

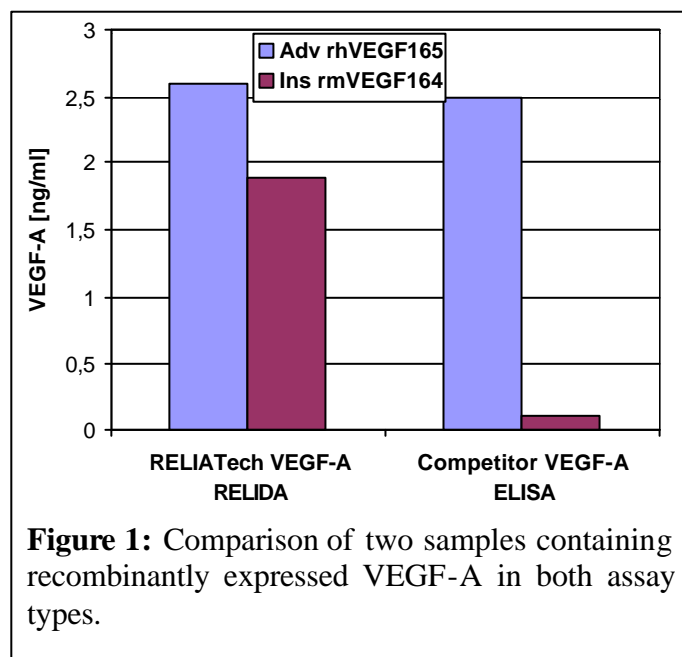
# The New RELIA*Tech* VEGF-A RELIDA®

## A perfect tool for the measurement of biologically active VEGF-A in your samples

In the last few years research has implicated VEGF-A in the progression of many different tumor-types. It has also been established as a marker with diagnostic value for other diseases characterized by vascular disorders and enhanced angiogenesis. In this respect it is important that the new RELIA*Tech* VEGF-A RELIDA (*receptor ligand detection assay*) offers a simple and accurate method for the quantitation of biologically active VEGF-A in many different sample types.

We compared the RELIDA to a prevalent VEGF-A ELISA sold by a competitor. Both assays have a different set-up, which is very important for the interpretation of the results obtained. While the competitors VEGF-A ELISA is based on antibodies for capturing and detection of sample VEGF-A, the RELIDA is receptor-based. The plates are pre-coated with the extracellular domains of a native, high-affinity VEGF-A receptor. Receptor-bound sample VEGF-A is subsequently detected by a polyclonal VEGF-A antibody.

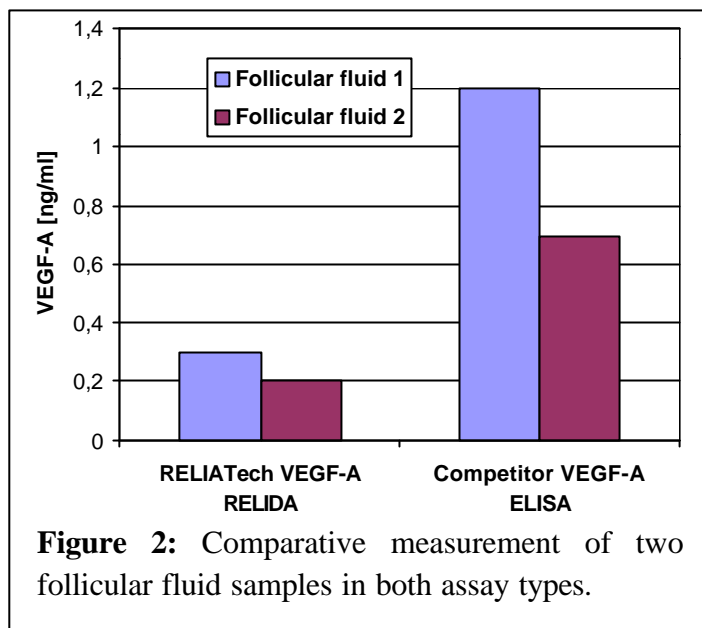
In figure 1 the measurement of two different samples containing recombinantly expressed VEGF-A is compared. Both assays detect the same amount of adenovirus-derived recombinant human VEGF<sub>165</sub> (Adv rhVEGF<sub>165</sub>) in the first sample, which would be expected as both assays have been developed for the detection of rhVEGF<sub>165</sub>. The RELIDA also detects insectcell-expressed recombinant murine VEGF<sub>164</sub> (Ins rmVEGF<sub>164</sub>) in the second sample. The receptor used for capturing of VEGF-A in the RELIDA recognises both human and murine forms, which are highly related. In contrast, the antibody-pair utilised in the VEGF-A



**Figure 1:** Comparison of two samples containing recombinantly expressed VEGF-A in both assay types.

ELISA discriminates well between human and murine VEGF-A. Therefore, no murine VEGF-A can be detected in the second sample with this assay.

Figure 2 shows the comparative measurement of two different follicular fluids in both assay-types. The striking difference that we observed during our experiments was that the antibody-based VEGF-A ELISA detected 3- to 4-fold higher VEGF-A levels in all samples than the receptor-based RELIDA. This discrepancy can be explained by the fact that both samples contained significant amounts of soluble VEGF-A receptor-1 (sVEGFR-1) as determined by means of the RELIA*Tech* sVEGFR-1 ELISA (DA-064). The values were 0.9 ng/ml for sample 1 and 0.5 ng/ml for sample 2. The sVEGFR-1 can bind to sample-VEGF-A, leaving only 20% to 30 % of the total



VEGF-A amount within a sample free and uncomplexed. Only this free part of VEGF-A is able to bind to the receptors coated onto the RELIDA plate. All sVEGFR-1 bound, or in any other form complexed VEGF-A, will not bind to the receptor molecules used in our RELIDA. Otherwise, the antibodies utilised in the VEGF-A ELISA can not discriminate between free and sVEGFR-1-bound VEGF-A. Therefore, the ELISA determines the total amount of VEGF-A within a sample.

VEGF-A is implicated in several pathological conditions that are provoked by enhanced angiogenesis or enhanced vascular permeability, e.g., tumour-growth, psoriasis, rheumatoid arthritis or diabetic retinopathies. Therefore, ELISA assays are valuable tools for research and diagnostic. While both assays used in this comparison can reliably detect and quantify VEGF-A in human samples, the major advantage of the RELIDA is that it enables the measurement of free and biologically active forms of VEGF-A, based on its novel design with native, high-affinity VEGF-A receptors as capture molecules. All other complexed or degraded and thus biologically inactive forms of VEGF-A will not be detected. Another advantage of our RELIDA is that it can also be used for the measurement of murine samples.

In combination with RELIA*Tech* sVEGFR-1 ELISA it is also possible to determine the ratio of VEGF-A to its naturally occurring antagonist sVEGFR-1 in serum samples. This ratio is important for the relapse free survival after surgical tumor excision and associated with a good or poor prognosis for cancer progression (Toi et al., Int. J. Cancer 2002; in press).

## **I. RELIDA: A new, receptor-based ELISA for the measurement of bioactive ligands!**

So far, the most commercially available ELISAs are based on mono- or polyclonal antibodies for capturing the desired “target protein”. Our **RELIDA** (*Receptor-Ligand-Detection-Assay*) is a novel kind of ELISA based not on an antibody but on a soluble receptor for binding of the “target protein”.

This system has several important advantages: *(i)* the recombinant soluble receptors normally have a similar high affinity for the ligand than the endogenous cell surface transmembrane receptors, *(ii)* in contrast to an antibody which bind to a certain epitop regardless of the integrity of the protein, only ligands able to bind to the receptor are detectable in this assay system and *(iii)* if there exist more than one ligand for a certain receptor and having the appropriate antibodies all ligands binding to the used soluble receptor can be measured. So, using sVEGFR-1 as capture receptor you would be able to measure the amount of bioactive VEGF-A and -B as well as PlGF.

In this respect we are pleased to announce that we were able to develop the first immunoassay for the detection of bioactive VEGF-A (**Cat.-No. DA-066**). It is important that the new RELIDA® will offer a simple and accurate method for the quantitation of biologically active VEGF-A in many different sample types [see Part II]. In addition, we also offer a classical ELISA for the detection of the soluble form of VEGFR-1 (**Cat.-No. DA-064**) which represents a naturally occurring antagonist of VEGF-A. This assay allows the detection of the total amount of sVEGFR-1 (free and complexed with ligand). Both products have been developed using state-of-the-art methods for sandwich-ELISA developments and have been validated under various conditions and are quantitatively calibrated to verified recombinant molecular standards.

The determination of both, bioactive VEGF-A as well as its naturally occurring antagonist sVEGFR-1 might be important for the following reason: The characterization of angiogenic activity such as embryonic development, placental vascularization, cancer and wound healing is measured by comparing the ratio of angiogenic stimulators (e.g. FGF-1, FGF-2, VEGF-A, Ang-1) to angiogenic inhibitors (e.g. sVEGFR-1, angiostatin, endostatin, thrombospondin). Several independently published data of both normal and pathogenic subjects have confirmed endogenous levels of VEGF-A and bFGF in pg/ml to ng/ml ranges as measured by commercially available sandwich ELISA kits. These factors have been thought to work unopposed to cause blood vessel formation. The finding that sVEGFR-1, a strong endogenous VEGF-A antagonist, is present in normal subjects

suggests a finely tuned balance of signal transduction, the workings of which can now be explored. Together with other similar assay systems, positive and negative angiogenic regulators can now be explored in many different physiological and pathological settings using human cell culture supernatants and biological fluids [see also Part III].

## **II. Characterizing the levels of biologically active vascular endothelial growth factor (VEGF-A) by a new type of ELISA**

In the last few years research has implicated VEGF-A in the progression of many different tumor-types. It has also been established as a marker with diagnostic value for other diseases characterized by vascular disorders and enhanced angiogenesis. In this respect it is important that the new RELIDA® (*receptor ligand detection assay*) will offer a simple and accurate method for the quantitation of biologically active VEGF-A in many different sample types.

### **Vascular endothelial growth factor**

Vascular endothelial growth factor (VEGF or VEGF-A) is a mitogen for vascular endothelial cells derived from arteries and veins, but it is devoid of consistent mitogenic activity for other cell types. VEGF-A is also known as vascular permeability factor (VPF), based on its ability to induce vascular leakage in the guinea-pig skin and in different models of vascular permeability. An increase in vascular permeability is a crucial step in tumor- and wound- angiogenesis. Besides the VEGF-related mitogen placenta growth factor (PlGF) other molecules of the VEGF family were described in the last years and consequently named VEGF-B to VEGF-E. The newly described family members VEGF-C and VEGF-D can also activate lymphatic endothelial cells. The human VEGF-A gene is organized in eight exons, separated by seven introns. Alternative splicing of the eight exons leads to the formation of at least five different molecular species of VEGF having 121, 145, 165, 189 and 206 amino acids. VEGF<sub>165</sub> is the predominant molecular species produced by a variety of normal and transformed cells. It is a basic, heparin-binding and homodimeric glycoprotein of 45kDa. VEGF<sub>121</sub> is also secreted by many cell types. But this isoform of VEGF-A does not bind to heparin and thus has a different pattern of distribution and bio-availability. VEGF-A binds to and activates two different receptor tyrosine kinases known as VEGFR-1 (Flt-1) and VEGFR-2 (KDR).

Among the mechanisms that have been proposed to participate in the regulation of VEGF-A gene expression, oxygen tension plays a major role both *in vitro* and *in vivo*. VEGF-A mRNA expression is rapidly induced in normal and transformed cultured cell types by exposure to low oxygen levels.

However, hypoxia increases the VEGF-A production not only by transcriptional activation, but also by enhancing the VEGF-A mRNA stability. Similarities exist in the mechanisms leading to the hypoxic regulation of VEGF-A and erythropoietin (Epo). VEGF-A transcription is also activated by oncogenes like H-ras and several transmembrane tyrosine kinases, such as epidermal growth factor receptor and ErbB2. Together these pathways account for a marked upregulation of VEGF-A in tumors compared to normal tissues and are often of prognostic importance and relevance.

### **The significance of the RELIDA®**

Many factors contribute to the regulation of angiogenesis. VEGF-A, as an endothelial cell specific growth factor, is the major key player involved in all varieties of physiological and pathological angiogenesis. Direct demonstration of the importance of VEGF-A for tumor growth has been achieved by using either dominant negative VEGF receptors to block *in vivo* proliferation of endothelial cells, or blocking antibodies to VEGF-A or to one of the VEGF-A receptors. Other pathological conditions as psoriasis, rheumatoid arthritis, the ovarian hyperstimulation syndrome and diabetic retinopathies are also associated with enhanced angiogenesis or enhanced vascular permeability induced by VEGF-A.

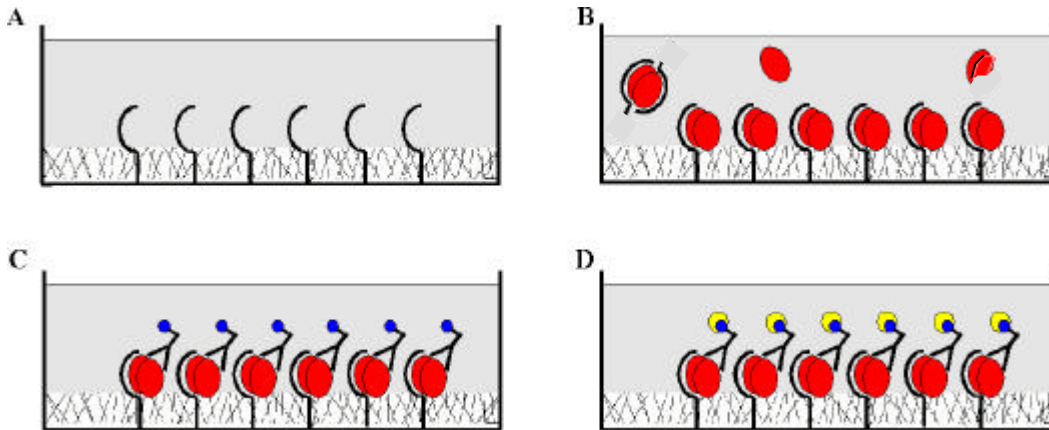
Compared to control sera the VEGF-A levels are elevated in both plasma and serum samples of tumor patients. Extremely high levels of VEGF-A can also be detected in the cystic brain fluids of brain tumor patients or in ascites fluid of patients. Platelets may be a major source of VEGF-A delivery to tumors, which they release upon aggregation. Several other studies have shown that high serum levels of VEGF-A are associated with a poor prognosis for cancer progression or a relapse free survival after surgical tumor excision. In this respect it is important to mention that this new VEGF-A RELIDA® offers a simple and reliable method for the quantitation of biologically active VEGF-A in serum or plasma samples, ascites fluids, intraocular-fluids or cell culture samples.

### **The principle of the RELIDA®**

This assay was designed to quantitate biologically active human vascular endothelial growth factor-A (VEGF-A) in cell culture supernatants or complex biological fluids. The type of the assay is unique based on its novel design with soluble VEGF-A receptors as capture molecules of active ligands. The soluble receptor molecules are used for high affinity solid phase binding of VEGF-A in the presence of biomatrix material. The assay type mimics the physiological ligand-receptor interaction and binds VEGF<sub>165</sub> with a  $K_D$  of about 30pM. All secreted isoforms of VEGF-A and also cell-associated isoforms (VEGF<sub>145</sub>, VEGF<sub>189</sub>), if these were solubilized by the action of proteases, can be measured with the RELIDA®. The assay will recognize only free, uncomplexed

and biologically active forms of VEGF-A that are not sequestered by soluble receptors. Sequestered, monomeric and degraded VEGF-A molecules will not be detected.

**Figure 1**



**Figure 1** depicts the assay principle: The „ready to use“ plates are precoated with a recombinant VEGF receptor and a specific proteoglycan to mimic a biological matrix and enhance VEGF-A binding (A). VEGF-A standard and samples are applied in a first incubation step (B), and bound VEGF-A is detected by a biotinylated antibody (C). After incubation with a horseradish-peroxidase- labeled streptavidine (D), the assay can be developed with TMB-substrate and measured in a common ELISA-plate reader.

The kit includes reagents for one 96-well ELISA plate. The newly developed RELIDA® has a sensitivity of 11 pg VEGF-A/ml sample. The range of detection is 16 pg/ml to 1000 pg/ml. The intra- and inter-assay variations were determined as 5.9 % and 17.7%, respectively.

### **III. Vascular Endothelial Growth Factor (VEGF-A) and its soluble receptor sVEGFR-1: two key players for physiological and pathophysiological blood vessel formation**

Normal tissue function depends on a regular supply of oxygen through the blood vessels. Understanding the formation of blood vessels has become the focus of a major research effort throughout the last decade. **Vasculogenesis** in the embryo is the process by which new blood vessels are generated *de novo* from primitive precursor cells. **Angiogenesis** is the process of new blood vessel formation from pre-existing vasculatures. It plays an essential role in development,

normal tissue growth, wound healing, the female reproductive cycle (placental development, ovulation, corpus luteum) and also plays a major role in various diseases [1]. Special interest is focused on tumor growth, since tumors cannot grow more than a few millimeters in size without developing a new blood supply. This process is described as **tumor angiogenesis** which is also essential for the spread and growth of tumor cell metastasis. One of the key molecules for angiogenesis and for the survival of the endothelium is vascular endothelial growth factor (VEGF-A) [2]. It is a specific endothelial cell mitogen and a strong vascular permeability factor (VPF) [3]. VEGF-A is a heparin-binding glycoprotein, secreted as a homodimer of 45 kDa by many different cell types. VEGF-A also causes vasodilation through the nitric oxide synthase pathway in endothelial cells and can activate migration in monocytes. Many different splice variants of VEGF-A have been described, but VEGF<sub>165</sub> is the most predominant protein and anchors with its heparin binding domain to extracellular matrix and to heparin sulfate. During the past few years, several other members of the VEGF family have been cloned, including VEGF-B, -C, -D and the Orf virus encoded VEGF-E [4,5]. In terms of vascular angiogenesis, which mainly is regulated by VEGF-A, lymphangiogenesis is mainly regulated by VEGF-C and -D [6].

### **Therapeutic implications:**

VEGF-A transcription is highly activated by hypoxia and by oncogenes [7] like H-ras and several transmembrane tyrosine kinases, such as epidermal growth factor receptor and ErbB2 [8]. Together these pathways account for a marked upregulation of VEGF-A in tumors compared to normal tissues and are often of prognostic importance and relevance [9,10]. VEGF-A can be detected in both plasma and serum samples of patients, with much higher levels in serum [11]. Extremely high levels can be detected in the cystic brain fluid of brain tumor patients [12,13] or in ascites fluid of patients. Platelets release VEGF-A upon aggregation and may be another major source of VEGF-A delivery to tumors [14]. Several other studies have shown that association of high serum levels of VEGF-A with poor prognosis in cancer patients may be correlated with an elevated platelet count [15]. Tumors can release cytokines and growth factors that stimulate the production of megakaryocytes in the marrow and elevate the platelet count. This can result in another, indirect increase of VEGF-A delivery to tumors [16]. Furthermore, VEGF-A is implicated in several other pathological conditions associated with enhanced angiogenesis or enhanced vascular permeability. Examples where VEGF-A plays an important role are psoriasis and rheumatoid arthritis [17], as well as the ovarian hyperstimulation syndrome [18]. Diabetic retinopathy is associated with high intraocular levels of VEGF-A, and inhibition of VEGF-A function may result in infertility by blockage of corpus luteum function [19]. Direct demonstration of the importance of VEGF-A in tumor growth has been achieved using dominant negative VEGF receptors to block in vivo

proliferation [20], as well as blocking antibodies to VEGF or to one of the VEGF receptors [21]. Interference with VEGF-A function has therefore become of major interest for drug development to block angiogenesis and metastasis. More than 110 pharmaceutical companies world-wide are involved in the development of such antagonists. Their approaches include antagonists of VEGF-A or its receptors, selective tyrosine kinase inhibitors, targeting of drugs and toxins to VEGF receptors and gene therapy regulated by the same hypoxia pathway that controls VEGF-A production. Targeting the VEGF signalling pathway may be of major therapeutic importance for many diseases [22] and serves as a basis for the design of future (anti)-angiogenic treatments.

### **The role of soluble VEGFR-1 (sVEGFR-1)**

There are three receptors in the VEGF family: VEGFR-1 (Flt-1), VEGFR-2 (KDR) and VEGFR-3 (FLT-4) [23]. VEGF-A signals through two receptors, VEGFR-1 and VEGFR-2, which are expressed predominantly but not exclusively on vascular endothelial cells. VEGFR-2 seems to be the principal signalling receptor for vascular endothelial cells, whereas VEGFR-1 is important for migration and may probably function as a decoy receptor, serving to regulate the bioavailability of VEGF-A in a given tissue [1]. VEGFR-3 was found to be predominantly expressed on lymphatic vessels during development and has an important function in lymphangiogenesis [24]. A naturally occurring soluble form of VEGFR-1 was discovered in 1990 and later the protein was isolated from the supernatant of vascular endothelial cells and monocytes [25,26] and is abundant in several biological fluids where angiogenesis is advancing [27]. The soluble receptor (sVEGFR-1) is generated by differential splicing and has a size of about 110 kDa with a unique C-terminus. It can be found in mouse as well and expression changes during placentation [28]. The physiological function is not fully understood, but several reports indicate, that the soluble form serves as a decoy receptor reducing VEGF-A availability and prevents overgrowth of endothelial cells into vessel lumen. It also neutralizes active VEGF-A lowering vascular leakage when it is released into the blood stream.

In several tissues VEGF-A and sVEGFR-1 are coexpressed and both genes can be induced by hypoxia [29]. High levels of sVEGFR-1 can be found in amniotic fluids of woman as a pregnancy-associated factor. The level depends on gestational age and is dependent on several diseases associated with pregnancy [30]. Soluble VEGFR-1 found in tissues and complex biological fluids are often complexed with VEGF-A. The placenta secreted sVEGFR-1 was found to be released into the maternal circulation where it binds VEGF-A; a finding first not observed in sera from men or non-pregnant women [31]. Meanwhile the presence of sVEGFR-1 was also shown in the serum and plasma of healthy male and female donors indicating that this soluble form of VEGFR-1 not only plays a role in pregnancy but might have a more general function in the “fine” regulation of VEGF-

A mediated activities *in vivo* [26]. The relationship between VEGF-A and sVEGFR-1 is an important marker for *in vitro* fertilisation protocols. High levels of VEGF-A may lead to ovarian hyperstimulation syndromes. Low levels of VEGF-A and excess sVEGFR-1 levels reduce the chance for successful follicle growth and maturation [32]. The sVEGFR-1 can be measured in nearly all follicle samples and has remarkably similar levels in both leading follicles.

In tumor tissues the relationship between VEGF-A and sVEGFR-1 may have an important prognostic value: excess of sVEGFR-1 seems to be a positive indicator for tumor-free survival in patients. In addition, the measurement of the amount of sVEGFR-1 in serum of patients with advanced renal cancer before and 1 month after anti-angiogenic therapy indicate that sVEGFR-1 may be of value in assessing anti-angiogenic treatments targeting different components of the vasculature or angiogenic therapy [33].

## Summary

In many vascular diseases VEGF-A and sVEGFR1 are important regulators for the generation of new blood vessels. The relationship between VEGF-A and sVEGFR-1 defines the amount of free, active VEGF-A that can bind to the signalling receptor proteins. Recent experimental evidence suggests that the relationship has important prognostic values in several situations where angiogenesis is ongoing. For example, physiological processes such as placental development, corpus luteum formation or pathophysiological situations like tumor angiogenesis or diabetic retinopathies. sVEGFR-1 is involved in the fine tuning of VEGF-A availability, but the exact regulation and release from endothelial cells is a matter of debate and will be further investigated [26,32].

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## **Characteristics of VEGF-A and sVEGFR-1:**

### **VEGF-A**

- Specific mitogen for endothelial cells, but normally not produced by them
- Regulates physiological and pathophysiological angiogenesis
- Acts as an potent vascular permeability factor
- Upregulated by hypoxia
- Key molecule for tumor angiogenesis and progression
- Maintenance and survival of endothelial cells  
No expression in quiescent endothelium/vessels

### **sVEGFR-1**

- Produced by activated endothelial cells and monocytes
- Generated by differential splicing and proteolytic processes
- Specific binding of VEGF-A, VEGF-B and PlGF
- Acts as a negative regulator of VEGF bioavailability

## **Clinical and pre-clinical observations**

### **VEGF-A**

- Elevated VEGF-A levels in tumor correlates with poor prognosis
- Blocking of VEGF or VEGF receptors prevents tumor growth
- Elevated levels in many vascular diseases, e.g. diabetic retinopathy
- VEGF gene transfer restores blood perfusion in ischemic organs
- Gene expression is highly upregulated in tumor tissues

### **sVEGFR-1**

- Release of sVEGFR-1 during pregnancy reduces the level of free VEGF in blood stream
- High levels of sVEGFR-1 in amniotic and follicle fluids
- Acts as a sink to neutralize excess VEGF/PlGF produced during development of placenta
- Relationship between VEGF-A and sVEGFR-1 is important for success of IVF
- Excess of soluble receptor in tumor tissue is a positive indicator of tumor-free survival
- Transfer of the sVEGFR-1 gene into tumor cells inhibits tumor growth
- Application of sVEGFR-1 prevents neovascularization in diabetic retinopathy models