and unfolded protein response. This is mediated through inositol-requiring enzyme 1 α (IRE1 α) and protein kinase R-like endoplasmic reticulum kinase (PERK) in human kidney epithelium [45]. Granner et al., [46] have shown the secretion of ANG is increased in human adult retinal pigment epithelium cells in response to treatment with bevacizumab, an anti-angiogenic therapeutic. However, as only secreted protein was assayed the regulatory point is unknown.

Regulated secretion of ANG (i.e. increased secretion without upregulation of expression) has been shown to occur in response to a variety of signals. Bone derived neurotrophic factor (BDNF) increases ANG secretion from vascular endothelial cells [47] and a wide range of signals can lead to increased ANG secretion from Mast cells. These include
lipopolysaccharides, nerve growth factor and peptidoglycans [48]. The role of BDNF in regulating the secretion of ANG from endothelial cells is unclear. ANG may have a role in neuroimmune modulation since Mast cells which secrete ANG have been associated with peripheral nerve fibres [49]. Similar tissue specific regulation of ANG secretion in response to environmental cues occurs in placental trophoblasts, in which vitronectin and fibronectin from the extracellular matrix increase both expression and secretion [50].

The pro-proliferative role of ANG is well characterised since it is shown to induce proliferation in endothelial cells [51]. After uptake by the cell and translocation to the nucleus, ANG accumulates in nucleoli where it binds to ANG binding elements (ABEs), upregulating rRNA transcription and is also involved in the processing of rRNA transcripts [52,53]. The increase in cell growth that we observe in SH-SY5Y upon overexpression of WT mAng1HA seems more likely due to improved post-passage stress response rather than a general increase in proliferation rate. We do not see robust nuclear translocation or nucleolar localisation associated with increased proliferation during re-uptake studies and proliferation rates become comparable between the mutant and untransfected SH-SY5Y within 48h of passage.

We did not observe any significant differences in apoptosis between wild type and ALS-variant mAng1HA expressing SH-SY5Y cell lines in the undifferentiated state. However, upon differentiation to a more mature phenotype [23] the ALS-variant C39W shows a significant increase in apoptosis while WT mAng1 results in increased neurite outgrowth.

We have previously shown that in the case of mutations which affect ribonucleolytic activity without significant structural changes, the variant ANG continues to function in pathways such as Pi3K signalling; ANG K40I continues to show robust microglial activation despite lacking RNase activity, while the structural mutant P112L is impaired [4]. It is possible that the partially unfolded C39W variant [10] may not be able to participate in non-RNase mediated intrinsic or extrinsic apoptotic pathways that ANG is associated with [54], while the
K40I variant continues to retain this function. Alternatively, the partially unfolded nature of the C39W variant may cause increased cell death through ER stress and UPR. This is supported by our observation that mAng1HA C39W is mis-localised to larger peri-Golgi aggregates in undifferentiated cells. UPR is increasingly being shown to be involved in the pathology of neurodegenerative disease [55] and has been shown to be active in ALS cases carrying SOD1 mutations [56].

Overexpression of the K40I variant does not lead to significantly increased apoptosis, however, expression of this variant does induce nuclear export of TDP43 similar to that seen with overexpression of C39W ALS variant. Mis-localisation of TDP43 in the cytoplasm is associated with stress response, particularly ER stress [57] and this pathologic distribution is observed in neurodegeneration and disease associated TDP43 variants are more prone to mislocalisation [58,59].

Our data suggests that ANG destined for secretion follows the conventional early secretory pathway from the ER to the Golgi apparatus and finally to the TGN. Anterograde transport of ANG from the ER to the cis-Golgi is disrupted by BFA inhibition of COPII vesicle formation, leaving ANG trapped in the resulting ER-Golgi super compartment. Intracellular trafficking of ANG during secretion is sensitive to microtubule disruption by nocodazole, leaving ANG trapped in perinuclear compartments and preventing trafficking to the neurites.

Lovastatin has been shown to reduce secretion of molecules such as matrix metalloproteinase 9 secreted by macrophages [60]. As an inhibitor of HMG CoA reductase, lovastatin is unlikely to affect the secretion of ANG though post-translational prenylational (ANG/mAng1 is not prenylated) nor is it likely to affect anterograde transport through membrane cholesterol depletion during short incubations used in our experiments. Therefore the reduction in anterograde transport of mAng1 to the TGN observed after lovastatin treatment
may be due to prenylation dependent vesicular trafficking factors such as the Rab family members [61–63].

Chloroquine has been shown to increase secretion of certain proteins. Treatment of normal cells (human prostate stromal cells, lung fibroblasts and epithelial cells) and in cancer cells (lung and prostate) resulted in increased secretion of Prostate apoptosis response-4 (Par-4) [64]. The opposite however has also been reported, such as decreased TGF-β secretion from glioblastoma cell lines [65]. Par-4 secretion was upregulated through its release from sequestration by Uveal autoantigen with coiled coil domain and Ankyrin repeats (UACA) in the ER and a post-Golgi Rab8b dependent mechanism [64,66]. TGF-β secretion found to be impaired due to the alkalisation of the compartment in which its pre-protein is processed to a mature form by a furin-like protease [65,67]. Beyond cleavage of the signal peptide, no post-translational processing of ANG has been reported to date. Therefore it is unlikely that inhibition of modifying enzymes due to the alkalisation of compartments will affect secretion of ANG. The increased distribution of small vesicles containing mAng1 throughout the cell may therefore indicate increased mobilisation and trafficking of TGN vesicles to the membrane associated with an increased rate of secretion.

Wortmannin acts as an inhibitor of PI3K at nanomolar concentrations [68,69] but at the higher concentrations used here it will also inhibit myosin light chain kinase and protein kinase C [70]. Wortmannin has been shown to perturb the regulated secretory pathway, for example it causes altered insulin secretion from pancreatic beta cells [71,72], reduced neurotensin secretion in small bowel endocrine cells [54] and reduced histamine secretion from the model mast cell line RBL-2H3 [49]. These effects are thought to occur through PI3-K mediated α-tubulin acetylation [73,74] [56]. Wortmannin has also been shown to inhibit autophagy due to PI3-K dependent membrane bound vesicle trafficking [75].
Autophagy as a key regulator of protein homeostasis is also involved in secretion. Autophagy-dependent secretion is a mechanism that allows cytosol-resident proteins lacking signal peptides such as IL-1β or FGF2, aggregate prone proteins such as α-synuclein and even whole organelle contents to be secreted [76]. Autophagy also mediates secretion via lysosomal compartments, such as secretion of cathepsin K from osteoclasts [77] or mutant huntingtin [78].

ANG contains a conventional signal peptide and as we do not observe mAng1HA in LAMP1 (lysosome) or RAB7 (late endosome) compartments it is unlikely either of these pathways are involved, however, after wortmannin treatment we find increased co-occurrence of mAng1HA in LAMP1 compartments. Another mechanism through which autophagy and secretion overlap is through maintenance of regulated secretory granule contents [79]. Autophagy mutant mice show impaired Paneth cell granule secretion and increased intracellular levels of their contents (e.g. Leptin) [80]. In senescing cells this mechanism condenses to a lysosome-like compartment associated with the trans-Golgi apparatus which selectively degrades secretory proteins in transit (e.g. IL-6 and IL-8) leading to a reduction in secretion [81]. Therefore the presence of ANG in LAMP1 compartments may represent ANG being rerouted for degradation due to reduced transport to the plasma membrane via wortmannin-sensitive mechanisms, a blockade of degradation in the lysosomal compartment, or simple mis-sorting [82]. As ANG secretion has been shown to be regulatable at the protein level, sorting excess for degradation may be the mechanism through which this occurs.

The only published pharmacological inhibitor of ANG secretion is the fungal metabolite terrein and its glucosides [83]. The mechanism of action of terrein is largely unclear, however, it has been previously characterised as an inhibitor of melanogenesis through ubiquitination of tyrosinase and its subsequent proteasomal degradation [84,85]. Terrein mediated suppression of ANG secretion slows proliferation in head and neck cancer cells and has been reported to halt migration in human breast cancer cells and proliferation in
oesophageal cancer cells [86–88]. Inhibition of secretion of zebrafish Rnasel (zebrafish ANG like molecules) by terrein causes excess branching of spinal motor axon and impaired vasculogenesis [89]. It seems plausible that terrein acts on ANG secretion in a similar manner to that seen in melanogenesis. The higher molecular weight species of mAng1 seen by Western blot may represent a pool of ubiquinated mAng1 destined for degradation in the absence of appropriate stimulation.

In summary, low level autocrine signalling mediated by mAng1 expressed from constitutive constructs is sufficient to enhance neurite outgrowth and stress mediation in the case of the wild type isoform while ALS-associated mutant variants increase stress and apoptosis in SH-SY5Y with a mature neuronal phenotype. At these levels negligible nuclear localisation is seen, only cytoplasmic, and no variant had any effect of proliferation therefore the localisation of mAng1 determines its post-secretion effects.

mAng1 secretion follows a conventional path. Trafficking from the ER to the TGN is COPII and microtubule dependent. Trafficking within the TGN is dependent on secondary prenylated factors and regulation of protein secretion occurs through a lysosomal dependent mechanism rather than through sequestration in storage granules. Manipulation of autophagy and degradation by lysosomal targeting may lead to further means of regulating mAng1 secretion in addition to terrein. Control of endogenous ANG secretion may have applications beyond slowing cancer progression. This could allowing suppression of toxic gain of function ANG variants in conjunction with therapeutics.
Acknowledgements

We are grateful to Professor Guo-Fu Hu (Tufts University, USA) for the kind gift of the 26-2F antibody and the Developmental Studies Hybridoma Bank for the 2H3 antibody. This work was funded by an NC3Rs (UK) project grant to VS.

Author contributions

RF carried out the experimental work, data analysis, participated in the design of the study and wrote the manuscript. VS conceived and designed the study, helped with data analysis and wrote the manuscript.

Conflict of Interest

The authors declare no conflict of interest
References


Figure Legends

Figure 1 –

Figure 1. Over-expressed HA-tagged Angiogenin is secreted and taken up by SH-SY5Y

(A) Growth curves of untransfected SH-SY5Y cells (yellow) and SH-SY5Y cells expressing mAng1HA variants: WT (purple), K40I (green) and C39W (red). SH-SY5Y cells mAng1HA WT cells appear to grow at a faster rate during earlier time points. No significant difference was found between growth rates of untransfected SH-SY5Y cells, K40I and C39W mAng1HA expressing SH-SY5Y cells. Mean reference corrected absorbances from three
independent growth curves of each cell line with four replicate samples for each 24h time point. SEM bars.

(B) ANG is secreted and can be seen adhered to the surface of the coverslips in SH-SY5Y cell cultures. SH-SY5Y cells express low levels of endogenous ANG. Immunostaining for endogenous hANG (anti-hANG) in SH-SY5Y cells with or without the addition of purified hANG in the culture medium (+hANG) shows ANG adhesion to the coverslips. Similar HA tag staining is seen in mAng1HA WT, C39W or K40I SH-SY5Y cell cultures, indicating secretion is occurring. Scale bar 25µm.

(C) Western blot against the HA tag shows the presence of mAng1HA variant proteins in concentrated conditioned culture medium, secreted by transgenic SH-SY5Y cells. The 17kDa band represents unmodified mAng1HA variants while the 21kDa species is an uncharacterised post-translationally modified form.

(D) Co-culture of untransfected SH-SY5Y cells with mAng1HA expressing SH-SY5Y cells on a permeable membrane.

(E) Immunostaining against the HA-tag present in recombinant WT, C39W and K40I-mAng1HA protein in co-cultured untransfected SH-SY5Y cells. The small punctate distribution is lost from the neurites and cell body. Multiple large accumulations are seen around the nucleus in co-culture with C39W or K40I mAng1HA. Less uptake is seen on co-culture with WT mAng1HA SH-SY5Y cells. Scale bar 25µm.
Figure 2 – Over-expression of ALS variant mAng1HA leads to cytosolic TDP43 in differentiated SH-SY5Y

(A) Immunostaining for neurofilament-m expression in differentiated untransfected SH-SY5Y cells, WT, C39W, K40I mAng1HA expressing SH-SY5Y cells. Scale bar 75µm.
(B) Violin plots of neurofilament positive SH-SY5Y neurite lengths measured in six randomly selected fields in three independent experiments. Neurites of WT-mAng1HA expressing SH-SY5Y cells are significantly longer than those of untransfected SH-SY5Y cells or C39W and K40I mAng1HA expressing SH-SY5Y cells. * P<0.05.

(C) Immunostaining for the HA-tag in differentiated cell lines expressing WT, C39W and K40I-mAng1HA protein. WT-mAng1HA staining appears concentrated towards the tips of neurites (closed arrows) while C39W and K40I appears in concentrated swellings closer to the cell bodies (open arrows). Neurite paths shown by dotted lines. Immunostaining for TDP43 shows a nuclear distribution in both PA6 and SH-SY5Y cells with no co-localisation with HA. Some redistribution of TDP43 into the cytoplasm can be observed in C39W and K40I expressing SH-SY5Y, indicated by *.. Scale bar 25µm.
Figure 3 – ALS variant mAng1HA over-expression increases apoptosis in differentiated SH-SY5Y

(A) Immunostaining for cleaved caspase 3 in undifferentiated and SH-SY5Y cells differentiated on PA6 with retinoic acid and dibutylryl-cAMP. Scale bar 100µm.

(B) The percentage of cleaved caspase 3 cells in undifferentiated cultures shows no significant differences in the number of apoptotic cells.

© The percentage of cleaved caspase 3 cells in differentiated cultures shows significantly more apoptotic SH-SY5Y cells mAng1HA C39W cells (* P<0.05). Cells positive for cleaved caspase 3 were counted in six randomly selected fields in three independent experiments. Total cell numbers were determined by counting DAPI positive nuclei.
Figure 4 – ALS-mAng1HA variants accumulate adjacent to the nucleus outside of the trans-Golgi.

(A) Immunostaining for TGN46 and HA tag in SH-SY5Y cells expressing mAng1HA WT, C39W and K40I. Closed arrows show co-localisation. Open arrows show larger proportions of the C39W-mAng1HA accumulations do not overlap with TGN46 (e-h) when compared to WT or K40I Scale bar 25μm.

(B) Immunostaining for TGN46 and HA tag in SH-SY5Y cells expressing mAng1HA WT, C39W and K40I treated with brefeldin A. Both TGN46 and mAng1HA staining appears dispersed throughout the cells but will very little co-localisation. Scale bar 25μm.
Figure 5 – Microtubule disruption leads to the dispersal of mAng1HA throughout the cells.

(A) Immunostaining for TGN46 and HA tag in SH-SY5Y cells expressing mAng1HA WT, C39W and K40I treated with nocodazole. Closed arrows show co-localisation. Open arrows show redistribution of the mAng1HA into many large accumulations throughout the cell and do not co-localise with TGN46. Scale bar 25µm.

(B) Immunostaining for TGN46 and HA tag in SH-SY5Y cells expressing mAng1HA WT, C39W and K40I treated with lovastatin. Closed arrows show dispersed TGN46 and unchanged mAng1HA distribution. Scale bar 25µm.
Figure 6 – Perinuclear accumulations of mAng1HA redistribute throughout the cell after chloroquine treatment.

(A) Immunostaining for EEA1 and HA tag in SH-SY5Y cells expressing mAng1HA WT, C39W and K40I. Scale bar 25µm.

(B) Immunostaining for EEA1 and HA tag in SH-SY5Y cells expressing mAng1HA WT, C39W and K40I after treatment with chloroquine. EEA1 compartments appear larger and closer together. The normally perinuclear mAng1HA accumulations are smaller and dispersed throughout the cell. Scale bar 25µm.
Figure 7 – mAng1HA is redirected to LAMP1 positive compartments after wortmannin treatment

(A) Immunostaining for LAMP1 and HA tag in SH-SY5Y cells expressing mAng1HA WT, C39W and K40I. LAMP1 can be seen in neurites shown in neurite insets from boxed areas WT, C39W and K40I. Infrequent co-localisation with HA staining is seen. Scale bar 25µm.

(B) Immunostaining for LAMP1 and HA tag in SH-SY5Y cells expressing mAng1HA WT, C39W and K40I after treatment with wortmannin. LAMP1 can be seen in neurites shown in...
neurite insets from boxed areas WT, C39W and K40I. Co-localisation with HA staining is seen much more frequently in the neurites in all three clones after treatment. Scale bar 25µm.
Table 1: Antibodies used for Immunostaining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
<th>Cat#</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEA1</td>
<td>1:200</td>
<td>Cell Signal Technologies</td>
<td>#3288</td>
</tr>
<tr>
<td>LAMP1</td>
<td>1:500</td>
<td>Abcam</td>
<td>Ab24170</td>
</tr>
<tr>
<td>PDI</td>
<td>1:200</td>
<td>Cell Signal Technologies</td>
<td>#3501</td>
</tr>
<tr>
<td>RAB7</td>
<td>1:200</td>
<td>Cell Signal Technologies</td>
<td>#9367</td>
</tr>
<tr>
<td>TGN46</td>
<td>1:500</td>
<td>Abcam</td>
<td>Ab16052</td>
</tr>
<tr>
<td>Human Angiogenin</td>
<td>1:1000</td>
<td>A gift from Dr. Guo-Fu Hu</td>
<td>Clone 26-2F</td>
</tr>
<tr>
<td>HA tag (HA.11)</td>
<td>1:1000</td>
<td>Covance</td>
<td>16B12</td>
</tr>
<tr>
<td>Cleaved caspase 3</td>
<td>1:500</td>
<td>Abcam</td>
<td>Ab2302</td>
</tr>
<tr>
<td>Neurofilament</td>
<td>1:5</td>
<td>DSHB</td>
<td>2H3</td>
</tr>
<tr>
<td>Alexa Fluor 488 goat anti-mouse</td>
<td>1:1000</td>
<td>Molecular probes</td>
<td>A-11001</td>
</tr>
<tr>
<td>Alexa Fluor 488 goat anti-rabbit</td>
<td>1:1000</td>
<td>Molecular probes</td>
<td>A-11034</td>
</tr>
<tr>
<td>Alexa Fluor 594 goat anti-rabbit</td>
<td>1:1000</td>
<td>Molecular probes</td>
<td>A-11037</td>
</tr>
</tbody>
</table>
Supplementary data for Ferguson and Subramanian
The secretion of the angiogenic and neurotrophic factor Angiogenin is COPII and microtubule dependent

Supplemental Figure 1 - Immunostaining for intracellular trafficking organelles in SH-SY5Y cells.
Distribution of organelle markers in untransfected SH-SY5Y cells shown by immunostaining. EEA1- early endosome marker, RAB7- late endosome marker and LAMP1- lysosome marker are found close to the nucleus (closed arrows) and in neurites (open arrows). The trans-Golgi network marker TGN46 is predominantly localised adjacent to the nucleus.
(closed arrows) but also throughout cell (open arrows). The endoplasmic reticulum marker PDI is found throughout the cell body. Scale bar 25µm.

Supplemental Figure 2 – PDI and mAng1HA distribution in SH-SY5Y
Immunostaining for PDI and HA tag in SH-SY5Y cells expressing mAng1HA WT (a-d), C39W (e-h) and K40I (i-l). Scale bar 25µm.
Supplemental Figure 3 – WGA redistribution confirms brefeldin A efficiency in SH-SY5Y
Fluorescently labelled WGA lectin staining for Golgi apparatus and immunostaining for PDI. Untreated (a-d) and treated with brefeldin A (e-h). Treatment results in a reduction in size of the Golgi apparatus and redistribution into the ER when compared to untreated. Scale bar 25µm.
Supplemental Figure 4 – mAng1HA does not co-localise with RAB7

Immunostaining for RAB7 and HA tag in SH-SY5Y cells expressing mAng1HA WT (a-d), C39W (i-l) and K40I (q-t). RAB7 can be seen in neurites shown in neurites in insets of boxed areas WT (e-h), C39W (m-p) and K40I (u-x). No co-localisation with HA staining is seen. Scale bar 25µm.