2017

IGCR1 is a novel cell-surface molecule

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

IGCR1 IS A NOVEL CELL-SURFACE MOLECULE

by

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B.A., Boston University, 2015

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2017

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ACKNOWLEDGMENTS

Thank you to my fellow labmates Rachel Ho, Marwa Ali, Nels Engblom, Dr. Rawan Tahboub, Dr. Zou Xuequing and Dr. Esma Esroy for both their help and their friendship, and my PI, Dr. Nader Rahimi, for his mentorship. This work was supported by grants R21CA191970 and R21CA193958 to N.R.

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ABSTRACT

Tumor angiogenesis, the ability of tumor cells to stimulate blood vessel growth, is one the most critical steps of tumor progression. To support the growth of the expanding tumor, the "angiogenic switch" is turned on, which is often triggered by hypoxia (*i.e.*, low oxygen)-mediated events such as expression of vascular endothelial growth factor (VEGF), causing normally quiescent endothelial cells to proliferate and sprout.

An emerging picture of angiogenesis suggests that while governed by complex mechanisms, cell adhesion molecules (CAMs) plays a pivotal role in the regulation of angiogenesis. Our laboratory recently identified multiple previously unknown proteins including, transmembrane and immunoglobulin domain containing 1 (TMIGD1) and immunoglobulin-containing and proline-rich receptor 1 (IGPR1). Immunoglobulin-containing and cysteine-rich receptor 1 (IGCR1) represents the third remember of IGPR-1 family proteins. To investigate the expression and function of IGCR1, we have developed a rabbit polyclonal anti-IGCR1 antibody and demonstrated that IGCR1 is expressed in the endothelial cells of human blood vessels. To examine possible function of IGCR1, we have generated porcine aortic endothelial (PAE) cells over-expressing IGCR1. We demonstrate that IGCR1 expression in PAE cells inhibited cell proliferation and capillary tube formation as measured by colorimetric MTT and matrigel tube formation assays, respectively. In contrast, over-expression of IGCR1 in PAE cells inhibited cell migration as measured by wounding assay. Taken together, this study

identifies IGCR1 as a novel regulator of angiogenesis. Given, angiogenesis is a highly coordinated cellular processes controlled spatially and temporally by a myriad of cell surface receptors and ligands, IGCR1 by modulating the rate of endothelial cell proliferation and migration, plays a significant role in the formation of blood vessels.

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LIST OF ABBREVIATIONS

CAM ..... Cell adhesion molecule

HIF ..... Hypoxia induced factor

IGCR1 ..... Immunoglobulin-containing and cysteine-rich receptor 1

IGPR ..... Immunoglobulin-containing and proline-rich receptor (family)

PDGF ..... Platelet Derived Growth Factor

PFKFB3 ..... Phosphofructo-2-kinase/fructo-2,6-bisphosphate-3

TMIGD1 ..... Trans Membrane Immunoglobulin-domain 1

VEGF ..... Vascular Endothelial Growth Factor

VEGFR ..... Vascular Endothelial Growth Factor Receptor

INTRODUCTION

Angiogenesis is the biological process of blood vessel formation from existing blood vessels. Angiogenesis can occur in one of two manners -described as sprouting and nonsprouting- each of which produce distinct blood vessel morphologies. Sprouting angiogenesis is a more common approach and results in new vessels branching out from the existing vessel, while nonsprouting, also called intussuception, occurs less frequently and results in one vessel splitting itself into two, which allows for new capillary formation while maintaining the same number of endothelial cells (Conway et. al, 2001). Angiogenesis is vital in embryonic development and plays a critical in normal physiological processes such as wound healing, the menstrual cycle and hair growth. Moreover, aberrant angiogenesis is a key hallmark of many human diseases ranging from cancer to inflammation and age-related macular degeneration (Folkman, 2006).

Regardless of the type of vessel formation, during angiogenesis, endothelial cells start to proliferate, migrate and form capillary tubes in response to vascular endothelial growth factor (VEGF), processes which require an enormous level of coordination among signaling factors, adjacent cellular junctions, the basement membrane and the extracellular matrix (Chung et. al, 2010). Vasculogenesis, the process of *de novo* blood vessel formation, which occurs during embryonic development, has its own distinct process, but of interest, vasculogenic tissue remains circulating even after birth and is possibly recruited for vessel growth (Asahara et. al, 1999; Kalka et. al, 2000). While major progress is being made to understand the signaling pathways that govern

angiogenesis, much of the cellular and molecular interactions that play into this process still have yet to be fully understood.

Role of VEGF and VEGF receptors in angiogenesis

Vascular endothelial growth factors (VEGFs) and their corresponding receptor (VEGFR) family compose a large group of ligand-receptors, whose interactions have been demonstrated to be required for numerous essential vascular processes, which include hematopoietic/endothelial cell differentiation (Shalaby et. al, 1995), endothelial/vascular smooth muscle cell differentiation (Yamashita et. al, 2000) and vasculogenesis (Carmeliet et. al, 1999), although their contribution to each respective process is simply one of many other signaling factors. On the contrary, VEGF/VEGFR interactions, chiefly those between the respective isoforms VEGFA (otherwise known simply as VEGF) and VEGFR2, are the major drivers of angiogenesis (Ferrara et. al 2003). VEGF expression is often prompted by hypoxic conditions, in which hypoxia-induced factors (HIFs) cease to be targeted for ubiquitination; as intracellular HIF concentration increases, enough HIFs enter the nucleus and bind to the VEGF transcription factor hypoxia response element (HRE) to increase its expression (Rahimi, 2012; Kimura et. al 2000).

In sprouting angiogenesis, VEGF/VEGFR2 binding first rearranges adhesive connections between endothelial cells and then prompts endothelial cells to proliferate in order to branch off the existing vessel (Gerber et al, 1998; Stapor et al, 2014). The foremost cell, aptly referred to as a “tip” cell (Jakobsson et. al 2010), leads the

progression of branching "stalk" cells into the avascular region along a chemotactic VEGF gradient (Ferrara, 2010). Endothelial cells are constantly vying to be the new tip cell, as selection is determined largely by the endothelial cell that can sense VEGF the most accurately; stalk cell selection is also related to VEGF signaling, in conjunction with Notch signaling (Jakobsson et. al 2010). Tip and stalk cells have also been shown to display different morphologies that correlate with their angiogenic roles (Phng and Gerhardt, 2009). Tip cells, specialized for leading the migration of stalk cells down the VEGF gradient, possess numerous filopodia with which they sense the surrounding environment; on the other hand, stalk cells proliferate, form tighter intercellular junctions and deposit basement membrane proteins (Phng and Gerhardt, 2009).

Additionally, endothelial cells use glycolysis as the preferred energetic pathway and regulators of glycolysis, particularly phosphofructo-2-kinase/fructo-2,6-bisphosphate-3 (PFKFB3) direct vessel sprouting (De Bock et al. 2013). Other ligand-receptor signaling has been demonstrated as part of angiogenesis, such as the angiopoietins/Ties, Notch receptor and PDGF, however much of angiogenic signalling remains poorly understood (Conway et. al, 2001).

Pathological Angiogenesis and Anti-Angiogenic Therapies

The process of pathological angiogenesis is much the same as physiological angiogenesis in that hypoxia remains the major stimulus and VEGF/VEGFR interactions remain the major drivers (Stapor et. al, 2014). Pathological angiogenesis, while prominent in cancer, is also prevalent in diseases such as macular degeneration and

diabetic retinopathy (Stapor et. al, 2014). Unlike in normal angiogenesis, there is no cessation of the signalling cascade in pathologic angiogenesis (Chung et. al, 2010). This is in part caused by tumor-secreted factors that continue the pro-angiogenic signal (Chung et al, 2010) and by an unresolved hypoxic condition due to either inadequate vasculature or an accelerated growth that exceeds angiogenesis (Stapor et. al, 2015).

Vessels formed in pathological angiogenesis are not normal. Vessels often form irregular lumens, increase their permeability and show more variable junctional complex compositions (Carmeliet and Jain, 2000). In some pathological circumstances, tumor cells themselves acquire vasculogenic properties and mimic healthy endothelial cells, referred to as "vascular mimicry" (Maniotis et. al, 1999; Folberg et. al 2000). Although still preliminary, another common theory of tumor angiogenesis posits that vasculogenic tissue that remains in circulation may be another source of abnormal vessels when recruited by tumor-secreted angiogenic factors (Stapor et. al, 2014).

The initial approach to treating pathological angiogenesis was to block the vessels supplying the tumor. This approach quickly presented two issues; tumor endothelial cells have a different cell-surface composition than healthy endothelial cells, making it more difficult for drugs to bind to their respective receptors. Second, worsening the hypoxic condition increases the risk of metastasis (Carmeliet and Jain, 2000). Many treatments now use vessel normalization, a general approach of first treating the tumor-formed blood vessel, then targeting the tumor with chemotherapy or other anti-cancer treatments. Anti-VEGF therapies are also a major focus of treating tumors, as cutting off their ability to recruit more vessels seems to be an effective treatment (Meadows and Hurwitz, 2012).

Cellular Adhesion Molecules and Angiogenesis

Cell adhesion molecules (CAMs) are cell-surface receptor proteins that allow cells to adhere to each other and sense their surroundings (Cavallaro,and Christofori. 2004), which in turn influences many critical cellular activities such as embryogenesis, antigen-antibody interactions and tissue repair. The CAMs afford cells adhesive qualities critical for proper attachment to the basement membrane and adjacent cells, and are grouped into one of five families: cadherins, integrins, selectins, the CD44 family and immunoglobulin (Ig) superfamily proteins (Okegawa et. al, 2004). The Ig superfamily CAMs are involved in cell adhesion and cell migration (Cavallaro,and Christofori. 2004). The Ig domains of these adhesion molecules function by trans-dimerization through homophilic or heterophilic interactions and can establish a cell's adhesive qualities this way (Takai et al., 2008).

Many CAMs also hold tumor-suppressor roles mainly because contact with the surrounding environment provides a consistent signal that "instructs" endothelial cells to remain static, a phenomenon referred to as "adhesion-mediated contact inhibition" (Moh and Shen, 2009; Okegawa et al, 2002). Disruption or loss of this signal, a common occurrence in tumor angiogenesis (Nair et al. 2005; Paschos et al. 2009), increases the metastatic potential of these cells not only by allowing their detachment from the surrounding environment, but also by promoting cell growth (Moh and Shen, 2009).

Identification of IGPR1, TMIGD1 and IGCR1 as novel CAMs involved in the regulation of cell adhesion and angiogenesis

Recent studies in our laboratory identified multiple Ig-containing adhesion molecules, including immunoglobulin-containing proline-rich receptor 1 (IGPR1) and transmembrane and immunoglobulin domain-containing 1 (TMIGD1), that influence cell migration, endothelial barrier function and in the latter case, protection of cells from oxidative cell injury (Arafa, et. al., 2015; Rahimi et. al., 2012, Wang et. al 2016).

The third of these CAMs is called immunoglobulin-containing cysteine-rich receptor 1 (IGCR1) and is a 45 kDa transmembrane protein that shares much of its amino acid sequence with IGPR1 and TMIGD1. IGCR1 also influences cellular adhesion, cell spreading and migration.

The primary goals of this study were to determine the expression and function of IGCR1 in endothelial cells. Specifically, the objectives of this study were to (a) determine the expression of IGCR1 in blood vessels and endothelial cells in culture (b) determine the biological importance of IGCR1 in angiogenesis, and (c) examine the signaling mechanisms of IGCR1 in angiogenesis.

MATERIALS AND METHODS

Antibodies and Reagents

The antibody rabbit polyclonal anti-IGCR1 was developed that specifically recognizes the extracellular domain of IGCR1 in house. Rabbit polyclonal anti-PLCγ1 and mouse polyclonal anti-c-myc as well as horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (cat # sc-2004) and horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit polyclonal anti-phospho-p44/42 MAPK, rabbit polyclonal anti-p44/42 MAPK and rabbit polyclonal-phospho-Akt and rabbit polyclonal anti-Akt were purchased from Cell Signaling Technology (Beverly, MA).

Immunohistochemistry

Human tissue samples were provided by Boston University Department of Pathology (Boston, MA). After formalin fixation and paraffin embedding (FFPE), 5μm sections were cut and mounted on slides. Sections were de-paraffinized, hydrated, and rinsed in distilled water (dH2O), then subject to heat-induced V antigen retrieval with 1X citrate buffer [10 mM citric acid and 0.05% Tween-20, pH 6.0] for 20 minutes at 98ºC. Slides were then rinsed in dH2O and blocked with 200-500μL 1X blocking buffer [made from 10X Power Block Universal Blocking Reagent] (cat # HK085-5K; BioGenex, Fremont, CA) for 30 minutes at room temperature in a humidified chamber. After removal of this 1X blocking buffer, rabbit anti-IGCR1 antibody was diluted in 1X

blocking buffer and added to each section for 60 minutes. The primary antibody solution was removed from the section and washed for five minutes three times in Western Rinse buffer.

One-Step Polymer-HRP (cat # HK595-50K; BioGenex) was then added to the sections and incubated for 30 minutes in a humidified chamber. Next, Polymer-HRP was removed from the sections and washed three times in Western Rinse. 3% hydrogen peroxide solution was added to each sample for 15 minutes after the third wash and then washed three more times in Western Rinse. Western Rinse was removed from each sample and then 100L DAB solution consisting of 20µL DAB Chromogen (cat # HK124-05K; BioGenex) and 1 mL Stable DAB Buffer (cat # HK520-50K; BioGenex) was added to each tissue sample. After a three minute incubation, samples were immediately immersed in dH2O and counterstained in Mayer's hematoxylin for 10 seconds. After the sections were dehydrated and mounted, microscopy images were taken at 40X.

Cell Culture

Human embryonic kidney-293 (HEK-293) cells and porcine aortic endothelial (PAE) cells were grown in Dulbecco's modified essential medium (DMEM) that contained 10 % fetal bovine serum (FBS). GPG cells were grown in 293 GPG growth medium, 10% FBS/DMEM that also contained 1 µg/mL tetracycline, 2 µg/mL Puromycin, 0.3 mg/mL G418, 10 mM HEPES, and 6 mL L-glutamine. All cells were placed in a 5% CO2 humidified chamber and incubated at 37°C. HEK and PAE cells expressing empty vector (pQ) or IGCR1 were generated using a pQ vector as described (Rahimi et al.,

2000).

Western Blot Analysis

Cells were washed three times with H/S buffer [25mM Hepes [pH 7.4]/150mM NaCl] and then lysed in EB lysis buffer [10mM Tris-HCl/10% Glycerol/ 5 mM EDTA (pH 7.4)/ 50 mM NaCl/50 mM NaF/1% Triton X-100/ 1 mM phenylmethylsulfonyl fluoride/2 mM sodium orthovanadate/aprotinin (20 mg/ml)]. The cell lysates were collected and then mixed with 5X Sample Buffer [bromophenol blue (0.25%)/ dithiothreitol (DTT) (0.5M)/ glycerol (50%), sodium dodecyl sulfate (SDS)(10%)/Tris – Cl (0.25M, pH 6.8)] and heat shocked at 95°C for five minutes.

Cell lysates were then resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on to polyvinylidene difluoride (PVDF) membranes and immunoblotted with antibodies targeting the proteins of interest. For some experiments, membranes were stripped using stripping buffer [β-Mercaptoethanol /1M Tris-HCl pH 6.8/20%SDS (690µl/100 ml)] and then re-probed with another antibody.

Antibodies were diluted in 1X TBS solution of 10% bovine serum albumin. The dilution for both rabbit polyclonal anti-IGCR1 antibodies was 1:2000, 1:1000 for anti-PLCγ antibody, 1:500 for anti-c-myc antibody, 1:1000 for anti-p44/42 MAPK, anti-phospho-p44/42 MAPK, anti-phospho-Akt, and anti-Akt antibodies. Secondary antibodies were used at 1:10,000 for both anti-rabbit-IgG HRP-linked antibody and antimouse-IgG HRP-linked antibody.

Cell Spreading Assay

An equal number of HEK and IGCR1/HEK cells were seeded into a 6-well tissue culture plate containing collagen matrix and incubated for 20 minutes, then fixed using Diff-Quik ® Stain Set purchased from Dade Behring [Fixer: 1.8 mg/L Triarylmethane dye in methyl alcohol; Stain 1: 1g/L Xanthene dye, buffer and sodium azide (0.01%); Stain 2: 1.25 g/L Thiazine Dye mixture (0.625 g/L Azure A and 0.625 g/L Methylene Blue) and buffer]. Cells were imaged at 10x magnification. This experiment was repeated twice.

Disulfide Dimerization Assay

One "group" of HEK and IGCR1/HEK cells were lysed as described above. A second group was also lysed but β-Mercaptoethanol was absent from the 5x Sample Buffer. Samples were resolved by SDS-PAGE, with two lanes of β-Mercaptoethanol-free 2x Sample Buffer separating the samples of each group, and developed according to the Western blot protocol above. Probing for IGCR1 used rabbit polyclonal anti-IGCR1 antibody 606. This experiment was repeated twice.

Trypsinization Assay

5 mL of trypsin/EDTA tissue culture medium was added to one plate of EV/PAE cells and the plate was imaged under 10x magnification every minute for 6 minutes total, then repeated with one plate of IGCR1/PAE cells. This experiment was repeated twice.

Cell Proliferation (MTT) Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay kit (CellTiter 96 Non-Radioactive Cell Proliferation Assay) purchased from Promega (Madison, WI) was used to measure endothelial cell survival. PAE cells ($2x10^3$ cells/mL) expressing empty vector and IGCR1 were seeded into four 24-well tissue culture plates in 10% FBS/DMEM media. After 3 hours, the 10% FBS/DMEM media was replaced with 0% FBS/DMEM media.

At day 0 (24 hours from seeding) an MTT yellow tetrazole dye was added to 6 wells at a concentration of 10μL/400μL of media and incubated for two hours at 37°C. Stop solution (Promega) was added to solubilize the insoluble 12-purple formazan that was produced as a result of metabolism during incubation, and the cells were incubated for one hour at 37°C. After incubation, 200μL of solubilized solution from the wells was transferred to a 96-well plate with four wells per group and underwent spectrophotometric analysis at 570 nm. This spectroscopy test was repeated for the next 6 wells on days 2, 4 and 6 and repeated in full twice.

Cell Migration (Wounding) Assay

PAE cells ($2x10^3$) expressing empty vector and IGCR1 were plated in DMEM media and incubated for 24 hours until they reached confluency above 90%. The wounding assay was performed by using the tip of a 5mL tissue culture pipette to create multiple “wounds” or scratches across the cell monolayer. Between 3-5 hours, the cell culture plates were viewed under microscope. Images were captured and documented.

This experiment was repeated twice.

Endothelial Cell Capillary Tube Formation Assay

PAE cells overexpressing IGCR1 and PAE cells expressing empty vector were seeded on matrigel with endothelial cell growth medium (Clonetics, San Diego, CA). After 24 hours, capillary tube formation was viewed under microscope and photographed as described (Meyer et al., 2008). Quantification of capillary tube formation was established by using ImageJ (National Institutes of Health, Bethesda, MD).

RESULTS

Characterization of anti-IGCR1 antibodies

To study the expression and function of IGCR1, we developed two rabbit polyclonal antibodies against the extracellular domain of IGCR1. The two IGCR1 antibodies, clones 605 and 606, were compared for specificity in Western blot analysis (**Figure 1a**). Since expression of IGCR1 in human cell lines is not known, we also generated a HEK-293 cell line over-expressing IGCR1 (herein called IGCR-1/HEK-293). Accordingly, we used cell lysates derived from HEK-293 cells expressing an empty vector (EV/HEK-293) or IGCR1and assessed the specificity of IGCR1 antibodies. Clone 606 anti-IGCR1 antibody (1:2,000) detected a specific protein band with an approximate molecular weight of 45kDa, which also corresponds to the predicted molecular weight of IGCR1 (**Figure 2**). The anti-IGCR1 antibody (clone 606) was further tested in western blotting in various dilutions ranging from 1:2000, 1:8000, 1:10,000 and 1:20,000. Clone 606 specifically recognized IGCR1 up to 1:20,000 dilutions (**Figure 1b**).

After validating the specificity of clone 606 in western blot analysis, we decided to test whether clone 606 also reacts with IGCR1 in immunohistochemistry staining. The result showed that clone 606 specifically stained endothelial cells of blood vessels in PFA fixed human tissue. (**Figure 1c**). This observation promoted us to investigate expression of IGCR1 in endothelial cells. Our analysis showed that IGCR1 is endogenously expressed in human transformed umbilical vein endothelial cells (HUVEC-TERT) and porcine aortic endothelial (PAE) cells (**Figure 3**).

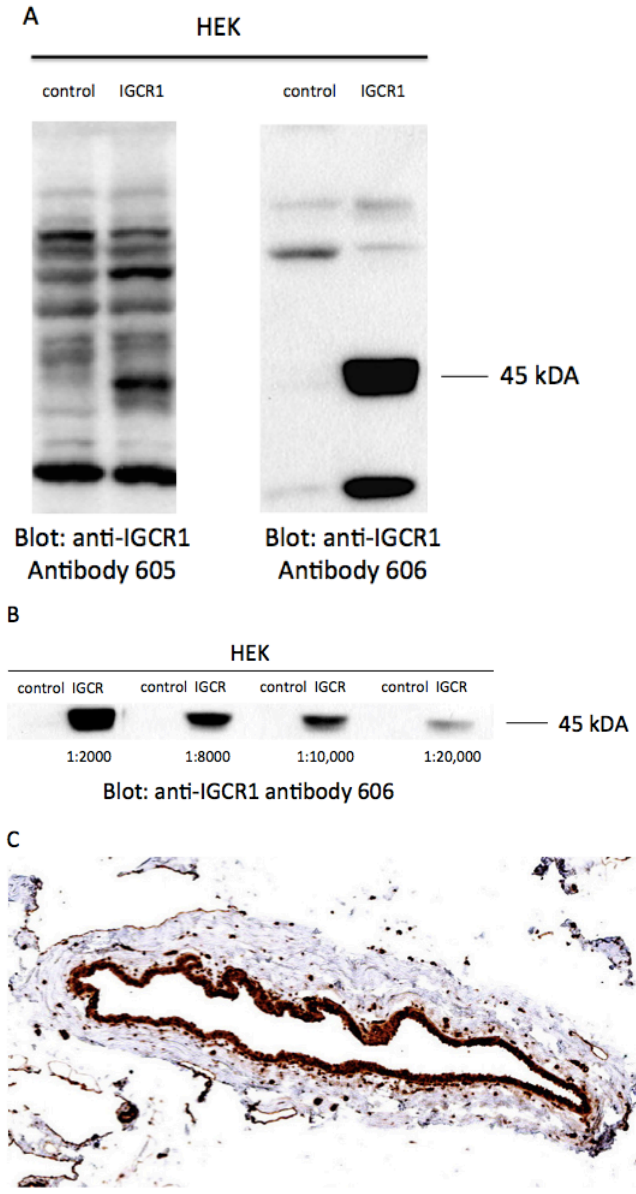

Figure 1. Antibody 606 is specific to IGCR1. (A) Antibodies 605 and 606 were compared for specificity. 605 has multiple nonspecific bands while 606 has fewer and is more specific. (B) Antibody 606 dilutions were compared and was found to be specific to IGCR1 up to 1:20,000 dilution. (C) Antibody 606 was used for immunohistochemistry staining of various tissues to identify areas of endogenously increased IGCR1. This large artery in the breast tissue was identified as one such area.

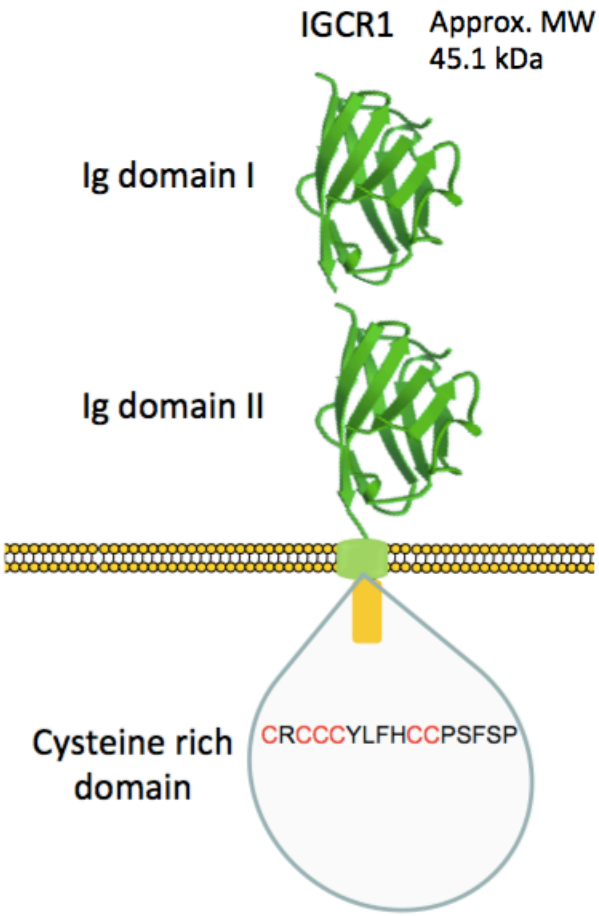

Figure 2. Ig-containing cysteine rich receptor 1 (IGCR1) amino acid sequence. The predicted molecular weight of IGCR1 is 45.1 kDa. IGCR1 contains two Ig domains, one transmembrane region and its intracellular region is rich in cysteine residues.

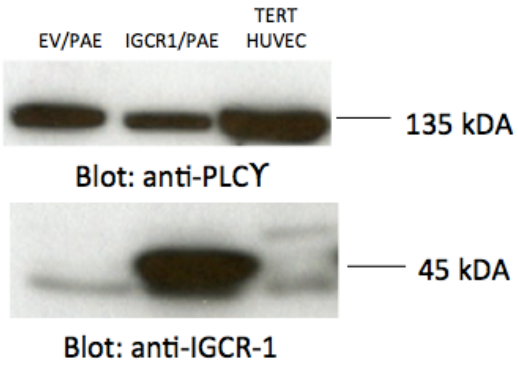

Figure 3. IGCR1 is expressed endogenously in endothelial cells. Protein loading control and expression of IGCR1 in transformed human umbilical vein endothelial cells, with transduced PAE cells as positive and negative controls.

IGCR1 regulates cell spreading and cell adhesion

To investigate the possible functions of IGCR1, we ectopically expressed IGCR1 in HEK-293 cells via a retroviral expression system (**Figure 4a**). Considering the role of other IGCR1-related cell adhesion molecules in cell spreading and adhesion (Rahimi, et al. 2012, Arafa, et al., 2015, Wang et al., 2016), we specifically examined whether expression of IGCR1 in HEK-293 cells could affect spreading of HEK-293 cells. Our initial observation showed that expression of IGCR1 in HEK-293 cells significantly altered the morphology of these cells (**Figure 4b, Figure 5**). Additionally, HEK-293 cells expressing IGCR1 were generally resistant to trypsinization, as assessed by their apparent change in their morphology and detachment from the cell culture plates (**Figure 4b**).

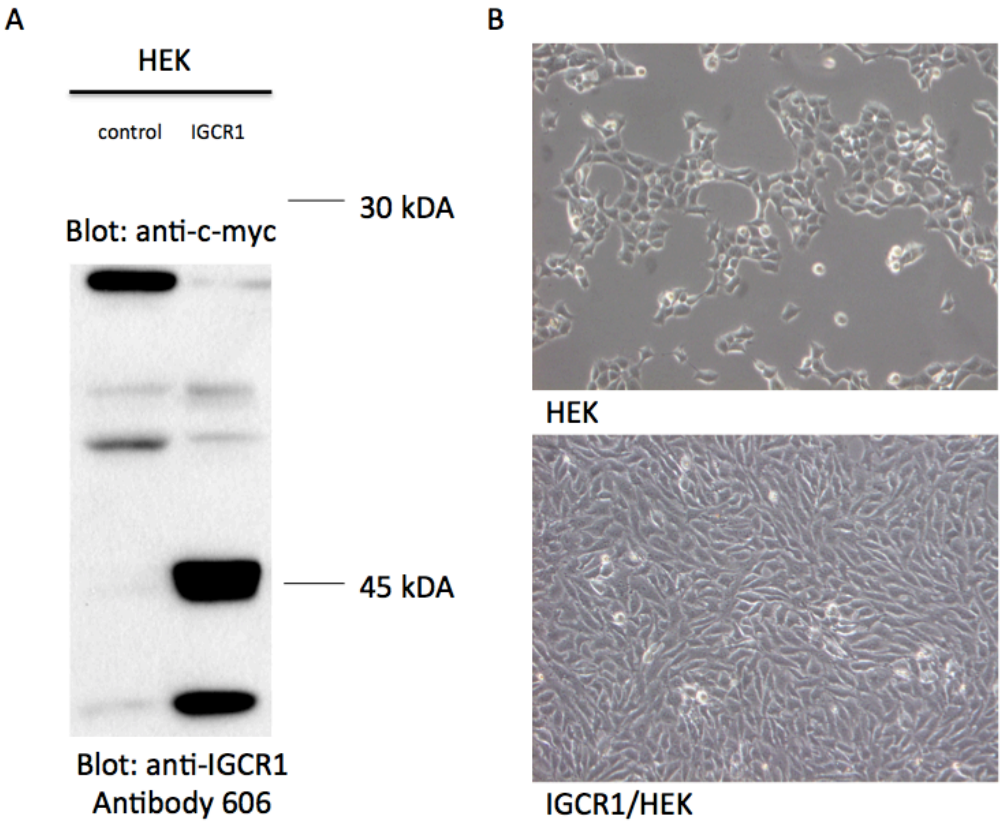

Figure 4. IGCR1 changes the morphology of HEK cells. (A) Western blot analysis of HEK and IGCR1/HEK. (B) Images of HEK and IGCR1/HEK cells under 10x magnification.

Expression of IGCR1 in HEK-293 cells regulates cell spreading

Having observed the specific effect of IGCR1 in HEK-293 cells' morphology and adhesion, we decided to specifically examine the effect of expression of IGCR1 in the spreading of HEK-293 cells. To this end, an equal number of HEK-293 cells and IGCR1/HEK293 cells were added to collagen-coated plates. After twenty minutes, cells were fixed, stained and imaged under 10x magnification (**Figure 5**). IGCR1/HEK-293 cells were found to have spread across the plate more than the HEK-293 control group. Morphologically, the IGCR1/HEK-293 cells also appeared to have longer and increased numbers of filipodia and lamellipodia, and their cell nuclei also appeared more euchromatic than the control (**Figure 5**).

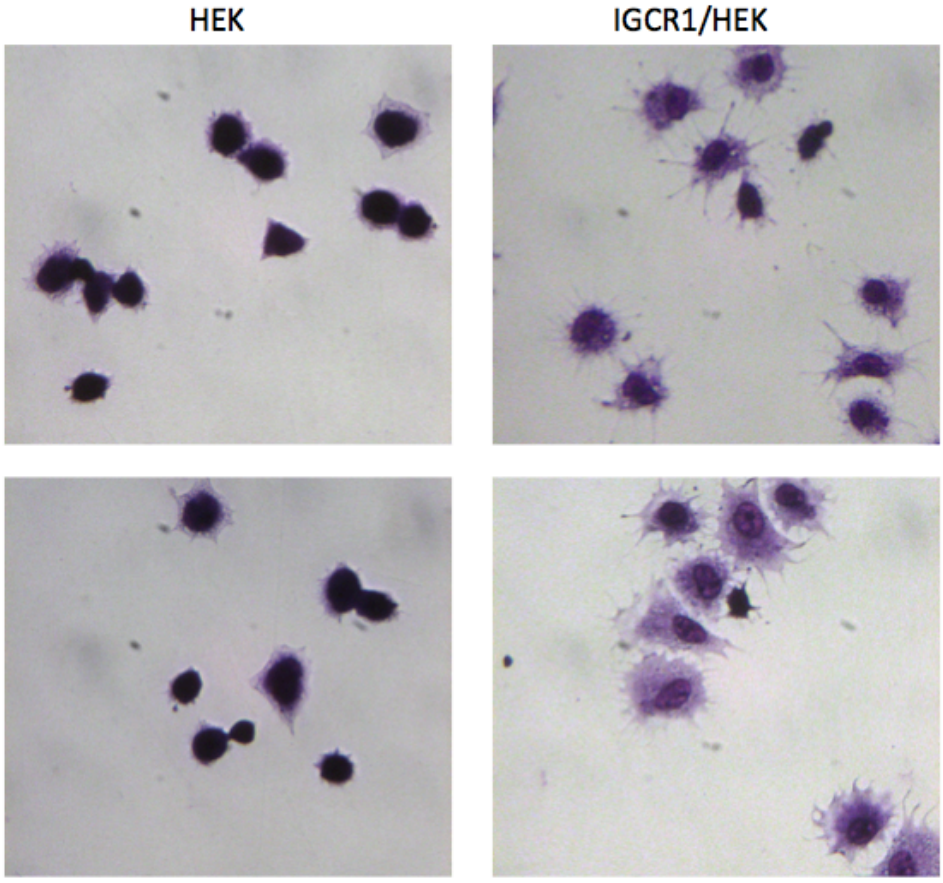

Figure 5. IGCR1 promotes cell spreading. An equal number of HEK cells and HEK cells expressing IGCR1 were added to collagen-coated plates and allowed to spread for 20 minutes. Both plates were then fixed, stained and imaged under 10x magnification.

Potential dimerization of IGCR1

Considering that usually immunoglobulin domain-containing cell adhesion molecules undergo disulfide bond formation, we decided to examine possible dimerization of ICGR1 expressed in HEK-293 cells (Barclay, 2003). To this end, HEK-293 control cells and IGCR1/HEK-293 cells were lysed and denatured in sample buffer with and without β-mercaptoethanol and resolved in SDS-PAGE, followed by western blot analysis (anti-IGCR1 antibody 606, 1:2000) (**Figure 6)**. If the Ig-containing domains did dimerize, the expected signal would move from about 45 kDa to about 90 kDa. However, the IGCR1 apparent molecular weight did not change in the β-mercaptoethanol, suggesting that IGCR1 does not undergo disulfide bond-mediated dimerization.

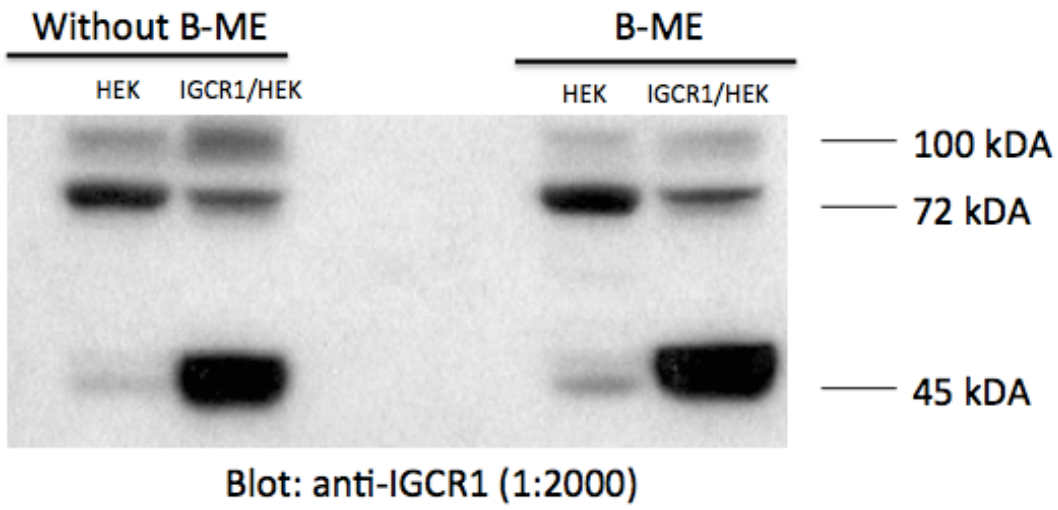

Figure 6. IGCR1 does not form disulfide bond mediated dimerization in HEK-293 cells. HEK-293 cells and HEK-293 cells expressing IGCR1 were lysed and denatured in sample buffer with and without β-mercaptoethanol and then probed for IGCR1.

Biological function of IGCR1 in PAE cells

To examine the effects of IGCR1 on the function of endothelial cells, we studied porcine aortic endothelial cells (PAE cells) ectopically expressing IGCR1. The expression of IGCR1 is shown (**Figure 8a**). Initially, we examined PAE cells expressing IGCR1 for its effect on cell adhesion. In particular, if the expression of IGCR1 in PAE cells was to increase the adhesive properties of these cells, it is expected that these cells would require a longer time to undergo detachment form the cell culture plates than their parental control cells. Our observation showed that detachment of PAE cells expressing IGCR1 in response to trypsin was much slower than the parental PAE cells (**Figure 7**)

The adhesive strength of cells is generally proportional to their trypsinization time in culture (Rahimi et al, 2012). PAE-EV cells require about 2-3 minutes of trypsinization in trypsin/EDTA medium. On the contrary, PAE cells overexpressing IGCR1 were qualitatively observed to need more time to trypsinize, about 3-4 minutes. Furthermore, even when trypsinized, PAE-IGCR1 cells adhered more strongly to the plate than the corresponding control group and only disengaged when repeatedly agitated with a pipette. To quantify this observation, a single plate of PAE cells were trypsinized, observed and imaged in 10x magnification until all had disengaged from neighboring cells and the plate, a period of about 6 minutes (**Figure 7a**). Images were taken every minute to track changes in morphology and adhesion. A plate of PAE-IGCR1 cells was subsequently trypsinized, observed and imaged in the same manner (**Figure 7b**).

Most cells in the PAE control group, as expected, had disengaged after about 2 minutes in the trypsin/EDTA medium and fully dislodged from the cell plate within 3

minutes. In contrast, PAE-IGCR1 cells were still predominantly attached to the plate after 2 minutes and showed little morphological change. At 4 minutes, the overexpressing cells did appear more rounded but were still associating closely with neighboring cells. After 5 minutes, eventually all cells were totally disengaged.

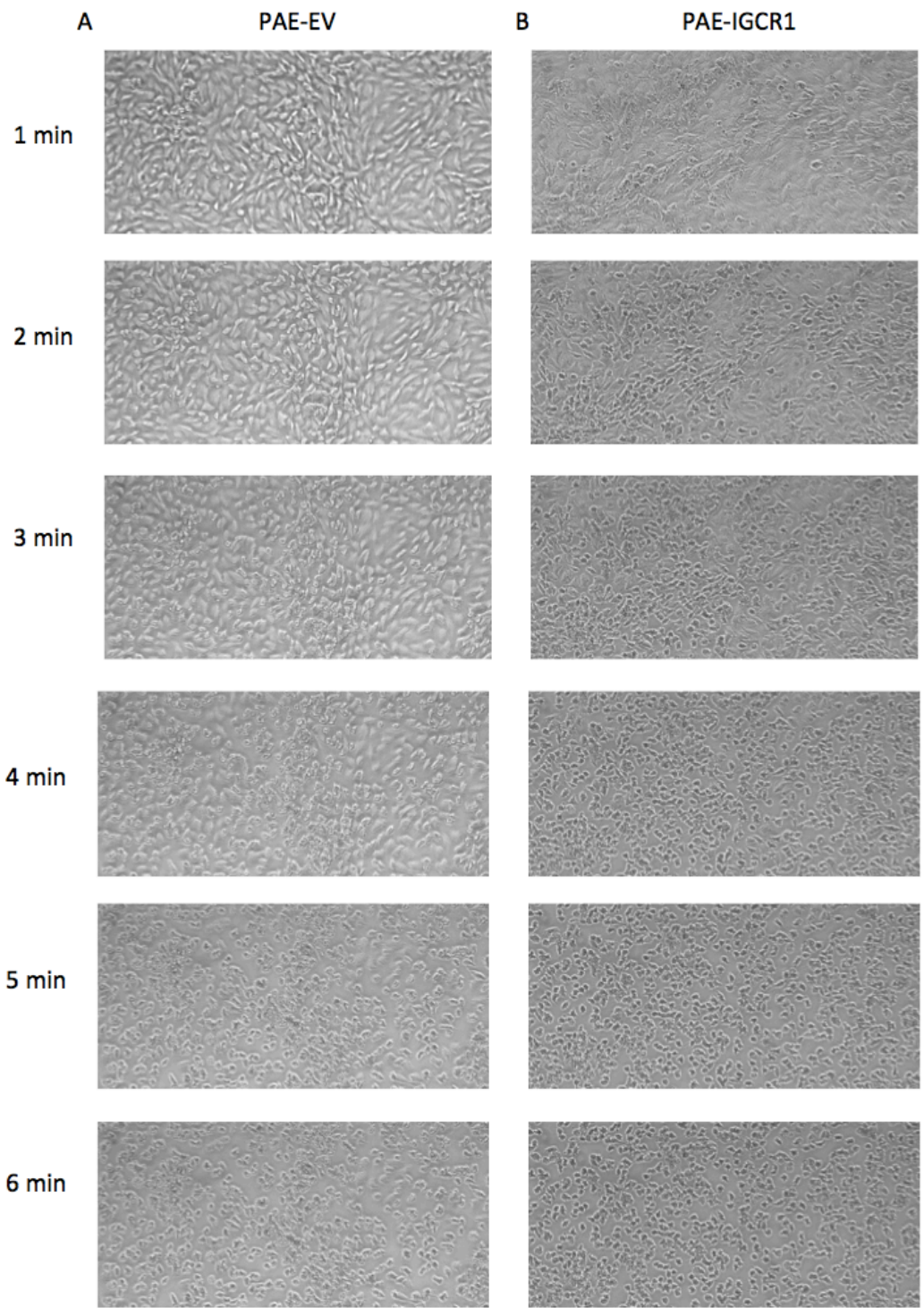

Figure 7. IGCR1 increases cell adhesion. Trypsin was added to (A) PAE-EV and (B) PAE-IGCR1 cell cultures, which were imaged every minute to track morphological changes.

IGCR1 regulates the angiogenic properties of PAE cells

Endothelial cells remain in a quiescent state for most of their lives, emerging from this state when they form new blood vessels (Stapor et al 2014). Therefore, it was postulated that as a CAM highly expressed by endothelial cells, IGCR1 could play a role in angiogenesis. Angiogenesis, while complex, consists of three main cellular processes: cell proliferation, cell migration and capillary tube formation (Stapor et al 2014). To study the effects of IGCR1 on angiogenesis, three assays for each of these processes were conducted on PAE cells transduced with empty vector and IGCR1. The results, stated in detail below and summarized in Table 1, likely represent IGCR1's total effect on angiogenesis.

Angiogenic process	Method used	Likely effect of IGCR1
Cell Proliferation and Cell Survival	MTT assay	Inhibition
Cell Migration	Wounding assay	Promotion
Capillary Tube Formation	Matrigel assay	Inhibition

Table 1. A summary of the angiogenic processes assayed and their findings.

IGCR1 inhibits proliferation of PAE cells

To determine IGCR1's effect on cell proliferation, PAE-EV and PAE-IGCR1 cells were subjected to an MTT assay. Cells were plated in Fetal Bovine Serum (FBS) conditions of 10% and 0% and were assayed every other day for six days (**Figure 8**). Proliferation was measured to be faster in the cells in the PAE-EV cells in both conditions. This observation may indicate that IGCR1 has a negative effect on cell proliferation. Also, the proliferation was more pronounced in the 10% FBS conditions for both cell lines, leading to the possibility that a ligand for IGCR1 is found in Fetal Bovine Serum.

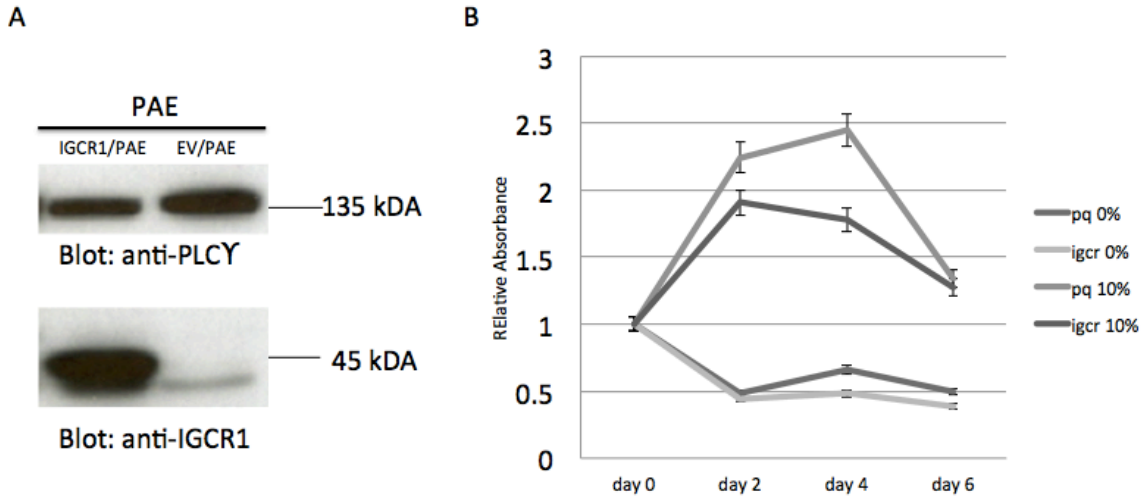

Figure 8. IGCR1 inhibits cell proliferation. (A) Expression of IGCR1 in PAE cells expressing empty vector vs. PAE cells overexpressing IGCR1 and protein loading control. (B) Cell proliferation as measured by MTT assay. Error bars indicate range of percent error.

IGCR-1 stimulates migration of PAE cells

To study IGCR1's effect on endothelial cell migration, PAE-EV and PAE-IGCR1 cells were subjected to a wound-healing assay. Cell plates were gently scratched to clear cells in a single line. Cells then migrated across this line and plates were monitored over a period of 4 hours for progression of the "wound healing" and imaged in 10x magnification between 3 to 4.5 hours (**Figure 9**). At 3.5 hours, PAE-IGCR1 cells had begun to migrate across the wound and at 4 hours, had almost totally "healed," as opposed to the PAE control group, which only started to migrate around 4.25 hours. IGCR1 thus appeared to increase the migratory properties of these cells.

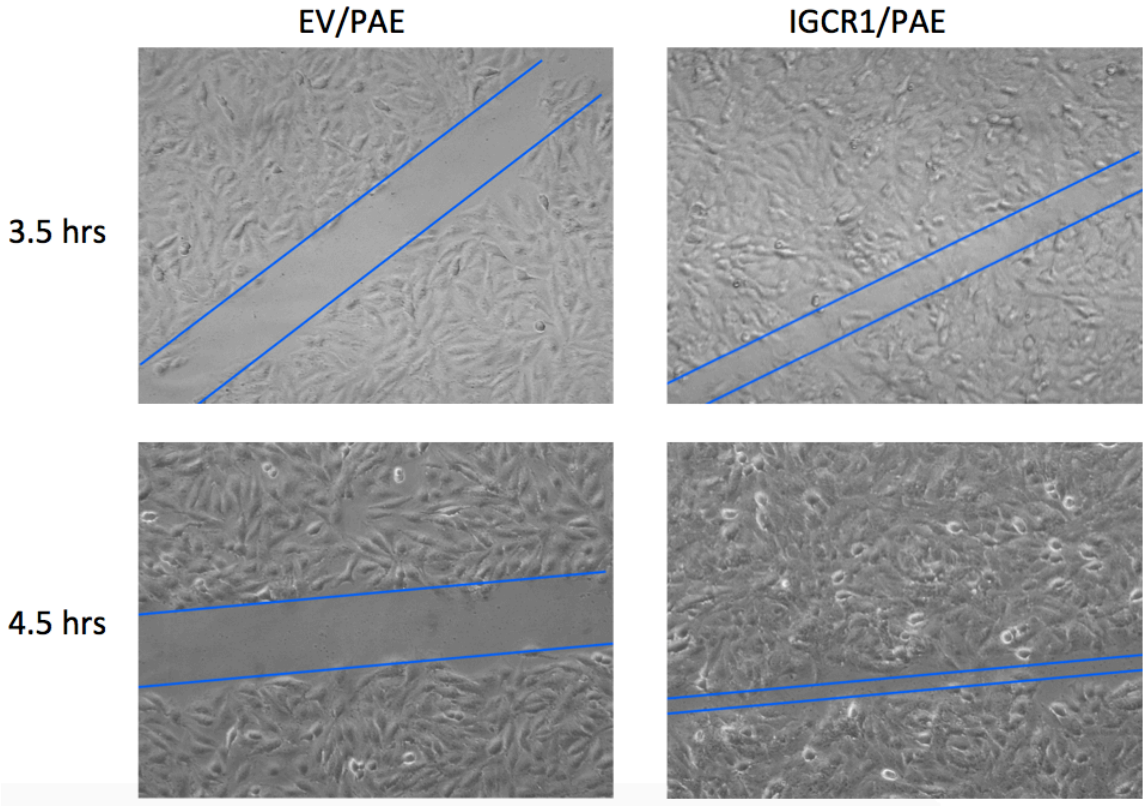

Figure 9. IGCR1 promotes cell migration. PAE cells expressing empty vector and IGCR1 were subjected to wounding assay and imaged over a period of 5 hours.

IGCR1 inhibits capliary tube formation of PAE cells

To study IGCR1's effect on endothelial cell migration, PAE-EV and PAE-IGCR1 cells were subjected to a capillary tube formation assay. An equal number of cells of each line were plated on a Matrigel matrix and incubated for 12 hours and imaged in 10x magnification. Tube formation in the PAE-IGCR1 group was almost nonexistent after 12 hours while the PAE-EV cells were able to form capillary tubes (**Figure 10**). The amount of capillary tubes formed was quantified using Image J, which demonstrated that endothelial cells overexpressing IGCR1 are barely able to form capillary tubes, and in the event that the tubes do form, they form more slowly and less frequently.

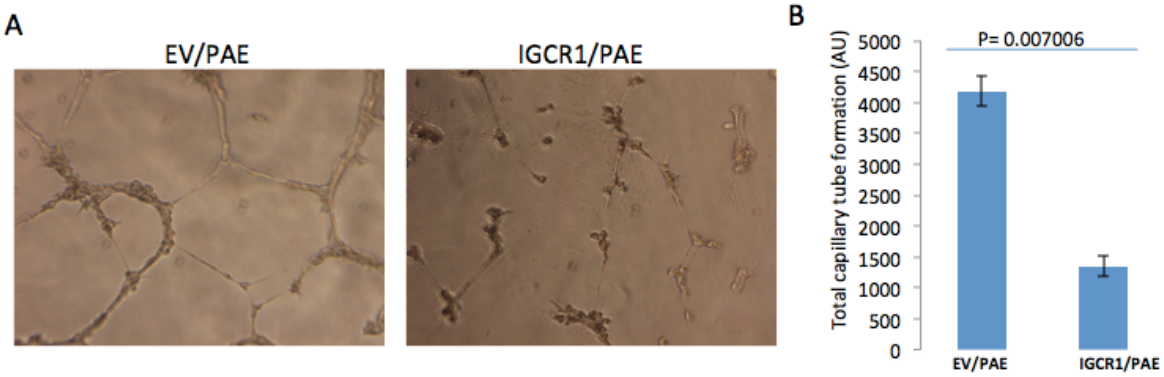

Figure 10 IGCR-1 inhibits capillary tube formation of PAE cells. (A) PAE cells expressing empty vector or IGCR1 were subjected to Matrigel assay and pictures were taken after 24 hours. (B) Quantitative analysis of the average length of the branches between PAE-EV and PAE-IGCR1.

IGCR1 regulates phosphorylation of PI3-kinase and MAPK pathways

After establishing that IGCR1 does seem to play a role in angiogenesis, a phosphorylation study was conducted using PAE cells expressing empty vector or IGCR1. For each group, cells were incubated for 6 hours in either 0% or 10% FBS, then lysed and run in a western blot. Probing for phosphorylation of major enzymes in the MAPK/ERK pathway and the PI3K-Akt pathway revealed that PAE-IGCR1 cells possessed more phosphorylated-ERK (p-ERK) than the empty vectors in both 0% and 10% FBS (**Figure 11a**). In addition, Akt was phosphorylated to a minor extent in both empty vector and IGCR1 cells growing in serum free conditions, PAE-EV cells growing in 10% FBS conditions particularly phosphorylated Akt, while there was a distinct lack of p-Akt in PAE-IGCR1 cells growing in 10% FBS conditions (**Figure 11b**).

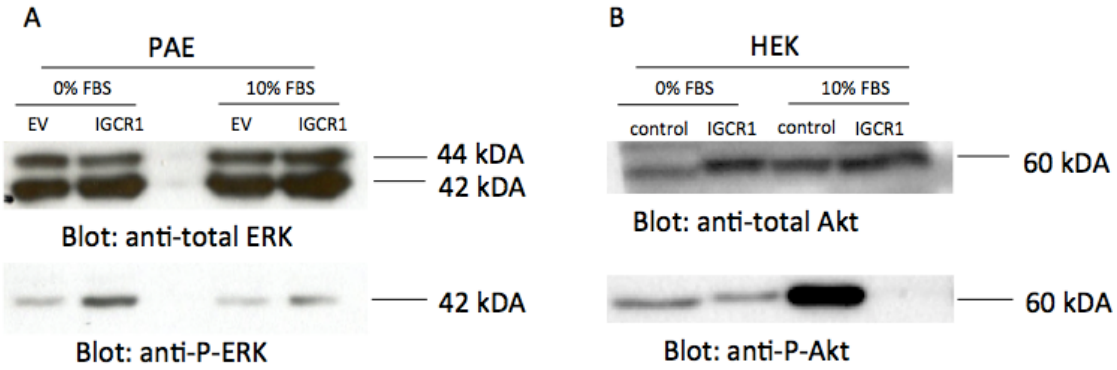

Figure 11. Akt and ERK phosphorylation in PAE-EV and PAE-IGCR1 cells. All antibodies were used in 1:1000 dilutions. (A) PAE cells expressing IGCR1

DISCUSSION

In this study, we demonstrate that IGCR1 is a novel protein that is expressed in the endothelial cells of human blood vessels and that its expression in endothelial cells modulates angiogenesis.

To characterize expression and possible function of IGCR1, we developed two rabbit polyclonal antibodies, clones 605 and 606, against the extracellular domain of IGCR1. Additionally, we created HEK-293 cells consistently expressing IGCR1 via a retroviral expression system.

Our characterization of anti-IGCR1 antibodies demonstrated that clone 606 anti-IGCR1 antibody specifically recognizes IGCR1 in western blot analysis.

Moreover, immunohistochemistry staining using clone 606 antibody revealed that the vasculature, particularly the tunica intima, strongly expressed IGCR1. Taken together with the qPCR data of human tissues, which showed high levels of IGCR1 mRNA in human arteries (Kobra Rezazadeh, unpublished data) and data presented in this present study IGCR1 is likely highly expressed by endothelial cells and may play a role in endothelial cell functions such as angiogenesis . Considering that endothelial cells are normally quiescent yet are highly activated in pathological circumstances, it is reasonable to posit that IGCR1 could play a role in endothelial cell function in normal physiological and pathological conditions associated with blood vessels (Chung et. al, 2010).

Before we examined the effect of IGCR1 in endothelial cells, we first investigated the general function of IGCR1 in human cells. To this end, IGCR1/HEK-293 and its parental control HEK-293 were used. Cell spreading is an inherent quality of all cells,

and the data presented in this study demonstrates that overexpression of IGCR1 in HEK cells increased their spreading. We noticed that after 20 minutes on collagen-coated plates HEK cells over-expressing IGCR1 not only spread more than the control cells, but also gained more filopodia and lamellipodia and associated with their neighboring cells more closely. Of note, the nuclei of these overexpressing cells appeared more euchromatic than the control, which is an indication of increased cell activity, consistent with the overall increase in motility.

Other members of the Ig superfamily cell adhesion molecules to which IGCR1 belongs are known to homophilically dimerize via disulfide bridges (Walter Wang, et al. 2016). Unexpectedly, we observed that IGCR1 does not form disulfide bond-mediated dimerization. Further pursuit of IGCR1 dimerization is a worthwhile endeavor and should be addressed in future research.

Next, we explored the role of IGCR1 in endothelial cell function. For this purpose, PAE cells were transduced using a retroviral method to express empty vector (EV) or IGCR1. Cell adhesion is an inherent quality of all cells, and is a critical characteristic for proper vascular function (Rahimi, et al. 2012, Arafa, et al., 2015, Wang et al., 2016). While IGCR1/HEK-293 cells were observed to adhere more strongly to each other and the cell culture plate, cell adhesion was specifically studied using PAE cells in order to determine IGCR1's possible effect specifically on endothelial cell adhesion. Cell adhesion is directly related to trypsinization time (Rahimi et al., 2012). When EV/PAE or IGCR1/PAE cells were trypsinized and imaged, the latter group adhered more strongly to the plate and did not begin to exhibit the token morphological

change desired during cell passaging until after 3 minutes had passed, while cells in the former had already retracted their connections before 3 minutes. Not as obvious from the static figures provided, the rate at which the two groups retracted their connections differed greatly. On the whole, the IGCR1/PAE cells were slower to disengage from their neighbors, demonstrating that IGCR1 appears to increase the adhesive properties of endothelial cells.

It is widely known that tumorigenic endothelial cells are able to delaminate and move through the vasculature in part due to not being as tightly adhered to each other or the basement membrane (Stapor et. al, 2014). As a corollary of this, many CAMs, by virtue of their function, hold tumor-suppressor roles. IGCR1's effect of increasing the adhesive properties of endothelial cells may suggest that it also assumes such a role.

To study the effects of IGCR1 on the overall process of angiogenesis, we separated aspects of angiogenesis into cell proliferation, migration and capillary tube formation and ran respective assays using IGCR1/PAE and EV/PAE cells. The results of a cell proliferation assay by MTT showed that IGCR1 not only has an inhibitory effect on cell proliferation in both starved and normal conditions, but also that the fetal bovine serum (FBS) used in cell culture media may contain a ligand for IGCR1. A wounding assay demonstrated that cell migration was also increased in IGCR1/PAE cells. The third aspect of angiogenesis tested was capillary tube formation and the findings of this study show IGCR1 has a significant inhibitory effect on this process.

Taken together with the findings of IGCR1's expression in the endothelium of mature vessels and that IGCR1 increases endothelial cell adhesion, IGCR1 most likely

functions in non-angiogenic vessels to maintain the endothelial cell barrier, and in angiogenesis as a mediator of cell migration.

To initially characterize IGCR1's intracellular signaling in endothelial cells, we sought to distinguish which key molecular pathways, Akt and ERK/MAPK, were activated by starved and normal conditions. Our data showed that ERK is highly phosphorylated in IGCR1/PAE cells, particularly in starved conditions, suggesting that perhaps IGCR1 exerts its effects on cell migration and cell spreading by altering ERK activation. Furthermore, our data showed that Akt phosphorylation, a key protein involved in cell proliferation and cell survival, is inhibited by IGCR1, suggesting that perhaps IGCR1 reduces the proliferation rate of endothelial cells by inhibiting AKT phosphorylation.

Further studies should further validate the anti-IGCR1 antibody using a blocking peptide, but also continue characterizing IGCR1's molecular signaling in response to different stimuli; the only stimulus tested here was the absence of FBS in the cell media. As results indicated differing responses in these two conditions, it is likely that FBS contains a ligand to IGCR1, and future research should identify this ligand or ligands. IGCR1's effects on cellular processes, particularly angiogenesis, would be further illuminated by knockdown studies. Lastly, and as mentioned above, further study into IGCR1's ability to increase cell adhesion has the potential to benefit many anti-angiogenic and anti-metastasis therapeutic approaches.

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