Identification of a gene expression profile associated with the regulation of angiogenesis in endometrial cancer

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Abstract. The publication of the human genome sequence provided direction in the search for novel diagnostic and therapeutic methods for the treatment of human diseases. The aim of the present study was to investigate the hypothesis that the expression profile of genes involved in the regulation of angiogenesis may be a marker in endometrial cancer that facilitates the diagnosis and prognosis of patients, as well as the identification of novel therapeutic targets. The current study included 36 patients with grade (G) 1 to 3 endometrial cancer, and a control group of patients consisting of females that qualified for the removal of the uterus. Out of these, 28 samples (control, 3; G1, 7; G2, 12; and G3, 6) were selected for microarray analysis. Molecular analysis of the endometrial samples involved the extraction of total RNA, purification of the obtained extracts and subsequent analysis of the gene expression profiles using an oligonucleotide microarray technique (GeneChip® Human Genome U133A plates). The results indicated that the mRNA expression profile of genes involved in the regulation of angiogenesis varies depending on the degree of histological differentiation of endometrial adenocarcinoma. Similar results were obtained from descriptive statistics characterizing the expression profile of 691 mRNAs associated with the regulation of angiogenesis in the groups of patients with endometrial adenocarcinoma. In addition, the results of the present study indicated that neuropilin2 (NRP2) may serve an important role in the activity of endothelial cells, and may affect vascular endothelial growth factor, and potentially plexins and integrins via regulation of their functions. An understanding of how these proteins interact remains to be determined; however, elucidating these interactions may provide an explanation for the mechanisms underlying angiogenesis. In conclusion, the results of the present study suggest that NRP2 may be a valuable target for investigation in future pharmacological studies involving angiogenesis in endometrial cancer.

Introduction

The publication of the human genome sequence increased the ability to identify novel diagnostic and therapeutic methods for the treatment of human diseases. The combination of morphological methods, the well-established biology of premalignant and invasive alterations, together with molecular biology techniques, which enables the expression of several hundred or even several thousand genes to be evaluated simultaneously, increases the precision of diagnosis and therapy and leads to the development of effective targeted molecular therapies for patients. The ability to use molecular criteria for the selection of an appropriate therapy for a particular patient has led to the rapid development of personalized medicine, particularly in oncology (1-3). Individualization of patient treatment involves adapting therapies to the individual characteristics of the particular patient and their disease. Personalized medicine increases the likelihood that an appropriate, effective and safe therapy is selected. For this reason, molecular biology techniques are increasingly becoming a widespread diagnostic tool in clinical practice (4,5). Molecular diagnostics facilitates the detection of neoplastic lesions in high-risk individuals at an early stage of disease, as alterations at the molecular level precede alterations at the phenotypic level. In addition, it enables the detection of drug resistance, which allows the precision of the treatment strategy to be improved.
Various mechanisms are associated with an altered expression profile of genes involved in the regulation of angiogenesis (6,7). Understanding these differences may be important for diagnosis and therapy. One current method of effective anticancer therapy is an anti-angiogenic therapy that targets products of genes involved in the regulation of angiogenesis. This treatment demonstrates a positive effect in numerous cancer types; however, extended duration of treatment often results in drug resistance (8,9). Maintaining anti-angiogenic therapy sometimes requires change of the drug as well as the molecular target due to drug resistance (10-12). Consequently, the search for novel targets for targeted therapy is ongoing (13,14).

Cancer of the uterus lining, also termed the endometrium, is one of the most commonly diagnosed gynecological cancers worldwide, and primarily affects postmenopausal women (15,16). According to the World Health Organization classification system, there are three histological grades (G) of endometrial cancer, including G1 (well differentiated), G2 (moderately differentiated) and G3 (poorly differentiated) (17). According to clinical, metabolic and endocrine characteristics, there are two types of endometrial cancer. Type I are estrogen-associated, low-grade (G1 and G2) and are often associated with obesity. They are diagnosed early, and therefore demonstrate a favorable prognosis. Type II are hormone-independent, high-grade (G3) and commonly occur in non-obese women. This type of endometrial cancer metastasizes early, and patients demonstrate a poorer prognosis when compared with type I endometrial cancers (18,19). The authors of the present study hypothesized that the expression profile of genes involved in the regulation of angiogenesis may present diagnostic, prognostic and therapeutic targets in endometrial cancer. The aim of the current study was therefore to identify and select genes involved in the regulation of angiogenesis, which have altered transcriptional activity depending on the degree of histopathological differentiation of endometrial adenocarcinoma, that may present diagnostic, prognostic and therapeutic targets.

Materials and Methods

Patients. The present study enrolled 36 patients, 30 with endometrial cancer with degrees of histopathological differentiation between G1 and G3 (the case study group), and a control group consisting of 6 patients without endometrial cancer that qualified for the removal of the uterus (hysterectomy) due pathologies of the uterus and adnexa. Patients in the control group that agreed to participate in the present study qualified for surgical removal of the uterus (abdominal hysterectomy) with the following medical indications: Uterine fibroids, benign tumors of the appendages, or reproductive organ prolapse. The patients were admitted into Regional Railway Hospital in Katowice from January to May 2015. The average age of patients in the study group was 65 and in the control group, 48. All patients provided written informed consent for the use of their samples in the present study. The criteria for exclusion from the case study group included patients that, according to medical history or postoperative pathological examination, were diagnosed with a form of cancer other than endometrial endometrioid adenocarcinoma. Additional exclusion criteria were the detection of endometrial hyperplasia with or without atypia during postoperative pathological examination, the use of hormone replacement therapy in the 5 years prior to the operation and extreme obesity (body mass index, >40). The histopathological assessments of tumor samples were performed in the Department of Pathomorphology, Medical University of Silesia (Katowice, Poland). A total of 28 samples (control, 3; G1, 7; G2, 12; and G3, 6) were selected for microarray analysis.

Ethical approval. The present study was approved by the Bioethical Committee of the Medical University of Silesia (Sosnowiec, Poland; no. KNW/0022/KBi/67/13). The study adhered to the tenets of the Declaration of Helsinki.

Molecular analysis. Molecular analysis of the endometrial samples from patients that had undergone a hysterectomy (study case group and control group) involved the extraction of total RNA using TRizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Extracted total RNA was then purified using an RNasy Mini kit (Qiagen GmbH, Hilden, Germany) using columns and an RNase-Free DNase Set (Qiagen GmbH) according to the manufacturer's instructions. Analysis of gene expression profiles was determined using an oligonucleotide microarray technique with GeneChip® Human Genome U133A plates (Affymetrix, Inc., Santa Clara, CA, USA). The first step of the analysis was cDNA synthesis (8 µg RNA was used as a template) with the use of SuperScript Choice System (Invitrogen; Thermo Fisher Scientific, Inc.). Then, the synthesis of biotinylated cRNA was performed using BioArray HighYield RNA Transcription Labeling kit (Enzo Life Sciences, Farmingdale, NY, USA). Subsequently, the Sample Cleanup Module kit (Qiagen GmbH, Germany) was used to perform fragmentation of the biotin-labeled cRNA. After the cRNA hybridized to the HG-U133A microarray, it was stained with streptavidin-phycocerythrin and scanned using GeneArray Scanner G2500A (Agilent Technologies, Inc., Santa Clara, CA, USA). The data was processed for signal values using Microarray Suite version 5.0 software (Affymetrix, Inc.). All of the procedures were performed as recommended by Affymetrix Gene Expression Analysis Technical Manual (20). Comparative analysis of the transcriptome was performed for 691 mRNA sequences of genes encoding proteins involved in the regulation of angiogenesis, which were selected based on the results of the literature, the NCBI database (Gene, http://ncbi.nlm.nih.gov/gene) and an Affymetrix NetAffx™ Analysis Center database (http://www.affymetrix.com/analysis/index.affx).

Statistical analysis. GeneSpring GX version 12.6.1 software (Agilent Technologies, Inc.) and PL-Grid Infrastructure (http://www.plgrid.pl/) were used for statistical analysis of the data after microarrays scanning. Microarray analysis was performed using the following specialized programs adapted to the analysis of the matrix experiment results: Microarray Suite 5.0 software (Affymetrix, Inc.), GeneSpring GX (version, 12.6.1; Agilent Technologies, Inc.) and PANTHER version 11.1 (Protein Analysis Through Evolutionary
Relationships, http://pantherdb.org) (21), Gene List Analysis tool. Initially, the degree of RNA degradation was assessed using 3'/5' ratio (signal intensity ratio of the 3' probe set over the 5' probe set) of the housekeeping genes GAPDH and β-actin. This parameter was termed RNA degradation index and is commonly used in Affymetrix U133 plates for RNA quality assessment. This index reflects the level of RNA integrity and accuracy of sample processing. A low index (3'/5' ratio<3) corresponds to high quality material, whereas a high index (3'/5' ratio>3) could indicate low quality material and/or problems during sample processing. The control of microarray hybridization with mRNA was based on the 8 exogenous controls, 8 probes complementary to the exogenous RNA added in varying concentrations to the hybridization cocktail by the manufacturer of the microarray (Affymetrix, Inc.). The following probes were used in the present study: AFFX-BioB_at, AFFX-BioC_at, AFFX-BioDn_at, AFFX-CreX_at, AFFX-r2-Ec-BioB_at, AFFX-r2-Ec-BioC_at, AFFX-r2-Ec-BioD_at, AFFX-r2-Pl-cre_at. Following the acceptance of the microarray for comparative analysis, the obtained results were normalized using the Robust Multi-array Average (RMA) Express program version 1.1.0 (http://rmaexpress.bmbolstad.com/) (22), and genes that were differentially expressed in the transcriptome depending on the severity of the disease (histopathological grade) were selected for molecular analysis using GeneSpring GX software (version12.6.1). One-way analysis of variance (ANOVA) with the Benjamini-Hochberg correction was performed to identify mRNAs that exhibited significantly altered expression in endometrial adenocarcinoma samples compared with the controls. The Tukey's test was used for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

The expression profiles of mRNA in endometrial samples with varying degrees of histological differentiation of endometrial adenocarcinoma were determined by microarray expression analysis using GeneChip® Human Genome U133A plates. Differentially expressed genes among the groups were first normalized using the RMA Express program. Hierarchical clustering of the results was then performed, which allowed a preliminary assessment of the similarity between mRNA expression profiles in the different groups (Fig. 1). Fig. 1 is a fragment of a dendogram showing expression of 15 mRNAs, it demonstrates that the mRNA expression profile of genes involved in the regulation of angiogenesis varies depending on the degree of histological differentiation of endometrial adenocarcinoma. The smallest differences relative to the control group were observed in the G1 group of endometrial adenocarcinomas, while the transcriptomes of G2 and G3 groups constituted a distinct subgroup (Fig. 1). Similar conclusions were drawn based on descriptive statistics characterizing the expression profile of 691 mRNAs associated with the regulation of angiogenesis in the control and endometrial adenocarcinoma groups (Fig. 2).

To determine which of the observed differences in mRNA expression were statistically significant, one-way ANOVA was performed. The results indicated that, out of the 691 mRNAs, 585 mRNAs were significantly differentially expressed in endometrial adenocarcinoma samples when compared with controls. Table I presents the level of significance for mRNAs that demonstrated significantly altered expression in endometrial adenocarcinoma samples when compared with controls. Multiple comparisons testing was subsequently performed using the Tukey post hoc test to obtain more detailed
The expression of a total of 691 mRNAs associated with the regulation of angiogenesis was compared between controls and patients with endometrial adenocarcinoma, 585 exhibited significantly altered expression levels. The results indicated that six mRNAs differentiated endometrial adenocarcinoma and controls (Table II). The results indicated that the number of mRNAs with significantly altered expression when compared with the controls for each histological grade were as follows: G1 vs. control, 27; G2 vs. control, 113; and G3 vs. control, 81 (P<0.05; Table II). Therefore, the number of mRNAs that demonstrated significantly altered expression in endometrial adenocarcinoma samples compared with the controls varied depending on whether they were G1, G2 or G3 samples.

A Venn diagram was constructed to demonstrate the number of overlapping mRNAs that exhibited significantly altered expression levels among endometrial adenocarcinoma samples of any histological grade and the controls (Fig. 3). The results indicate that six mRNAs differentiated endometrial adenocarcinoma samples from the control regardless of histological grade (Fig. 3). The number of mRNAs that were significantly differentiated between specific histological grades and controls (significantly altered expression in only one histological grade vs. controls) were as follows: G1 vs. control, 15; G2 vs. control, 43; and G3 vs. control, 15 mRNA (Fig. 3).

This next step of the analysis was completed by performing an over-representation test using PANTHER software (Gene List Analysis). This allowed for the identification of differentially expressed mRNAs that are essential for tumor angiogenesis (Table III). The results demonstrated that in G1 endometrial adenocarcinoma, three genes, including endoglin (ENG), EGF like repeats and discoidin domains 3 (EDIL3) and neuropilin 2 (NRP2) demonstrated a significant increase in expression when compared with the control group (Table III). In addition, a significant increase in the mRNA expression levels of semaphorin (SEMA) 3B in G2 endometrial adenocarcinomas, and SEMA3F expression in G3 endometrial adenocarcinoma was observed when compared with the control group (Table III). The remaining mRNAs identified by the over-representation test that were determined to be important for tumor angiogenesis, occurred in G2 and G3 endometrial adenocarcinoma at an expression level that was significantly lower when compared with the control. A characteristic feature was that the mRNA level was gradually reduced with the increase of histopathological differentiation of endometrial adenocarcinoma (Table III). The overlapping mRNAs between the G2 vs. control and G3 vs. control groups included the transforming growth factor β receptor 3 (TGFBR3) isoform of ENG, formed due to post-transcriptional modification of the TEK receptor tyrosine kinase, vascular endothelial growth factor C (VEGFC), SEMA5A and homeobox A5. The over-representation test did not identify mRNA sequences that differentiated all histological grades of endometrial adenocarcinoma (G1 vs. control, G2 vs. control and G3 vs. control) from controls. Similarly, no mRNAs that were common among G1 and G2 vs. control or G1 and G3 vs. control groups were identified as essential for tumor angiogenesis (Table III).

### Discussion

Microarray technology facilitates detection of the expression levels of several thousand genes with one experiment, and is therefore a useful tool for determining the influence of changes in gene expression levels in the development of human disease, such as cancer (23). The results of the present study suggest that the expression of genes involved in the regulation of angiogenesis may present a useful diagnostic marker.
that facilitates differentiation between low and high grades of endometrial adenocarcinoma.

Neoangiogenesis is associated with the expression of certain markers of angiogenesis, including VEGF, ENG (transmembrane glycoprotein receptor for TGF-β) and NRP (a co-receptor for VEGF) (24-27). Conventionally, the prediction of malignancy and treatment of cancer patients is based primarily on the determination of the clinical stage. Understanding the cellular processes responsible for the metastasis of cancer, as well as the molecular markers of invasiveness, may be useful to improve the diagnosis and prognosis of the disease. In addition, this understanding may facilitate the development of drugs targeting the factors responsible for the invasiveness of cancer and the enable the development of novel therapeutic regimens. ENG serves an important role in tumor progression via the regulation of angiogenesis, cell migration and metastasis. It is present on the surface of endothelial cells of tumor blood vessels and particular types of tumor cells. ENG is an auxiliary co-receptor of TGF-β that is responsible for cell proliferation, differentiation, migration and adhesion (28-31). It is known that TGF-β1 functions as an inhibitory factor during tumor development. In addition,
it induces inflammation and releases angiogenic factors from inflammatory cells in vivo (32). It was previously demonstrated that inhibition of ENG stimulates cell growth induced by TGF-β1 and inhibits cell migration (33). By contrast, ENG is an identified component of the endothelial nitric oxide synthase signaling pathway, which modulates the activity of cyclooxygenase-2 (34,35). ENG appears to modulate the transition from endothelial progenitor cells to active endothelial cells (36).

Angiogenesis is essential for numerous physiological and pathological processes, such as tumor progression, as vascularization is required for tumor growth and metastasis (37). When there is an insufficient blood supply, cancer cells undergo apoptosis/necrosis. However, due to the observed distribution of ENG in all tissues and the other proven functional involvement with TGF-β (38,39), it was demonstrated to be involved in angiogenesis (25,40). The participation of ENG in angiogenesis corresponds with the observed death of ENG−/− knockout mice by vascular malformations (40). An association between the levels of ENG and cell proliferation markers, such as Ki-67 were observed (25). Using immunohistochemistry, increased expression of ENG has been observed in endothelial cells undergoing active angiogenesis, including those in the tumor, when compared with normal endothelium (41,42). The function of ENG may be contradicting as it is required for tumor neoangiogenesis; however, the overexpression of this gene may act as a suppressor of invasion and metastasis (43). ENG as a part of the TGF-β receptor complex modulates TGF-β receptor signaling. The complex relays contradicting signals from TGF-β that has a paradoxical role in cancer development. It may inhibit cell growth and induce apoptosis or differentiation during the premalignant phase of carcinogenesis. Conversely, TGF-β may modulate processes such as cell invasion, angiogenesis, immune regulation after the cancer cells lose inhibitory growth responses, which allows them to become malignant (44). Previous studies observed that downregulation of endoglin was associated with malignant progression in prostate carcinoma cell lines and enhanced migration and invasion of nontumorigenic prostate cell lines, while overexpression had the opposite effect (45,46). The present study, revealed that ENG was overexpressed in the G1 vs. control group [FC=(+)1.66033]. Therefore, it may be assumed that the increase in ENG mRNA levels may lead to inhibition of cell growth, and will thus function to inhibit tumor growth. From the G2 vs. control and G3 vs. control transcriptome groups, PANTHER highlighted ENG as a differentially expressed gene. However, in this case, decreases in mRNA expression levels [FC=(−)2.7451] were observed. As a consequence, TGF-β may induce tumor cell growth and the likelihood of tumor progression may increase.

The expression of NRP2 is observed in healthy, mature and developing vasculature systems, on endothelial cells, vascular smooth muscle cells (47,48) and in the vascular endothelium surrounding tumors (47,49-51). This is an important observation concerning the role of NRP2 in the promotion of angiogenesis in cancer. Previous studies have indicated that NRP2 play antiangiogenic roles by interacting with class 3 SEMAs (52). SEMA3 family proteins bind to the b1/b2 domains, whereas VEGF family proteins bind to

the b1/b2 domains. Previous studies revealed that anti-Nrp1 antibody (anti-Nrp1A), that blocks the SEMA3 binding domain, does not block VEGF binding, whereas the antibody blocking the VEGF binding domain (anty-Nrp1B) does not block binding to SEMA3 (9,53). There is sufficient evidence to indicate that SEMAs are involved in the regulation of apoptosis, cell migration, tumor growth and angiogenesis (54-57). SEMA3 particularly affects endothelial cells (54,58,59). NRP2 preferentially binds to SEMA3B, 3C, 3D and 3F (54,55,60). By inhibiting the interaction between VEGF-NRP, the SEMA3F isofrom inhibits VEGF-dependent cell proliferation and migration (54). The results of the present study demonstrated that NRP2 was overexpressed in the G1 group when compared with the control group [FC=(+)1.8525], and was the most over-represented gene. If the information presented is reliable, it is possible that cancer cells at this stage demonstrate increased pro-angiogenic interactions between VEGF and NRP2 in G1 compared with control, which may promote increased angiogenesis and subsequent cancer progression.

It is considered that at least two protein members of the class 3 SEMA family, one of which includes SEMA3F, exhibit anti-angiogenic effects involving NRPs (65). Microarray analysis performed in the current study, demonstrated that SEMA3B and 3F of the class 3 SEMA family, were overexpressed in endometrial adenocarcinoma samples when compared with controls. SEMA3B was identified as the over-represented gene in the G2 vs. control groups, and SEMA3F was the over-represented gene in the G3 vs. control groups. Overexpression of SEMA3F may inhibit cellular migration, which may result in a reduction of angiogenesis and metastasis (63,64).

It has been previously demonstrated that SEMA3F inhibits angiogenesis, cell proliferation and cell survival, which are induced by VEGF and basic fibroblast growth factor (bFGF) (66). The lack of bFGF-binding NRPs, coupled with the fact that SEMA3F does not inhibit binding of bFGF to its receptor, indicate that SEMA3F may function through NRP2 (66-68). Additional studies have demonstrated that SEMA3F inhibits tumor angiogenesis in vivo. Previous studies have suggested that VEGF promotes angiogenesis via NRP2, while SEMA3F is an inhibitor of angiogenesis (66). These features of NRP and SEMA3Findicate that they may be potential targets for anti-angiogenic therapy. The direct effect of VEGF on endothelial cells has long been established; however, the role of the NRP in this process is currently under investigation. Angiogenic induction through the binding of VEGF to NRP2 has been characterized to a limited extent, and the evidence presented so far suggests that the regulation occurs in a different manner to that of VEGF-NRP1 (46). Favier et al (65) investigated the role of NRP2 in primary human endothelial cells, and the results indicated that NRP2 interacts with VEGFR2 and VEGFR3. By assessing the overexpression or suppression of NRP2, an essential role of NRP2 in the survival and migration of endothelial cells induced by VEGFA and VEGFC was demonstrated. In
addition, this previous study demonstrated that NRP2 functions as a co-receptor for VEGFR. As VEGFR2 is always present on endothelial cells, whereas VEGFR3 is expressed, it was demonstrated that NRP2 interacts with VEGFR3 and VEGFR2. VEGFR2 is responsible for the full spectrum of action of VEGF factors on endothelial cells. Unlike VEGFR1, expression of the VEGFR2 gene is not dependent on hypoxia (69). Expression of VEGFR2 occurs primarily in vascular endothelial cells and in megakaryocytes, platelets and hematopoietic stem cells (70-72). The expression of VEGFR3 regulates the development and growth of the lymphatic system (73). Embryos with a defective VEGFR3 gene succumb at an early stage of development due to malformation of the cardiovascular system (74). In adults, expression of this isoform occurs exclusively in the endothelial lymphatic vessels. In addition, its expression was identified in the blood vessels of various tumors during neovascularization, which is associated with the spread of the tumor process.

If the interaction between VEGFR and NRP2 is supported by a co-ligand, it is possible that NRP2 may function as a co-receptor for VEGFR2 and VEGFR3. The results obtained by Favier et al (65) demonstrated that NRP2 functions as a co-receptor for VEGFR in response to VEGFC. The present study demonstrated that VEGFC was strongly silenced in G2 and G3 endometrial adenocarcinomas. This may be due to the marked decrease in the concentration of a specific mRNA, which affects the decrease in survival and migration of endothelial cells.

SEMA5A demonstrates oncogenic and tumor suppressive functions in a number of cancers. High expression of SEMA5A and its receptor, plexin-B3, is associated with the aggressiveness of pancreatic and prostate cancers. It was demonstrated that their expression was the primary factor underlying the involvement of SEMA5A in the invasion, migration, proliferation and growth of tumors (64,75,76). In vitro studies have demonstrated that one of the consequences of SEMA5A expression is a decrease in the level of apoptosis of endothelial cells (75,77). Furthermore, downregulation of SEMA5A at the transcriptional and translational levels was observed in lung cancer samples, which is generally associated with low survival rates (78). The use of microarrays in the present study demonstrated that SEMA5A is silenced [FC= (-)3.5141] in G2 and G3 endometrial adenocarcinomas when compared with controls. Based on these observations, it is possible that SEMA5A may not be associated with cancer aggressiveness.

EDIL3, also termed DEL-1, was one of the first extracellular matrix proteins determined to be involved in vascular morphogenesis. EDIL3 has been investigated extensively, and is associated with the regulation of angiogenesis and cell adhesion. It is an embryonic endothelial cell protein, which is not expressed following birth; however, it is expressed in numerous tumor types (79,80). The current study demonstrated that EDIL3 was overexpressed in the G1 vs. control groups (FC= (+)1.7940). Overexpression of EDIL3 reduces apoptosis of tumor cells and leads to increased tumor vascularization, which promotes tumor growth (80,81). The potential modulation of tumor vasculature by EDIL3 may be a potential target for anti-angiogenic therapy of cancer (82,83).

In conclusion, the results of the present study provide potential novel results that may facilitate diagnosis and treatment of patients with endometrial cancer. The scientific value of the present study has been discussed; however, certain limitations have to be considered. The small total number of collected and selected samples analyzed, may affect the impact of the current study. In order to improve the results further, a study that includes a higher number of patients would be desirable. The results of the present study indicate that NRP2 may demonstrate an important role in the activity of endothelial cells, including VEGF, and potentially plexins and integrins. An understanding of how these proteins interact remains to be established. In the future, this may lead to an improved understanding of the mechanisms underlying angiogenesis. The results of the present study indicate that NRP2 may be a worthwhile target for future pharmacological studies.

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