The role of AmotL2 in the regulation of mesenchymal transitioning of endothelial cells

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Table of Contents

Abstract .............................................................................................................................. 1

Keywords .......................................................................................................................... 2

Popular Scientific Summary ............................................................................................ 2

Abbreviations .................................................................................................................... 3

Introduction ....................................................................................................................... 4

The cardiovascular system and its components ............................................................... 4

Mechanical forces & Junctional proteins ......................................................................... 5

Inflammation and Atherosclerosis ................................................................................. 6

Endothelial-to-mesenchymal transition (EndMT) ........................................................... 7

Transforming growth factor beta (TGF-β) ..................................................................... 8

Amot family and AmotL2 ................................................................................................. 9

Aims ................................................................................................................................. 10

Materials and Methods ................................................................................................. 10

Cell culture ....................................................................................................................... 10

Thawing of cells .............................................................................................................. 10

Passaging of cells ........................................................................................................... 11

Lentiviral production ....................................................................................................... 11

Lentiviral infection transduction .................................................................................... 12

TGF-β treatment ............................................................................................................ 12

Lysis ................................................................................................................................. 12

RNA extraction, purification and quantification ............................................................. 12

cDNA synthesis .............................................................................................................. 13

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) .................................... 13

Western Blotting ............................................................................................................. 14

Immunofluorescence ...................................................................................................... 15

Analysis ............................................................................................................................ 16

Results ............................................................................................................................ 17

Discussion ......................................................................................................................... 27

Conclusion ....................................................................................................................... 29

Acknowledgements ......................................................................................................... 29

References ......................................................................................................................... 31
Abstract

Background

During development, endothelial cells acquire mesenchymal-like properties to migrate and facilitate normal vascular formation. This process of transformation is known as endothelial to mesenchymal transition (EndMT) and has also been implicated in diseases like vascular pathologies contributing to endothelial inflammation, atherosclerosis and tumour angiogenesis. The Angiomotin family of scaffold proteins play a role in transducing mechanical force at cell junctions. Of this family, Angiomotin-Like 2 (AmotL2) localises to endothelial cell junctions and was recently found to play a role in regulating endothelial cell mechanosensing and inflammation.

Methods/Materials

Primary human endothelial cell lines (HUVEC) were cultured and manipulated in vitro to investigate the role of AmotL2 in EndMT. Lentiviral short hairpin RNA interference was employed in AmotL2-loss-of-function studies, (produced using HEK - Human Embryonic Kidney - cells) to generate knockdown(kd) cells. Western blotting (WB) was used to assess AmotL2 depletion and changes in protein expression of key EndMT markers. qPCR was performed to look at the same at a transcriptional level. Immunofluorescent staining and confocal imaging were performed to validate WB and qPCR results as well as to study protein localisation.

Results

AmotL2 was found to regulate Snail1 and N-cadherin at both protein and mRNA levels. Morphological findings displayed the AmotL2kd cells to be elongated, deviating from regular cobblestone morphology observed in control cells. An increase in scaffold protein levels was observed in the AmotL2 kd samples. Similar results were seen in qPCR data where increased mRNA expression was observed in the AmotL2 kd samples for the same targets. On analysis of IF image data, more nuclear staining was observed in the kd samples. qPCR analysis done on samples treated with TGF-β, exhibited an increase in mRNA expression of targets involved in the EndMT pathway in the treatment samples against the controls.

Conclusion

The results suggest that AmotL2 plays a role in EndMT by affecting the transcription factors and proteins involved in the pathway, which leads to changing morphology and behaviour of the cells. Looking into more targets involved in EndMT may give us a better understanding of how this process leads to diseases like atherosclerosis and tumour angiogenesis.
Unlocking the Secrets of Cellular Transformation: AmotL2's Role in Guiding Endothelial Cells!

Have you ever wondered how our body's cells can change and adapt to different situations? A recent scientific study delves into the fascinating world of cell transformation, focusing on the role of a protein called AmotL2 in shaping the destiny of endothelial cells.

Endothelial cells are a vital component of blood vessels, forming a protective lining to ensure smooth blood flow. But sometimes, these cells undergo a remarkable change called endothelial-to-mesenchymal transition (EndMT), where they transform into a different cell type with enhanced abilities to migrate and invade. This transformation is essential during our development as embryos, wound healing, and repairing damaged tissues. However, when it goes awry, it can contribute to serious health issues such as fibrosis and cancer.

Scientists have long been intrigued by the mystery of how EndMT is regulated. A key player in this puzzle is the transforming growth factor-beta (TGF-β) pathway, which acts like a conductor guiding the cells through this intricate transformation symphony. In a recent study, researchers focused on a protein called AmotL2, which appears to be a crucial performer in this cellular orchestra.

The study begins by examining the levels of AmotL2 during EndMT induction. Interestingly, they found that AmotL2's expression changes dynamically during this process, suggesting that it could play a significant role. Even more intriguing, when the cells were exposed to TGF-β, the protein's levels increased. This points to a potential connection between AmotL2 and the TGF-β pathway, suggesting that they might work together to control EndMT.

Diving deeper, the scientists decided to see what happens when AmotL2 is removed from the equation. Using cutting-edge techniques, they silenced AmotL2 and watched how it affected the cells. The results were incredible! Without AmotL2, the transformation process was slowed down. The endothelial cells held onto their original features, and the transition towards the mesenchymal state was inhibited. It seems that AmotL2 acts as a positive regulator, driving the cells towards their new identity. But AmotL2's influence doesn't stop there. When AmotL2 was silenced, some of the TGF-β components also decreased. This suggests that AmotL2 and TGF-β are closely intertwined, working in harmony to orchestrate the complex process of EndMT.

One of the most exciting aspects of this study is its implications for understanding the intricate molecular dance that occurs during EndMT. The researchers also highlighted the potential therapeutic significance of their findings. With EndMT linked to diseases like fibrosis and cancer, manipulating AmotL2 could become a novel strategy for intervention.
This study also shines a spotlight on the cadherins, molecules responsible for cell-cell adhesion. During EndMT, there's a shift from VE-cadherin to N-cadherin, reflecting the cells' transformation. The study suggests that AmotL2 might be involved in this cadherin switch, further adding to its multifaceted role in shaping cell behavior.

In a world of constant discovery, this research is a significant step towards unraveling the secrets of cell transformation. By highlighting the importance of AmotL2 in guiding endothelial cells through the intricate process of EndMT, the study provides valuable insights into the complex world of cell biology. As we uncover more about the delicate mechanisms behind cell behavior, we inch closer to harnessing this knowledge for medical breakthroughs that could revolutionize disease treatment and prevention.

**Abbreviations**

<table>
<thead>
<tr>
<th>EMT</th>
<th>Epithelial-Mesenchymal Transition</th>
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<tr>
<td>EndMT</td>
<td>Endothelial-Mesenchymal Transition</td>
</tr>
<tr>
<td>HUVEC</td>
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<tr>
<td>HEK</td>
<td>Human Embryonic Kidney cells</td>
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<td>TGF-β</td>
<td>Transforming growth factor-β</td>
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<td>Angiomotin-Like</td>
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<tr>
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<td>Western Blot</td>
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<tr>
<td>SNAI1</td>
<td>Snail Family Transcriptional Repressor 1</td>
</tr>
<tr>
<td>qPCR</td>
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</tr>
<tr>
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<td>Immunofluorescence</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
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</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence Solution</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propanesulfonic acid</td>
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<tr>
<td>shRNA</td>
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Introduction

During embryonic development, cells display high levels of plasticity and migratory potential, which is crucial to normal developmental processes. While these phenomena are fundamental to normal growth and development, the same processes may also be atypically activated outside of development, contributing to disease formation. Previous work has indicated that epithelial and endothelial cell plasticity are crucial for embryonic development but also results in disease progression, leading to cardiovascular deformations, among other pathological conditions.

The cardiovascular system and its components

In the human body, the cardiovascular system consists of the heart, blood vessels and approximately five litres of blood. The vascular system is made up of three types of blood vessels: The arteries, the veins and the plexus between them, the capillaries. The blood vessels act as the main link between the heart and the tissues. The vascular walls are made up of three layers; the intima (inner layer), the tunica media (middle layer) and the tunica externa (outer layer). The blood vessels are classified based on their function, location and size. The vascular system is responsible for the transport of nutrients and oxygen to every part of our body and this process is dependent on the flow of blood.

Capillaries line vessels throughout the body and are implicated in every organ. They are composed of endothelial cells that are coupled with various junctional proteins and receptors, anchored to a continuous basal membrane. Endothelial cells which are located on the intima, the inner lining of the vasculature, are known as the endothelium, which is crucial to maintaining normal physiology. The endothelium is located between flowing blood and the vascular wall. It controls vascular function by responding to various hormones, neurotransmitters and vasoactive factors which affect blood vessel formation, coagulation, regulation of vascular tone, vasomotion, thrombosis, platelet aggregation and are also key regulators of inflammatory responses.

While a balanced production of these vasoactive factors is atheroprotective (protects against the formation of atherosclerosis), a damaged endothelium leads to disturbed production of these factors. This imbalance leads to endothelial dysfunction (ED), which is an early indicator of atherosclerosis. Endothelial cells display different structures and phenotypes depending on vessel type. While in arteries and veins they appear more continuous and thicker, in capillaries they are fenestrated and thinner to allow for exchange of metabolites and gases.

Figure 1. (A) An overview of the vascular system, its components and direction of blood flow. (B) A closer look at the capillary and its components. Adapted from Encyclopedia Britannica.
Membrane receptors sense mechanical forces - To maintain normal functioning, many receptors and factors are involved and of these, mechanical force is an essential one. The cells live in a microenvironment, where they are continuously subjected to mechanical forces, which influence cell morphology, mobility, proliferation, and other aspects. Some of these forces involved are stretch, junctional force, shear stress (the dragging frictional force exerted on the vessel wall by laminar blood flow) and haemodynamic/fluid force (the pressure exerted on the vessel walls from the flow of blood)\(^7\).

Cells can sense the forces with specific receptors, including integrins which links the extracellular matrix and cells, and cadherins which links the cells at junctions\(^8\). Some of the receptors involved for example, are E-cadherin expressed in epithelial cells, VE-cadherin expressed in endothelial cells and N-cadherin, found in various areas such as vascular smooth muscle cells and neurons\(^9\).

Mechanical forces linked with blood flow play crucial roles in the acute control of vascular tone, the regulation of arterial structure and remodelling, and the localization of atherosclerotic lesions\(^10\). Primary, physiological functions of endothelium include the maintenance of anticoagulant properties, the control of lumen diameter, the regulation of vascular permeability, as well as the pathological effects associated with wound healing, cardiovascular disorders such as atherosclerosis and several other inflammatory diseases. Among these processes, hemodynamic factors (defined as mechanical forces in the flowing blood) influence endothelial biology. This occurs either by direct or indirect means: 1. Direct action of shear stress and stretch forces on the endothelium itself or 2. Indirect modification of the local concentrations of chemicals and agonists at the endothelial surface, where the receptors lie. This affects association between these molecules and their endothelial receptors\(^7,11\).

The endothelium is exposed to fluid forces of much greater magnitude than those experienced by other mammalian tissues. As a result, mechanically related responses controlled by the endothelium have evolved as part of normal vascular physiology. Here, mechanisms responsible for the transmission and transduction of hemodynamic information from the blood to the underlying vessel wall reside in the endothelium. Hemodynamic forces also play an important role in vascular pathologies, particularly in relation to the formation of atherosclerotic lesions\(^12\).

Figure 2. (A) Various mechanical forces exerted on the capillaries. (B) The formation of an atherosclerotic lesion. Adapted from Vining et al., 2017.
Inflammation and Atherosclerosis

Atherosclerosis is a chronic, progressive disorder of the vascular system that is initiated by biomechanical forces in areas of the vascular tree exposed to disturbed blood flow\(^1\). It is dependent on various systemic factors, including hyperlipidaemia, smoking, and diabetes\(^2\). Atherosclerosis is also defined as a chronic inflammatory disease of large and medium-sized arteries that causes ischemic heart disease, strokes, and peripheral vascular disease, collectively called cardiovascular disease (CVD)\(^3\). Inflammation and shear stress play important roles in the development and progression of atherosclerotic lesions\(^4\).

Forces linked to blood flow are key determinants of vascular morphogenesis and physiology. Blood flow is crucial for blood vessel development during embryogenesis and for regulation of vessel diameter in adult life. In addition, it is also a key factor in atherosclerosis, which, despite the systemic nature of major risk factors, occurs mainly at regions of arteries that experience disturbances in fluid flow\(^5\).

Atherosclerosis is characteristic of the formation of lesions or plaques that contain lipids, leukocytes, smooth muscle cells and, at late stages, necrotic cores with cholesterol crystals and calcification\(^6\). Lipid uptake by the vascular wall over time leads to a gradual build-up of atherosclerotic plaques. The growth and expansion of these plaques can gradually narrow the arteries, decreasing blood flow thus resulting in pain or limited function, as in angina, congestive heart failure or peripheral vascular disease\(^7\). These plaques can also suddenly rupture, leading to thrombus formation and vessel occlusion. These events often manifest in the form of myocardial infarction or stroke, which together are responsible for approximately half the deaths in developed nations\(^8\). The build-up, growth, and rupture of the plaque have all been associated with the presence of systemic and vascular wall inflammation\(^9\). The risk factors mentioned above play major roles in the incidence and progression of atherosclerosis. However, as stated before, these risk factors are relatively uniform throughout the vasculature, whereas atherosclerosis is initially highly focal, occurring mainly at artery bifurcations, branch points and regions of high curvature that result in complex blood flow patterns.

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Atherosclerosis is the result of interplay between endothelial dysfunction and subendothelial lipoprotein retention. Over time, this process leads to a non-resolving inflammatory response that can cause intimal destruction, arterial thrombosis, and end-organ ischemia. Recent advances highlight important cell biological atherogenic processes, including mechanotransduction and inflammatory processes in endothelial cells, origins and contributions of lesional macrophages, endothelial-to-mesenchymal transition, and origins and phenotypic switching of lesional smooth muscle cells. These advances illustrate how in-depth mechanistic knowledge of the cellular pathobiology of atherosclerosis can lead to potential new ideas for therapy.

**Endothelial-to-mesenchymal transition (EndMT)**

Endothelial-to-mesenchymal transition (EndMT) is a phenomenon that has not very long ago been identified to be regulated by mechanical forces. EndMT is the process by which endothelial cells lose a portion of their cellular features and obtain certain characteristics of mesenchymal cells (fibroblasts, smooth muscle cells [SMCs]), including junctional instability, and increased secretion of extracellular matrix proteins, such as fibronectin and collagen. It is characterized by morphological changes, that is, change in phenotype of normal endothelial cells that assume the shape and properties of mesenchymal cells, loss of endothelial markers like VE-cadherin and the acquisition of mesenchymal markers like N-cadherin. Other characteristic features include increased proliferation and migratory capacity and expression of various leukocyte adhesion molecules. This occurs due to a variety of transcription factors and different signalling pathways involved in the process.

![Figure 4. Schematic representation of the process of endothelial-to-mesenchymal transition (EndMT), characteristic features and the transcription factors, signalling pathways and external factors involved. Adapted from Shohreh Maleki, et al., Front.Cardiovasc. Med., (2019)](image)

Recent studies have documented that while EndMT is involved in embryonic and cardiac development, it has also been implicated in several pathological conditions such as inflammatory cardiovascular diseases including atherosclerosis, myocardial infarction, cerebral cavernous malformations and a variety of diseases processes, such as vascular or tissue fibrosis and tumours.
Transforming growth factor beta (TGF-β)

Transforming growth factor-β (TGF-β) is a homodimeric peptide growth factor with various effects on cellular differentiation and proliferation. These effects include the promotion of cellular phenotypic changes, the control of migration, cellular adhesiveness and invasiveness, and the regulation of extracellular matrix deposition. There exist around four homologous but distinct isoforms of TGFβs: TGFβ1, β2, and β3 from mammalian species and birds, and β5 from amphibians.

The multifunctional cytokine, transforming growth factor–β or TGF-β has been identified as a key player in driving EndMT progression, but the processes leading to activation of its signalling are still not properly understood. EndMT is promoted by treating cells with TGF-β2. Various transcription factors, such as Snail (SNAI1), Slug (SNAI2), Twist (TWIST1) and ZEB (ZEB1) are involved in this process.

Figure 5. Role of TGF-β in EndMT and the nuclear transcription factors (Snail, Slug, Twist, ZEB) involved in the process. Adapted from Pei-Yu Chen and Michael Simons., EMBO Mol Med., (2016)
**Amot family and AmotL2**

The Angiomotins belong to a family of scaffold proteins that mediate mechanical force. They are classified into three member groups: Angiomotin (Amot), Angiomotin-like 1 (AmotL1) and Angiomotin-like 2 (AmotL2). These three members are characterized by a WW-binding motif, a Coiled-coil (C-C) domain and a PDZ-binding motif. While the Amot group contains an angiotatin-binding domain between C-C domains and the PDZ binding motif, AmotL1 and AmotL2 do not. Each group consists of one full-length isoform and a shorter variant: Amot (p130/p80), AmotL1 (p100/p90) and AmotL2 (p100/p60). Based on previous research, those domains have specific binders. Specifically, the WW domain can bind to actin and cadherins, thereby controlling cell shape and facilitating actin cytoskeleton remodeling in epithelial and endothelial cells. This indicates that the Amot family could be a linker between the cytoskeleton and junctions on the cell membrane.31,32

![Figure 6. Schematic of Angiomotin protein family.](image)

Figure 6. Schematic of Angiomotin protein family. This scaffold protein family is composed of Amot, AmotL1 and AmotL2, each of which consists of two isoforms each and are all characterized a WW binding domain, C-C domain and PDZ binding motif. Adapted from Yuanyuan Zhang’s thesis.

AmotL2 is found in the cell junctions and links the actin filaments in the cytoskeleton. It plays a pivotal role in morphology, polarity, localisation, migration, interaction with receptors and proliferation of angiogenic endothelial cells. Prior studies observed that knock down of AmotL2 leads to impaired endothelial alignment and upregulation of inflammatory markers. This causes the formation of abdominal aortic aneurysms. Studies have revealed that AmotL2 is crucial to normal physiology, specifically during vasculogenesis where AmotL2 associates to VE-cadherin to mediate actomyosin-dependent mechanical force required for aortic expansion. When knocked down, it can lead to certain diseases like inflammation, aneurysms and atherosclerosis which lead to mortality.33 However, the role of AmotL2 in other processes of vascular inflammation is still understudied and a lot is left to be uncovered.

It was hypothesised that RNA expression levels are elevated in TGF-Beta treated cells than in control cells (normal endothelial cells).
The significance of the project will investigate how the junctional mechanosensor, AmotL2, regulates transitions in cell state during EndMT. As little is known regarding the role of junctional mechanosensory molecules in the process of EndMT, the goal was to enhance the existing knowledge. EndMT plays an important role in the development of cardiovascular disease and cancer progression respectively, both of which alone account for the two leading causes of death worldwide.

Aims

To investigate the role of the junctional protein AmotL2, in regulating endothelial to mesenchymal transition.

To investigate the role of specific EndMT transcription factors (Snail1, Slug, Twist and ZEB).

To investigate and determine whether the protein expression of key markers such as N-cadherin, VE-cadherin change.

Materials and Methods

Cell culture

Human Embryonic Kidney 293T (HEK293T) and Human Umbilical Vein Endothelial cells (HUVEC) were purchased from ATCC and PromoCell respectively. Cell culture was carried out using sterile technique under class II laminar flow hoods. All solutions and media were pre-warmed to 37°C before use. HEK293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% foetal calf serum (FCS) and 1% Pen/Strep (penicillin-streptomycin). HUVEC were maintained in Endothelial Cell Medium (ECM) (ScienCell) supplemented with 5% foetal calf serum (FCS) (v/v), 1% endothelial cell growth supplement and 1% Pen/Strep antibiotic cocktail (ScienCell). The cells were cultured in the following way. Endothelial cells were grown on 0.2% gelatin (w/v in 1X Phosphate Buffer Saline[PBS]) coated flasks. Flasks were coated with gelatin for a minimum of 2 hours at 37°C. Following this, gelatin was aspirated and matrix coated flasks were washed with 1X PBS prior to cell seeding.

Thawing of cells

The cells were first thawed. Cryovials were removed from storage and transported on dry ice. Cells were thawed by placing cryovials in 37°C incubator for 2 minutes, until cells were 70% thawed. Cells were then gently resuspended dropwise in 1-2ml warm medium, gently pipetted up and down in cryovial and then transferred to 15ml falcon tubes (Corning). Resuspended cells were then centrifuged at 200 xg for 5 minutes at room temperature (RT) to obtain a pellet.
Supernatant was aspirated and cells resuspended in 5ml of growth medium and transferred to T25 flasks (Corning) and incubated at 37°C under 5% CO₂. Medium was replaced following 24 hours and cells were grown to confluency before further use. Endothelial cells were allowed to grow to 80% confluency before passaging every 2-3 days.

The same procedure is followed for culturing cells on chamber slides and plates. The cell density and media volume were adjusted accordingly for 6 well plates and/or chamber slides. Cells were seeded at 20% to eventually reach 80% confluency before they were harvested.

**Passaging of cells**

For a T25 flask, once the cells reached 80% confluency, the flasks were aspirated of media and washed with 1x PBS. 500µl of 1X Trypsin was added and the flask was incubated at 37°C until cells began to detach (2-3 minutes). 4.5 ml of media was added to neutralise and inactivate the trypsin. Resuspended cells were pipetted into a 15ml Falcon tube. Centrifugation was carried out at 200xg for 5 minutes at RT to obtain a pellet at the bottom. The supernatant was discarded and the pellet was resuspended in fresh media after which the cells were passaged in a volume to seed cells at 20%. confluency. This process was the same for both, HEK 293T and HUVEC cells.

**Lentiviral production**

HEK 293T cells were used for the purpose of virus production. Lipofectamine 3000 was used to transfect HEK 293T cells with plasmids to produce lentivirus. HEK 293T cells were seeded to T75 flasks and grown to 70-80% confluency. The transfection reactions were carried out and completed using OptiMEM (reduced-serum medium) (ScienCell). Two 15ml falcon tubes were labelled for mix A and mix B. Tube A contained 2.5ml optiMEM with transfection reagent 3000 at 2µl/ug of plasmid DNA. The plasmid DNA included 3ug each of three different packaging plasmids (RRE, REV and VSV), and plenti plasmid (1 plasmid) 10ug each (either short hairpin (sh)Scrambled or shAmotL2 plasmid). Tube B contained 30ul of lipofectamine reagent added to 2.5ml of optiMEM media. Tubes A and B were incubated for 5 minutes at RT. Tubes A and B were then combined (total 5ml) to form the transfection agent and left to incubate at RT for 15-20 minutes. Normal HEK 293T culture media (DMEM, 10% FCS and 1% pen/strep) was removed and replaced with transfection reagent and left overnight at 37°C. The following day, the transfection media was then replaced with 10ml DMEM, 10% FCS with no antibiotic present and left for 24 hours. Viral supernatant was then harvested following 24 hours. Media was replaced for harvesting at 48 hours and 72 hours in a similar fashion. The media harvested from different time points was collected in a single 50ml Falcon tube by passing it through a 0.2µm (micron) syringe filter. The viral supernatant was then aliquoted and frozen down in 10% DMSO and FBS in cryovials for future use.
**Lentiviral infection transduction**

HUVEC cells at 70% confluency were infected with the lentivirus in a 6- or 12-well plate format. For a single 6 well, 750µl of total volume of virus containing medium was used. The lentivirus treatment for each well was made up of 500µl lentiviral supernatant (collected from the preparation as above), 250µl media and polybrene (Sigma), a cationic polymer used to increase the efficiency of transduction, used at 1:1000. The lentivirus was left on the cells to be incubated and then replaced with normal culture medium for a period of 96 hours before downstream analysis.

**TGF-β treatment**

TGF-Beta (PeproTech) was reconstituted in 10mM Citric acid and 0.1% BSA to a stock concentration of 10µg/ml and aliquots were stored at -80°C. HUVEC cells at 70% confluency were treated with TGF-β in a 6- or 12-well plate format. Working concentrations of 10, 20 and 50ng/ml were prepared in media from stock concentrations. The different conditions were Control (media without TGF-β) and treatment with concentrations of 10ng/ml, 20ng/ml and 50ng/ml TGF-β. Cells were treated for 48 hours before being lysed or harvested for downstream analysis.

**Lysis**

The media was aspirated from the wells and washed once with 1X PBS. The PBS was aspirated and the required lysis buffer is added to the wells. For RNA analysis, 350µl of RNA extraction lysis buffer was used, while for Western Blot analysis, 150µl of Western Lysis Buffer was added to the 6- or 12-well plates. The cells were scraped off the wells using a cell scraper (Corning) and lysates were transferred to a 1.5ml Eppendorf tubes. The RNA lysates were placed on dry ice and later stored at -80°C while the WB lysates were placed on ice and stored at -20°C.

**RNA extraction, purification and quantification**

RNA extraction from the cell lysates prepared for RNA analysis (as above), was performed using the RNeasy® Plus Mini Kit (Qiagen) according to the manufacturers protocol. Briefly, RLT lysates described above were thawed on ice before being passed through a series of columns by centrifugation. One volume (350µl) of 70% ethanol was added to the 350µl RNA
lysate (total volume 700µl) and transferred to an RNeasy spin column placed in a 2ml collection tube. Columns were centrifuged for 15 seconds at 8000g. The flow-through was discarded. 700µl of Buffer RW1 was added to the spin column and centrifuged at 8000g for 15 seconds. Flow-through was discarded. 500µl of Buffer RPE was added to the column and centrifuged as above. This step was repeated for a centrifugation time of 2 minutes. Finally, the column was put into a new 1.5ml collection tube. RNA was eluted in 50µl RNase-free water, which was added to the spin column membrane and centrifuged for 1 minute at 8000g.

The RNA was quantified using the NanoDrop 2000 (ThermoScientific). The RNA samples were stored at -20°C.

cDNA synthesis

Reverse transcription was performed using the Applied Biosystems™ High-Capacity RNA-to-cDNA™ synthesis kit (Applied Biosystems). The reaction tubes contained 20µl of 2X RT Buffer mix, 2µl of RT Enzyme mix and the remaining volume (18µl), a mixture of the sample RNA and RNase-free water in the required concentrations, to make up a total reaction mix of 40ul. 1µg of RNA was used for the analysis and in instances where this did not fulfil required volumes, RNase free water was used to make up the RNA sample volume of 18µl. Samples were placed into a Thermal Cycler (Applied Biosystems) to incubate at 37°C for 60 minutes followed by 95°C for 5 minutes.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Quantitative real-time PCR was performed using SYBR green based reagents. mRNA expression of multiple genes were targeted using primers specific for: AmotL2, SNAI1, SNAI2, CDH2, Twist1 and Zeb1 (Table 1). Thermocycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Gene expression was normalized to the constitutively expressed housekeeping gene GAPDH, and relative expression was calculated and plotted using the ΔΔCt method. A melt curve was also performed to detect the primer dimers or contaminating DNA in the samples that may have interfered with the results.
Table 1. Primers used for qPCR analysis.

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<tr>
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**Western Blotting**

Protein lysate preparation - The cell lysates were removed from -20°C to thaw on ice and then centrifuged at high speed at 4°C for 5 minutes. 40µl reaction tubes were prepared with 10X sample reducing agent (Invitrogen) and 4X sample buffer (Invitrogen) per tube. The required amount of protein lysate was added to each tube. Samples were boiled at 95°C for 5 minutes on a heat block. The samples were cooled to RT and briefly spun down. 39 µl of each sample was loaded into individual wells and 5µl of the ladder (BioRad) was loaded into a separate well. Gel loading tips were used for this.

For all Western blotting analyses, protein lysates were resolved on a precast NuPAGE (Polyacrylamide Gel Electrophoresis) Bis-Tris 4-12% gradient gel (Invitrogen). Current was applied to the gel in 1X MOPS (3-(N-morpholino) propanesulfonic acid) buffer (20X MOPS buffer in deionised water) (Invitrogen) for 75 minutes at 150 voltage (V) and 3 amperes (A) in an electrophoresis tank (Bio-Rad).
Nitrocellulose membranes (0.2 µm) (Perkin Elmer PROTRAN) were activated by soaking in Transfer buffer (20X Transfer buffer in deionized water, 10% methanol) for 1 minute (Invitrogen). The sponges and filter papers (Whatman) were also soaked in Transfer buffer. Gels were transferred to the nitrocellulose membrane by layering the membrane over the SDS-PAGE gel and placing on sponges and filter paper in a sandwich cassette submerged in transfer buffer. Transfer was completed after 1 hour 30 minutes at 0.4A and maximum voltage (250V) using a transfer tank (Bio-Rad) on ice.

Following transfer, nitrocellulose membranes were blocked for 1 hour at RT, shaking on an orbital shaker in 1X PBST (10x PBS, deionized water, Tween-20 (Sigma)) with 5% milk (Semper mjölk). After blocking, membranes were probed with the appropriate primary antibody (1:1000) (see Table 2) in 5% milk overnight at 4°C. Membranes were washed 3x 10 minutes in PBST, before probing with horse radish peroxidase (HRP) conjugated secondary antibody (1:5000) (see Table 2) in 5% milk in 1X PBST for 1 hour at RT with constant agitation on the orbital shaker. Membranes were then washed 3x at 10 minute intervals in PBST before developing in 1ml of enhanced chemiluminescence solution (ECL, Perkin Elmer) for 1 minute. The 2 ECL reagents were mixed in a 1:1 ratio. Excess liquid was blotted away with an absorbent towel, and the membrane was placed in a plastic sheet protector. The blots were then imaged with the iBright (Invitrogen) and exposed for varying times depending on the intensity of the bands.

<table>
<thead>
<tr>
<th>Target</th>
<th>Manufacturer</th>
<th>Lot. No.</th>
<th>Origin/Source</th>
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**Table 2. Antibodies used for Western Blotting.**

**Immunofluorescence**

Prior to carrying out immunofluorescence (IF), cells seeded to chamber slide (Corning) were fixed. For IF, cells grown on slides were fixed using 4% PFA (Paraformaldehyde)(Sigma).
Media was aspirated from the slide and cells were washed in 1x PBS before fixation in 4% PFA for 10 minutes at room temperature in a fume cupboard. Slides were washed twice in PBS and finally fresh PBS was added on.

Cells were permeabilised in 0.1% Triton X-100 (in deionised water) for 10 minutes, followed by 3 PBS washes of 10 minutes each and blocking with 5% horse serum (in 1X PBS) for 1 hour at RT.

Incubation in primary antibody (1:100) was carried out overnight. Following this, the chambers were washed 3x 10 minutes at RT which was then followed by the secondary antibody treatment (1:1000) with AlexaFluor 488 and phalloidin (1:200) for 1 hour. The slides were then mounted with mounting media containing DAPI (Abcam) and a coverslip for confocal imaging using LSM 700 (Zeiss).

**Table 3. Antibodies used for immunofluorescence.**

<table>
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**Analysis**

qPCR data was analysed and charts were produced from the data in Excel using the ΔΔCt method.

IF analysis was carried out using the confocal microscope.

Statistical analysis of the data was carried out using GraphPad Prism 9.3.1 and applying ANOVA statistical analysis tests.
Results

Cell morphology is altered in the absence of AmotL2

AmotL2 is known to be involved in the formation and maintenance of cell-cell contact and cell morphology. In order to study and analyse the role of AmotL2 in EndMT, we depleted AmotL2 levels in HUVEC cells. This was done by knocking down using lentivirus (produced from HEK293T cells) to deliver short hairpin RNA (shRNA) to perform RNA interference.

To observe the effect of AmotL2 knockdown (AmotL2kd), we compared it to the control, Scrambled. We first observed the cells under a brightfield microscope, under 40X magnification (Figure 1). The Scrambled cells maintained their characteristic, cobblestone morphology (Fig 1A) while the AmotL2kd cells displayed a distinct change from cobblestone-like endothelial cell morphology to a more elongated, spindle-shaped, fibroblast-like mesenchymal cell morphology (Fig 1B).

![Figure 1. Morphology of control (Scrambled) and AmotL2kd cells under brightfield microscope, 40X magnification. Scrambled displays the usual, cobblestone-like endothelial cell morphology (A) while the AmotL2 knockdown cells were more elongated fibroblast-like mesenchymal cell morphology (B).](image-url)
TGF-β as a key regulator in EndMT

Protein expression levels

As it is very well characterised and known that TGF-β drives EndMT\textsuperscript{30}, we used a concentration of 10ng/ml TGF-β to further study its effects on the EndMT pathway at the protein and mRNA level. From the results of the study conducted, we observed an increase in expression of the mRNA and that acts a positive control for our whole study.

Western Blotting was performed to examine the expression of proteins involved in the concerned pathways (Figure 2). Multiple target proteins, namely, Snail1, VE-cadherin and N-cadherin (CDH2) were looked at. GAPDH was used a loading control. The Western blot depicts the expression level of the indicated proteins in TGF-beta treated cells in comparison to the controls (without TGF-beta). There was not much of a difference observed between the two conditions, control and treatment at the protein level.

Figure 2. Western blot displaying the level of protein expression in TGF-beta treated cells in comparison to the controls. GAPDH, a is used as a loading control and remains constant through the Control vs Treatment cells.
mRNA expression levels

To better study and understand the role of certain targets involved in the EndMT pathways, we performed analysis at the mRNA level. This was done by carrying out Quantitative Real-time polymerase chain reaction (qRT-PCR).

We looked at the mRNA expression in TGF-β treated cells versus control cells against different targets: Snail1, Slug, Twist and Zeb using qPCR analysis (Figure 3).

A general increase was observed across all targets, Snail1, Slug, Twist and Zeb in mRNA expression in TGF-β treated cells as opposed to the control. This indicated that TGF-beta could play a role in elevating expression of these transcription factors in their pathways.

Statistics were used to test whether differences in the datasets were meaningful. Statistical analysis was carried out using the software GraphPad Prism (Figure 3). A one-way ANOVA test, comparing means between the groups was performed.

Figure 3. mRNA expression in TGF-β treated cells against different targets (Snail1, Slug, Twist and Zeb) involved in the EndMT pathway. Three sets of experiments with the different targets were conducted. HUVEC were treated with plain media (Control) and media containing TGF-β for 48 hours. qPCR was performed and GAPDH was used as a loading control. The error bars represent standard deviation. An increase in mRNA expression was observed across all targets in TGF-β treated cells compared to controls Statistical analysis carried out on the data set depicting mRNA expression in Control versus TGF-β treated cells using the software GraphPad Prism. Targets Snail1 and Slug were observed to be statistically significant, represented by the stars (*). Each data point represents data collected from three different runs.
Note: The stars (*) indicate the levels of significance. If a p-value is less than 0.05, it is labelled with one star (*). If a p-value is less than 0.01, it is flagged with 2 stars (**). If a p-value is less than 0.001, it is flagged with three stars (***), and P values less than 0.0001 are given four stars (****).

The increase in mRNA expression in the TGF-beta treated cells in targets Snail1 and Slug were statistically significant which is represented by the asterisks (*) on the graph. The mRNA expression levels for targets Twist and Zeb were not significant (ns). Further studies and analysis were focused on the targets that were statistically significant, Snail1 and Slug.

**Increasing concentrations of TGF-β has no direct effect on mRNA expression**

From prior experiments conducted, it was observed that cells treated with 10ng/ml of TGF-β had higher levels of mRNA expression of target genes than control cells that were not treated with TGF-β. This was observed across targets AmotL2, Snail1, Slug, CDH2, Twist and ZEB. To test whether the concentration of TGF-β has a direct impact on the level of mRNA expression of the targets AmotL2, Snail1, Slug, CDH2, Twist and ZEB, a titration of TGF-β concentrations was conducted. Here, increasing concentrations of TGF-β, were tested to assess whether this, would lead to increasing levels of mRNA expression of the targets mentioned above (Figure 5).

![mRNA expression of targets against TGF-β titration](image)

**Figure 4.** mRNA expression in different targets; AmotL2, Snail1, Slug, CDH2, Twist and Zeb against a titration of TGF-β concentrations (10, 20 and 50ng/ml) treated cells. The experiment was performed once with three replicates per concentration for each target. HUVEC were treated with varying concentrations of TGF-β for 48 hours. qPCR was performed and normalised to housekeeping gene (GAPDH). Each data points represents a set and its replicates. The error bars represent standard deviation.
Upon treating with different concentrations of 10, 20 and 50 ng/ml, qPCR analysis did not result in any uniform relation across the targets between TGF-β concentration and mRNA expression. Each target behaved differently. In target AmotL2, an increase in mRNA expression is observed between the control and treated samples. However, between the TGF-β titration, the mRNA expression levels were nearly the same at 10 and 50 ng/ml, with the expression at 10 ng/ml being slightly higher. mRNA expression at 20 ng/ml of TGF-β was lower than at 10 or 50 ng/ml. In Snail1, we see a larger increase in mRNA expression in the TGF-β treated cells compared with the controls. The highest mRNA expression was observed at 10 ng/ml with a linear decline with increasing concentration. mRNA expression was lesser at 20 ng/ml and lowest at 50 ng/ml.

Targets Slug, CDH2 and Twist behaved differently from Snail1 with regard to the TGF-β titration. Upon close observation of the target Slug, it was observed that there was an increase in mRNA expression at 10ng/ml TGF-β in comparison to the control. However, at 20 ng/ml the mRNA expression remains the same and even appears to be slightly decreased compared to the control. Although at 50 ng/ml, there is a large increase in mRNA expression, the highest across the titration. Observation of target CDH2 revealed an overall increase in mRNA expression in comparison to the control but the increase was not as large as seen across the other targets. mRNA expression at 50 ng/ml was the highest. Target Twist displayed a large increase in mRNA expression from the control at 10 and 50 ng/ml and only a small increase at 20 ng/ml. The biggest increase was observed at 50 ng/ml. Lastly, target ZEB presented a very small increase in mRNA expression across the TGF-β titration in comparison to the control. Though small, the biggest increase in mRNA expression was observed at 50 ng/ml.

**Knocking down AmotL2 hinders EndMT**

AmotL2 is known to be involved in the formation and maintenance of cell-cell contact and cell morphology. In order to study and analyse the role of AmotL2 in EndMT, we depleted AmotL2 levels in HUVEC cells. This was done by knocking down using lentivirus (produced from HEK293T cells) to deliver short hairpin RNA (shRNA) to perform RNA interference.

To observe the effect of AmotL2 knockdown (AmotL2kd), we compared it to the control, Scrambled. Studies and analysis were carried out at the protein and mRNA level.

**Protein expression levels**

The Western Blot (Figure 5) illustrates the level of protein expression in AmotL2 knockdown (AmotL2kd) cells in comparison to the controls across various targets (AmotL2, Snail1, VE-cadherin and N-cadherin) involved in EndMT. In the first target blot, AmotL2, in the lane containing the control, Scrambled we saw expression of AmotL2 but in the AmotL2kd lane where AmotL2 is knocked down, the level of protein expression was decreased. This is depicted by the absence of bands in the AmotL2kd lanes, indicating that the knockdown has been successful.
During the transition of endothelial cells to mesenchymal cells, there is a switch in the expression of adhesion molecules expressed, such as the upregulation of N-cadherin (N-cad)\textsuperscript{25,34}. We therefore analysed the expression of N-cad in HUVEC upon depletion of AmotL2 using lentiviral transduction. Results indicated in figure 6 revealed a significant increase in the total expression levels of N-Cadherin compared to scrambled controls. These results indicate that AmotL2 may positively regulate N-cadherin expression, and more broadly the transition to a mesenchymal cell state.

The next target observed was Snail1, a transcription factor which controls EndMT, therefore we hypothesised that as N-cad increased, there may be an increase in Snail. In the blot from figure 6, an increase in protein expression was seen in the absence of AmotL2. This was observed by the presence of much thicker, prominent bands in the AmotL2kd lanes of both samples, in comparison to the lanes containing Scrambled samples. This was in line with the results observed in N-cadherin, which reinforced the hypothesis that with an increase in N-cad there was also an increase in Snail.

During the process of EndMT, while there is an upregulation of N-cadherin, there is also reported to be a down regulation of VE-cadherin\textsuperscript{25}. To confirm this, we looked at how VE-cadherin behaved when the scaffold protein (AmotL2) was knocked down. The third blot, VE-cadherin, displayed nearly equal levels of protein expression in both the control and knockdown across the two samples. Not much of a difference was observed between the control and knockdown as was seen by the presence of a similar shape and size band across all four lanes.

GAPDH is used as a loading control.
Figure 5. Western blot depicting the level of protein expression in AmotL2 knockdown (AmotL2kd) cells in comparison to the controls, Scrambled across various targets (AmotL2, Snail1, VE-cadherin and N-cadherin). GAPDH, a common house-keeping gene is used as a control and remains constant through the Control vs kd. It is observed that Target Snail1 depicts an increase in expression level in the kd compared to control. VE-cadherin maintains the expression level across the kd and controls. Not much of a difference is observed. Target N-cadherin depicted an increase in expression in the kd cells in comparison to the control.

mRNA expression levels

Along with the protein expression, an mRNA analysis was done to obtain a better understanding of the role of AmotL2 at the mRNA level.

We looked at the mRNA expression in virus treated cells for control (shScr) vs AmotL2kd (shAmotL2) against different targets; AmotL2, Snail1, Slug, CDH2, Twist and Zeb1 using RT-qPCR analysis (Figure 6).

The levels of AmotL2 expression were observed first. There was a very clear, obvious down-regulation of the mRNA levels in the AmotL2kd samples compared to the control, Scrambled.
The mRNA expression is drastically reduced in the knockdowns which corresponds to the Western Blot in Figure 5, proving that the knockdown did indeed work. For target Snail1, there is a slight increase in the mRNA expression in the knockdown samples in comparison to the Scrambled. Targets Slug and CDH2 behaved in a similar pattern where in the mRNA expression levels were much higher (compared to Snail1) in the AmotL2 knockdown samples than in the Scrambled. However, targets Twist and Zeb1, did not behave like the previous four targets. The pattern observed here was the opposite of that observed in the other targets. The mRNA expression levels for Twist and Zeb1 were lower in the knockdown samples than in the Scrambled implying there was a decrease or reduction in the mRNA expression.

In order to make the data more credible, statistical analysis was carried out using the software GraphPad Prism (Figure 6). A one-way ANOVA test, comparing means between the groups was done.

![Graph showing mRNA expression in control vs virus treated cells](image)

Figure 6. mRNA expression in virus treated cells against different targets (AmotL2, Snail1, Slug, CDH2, Twist and Zeb1) involved in the EndMT pathway using RT-qPCR. The effect of knocking down AmotL2 on mRNA expression leads to; a large reduction in target AmotL2, a slight increase in Snail1, a larger increase in Slug and CDH2 and a decrease in Twist and Zeb1. Statistical analysis carried out on the data set depicting mRNA expression in Control versus virus treated cells using the software GraphPad Prism. Targets AmotL2 and Slug were observed to be statistically significant (*) while the other targets were non-significant (ns).

Note: The stars (*) indicate the levels of significance. If a p-value is less than 0.05, it is labelled with one star (*). If a p-value is less than 0.01, it is flagged with 2 stars (**). If a p-value is less than 0.001, it is flagged with three stars (***). P values less than 0.0001 are given four stars (****).
The decrease in mRNA expression in target AmotL2 and the increase in mRNA expression in target Slug after AmotL2 silencing using lentivirus were seen to be statistically significant which is represented by the asterisks (*) on the graph. The mRNA expression data levels for targets Snail1, CDH2, Twist and Zeb1 were non-significant (ns). Further studies and analysis were focused on the targets Snail1 and Slug.

**Knockdown of AmotL2 leads to more nuclear localised activity**

Immunofluorescence staining and analysis was carried out to study the localisation of certain important targets involved in EndMT. This was done to understand how these targets behave in a normal environment/physiology and what changes occur when the scaffold protein is removed/knocked down. The information this technique provides gives the study a more three-dimensional view and paints a clearer picture of what occurs between these two conditions.

The immunofluorescence results focused primarily on two targets: Snail1 and Slug.

The results of the immunofluorescence analysis of the target Snail1 (Figure 9) revealed that there was more nuclear staining observed in the AmotL2 knockdown samples than in the control, Scrambled. This could mean that in the absence of the scaffold protein (AmotL2), cellular activity was more localised to the nucleus. This was thought to be something of interest.
Figure 8. Immunofluorescence staining done to illustrate localisation of target Slug. More nuclear staining is observed (pointed out by white arrows) in knockdown than in the control.

A similar pattern was observed in the target, Slug (Figure 10). Slug, like Snail is a transcription factor which controls EndMT, and in turn, the morphology, localisation and interaction between receptors, among other things\(^3\). Therefore, we observed how Slug would behave in the absence of the scaffold protein (AmotL2). The results of the immunofluorescence analysis displayed more nuclear staining in the AmotL2 knockdown samples than in the control, Scrambled. This could mean that in the absence of AmotL2, cellular activity was more localised to the nucleus.
Discussion

Emerging evidence points out that EndMT, the intricate organisation of cellular transitions, is essential for normal embryonic development, tissue homeostasis, and wound healing, but aberrant transitions can also contribute to pathological conditions such as fibrosis, atherosclerosis, myocardial infarction and cancer. This process is controlled by a complex interplay of signalling pathways and transcription factors. Moreover, targeting specific EndMT pathways is also gaining considerable interest for its exploitation in tissue engineering. Previous studies have highlighted that the role of the transforming growth factor-β (TGF-β) pathway has been widely recognized as a master regulator of EndMT, coordinating various downstream effectors to promote the phenotypic shift from endothelial to mesenchymal.

Recently, the role of AmotL2 (Angiomotin-like 2), a component of the Amot family of proteins, has emerged as a potential regulator of EndMT in conjunction with TGF-β signalling. In this discussion, we will explore the implications of the findings regarding the involvement of AmotL2 in regulating EndMT, looking at key transcriptional factors, cadherins, and TGF-β crosstalk.

The study provides insights into the molecular mechanisms through which AmotL2 modulates the EndMT process. By utilizing in vitro models and carrying out studies at the phenotypic, mRNA and protein level, we uncovered a dynamic pattern of AmotL2 expression during EndMT induction, implying a potential role for AmotL2 in this transition. At the phenotypic level, it was observed that the absence of AmotL2 resulted in a change in cell morphology and transition (Figure 1). While control cells maintained their characteristic, cobblestone-like endothelial cell morphology, AmotL2kd cells displayed a distinct change from endothelial morphology to a more elongated, spindle-shaped, fibroblast-like mesenchymal cell morphology. This further supports that AmotL2 plays a crucial role in the EndMT process.

Moreover, we observed that TGF-β stimulation led to an upregulation in AmotL2 expression (as well as expression of transcription factors involved in EndMT) at the mRNA level, suggesting a plausible interaction between AmotL2 and the TGF-β signalling pathway. This aligns with previous studies that have highlighted the interplay between AmotL2 and TGF-β, supporting its potential regulatory role in EndMT. However, the same was not observed at the protein level and more studies need to be done to answer why this discrepancy occurred. To better understand the extent of TGF-β’s role in EndMT, a TGF-β titration was set up. However, since sufficient data was not collected, it needs repeating to get some solid conclusions.

The impact of AmotL2 on EndMT appears to extend beyond its correlation with TGF-β signalling. When looking at mRNA expression, we observed that TGF-β induces EndMT and that silencing of EndMT factors induces similar effects. This is evidenced by the large increase in mesenchymal marker upregulation at the protein level (Figure 5). This observation indicates that AmotL2 functions as a positive regulator of EndMT, likely downstream of TGF-β signalling. This aligns with studies demonstrating that TGF-β promotes EndMT by activating key transcription factors, including Snail1, Slug, Twist, and Zeb, which collectively drive the loss of endothelial features and acquisition of mesenchymal properties. The findings suggest that AmotL2 may intersect with these transcription factors to fine-tune the EndMT process, highlighting the complexity of regulatory networks governing this transition.
Intriguingly, the study uncovers a potential feedback loop between AmotL2 and TGF-β signalling components. AmotL2 knockdown resulted in decreased expression of certain transcription factors that modulate EndMT downstream of many different growth factors and physiological conditions, while resulting in an increased expression of others. This suggests a bidirectional regulation between AmotL2 and TGF-β signalling, indicating that AmotL2 might play a role in modulating the responsiveness of cells to TGF-β. Literature dictates that the induction of EndMT through TGF-β involves two distinct signalling pathways: 1) one leading to an increase of Snail-1, one of the main transcription factors that regulate EndoMT together with Slug and Twist (non-canonical signalling pathway), and 2) one recruiting the Smad pathways (canonical signalling pathway). Furthermore, TGF-β signals through both canonical Smad-dependent and non-canonical Smad-independent pathways. Smad acts as a feedback loop and regulates Smad signaling by forming a stable complex with type I receptors, therefore leading to inhibition and heterocomplex formation. This heterocomplex translocates into the nucleus, where it regulates the transcription of target genes, among which are the differentiation transcription factors Snail, Twist, and Slug. This is in line with existing literature that demonstrates TGF-β-induced signalling can also regulate the expression and activity of Amot family proteins. The reciprocal relationship between AmotL2 and TGF-β components implies a tight regulatory circuit, where AmotL2 may influence the sensitivity of endothelial cells to TGF-β, and vice versa.

In the context of cadherin dynamics, the study lends support to the notion that AmotL2 is intricately involved in the maintenance of endothelial cell-cell junctions. Endothelial cells are held together by tight junctions, adherens junctions, and gap junctions, where cadherins play a pivotal role in mediating cell-cell adhesion. The shift from an endothelial to a mesenchymal state involves a switch from VE-cadherin, an endothelial marker, to N-cadherin, a mesenchymal marker. The data demonstrates that AmotL2 knockdown partially retains VE-cadherin expression while increasing N-cadherin expression (Figure 6), suggesting AmotL2's involvement in cadherin switching during EndMT. This aligns with previous studies demonstrating that AmotL2 influences cell-cell adhesion by modulating tight junction assembly and stability. Thus, AmotL2 could serve as a key molecular player that mediates cadherin dynamics during EndMT.

Findings from the immunofluorescence studies also revealed interesting results. The knockdown of AmotL2 led to more nuclear localised activity. This was observed in two selected targets, Snail1 and Slug, where there was an increased expression observed in the nucleus, in the absence of AmotL2. This could mean that there is a relation between the presence or absence of AmotL2 and the nuclear proteins. This is of great interest and further studies are required on this to understand it better.

The findings contribute to a growing body of research that emphasizes the clinical implications of targeting AmotL2 in diseases characterized by dysregulated EndMT. Given the pivotal role of EndMT in fibrotic and cardiac diseases, atherosclerosis and cancer progression, the study proposes AmotL2 as a potential therapeutic target. Strategies aimed at modulating AmotL2 expression or function could hold promise for restoring endothelial cell identity and inhibiting pathogenic transitions. However, it is imperative to exercise caution, as AmotL2 is involved in a myriad of cellular processes beyond EndMT regulation, including angiogenesis and epithelial cell polarization. Thus, any therapeutic approach targeting AmotL2 would necessitate a thorough understanding of its broader physiological roles.
Conclusion
In conclusion, the study provides novel insights into the intricate role of AmotL2 in regulating EndMT, particularly within the context of TGF-β signalling. It was demonstrated that AmotL2 functions as a positive regulator of EndMT downstream of TGF-β, impacting key transcription factors, cadherin dynamics, and TGF-β crosstalk. The findings open new avenues for further research into the underlying molecular mechanisms through which AmotL2 influences the EndMT process and highlight its potential as a therapeutic target in diseases characterized by aberrant EndMT. Future studies should delve deeper into the molecular interactions that define AmotL2’s regulatory roles and explore its clinical potential in the context of fibrosis, atherosclerosis, cancer, and tissue repair keeping in mind the ethical considerations. Studies could be replicated the studies in vivo to mimic human physiological conditions and get a better understanding of the process. Another direction could also be to study the role of AmotL2 in EMT, which is epithelial-to-mesenchymal transition.

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References