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Knockdown of AAMP Impacts on HECV Cell Functions *In Vitro* and Affects the Expression of VE-Cadherin

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Abstract

Background: Angio-Associated Migratory Cell Protein (AAMP) is a 52 kDa protein expressed in a variety of human cell lines. Previous studies have demonstrated that AAMP is involved in endothelial cell adhesion, migration, and tube formation and plays roles in signalling pathways, such as RhoA-ROCK

Methods: In this study, we knocked down the expression of AAMP in HECV cells using hammerhead ribozymes and assessed the influence of AAMP on endothelial cells. In order to explore potential mechanisms, Wnt/β catenin inhibitors (FH535 or IWP2) were also used in a number of the functional assays and the immunofluorescent staining patterns of several key molecules were explored.

Results: Knockdown of AAMP expression was observed in the HECV cell line following transfection with the ribozyme transgene. Cellular migration and attachment, assessed using ECIS methods, and tubule formation were significantly inhibited by the knockdown of AAMP. Additionally, cell growth and cell matrix adhesion was also substantially reduced following AAMP knockdown, though this did not reach significance. Cell aggregation levels showed no statistical difference between AAMP knockdown cells and control cells. Interestingly, immunofluorescence staining showed AAMP knockdown cells had a reduced expression of VE-cadherin. Some overlap of function was seen between AAMP knockdown and the FH535 inhibitor.

Conclusion: AAMP appears to influence endothelial cell migration and tubule formation and potentially, to a lesser effect, cell matrix adhesion and growth and also affects the expression of VE-cadherin. This data suggests that AAMP may play a role in angiogenesis. Assays performed with the FH535 inhibitor suggest a potential relationship between AAMP and Wnt/β catenin signalling.

Keywords: Angio-Associated Migratory Cell Protein AAMP; Angiogenesis; Endothelial; VE-cadherin; Wnt inhibitor

Introduction

The angiogenesis process is vitally important for growth, development and maintenance of a normal physiological state, playing key roles in essential processes such as reproduction, wound healing, and development [1,2]. Despite this, imbalance of the angiogenic process is observed in a number of disease states. One key example is seen in cancer, where tumour angiogenesis is essential in facilitating advanced tumour growth and increased metastatic potential, a realisation that has led to the development of anti-angiogenic therapies [1,2].

In 1995, Beckner *et al.* [3] isolated a 52kDa protein from a human melanoma cell line, termed Angio-Associated Migratory Cell Protein (AAMP), during a search for motility associated cell surface proteins. The cytoplasmic and membrane located protein contains two immunoglobulin-like domains, a WD40 repeat and a heparin binding consensus sequence [3]. The WD40 repeat motif is found in a wide range of proteins with diverse roles in signal transduction, transcriptional activation, cytoskeletal regulation and cell cycle control [4]. The homology of AAMP with members of the immune globular super family suggests AAMP may have similar roles to these members, which include recognition and binding, cell-cell interaction and cell signalling [5-9].

AAMP has a wide expression pattern, being observed in numerous endothelial and aortic smooth muscle cells, activated T-lymphocytes, renal proximal tubular cells, dermal fibroblasts, glomerular mesangial cells, rat myocytes and a number of cancerous cell lines including human melanoma cells, prostate and breast carcinoma cells and benign mammary cells [3,10-16]. A number of studies have focused on this molecule in cancer, demonstrating an enhanced expression of AAMP in invasive gastrointestinal stromal tumours [9]. Enhanced expression of AAMP was also seen in breast Ductal Carcinoma in Situ (DCIS) with necrosis and found to be elevated in the T47D cell line subjected to hypoxia [17]. Recently, a study conducted in our laboratories has explored the importance of AAMP in a clinical cohort of breast cancer patients and the impact of targeting AAMP in a number of breast cancer cell lines [16]. Our data suggests that AAMP levels are elevated in tumour compared to normal samples and higher expression of AAMP is generally associated with a poorer patient prognosis [16]. Additionally, knockdown of AAMP in MCF-7 and MDA-MB-231 breast cancer cell lines brought about reductions in aggressive traits such as growth, matrix adhesion and cellular invasion, though the impact on these traits was different between the two cell lines [16,17]. Taken together, these studies implicate a role for AAMP in cancer progression.

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A role for AAMP has also been implicated in the process of angiogenesis, where anti-recombinant AAMP can inhibit endothelial tube formation on Matrigel and the cellular motility of endothelial and smooth muscle cells [11,18-20]. Similarly, it has been suggested that the extracellular form of AAMP has a positive role in angiogenesis [18]. Further studies have implicated AAMP in the translocation and activation of RhoA in both endothelial and smooth muscle cells and subsequently, influence downstream signalling of RhoA through its effector Rho-Associated Kinase (ROCK) and hence cell migration [20]. AAMP has also been identified as a novel binding partner of thromboxane A2 receptor alpha and beta (TPα and TPβ), potentially implicating it in vascular pathologies [14].

In this study, we knocked down the expression of AAMP in HECV human endothelial cells, using hammerhead ribozyme transgenes, and assessed the influence of AAMP on endothelial cell growth, adhesion, migration, tubule formation and cell aggregation *in vitro*. Wnt/β catenin inhibitors (IWP2 and FH535) were also used in the functional assays and VE-cadherin and catenin (α, β and γ) expression patterns were examined to explore potential mechanism of action of AAMP.

Materials and Methods

Cell culture

The human HECV endothelial cell line was purchased from Interlab, (Interlab, Naples, Italy).Cells were routinely cultured in DMEM/Ham's F12 with L-Glutamine medium (Sigma, Dorset, UK), supplemented with antibiotics and 10% foetal calf serum (Sigma, Dorset, UK), and incubated at 37.0˚C, 5% CO_2 and 95% humidity. Low passage cells (passage 5) were used to generate stocks of control and transfected cells. Cells were frequently revived from initial stocks to ensure experimental consistency.

Generation of AAMP knockdown in HECV cell lines

Anti-human AAMP hammerhead ribozyme transgenes were designed and cloned into a pEF6/V5-His-TOPO plasmid vector (Invitrogen, Paisley, UK) as described previously [16]. Plasmids containing either AAMP specific ribozyme transgenes or empty pEF6 control plasmids were transfected into HECV cells using electroporation and subjected to a selective period (5 μg/ml Blasticidin) before being maintained in maintenance medium (0.5 μg/ml Blasticidin). Transfected HECV cells containing the ribozyme transgene plasmids were routinely tested to confirm knockdown of AAMP expression. Cells were respectively labelled as $HECV^{WT}$ (wild type), $HECV^{AAMPrib}$ (HECV cells containing the ribozyme transgene to AAMP) and $HECV^{pEF6}$ (control cells containing empty pEF6 plasmid).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

TRI-reagent (Sigma, Dorset, UK) was used to extract RNA from transfected and control cells. Subsequently, RNA was quantified and standardised to 250 ng before proceeding with reverse transcription (iScript™ cDNA synthesis kit; Bio-Rad, Hemel Hempstead, UK). PCR was then undertaken on the resulting cDNA using the following cycling conditions: 94˚C for 40 sec, 55˚C for 40 sec, 72˚C for 60 sec. PCR was conducted over 34 cycles with an initial 5 min denaturing step (94˚C) and a final 10 min extension step (72˚C). PCR products were separated electrophoretically on an agarose gel before being stained in ethidium bromide and visualised under UV light. The result was captured and the band density was calculated using Image J software for semi-quantitative analysis. Primer sequences are provided in Table 1.

SDS-PAGE and Western blotting

Protein was extracted from transfected and control HECV cells grown in a 75 cm^2 flask following detachment with a cell scraper, addition of lysis buffer (HCMF buffer containing 0.5% SDS, 1% Triton X-100, 2 mM $CaCl_2$, 100 μg/ml phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin and, 10 mM sodium orthovanadate) and incubation on a rotor wheel for 1 hour. Samples were subsequently micro-centrifuged at 13,000g to remove insolubles, before being quantified (Bio-Rad DC Protein assay kit; Bio-Rad laboratories, Hemel Hempstead, UK), standardised to 2 mg/ml, diluted in Laemmli 2x concentrate sample buffer (Sigma, Dorset, UK) and boiled for 5 minutes. Samples were electrophoretically separated on a 10% acrylamide gel before being transferred onto a Nitrocellulose membrane (Santa Cruz Biotechnologies, Sant Cruz, CA, USA). The membrane was subsequently probed using the respective primary antibodies, (AAMP and GAPDH; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) at a concentration of 1:70 and specific peroxidase conjugated secondary antibodies (Sigma, Dorset, UK) at a concentration of 1:350, in line with the Snap ID protein system guidelines (Millipore, Watford, UK). Protein bands were documented using a gel documentation system (UVI Tech, Cambridge, UK).

In vitro growth assay

The impact of AAMP knockdown on HECV cells was assessed using an *in vitro* growth assay. As previously described, three 96-well plates were set up and labelled as Day 1, Day 3, and Day 5 and cells were seeded into each well at a density of 2,000 cells/well [16]. After incubating for 1, 3 and 5 days, media was removed and cells were fixed in 4% formaldehyde (v/v) and stained with 0.5% (w/v) crystal

Gene of interest	Primer name	Primer sequence (5'-3')	Optimal annealing temperature (˚C)
AAMP Ribozyme	AAMP Ribozyme 1F	CTGCAGACCCTCAGTCCCTTTCAGTACATGCTGATGAGTCCGT-GAGGA	55
	AAMP Ribozyme 1R	ACTAGTGGGACCTGAAGCAGGGAAGCCCTATTTCGTCCTCACG-GACT	55
AAMP	AAMP F11	TCGAGGTGGTAGAACTTGAT	55
	AAMP R11	AGGTCTTGCAGTCACCATT	55
	AAMP F12	ACTAAGGAGGAGGTCTGGTC	55
	AAMP R12	ACTGATGCCTAAGAGTCTGC	55
GAPDH	GAPDH F8	GGCTGCTTTTAACTCTGGTA	55
	GAPDH R8	GACTGTGGTCATGAGTCCTT	55

Table 1: Primers/Ribozyme transgenes used in the present study

violet. Acetic acid (10%, v/v) was used to extract the crystal violet stain taken up by the cells and cell density was determined by measuring the absorbance of this extracted crystal violet solution at a wavelength of 540 nm using an ELx 800 spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT, USA). The percentage increase in cell density between day 1 and either day 3 or day 5 was then calculated.

In vitro matrigel adhesion assay

In vitro Matrigel adhesion assays were used to compare any differences in cell adhesion abilities of AAMP knockdown cells and control cells as described previously [21]. Briefly, a 96-well plate was pre-coated with 5 µg of Matrigel in 100 µl serum free media per well. After being dried and re-hydrated, 45,000 cells were seeded into each well and incubated for 45 minutes. Following incubation, non-adherent cells were removed by vigorous washing with BSS. Adherent cells were then fixed in 4% formaldehyde (v/v), and stained with 0.5% (w/v) crystal violet. Images of adherent cells were then captured under X20 objective magnification and counted using the Image J software.

Electric cell-substrate impedance sensing (ECIS) based cellular attachment assay and motility assay

The ECIS model system (Applied Biophysics Inc, NJ, US) was used for analysis of cellular attachment and migration as described previously [22]. A 96W1E plate was used in the present study. Following stabilisation of the 96W1E array, the same number of $HECV^{WT}$, $HECV^{AAMPrib}$ and $HECV^{pEF6}$ (100,000 cells per well) were seeded in 200 µl of medium per well. Impedance and resistance of the cell layer in the first 3 hours was recorded for the attachment assay. Following attachment of seeded cells to the array and establishment of a confluent monolayer within the well (10 hours after seeding), the monolayer was electrically wounded at 6 V for 30 seconds. Electrical wounding of the monolayer within the array results in a disruption of the monolayer (250 µm diameter wound). Subsequently, the impedance and resistance of the cell layer were immediately recorded over a period of 4 hours for detection of cellular migration as surrounding cells migrate to re-colonise the wound

In vitro matrigel tubule formation assay

Matrigel endothelial cell tubule formation assays were set up, based on a previously described method [23], to assess the involvement of AAMP knockdown on HECV angiogenic potential, in the presence and absence of a series of treatments with IWP2 (2.5 nM, 25 nM, 250 nM) and FH535 (0.1 µM, 1 µM, 10 µM). Briefly, 500 µg of Matrigel in serum-free medium was added to each well of a 96-well plate and placed in an incubator for a minimum of 40 minutes to set. Once the Matrigel had set, 35,000 HECV control or transfected cells were seeded onto the Matrigel layer, and either normal media or media containing inhibitors was added to the appropriate wells. Cells were incubated for 4-5 hours. Following incubation, any tubules that had formed were visualized under the microscope and images captured. Total tubule perimeter/field in these images was later quantified using Image J software.

In vitro cell aggregation assay

Cell aggregation was assessed using a previously described method [24]. Cells were first briefly washed in BSS and then detached in HCMF buffer (160 mM NaCl, 0.6 mM Na_2HPO_4, 0.1% (w/v) glucose, and 0.01 M HEPES, pH 7.4) containing 0.01% trypsin and 2 mM $CaCl_2$ for 30 mins at 37°C incubation. Cells were pelleted and washed in HCMF buffer containing $CaCl_2$ before being counted. Each tube was seeded with 1ml of cells at a concentration of 1×10^5/ml in HCMF buffer containing $CaCl_2$ and a different concentration of Wnt inhibitors were added (IWP2 at 25 nM and FH535 at 1 µM) before being placed on a shaker. Fifty micro litres of medium from each tube was taken and added to 50 µl of 4% Formalin to fix at the following time points: 0 min, 30 min, 60 min and 90 min. Cells were counted and aggregation index was calculated using the formula $(N_0-N_t)/N_0\times100\%$, where N_0 is the number of particles at the 0 min time point, and N_t is the number at the chosen time.

Immunofluorescence staining

Immunofluorescence staining was used to assess any alterations in the expression patterns of VE-cadherin, α-catenin, β-catenin and γ-catenin brought about by AAMP knockdown and any subsequent alterations caused by the addition of Wnt inhibitors (IWP2 at 25 nM and FH535 at 1 µM). Briefly, 20,000 cells per well were seeded into a 16 well Chamber slide (LAB-TEK Fisher Scientific UK, Loughborough, Leics, UK) together with the inhibitor (IWP2 at 25 nM or FH535 at 1 µM) or normal medium. Cells were incubated at 37˚C, 5% CO_2 overnight. Following incubation, the medium was aspirated and cells were fixed in ice-cold ethanol at -20˚C for at least 20 minutes before being rehydrated in BSS. Cells were then permeabilized with 0.1% TritonX100 for 2 minutes. Cells were placed in TBS buffer containing 5% horse serum for 20 minutes to block non-specific binding. Following this blocking step, cells were washed with TBS buffer twice. The primary antibody was then added at a concentration of 1:100, made up in TBS buffer containing horse serum for 1 hour. The primary antibody was then removed through three washes in TBS buffer. FITC conjugated secondary antibodies at a concentration of 1:100, made in TBS bufferwith horse serum, were added for 1hour in the dark. Cells were then washed three times with TBS buffer before being mounted with Fluorosave (Calbiochem-Nova Biochem ltd) Nottingham, UK). Images were viewed and captured using a fluorescent microscope. Antibodies used in the above study were as follows: anti-VE-cadherin (sc-101580, anti-mouse; Santa Cruz Biotechnologies), anti-α-catenin (606-259-1550, anti-mouse, Sigma), anti-β-catenin (059K4754, anti-rabbit, Sigma), anti-γ-catenin (P-8087, anti-mouse, Sigma), FITC anti-rabbit (F1262, Sigma), FITC anti-mouse (F3008, Sigma).

Statistical analysis

Statistical analysis of data was carried out using Sigma Plot 11 statistical software package. Experimental procedures were independently repeated a minimum of three times and data was analysed using one way ANOVA Holm Sidak post hoc test, ANOVA on Ranks Tukey post hoc test or a two way ANOVA test. Data was considered to be significant at $p < 0.05$.

Results

Ribozyme transgene manipulation of AAMP expression

RT-PCR indicated that transcript expression of AAMP was successfully knocked down in $HECV^{AAMPrib}$ cells in comparison to the expression levels seen in wild-type cells ($HECV^{WT}$) and in empty plasmid control cells ($HECV^{pEF6}$) (Figure 1A). This trend was further confirmed through semi quantitative analysis of band density, demonstrating large reductions in expression following normalisation against GAPDH levels (Figure 1B). Additionally, Western blotting was used to assess AAMP protein expression in both the control and ribozyme transgene transfected HECV cell lines. Similar to the trends seen at the transcript level, AAMP protein expression was observed in the control cell line ($HECV^{pEF6}$) and displayed large reductions in expression levels following transfection with the AAMP ribozyme

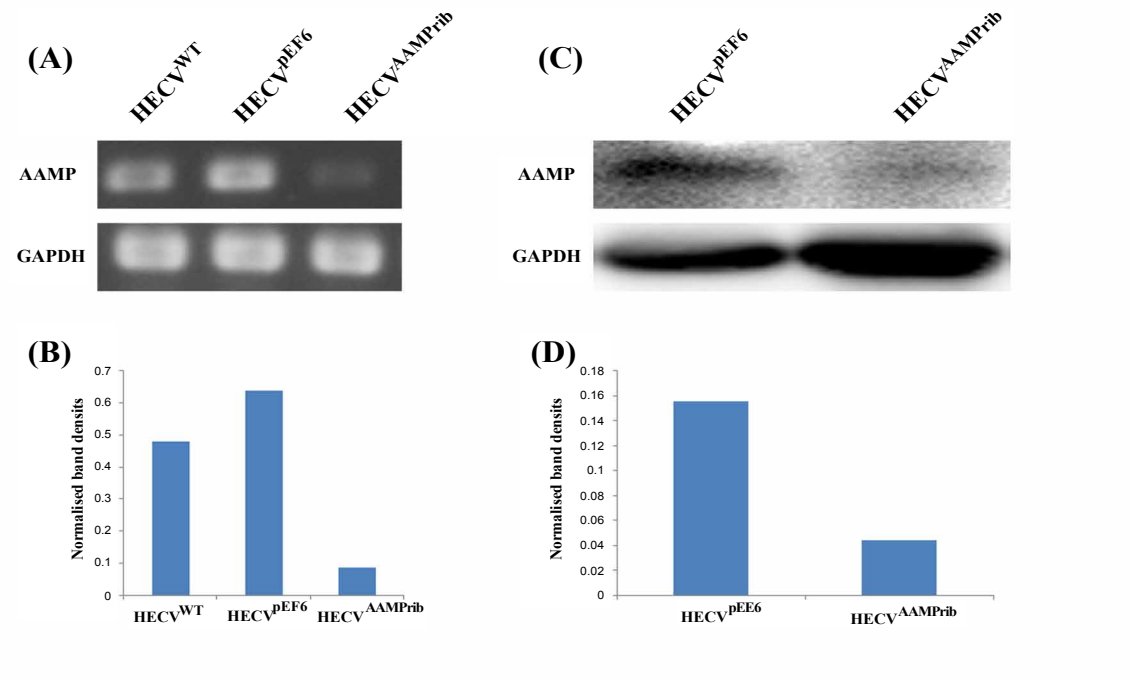

Figure 1: Ribozyme transgene knockdown of AAMP in HECV endothelial cells
(A/B) RT-PCR and semi-quantitative analysis of GAPDH normalised band densitometry, demonstrating substantial reductions in AAMP transcript levels, in comparison to wild type and plasmid control cells, following transfection with the AAMP ribozyme transgene.
(C/D) Similarly, a reduction in AAMP protein levels was also observed using Western blot analysis and quantification of protein bands. Representative images shown.

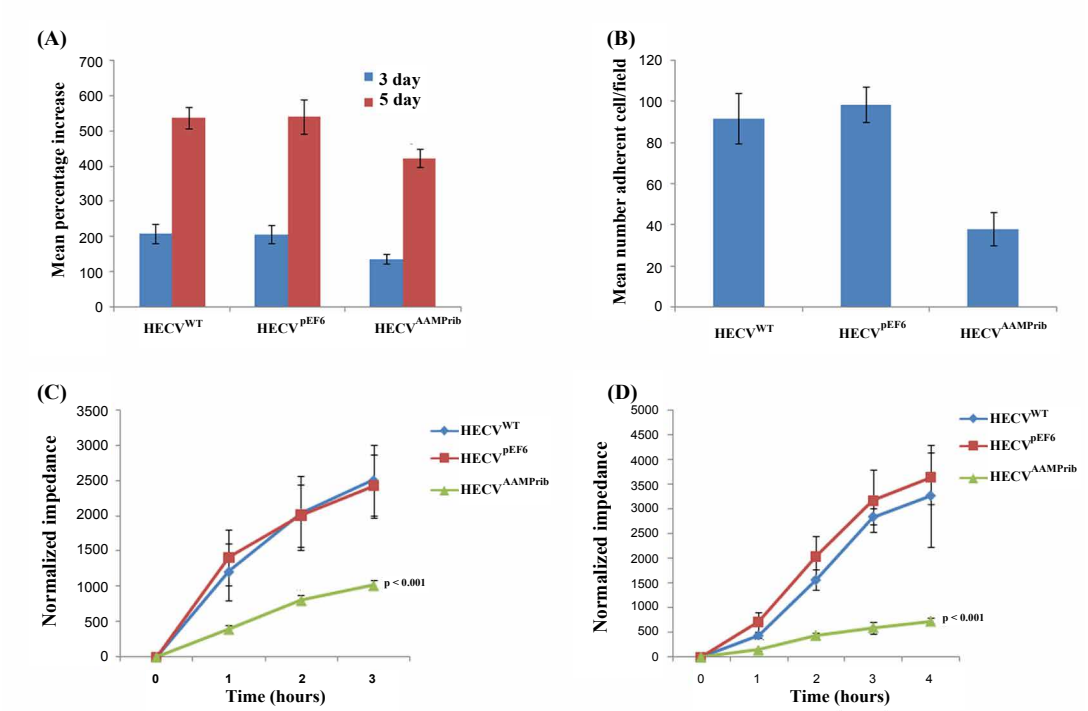

Figure 2: Impact of AAMP knockdown on cell function
(A) Knockdown of AAMP brought about a general reduction in HECV cell growth rate over both 3 and 5 day incubation periods, this did not reach significance.
(B) Targeting AAMP also reduced the capacity of HECV cells to adhere to an artificial Matrigel basement membrane, though again this did not reach significance.
(C) However, $HECV^{AAMPrib}$ cells were less able to attach to the array substrate in an ECIS attachment model system in comparison to $HECV^{pEF6}$ and $HECV^{WT}$ control cells over the experimental course ($p < 0.001$) and also demonstrated a significantly reduced migratory ability following electrical wounding of the monolayer using the ECIS system than $HECV^{pEF6}$ and $HECV^{WT}$ control cells over the experimental course ($p < 0.001$) (D).

transgene ($HECV^{AAMPrib}$) (Figure 1C), which was again confirmed through semi-quantitative band densitometry (Figure 1D).

Knockdown of AAMP impacts on HECV cell growth

The effect of AAMP on the growth of HECV cells was examined using an *in vitro* cell growth assay. Knockdown of AAMP appeared to influence the growth rate of the HECV cell line. Close to significant differences were observed between the growth rates of control cells and those containing the ribozyme transgene ($HECV^{pEF6}$, $HECV^{WT}$ and $HECV^{AAMPrib;}$ p=0.083 over 3 day incubation and p=0.06 over 5 day incubation periods) (Figure 2A).

Loss of endogenous AAMP dramatically reduces HECV cell adhesion

The cell-matrix adhesive capacity of HECV cells over a 45 minute incubation period was examined using an *in vitro* Matrigel adhesion assay. Loss of endogenous AAMP resulted in a dramatic decrease in matrix-adhesion levels in $HECV^{AAMPrib}$ cells compared to the control $HECV^{WT}$ and $HECV^{pEF6}$ cells though statistical analysis between the groups did not quite reach significance (p=0.05) (Figure 2B).

Knockdown of AAMP impacts HECV cell attachment and motility abilities

The effects of AAMP expression on cell attachment and motility were assessed using an ECIS assay. Knockdown of AAMP, similar to the Matrigel adhesion assay, dramatically reduced HECV cell attachment in comparison to control cell lines. Using this methodology, statistical differences in attachment can be seen between the knockdown cells and control cells over the course of the experiment (p<0.001; $HECV^{AAMPrib}$ vs. $HECV^{pEF6}$, p<0.001; $HECV^{AAMPrib}$ vs. $HECV^{WT}$, p<0.001) (Figure 2C). Knockdown of AAMP also impacted on the migratory capacity of HECV cells following electric wounding. Significant differences were observed between $HECV^{AAMPrib}$ cells and the wild type and pEF6 plasmid control cells over the course of the experiment (p<0.001; $HECV^{AAMPrib}$ vs. $HECV^{pEF6}$, p<0.001; $HECV^{AAMPrib}$ vs. $HECV^{WT}$, p<0.001)(Figure 2D).

Tubule formation of HECV cells was altered by AAMP knockdown

The impact of AAMP knockdown on tubule formation was examined in both the presence and absence of several concentrations of the Wnt/β catenin inhibitors, IWP2 and FH535 inhibitors. Significant differences in tubule formation levels were observed within the treatment groups (p<0.001). $HECV^{pEF6}$ cells without treatment formed tubule-like structures when seeded onto Matrigel. AAMP knockdown cells were seen to possess only a weak capability to form tubules on Matrigel and significant decreases in tubule formation levels, following quantification of tubule perimeters, were observed between $HECV^{AAMPrib}$ and $HECV^{pEF6}$ cells (p<0.001) (Figure 3A/B). Treatment with the IWP2 inhibitor, at the range of concentrations tested in this study did not seem to have any substantial effects on the level of tubule formation in either the control $HECV^{pEF6}$ or $HECV^{AAMPrib}$ cell lines, with no significant differences being observed in comparison to the respective untreated cells. A similar trend was seen with the FH535 inhibitor at the lower concentrations (0.1 μM and 1 μM). However, at the highest tested concentration of 10 μM, the FH535 inhibitor appeared to have differential effects on $HECV^{pEF6}$ and $HECV^{AAMPrib}$ cells. At this concentration, FH535 could significantly reduce the level of tubule formation in the $HECV^{pEF6}$ control (p<0.001 vs. untreated $HECV^{pEF6}$) whilst having no further effect on $HECV^{AAMPrib}$ cells (p=0.642 vs. untreated $HECV^{AAMPrib}$).

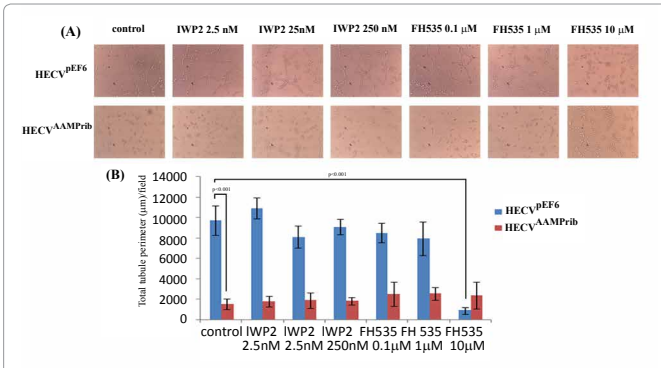

Figure 3: Impact of AAMP knockdown on HECV tubule formation
Targeting AAMP in the HECV cell line significantly reduced the angiogenic tubule formation capacity in comparison to control $HECV^{pEF6}$ cells (p < 0.001). The addition of the IWP2 and FH535 Wnt/β catenin signalling inhibitors had no significant effects on either control or transfected cells except at the highest 10μM concentration of FH535 where, at this concentration, FH535 brought about a significant decrease in $HECV^{pEF6}$ tubule formation in comparison to untreated $HECV^{pEF6}$ cells (p < 0.001).
(A) Representative images of tubule formation assays for each group.
(B) Mean quantified total tubule perimeter of all tubules within the microscopic field

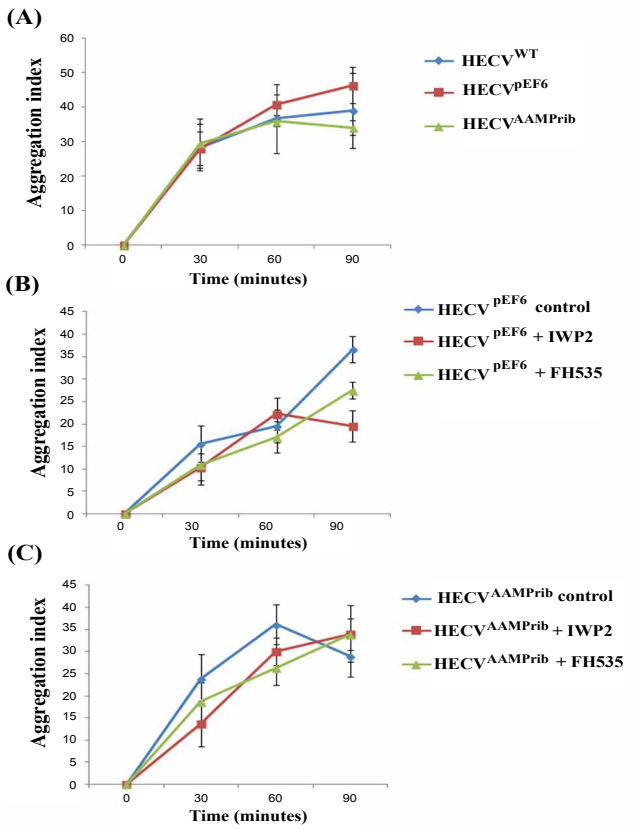

Figure 4: AAMP knockdown does not alter HECV cell aggregation
Aggregation assays indicate that knockdown of AAMP did not significantly alter the rates of cell aggregation and no significant differences were observed between $HECV^{pEF6}$, $HECV^{WT}$ and $HECV^{AAMPrib}$ cells (A). Additionally, treatment of $HECV^{pEF6}$ cells (B) or HECVAAMPrib cells (C) with either of the IWP2 or FH535 inhibitors did not have any significant impact on cell aggregation rates of either cell line.

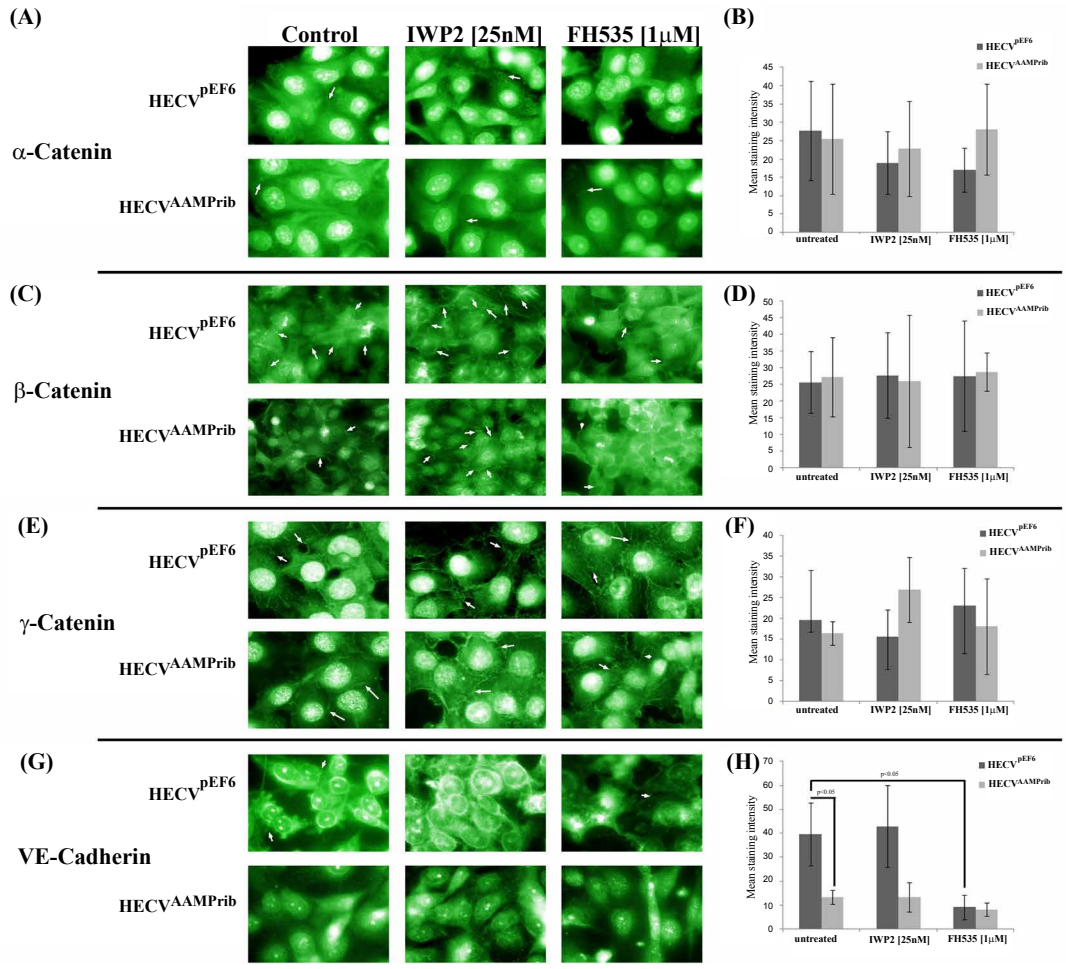

Figure 5: Immunoflourescent staining demonstrating the relationship between AAMP and cadherin/catenins Staining profiles (A,C, E,G) and quantified staining intensities (B,D,F,H) for α, β, γ catenin and VE-cadherin in HECV^{pEF6} and HECV^{AAMPrib} in the presence and absence of the IWP2 and FH535 inhibitors. Similar staining profiles and intensities were observed for α, β and γ catenin in both HECV^{pEF6} and HECV^{AAMPrib} cells. HECV^{AAMPrib} cells displayed lower levels of VE-cadherin staining that HECV^{pEF6} cells and quantification of intensities showed a significantly decreased level of VE-cadherin in HECV^{AAMPrib} cells compared to HECV^{pEF6} control cells ($P < 0.05$). The staining profile of VE-cadherin in HECV^{pEF6} cells was also affected following treatment with FH535 inhibitor, which similarly brought about a significant decrease in staining intensity compared to untreated HECV^{pEF6} cells ($p < 0.05$). Arrows indicate example of staining.

AAMP knockdown had no impact on cell aggregation

An aggregation assay was performed to assess the involvement of AAMP in cell-cell adhesion. No significant differences in cell-cell aggregation, over the experimental course, were observed between HECV^{WT}, HECV^{pEF6} and HECV^{AAMPrib} cells (p=0.63) (Figure 4A). In addition, neither IWP2 (25nM) or FH535 (1 μM) treatment was seen to have any impact on cell aggregation in either the control or the ribozyme transfected cell line over the course of the experiment (p=0.054 within the HECV^{pEF6} treatment groups, Figure 4B and p=0.565 within the HECV^{AAMPrib} treatment groups, Figure 4C).

VE-Cadherin expression is reduced following AAMP knockdown

Immunoflourescent staining was used to examine the staining profile and localisation of α, β and γ catenin, together with VE-cadherin in control and ribozyme transfected HECV cells (Figure 5). Expression patterns and staining intensities of the catenins were similar in both the HECV^{pEF6} and HECV^{AAMPrib} cells and few differences in staining intensities were observed in the images and following quantification of staining intensity (Figure 5A-F). The results of the immunofluorescence staining indicated that AAMP knockdown cells had a lower level of VE-cadherin expression compared with its pEF6 control which was further confirmed following quantification of staining intensity (Figure 5G and H). A statistically significant difference in VE-cadherin staining was discovered within the knockdown and treatment groups (p=0.004). Levels of VE-cadherin expression were significantly reduced in HECV^{AAMPrib} cells compared to HECV^{pEF6} cells (p<0.05). Similarly, treatment of HECV^{pEF6} cells with FH535 resulted in a similar reduction in VE-cadherin, and differences in VE-cadherin staining patterns could be observed between HECV^{pEF6} cells treated with or without FH535 (p<0.05). However, treatment with IWP2 did not change VE-cadherin staining intensity in either HECV^{AAMP}rib or HECV^{pEF6} cells compared to the respective untreated control (p>0.05).

Discussion

The AAMP gene was originally identified during research into

motility-associated proteins and was found to be expressed extensively in all the tested types of endothelial cells and other different cell lines and tissues [3,10-16]. Previous studies show that loss of AAMP result in a reduction in endothelial cell adhesion, migration and tube formation. In the present study, we verified the expression of AAMP in the human HECV endothelial cell line and subsequently used a ribozyme transgene system to knockdown AAMP expression in this cell line. Our results indicate that knockdown of AAMP, whilst not significant, brings about a general reduction in proliferation rates of HECV endothelial cells over 3 and 5 day growth periods. Migration and tubule formation are significantly inhibited when AAMP expression is reduced. Similarly, cell-matrix adhesion rates are inhibited following AAMP knockdown, with significant reductions observed using the ECIS model and substantial reductions observed using the Matrigel adhesion assay, though these did not quite reach significance (p=0.05). These results are in line with previous research reports [3,13,15,20]. Our study further explored the relationship between AAMP and Wnt/β catenin signalling using the FH535 (suppresses both Wnt/β-catenin and Peroxisome Proliferator-Activated Receptor (PPAR) signalling) and IWP2 (an inhibitor of Wnt processing and secretion) compounds. In the tubule formation assays, knockdown of AAMP significantly reduced the formation of tubule like structures compared to the control $HECV^{pEF6}$ cells. Further treatment with the IWP2 inhibitor had little additional effects on either $HECV^{pEF6}$or $HECV^{AAMPrib}$ cells. Treatment of these cells with FH535 did bring about differential effects between the two lines, where treatment with the highest (10 μM) concentration could significantly reduce $HECV^{pEF6}$ tubule formation (vs. untreated $HECV^{pEF6}$ cells) but had no further effect on AAMP knockout HECV cells. This result may suggest some relationship between AAMP and these pathways, however this trend was only observed at the higher inhibitor concentration and further work will be required to fully clarify this relationship.

Perhaps the most important observation in the present study is the relationship between the expression of AAMP and VE-cadherin seen using immunofluorescence staining. Reduced expression of VE-cadherin was observed in the $HECV^{AAMPrib}$ cells compared to the $HECV^{pEF6}$ cells. VE-cadherin plays a vital role in endothelial cell binding. Endothelial cell homotypic adhesion is mediated by two types of adhesive structures, tight junctions and adherens junctions, where VE-cadherin, an endothelial-specific member belonging to the cadherin family, plays a key role in mediating the adhesion through adherens junctions [25]. VE-cadherin is linked to a large number of intracellular partners which mediate its anchorage to the actin cytoskeleton from one side and from the other transfer intracellular signals. This potential relationship, between AAMP and VE-cadherin expression, may in part be responsible for the change in endothelial adhesion and motility caused by the knockdown of AAMP. Previous studies have also reported that VE-cadherin may form a multi-protein complex with Vascular Endothelial Growth Factor (VEGF) receptor 2 (VEGFR2) and limit its internalization and signalling activity [26]. Similarly, VE-cadherin can interact with transforming growth factor beta (TGFβ) receptor complex, platelet derived growth factor β receptor or Fibroblast Growth Factor (FGF) receptor 1 and others [27-29]. These interactions contribute to the control of endothelial proliferative signals and induction of vascular stability. This may again partly explain the impact on HECV cell growth following AAMP knockdown in our study. VE-cadherin directly binds inside the cells to β-catenin, an intracellular protein which is a crucial downstream element of the so-called canonical Wnt pathway, but it is also firmly bound to VE-cadherin [30]. β-catenin transcriptional activity is usually associated to cell proliferation. Our immunofluorescence staining data suggests that VE-cadherin was not only reduced with AAMP knockdown, but also by FH535, however, the mechanism of how FH535 impacts on VE-cadherin needs further investigation. It is again interesting to note that at the higher concentration, FH535 inhibition was able to reduce tubule formation levels of $HECV^{pEF6}$ control cells to a similar level to $HECV^{AAMPrib}$ cells and again may suggest some relationship. Whether AAMP has some functional relationship with Wnt/β-catenin requires further investigation.

Consistent with recent reports, AAMP knockdown reduces HECV endothelial cell adhesion, migration and tubule formation. In our recent study, *in vitro* growth ability may also be linked with AAMP. Our study also demonstrated that reduced AAMP is accompanied with low expression of VE-cadherin, and may also have some link with Wnt/β-catenin. The mechanism of how AAMP plays a role in these signalling pathways needs further research. The results presented here implicate a role for AAMP in a number of traits essential for the process of tumour angiogenesis and potentially implicate a relationship with Wnt/β-catenin signalling. Further elucidation of this relationship and mechanism may highlight potential anti-angiogenic therapeutic strategies.

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