Effect of vascular endothelial growth factor A in PROX1 silenced colorectal cancer xenografts

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Tiivistelmä Referat – Abstract

In our research we studied if the overexpression of VEGF-A would save the impaired tumor growth in PROX1 silenced colorectal cancer xenografts.

We conducted both small hairpin PROX1 and VEGF-A gene transfers to SW1222 colorectal cancer cells using lentiviral vectors. SW1222 cell lines were then injected to Nod scid gamma mice and grown for 14 days for analysis.

We also established 3D cocultures of genetically modified SW1222 cell line together with lymphatic endothelial cells or blood endothelial cells to analyze lymphangiogenesis and angiogenesis *in vitro*.

We found out that overexpression of VEGF-A rescues the growth of PROX1 silenced tumors. Large necrotic areas in the central of the PROX1 silenced tumors remain even though VEGF-A overexpression induces greater vascularity in these xenografts.

In 3D cocultures silencing of PROX1 did not affect *in vitro* lymphangiogenesis or angiogenesis. (135)

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1 Introduction

The host laboratory has previously shown by histological and immunohistochemical methods that silencing of PROX1 in colorectal cancer (CRC) xenografts implanted ectopically into mouse increased apoptosis in the hypoxic tumor core. However, the tumor periphery continues to thrive. (49) The aim of our study was to see if vascular endothelial growth factors (VEGFs) could compensate for the loss of PROX1 in colorectal cancer cell progression *in vivo* and *in vitro*.

An outline of study objectives

The first objective was to transfer VEGF-C or VEGF-A gene to CRC cells (SW1222 cell line) to obtain their overexpression. Afterwards the CRC cells were introduced with lentiviral shPROX1 or SCR short hairpin RNA construct to establish PROX1-silencing in that cell line. After successful gene transfer, the cells were cultured in order to have enough cells (1.5 x 10 exp6) for mouse xenograft transplants.

The examined cell lines were injected into the skin of NOD-SCID immunodeficient mice for the *in vivo* studies. The tumors were surgically removed and their weights and volumes were measured. Histological sections were made and analyzed by immunohistochemical methods for evaluation of blood and lymphatic vasculature.

An additional objective was to establish and optimize cocultures of the examined cancer cell line and blood and lymphatic endothelial cells (BEC and LEC, respectively). This would allow a closer modeling of the paracrine interactions of these cells *in vivo*. At the start of the study it was not clear what kind of cocultures would work with our research. One issue to solve was to try different combinations and find out proper conditions for our 2D and 3D cocultures between SW1222 cell line and lymphatic endothelial cells.

With the cocultures we wanted to study interactions of control and PROX1-silenced cancer cells with endothelial cells (EC) *in vitro* in the presence of excess concentrations of VEGFs. More precisely the goal was to study how VEGFs contributed to the interactions between cancer cells and ECs, migration, mitogenesis, cytoskeleton morphology and formation of tubule like structures and if any effects of PROX1 could be detected *in vitro.*

3D culture permits *in vitro* studies that resemble more the actual microenvironment events *in vivo* than does the regular 2D culture. In three dimensional cell cultures the cells are able to interact with each other and with the extracellular matrix in three dimensions. In 2D cultures the interaction is limited to laterally with each other and the underlying plastic petri dish, which does not efficiently model cell interaction *in vivo*. (1) Angiogenesis and lymphangiogenesis are complex processes with growth factor interactions and endothelial cell migration. Therefore 3D culture was the method of choice for *in vitro* studies.

2 Review of the literature

2.1 Colorectal cancer

Colorectal cancer (CRC) is the second most common cause of cancer related death in developed countries. Incidence rate is also rapidly increasing in developing countries as standard of living grows. (2) Mutations in the intestinal crypt (stem) cells are the proximal drivers of colorectal adenocarcinomas (3).
In most colorectal cancers the initiating event is an inactivation mutation of APC (adenomatous polyposis coli) protein in the APC/ß-catenin/TCF pathway (4, 5) A truncating mutation in the tumor suppressor APC gene induces the development colorectal polyps, which are benign overgrowths of the epithelial cells in the gut. These adenomas have a significantly elevated risk of progressing into malignant adenocarcinoma. This is well illustrated in patients with inherited loss of the one of the APC-genes. (4, 6) A person who suffers

from this condition, called familiar adenomatous polyposis (FAP) develops a large number of polyps at a very young age. FAP-diagnosis means almost certain development of colorectal cancer at some point in person's life, and for this reason, the colon is usually removed from the patient. CRC is usually diagnosed when the patients are 35-40 years of age. (4, 7) It is estimated that 5-10% of colorectal cancers are not sporadic. Besides FAP, hereditary nonpolyposis colorectal cancer (HNPCC) known also as the Lynch syndrome is another common hereditary form of CRC. HNPCC patients have a mutation in a gene that codes for a component of the DNA mismatch repair system. A somatic mutation in the other functioning allele compromises the repair system. Malfunction in DNA mismatch repair system causes cell to develop mutations at an accelerated rate, which is the basis for the formation of HNPCC-associated cancers. (8) CRC generally progress in a designated manner where certain tumor suppressor genes and oncogenes mutate in an orderly fashion. APC is called the gatekeeper gene in colorectal cancers since an inhibitory mutation of APC, activating the WNT signaling pathway, is the initiating event of CRC. Activating mutations of oncogenic K-RAS gene encoding a GTPase involved in transmitting growth signals commonly accompany APC mutation later on. Subsequent mutations in non-hypermutated CRCs occur in the TGF-ß/SMAD signaling pathway, as well as in the p53, PIK3CA, FBXW7, TCF7L2 and NRAS genes. (9, 10)

2.2 The APC/ß-catenin/TCF pathway

The APC/ß-catenin/TCF pathway is the so-called canonical pathway in WNT-signaling. One important signaling molecule of this pathway is the glycoprotein WNT3a that binds to a coreceptor complex at the plasma membrane. This consists of a frizzled family-protein and a low-density lipoprotein receptor-related protein 5 or 6 (LRP-5/6). The canonical WNT pathway signaling cascade connects to a multimeric protein complex inside the cell, known as the destruction complex, which consists of glycogen synthase kinase (GSK3), casein kinase (CKI), axin and APC. In the absence of a Frizzled/LRP-5/6-receptor binding ligand, this destruction complex binds to ß-catenin, which allows GSK3 to phosphorylate ß-catenin. (11, 12) The phosphorylation of ß-

catenin induces its ubiquitin-mediated proteolysis, whereby it is degraded. WNT binding to frz/LRP-5/6-receptor activates frizzled, which recruits the disheveled (Dvl) family protein in the cytoplasm. Dvl is involved in the disassembly of the destruction complex, thus rescuing beta-catenin from degradation and allowing its accumulation in the cells. (12, 13) the thus accumulated ß-catenin is transported to the nucleus, where it complexes with transcription factors of the TCF/LEF-family. As a result, a transcription of multiple WNT regulated genes is activated. (11-13) In colorectal cancer, activation of the APC/ß-catenin/TCF pathway regulates cell differentiation, proliferation, apoptosis, migration and colorectal progenitor cell populations through the c-myc transcription factor (14, 15).

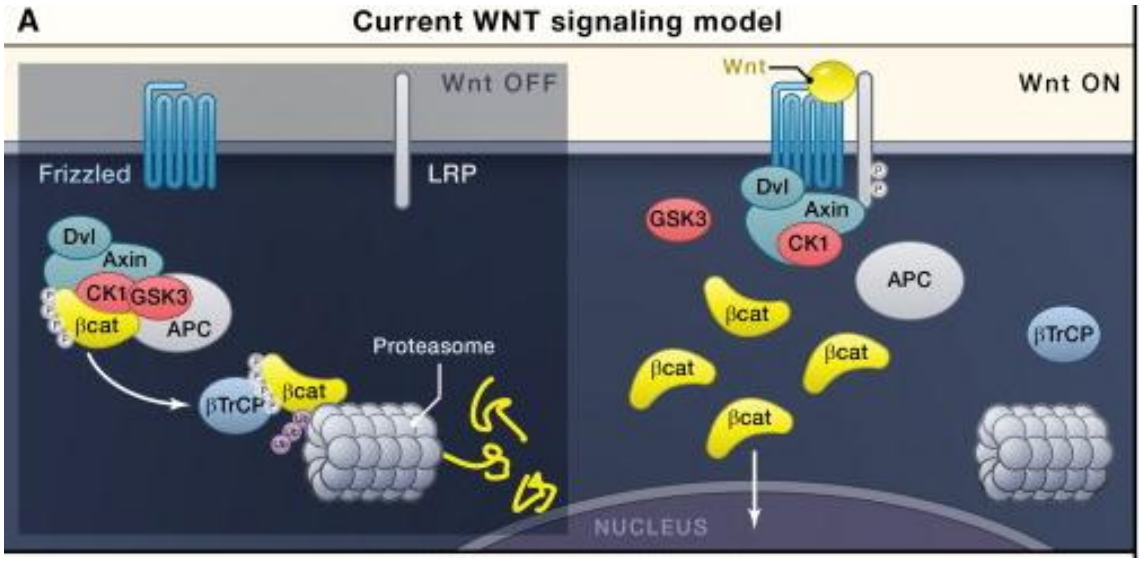

Figure 1. Activation and inactivation of the canonical WNT pathway(16).

2.3 PROX1

2.3.1 Discovery

PROX1, whose homologue was first discovered in Drosophila as the Prospero gene, is an highly conserved homeobox transcription factor that regulates cell differentiation of many different organs in the embryo such as in the central nervous system (CNS), eye lens cells, pancreas, liver and heart. Prox1 deletion

in mouse embryos leads to developmental defects and death of the embryo before birth. (17)

2.3.2 PROX1 in embryogenesis

In the CNS PROX1 expression is activated through APC/ß-catenin/TCF pathway. Prox1 is necessary for the differentiation of neural stem cells in the adult hippocampus and initiation of neurogenesis (18). Expression of Prox1 diminishes the neuronal progenitor cell pool by inducing cell differentiation. Sox1 regulates the extent of this cell pool by negatively regulating the rate of Prox1 expression. (19) Prox1 can also inhibit Notch1 signaling, which leads to decreased neurogenesis (17). In the eye of mouse embryos Prox1 is responsible for lens fibers differentiation and elongation (20). Prox1 also regulates differentiation of progenitor cells in the retina (17). Also the liver and pancreas express Prox1 during embryonic development. Prox1 is responsible for hepatocyte migration to hepatic lobes in the development of the liver. Prox1 knockout mice have prominently smaller sized liver and pancreas. (17)

2.3.3 PROX1 in the development of lymphatic tissue

PROX1 is responsible for lymphatic system development as it is essential for the differentiation of blood vascular endothelial cells to lymphatic endothelial cells (21-23). Mouse embryos where Prox1 has been deleted do not have lymph sacs or lymphatic vessels (24, 25). The differentiation of the lymphatic system begins when Sox18 induces the expression of Prox1 in the embryonic cardinal veins in mice (26). Prox1 positive cells begin to show expression of VEGFR-3, LYVE-1 and other cell surface proteins specific for lymphatic endothelial cell identity. The VEGFR-3 is essential for proper lymphangiogenesis during embryonic development (27, 28). VEGF-C is secreted by nearby cells, binds to VEGFR-3 and establishes lymphatic endothelial cell sprouting from embryonic veins, promoting also lymphatic endothelial cell survival and proliferation (25). Prox1 is also required for maintaining the identity of differentiated lymphatic endothelial cells (23).

2.3.4 Role of PROX1 in cancer

PROX1 is a tumor suppressor gene in many human cancers, such as certain brain tumors, hepatocellular carcinomas and cancer of the biliary duct. PROX1 levels are suppressed in these cancer types (17). Conversely in some cancers the role of PROX1 is quite the opposite and PROX1 actually shows oncogenic properties. For example, in colorectal cancer PROX1 levels increase considerably when compared to the normal intestine, where PROX1 expression is restricted to specific enteroendocrine cells and their progenitors. Indeed, PROX1 seems to promote the progression of intestinal adenomas to malignant CRC. PROX1 expression seems to modulate cytoskeletal organization and to decrease the adhesion of the cells to pericellular structures. This may contribute to tumor progression induced by PROX1. In fact, PROX1 is a target gene of the TCF/ß-catenin-pathway, which is overactivated in most colorectal cancers. (29) The significance of PROX1 in CRC has been proposed to be associated with the tumor's ability to metastasize. Here one should note that PROX1 also plays a role in the lymphatic system, which provides one major route of metastatic spread. (17)

2.4 The VEGF Family

There are several families of growth factors and their receptor protein tyrosine kinases that regulate angiogenesis. These include angiopoietin/Tie, ephrin/Eph, fibroblast growth factor/FGF receptor families as well as the most intensively studied angiogenic ligand/receptor family, the VEGFs and VEGFRs. The members of the VEGF family are secreted glycoproteins and the family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PIGF) (30, 31). There are also VEGF homologues known as VEGF-E (produces by Orf-viruses) and VEGF-F (found in snake venom) (31).

2.5 VEGF-A

2.5.1 Effects of VEGF-A on endothelial cells

VEGF-A is the major growth factor involved in vasculogenesis in embryos and angiogenesis in both embryos and in adults. Paracrine VEGF-A activates angiogenesis by promoting the proliferation, migration, specialization, and survival of endothelial cells. VEGF-A also has a role creating fenestrae in the endothelium and it promotes endothelial permeability to plasma proteins *in vivo*. (32) VEGF-A mediates these effects on the endothelial cells mainly through VEGF receptor 2 (VEGFR-2). (31-33) VEGF-A also binds to VEGFR-1, which does not directly activate angiogenesis in most settings. (31, 34, 35) Besides ECs, VEGFR-1 is also expressed in some other cell types, such as macrophages, where its activation is needed cell migration in chemotaxis towards VEGF-A(31). Hypoxia is the strongest inducer of VEGF-A expression. In hypoxic conditions hypoxia-inducible transcription factor 1 binds to the VEGF-A gene and the levels of this otherwise fast-degraded protein are stabilized. VEGF-A expression is also induced by TGF-ß, FGF-2, Il-1ß and Il-6.(34, 36)

2.5.2 Activation of VEGF receptor

VEGF receptors have tyrosine kinase activity. When VEGF-A binds to VEGFR-2 on the receptor at the plasma membrane, the receptor undergoes dimerization, which induces the trans- or autophosphorylation of tyrosine residues in the cytoplasmic part of the receptor. Phosphorylation of tyrosine residues generates a docking site for mediator phospholipase C gamma (PLC γ) and certain other proteins. PLCγ hydrolyzes PIP2 (phosphatidylinositol), which releases DAG (diacylglycerol) and IP3 (inositol 1,4,5-triphosphate), these act as second messengers (37).

Rise in intracellular IP3 concentration results in the rise of intracellular calcium. DAG in turn in corporation with the risen intracellular calcium possibly activates

RAS/RAF/ERK/MAPK pathway through PKC (protein kinase C). This Map kinase pathway ends in the expression of genes inducing the proliferation of endothelial cells. Survival of the endothelial cells is in part promoted through the PKB/AKT pathway activated by VEGFR-2. (37) In addition to VEGFR-1 and VEGFR-2, VEGF-A also binds to other class of receptors known as neuropilins (NRP). NRP-1 improves VEGF-A binding to VEGFR-2 and it is thought that NRP-1 is a coreceptor for VEGF-A in angiogenesis. (32, 35)

2.5.3 VEGF-A in embryogenesis

VEGF-A has a major role in embryonic vasculogenesis. Embryos lacking one VEGF-A allele die due to dysfunctional formation of blood vessels. (31, 36) VEGF-A is also important postnatally as it is required for the body growth, organ development and endochondral bone formation.(36)

2.5.4 Physiological effects of VEGF-A

In adults, low levels of VEGF-A are produced in almost every tissue of the human body(34). It is commonly known that VEGF-A has an angiogenic role in wound healing(30, 32, 34-36). VEGF-A also facilitates the bone repair process. In addition, VEGF-A has other important roles in blood vessel regulation. Increased VEGF-A is expressed in skeletal muscles after exercise and vascular growth is to some extent associated with physiological muscle hypertrophy. In females, VEGF-A mediates angiogenesis in the female reproductive system such as in the breast, uterus and ovaries. (34) Newly formed vessels in adults also are dependent of stimulation by VEGF-A and without sufficient blood flow or VEGF they will degenerate(34, 35).

2.5.5 VEGF-A in disease

Dysregulation of VEGF-A expression is a contributing factor in many pathological conditions in adults. These include atherosclerosis, diabetic retinopathy, psoriasis, and age-related macular degeneration. Overactivation of angiogenesis is an important hallmark of cancer. (34) VEGF-A plays a strong role in tumor growth. Depletion of VEGF-A from mouse xenografts reduces tumor growth and vice versa, overexpression of VEGF-A accelerates tumor growth. (36) Recent studies suggest that VEGF-A has a distinct role in neuronal development. VEGF-A induces neuronal migration, survival, axon guidance and neural stem cell differentiation to neurons or neurogenesis.(38)

2.5.6 VEGF-A isoforms

There exists four different isoforms of the VEGF-A: VEFG-A121, VEGF-A145, VEGF-A165 and VEGF-A189, which are produced by alternative pre-mRNA splicing. The isoforms differ by their ability to bind to heparin sulfate proteoglycans. Due to differences in their binding affinities, they are thought to localize heterogeneously around the secreting cells. The heparin-binding domains regulate differences in the affinities to VEGF cell surface receptors. Functionally different isoforms of VEGF-A regulate the vascular network pattern, including vessel size, quality and number of branches.(32)

2.6 VEGF-C

VEGF-C is made as a precursor protein, which undergoes proteolytic processing to gain receptor-activating properties. Its pro-form binds to VEGFR-3(39); this is then processed by a metalloprotease-CCBE1 protein complex to activate the signal transduction(40). The proteolytically processed short form of VEGF-C, which is proteolytically from the pro-VEGF-C, shows affinity also to VEGFR-2 (28) and to a soluble for of VEGFR-2 (sVEGFR-2) (37). sVEGFR-2 can compete with VEGFR-2 for VEGF-C binding, which inhibits the proliferation of lymphatic endothelial cells(37). VEGF-C is shown to interact with NRP-2 in addition to VEGFR-3. NRP-2 and VEGFR-3 might have coreceptor properties in

similar way as VEGF-A and NRP-1. (17) VEGFR-3 is highly expressed in lymphatic endothelium and VEGF-C induces lymphatic vessel growth through this receptor. (28, 36, 39) VEGFR-3 activation is needed for the survival and migration of the lymphatic endothelial cells destined for lymphatic vessel formation. Naturally, activation of VEGFR-3 also promotes lymphangiogenesis. VEGF-C is essential for the development of lymphatic vessels as mice with both deleted alleles of VEGF-C gene die before birth from developmental faults of the lymphatic system. (28)

2.7 VEGF-D

VEGF-D is also a ligand of VEGFR-2 and VEGFR-3. VEGF-D can promote blood vascular endothelial cell mitogenesis and thus angiogenesis. VEGF-D also promotes migration and survival of lymphatic endothelial cells through VEGFR-3-induced lymphangiogenesis. (36) VEGF-D is not as important as VEGF-C in embryonic development as its deletion causes only minor lymphatic defects in embryos(25, 41).

2.8 VEGF-B

Heart, skeletal muscle and brown fat and vascular smooth muscle show expression of VEGF-B. VEGF-B binds only to VEGFR-1 and NRP-1 and it is poorly angiogenic. (31, 42) Overexpression of VEGF-B in the heart leads to cardiomyocyte hypertrophy and changes in mitochondrial and lipid metabolism. This indicates that VEGF-B has as yet poorly studied, but interesting and unusual functions for a member of the VEGF family.(42)

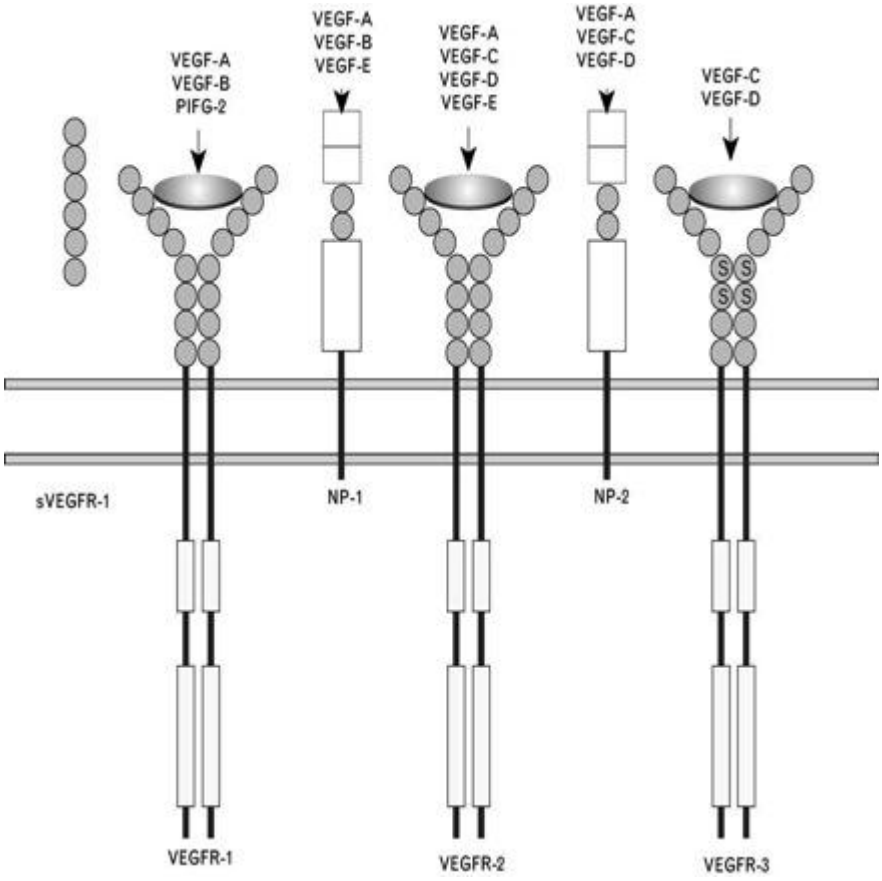

Figure 2. VEGF growth factors and their binding to VEGF receptors (43).

2.9 RNA interference

RNA interference is a mechanism in which short strands of RNA known as microRNAs (miRNA) can regulate mRNA stability and translation. MiRNAs have a nucleotide sequence that is complementary to their target mRNAs.

Transfection of short hairpin RNAs (shRNAs) is a mechanism for long lasting silencing of target genes. A vector (e.g. lentivirus) encoding the desired shRNA structure is introduced into target cells. The lentiviral vector integrates the shRNA gene into the genome of the target cell. The transcribed double-stranded shRNA is transported out of the nucleus by exportin and processed by the dicer enzyme in the cytoplasm. The processed shRNA binds to the so-called RNA-induced silencing complex (RISC). In the RISC, a family of argonaute proteins (Ago) catalyzes the recognition of the target mRNA complementary to the sequence of the shRNA. The RISC with its attached

shRNA binds to the target mRNA and two alternative events may occur. Either a type of Ago protein acts as an endonuclease protein and cleaves the target mRNA or RISC-shRNA complex suppresses the translation of the mRNA. Both events “silence” the translation of the target gene’s protein.(44)

3 Materials and methods

3.1 Cells

In this research project, we mainly used a previously made stable clone of the SW1222 CRC cells that expresses high amounts of PROX1. The SW1222 cell line is commonly used in laboratories and it is originally derived from a relatively differentiated human colorectal adenocarcinoma that can form glandular structures in 3D cultures(45)

Lentiviruses were used for gene transfers to produce additional populations of the SW1222 cells as shown. (Table 1.)

shPROX1 + VEGF-A	SCR + VEGF-A
shPROX1 + Luc	SCR + Luc

Table 1. Different SW1222 cell lines created by lentiviral gene transduction

VEGF-A means the overexpression of VEGF-A gene. Luc stands for luciferase and it was used as a control for VEGF-A gene. ShPROX1 stands for small hairpin silenced PROX1 gene. Scrambled (SCR) small hairpin was used as a control for shPROX1.

Together with my collaborator, Ville Hyvönen, we cultured also BECs and LECs using published methods(46).

3.2 Gene transduction

Lentivirus vectors with VSVG-surface protein were used for gene transfers. Although the vectors are unable to replicate, they are capable of gene transduction into human cells. Lentiviral work was performed in Biosafety level 2 cell culture room to avoid contamination.

Lentiviruses transfer their genes to fairly random parts of the cell genome. Lentivirus constructs containing the VEGF-A gene and shPROX1 gene were produced by others. Transductions were performed by adding 10-40 x 10^6 particles/ml of concentrated viral vector to recently subcultured cells. Approximately 8 µg/ml polybrene was added to enhance the transduction rate. Polybrene decreases the electric repulsion between the target cells and the virus particles, which is thought to increase the viruses' penetration rate. The viral vectors were let to infect the cancer cells for approximately 24 hours.

In addition to the examined genes, the lentivirus constructs also included gene encoding the enhanced green fluorescent protein (EGFP), which is a widely used gene to mark successfully transduced cells. Cells where the EGFP gene has integrated into host DNA produce the EGFP protein, which can be observed in a fluorescent microscope. Expression and silencing of the desired proteins was verified by immunofluorescence staining with antibodies against VEGF-A and PROX1, respectively.

3.3 Cell culture

Cells were cultured on sterile cell culture plates in an incubator set at 37 ℃, equipped with a humidified atmosphere containing 5% carbon dioxide. We used the high glucose (4,5g/l) DMEM-medium (Lonza) supplemented with L-glutamine (Sigma) 10% Fetal Bovine serum (Gibco), 1,0ml penicillin + streptomycin for the cell culturing. Cells were subcultured approximately 3 times a week and 70 % confluence.

LECs and BECs were cultured in Endothelial Cell Basal Medium MV (PromoCell) supplemented with L-glutamine (Sigma), 10% fetal bovine serum (Gibca), penicillin + streptomycin and fungizone. Also 100 ng/ml VEGF-C was added to LEC endothelial medium in order to induce LEC proliferation. LEC and BECs were incubated in 37℃with 5% carbon dioxide. LECs and BECs used in cocultures were in cell passage 3 or 4.

3.4 Subculturing of cells

The culture medium was removed from the plate and the plates were then washed quickly with PBS or trypsin in order to get rid of remaining fetal calf serum that can inhibit trypsin. The cells were incubated in 0,05% trypsin/PBS. 1-7ml of trypsin was added to the cell plates depending on the size of the plate. The function of trypsin is to cleave cell adhesion molecules attaching the cells each other and the plate so that the cells can be detached. The trypsin incubation in 37℃, 5%CO_2 was for 45-60 minutes for SW1222 cells and 2 minutes for LECs and BECs.

After the incubation, the cells were extracted from the culture plates by pipetting them back and forth repeatedly. This mechanic force detaches the cells from each other. Microscope was used to check that the cells were detached from the plate surface and separated. The trypsin solution containing the cells was transferred to falcon tubes and centrifuged 1500 rpm for 5 minutes in order to pellet the cells to the bottom of the tube.

The supernatant containing the trypsin was removed from top of the pellet with pipet. Pellet was resuspended in additional medium. The medium that contained the cells was divided to different plates in convenient proportion. The cells were then suspended to new medium, which was added to plates and the plates were shaken slightly to spread cells evenly on the plate.

3.5 Cocultures of tumor cells with endothelial cells

In vitro cocultures were established between SW1222 carcinoma cells and LECs or BECs in order to examine the effect of VEGF overexpression in the PROX1 silenced SW1222-cells. In addition to 2D cocultures, 3D cocultures were established by culturing cells on top of the Geltrex™-matrix, which gave the best results among the ones tried.

Geltrex is composed of extracellular basement membrane matrix purified from Engelbreth-Holm-Swarm (EHS) tumors. Growing cells on top of basement membrane resembles better the *in vivo* interactions between cells than the regular 2D cultures.

3D cocultures were carried out in the BD BioCoat™ matrigel™ invasion chamber (BD biosciences). Cells were detached from plates and counted. Medium containing the appropriate amount of SW1222 cells and LECs was combined, diluted to 1 ml of endothelial medium and seeded to a chamber. 10 000 SW1222 cells and 50 000 LECs were used for one chamber. The cells in the cocultures were allowed to proliferate for 2 days, after which they were imaged with an axiovert microscope. The actin cytoskeleton was then stained with fluorescent phalloidin and imaged in a confocal microscope.

2D cocultures were established in regular 48-well plates with 4000 SW1222 cells and 20 000 LECs. They were imaged in the fluorescence and confocal microscopes after phalloidin and Pecam-1 staining.

3.6 Immunofluorescent staining of coverslips

Cells were subcultured with plating into 12-well plate containing coverslips and allowed to attach overnight. They were then fixed with 4 % paraformaldehyde (PFA) for 10 minutes, washed with PBS three times for five minutes. Plasma

membranes of the cells were made permeable to antibodies with detergent 0,1% TritonX100 in PBS solution. The blocking solution, made of 1% BSA (bovine serum albumin) in PBS, was also introduced to the cells at the same time. The function of the blocking solution is to prevent nonspecific binding of the antibody and thus decrease background noise in the staining. The cells were incubated for 15 minutes in these solutions.

The primary antibody attaches to the proteins of interest. Primary antibodies were diluted in 1% BSA-PBS solution. The PROX1 antibody (made in goat, R&D) was applied at 0,5 ug/ml, VEGF-A antibody (made in goat, R&D) was 0,025 ug/ml. Pecam-1 (antibody against CD31, made in Rat, BD biosciences), which attaches to endothelial cells, was 1,25 ug/ml

Primary antibodies were introduced to the cells and incubated for 60 minutes. After the incubation, unbound antibody was washed out with PBS, 3 times for 5 minutes.

Secondary antibodies, also diluted in 1% BSA-PBS, were centrifuged at +4°C with 14 000 rpm for 9 minutes. We used antibody against goat (made in donkey, life technologies A11058) applied at 4 ug/ml.

Secondary antibody is a protein against the primary antibody and therefore it attaches to the primary antibody. Fluorescent molecule is attached to the secondary antibody consequently antibody-complex can be seen with the immunofluorescent microscope. The washes were done as above.

We also used conjugated Texas Red-X Phalloidin (Life Technologies). Phalloidin is a mushroom toxin that binds to F-actin molecules inside cells. Fluorescent Texas Red-X is conjugated to phalloidin. Texas Red-X Phalloidin was applied at 0,4 units/ml in 1%BSA-PBS and incubated for 30 minutes.

Coverslips were then carefully removed from well plates and transferred to microscope slides on top of a drop of Vectashield mounting medium containing

DAPI, a staining color that binds to DNA and can be observed under proper UV-light illumination in a fluorescence microscope.

3.7 Mouse xenograft experiments

For tumor xenograft modeling, we used immunodeficient Nod scid gamma (NSG) mice, which lack mature B cells, T cells and functional NK cells. In order to injected cancer cells to survive and form tumors, the rodents' immune system must be compromised so that it will not eliminate foreign cells.

Cells were resuspended to geltrex for injections after splitting and counting. We injected cells subcutaneously, one cell line to other side and its control to the other side. Overall, 6 mice were used and 1,5 million cells/injection. The mice were anesthetized before the injections. Of note, all work with mice was done by or in the presence of my instructor Ville Hyvönen, who has the appropriate training and licenses for working with mice.

Mice were put to sleep by anesthetics and the tumors were surgically removed from the mice after 14 days. Tumors were weighed and their diameter was measured before the tumors samples were embedded in paraffin for overnight. Tumor samples were then sectioned to thin sections. Sections were stained for E-cadherin (2 ug/ml dilution, made in mouse, Abcam) and Endomucin (2,5 ug/ml dilution, made in rat, R&D)

Most of the stainings and analysis of the tumor sections were done by Ville Hyvönen after my collaboration with the project ended.

3.8 Immunofluorescent staining of paraffin sections

Paraffin sections were deparaffinized and rehydrated by the following procedure. Sections were incubated in xylen for 5 minutes 3 times, in absolute EtOH for 2 minutes 2 times, in 95% EtOH for 2 minutes 2 times, in 70% EtOH for 2 minutes 1 time and rehydrated with deionized water for 4 minutes 2 times. Sections were then boiled in microwave for 15 minutes in pH 9 antigen retrieval

solution diluted 1:10 in ionized water and washed with PBS afterwards. Sections were incubated in 0,1% TritonX100 and blocking solution made of 1% BSA for 30 minutes. Primary antibodies diluted in blocking solution were added on Sections were incubated with primary antibodies overnight at +4 ℃. On the next day sections were washed 3 times for 5 minutes with TNT washing buffer (0,1 M Tris pH7,4, 0,15 M NaCl, 0,05% Tween 20) Sections were then incubated with the respective species specific fluorescent secondary antibodies (4 ug/ml, Life technologies) for 1 hour. After incubation sections were washed with TNT 3 times, once with water, and mounted with mounting media with DAPI to visualize cell nuclei (Vectashield).

3.9 Imaging

Immunofluorescence pictures were taken with Zeiss Axioplan 2-epifluorescence microscope and non-fixed co-cultures were imaged with Zeiss Axiovert 200 inverted epifluorescence microscope. This microscope transmits ultraviolet light that can excite electrons of the sample. Fluorescent proteins emit light depending on the wavelength of the exciting UV-light. Different filters were used to separate different fluorescent proteins. FITC filter was used for both DAPI-nuclear staining and EGFP. Alexa 594 was used for fluorescent antibodies. Confocal images were taken with Zeiss LSM 780.

3.10 Statistical analysis

Statistical comparison of two groups was done by two-tailed paired t-test using Microsoft excel. The Statistical significance is marked by *$p<0,05$ and ***$p<0,005$.

4 Results

4.1 Effect of VEGF-A in *in vitro* cocultures

As expected, in 3D cocultures with VEGF-A overexpressing SW1222 cells, LECs were clearly more organized into tubules and formed connections between the colorectal cell colonies. In the control cultures, the LECs formed web-like structures around the cancer cell colonies and tubule formation was less clear (Figs. 3 and 4). Cocultures of SW1222 cells with BECs showed a similar tube formative response to VEGF-A (Fig. 5).

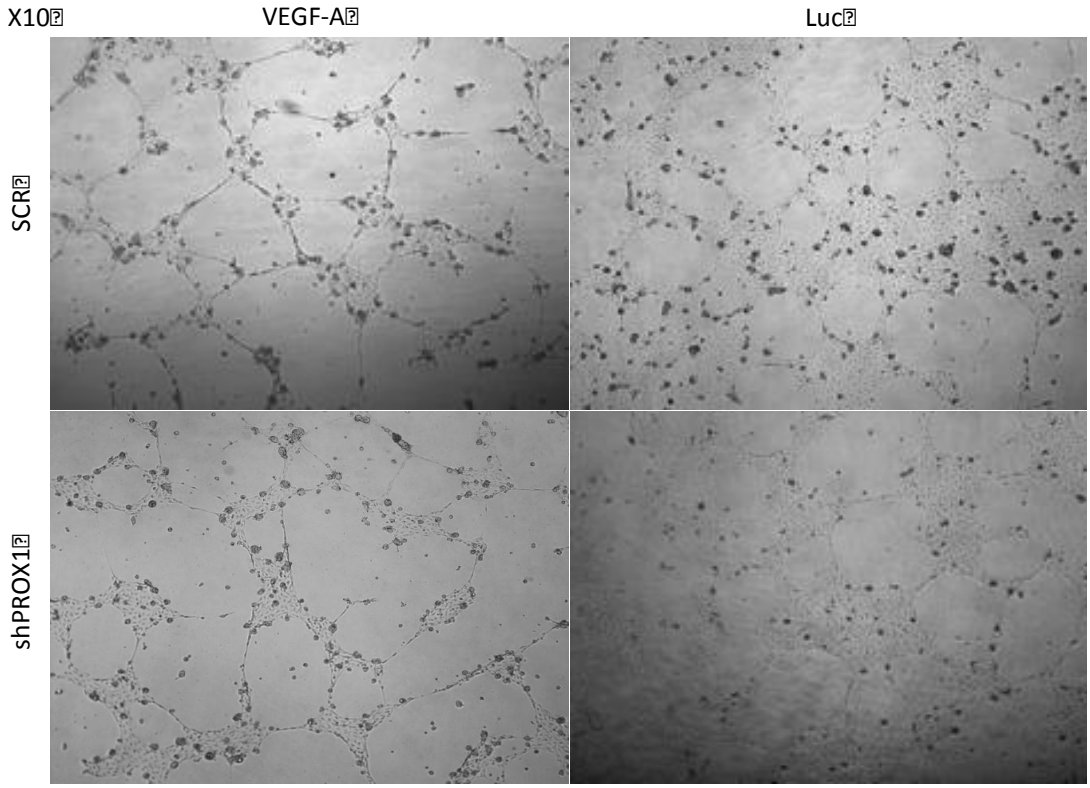

Figure 3. Phase contrast images of SW1222/LEC 3D cocultures.

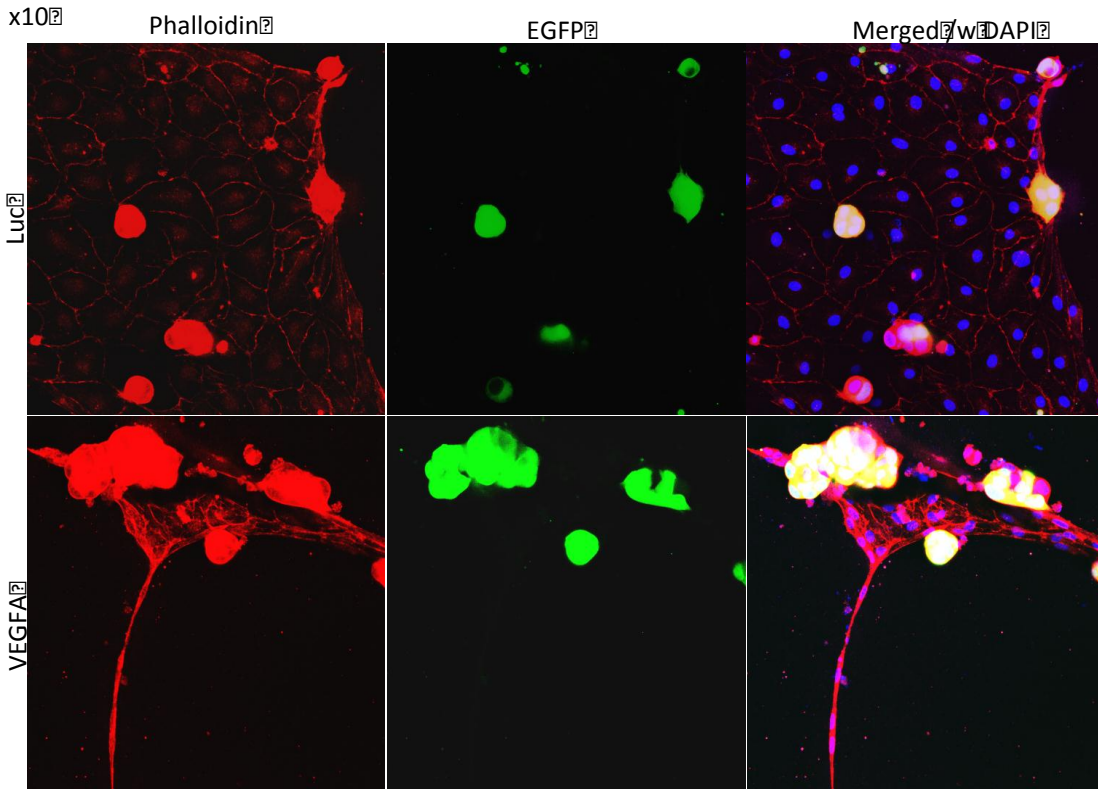

Figure 4. Confocal image of 3D SW1222/LEC cells (green) in coculture with LECs; phalloidin staining in red.

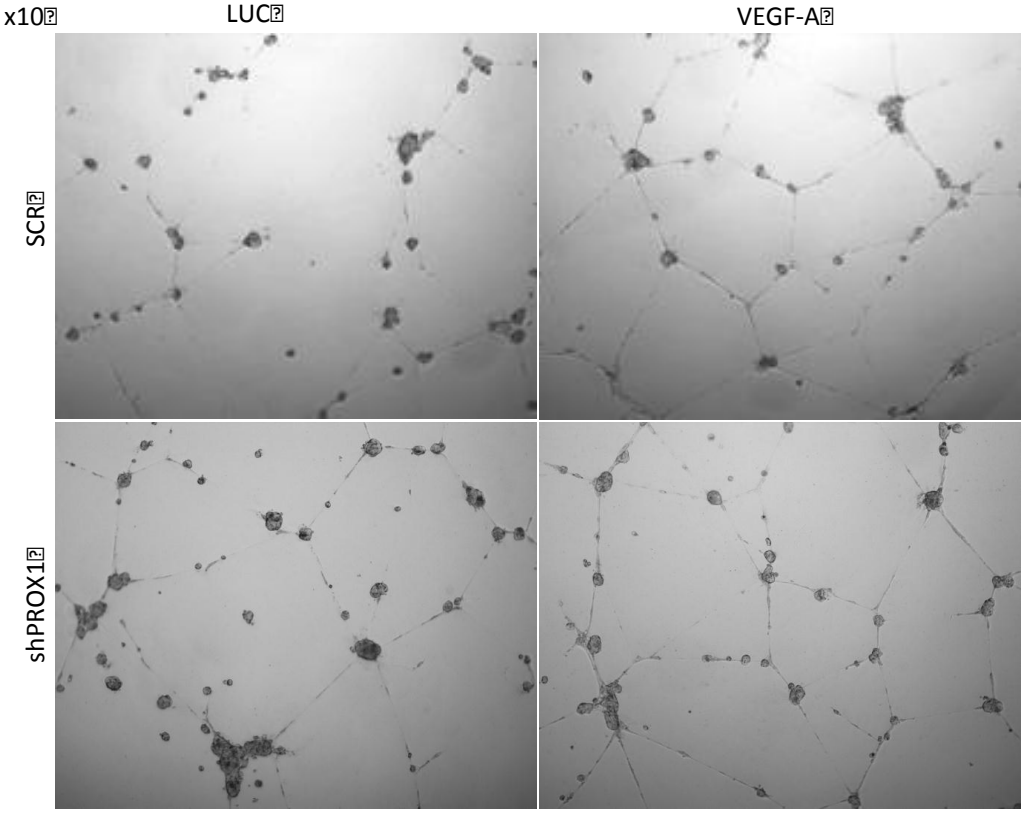

Figure 5. Phase contrast images of the SW1222/BEC 3D cocultures.

PROX1 silencing did not affect tubule formation in the presence of VEGF-A (Figs. 3 and 5). No difference was observed between the cocultures with PROX1 silenced or control SW1222 cells.

We noticed that VEGF-A overexpressing CRC colonies were larger than their controls in the 3D cocultures with LECs. (Fig. 6) This was also true when the BECs were used (Fig. 7).

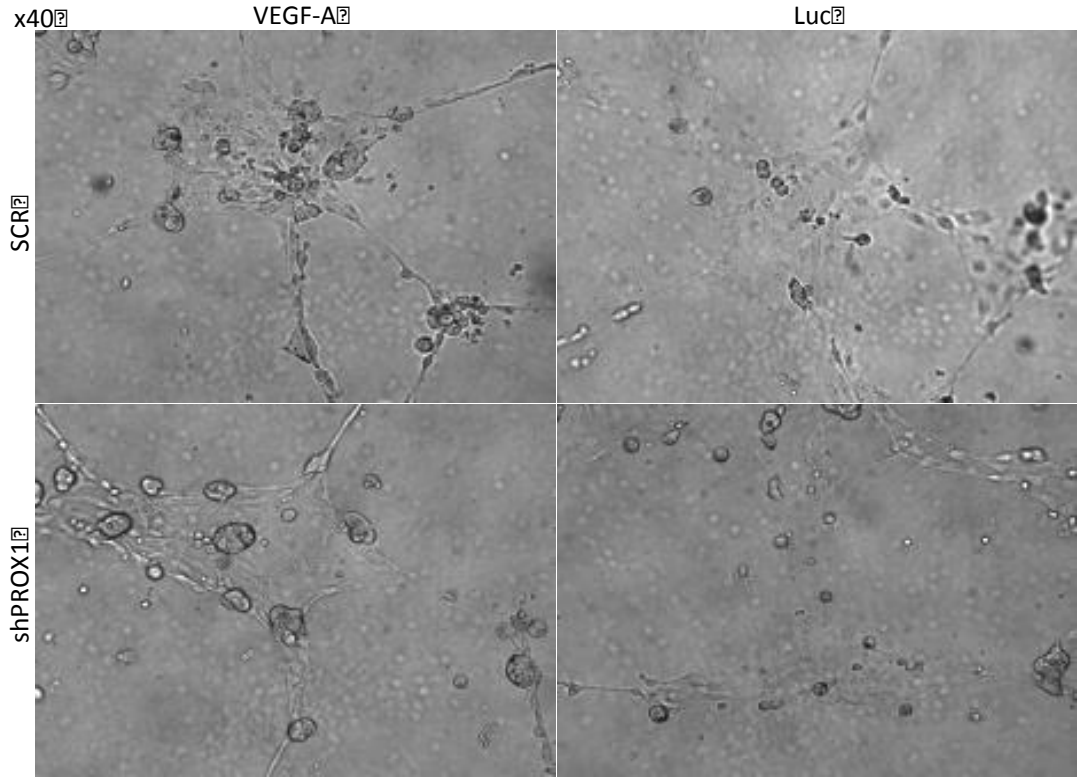

Figure 6. Phase contrast images of the SW1222/LEC 3D cocultures

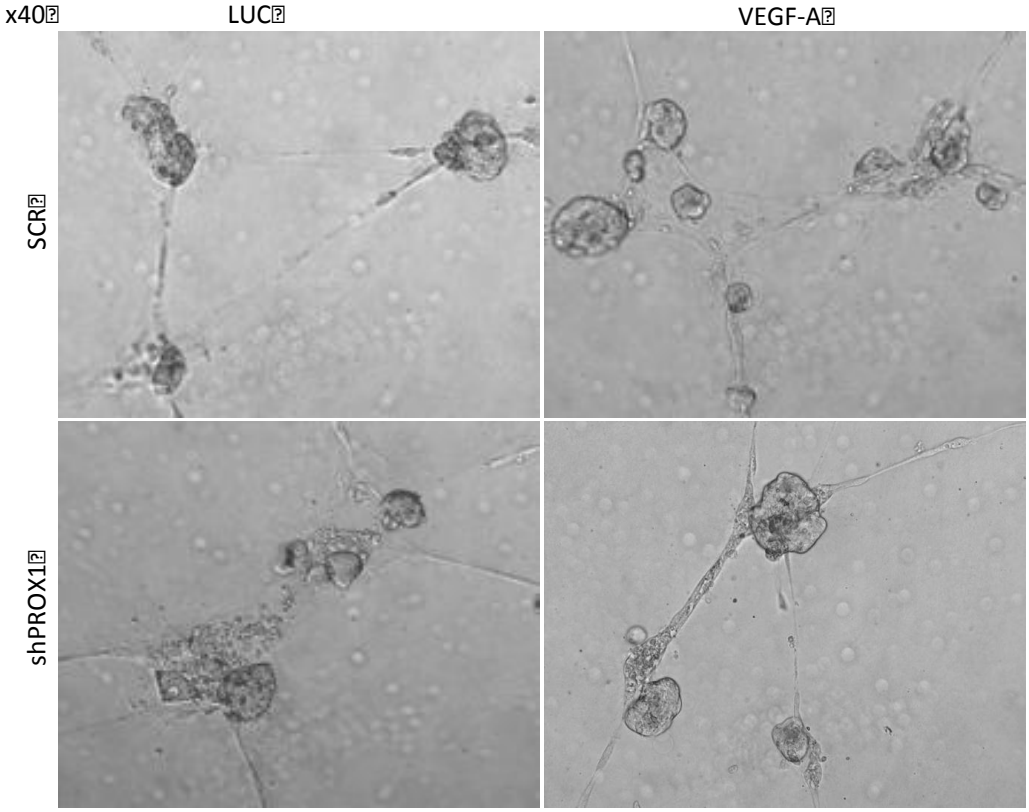

Figure 7. High-power magnification of the SW1222/BEC 3D cocultures.

We did not find any difference between the cell lines in 2D cocultures with LECs (data not shown). The LECs and SW1222 cells were both proliferated in these cultures, but in contrast to the matrigel cultures, the LECs were located around the cancer cells randomly without organized structures. Based on results with LECs we did not study 2D cocultures with BECs.

4.2 Effect of VEGF-A in tumor xenografts

As can be seen in Fig. 8, the SCR-VEGF-A tumors were significantly larger than the SCR-Luc tumors. The shPROX1-LUC tumors were also smaller than the control SCR-LUC tumors. There was no statistically significant difference between the weight of the shPROX1-VEGF-A and SCR-LUC tumors.

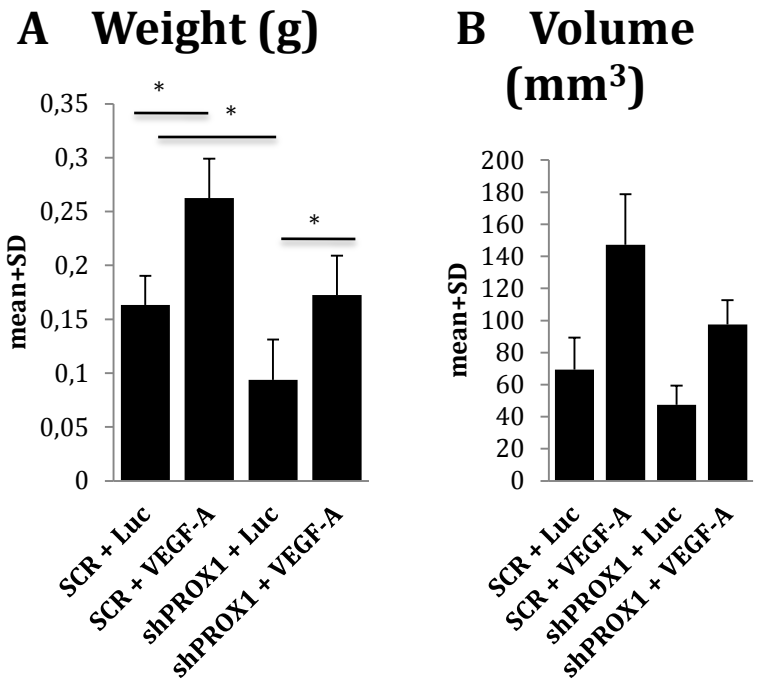

Figure 8. (A) Average weights of the xenotransplant tumors made of transfected SW1222 cells (the standard error of the mean and statistical significance is indicated). (B) Volumes of the respective tumors.

VEGF-A overexpressing tumors were significantly more densely vascularized than their controls as expected. VEGF-A overexpression induced angiogenesis in the PROX1 silenced tumors. PROX1 silencing decreased angiogenesis compared to scrambled shRNA. (Figs. 9 & 10)

The PROX1 silenced tumors showed a greater area of necrosis in the core of the tumors even with VEGF-A overexpression (Fig. 11).

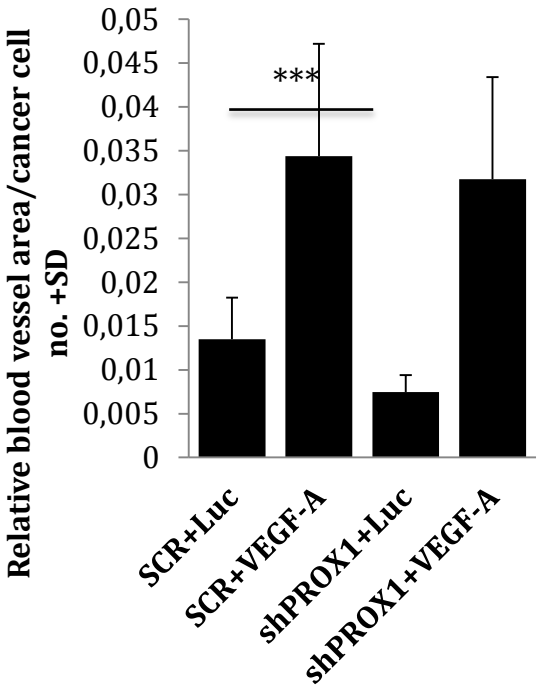

Figure 9. Average relative blood vessel area / number of cancer cells in comparable tumor areas.

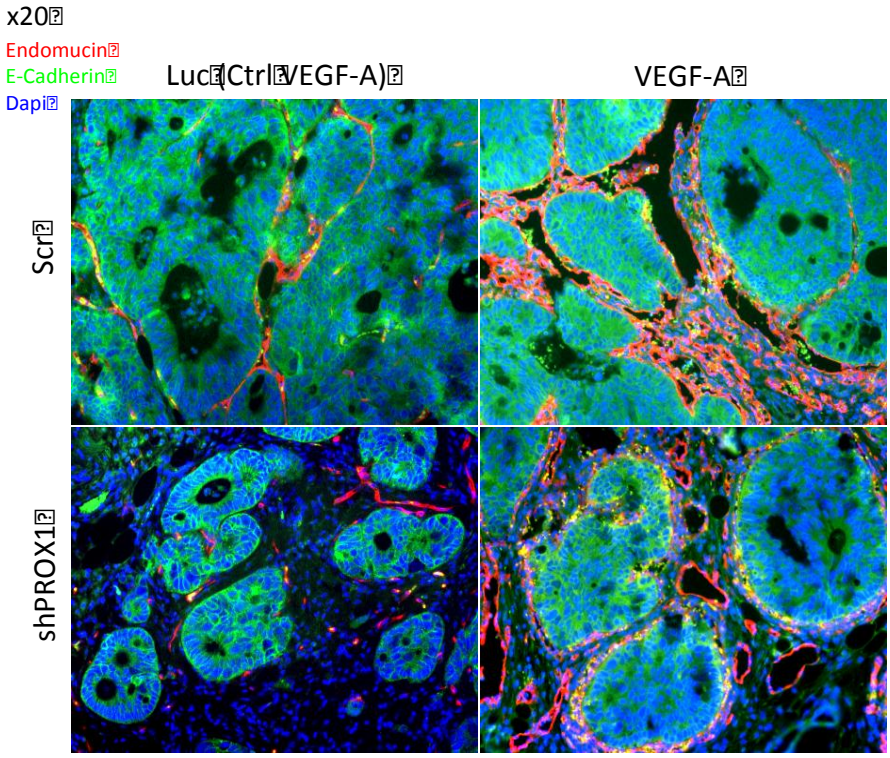

Figure 10. Endomucin/E-cadherin/Dapi staining of tumor sections.

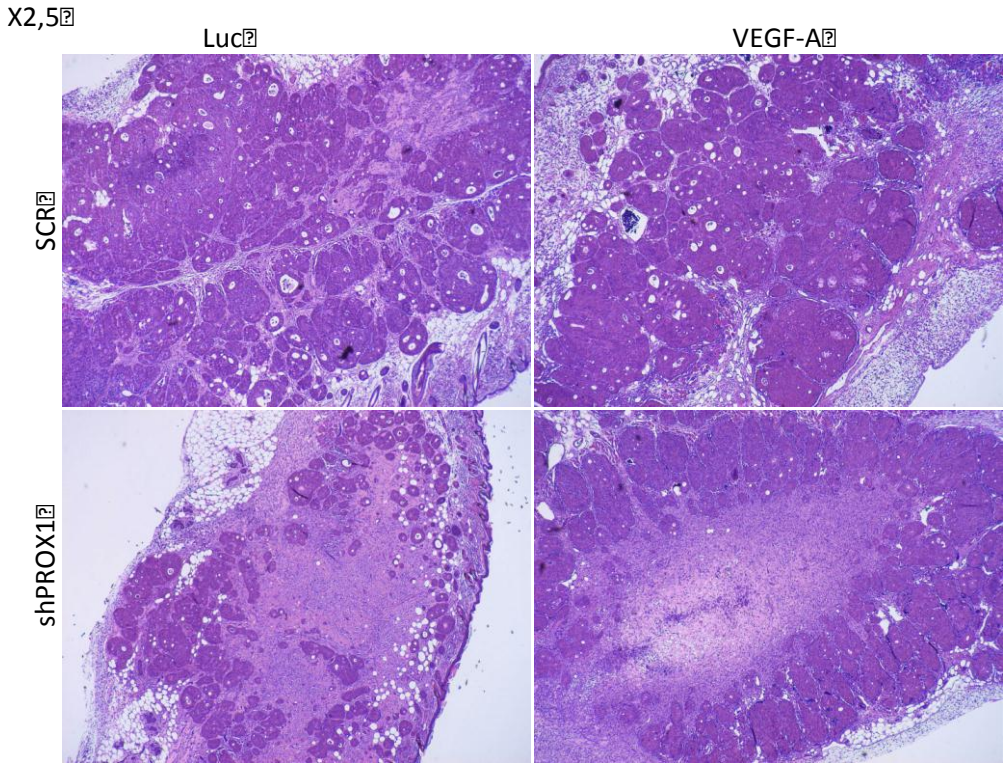

Figure 11. Histology image of tumor sections.

5 Discussion

Our study suggests that overexpression of VEGF-A increases the growth of the tumor xenografts in terms of tumor weight during a 14 day follow-up. Interestingly, even though the overexpression of VEGF-A increased the vascularity of the PROX1 silenced tumors, the core of the shPROX1 tumors still showed a larger necrotic area. In fact, further studies have recently shown that PROX1 helps the cells to adapt against hypoxia by promoting autophagy (49).

In the *in vitro* studies we found out that PROX1 silencing did not stop lymphatic endothelial cells or blood endothelial cells from forming structured tubules, which is an indication of lymphangiogenic activity. This observation *in vitro* correlates directly with the *in vivo* results where PROX1 silencing did not inhibit tumor angiogenesis when VEGF-A was overexpressed.

The intriguing observation that colorectal cancer cell colonies were bigger in cocultures with excess of VEGF-A could have engendered a project to find out what signaling molecules are involved in the cross-talk between the LECs and cancer cells. Indeed, a paper with a finding that endothelial cells secrete soluble Jagged-1 that promotes colorectal cancer stem cells via Notch was published in early 2013 (47).

During my project, I also made a SW1222 cell line with VEGF-C overexpression similar to the VEGF-A overexpression. *In vitro* studies with the VEGF-C transfected SW1222 cells showed a similar LEC tube formation as VEGF-A (data not shown). A continuation to this project would mean repeating the same *in vivo* experiment with VEGF-C instead of VEGF-A. VEGF-C expression induces tumor metastasis and progression, whereas VEGF-C downregulation inhibits tumor growth and metastasis, depending on the tumor type (48). It would be interesting to see how VEGF-C overexpression influences the xenografts in relation to PROX1-silencing. It would be also interesting to study VEGF-C overexpression, PROX1 silencing and its expected antagonistic effect on tumor metastasis.

One potential problem in our study was the mediocre rate of PROX1 silencing. Gene transduction rate is seldom perfect: Furthermore, we did not have a gene construct with a good selection marker (e.g. antibiotic resistance gene for antibiotic selection) to accomplish very strong shPROX1 rate in the transfected cells. Furthermore, the PROX1 silenced cells slowly tend to be selected out by the survival of the fittest in cell culture, which further reduces the population of PROX1 silenced cells. This might have reduced the contrast between the SCR and PROX1 transfected cells in our experiments.

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