Localization and functional study of VEGF receptors in normal and adenomatous pituitary: evidence for a non-angiogenic role of VEGF

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ABBREVIATIONS

ACRO	Somatotrophinoma
ACTH	Adrenocorticotropic hormone
Akt	Protein kinase B
Bcl-2	B-cell leukemia 2
CREB	cAMP-response-element-binding
CUSH	Corticotrophinoma
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
ERK	Extracellular signal regulated kinase
FGF	Fibroblast growth factor
Flk-1	Fetal liver kinase 1
KDR	kinase insert domain containing receptor
Flt-1;-4	*fms*-like tyrosine kinase receptor-1, -4
FSH	Folliculo-stimulant hormone
GAPDH	Gyceraldehyde-3-phosphate dehydrogenase
GH	Growth hormone
GSK3-β	Glycogen synthase kinase-3 subunit β
HGF	Hepatocyte growth factor
IGF-1	Insulin like growth factor-1
IHC	Immunohistochemistry
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
ISH	*in situ* hybridization
LOH	Loss of heterozygosity
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
LYVE-1	Lymphatic vessel endothelial hyaluronan receptor-1
MAPK	Mitogen activated protein kinase
MEK	MAPK-1
MEN-1	Multiple endocrine neoplasia type 1

NFPA	Non functioning pituitary adenoma
NP	Normal pituitary
PDGF	Platelet derived growth factor
PDGF-R	Platelet derived growth factor receptor
PDK-1	Phosphoinositide dependent kinase-1
PI	Proliferation index
PI3K	Phosphatidylinositole 3´-kinase
PI(4,5)P2	Phosphatidylinositole-4, 5 bisphosphate
PI(3,4,5)P3	Phosphatidylinositole-3,4,5 trisphosphate
PlGF	Placenta growth factor
PROL	Prolactinoma
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PTTG	Pituitary transforming gene
Rb	Retinoblastoma
RIA	Radioimmunoassay
RT-PCR	Reverse transcriptase-Polymerase chain reaction
TGF-α	Transforming growth factor-α
TGF-β	Transforming growth factor-β
THYR	Thyreotrophinoma
TNF-α	Tumour necrosis factor-α
TSH	Thyreo-stimulant hormone
TSP	Thrombospondin
VEGF	Vascular endothelial growth factor
VEGFR-1, -2, -3	Vascular endothelial growth factor-1, -2, -3
Zac	Zinc finger protein inducing Apoptosis and cell Cycle arrest

1 INTRODUCTION

1.1 Anatomy of the pituitary gland

The pituitary gland is a small oval body positioned on a saddle-shaped depression of the sphenoid bone (sella turcica) which plays a central role in the hormonal regulation of several processes in the human physiology. Its function is mainly regulated by another structure of the brain, the hypothalamus, a brain area localized just above the pituitary gland.

This gland consists of three different parts: the endocrine part which constitutes the anterior lobe or adenohypophysis and derives embriologically from an evagination of the oral cavity (Rathke´s pouch) and the neuronal part known also as posterior lobe or neurohypophysis, derived by a downward extension of the hypothalamic area, which forms the hypophyseal stalk. Between the anterior and posterior lobes there is a third part, the intermediate lobe, which in rodents plays a significant role in the regulation of pigmentation but in human is reduced to some cells without any specific function.

The neurohypophysis consists of nervous fibres and neuroglial cells called pituicytes. The fibres are the axons of neurons belonging to the hypothalamic paraventricular and supraoptic nuclei. Pituicytes secrete, directly in the fenestrated capillaries supplying the neurohypophysis, two hormones: the oxytocin, necessary to induce uterus contractions during delivery and the antidiuretic hormone, which regulates the renal water reabsorption.

The anterior pituitary gland is composed of both endocrine and non-endocrine cells. There are five different types of endocrine cells that, under the control of releasing and inhibiting hypothalamic factors, synthesize six different hormones. The most prevalent are the somatotrophs (around 50%) whose product is the growth hormone (GH), then come the corticotrophs (around 20%) which secrete the adrenocorticotrophic hormone (ACTH), the thyreotrophs (5%) which are known to secrete thyroid-stimulating hormone (TSH), the lactotrophs

that release prolactin and the gonadotrophs producing follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The activities of these hormones are disparate, as it can be seen from the physiologic responses they induce. GH promotes growth of the skeleton and soft tissues and has important metabolic effects. It acts directly on peripheral GH receptors or indirectly by inducing insulin-like growth factor I (IGF-I) synthesis in the liver. ACTH induces glucocorticoid secretion from adrenal cortex and is a split product of proopiomelanocortin, a precursor protein from which also β-lipotropic hormone, endorphins, encephalin, corticotropin-like immunoreactive peptide and melanocyte stimulating hormone, are produced. TSH is important for the physiological growth and function of the thyroid gland, whereas prolactin is necessary for the initiation and maintenance of lactation. Finally FSH and LH, collectively referred as gonadotrophins, are respectively known to promote follicular growth in the ovaries and spermatogenesis and to induce the development of corpus luteum.

Approximately 5–10% of all pituitary cells is represented by a non-endocrine cell type: the folliculo-stellate cells (FS-cells) [Rinehart and Farquhar, 1953]. The name is due to their star-shaped morphology and ability to form tiny follicles. They do not secrete pituitary hormones and are connected to each other and to hormone-producing cells with gap junctions [Morand et al., 1996]. They also exhibit a phagocytic activity and are known to work as scavenger cells. Although the origin of this cell type is still unclear, they are immunopositive for S100 protein [Nakajima et al., 1980] and glial fibrillary acidic protein [Velasco et al., 1982], which are markers for cells of neuroectodermal origin. FS-cells produce many cytokines or growth factors that control the function of neighbouring cells, such as, interleukin-6 (IL-6) [Vankelecom et al., 1993], leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF) and vascular endothelial cell growth factor (VEGF) [Renner et al., 1998; Renner et al., 2004]. Nowadays, there is increasing evidence that anterior pituitary cells are not only regulated in a classical endocrine manner by hypothalamic factors and circulating peripheral hormones, but also by locally produced factors that act through auto- or paracrine mechanisms [Renner et al., 1998; Ray and Melmed, 1997].

1.2 Vascularization of normal anterior pituitary gland

Unlike the majority of other tissues, the anterior pituitary gland, receives a dual blood supply (Fig. 1). The main vascular source is the hypothalamo-hypophyseal portal system. At the same time, a direct arterial supply deriving from the systemic vasculature gives rise to the inferior hypophyseal artery, capsular vessels and middle hypophyseal artery. It is thought that this direct arterial input from the internal carotid artery and its basal branches may provide oxygen and nutritional substances to the pituitary cells, whereas the hypothalamic-hypophyseal portal veins provide a mean of communication between the hypothalamus and the adenohypophysis [Gorczyca and Hardy, 1988]. In fact, it is through these vessels that the different releasing and inhibiting factors produced by the neuroendocrine cells of the hypothalamus and released in the blood flow of the median eminence capillaries, reach the endocrine cells of the anterior lobe and control their hormone secretion. The sinusoid-capillary network of the anterior lobe has a fenestrated layer of endothelial cells, as in all endocrine organs, which allows hormones and growth factors to diffuse into the surrounding tissue and in the systemic bloodstream and vice versa.

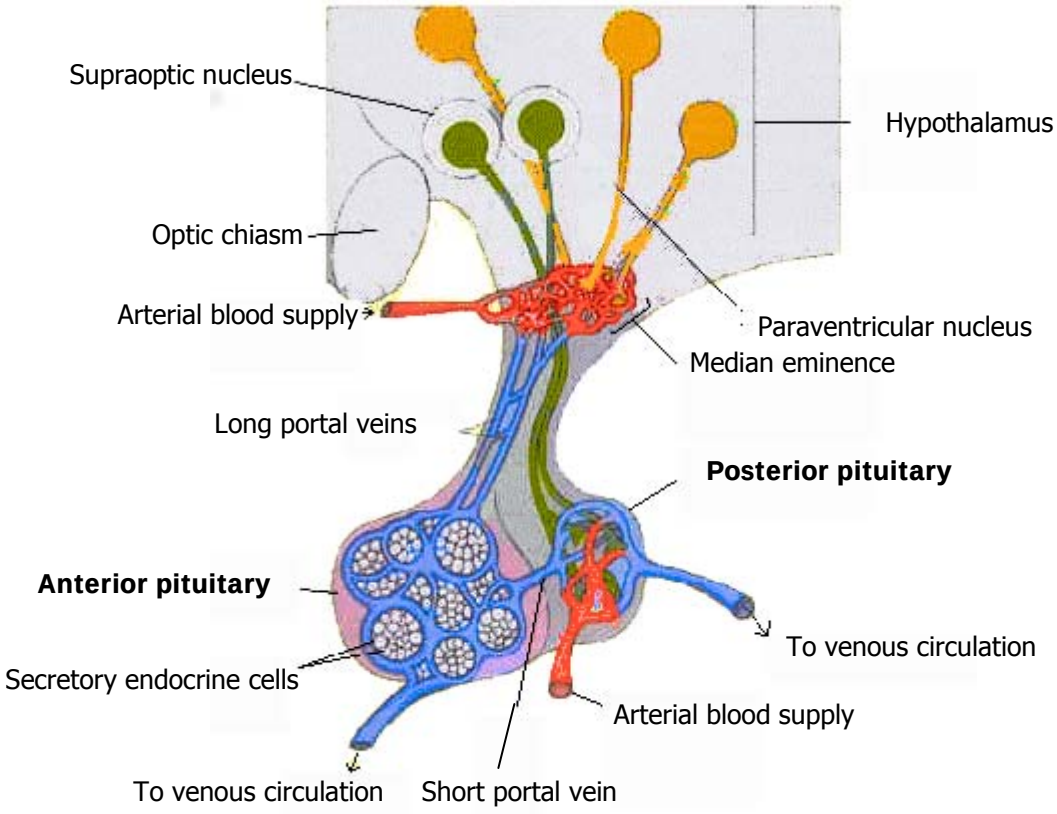


Figure 1. Vascularization of the pituitary gland. A branch of the hypophyseal artery ramifies into a capillary bed in the lower hypothalamus, where hypothalamic hormones destined for the anterior pituitary are secreted. Blood from these capillaries goes into hypothalamic-hypophyseal portal veins that branch again into another series of capillaries within the anterior pituitary. The vessels of this network, which carry the secreted pituitary hormones, join together and end into veins that, after collecting capillary blood from posterior pituitary gland, drain into the systemic venous blood.

1.3 Pituitary adenomas

Pituitary adenomas are benign neoplasms, accounting for approximately 15% of intracranial tumours. An occult adenoma is discovered in about 25% of unselected autopsies. Results obtained from X-chromosome inactivation studies and LOH analysis showed that these tumours are monoclonal, which means that they initiate from a single transformed cell [Herman et al., 1990]. Pituitary tumours are usually benign and do not metastasize, although some of them can become invasive, leading to bone destruction and infiltration within the cavernous sinus. They can cause severe clinical symptoms due to their critical

location and expanding size, like headache and visual disorders, and to inappropriate pituitary hormone production. The clinical features associated with hormonal hypersecretion reflect specifically the type of endocrine cell from which the adenoma arises. Therefore, somatotrophinomas (ACRO) overexpress GH, causing gigantism in children and acromegaly in adults, which is associated with increased risk of hypertension, cardiac disease and diabetes; transsphenoidal surgical resection is the therapy chosen if the treatment with somatostatin analogues is not effective in suppressing GH secretion and reducing tumour volume. Prolactinomas (PROL), which are the most common of all functioning pituitary adenomas, cause amenorrhea, infertility and galactorrhea in female patients and impotence or infertility in males, because of the prolactin hypersecretion. Most prolactinomas show good response to dopamine agonists, although there are few cases with resistance. Reduction of dopamine- binding sites and absence of dopamine D2 receptors was demonstrated in prolactinomas from patients resistant to dopamine treatment [Caccavelli et al., 1994]. Corticoptrophinomas (CUSH) lead to ACTH hypersecretion with consequent adrenal steroid overstimulation; these patients present truncal obesity, striae, muscle wasting, hirsutism, cardiovascular complications, osteoporosis and psychiatric disturbances. Transphenoidal surgery is the treatment of choice. Pure gonadotrophinomas secreting intact FSH or LH are quite rare and may cause sexual dysfunction and hypogonadism. Thyreotrophinomas (THYR) cause a mild increase in thyroxine levels with inappropriate TSH secretion and consequential hyperthyroidism. Transphenoidal surgery is the treatment of choice, although octreotide administration can often normalise TSH levels and induce tumour shrinkage [Beck-Peccoz et al., 1996]. Non functioning pituitary adenomas (NFPA), also known as hormone inactive, do not secrete hormones and therefore have no typical hormone excess-related presentation. At diagnosis, patients have usually already very large tumours, presenting mass effects such as visual problems, neurological symptoms or disturbances related to hormone hyposecretion. Transphenoidal surgery is the best way to treat these tumours.

The functional classification outlined above is not the only one used for pituitary adenomas. They can also be classified according to tumour size and local invasion (anatomical classification), or according to histological and

cytological features (histological classification). The anatomical classification (Hardy´s classification; [Hardy, 1979]) divides pituitary adenomas in four grades: grade I comprises the microadenomas with a diameter smaller than 10 mm; grade II refers to macroadenomas, bigger than 10 mm in diameter, that may exhibit suprasellar extension but no invasion to the surrounding bony structures; grade III adenomas are locally invasive tumours and grade IV refers to large invasive tumours that can invade, not only the bones, but also the hypothalamus and the cavernous sinuses and can metastasize.

The histological classification is based on a combination of the results of immunohistochemistry and electron microscopy, which allows the identification of the pathologic cell types on the basis of their cellular hormonal content and their ultra-structural features. Thanks to this method it was possible to identify that among pituitary adenomas there are some tumours producing more than one hormone, i.e. some somatotrophinomas secrete both GH and prolactin (mixed GH-PRL adenomas and mammosomatotrophinomas), whereas some prolactinomas show GH reactivity (acidophilic stem cell adenomas). The most interesting findings are related to non functioning pituitary adenomas; most of these tumours were found to display reactivity for FSH, LH and/or α–subunit (common subunit shared by gonadotrophins and TSH), showing that the non functioning pituitary tumours lacking hormone reactivity are a minority. Furthermore, some non functioning pituitary tumours display immunoreactivity for ACTH (silent ACTH adenomas).

In this study, the pituitary tumours investigated have been classified according to the clinical presentation and subdivided in four grades according to the anatomical localization and degree of invasion of the surrounding structures.

1.4 Cell cycle and tumour development- general overview

Cell cycle is the process by which a cell growths, replicates its genome and finally divides in two daughter cells. It is an event strictly controlled at different crucial points (restriction points), since its deregulation can lead to uncontrolled cell proliferation and consequently to tumour development. The cell cycle consists

of different phases: G1 phase, between the end of mitosis and beginning of DNA synthesis (S phase), in which the cell gets ready to enter into S phase by producing all the enzymes necessary for the replication of its genome; G2 phase occurs instead between DNA synthesis and mitosis (M phase), and during this period the cells produce the factors needed for the correct segregation of the chromosomes in the two daughter cells. In absence of mitogenic signals (e.g. growth factor binding to its receptor), the cell leaves the cell cycle and enters a quiescent phase called G0, where it can growth and differentiate.

The most important restriction point in mammalian cells is in G1 phase. During the early G1 phase and after receiving a mitogenic signal, members of the cyclins D family bind and therefore activate the cyclin dependent kinases CDK 4/ 6 [Baldin et al., 1993], that will phosphorylate the members of the retinoblastoma (Rb) protein family. The full phosphorylated form of these proteins (cyclin E/ CDK2 complex completes Rb phosphorylation in the late G1 phase) releases the E2F transcription factor [Weintraub et al., 1992], which can subsequently bind to the promoters of several growth-promoting genes, such as c-myc, c-mib, thymidine kinase, DNA polymerase α and cyclin A [Sala et al., 1994]. The latter protein sequesters CDK-2 from cyclin E and allows the progression through the S phase to G2 phase [Jeffrey et al., 1995]. During this phase, the activated cyclin B/ CDK1 complex induces the expression of genes involved in the production of the metaphase promoting complex, which plays an important role in mitosis [Pines and Hunter, 1989].

The negative control on cell cycle progression is exerted by the cyclin dependent kinase inhibitors, which bind to CDK and inhibit their activity [Pavletich, 1999]. The members of the INK family (p16, p15, p19) inhibit CDK 4/ 6 in the early G1 phase [Serrano et al., 1999], whereas members of the WAF/KIP family (p21, p27, p57) inhibit CDK2 activity in the late G1 phase [Nakayama and Nakayama, 1998].

Other factors are involved in the negative regulation of cell cycle progression. In the M phase for example, the protein securin binds and therefore prevents the protease separin to digest the protein cohesin, important for tethering the sister chromatides during metaphase. The degradation of securin triggers the anaphase and occurs only after activation of Anaphase Promoting Complex [Zou et al.,

1999]. The product of the pituitary tumour transforming gene (PTTG) is identical to the securin protein [Kakar and Jennes, 1999]. Members of the p53 family play also a role in cell cycle progression control. These transcription factors are induced by oncogenes, DNA damage and other stress signals, and promote the transcription of several genes involved in cell cycle control (e.g. p21), DNA repair and programmed cell death (apoptosis) [reviewed in Vousden and Lu, 2002].

Tumour development and growth are thought to be the outcome of a series of different mutations occurring in a somatic cell, leading to progressive acquisition of proliferative advantage compared to non-mutated cells. These mutations can affect different genes essential for cell survival, belonging to two major categories: proto-oncogenes and tumour suppressor genes. Proto-oncogenes have an important role in the regulation of cell proliferation, differentiation and apoptosis. They usually act as positive regulators of cell growth and in cancer they can be activated by gain-of-function point mutations, or overexpressed because of gene amplification, translocation of the proto-oncogene in an area of actively transcribed chromatin, increase in promoter activity or protein stability. Other causes of oncogene activation in cancer can be translocation of the proto-oncogene near to another gene, whose fusion lead to the production of a protein with transforming properties [reviewed in Munger, 2002]. Mutations affecting proto-oncogenes are defined as dominant since the mutation in one allele is sufficient to determine the phenotype.

On the other hand, tumour suppressor genes inhibit cell proliferation and are mostly involved in the inhibition of cell cycle progression, induction of cell differentiation and programmed cell death and in the assemblement of the mitotic machinery. They acquire transforming potential loosing their anti-proliferative properties. This can be due to loss-of-function mutations, loss of a big chromosomic portion or to epigenetic alterations, like hypermethylation of cytosine-guanine islands in promoters with consequent gene silencing [Baylin et al., 1998]. Mutations occurring in tumour suppressor genes are recessive, so in order to loose their growth inhibiting phenotype, both alleles must be inactivated.

1.5 Pituitary tumour genesis

Pituitary tumour development results from both intrinsic alterations of the pituitary gland cells, as well as from deregulation of regulatory factors like hypothalamic releasing and inhibitory hormones, peripheral hormones and paracrine growth factors.

There are two main theories about pituitary tumour genesis. One implies the occurring of a mutational transforming event in a single cell that then will proliferate under the effect of hormones and growth factors, originating a monoclonal tumour. The second theory supports the fact that hypothalamic hormones and growth factors induce the proliferation of target pituitary (hyperplasia) in which a mutational event in a hyperplastic cell will lead to tumour formation. Rarely, a further mutational event can induce a malignant transformation leading to pituitary carcinoma development. LOH and X-inactivation experiments support the monoclonal theory, although there are reports of different LOH patterns in recurring pituitary tumours, meaning that they are different clonal entities from the original tumours, even though they are still monoclonal themselves [Clayton and Farrel, 2001].

The alterations that occur in pituitary cells and trigger pituitary tumour growth involve, as for other tumours types, activation or overexpression of proto-oncogenes or inactivation or loss of tumour suppressor genes. Overexpressed oncogenes described in pituitary tumour genesis are: G proteins [Bertherat et al., 1995; Weinstein et al., 1991], cyclin D1 [Hibberts et al., 1999], growth factors and their receptors (EGF, TGF-α, with their common receptor EGFR [Kontogeorgos et al., 1996; Theodoropoulou et al., 2004 a], FGF-2 with its receptors FGFR-1, -2, -3 [Ezzat et al., 1995; Ezzat et al., 2002]), PTTG [Pei, 2000; McCabe et al., 2002; McCabe et al., 2003]. While, concerning the tumour suppressor genes lost or deregulated in pituitary adenomas: MEN-1 encoding for the protein menin [Theodoropoulou et al., 2004 b], Rb [Pei et al., 1995; Simpson et al., 2000], p16 [Woloschack et al., 1997], p27 [Lidahr et al., 1999] and Zac

encoding for a zinc finger transcription factor [Pagotto et al., 2000], have been reported.

1.6 PI3K/Akt signalling pathway and its involvement in tumour development

The PI3K/Akt signalling pathway is activated by many types of cellular stimuli and regulates fundamental cellular functions, such as proliferation, growth and survival (Fig. 2) [Datta et al., 1999; Vivanco and Sawyers, 2002]. The development and progression of cancer are the outcomes of a disturbance in the balance between cell proliferation and apoptosis and PI3K/Akt signalling is involved in both these events [Testa and Bellacosa, 2001; Nicholson and Andersons, 2002]. Tyrosine kinase receptors activate this pathway by binding the PI3K regulatory subunit (p85) to the phosphorylated tyrosine of their cytoplasmatic domain [Vanhaesebroeck and Waterfield, 1999]. This leads to the activation of the PI3K catalytic subunit (p110) which converts the plasmatic membrane phospholipid phosphatidylinositole-4,5 bisphosphate (PI(4,5) P2) to phosphatidylinositole-3,4,5 trisphosphate (PI(3,4,5) P3). The major negative regulator of this pathway is the PTEN phosphatase that catalyzes the opposite reaction [Myers and Tonks, 1997]. PI (3,4,5) P3 causes a conformational change in Akt, resulting in the exposure of its two main phosphorylation sites (Thr 308 in the kinase domain and Ser 473 in the C-terminal regulatory domain) [Alessi et al., 1996]. PDK1 phosphorylates Akt at Thr 308 and stabilizes its active conformation. Akt is fully activated after phosphorylation at Ser 473 within the C-terminus, although the identity of the kinase catalyzing this step is still unknown.

Activated Akt modulates the function of several substrates involved in the regulation of cell proliferation and apoptosis [Yao and Cooper, 1995], such as GSK-3β [Cross et al., 1995] and cyclin dependent kinase inhibitors like p21 [Zhou et al., 2001] and p27 [Liang et al., 2002]. Inhibition of GSK-3β by Akt-mediated phosphorylation leads to a decrease in the degradation rate of cyclin D1 [Diehl et al., 1998], which is then free to bind to CDK4-6, generating active holoenzymes helping the inactivation of the Rb protein growth-suppressive function [reviewed

in Weinberg, 1995]. In addition, Akt is known to block the anti-proliferative action of p21 and p27, by phosphorylating them and retaining them in the cytoplasm, where they cannot form a complex with and deactivate CDK1-cyclin D complex. Concerning the Akt involvement in the control of apoptosis, it was reported that Akt phosphorylates CREB protein [Du and Montminy, 1998] which then induces the transcription of anti-apoptotic genes like Bcl-2 [Pugazhenthi et al., 2000].

PI3K signalling pathway can be overactivated by several events like: overexpression or constitutive activation of tyrosine kinase receptors, as it was demonstrated in breast, ovarian, colon cancers and glioblastomas overexpressing ErbB2, PDGFR-α and FGFR-1 [reviewed in Blume-Jensen and Hunter, 2001]; by activating mutations or gene amplification of its components, as it was shown for PIK3CA gene (encoding P110 α catalytic subunit of PI3K) which is overexpressed in human colon, gastric, breast and lung cancers [Samuels et al., 2004], and for the different Akt isoforms genes, which are amplified in glioblastoma and pancreatic cancers [Knobbe and Reifenberger, 2003; Cheng et al., 1996], or by inactivating mutations in PTEN which is the negative regulator of the pathway, as reported in human colorectal cancer [Goel et al., 2004].

PI3K is a necessary signalling component of VEGF-mediated cell cycle progression in human umbilical endothelial cells [Thakker et al., 1999]. In different cell types, activation of this pathway by VEGF promotes the growth and survival in melanoma cells [Graells et al., 2004] and in acute myeloid leukemia cells [List et al., 2004].

There are not many data about the proliferative and anti-apoptotic effects of the PI3K pathway in pituitary tumour cells, except that IGF-1 induced activation of the PI3K/Akt pathway inhibits apoptosis in cultured rat pituitary cells [Fernandez et al., 2004].

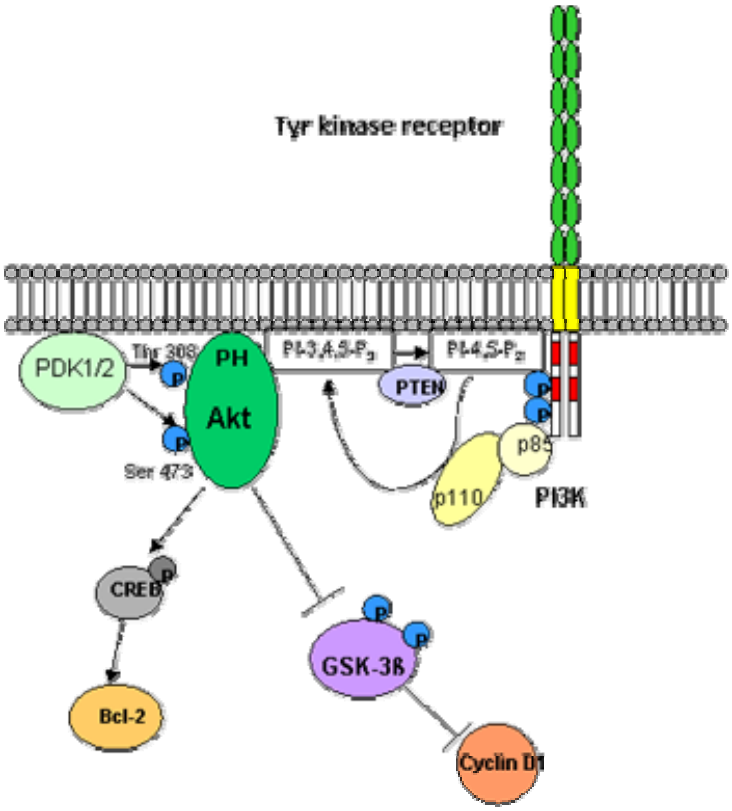


Figure 2. The PI3/Akt signalling pathway. The binding of the growth factors to their receptor tyrosine kinase stimulates the PI3K comprised of p85 and p110 subunits. PI3K converts PI(4,5) P2 to PI(3,4,5)P3, whereas PTEN reverses this reaction. Akt translocates to the cell membrane and interacts with PI3(3,4,5)P3 via its PH domain, being phosphorylated at two residues (Thr304 and Ser473) by PDK1/2. Once active, Akt controls fundamental cellular processes, such as, the cell cycle and cell survival by phosphorylating and activating (i.e. CREB) or deactivating (i.e. GSK3-β) several signalling components.

1.7 Angiogenesis and lymphangiogenesis- general overview

1.7.1 Angiogenesis

Capillaries are the simplest and smallest units of the vascular apparatus. Although there are several varieties of capillaries each designed for a particular function, they are all composed of a single layer of endothelial cells surrounded by a basal membrane that is encircled by one or two layers of pericytes. Larger vessels show multiple layers of cellular and extracellular materials: tunica intima which consists of the endothelium, the basal membrane and internal elastic

tissue; the tunica media composed by a layer of smooth muscle, collagen III reticular fibres, elastin and proteoglycans and the tunica adventitia that consists in external elastic tissue and fibrous connective tissue. The thickness of the tunica media is greater in arteries than in veins, since they must stand higher blood pressure. The blood vessel endothelial cells share many common features (flat morphologic shape, tight intercellular junctions and continuous basement membrane) but they also display some remarkable differences according to their localization in the organism, e.g. capillaries in heart, muscles, skeleton, brain have continuous endothelium, whereas capillaries in endocrine and exocrine glands, choroid plexus and intestinal villi have fenestrations [Pasqualini et al., 2002].

There are two main processes that lead to vessel formation: vasculogenesis and angiogenesis. Vasculogenesis takes place during embryogenesis and involves the differentiation of endothelial cells from angioblasts to endothelial cells that assemble into a vascular labyrinth [Risau, 1997].

Angiogenesis is defined as the development of new blood vessels from pre-existing vasculature. It is a complex multistep process, involving stimulation of various pro-angiogenic growth factors production and reduction of angiogenesis inhibitors. Known pro-angiogenic growth factors are: VEGF [Ferrara, 1995], FGF-2 [Slavin, 1995], TGF-α [Schmitt and Soares, 1999], proliferin [Jackson et al., 1994], PDGF [Lindahl et al., 1999], IL-8 [Heidemann et al., 2003], HGF [Matsumoto and Nakamura, 1996]. While, angiogenesis can be inhibited by: angiostatin [Cao et al., 1998] and endostatin [O´Really et al., 1997], which are proteolytic fragments of plasminogen and collagen XVIII respectively, thrombospondins [Lawler, 1986] and LIF [Ferrara et al., 1992 b].

The process starts when activated endothelial cells release specific proteases, which lead to degradation of the extracellular matrix surrounding the vessels, followed by migration and proliferation of endothelial cells. They are then reorganized into tubular structures which make connections with other newly formed vessels, leading to the formation of an anastomotic network [Risau, 1997]. Physiological angiogenesis during adult life is mainly restricted to the female reproductive cycle and wound healing, while this process is usually inhibited in adult normal tissues [Canfield and Schor, 1995]. In pathological

conditions, like psoriasis [Nickoloff et al., 1994], retinal neovascularization [Sharp, 1995], arthritis [Colville-Nash and Scott, 1992] and cancer [Folkman, 1990; Hanahan and Folkman, 1996], angiogenesis becomes activated. This process plays a crucial role in tumour growth and development by supplying nutrients and oxygen to malignant cells and removing the catabolic substances [Folkman and Shing, 1992]. Moreover, the degradation of extracellular matrix, which takes place during angiogenesis through the action of matrix metalloproteinases, allows tumour invasion of surrounding structures, and the new blood vessels provide a way for metastatic tumour cells to enter the systemic circulation [Folkman, 1990; Gasparini and Harris, 1995]. In both physiological and pathological contexts, the extent of angiogenesis can be represented as microvessel density measured by counting vessels identified by positive immunostaining with antibodies to different blood vessel endothelial cell markers such as CD31, Factor VIII-related antigen, CD34 and Ulex europaeus agglutinin-1.

1.7.2 Lymphangiogenesis

The lymphatic system consists of a one-way, open-ended, complex network of vessels collecting and transporting lymph from peripheral tissues to the venous system. Lymph is composed of interstitial fluid components, metabolites and plasma proteins extravasated from blood capillaries and cells deriving from the immune system [Karkkainen et al., 2002]. Lymphatic vessels are lined by one layer of endothelial cells that are non-fenestrated and slightly allow the passage of large macromolecules, pathogens and migrating cells of the immune system. These vessels harbour a discontinuous or completely absent basal membrane, are non-contractile and are bound to extracellular matrix by elastic fibres which play a role in the lymphatic flow [Swartz, 2001]. The growth of lymphatic vessels is called lymphangiogenesis and occurs after tissue injury, obstruction or damage of lymphatic vessels, in the adult tissues. During the embryonic development, these vessels are thought to sprout from the embryonic veins in the jugular and perimesonephric regions, after the blood vascular system has been established [Kaipanen et al., 1995]. Mesodermal lymphangioblasts may also contribute to the

development of the lymphatic system [Wilting et al., 2000]. Typical lymphatic markers are VEGFR-3, that becomes restricted to the lymphatic endothelium during development [Kaipanen et al., 1995], the podocyte cell-surface mucoprotein podoplanin [Breiteneder-Geleff et al., 1997] and LYVE-1 that is related to CD44, which is the major receptor for hyaluronic acid in epithelial, mesenchimal and lymphoid cells [Banerji et al., 1999]. VEGF-C, which is a member of the VEGF family, binds specifically to VEGFR-3 and induces lymphatic endothelial cell proliferation *in vitro* [Oh et al., 1997] and lymphatic hyperplasia *in vivo* [Jeltsch et al., 1997].

Apart from roles in immunity and fluid homeostasis, the lymphatics are an important way for the early metastastatic spread of tumours. The occurrence of intratumour lymphangiogenesis has been demonstrated in several works [Dadras et al., 2003; Stacker et al., 2001; Skobe et al., 2001].

1.8 The vasculature and angiogenesis of pituitary adenomas

A direct extraportal arterial supply was identified in many pituitary adenomas and it is hypothesized that it may predispose to pituitary tumour development, since systemic blood contains low levels of hypothalamic hormones [Gorczyca and Hardy, 1988; Schechter et al., 1988]. From the ultrastructural point of view, the adenomatous capillaries were reported to have a thickened and swollen endothelium, devoid of fenestrations, with few cytoplasmic organelles, numerous pinocytic vesicles, multilayered and fragmented basal membrane and few sprouting capillaries [Erroi et al., 1986; Schechter, 1972].

Angiogenesis in pituitary adenomas, as well as, in other endocrine neoplasms, probably reflects the basic observations that tumours require neovascularization if they grow beyond 2 mm^3 [Jugenburg et al., 1995]. However, because normal endocrine cells are usually highly vascularized, the changes that occur during neoplastic development may be different from that taking place in some other tissues that are normally less vascularized [Lloyd et al., 2003]. Actually, reduced vascularization in pituitary tumour parenchyma,

compared to normal pituitary gland was reported by different groups [Schechter, 1972; Jugenburg et al., 1995; Turner et al., 2000 a]. This finding is quite unusual for tumours, but it may explain the slow growth rate of pituitary adenomas. An alternative explanation could be that the low growth rate of these tumours may not influence significantly the metabolic demand, so that vascularization does not limit their growth. Still, several studies have shown that even pituitary angiogenesis can be related to tumour behaviour and outcome. One of the first studies about angiogenesis in pituitary adenomas showed that in Fisher 344 rats, estrogen-induced prolactin-secreting pituitary tumours develop a new arterial supply, bypassing the normal hypothalamo-hypophyseal portal system and, in this way, escaping the dopamine-mediated inhibition of prolactin secretion [Elias and Weiner, 1984]. In addition, it has been reported that human macroprolactinomas are significantly more vascular than microprolactinomas [Turner et al., 2000 a]. The same observation was done comparing invasive prolactinomas to non-invasive tumours [Turner et al., 2000 b] and pituitary carcinomas to pituitary adenomas [Jugenburg et al., 1995; Turner et al. 2000 c; Vidal et al., 2001].

1.9 VEGF and its receptors

1.9.1 VEGF family

VEGF is one of the most important angiogenic factors. It is a member of the VEGF family together with VEGF-B, VEGF-C, VEGF-D, VEGF-E (a sheep parapoxvirus open reading frame encoding a VEGF-related protein [Lyttle et al., 1994]) and Placenta Growth Factor (PlGF) (Fig. 3). In humans, the most prevalent form of VEGF (also known as VEGF-A) is actually translated from six VEGF mRNA species encoding different VEGF isoforms of 121, 145, 165, 183, 189, and 206 amino acids ($VEGF_{121–206}$) that are produced by alternative splicing. These isoforms differ in their ability to bind to heparan sulphate proteoglycans in the extracellular matrix [Leung et al., 1989; Tischer et al., 1989]. $VEGF_{121}$,

$VEGF_{165}$ and $VEGF_{189}$ are the major secreted forms [Robinson and Stringer, 2001] and since mice expressing only $VEGF_{164}$ (murine homologue of $VEGF_{165}$) are viable and healthy [Stalmans et al., 2002], $VEGF_{165}$ can be considered as the principal effector of VEGF actions. VEGF plays a key role in both physiological and pathological angiogenesis by increasing the proliferation and migration of endothelial cells [Ferrara and Bunting, 1996], but also the permeability of vessels by inducing pores and fenestration in the endothelium [Esser et al., 1998]. In addition, VEGF is a potent survival factor for endothelial cells during physiological and tumour angiogenesis and it was shown to induce the expression of the antiapoptotic protein Bcl-2 in these cells [Gerber et al., 1998]. Besides its effect on angiogenesis, VEGF can play a role in other physiological processes: it induces mobilization of haematopoietic stem cells from the bone marrow, monocyte chemoattraction, osteoblast-mediated bone formation and neuronal protection [Ferrara et al., 2003; Storkebaum et al., 2004]. Furthermore, it stimulates inflammatory cell recruitment and promotes the expression of proteases implicated in pericellular matrix degradation that occurs in angiogenesis [Pepper et al., 1991; Unemori et al., 1992].

During embryogenesis, VEGF is initially mainly expressed at sites of active vasculogenesis and angiogenesis [Weinstein, 1999] in the anterior part of the mouse embryo and directs the migration of VEGFR-1 and VEGFR-2 positive cells in the embryonic tissues [Hiratsuka et al., 2005]. The outstanding role of this growth factor during embryogenesis is confirmed by the finding that homozygous VEGF knockout mice die at day 8 -9 of embryonic life from defects in the development of blood islands, endothelial cells and vessels [Ferrara, 2004].

1.9.2 VEGF receptors

VEGF activities are mediated by high-affinity receptor tyrosine kinases expressed primarily in endothelial cells. These are: VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), which are mainly expressed by blood vessel endothelial cells and VEGFR-3 (Flt-4) expressed in lymphatic endothelial cells (Fig. 3) [de Vries et al., 1992; Terman et al., 1992; Kaipainen et al., 1995]. These receptors are characterised by seven extracellular immunoglobulin-like domains, which bind the

growth factor, followed by a single membrane-spanning region and a conserved intracellular tyrosine kinase domain interrupted by a kinase insert sequence [Shibuya et al., 1990; Matthews et al., 1991; Terman et al., 1991; Pajusola et al., 1992]. These receptors are themselves enzymes and once activated from the ligand binding, they dimerize and undergo autophosphorylation. This step enhances the capacity of the receptor to directly activate other target proteins by phosphorylating them on specific tyrosine residues.

Each VEGF receptor binds specifically the different members of VEGF family: VEGF-A binds with high affinity to VEGFR-1 and VEGFR-2, VEGF-B and PlGF bind selectively to VEGFR-1, VEGF-C and VEGF-D bind to VEGFR-2 and VEGFR-3 and VEGF-E binds exclusively to VEGFR-2 (Fig. 3) [Vaisman et al., 1990;Park et al., 1994; Mustonen and Alitalo, 1995].

VEGF and its receptors are the primary factors establishing the typical angiogenic phenotype found in tumours. Their expression correlates with the degree of vascularization of many experimental and clinical tumours, as detected by ISH and IHC [Brown et al., 1993; Brown et al., 1995; Hatva et al., 1995; Plate et al., 1992; Takahashi Y. et al., 1995]. Tumour growth- dependent hypoxia and activated oncogenes up-regulate VEGF levels in the neoplastic cells, and hypoxia, in combination with the locally increased VEGF concentrations, up-regulates VEGFR-1 and VEGFR-2 on tumour endothelial cells [Kremer et al., 1997; Plate et al., 1992; Shweiki et al., 1992]. VEGF receptors were also detected at high levels in several tumour cell types. Prostate carcinoma [Jackson et al., 2002], malignant mesothelioma [Strizzi et al., 2001], pancreatic [Von Marschall et al., 2000], gastric [Tian et al., 2001], bladder [Wu et al., 2003] and breast cancer cells [De Jong et al., 1998] can express high levels of VEGFR-1 and VEGFR-2. VEGF was found to induce proliferation in all these cells, suggesting a direct autocrine/paracrine effect of VEGF on tumour cell growth.

1.9.2.1 VEGFR-1

VEGFR-1 is mostly expressed by endothelial cells, but it can also be found in other cell types, like trophoblast cells, monocytes and renal mesangial cells [Charnock-Jones et al., 1994; Barleon et al., 1996; Takahashi T. et al., 1995].

The function and signalling properties of VEGFR-1 can be different depending on the developmental stage and the cell type considered.

During vascular development, VEGFR-1 works as ´decoy` receptor, since the number of endothelial progenitors lacking VEGFR-1 increases [Fong et al., 1999] implying a negative regulatory role for this receptor. Nevertheless, the VEGFR-1 ´decoy´function was suggested to take place also during angiogenesis in adult. The tyrosine autophosphorylation of this receptor in response to VEGF was found to be weak [Waltenberger et al., 1994] and the binding affinity of VEGF for VEGFR-1 is ten times higher than for VEGFR-2 [Terman et al., 1992; Quinn et al., 1993]. VEGFR-1 also exists in a soluble form that lacks the transmembrane and intracellular part of the receptor and can sequester VEGF preventing its binding to other receptors [Carmeliet et al., 2001].

Several studies report VEGFR-1 having an inhibitory activity: a repressor motif has been described in its juxtamembrane region that impairs PI3K activation and endothelial cell migration in response to VEGF [Gille et al., 2001]. Furthermore VEGFR-1 was found to inhibit VEGFR-2 induced endothelial cell proliferation [Zeng et al., 2001]. Nonetheless VEGFR-1, when stimulated with PlGF, is able to induce tyrosine receptor autophosphorylation, mitogenicity in endothelial cells and expression of enzymes involved in the degradation of the extracellular matrix [Landgren et al., 1998]. Moreover its activation by PlGF was reported to result in intermolecular transphosphorylation of VEGFR-2, thereby amplifying VEGF-driven angiogenesis through VEGFR-2 [Autiero et al., 2003]. In porcine aortic endothelial cells, VEGFR-1 was able to transduce signals for increased DNA synthesis and proliferation [Ito et al., 2001].

Activation of VEGFR-1 by PlGF, can induce, in non-endothelial cells, a broad spectrum of signals resulting in: chemotaxis and expression of inflammatory cytokines, through PI3K/Akt activation in monocytes [Selvaraj et al., 2003], reconstitution of haematopoiesis through recruiting hematopioetic stem cells [Hattori et al., 2002] and upregulation of matrix metalloproteinases by vascular smooth muscle cells [Wang and Keiser, 1998].

1.9.2.2 VEGFR-2

Mitogenesis, chemotaxis, cell survival and changes in the morphology of endothelial cells are mainly mediated by VEGFR-2 [Quinn, 1993; Waltenberger, 1994]. The mitogenic signal is induced by activation of the Raf-Mek-Erk pathway [Takahashi et al., 1999], while the antiapoptotic effects and chemotaxis are mediated by PI3K/Akt activation [Gerber et al., 1998 b; Gille et al., 2001]. VEGF binding to VEGFR-2 also results in activation of several integrins, which are adhesion molecules involved in angiogenesis, in a PI3K/Akt dependent manner [Byzova et al., 2000]. Apart from being expressed in endothelial cells, VEGFR-2 is also found in haematopoietic stem cells, where it increases their survival [Larrivee et al., 2003], and in retinal progenitor cells, where it plays a critical role in neurogenesis and vasculogenesis [Katoh et al, 1995; Yang and Cepko, 1996].

The key role of VEGFR-2 in developmental angiogenesis and haematopoiesis has been confirmed by the observation that knockout mice for VEGFR-2 die at 8,5-9,5 days of embryonic life, due to lack of development of blood islands, embryonic vasculature and haematopoietic cells [Shalaby et al., 1997].

1.9.2.3 Neuropilin-1

The functional VEGF–VEGFR-2 complex includes neuropilins [Soker et al., 1998], which are ubiquitous membrane-bound molecules, also implicated in axon guidance by binding to the collapsin/ semaphorin family members [Neufeld et al., 2002]. Neuropilin-1 is the most important member of the family and is essential for the development of vascular system [Kawasaki et al., 1999]. However, neuropilins do not have a direct signalling function, since endothelial cells expressing neuropilin-1 but not VEGFR-2, are not able to respond to any VEGF isoform. This observation suggests that neuropilin-1 is not a signalling receptor for VEGF but it rather acts as a coreceptor for VEGFR-2, stabilizing the VEGF–VEGFR-2 complex and enhancing the angiogenic activity of VEGF [Soker et al., 1998]. A truncated soluble neuropilin-1 form, lacking the transmembrane and cytoplasmatic domains, was identified, and functions as a $VEGF_{165}$ antagonist, preventing its binding to the other VEGF receptors [Gagnon et al., 2000].

1.9.2.4 VEGFR-3

VEGFR-3 is characterized by distinct structural features, including a cleavage within the fifth extracellular immunoglobulin loop and a disulfide bridge keeping together the two parts of the extracellular domain [Pajusola et al., 1994]. During embryonic life, VEGFR-3 deficient mice die at the 10,5 day of gestation, because although vasculogenesis and angiogenesis are normal, large blood vessels are abnormally organized with defective lumens, leading to fluid accumulation in the pericardial cavity and cardiovascular failure. Thus, VEGFR-3 has an essential role in the development of the embryonic cardiovascular system, before the emergence of the lymphatic vessels [Dumont et al., 1998]. On the other hand, in the post-natal life, VEGFR-3 expression is detected primarily in lymphatic endothelial cells, but also in fenestrated capillaries of several organs including the bone marrow, splenic and hepatic sinusoids, kidney glomeruli and endocrine glands (pituitary gland included), where it is supposed to be involved in regulating the permeability of vessels [Partanen et al., 2000].

Activation of VEGFR-3 by VEGF-C and VEGF-D binding induces proliferation, migration and survival in lymphatic endothelial cells [Makinen et al., 2001]. However, the activities of this factor are not restricted to lymphatic endothelial cells, since it was observed that VEGFR-3 is, to some extent, also expressed in the blood vessel endothelial cells lining the capillaries adjacent to epithelial tissues [Witmer et al., 2002]. This expression is induced in proliferating blood vessel endothelial cells [Hamada et al., 2000], for example in the granulation tissue of healing wounds, and in the capillary endothelium of human gliomas and colon carcinomas that are known to be devoid of lymphatic vessels [Witmer et al., 2001]. A considerable body of literature suggests that in tumours, VEGFR-3 and its ligands VEGF-C and VEGF-D expression correlates with metastasis to regional lymph nodes and poor prognosis. For example VEGFR-3 expression is associated with tumour progression and may play an important role in facilitating lymphatic spread of prostatic carcinoma [Li et al., 2004], while in cervical carcinogenesis a switch to the lymphangiogenic phenotype may occur at the most aggressive stage [Van Trappen et al., 2003]. In lung cancer, VEGF-C and VEGFR-3 are related to the lymphangiogenesis and angiogenesis, as well as to its occurrence and development [Li et al., 2003].

Similar to the other VEGF receptors, VEGFR-3 was found to be expressed directly by some tumour cells. In small cell lung carcinoma cell line, VEGFR-3 was detected and found to be phosphorylated by VEGF-D, which also induced cell proliferation [Tanno et al., 2004]. In human colorectal cancer expressing VEGF-C, a positive correlation was reported between VEGFR-3 expression in tumour cells and poor overall survival [Witte et al., 2002].

Altogether these reports suggest that VEGFs promote cancer growth not only by stimulating angiogenesis, but also by acting on receptors present on the cancer cells themselves.

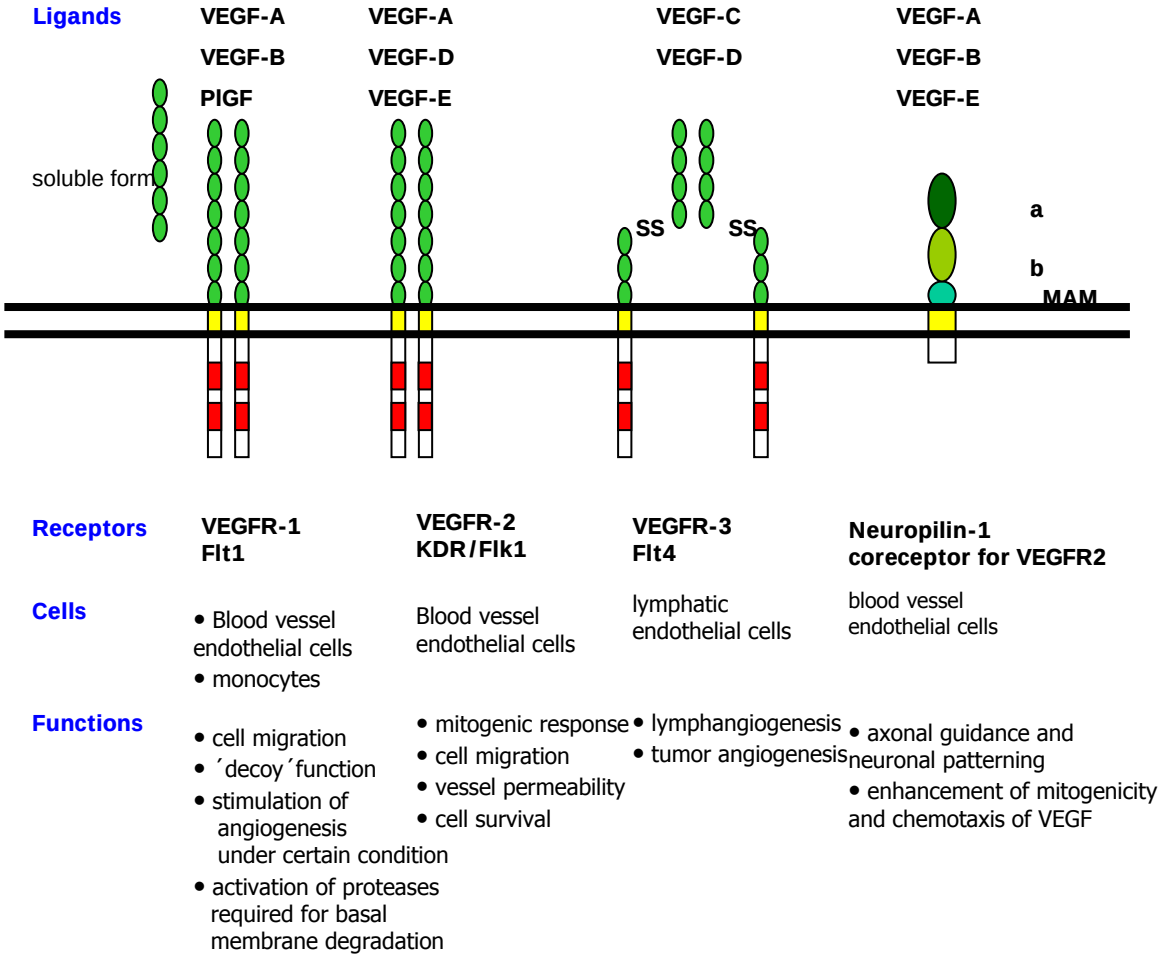


Figure 3. VEGF receptors and their ligands. VEGFR-1 and VEGFR-2 have seven extracellular immunoglobulin homology domains whereas in VEGFR-3, the fifth immunoglobulin domain is cleaved into disulfide-linked subunits. The extracellular domain of VEGFR-1 is also expressed as a soluble protein. Members the VEGF family: VEGF-A, -B, -C, -D, -E and PlGF bind in specific patterns to the different VEGF receptors. PlGF and VEGF-B are selective ligands for VEGFR-1,

whereas VEGF-A binds to both VEGFR-1 and VEGFR-2. VEGF-C and VEGF-D interact with both VEGFR-3 and VEGFR-2. On the other hand, VEGF-E binds only VEGFR-2. The non-tyrosine kinase transmembrane protein neuropilin-1 acts as a co-receptor for certain isoforms of VEGF-A. Although both VEGFR-1 and VEGFR-2 are expressed in blood vessel endothelium, biological angiogenic activities are transduced mainly through VEGFR-2. VEGFR-1 is also expressed on monocytes and its activation leads to chemotaxis and activation of proteases required for basal membrane degradation. VEGFR-3 is mainly expressed in lymphatic endothelium and is involved in lymphangiogenesis.

1.10 VEGF and its receptors in normal and adenomatous pituitary

There are many studies reporting that VEGF is secreted by normal and neoplastic anterior pituitary cells [Lloyd et al., 1999; Vidal et al., 1999; Ochoa et al., 2000; Onofri et al., 2004; Lohrer et al., 2001].

Although unanswered questions remain, regarding the functional meaning of VEGF expression in the normal adenohypophysis, it is conceivable that VEGF plays a role both in the formation of pituitary portal vessels during foetal life and in maintenance of their differentiated state in adult animals [Ferrara et al., 1992 a]. Originally, VEGF was identified to be secreted by pituitary FS- cells [Gospodarowicz et al., 1989] but was later also detected in secretory granules of the normal pituitary cells. Since VEGF co-localized with all the adenohypophyseal hormones, it was also suggested that it might affect the endocrine activity of pituitary cells. The anterior pituitary endocrine cells that showed the highest percentage of VEGF immunostaining were somatotrophs, corticotrophs and FS-cells [Vidal et al., 1999]. The subcellular distribution of VEGF indicates that it can be simultaneously released with the various pituitary hormones and affects perhaps vascular permeability facilitating the transport of pituitary hormones across the capillaries [Vidal et al., 2002].

Expression of VEGF was reported in various pituitary adenoma cell lines and even in pituitary adenoma cells in primary culture [Lohrer et al., 2001; Borg et al., 2005]. VEGF is involved in pituitary neovascularization in estrogen-treated rats and in the pathogenesis of prolactinomas [Banerjee et al., 1997; Banerjee et al., 2000]. Indeed, the overexpression of VEGF and its receptor Flk-1/KDR was

linked to prolactinoma development in this animal model [Banerjee et al, 1997]. In humans, it was reported that VEGF protein expression is higher in pituitary carcinomas than in adenomas [Lloyd et al., 1999]. Concerning the expression of VEGF in pituitary adenomas, there are conflicting reports. One study showed that VEGF mRNA expression is higher in pituitary adenomas compared to the normal pituitary gland, probably due to PTTG action [McCabe et al., 2002]. On the other hand, Lloyd and colleagues reported a stronger VEGF immunoreactivity in normal pituitary compared to pituitary adenomas [Lloyd et al., 1999], which might be consistent with the decreased vascularity detected in pituitary adenomas [Schechter, 1972; Jugenburg et al., 1995; Turner et al., 2000 a]. A third group reported no significant difference in VEGF immunostaining between normal and tumoural human pituitary tissues [Viacava et al., 2003].

The published literature does not contain many studies about VEGF receptors expression in normal and tumoural pituitary gland. VEGFR-2 was detected by IHC in the blood vessel endothelial cells of rodent normal and adenomatous pituitary, and its expression was significantly elevated after estrogen treatment [Banerjee et al., 1997]. Another study located VEGFR-2 in both endothelial and anterior pituitary cells, especially in GH- and prolactin-secreting cells, as well as, in GH3 mammosomatotrophinoma cell line [Vidal et al., 2002]. No immunohistochemical studies on VEGFR-2 localization in human normal and adenomatous pituitary are available. The study of a large cohort of human pituitary adenomas, reported VEGFR-2 overexpression in all the tumours analyzed (especially NFPA) compared to normal pituitaries [McCabe et al., 2002]. A brief report showed VEGFR-2 immunoreactivity in ACTH and LH/FSH secreting cells in normal human pituitary, as well as, in pituitary adenomas (mainly prolactinomas and gonadotrophinomas) [Lloyd et al., 2003].

VEGFR-1 was initially identified in blood vessel endothelial cells of sheep pituitary [Jabbour et al., 1997], but later studies reported VEGFR-1 immunoreactivity also in ACTH-secreting cells of the normal human pituitary. In pituitary adenomas, VEGFR-1 was found in corticotrophinomas, gonadotrophinomas and NFPA tumour cells [Lloyd et al., 2003]. VEGFR-1 transcription was found to be significantly downregulated by TGF-β treatment in the human gonadotrophinoma cell line HP75 [Horiguchi et al., 2004].

The number of studies on VEGFR-3 and neuropilin-1 in the pituitary gland is still quite restricted. This VEGFR-2 co-receptor was found to be overexpressed in estrogen-induced rat pituitary tumour cells and in the GH3 cell line, compared to normal rat pituitary cells, and this was associated with substantially enhanced tumour angiogenesis [Banerjee et al., 2000]. VEGFR-3 expression was detected in the blood capillary endothelial cells of the human foetal and adult anterior pituitary, as well as, in endothelial cells of fenestrated capillaries of tissues like thyroid and parathyroid glands, adrenal glands, spleen and kidneys, in which extensive molecular exchange occurs across the blood vessel wall [Partanen et al., 2000].

2 AIM OF THE STUDY

There is a large body of evidence claiming that angiogenesis plays a role in tumour growth and development through VEGF, which is one of the most powerful angiogenic factors.

However, the few studies available, about the expression of VEGF and its receptors (VEGFR-1, VEGFR-2, neuropilin-1 and VEGFR-3) in normal and adenomatous pituitary, are controversial.

The aim of this work is to investigate VEGF and its receptors expression in human normal pituitary and in different pituitary adenomas. In order to achieve these goals, the expression of VEGF and its receptors is examined in human normal and adenomatous pituitary tissues at RNA and protein levels through RT-PCR, ISH and IHC analysis.

The IHC results obtained for the expression of the different VEGF receptors are then correlated with the expression of their specific ligands and with several biological parameters like PI, blood and lymphatic vessel counts and tumour grade.

The role of VEGFR-1 in pituitary endocrine cell function and growth is investigated in a pituitary adenoma cell line and the signalling pathways involved are analyzed.

3 MATERIAL AND METHODS

3.1 Reagents

Product	Company
ABC kit	Vector Laboratories (Burlingane, CA, USA)
AP-ABC kit	Vector Laboratories (Burlingane, CA, USA)
Acetic acid	MERCK (Darmstadt, Germany)
Acridine orange	Sigma (St. Louis. MO, USA)
Agar	Life Technologies (Paisley, UK)
Ammonium persulfate	Sigma (St. Louis. MO, USA)
Amphotericin B	Biochrom (Berlin, Germany)
Ampicillin	Roche (Mannheim, Germany)
Ampuwa water	Frisenius (Germany)
Autoradiography photoemulsion NTB2	Kodak (Stuttgart, Germany)
Beta-mercaptoethanol	MERCK (Darmstadt, Germany)
Biomax MR films	Kodak (Stuttgart, Germany)
Boric acid	Roth (Karlsruhe, Germany)
Bovine serum albumin (BSA)	Invitrogen Corp. (Paisley, UK)
Protein assay Dye Reagent	Biorad (Munich, Germany)
Chloroform	Sigma (St. Louis. MO, USA)
Collagenase	Worthington Biochemical Corp. (Lakewood, NJ, USA)
Competent Bacteria	Promega Corp. (Madison, WI, USA)
Developer solution	Kodak (Stuttgart, Germany)
Dextran sulphate	Sigma (St. Louis. MO, USA)
Diaminobenzidine (DAB)	Sigma (St. Louis. MO, USA)
Diethyl-pyrocarbonate (DEPC)	Sigma (St. Louis. MO, USA)
Dimethyl sulfoxide (DMSO)	Sigma (St. Louis. MO, USA)
Dithiothreitol (DTT)	Sigma (St. Louis. MO, USA)
DNAse I	Invitrogen Corp (Paisley, UK)
dNTP Mix	MBI Fermentas (Vilnius, Lithouania)
Dulbecco's modified Eagle medium (DMEM)	Invitrogen Corp (Paisley, UK)
EcoRI	Roche (Mannheim, Germany)
Ethylenediaminotetracetic acid (EDTA)	MERCK (Darmstadt, Germany)
Ethidium bromide	Sigma (St. Louis. MO, USA)

Foetal calf serum	Gibco (Karlsruhe, Germany)
Ficoll 400	Sigma (St. Louis. MO, USA)
Fixer solution	Kodak (Stuttgart, Germany)
Formamide	Sigma (St. Louis. MO, USA)
Guanidine thiocyanate	Fluka Chemie AG (Buchs, Switzerland)
^{3}H-Thymidine	Amersham Biosciences (Uppsala, Sweden)
Herring sperm DNA	Roche (Mannheim, Germany)
Hexanucleotide Mix	Roche (Mannheim, Germany)
Hydrochloric acid	MERCK (Darmstadt, Germany)
Hydrogen peroxyde	Roth (Karlsruhe, Germany)
Isoamylalcohol	MERCK (Darmstadt, Germany)
Isopropanol	Sigma (St. Louis. MO, USA)
KH_2PO_4	MERCK (Darmstadt, Germany)
Levamisole	Sigma (St. Louis. MO, USA)
L-Glutamine	Biochrom AG (Berlin, Germany)
Lumi-Light Western Blotting Substrate	Roche (Mannheim, Germany)
LY294002	Calbiochem (La Jolla, CA, USA)
Magnesium chloride	MERCK (Darmstadt, Germany)
Marker 1kb Plus	Life Technologies (Paisley, UK)
MEM-Vitamins	Biochrom (Berlin, Germany)
Milk powder	Roth (Karlsruhe, Germany)
Nitrocellulose membrane Hybond-ECL	Amersham Biosciences (Uppsala, Sweden)
Paraformaldehyde (PFA)	MERCK (Darmstadt, Germany)
PBS	Gibco/invitrogen (Carlsbad, CA, USA)
Penicillin+Streptavidine mix	Biochrom AG (Berlin, Germany)
Peptone	ICN Pharmaceuticals (Aurora, OH, USA)
pGEM®-T Easy Vector	Promega Corp. (Madison, WI, USA)
Phenol	Roth (Karlsruhe, Germany)
Phosphate based buffer PBS	Life Technologies (Paisley, UK)
Placenta Growth Factor (PlGF)	R & D Systems (Minneapolis, MN, USA)
Polyacrylamide	Invitrogen Corp (Paisle, UK)
poly-L-lysine-coated microscope slides (SuperFrost® Plus)	Menzel-Gläser (Braunschweig, Germany)
Polyvinylpyrrolidone	Sigma (St. Louis. MO, USA)
Pothassium chloride (KCl)	MERCK (Darmstadt, Germany)
Qiaquick Nucleotide Removal kit	Qiagen (Hilden, Germany)
Reisin	Bio-Rad (Hercules, CA, USA)
RNAse A	Roche (Mannheim, Germany)
RNAsin (RNAase inhibitor)	Promega Corp. (Madison, WI, USA)
Rneasy Mini kit	QIAGEN (Hilden, Germany)
Roti-Histokitt	Roth (Karlsruhe, Germany)
Roti-Histol	Roth (Karlsruhe, Germany)

Reverse transcriptase (SuperScript II ™)	Invitrogen (Carlsbad, CA, USA)
SacI	New England Biolabs (Beverly, Massachussets, USA)
SacII	New England Biolabs (Beverly, Massachussets, USA)
Sodium acetate dihydrate	MERCK (Darmstadt, Germany)
Sodium acetate trihydrate	MERCK (Darmstadt, Germany)
Sodium chloride (NaCl)	Roth (Karlsruhe, Germany)
Sodium citrate dihydrate	MERCK (Darmstadt, Germany)
Sodium dihydrogen phosphate mono-hydrate (NaH_2PO_4-H_2O)	MERCK (Darmstadt, Germany)
Sodium hydrogen phosphate dihydrate (Na_2HPO_4-$2H_2O$)	MERCK (Darmstadt, Germany)
Sodium peroxyde (NaOH)	MERCK (Darmstadt, Germany)
SP6 RNA polymerase	Roche (Mannheim, Germany)
^{35}S-UTP	Hartmann Analytic (Braunschweig, Germany)
T4 DNA Ligase	Promega Corp. (Madison, WI, USA)
T4 DNA Ligase Buffer 2X	Promega Corp. (Madison, WI, USA)
T7 RNA polymerase	Roche (Mannheim, Germany)
Taq DNA polymerase	MBI Fermentas
TEMED	Sigma (St. Louis, Mo, USA)
Tissue-Tek®	Sakura Finetek Europe (Zoeterwoude, The Nederlands)
Toluidin Blue	Sigma (St. Louis, Mo, USA)
Transferrin	Sigma (St. Louis, Mo, USA)
Trichloroacetic acid	Roth (Karlsruhe, Germany)
Triethanolamine	Sigma (St. Louis, Mo, USA)
Triiodothyronine	Henning (Berlin, Germany)
Tris-Glycine 10% gel	Anamed (Darmstadt, Germany)
Tris pure	ICN Pharmaceuticals (Aurora, OH, USA)
Triton X-100	Roth (Karlsruhe, Germany)
tRNA	Roche (Mannheim, Germany)
Trypsin	Sigma (St. Louis, Mo, USA)
Tween 20	Sigma (St. Louis, Mo, USA)
Ultima Gold Scintillation Solution	Packard Bioscience (Gromingen, Netherlands)
Vector Red kit	Vector Laboratories (Burlingane, CA, USA)
Vascular Endothelial Growth Factor (VEGF-A)	R & D Systems (Minneapolis, MN, USA)
X-Gal	Roche (Mannheim, Germany)
Yeast extract powder	ICN Pharmaceuticals (Aurora, OH, USA)

3.2 Solutions

Collagenase Mix	1000 U/ml Collagenase : 4g/ 100ml solution Trypsin inhibitor : 10 mg/ 100ml solution Hyaluronidase : 100 mg/ 100ml solution BSA : 400 mg/ 100ml solution Dnase : 500 µl/ 100ml solution
DEPC water	200 µl DEPC/l deionized water Leave under the fume hood overnight Autoclave
Formamide deionized	Add Reisin: 5ml/ 50ml Formamide
Formamide / 4xSSC buffer	Formamide deionized: 50ml/ 100ml solution SSC 20x sterile: 20ml/ 100ml solution DEPC water: 30ml/ 100 ml solution
HDB buffer	Hepes: 5,95 g/l NaCl : 8 g/l KCl: 0,37 g/l $Na_2HPO_4.H_2O$: 0,12 g/l Glucose: 1,982 g/l Amphotericine B 25µg/ml: 10 ml Penicillin/Streptomycin 10^5U/l : 10 ml Ad just pH to 7,3 with NaOH Store at +4°C

Hybridization mix	Deionized formamide: 15 ml/ 30 ml solution Tris-HCl 1M pH 8,0: 0,6ml/ 30 ml solution NaCl 5M: 1,8 ml/ 30 ml solution EDTA 0,5M pH 8,0: 300 µl/ 30 ml solution Dextran Sulphate: 6 ml / 30 ml solution Polymers 10X: 3 ml/ 30 ml solution t RNA 10 mg/ml: 1,5 ml/ 30 ml solution Herring sperm DNA 10 mg/ml: 600 µl/ 30 ml solution DTT 5M: 1,2 ml/ 30 ml solution
LB medium	Peptone :10 g/l Yeast extract: 5 g/l NaCl : 5 g/l NaOH 1M : 2 ml/l Adjust to pH 7.0
NTE Buffer	5 X NaCl: 146,1 g/l Tris-HCl pH 8,0 1 M: 50 ml/l EDTA 0,5M pH 8,0: 50 ml/l Autoclave
Paraformaldehyde (PFA)	4% paraformaldehyde: 4 g/100 ml Sodium phosphate buffer: 20 ml/100ml Ampuwa water: 80 ml Add 1M NaOH to pH 7.4 Heat at 56°C to dissolve Filter and cool before usage Store at +4°C for maximum 2 days

Phosphate based buffer (PBS)	1x NaCl: 8 g/l KCl: 0.2 g/l $Na_2HPO_4.2H_2O$: 1.44 g/l KH_2PO_4: 0.2 g/l Adjust to pH 7.4
Polymers	10X Ficoll 400: 0,2 g/ 10 ml Polyvinl pyrrolidone: 0,2 g/ 10 ml BSA: 0,2 g/ 10 ml Dissolve in 10 ml DEPC water
Sodium acetate	2M Sodium acetate trihydrate: 27.2 g/ 100ml DEPC: 20 µl Add acetic acid to pH 4.0 Leave at room temperature overnight and the next day autoclave
Sodium phosphate buffer	50mM $Na_2HPO_4.2H_2O$: 7.06 g/l NaH_2PO_4. H_2O: 1.32 g/l Adjust to pH 7.4
Solution D	4M Guanidium thiocyanate: 250 g/337 ml 0,75 M Sodium citrate pH 7.0: 17,6 ml/337ml 10% Sarcosyl: 26,4 ml/337 ml dissolve in 293 ml DEPC To complete the medium add: 180µl beta-mercaptoethanol/25ml solution just before use

SSC	20x NaCl : 175 g/l Sodium citrate dihydrate: 88.23 g/l Adjust to pH 7.0 Filter and autoclave before use
Triethanolamine/ acetic anhydride	0,1 M Triethanolamine: 3,3 ml/ 250 ml NaCl: 2,25 g/ 250 ml DEPC water: 250 ml Adjust to pH 8,0 Acetic Anhydride: 625 µl added in the last minute
Tris borate EDTA buffer (TBE)	10x Boric acid (H_3BO_3): 61.83 g/l EDTA: 37.2 g/l Tris pure: 30.03 g/l Adjust to pH 8.0
Tris buffer	Tris pure: 12.114 g/l Adjust to pH 7.6
Tris-based buffer (TBS)	1x Tris pure: 2.42 g/l NaCl: 8 g/l Adjust to pH 7.6
Tris-HCl	1M Tris pure: 121.14 g/l Add 25% HCl to a pH 8.2

3.3 Human tissues

This study was performed after approval of the ethics committee of the Max Planck Institute and informed consent was received from each patient or their relatives.

The 3 normal pituitary glands (NP) were obtained from the autopsy performed within 12 h after accidentally occurred death of 3 healthy persons: 2 males (age 67 and 47) and 1 female (age 37). The pituitary tumours studied were obtained from trans-sphenoidal surgery of 39 patients: 17 males and 22 females with 47,1 $\pm$ 14,1 average age (range 26-77 years), classified according to clinical presentation in 11 somatotrophinomas (ACRO), 3 corticotrophinomas (CUSH), 17 non functioning pituitary adenomas (NFPA), 6 prolactinomas (PROL), 2 thyreotropinomas (THYR). All the tumours were benign and tumour grade was determined according to a modified Hardy´s classification [Boggild et al., 1994] following the medical reports after nuclear magnetic resonance and after surgery: 2 grade I, 11 grade II and 26 grade III cases were identified (Tab. 5).

Tissues fragments of normal pituitaries and pituitary adenomas were shock-frozen on dry ice and stored at -80°C until use.

3.4 RNA isolation

RNA was isolated from normal human pituitaries and from human pituitary adenomas using the guanidium isothiocyanate protocol. The tissue piece was first homogenized in 800 µl of solution D added with β–mercaptoethanol, using the Ultra-TURRAX T8 (IKA Labortechnic) tissue homogenizer.

Guanidium isothiocyanate and β–mercaptoethanol inhibit the RNAase action activated by cell disruption, preventing in this way RNA degradation. Eighty µl of sodium acetate 2 M pH 4,0 were added afterwards to precipitate RNA, followed by 800 µl of saturated phenol and 160 µl of a choloroform-isoamyl alcohol (49:1) solution. After 15 minutes incubation on ice the samples were centrifuged at

13000 rpm for 20 minutes at 4°C; this step led to the formation of two phases, the upper one containing RNA and the lower one containing DNA and proteins. The upper phase was then transferred to a new tube together with the same volume of ice-cold isopropanol. Incubation of the sample at –20°C at least for 2 hours was necessary for RNA precipitation. After centrifugation of the sample at 13000 rpm for 10 min at 4°C, the supernatant was discarded and the pellet was washed with ice-cold ethanol 70%. After 10 min centrifugation at 13000 rpm the supernatant was again discarded and the pellet left to dry at room temperature and then dissolved in an appropriate amount of DEPC-treated water.

The samples absorbance was measured with a photometer and RNA concentration calculated according to the following formula: (A_{260}x40x60)/1000= µg/µl RNA in which A_{260} is the sample absorbance at 260 nm, 40 is the concentration in µg/µl of RNA giving A_{260} value equal to 1 and 60 is the dilution factor used to measure the sample concentration (1 µl RNA+ 59 µl DEPC water). The lack of DNA contamination was assessed performing a PCR reaction for a housekeeping gene like GAPDH or β– actin, using the RNA sample: if no DNA contamination is present, no band is visible after loading the PCR product on an ethidium bromide gel (as described below).

RNA extraction from the different pituitary adenoma cell lines used in this study was performed as described above. Cells were washed with PBS, scraped with 800 µl of solution D and collected in an eppendorf tube; from this point on the protocol was identical to that one just described.

3.5 Reverse Transcriptase- Polymerase Chain Reaction

Retrotranscription of RNA samples was performed incubating 1µg RNA with 1 µl of dNTP mix 2 mM, 2 µl of 62.5 U/ml random primers (Hexanucleotide mix), 2 µl of dithiothreitol (DTT) 10 mM and 1 µl of 200 U reverse transcriptase (SuperScript II) all diluted in 4 µl of 1x first strand buffer and DEPC-water to get a final volume of 20 µl, for 1 hour at 45°C. Reaction was stopped by boiling the samples at 95°C for 5 minutes.

One µl of c-DNA samples obtained was used for PCR reaction and incubated with 1.5 µl PCR buffer 10x, 0.9 µl Mg Cl_2 25 mM, 1.5 µl dNTP mix 2mM, 0.5 µl amplification primer sense 10 pmol/µl, 0.5 µl amplification primer anti-sense 10 pmol/µl, 0.15 µl *Thermus aquaticus* (Taq) DNA polymerase and 8.95 µl autoclaved distilled water. The PCR reaction consisted of 35 cycles each containing the following steps: denaturation at 94° C for 1 min, annealing at 55° C- 65° C according to the employed primers specific annealing temperature (see Tab. 1) for 1 min and finally elongation of the PCR fragment at 72° C for 1 min.

The amplified fragments were electrophoresed in ethidium bromide agarose gel 1 - 1,5% according to the size of the product (1% for 500- 1100 bp fragments, 1,5% for 200- 500 bp fragments), in 1 X TBE buffer for 15-20 min at 80 V and then visualized under UV light. The 1 kb Plus DNA Ladder marker was used to determine the fragments size.

In the table 1 the primers used are listed together with the corresponding sequence, annealing temperature and expected length of the amplified fragment. Each sequence was checked with the NCBI BLAST program in order to exclude eventual annealing with other genes different from the ones studied. All primers were synthesized by MWG Biotech, reconstituted with sterile distilled water to a concentration of 100 µM and stored at -20 ° C. The annealing temperature for each pair of primers was optimized by PCR in a range of 55, 60 and 65 °C using cDNA from normal human pituitary as a template. The optimal temperature was the one which was yielding an intense signal with no secondary amplification fragments.

Table 1. Primers used for RT-PCR reactions.

Primers	Sequence (5´-3´)	Ta (°C)	Amplified fragment (bp)
β–**actin** human	ACGGGGTCACCCACACTGTGC sense CTAGAAGCATTTGCGGTGGACGATG antisense	60	660
GAPDH rat	ATGGTGAAGGTCGGTGTGAACG sense GTTGTCATGGATGACCTTGGC antisense	60	495
VEGF-A human	CCTGGTGGACATCTTCCAGGAGTACC sense TGTGCTGTAGGAAGCTCAT antisense	60	209
VEGFR-1 (flt-1) human	TGCTTGAAACCGTAGCTGG sense GGTGCCAGAACCACTTGATT antisense	60	378
VEGFR-2 (flk-1/KDR) human	CTGGCATGGTCTTCTGTGAAGCA sense AATACCAGTGGATGTGATGCGG antisense	60	790
VEGFR-3 human	CAGGATGAAGACATTTGA sense AAGAAAATGCTGACGTATGC antisense	60	190
Neuropilin-1 human	GAAAGATAGCCCCTCCTCC sense CCACAGTAACGCCCAATG antisense	60	372
VEGFR-1 (flt-1) rat	CCCGGTTTGCTGAACTTGTGG sense GGCATTTGGTGAAAGCTCCTC antisense	60	271
VEGFR-2 (flk-1/KDR) rat	GCCAATGAAGGGGAACTGAAGAC sense TCTGACTGCTGGTGATGCTGTC antisense	60	537
VEGFR-3 rat	CCAAGGCCTGGCAAATGGTTAC sense AACACATAGGTGCTGGCAGCTG antisense	60	339
Neuropilin-1 rat	GGCTGCCGTTGCTGTGCGCCA sense ATAGCGGATGGAAAACCCTGC antisense	60	383

1st column: name of the gene, 2nd column: sense and antisense primers sequences, 3rd column: annealing temperature, 4th column: expected fragment size.

3.6 *In situ* hybridization with radioactive riboprobe

3.6.1 Principle

ISH is a method which allows the localization of a specific sequence of nucleic acid on a morphologically preserved tissue section, thanks to the ability of

nucleic acid single strands to bind with a complementary sequence. In this specific case, the sequence of interest is recognized by a probe of complementary RNA, called riboprobe, labelled with a radioisotope (usually ^{35}S) which binds its cognate mRNA directly on the tissue section. Radiolabeled probes are then visualized by exposure of the tissue section on a photographic film or by dipping of the hybridized slides into a photographic emulsion sensible to radioactive emission. The slides are then stored in the dark at +4°C to allow the slide emulsion to become exposed and then developed in the same way as normal photographic film. The areas in which the riboprobe has bound to the mRNA in the tissue are visible as silver grains signals, on microscope. This method is particularly useful for investigating gene expression on a cellular level.

In general, pre-treatment of the tissue sections on the slides is carried out to reduce background staining and to facilitate the probe access into the cells. For this purpose is chloroform treatment usually performed to remove lipids followed by acetylation with acetic anhydride (0.25%) in triethanolamine to decrease background and to inactivate RNAases.

The composition of the hybridization solution is critical in controlling the efficiency of the hybridization process. Hybridization depends on the ability of the oligonucleotide to anneal to a complementary mRNA strand just below its melting point (Tm). The value of the Tm is the temperature at which half of the oligonucleotide is present in a single stranded form and depends mainly upon its specific guanine-cytosine content. The melting temperature is also dependent upon the length of the sequences to be annealed, pH, monovalent cation concentration and presence of organic solvents. Typical chemical compounds used to prepare the hybridization solution are: dextran sulphate that, becoming strongly hydrated, can reduce the amount of water for dissolving the nucleotides, increasing therefore the probe concentration in solution and resulting in higher hybridization rates. Formamide and dithiothreitol (DTT) are organic solvents able to reduce the thermal stability of the chemical molecular bonds, allowing hybridization to be carried out at lower temperatures. NaCl and Tris HCl buffer are employed since monovalent cations interact mainly with the phosphate groups of the nucleic acids, decreasing the electrostatic interactions between the two strands. EDTA is a chelator and removes free divalent cations from the

hybridization solution, avoiding them to stabilize duplex DNA. Finally, other components are added to decrease the chance of nonspecific binding of the probe and include: ssDNA (hydrolyzed salmon sperm DNA) and tRNA, which acts as a carrier for RNA.

After hybridization, the slides are washed to remove unbound probe or probe which has loosely bound to imperfectly matched sequences. Washing is carried out close to the stringency condition at which the hybridization takes place with a final low stringency wash.

3.6.2 Riboprobes characteristics- general overview

Single strand labelled RNA probes are generated by *in vitro* RNA polymerase transcription of a linearized plasmid, containing RNA polymerase promoters from two different bacteriophages, in which the cDNA of the studied sequence is cloned.

The target sequence has to be selected in the mRNA sequence of the gene investigated and to be around 1.0 kb long (enough to give a strong and specific radioactive signal but not too much in order to avoid problems in entering the cells). It does not have to contain the restriction sites of the endonucleases used for the linearization of the plasmid and it must contain one restriction site of one of the endonucleases which are in the polylinker site of the vector; this is necessary to assess the orientation in which the fragment has been inserted (explained in the next paragraph).

RNA probes (cRNA probes or riboprobes) have the advantage that RNA-RNA hybrids are very thermostable and are resistant to digestion by RNases. This allows the possibility of post-hybridization digestion with RNase to remove non-hybridized RNA and therefore reduces the possibility of background staining.

According to the orientation of the inserted fragment, the use of the two different RNA polymerases allows to obtain two different single strand riboprobes: the sense (with the same sequence of the target mRNA) and the antisense (with the complementary sequence to the target mRNA) (Fig. 5); the hybridization with the labelled sense probe is considered as negative control and it measures the non-specific probe binding, only due to the chemical properties

of the probe. If the sense probe detects nothing, this means that any signal detected by the antisense probe is due to sequence-specific binding to mRNA and not to the binding to other targets within the cell.

3.6.3 Protocol for riboprobes generation

VEGFR-1 cDNA (920 bp fragment, GenBank accession AF063657, nucleotides 617-1537) was generated by RT-PCR from the total human normal pituitary RNA using the primers shown in the table 2. VEGFR-2 cDNA (1031 bp fragment, GenBank accession AF035121, nucleotides 1406-2437) was obtained in the same way, employing the primers for VEGFR-2 (Tab. 2). The PCR reaction was identical to the one described above except that in this program, one single step of denaturation at 94 °C for 5 min and one single step of annealing at 72 °C for 5 min, were added before and after the starting of the cycles, respectively.

Both fragments were then cloned into pGEM®-T Easy Vector (see Fig. 4 for an overview of the plasmid structure and of all protocol steps); the use of this plasmid makes the fragments ligation shorter than it would be with the traditional vectors since it is linearized and contains a thymidine in both 3´-ends. These overhanging 3´-T at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmid by preventing recircularization of the vector and by providing a compatible overhang for PCR products, since most of the DNA polymerases usually add a single deoxyadenosine to the 3´-ends of the amplified fragments independently from the template sequence.

The standard ligation reaction was performed using the following reagents for both fragments:

T4 DNA Ligase buffer 2X	5 µl
pGEM®-T Easy Vector	1 µl
PCR product	3 µl
T4 DNA Ligase	1 µl

The tube containing the reaction mixture was incubated overnight at +4°C.

For bacteria transformation, 5 µl of the ligation reaction mixture were mixed with 20 µl of competent bacteria and incubated on ice for 30 min. The mixture

underwent then a heat shock in a 42°C waterbath for 40 sec in order to let the plasmid enter into the cells and then after 2 more min on ice, 500 μl of LB medium without ampicillin were added and the vial was incubated for 45 min at 37°C. The transformed bacteria were then seeded on agar plates containing X-gal (20 mg/ml) and ampicillin (100 mg/ml) and incubated overnight at 37°C. These plates allow the growth only of the bacteria containing the plasmid, since it bears the gene for the ampicillin resistance. They are also useful to identify the colonies containing the transformed vector because the fragment insertion site in the plasmid is localized in the middle of the β-galactosidase gene, which is necessary to obtain the blue-coloured metabolic product of X-gal. For this reason, the colonies containing the plasmid transformed with the studied fragment are white.

The following day, some of the white colonies were selected and amplified overnight at 37°C, in 2 ml of LB medium containing ampicillin. The plasmid DNA was then extracted and digested with EcoRI endonuclease (which cuts at both sides of the inserted sequence, see Fig. 4) to check if the selected clones really contained the plasmid with the inserted fragment. One of these colonies was finally amplified in 200 ml LB medium containing 100 mg/ml ampicillin overnight at 37°C. Plasmid isolation was performed using the QIAGEN plasmid purification system. Digestion with NdeI or PstI endonucleases was then carried out for VEGFR-1 and VEGFR-2 fragments respectively, in order to determine their orientation inside the vector and to employ the suitable linearizing enzymes and RNA polymerases (Fig. 4).

For linearization and generation of each riboprobe, the following restriction enzymes and RNA polymerases were used: VEGFR-1 sense: SacI, T7; VEGFR-1 antisense: SacII, SP6; VEGFR-2 sense: SacII, SP6; VEGFR-2 antisense: SacI, T7. After plasmid linearization, antisense and sense riboprobes were synthesized and labelled with ^{35}S according to the following protocol:

Buffer 10X	3 µl
dNTPs mix	3 µl
0,5M DTT	1 µl
Linearized plasmid	volume corresponding to 1,5 µg
1 mCi α–^{35}S-UTP	13 µl
RNAsin	1 µl
SP6 or T7 RNA polymerase	1µl
Distilled water	to a final volume of 30 µl

The mix was incubated for 3 hours at 37°C and 0,5 µl of the RNA polymerase were added again after 1 hour. To remove any DNA contamination, DNAse treatment was performed at the end of the incubation time by adding 2 µl of the enzyme for 15 min at 37°C.

After cleaning the riboprobe with Rneasy Mini kit from QIAGEN in order to remove nucleotides, enzymes, salts and all the compounds that had not been incorporated to the riboprobe, the radioactivity of each sample was determined in scintillation solution with a β–counter apparatus and 35,000-70,000 cpm/µl of antisense or sense (control) ^{35}S-labeled riboprobe were used for the hybridization of the slides.

Table 2. Primers used for generation of the riboprobes used in ISH studies.

Primers	Sequence (5´-3´)	Ta (°C)	Amplified fragment (bp)
VEGFR-1 (flt-1) human	CTGTGAAGCAACAGTCAATGG sense CTATTATTGCCATGCGCTGAG antisense	58	920
VEGFR-2 (flk-1/KDR) human	GAATACCCCTTGAGTCCAATC sense CTGAGTCTTCTACAAGGGTCT antisense	58	1031

1st column: name of the gene, 2nd column: sense and antisense primers sequences, 3rd column: annealing temperature, 4th column: expected fragment size.

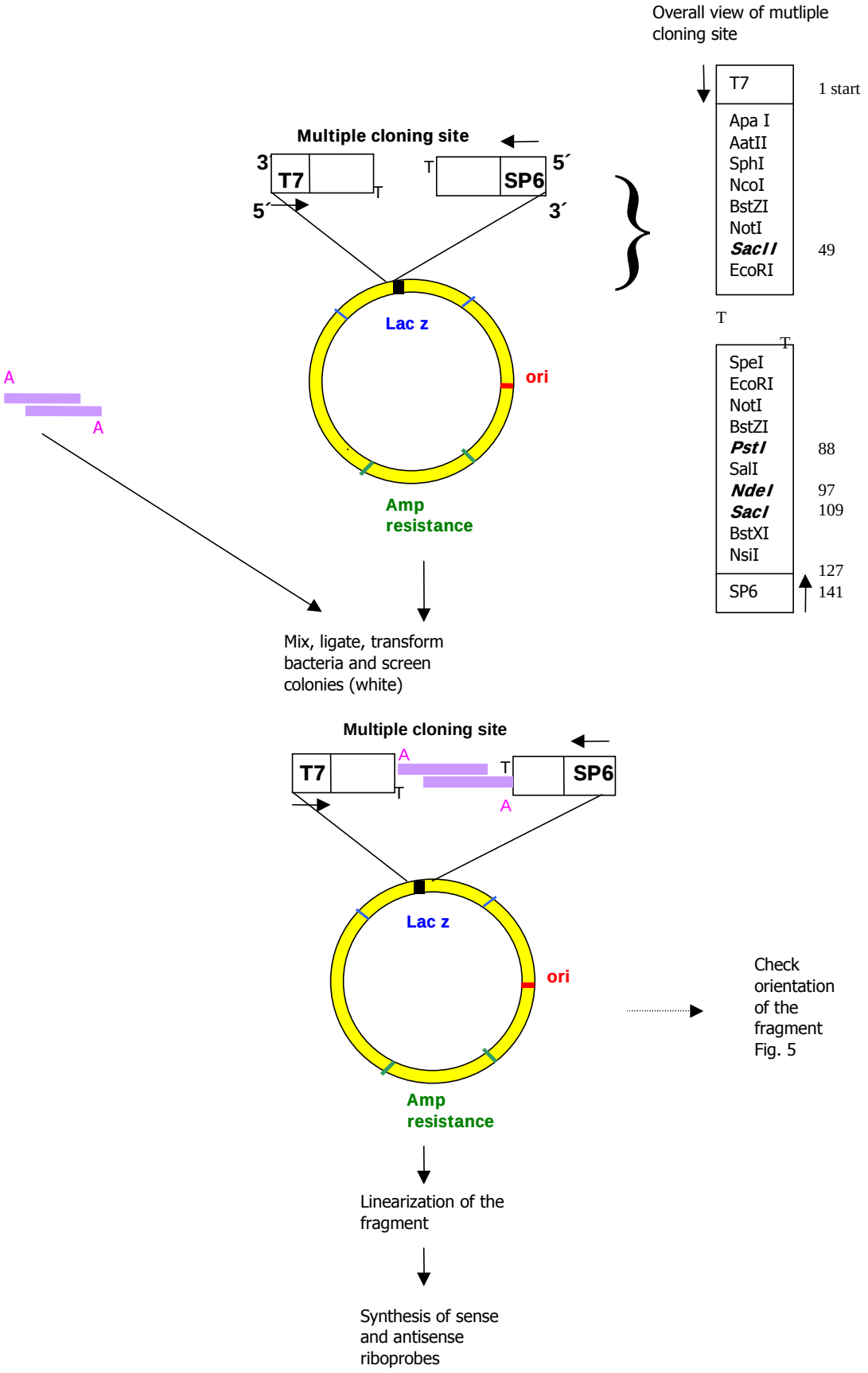
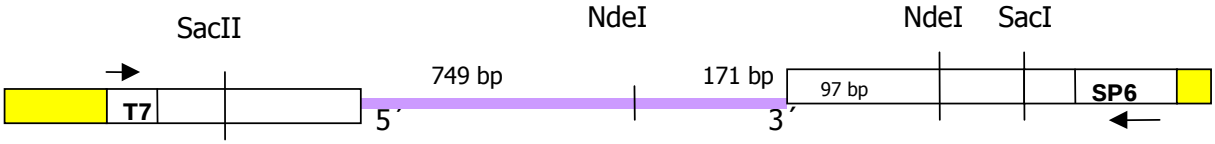


Figure 4. Structure of pGEM®-T Easy Vector before and after fragment (in violet) insertion. The multiple cloning site is inserted in the LacZ gene which allows the screening of the colonies transformed with plasmid containing the fragment, after the ligation reaction. The

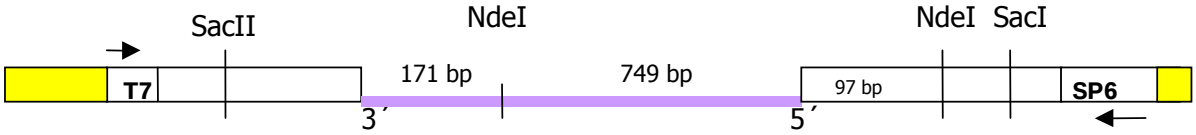
multiple cloning site enlargement (in white) shows the overhanging 3´-T and several restriction enzymes cutting sites, in bold are the ones used to determine the orientation of VEGFR-1 and -2 fragments (NdeI e PstI) and for plasmid linearization(SacI and SacII). Promoters for T7 and SP6 RNA polymerases flank the multiple cloning site. The plasmid contains also a bacterial replication origin site to be replicated inside bacteria cells and an ampicillin resistance gene that allows the growth of plasmid-containing bacteria only. How to check fragment orientation, before linearization and riboprobe synthesis, is shown in fig. 5

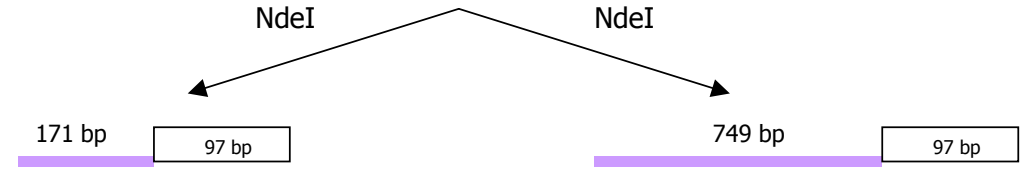
A

• orientation 5´-3´



• orientation 3´-5´





268 bp band
Flt-1 cDNA is inserted in 5´-3´orientation

846 bp band
Flt-1 cDNA is inserted in 3´-5´orientation

B

Vector linearization and riboprobe synthesis

• orientation 5´-3´

linearization with SacI → T7 promoter remains → T7 polymerase synthsizes antisense (3´-5´) probe

linearization with SacII → SP6 promoter remains → SP6 polymerase synthesizes sense (5´-3´) probe

• orientation 3´-5´

linearization with SacI → T7 promoter remains → T7 polymerase synthesizes sense (5´-3´)probe

linearization with SacII → SP6 promoter remains → SP6 polymerase synthesizes antisense (3´-5´) probe

Figure 5. Different possible orientation of the inserted fragment and different enzymes employed for linearization and riboprobe synthesis, according to fragment orientation. (A) Both possible fragment orientations (5´-3´ and 3´-5´) inside the plasmid and bands obtained after NdeI digestion: a 268 bp band indicates a 5´-3´ fragment orientation, whereas a 846 bp band indicates a 3´-5´ fragment orientation. The inserted fragment is shown in violet, the polylinker area in white and the rest of the plasmid in yellow. (B) Linearization enzymes and RNA polymerases are chosen according to the fragment orientation. The linearizing enzyme opens the plasmid and the RNA polymerase promoter next to its restriction site is separated from the fragment and lost.

3.6.4 Protocol for cryostat sections

For ISH study, 8-µm sections of shock-frozen tumour tissues were thaw mounted onto SuperFrost Plus slides and stored in –80°C until use. Before starting the experiment, slides were equilibrated to room temperature for 30 min and then fixed in 4% paraformhaldehyde in PBS; after a passage into 0,25% acetic anhydride in 0,1 M triethanolamine-HCl pH 8,0/ 0,9% NaCl for 10 min and two washes in 2X SSC, sections were dehydrated in ethanol: 1 min 60 % ethanol, 1 min 75% ethanol, 1 min 95% ethanol and 1 min 100% ethanol; they were then delipidated with 5 min chloroform treatment and after two passages in 100% ethanol and 95% ethanol, two min each, finally air dried. The slides were then hybridized with 45 µl (35,000-70,000 cpm/µl) of antisense or sense (control) ^{35}S-labeled riboprobe for VEGFR-1 or VEGFR-2 mRNA diluted in hybridization buffer and incubated overnight at 65°C in a slides chamber humidified with deionized formamide in 20X SSC. The following day, the slides were washed four times in 4X SSC for 5 min, treated with RNase A (20 µg/ml) at 37°C for 30 min, then twice in 2X SSC with 1mM DTT and finally washed again twice, for 30 minutes, at 65°C in 0.1X SSC to remove nonspecific label. After dehydration in ethanol, the slides were exposed on Biomax MR film for 2 days and then dipped in autoradiography emulsion (diluted 1:1 with distilled water). The slides were exposed for 4 weeks at 4°C in light-tight desiccated slide boxes, photographically processed, counterstained in toluidine blue, fixed in Roti-Histol and coverslipped using Roti-Histokitt.

3.7 Immunohistochemistry

3.7.1 Principle

The technique of IHC is useful to visualize the localization and expression intensity of a specific protein in a tissue section. The basic principle of this method is the ability of antibodies to recognize in a specific way particular areas of a protein, called antigen epitopes. To make the reaction visible at microscope

observation, it is necessary to amplify the signal of the primary antibody (raised against the protein of interest) and this purpose can be achieved using a secondary antibody raised against the antibodies of the animal in which the primary antibody was produced. For example, one of the primary antibodies employed in this study was anti- human VEGFR-1 made in rabbit, which was recognized by a secondary antibody anti-rabbit made in goat. To further amplify the signal, the secondary antibody is linked to biotin, a molecule that shows a very strong affinity to avidin; this latter molecule is associated to an enzyme able to convert a colourless substrate (chromogen) into a coloured product that precipitates on the slide at the site of the reaction. In the present work, the enzymes employed were: peroxidase, which produces a brown precipitate acting on the chromogen diaminobenzidine (DAB) (Fig. 6A) and alkaline-phosphatase that gives a red colour acting on the Vector Red substrate (Fig. 6B).

3.7.2 Primary Antibodies

The primary antibodies and dilutions used are listed in the Tab. 3. The antibodies were tested and dilutions were optimized in human normal pituitary glands, which were used as control tissues.

Table 3. Primary antibodies used for IHC studies.

Antigens	Primary antibodies	Secondary biotinylated antibodies	Incubation time in chromogen
VEGF-A	Goat anti-human (1:200 dil.) (Santa Cruz Biotechnology, Santa Cruz, CA)	Horse anti goat (Vector Laboratories Inc., Burlingame, CA)	5 min
VEGF-C	Rabbit anti-human (1:100 dil.) (Zymed, S. Francisco, CA)	Goat anti rabbit (Vector Labs.)	5 min
VEGFR-1 (Flt-1)	Rabbit anti-human (1:100 dil.) (Santa Cruz Biotechnology)	Goat anti rabbit (Vector Labs.)	2 min 30 sec
VEGFR-2 (Flk-1/KDR)	Rabbit anti-human (1:100 dil.) (Santa Cruz Biotechnology)	Goat anti rabbit (Vector Labs.)	4 min
Neuropilin-1	Goat anti-human (1:500 dil.) (Santa Cruz Biotechnology)	Horse anti goat (Vector Labs.)	6 min
VEGFR-3 (Flt-4)	Rabbit anti-human (1:800 dil.) (Santa Cruz Biotechnology)	Goat anti rabbit (Vector Labs.)	5 min
CD31 (PECAM-1)	Mouse anti-human (1:500 dil.) (Dako Cytomation, Glostrup, Denmark)	Horse anti mouse (Vector Labs.)	45 sec
LYVE-1	Rabbit anti-human (1:800 dil.) (Upstate, Lake Placid, NY)	Goat anti rabbit (Vector Labs.)	1 min
Ki-67 (MIB-1)	Mouse anti-human (1:100 dil.) (Dako Cytomation)	Horse anti mouse (Vector Labs.)	1 min
ACTH	Mouse anti-human (1:1000 dil.) (Dako Cytomation)	Goat anti mouse (Vector Labs.)	30 min
LH	Mouse anti-human (1:1000 dil.) (Immunotech, Marseille, France)	Goat anti mouse (Vector Labs.)	30 min
PRL	Mouse anti-human (1:1000 dil.) (Immunotech)	Goat anti mouse (Vector Labs.)	30 min
TSH	Mouse anti-human (1:800 dil.) (Immunotech)	Goat anti mouse (Vector Labs.)	30 min
FSH	Mouse anti-human (1:800 dil.) (Immunotech)	Goat anti mouse (Vector Labs.)	30 min
GH	Mouse anti-human (1:800 dil.) (gift from Dr. C.J. Strasburger, Berlin, Germany)	Goat anti mouse (Vector Labs.)	30 min

1st column: name of the antigen detected; 2nd column: characteristic of the primary antibodies, working dilution and producing companies; 3rd column: characteristic of the secondary antibodies and producing companies; 4th column: incubation time of the chromogenic reaction.

3.7.3 Mono immunohistochemistry- protocol for cryostat sections

For IHC detection, 8-µm sections of shock-frozen tumoural and normal pituitary gland tissues were thaw mounted onto SuperFrost Plus slides, fixed in 4% paraformhaldehyde freshly prepared in PBS and stored in 96% ethanol, at 4°C until use.

Different primary antibodies (listed in Tab. 3) were used to detect intratumoural VEGF receptors (anti VEGFR-1, -2, -3, neuropilin-1), VEGF-A and -C (anti VEGF-A, -C) expression, blood and lymphatic microvessels density (anti CD-31 and anti LYVE-1) and PI (anti Ki-67), which gives an indication about the percentage of tumour cells entering the cell cycle. After a wash in 1X TBS, slides were first incubated for 30 min in serum (diluted 1:10 in 1X TBS buffer pH 7,6) of the same animal in which the specific biotinylated secondary antibody was raised; this step is necessary to prevent the non-specific binding of the primary antibody. For example, before the detection of VEGFR-1, which is recognized by a secondary antibody made in goat, slides were incubated in goat serum. In this way, anything that would bind goat IgG is blocked. Slides were then incubated overnight at 4°C with different primary antibodies diluted as listed in Tab. 3. After three washes in TBS buffer, the corresponding biotinylated secondary antibody, diluted 1:300, was added at room temperature for 30 min. The slides were again rinsed three times in TBS buffer and incubated for 30 min with the avidin-biotin-peroxidase complex (ABC complex) at room temperature. The ABC complex was prepared 30 min before use in order to allow the complex formation. The colour development was performed using 1 mg/ml DAB with 0,01% hydrogen peroxide, applied for the suitable incubation time listed in Tab. 3. Since DAB is light-sensitive, these final steps were performed in the dark. After three more washes, slides were finally counterstained for 15 min in tolouidine blue, which stains nuclei in light blue permitting a more clear visualization of the tissue structure. Excess of colour was removed with two washes in distilled water and one final wash in 70% ethanol containing 5 drops of acetic acid. After dehydration in 96% ethanol and in 100% ethanol, slides were fixed in Roti-Histol and coverslipped using Roti-Histokitt. Negative controls were performed omitting the primary antibody and no staining has been detected in negative control sections.

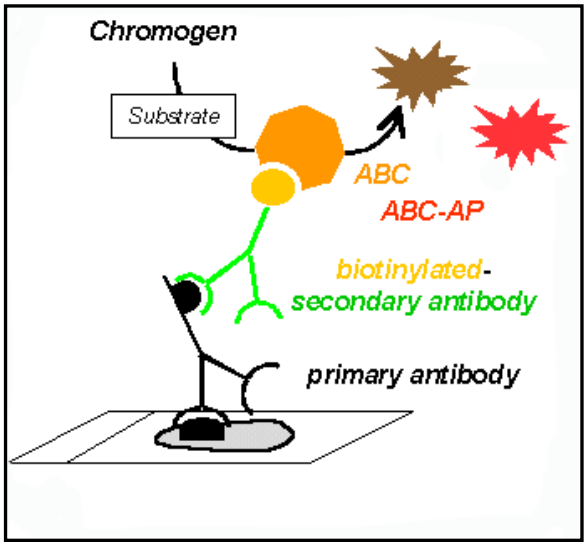
3.7.4 Double immunohistochemistry

For the co-localization of two different antigens on a tissue section, double IHC is the eligible technique, since it produces pictures in which the two antigens

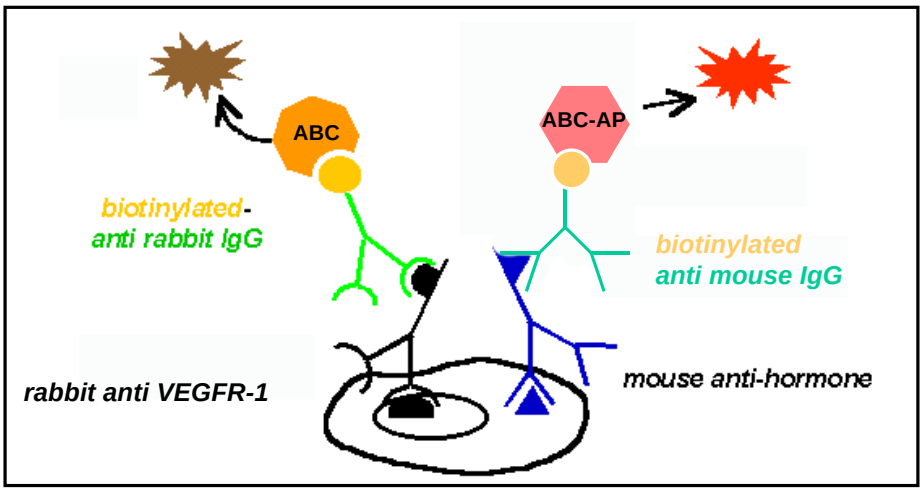
of interest can be identified in two different colours. This is possible, performing one IHC after the other and employing, for one of the two antibodies, a different ABC complex called AP-ABC which is linked to the enzyme alkaline phosphatase. This enzyme acts on the components of the Vector red kit, producing a red colour. In this case the choice of the primary antibodies is very important in order to avoid cross-reactions, usually one monoclonal antibody (made in mouse) and one polyclonal antibody (made in another animal) are employed and the antibody which recognizes the most expressed protein is coupled to AP-ABC complex, since the colour developed by Vector red is less intense than the one obtained by DAB (Fig. 6 B).

In this study we wanted to see which hormone-secreting cells of the human normal adenohypophysis were expressing VEGFR-1.

After immunohistochemical staining of VEGFR-1 with DAB, different sections of human normal adenohypophysis were incubated, for 2 h at room temperature, with diluted monoclonal antibodies anti ACTH, LH, prolactin diluted 1:1000 and TSH, FSH and GH diluted 1:800. Staining was detected using the avidine-biotine-alkaline phosphatase complex, prepared in the same way as ABC complex and Vector Red as chromogen. The Vector Red was prepared according to manufacturer´s instructions, using 2 ml Tris-HCl solution (100 mM, pH 8,2-8,5), 500 µl levamisole (for blocking endogenous alkaline phosphatase activity) and 1 drop of each of the three compounds of the kit; the mixture was then applied on the slides for 30 min. After processing, the slides were finally counterstained with toluidine blue, fixed in Roti-Histol and coverslipped using Roti-Histokitt.



A



B

Figure 6. The mono- and double-IHC principles. (A) Mono IHC. The antigen of interest is recognized by a specific primary antibody that is recognized by a biotinylated secondary antibody, the biotin is bound by avidin conjugated with peroxidase (ABC complex) or alkaline phosphatase (AP-ABC complex), which using respectively as substrate DAB and Vector red catalyze a chromogenic reaction yielding a coloured product (brown or red). (B) Double IHC. Two antigens are investigated on the same tissue (i.e. VEGFR-1 and the different pituitary gland hormones); they have to be recognized by two biotinylated secondary antibodies made in different host animals in order to avoid cross-reaction. The biotin is bound to avidin conjugated with peroxidase for VEGFR-1 and with alkaline-phosphatase for the different pituitary hormones; in this way VEGFR-1 signal is brown (DAB chromogenic reaction) and hormone signal is red (Vector red substrate chromogenic reaction).

3.7.5 Immunoreactivity evaluation

The immunohistochemical staining for VEGF-A, VEGF-C and VEGFR-1 was detected in the cytoplasm of endocrine cells, both in normal and adenomatous pituitaries and the expression of these two antigens was evaluated counting the positive cells out of 100 cells, in three different areas, for each tissue section.

The immunohistochemical signals for VEGFR-2, VEGFR-3, neuropilin-1, CD31 and LYVE-1 were observed in the endothelial cells of vessels. For determining the number of positive vessels, stained vessels were counted inside an area delimited by an eyepiece grid 12,5 X 12,5 mm divided in 10 X 10 squares (Zeiss, Munich, Germany) at a magnification 200X (20X objective and 10X ocular). For each antigen, the number of vessels was determined counting the positive vessels in three different areas of each tumour [Perez-Castro et al., 2003; Graciarena et al., 2004]. The same guidelines were applied for VEGFR-3 positive vessels and for vessels expressing LYVE-1 antigen. Vessels were defined as any positively stained single cell or cluster of cells or structure clearly separated from adjacent microvessels. VEGFR-2 and neuropilin-1 IHC extents have been expressed as percentage of positive vessels for these two antigens compared to CD31 positive vessels (the IHC analysis for these antigens has been performed in serial sections), whereas the VEGFR-3 and LYVE-1 extents are expressed as the average number of positive vessels counted in three different areas.

Ki-67 is a growth-associated nuclear antigen and the PI was calculated counting the positive nuclei out of 100 cells, in three different areas of the tissue section.

3.8 Cell cultures

3.8.1 Primary rat pituitary cell culture

The pituitary primary cell culture was obtained (as explained in Renner et al., 1998) from adult male Sprague-Dawley rats (180-250 g). They were kept for 5

days in our animal house in standard conditions: 12 hours light/dark rhythm, temperature 21°C, water and standard food. Pituitary glands were obtained after decapitation performed quickly after CO_2 narcosis. The tissue was washed with HDB buffer. Sliced fragments were enzymatically dispersed in a buffer containing 4 g/l collagenase, 10 mg/l DNAse II, 0.1 g/l soybean trypsin inhibitor, and 1 g/l hyaluronidase (37°C, approximately 45 minutes). Dispersed cells were centrifuged and resuspended in Dulbecco´s Modified Eagle´s Medium (DMEM) supplemented with 2 mM essential vitamins, 40U/l insulin, 20 ng/l natrium selenate, 5 mg/l transferrin, 30 pM triiodothyronine (T_3), 10% fetal calf serum, 2 mmol/l L-glutamine, 2.5 ng/l amphotericine B and 10^5 U/ml penicillin-streptomycin. Cell viability was determined by fluorescence microscopy after staining with acridin orange and ethidium bromide. Acridin orange enters the membranes of normal cells, yielding green fluorescence in viable cells. Ethidium bromide does not pass the healthy cell membrane and enters only in dead cells with damaged membranes, yielding a red fluorescence. Cell viability of pituitary cells was determined as the percentage of green cells in the total number of cells (counted in a Neubauer chamber) and was over 95%. Cells were distributed in 96-well plates and incubated at 37°C under 5 % CO_2. The stimulation was performed 48 hours after preparation.

3.8.2 Immortalised pituitary cell lines

Rodent and human cell lines were grown routinely in the suitable medium, different for each cell line and under the same conditions in an incubator at 37°C, with 5% CO_2. Corticotrophinoma mouse AtT20, mammosomatotrophinoma rat GH3 and FS-like mouse TtT/GF cells were grown in DMEM supplemented with 10% fetal calf serum (FCS), 2 mmol/l L-glutamine, 2,5 ng/ml amphotericin B and 10^5 U/ml penicillin-streptomycin; the same medium was adopted for gonadotrophinoma HP75 cells except that these cells need 2,5% FCS and 15% horse serum. Somatotrophinoma rat MtT/S cells were cultured in the same medium described before for the primary rat pituitary cell culture. The expression of VEGF receptors was studied by RT-PCR in MtT/S cells, AtT20 cells, GH3 cells,

TtT/GF cells and HP75 cells. Functional studies on cell growth and signaling were performed in MtT/S cells.

3.8.3 Cell Proliferation Assay

Proliferation of MtT/S cells induced by exogenously added VEGF-A or PlGF was measured using the [^{3}H]-thymidine incorporation method; the radioactivity, incorporated in the DNA of the stimulated cells during S phase of the cell cycle, is an indicator of the cells growth rate.

The cells were seeded (20000 cells/well) in 48- well plates. After 24 h, the cells were made quiescent by overnight incubation with serum-free medium; this step synchronizes all the cells to the cell cycle phase G0. The quiescent cells were treated with various concentrations of VEGF or PlGF (0, 0.1, 1, 10, 50, 100 ng/ml) in serum-free medium for 96 h. In order to study the effect of LY294002 (a specific inhibitor of PI3K), four stimulation condition were planned. After overnight serum deprivation, the cells were treated as follows: control and the second condition with 0,1% DMSO (since LY294002 is dissolved in DMSO), the third and fourth condition were treated with LY294002 30µM for 1 h, then to the cells in conditon number two and four, 50 ng/ml PlGF were added for 96 h. During the last 3 h of incubation, 0.5 µCi/ml [^{3}H]thymidine were added, then medium was removed and after one wash in cold PBS, cells were precipitated with ice-cold 10% tricholoroacetic acid (1 h, 4°C) and washed with cold PBS. Then DNA was hydrolyzed overnight with 0,5 M NaOH/0,1% Triton X-100 and the radioactivity measured in a liquid scintillation counter.

3.9 VEGF-A ELISA (Enzyme linked immunosorbent assay)

Enzyme-Linked Immunosorbent Assay (ELISA) is a useful and powerful method in estimating ng/ml to pg/ml ordered substances in solution, such as serum, urine and cell culture supernatant. It employs the quantitative sandwich immunoassay technique. Standards and samples are pipetted into the wells of a microplate pre-coated with affinity purified polyclonal antibody specific for mouse

VEGF-A and any mouse VEGF-A present in the supernatants is bound by immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody, specific for mouse VEGF is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a coloured product whose intensity is measured by a microplate reader capable of measuring absorbance at 450 nm. The intensity of the colour measured is proportional to the amount of mouse VEGF bound in the initial step. The sample concentration values are then calculated from the standard curve.

MtT/S cells were seeded in 48-well-plate at 50000 cells/well, serum deprived overnight and, after 24 h, supernatant was collected from three different wells. VEGF-A concentration was measured in MtT/S cell supernatants by a Quantikine ELISA kit specific for mouse VEGF-A (R&D Systems) according to the manufacturer´s instructions. The detection range was 3-2500 pg/ml.

3.10 Hormones measurement by RIA

Radioimmunoassay (RIA) is a highly sensitive and quantitative technique used for the measurement of substances such as enzymes, proteins, hormones, that exist in very low concentrations. In this study, the RIA has been used to measure the concentration of rat ACTH, PRL and GH secreted in the medium by primary rat pituitary cultures.

RIA uses radiolabeled antigens (Ag) to detect Ag-Ab reactions. The procedure follows the basic principle of radioimmunoassay where there is competition between a radioactive and a non-radioactive antigen for a fixed number of specific antibody binding sites. The antigens are labeled with the I^{125} (iodine-125) isotope, and the presence of Ag-Ab reactions is detected using a gamma counter.

So the first step for starting the RIA is developing an antibody that is highly specific for the hormone being measured. An N-terminal specific antibody against rat ACTH was raised in rabbits using an antigen produced by the two-step carbodiimid method (explained in Stalla et al., 1989). Standards were purchased

from Bachem (Bubendorf, Switzerland). The rat GH and rat prolactin antibodies were included in the specific RIA reagent kits provided by the National Hormone and Peptide Program (Baltimore, MD), containing the specific antigens, antiserums and standards. A small quantity of the antibody was mixed with a certain quantity of the sample (cell culture supernatant) containing the hormone to be measured. At the same time, a certain amount of tracer (standard Antigen labeled with the radioactive isotope I^{125}) was added to the mixture. The samples were incubated 1 hour at 37°C, allowing time for the hormone (Ag) to bind to the antibody. The mixture was prepared in such quantities that there was not enough antibody to bind with both the labelled hormone and with the hormone to be measured, so the natural hormone and the labeled hormone had to compete for binding sites. The quantity of each hormone bound was proportional to their concentration and the amount of labeled hormone (tracer) bound to the specific antibody was inversely proportional to the concentration of the natural hormone. After binding had reached the equilibrium, the quantity of radioactive hormone bound to the antibody was measured in a gamma counter. As explained above, the amount of radioactivity present in the test was inversely proportional to the amount of hormone in the sample.

Quantification of the unknown free hormone in the sample was achieved by comparing their activity with a standard curve prepared by using increasing amounts of known concentrations of the hormone.

Before hormone measurements, the primary rat pituitary cell culture were serum-deprived overnight, stimulated for 24 h with 0.1, 1, 10, 50, 100 ng/ml VEGF in serum-free medium and afterwards the supernatants were collected for RIA analysis.

3.11 Western immunoblotting

Western blot is a technique for detection of proteins in different samples such as, tissue or cell extracts, serum, liquor or cell culture supernatants. Mostly, the proteins are separated by electrophoresis, transferred to different types of membranes and detected by various methods, among them immunological

methods. The latter allow the measurement of the relative amount of a specific protein present in the above-mentioned samples. This is possible by using a primary specific antibody directed against the studied protein which will be detected by a secondary antibody (horseradish peroxidase-conjugated) and visualized on a nitrocellulose membrane, after incubation with a substrate developing a luminescent product.

For the western blot experiments, $1x10^6$ MtT/S cells were plated in a 10 cm-diameter Petri dish containing the specific medium described before added with 10% FCS; a dish was prepared for each different condition chosen for stimulation. After overnight serum deprivation, each dish was stimulated with 50 ng/ml PlGF for 30 min, 1 h, 3h and 6 h to study the phosphorylation of the PI3K transduction pathway components (PDK1, PTEN, Akt (Thr308 and Ser473), GSK3-β); whereas for detection of Bcl-2 and cyclin D1, the cells were treated with 50 ng/ml PlGF and then collected 24h, 48h, 72h, 96 h after the stimulation. For the experiment with LY294002, four Petri dishes (one for each condition) were prepared with $1x10^6$ MtT/S cells per dish. After overnight serum deprivation, the plates were treated as follows: control plate and the second plate with 0,1% DMSO (since LY294002 is dissolved in DMSO), the third and fourth plate were treated with LY294002 30μM for 1 h, then to the plate number two and four 50 ng/ml PlGF were added for 30 min.

The cells were washed with cold PBS, removed from the dish with a plastic scraper and the proteins were extracted breaking the cell membranes by pipeting up and down through a very small (insulin) syringe, in proteases inhibitor cocktail diluted 1:100 in PBS, working always on ice; the volume of the inhibitor cocktail was decided according to the dimension of the membranes pellet obtained after centrifugation. The protein samples concentration was determined with Bradford dye assay [Bradford, 1976]. The method is based on the proportional binding of the dye Coomassie to proteins and colorimetric reaction of this binding; as the protein concentration increases, the color of the test sample becomes darker. The protein concentration of a test sample is determined by comparison to that of a series of protein standards known to reproducibly exhibit a linear absorbance profile in this assay.

Fifty µl of the protein mixture obtained for each sample were separated using a pre-cast Tris-Glycine 10% gel in an Invitrogen electrophoresis apparatus, according to the manufacturer´s instructions. This procedure separates the proteins according to their size.

The protein bands were then transferred on a nitrocellulose membrane (Hybond ECL), through an electrophoresis procedure in which the gel was on the negative side of the apparatus and the nitrocellulose membrane on the positive side, in this way the negative-charged proteins are driven from the gel to the positive-charged membrane, in the same position.

The nitrocellulose membrane was then blocked for 2 h at room temperature in a 1X TBS solution containing 5% milk powder and 0,1% Tween, with gentle shaking. Then it was incubated over night at 4°C with the primary antibody diluted (see Tab. 4) in a 1X TBS solution containing 2,5% milk powder and 0,1% Tween, with gentle shaking. After 3 washes in 1X TBS 0,1% Tween 10 min each, the membrane was incubated with the secondary antibody diluted 1:1000 in 1X TBS 2,5% milk 0,1% Tween solution for 1 h at room temperature. Three further washes in 1X TBS 0,1% Tween, 10 min each, were then performed before incubating the membrane in the Lumi-light Western Blotting Substrate solution, prepared according to the manufacturer´s instructions. An x-ray film was exposed to the membrane in an autoradiography cassette, to detect the light given off by the enzyme reaction. After 30 min, the film was removed and developed to visualize the immunoreactivity bands. The bands were present wherever there was a protein-primary antibody-secondary antibody-enzyme complex, or in other words, wherever the studied protein was.

Table 4. Antibodies used in the western blotting studies.

Antigens	Primary antibodies	Secondary horseradish peroxidase conj. antibodies
Phospho-PDK1	Rabbit anti-human, rat, mouse (1:1000 dil.) (Cell Signalling Tech., Beverly, MA)	Donkey anti-rabbit (Amersham Biosciences, Bucks, UK)
Phospho-PTEN	Rabbit anti-human, rat, mouse (1:1000 dil.) (Cell Signaling Tech.)	Donkey anti-rabbit (Amersham Biosciences)
Phospho-Akt (Thr308)	Rabbit anti-human, rat, mouse (1:500 dil.) (Cell Signaling Tech.)	Donkey anti-rabbit (Amersham Biosciences)
Phospho-Akt (Ser473)	Rabbit anti-human, rat, mouse (1:500 dil.) (Cell Signaling Tech.)	Donkey anti-rabbit (Amersham Biosciences)
Phospho-GSK-3β	Rabbit anti-human, rat, mouse (1:1000 dil.) (Cell Signaling Tech.)	Donkey anti-rabbit (Amersham Biosciences)
Cyclin D1	Mouse anti-human (1:1000 dil.) (BD Biosciences, San Diego, CA)	Donkey anti-mouse (Amersham Biosciences)
Bcl-2	Mouse anti-human (1:1000 dil.) (BD Biosciences)	Donkey anti-mouse (Amersham Biosciences)

1st column: name of the antigen detected; 2nd column: characteristic of the primary antibodies, working dilution and producing companies; 3rd column: characteristic of the secondary horseradish peroxidase conjugated antibodies and producing companies.

3.12 Statistics

The statistical analysis of the immunohistochemical expression of different VEGF receptors compared to the biological parameters of the tumours investigated was performed with the Fisher exact test. The same statistical test was employed to analyze the correlation between VEGF-A and VEGFR-1, -2, -3, neuropilin-1 expression and between VEGF-C and VEGFR-3. Statistical significance was considered at $p<0,05$.

Hormone secretion and cell proliferation experiments were all performed in quadruplicate wells and results are expressed as mean ± standard error.

For statistical analyses of stimulation experiments, the mean values were compared by one-way ANOVA. P values smaller than 0,05 were considered significant. The significance grades are marked with stars as follows: * $p< 0,05$, ** $p<0,005$, *** $p<0,001$.

4 RESULTS

4.1 Characteristics of the normal and adenomatous pituitary samples studied

The normal and adenomatous pituitary tissues included in this study were analyzed by IHC to investigate PI, which represents the number of cells entering the cell cycle, as well as, the number of blood (CD31 positive) and lymphatic (LYVE-1 positive) vessels (Tab. 5).

In the three NP analyzed, the PI values were not higher than 0,6%, whereas in the pituitary adenomas group, the PI values ranged between 0 and 10,6%: in 21 cases the PI value was lower than 1%, in 10 cases it was between 1 and 2% and in 8 cases it was greater than 2%.

Concerning blood vessels count in NP, two cases showed a vessels count higher than 30 vessels and one between 21 and 30 vessels.

The blood vessels count analysis revealed a number of vessels lower than 10 in 9 cases, between 10 and 20 in 12 cases, between 21 and 30 in 8 cases and more than 30 in 13 cases (Tab. 5).

No lymphatic vessels were detected in the NP and in any tumour investigated, the lymphatic vessels count did not exceed the average of 14,6 (Tab. 5). The majority of the pituitary tumours investigated, did not show LYVE-1 immunopositive vessels, while 11 tumours out of 35 (in 4 cases the determination of lymphatic vessels count was not possible) showed positive LYVE-1 vessels. Five cases out of these 11 had no more than 6 vessels in the whole tissue section. It is of interest that, 8 out of these 11 LYVE-1 positive cases were classified as grade III, and one tumour demonstrated the highest PI (10,6%).

No other significant relation was found among tumour type, tumour grade, PI, blood and lymphatic vessels counts.

Table 5. Clinico-biological characteristics of the normal and adenomatous pituitary tissues included in this study.

Tissue	Gender	Age	Grade	PI (%)	Blood vessels Count (CD31+)	Lymphatic vessels Count (LYVE-1+)
NP1	M	67	-	0,3	21-30	0
NP2	M	46	-	0,6	>30	0
NP3	F	37	-	0,6	>30	0
ACRO1	M	29	III	1,3	21-30	14
ACRO2	F	28	II	0	>30	14,6
ACRO3	M	41	II	0	10-20	0
ACRO4	F	52	III	2	10-20	0
ACRO5	M	70	III	7,6	<10	0
ACRO6	F	51	II	4,6	10-20	n.d.
ACRO7	F	77	III	0	>30	0
ACRO8	F	60	III	0,6	>30	0
ACRO9	M	44	II	0	21-30	0
ACRO10	F	47	I	0	>30	0
ACRO11	F	50	II	0	10-20	0
CUSH1	F	46	III	2,6	<10	0
CUSH2	F	52	II	1	10-20	0
CUSH3	F	29	III	0	<10	n.d.
NFPA1	M	48	III	1	10-20	0
NFPA2	F	49	III	2	<10	8
NFPA3	F	64	III	0,5	21-30	0
NFPA4	M	35	III	0	<10	8,3
NFPA5	M	68	III	0,5	>30	0
NFPA6	M	53	III	0	>30	n.d.
NFPA7	M	39	III	0	<10	3 *
NFPA8	M	61	III	0	21-30	0
NFPA9	F	59	III	0	<10	0
NFPA10	F	55	III	2,3	>30	0
NFPA11	F	52	II	1,3	>30	0
NFPA12	F	30	III	2	21-30	0
NFPA13	F	66	III	2,6	10-20	2
NFPA14	M	61	II	0	10-20	0
NFPA15	F	48	III	9	>30	0
NFPA16	M	75	II	0	21-30	0
NFPA17	F	32	II	1	10-20	n.d.
PROL1	F	37	III	0,6	<10	2 *
PROL2	M	28	III	0	21-30	0
PROL3	M	28	III	4,3	10-20	0
PROL4	M	43	II	1,6	>30	3 *
PROL5	F	26	I	0	>30	9,6
PROL6	M	43	III	10,6	10-20	4,3
THYR1	F	29	III	1,3	<10	3 *
THYR2	M	32	III	0,7	10-20	0

1 st column: clinical diagnosis (see abbreviation list for the meaning of abbreviations); 2nd column: gender (M: male, F: female); 3 rd column: age at the time of autopsy/tumour resection; 4 th column: tumour grade according to Hardy classification directions; 5 th column: PI values (% of ki-67 positive cells); 6 th column: blood vessels number (number of CD31 positive vessels); 7 th column: lymphatic vessels number (number of LYVE-1 positive vessels); PI, blood and

lymphatic vessels numbers were determined as described in Materials and Methods; * number of LYVE-1 positive vessels in whole tissue; nd, not determined.

4.2 Expression of VEGF and its receptors in normal and adenomatous pituitary by RT-PCR

To have a first overview of VEGF and VEGF receptors expression in normal and adenomatous pituitary, RT-PCR was performed using specific primers for VEGF, VEGFR-1, VEGFR-2, VEGFR-3 and neuropilin-1 listed in Tab. 1.

The results obtained are summarized in Tab. 6. All the three normal human pituitary tissues expressed VEGF, VEGFR-1, VEGFR-2, VEGFR-3 and neuropilin-1 transcripts. All tumours expressed VEGF mRNA, whereas 20 out of 21 expressed VEGFR-1 mRNA and only 1 case did not express this receptor mRNA. The results of RT-PCR analysis for VEGFR-2 15 out of 21 showed a strong expression of VEGFR-2 mRNA, whereas 3 out of 21 showed a weak expression and 3 out of 21 did not express VEGFR-2 mRNA. Neuropilin-1 mRNA was expressed strongly in 6 out of 9 tumours, weakly in 2 out of 9 cases and lacked in 1 case out of 9. The results of RT-PCR analysis for VEGFR-3 showed that the mRNA expression of this receptor was strong in 5 cases out of 8 and absent in the remaining 3 cases (Tab. 6).

The RT-PCR method is good for a preliminary screening of the investigated genes in a group of samples; however, it has some limitations, since it gives information only about the presence of the mRNA of the studied genes, but is not useful to detect the localization of the genes product or to get information about their translation into protein. This is the reason why we performed the RT-PCR analysis only in some pituitary tumour samples and we preferred to keep the other samples for ISH and IHC studies.

Table 6. Results of the RT-PCR performed for VEGF, VEGFR-1, VEGFR-2, VEGFR-3, neuropilin-1 in normal and adenomatous pituitary samples.

Tissue	VEGF	VEGFR-1	VEGFR-2	Neuropilin-1	VEGFR-3
NP1	+	+	+	+	+
NP2	+	+	+	+	+
NP3	+	+	+	+	+
ACRO1	+	+	+	n.d.	n.d.
ACRO2	n.d.	n.d.	n.d.	n.d.	n.d.
ACRO3	+	+	+	n.d.	n.d.
ACRO4	n.d.	n.d.	+/-	-	-
ACRO5	n.d.	n.d.	n.d.	n.d.	n.d.
ACRO6	n.d.	n.d.	n.d.	n.d.	n.d.
ACRO7	+	+	+	n.d.	n.d.
ACRO8	+	+	-	n.d.	n.d.
ACRO9	n.d.	n.d.	n.d.	n.d.	n.d.
ACRO10	n.d.	n.d.	n.d.	n.d.	n.d.
ACRO11	n.d.	n.d.	n.d.	n.d.	n.d.
CUSH1	n.d.	+	+/-	+/-	-
CUSH2	+	+	+	n.d.	n.d.
CUSH3	n.d.	+	+	n.d.	n.d.
NFPA1	n.d.	n.d.	n.d.	n.d.	n.d.
NFPA2	n.d.	n.d.	n.d.	n.d.	n.d.
NFPA3	n.d.	+	+	n.d.	n.d.
NFPA4	n.d.	n.d.	n.d.	+	+
NFPA5	n.d.	n.d.	n.d.	n.d.	n.d.
NFPA6	n.d.	n.d.	n.d.	n.d.	n.d.
NFPA7	+	+	+	+	-
NFPA8	+	+	+	n.d.	n.d.
NFPA9	n.d.	n.d.	n.d.	n.d.	n.d.
NFPA10	+	+	+	+	+
NFPA11	+	+	+	+	n.d.
NFPA12	+	+	+	n.d.	n.d.
NFPA13	+	+	+	+/-	+
NFPA14	n.d.	n.d.	n.d.	n.d.	n.d.
NFPA15	+	+	+/-	n.d.	n.d.
NFPA16	+	+	-	n.d.	n.d.
NFPA17	n.d.	n.d.	n.d.	n.d.	n.d.
PROL1	n.d.	n.d.	n.d.	n.d.	n.d.
PROL2	+	+	+	n.d.	n.d.
PROL3	+	+	+	+	+
PROL4	n.d.	+	+	+	+
PROL5	n.d.	n.d.	n.d.	n.d.	n.d.
PROL6	n.d.	-	n.d.	n.d.	n.d.
THYR1	n.d.	n.d.	n.d.	n.d.	n.d.
THYR2	+	+	-	n.d.	n.d.

See abbreviation list for the meaning of abbreviations

+ strong expression; +/- faint expression; - no transcript; n.d., not determined.

4.3 VEGF-A expression in normal and adenomatous pituitary by IHC

VEGF-A immunostaining was detected in endocrine cells cytoplasm of both normal pituitaries and pituitary adenomas. All the 3 NP expressed VEGF-A in a similar extent (31-60% of the endocrine cells). Nineteen out of 39 tumours showed VEGF-A immunoreactivity in more than 61% of the endocrine cells, 9 tumours in 31-60%, 5 in 10-30% and 1 had less than 10% VEGF-A positive cells; 5 tumours were negative for VEGF-A. All the cases of CUSH (3/3), PROL (6/6) and THYR (2/2) were positive for VEGF-A immunohistochemistry, whereas 8 out of 11 ACRO and 15 out of 17 NFPA showed VEGF-A immunostaining. Nineteen cases of the 39 pituitary adenomas investigated showed a number of VEGF-A positive cells higher than 61%, which is higher than the VEGF-A extent observed in NP (Tab. 7).

4.4 VEGFR-1 expression in normal and adenomatous pituitary by IHC

In all the 3 human NP studied, VEGFR-1 immunoreactivity was detected in 30% of endocrine cells (Fig. 7A); double IHC revealed VEGFR-1 immunoreactivity in ACTH-, FSH-, GH-, LH- and PRL- immunopositive cells (Fig. 8A, B, C, D, E). In pituitary adenomas, VEGFR-1 immunostaining was detected in endocrine cells, but not in vessels (Fig. 9A). Twenty-four out of 39 tumours stained positive for VEGFR-1: in 11 out of these 24 cases the number of immunopositive cells was higher than 61%, in 4 cases the percentage of positive cells was in the range 10-30%, while in 9 cases VEGFR-1 immunoreactivity was detected in less than 10% of the endocrine cells. Half of the VEGFR-1 immunopositive tumours had higher number of VEGFR-1 expressing cells compared to the normal pituitary glands. VEGFR-1 expression was detected in 7 out of 11 ACRO, 2 out of 3 CUSH, 11 out of 17 NFPA, 3 out of 6 PROL and 2 out of 2 THYR (Tab. 7). ISH analysis confirmed the data derived by IHC (Tab. 7) and VEGFR-1 mRNA was localized in endocrine cells (Fig. 10A).

4.5 VEGFR-2 expression in normal and adenomatous pituitary by IHC

Immunohistochemical analysis for VEGFR-2 revealed its expression in blood vessel endothelial cells of both normal (Fig. 7B) and adenomatous (Fig. 9B) pituitaries; these data were confirmed by ISH (Tab. 7, Fig. 10B).

Comparison of the number of CD31 positive vessels with VEGFR-2 positive vessels demonstrated that not all the vessels, in normal and adenomatous pituitaries, expressed VEGFR-2. For this reason VEGFR-2 immunostaining was expressed as percentage of vessels positive for VEGFR-2 compared to vessels positive for CD31. In the normal pituitaries the percentage of VEGFR-2 positive vessels was higher than 61%. VEGFR-2 immunoreactivity was found in: 2 out of 11 ACRO, 2 out of 3 CUSH, 10 out of 17 NFPA, 2 out of 6 PROL but all THYR were negative for VEGFR-2. More in detail, 21 pituitary adenomas out of 39 did not express VEGFR-2, whereas in 12 of the positive cases, the percentage of VEGFR-2 positive vessels was higher than 61%, in 3 cases between 31 and 60%, in 1 case between 10 and 30% and in 2 cases lower than 10% (Tab. 7).

4.6 Neuropilin-1 expression in normal and adenomatous pituitary by IHC

IHC revealed neuropilin-1 expression in blood vessel endothelial cells in normal (Fig. 7C) and adenomatous (Fig. 9C) pituitary. As it was observed for VEGFR-2, not all the CD31 positive vessels were positive for neuropilin-1, thus immunostaining for this VEGF receptor was expressed as percentage of positive vessels compared to CD31 positive vessels. All the NP analyzed showed neuropilin-1 immunostaining, in 31 to 60% of the CD31 immunopositive vessels. In the different pituitary adenomas, neuropilin-1 was present in 5 out of 11 ACRO, 1 out of 3 CUSH, 7 out of 17 NFPA, 3 out of 6 PROL and 1 out of 2 THYR. Immunohistochemical analysis for neuropilin-1 in pituitary adenomas revealed that more than half tumours were negative. Four tumours had less than 10% neuropilin-1 immunopositive vessels, 4 were in the range of 10-30% neuropilin-1

positive vessels, 7 had 31-60% neuropilin-1 positive vessels, while 2 cases had more than 61% of neuropilin-1 positive vessels. These two pituitary adenomas were the only ones with the number of neuropilin-1 positive vessels higher than in the normal pituitaries (Tab. 7). Altogether these data indicate that in the majority of pituitary adenomas, neuropilin-1 expression is lower than in the normal pituitary.

Table 7. Expression of VEGF-A, VEGFR-1, VEGFR-2 and neuropilin-1, investigated by IHC and ISH, in human normal and adenomatous pituitaries.

Tissue	Grade	PI (%)	Blood Vessel Count (CD31+)	VEGF-A IHC	VEGFR-1 IHC	VEGFR-1 ISH	VEGFR-2 IHC	VEGFR-2 ISH	Neuropilin-1 IHC
NP1	-	0,3	21-30	+++	++	+	++++	+	+++
NP2	-	0,6	>30	+++	++	n.d.	++++	n.d.	+++
NP3	-	0,6	>30	+++	++	n.d.	+++	n.d.	+++
ACRO1	III	1,3	21-30	+++	++	n.d.	-	-	-
ACRO2	II	0	>30	++++	++++	n.d.	-	n.d.	++
ACRO3	II	0	10-20	++	+	+	-	-	+
ACRO4	III	2	10-20	-	++++	n.d.	-	n.d.	-
ACRO5	III	7,6	<10	-	-	n.d.	-	n.d.	-
ACRO6	II	4,6	10-20	++	+	n.d.	+++	n.d.	-
ACRO7	III	0	>30	-	++	+	-	n.d.	++
ACRO8	III	0,6	>30	++	++++	n.d.	++	n.d.	++
ACRO9	II	0	21-30	+++	-	n.d.	-	n.d.	++
ACRO10	I	0	>30	++++	-	n.d.	-	n.d.	-
ACRO11	II	0	10-20	++++	-	n.d.	-	n.d.	-
CUSH1	III	2,6	<10	++++	++++	+	++++	+	++++
CUSH2	II	1	10-20	++++	-	n.d.	+	n.d.	-
CUSH3	III	0	<10	+++	++	n.d.	-	n.d.	-
NFPA1	III	1	10-20	+++	++++	n.d.	++++	n.d.	+++
NFPA2	III	2	<10	++	++++	n.d.	-	n.d.	-
NFPA3	III	0,5	21-30	++++	++++	+	-	-	-
NFPA4	III	0	<10	++++	-	n.d.	++++	n.d.	-
NFPA5	III	0,5	>30	++++	-	n.d.	-	n.d.	-
NFPA6	III	0	>30	+++	+	n.d.	+++	n.d.	-
NFPA7	III	0	<10	++++	++++	+	-	-	-
NFPA8	III	0	21-30	+++	+	+	-	-	-
NFPA9	III	0	<10	++++	-	n.d.	++++	n.d.	-
NFPA10	III	2,3	>30	++++	-	-	+	+	-
NFPA11	II	1,3	>30	-	-	-	+++	+	+
NFPA12	III	2	21-30	+++	++	n.d.	++++	n.d.	++++
NFPA13	III	2,6	10-20	++++	+	n.d.	++++	n.d.	+++
NFPA14	II	0	10-20	-	++++	n.d.	-	n.d.	-
NFPA15	III	9	>30	++++	-	n.d.	++++	n.d.	+++
NFPA16	II	0	21-30	++++	+	n.d.	++++	n.d.	+++
NFPA17	II	1	10-20	+++	-	n.d.	++++	n.d.	+
PROL1	III	0,6	<10	++++	+	n.d.	++++	n.d.	-
PROL2	III	0	21-30	++++	+	+	-	-	-
PROL3	III	4,3	10-20	++++	++++	n.d.	++++	+	+++
PROL4	II	1,6	>30	+++	-	-	-	-	+
PROL5	I	0	>30	+	-	-	-	-	-
PROL6	III	10,6	10-20	++	-	n.d.	++++	+	+++
THYR1	III	1,3	<10	++++	+	n.d.	-	n.d.	-
THYR2	III	0,7	10-20	++++	++++	+	-	-	+++

Immunostaining intensity for VEGF-A and VEGFR-1 was assessed according to an arbitrary scale: - no immunoreactivity, + <10%, ++ 10-30%, +++ 31-60%, ++++ 61-100% immunopositive cells. Immunoreactivity for VEGFR-2 and neuropilin-1: - no immunoreactivity, + <10%, ++ 10-30%, +++ 31-60%, ++++ 61-100 % of CD31 positive vessels which show immunostaining for VEGFR-2 and neuropilin-1. The results of ISH were scored as positive (+) or negative (-) only; n.d. not determined.

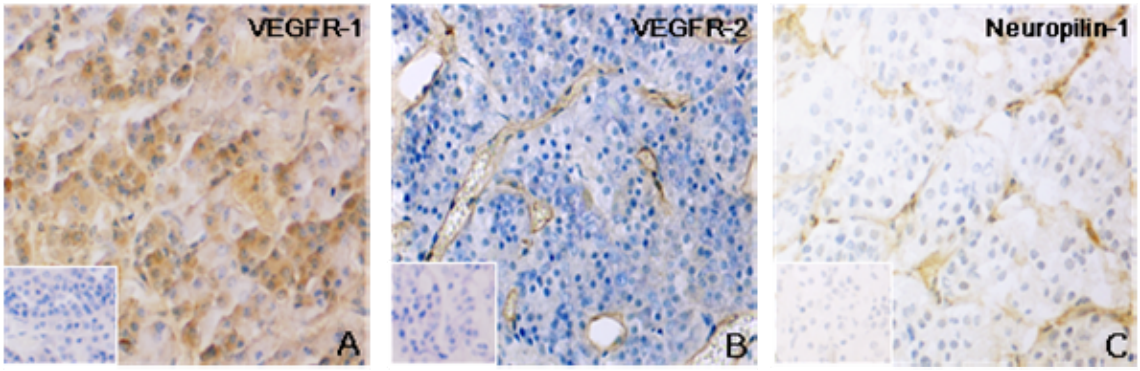


Figure 7. Localization of VEGFR-1, VEGFR-2 and neuropilin-1 in normal human anterior pituitary. Immunostaining of VEGFR-1 was detected in endocrine cells (A); VEGFR-2 (B) and Neuropilin-1 (C) immunostaining were detected in endothelial cells. Inserts: negative control omitting the primary antibody. Nuclei were counterstained with toluidine blue. Magnification 200X.

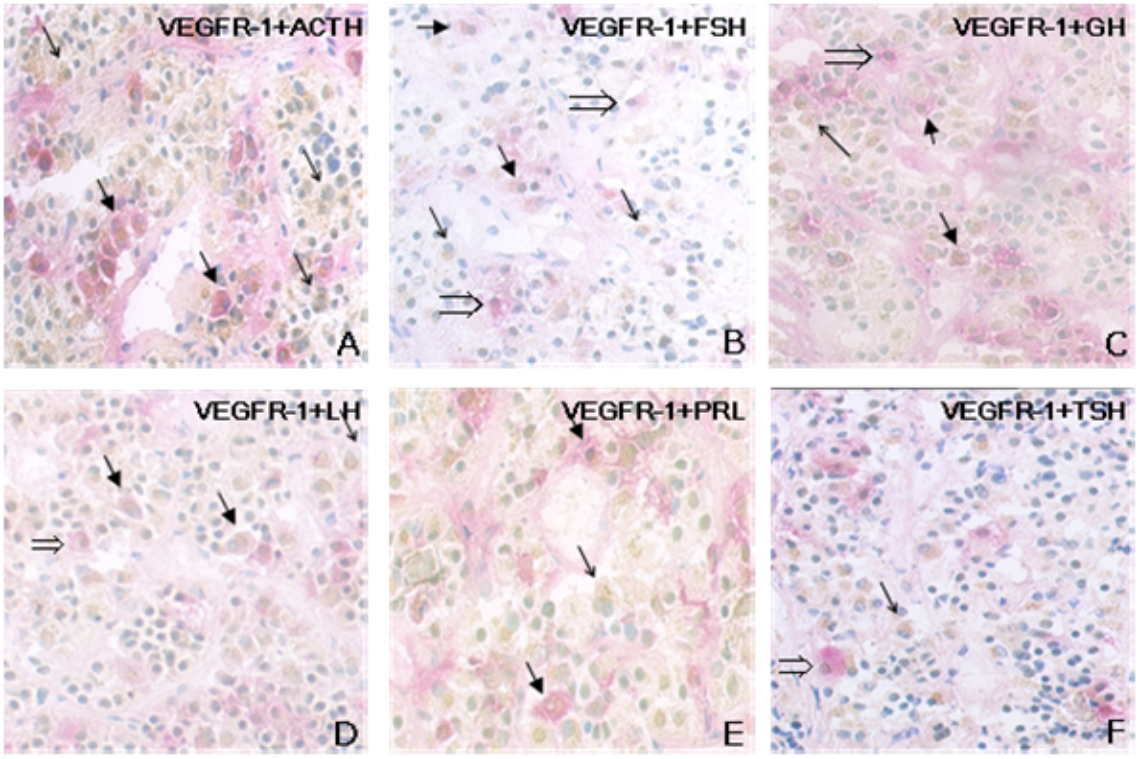


Figure 8. Co-localization of VEGFR-1 and pituitary hormones in normal human anterior pituitary. VEGFR-1 immunostaining (in brown) and co- localized with pituitary hormones immunostaining (in red) for ACTH (A), FSH (B), GH (C), LH (D), PRL (E), but not with TSH (F). Open arrows indicate cells which express VEGFR-1 only, double arrows mark the cells expressing only pituitary hormones and the full arrows show the cells which express VEGFR-1 and one of the pituitary hormones. Nuclei were counterstained with toluidine blue. Magnification X 200.

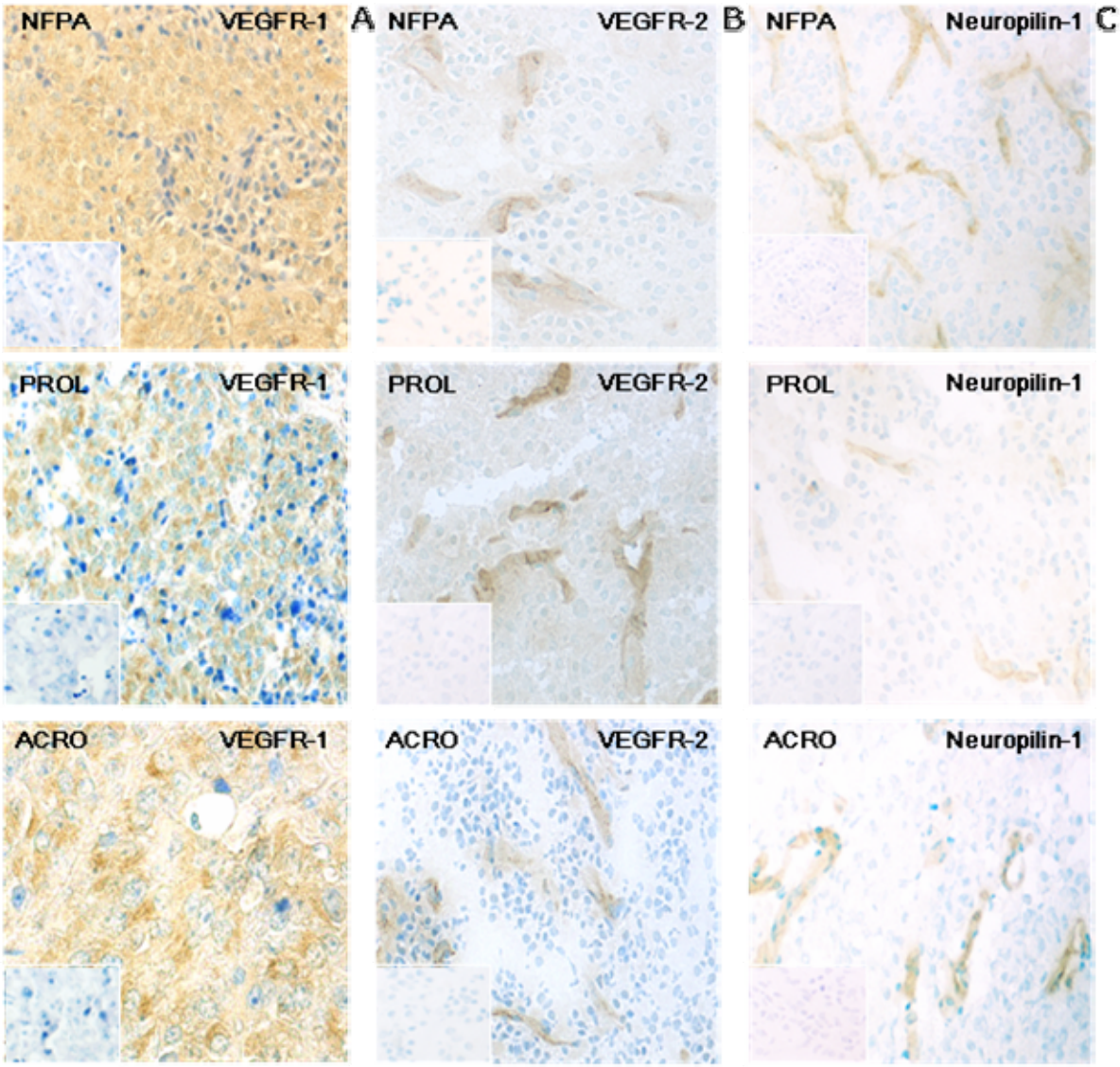


Figure 9. Localization of VEGFR-1, VEGFR-2 and neuropilin-1 in some representative pituitary adenoma types (NFPA, PROL, ACRO). (A) VEGFR-1 immunoreactivity in endocrine cells of a NFPA, PROL, ACRO. (B) VEGFR-2 immunoreactivity in endothelial cells of a NFPA, PROL, ACRO. (C) Neuropilin-1 immunoreactivity in endothelial cells of a NFPA, PROL, ACRO. Inserts: negative controls omitting the primary antibodies. Nuclei were counterstained with toluidine blue. Magnification X 200.

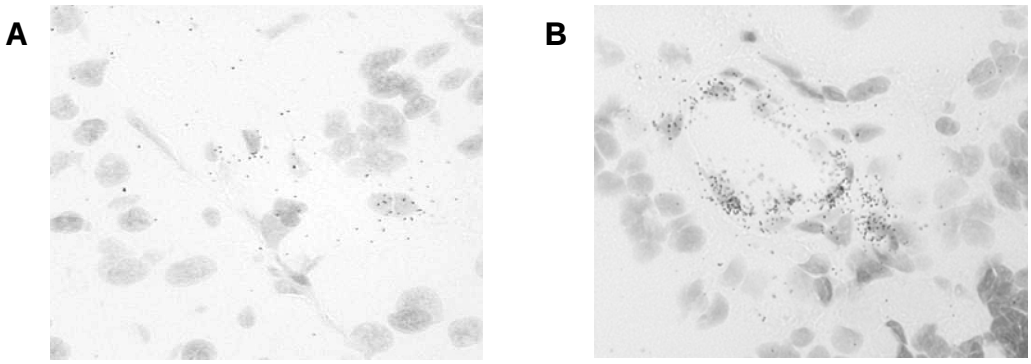


Figure 10. ISH for VEGFR-1 and VEGFR-2 in a representative pituitary adenoma (NFPA). VEGFR-1 ^{35}S-labeled riboprobe signal (black grain) is localized in tumour cells (A), while VEGFR-2 ^{35}S-labeled riboprobe signal (black grain) is detected in vessels endothelial cells (B). Magnification X 400.

4.7 Correlation between VEGF-A, VEGFR-1, VEGFR-2 and neuropilin-1 expression and clinico-biological parameters of the tumours studied

The data obtained from IHC evaluation were analyzed for possible correlation between VEGF-A, VEGFR-1, VEGFR-2 and neuropilin-1 expression and different clinico-biological parameters of the tumours investigated, such as: tumour grade, PI and blood vessel count. VEGF-A expression was also compared to the expression of its receptors VEGFR-1, VEGFR-2 and neuropilin-1 (Tab. 8A). The statistical analysis of the IHC results, performed with the Fisher´s exact test, showed no correlation between VEGF-A and VEGFR-1 expressions and any of the parameters investigated (Tab. 8B). On the other hand, VEGFR-2 and neuropilin-1 significantly correlated with PI (p=0,037 and p=0,009 respectively) (Tab. 8B). VEGF-A expression showed no significant association with the expression of any of its receptors (Tab. 9).

Table 8. Relationship between the expression of VEGF-A, VEGFR-1, VEGFR-2 and neuropilin-1 and tumour grade, PI and blood vessel count.

A

	VEGF-A		VEGFR-1		VEGFR-2		neuropilin-1	
	Low (-/++)	High (+++/ ++++)	Low (-/++)	High (+++/ ++++)	Low (-/++)	High (+++/ ++++)	Low (-/++)	High (+++/ ++++)
Grade								
I/II	5	8	11	2	9	4	12	1
III	6	20	17	9	15	11	18	8
PI								
≤ 2%	8	23	22	9	22	9	27	4
> 2%	3	5	6	2	2	6	3	5
Blood Vess Count								
≤ 20	7	14	13	8	11	10	15	6
> 20	4	14	15	3	13	5	15	3

B

	VEGF-A	VEGFR-1	VEGFR-2	neuropilin-1
Grade	0,453	0,276	0,728	0,225
PI	0,663	1,000	**0,037**	**0,009**
Blood vess count	0,382	0,569	0,323	0,464

The immunoreactivities for VEGF-A and its receptors were subdivided in two categories: `low´ were considered the tumours scored -/++, whereas `high´ were the tumours scored with +++/++++. The tumours were classified in two groups according to tumour grade: grade I/II and grade III, according to PI values: less than or equal to 2% and more than 2% and according to blood vessels count: less than or equal to 20 or more than 20 counted vessels (A). The Fisher exact test was used for statistical analysis and statistical significance was considered at $p< 0,05$; the p values are shown in the lower table (B). The association between VEGFR-2 expression and PI and between neuropilin- 1 expression and PI are statistically significant.

Table 9. Association between VEGF-A expression and the expression of its receptors VEGFR-1, VEGFR-2 and neuropilin-1.

	VEGF-A			VEGF-A			VEGF-A	
VEGFR-1	Low (-/2+)	High (3+/4+)	VEGFR-2	Low (-/2+)	High (3+/4+)	neuropilin-1	Low (-/2+)	High (3+/4+)
Low (-/2+)	7	21	Low (-/2+)	7	16	Low (-/2+)	10	20
High (3+/4+)	4	7	High (3+/4+)	4	12	High (3+/4+)	1	8
	p=0,694			p=1,000			p=0,399	

According to Fisher exact test, there is no significant correlation between VEGF expression and its receptors.

4.8 VEGF-C expression in normal and adenomatous pituitary by IHC

VEGF-C immunoreactivity was detected in cytoplasm of endocrine cells both in NP (Fig. 11A) and in adenomas (Fig. 11 C, D, E, F). In all the 3 NP studied, the percentage of positive cells was less than 10%. Most (22 cases out of 32) of the tumours analyzed did not show any VEGF-C immunoreactivity; of the 10 positive tumours, 8 displayed more than 80% of VEGF-C positive cells, while 2 expressed VEGF-C in less than 10% of the cells (Tab. 10) (see also Fig. 11C-D). VEGF-C immunoreactivity was observed in: 3 out of 7 ACRO, 1 out of 2 CUSH, 4 out of 15 NFPA, 1 out of 6 PROL and 1 out of 2 THYR.

4.9 VEGFR-3 expression in normal and adenomatous pituitary by IHC

VEGFR-3 immunoreactivity was detected in endothelial cells of both normal (Fig. 11B) and adenomatous human pituitary (Fig. 11 G, H, I). All the 3 NP investigated displayed a small number of VEGFR-3 immunopositive vessels (Tab. 10).

Twenty-two out of 38 tumours showed VEGFR-3 positive vessels with numbers ranging from 14,6 to 3,3. Two cases had only 2 and 5 immunopositive vessels in the whole tissue. The positive cases observed were: 4 out of 11 ACRO, 1 out of 3 CUSH, 11 out of 17 NFPA, 5 out of 6 PROL and 1 out of 2 THYR (Tab. 10).

Since VEGFR-3 is also known to be expressed in lymphatic vessels [Kaipainen et al., 1995], the number of vessels positive for VEGFR-3 should be compared to the number of vessels expressing the lymphatic marker, LYVE-1. However, since normal pituitary and most of the pituitary adenomas, had no LYVE-1 positive vessels, it is possible that the VEGFR-3 immunopositive endothelial cells were not belonging to lymphatic vessels.

Table 10. Expression of VEGF-C, VEGFR-3 and LYVE-1 investigated by IHC in human normal and adenomatous pituitaries.

Tissue	Grade	PI(%)	Blood Vessels Count (CD31+)	VEGF-C	VEGFR-3	Lymphatic vessels Count (LYVE-1+)
NP1		0,3	21-30	+	12 *	0
NP2		0,6	>30	+	4 *	0
NP3		0,6	>30	+	4 *	0
ACRO1	III	1,3	21-30	n.d	11,3	14
ACRO2	II	0	>30	-	0	14,6
ACRO3	II	0	10-20	-	0	0
ACRO4	III	2	10-20	++++	0	0
ACRO5	III	7,6	<10	++++	0	0
ACRO6	II	4,6	10-20	n.d.	0	n.d.
ACRO7	III	0	>30	++++	5,6	0
ACRO8	III	0,6	>30	-	7	0
ACRO9	II	0	21-30	-	13,3	0
ACRO10	I	0	>30	n.d.	0	0
ACRO11	II	0	10-20	n.d.	0	0
CUSH1	III	2,6	<10	++++	6	0
CUSH2	II	1	10-20	-	0	0
CUSH3	III	0	<10	n.d.	0	n.d.
NFPA1	III	1	10-20	+	0	0
NFPA2	III	2	<10	-	11	8
NFPA3	III	0,5	21-30	-	0	0
NFPA4	III	0	<10	-	6,3	8,3
NFPA5	III	0,5	>30	-	14,6	0
NFPA6	III	0	>30	-	n.d.	n.d.
NFPA7	III	0	<10	++++	5 *	3 *
NFPA8	III	0	21-30	-	12	0
NFPA9	III	0	<10	++++	7	0
NFPA10	III	2,3	>30	+	0	0
NFPA11	II	1,3	>30	-	4,3	0
NFPA12	III	2	21-30	-	4,6	0
NFPA13	III	2,6	10-20	-	4,3	2
NFPA14	II	0	10-20	n.d.	0	0
NFPA15	III	9	>30	-	10,3	0
NFPA16	II	0	21-30	-	0	0
NFPA17	II	1	10-20	n.d.	13,3	n.d.
PROL1	III	0,6	<10	-	2 *	2 *
PROL2	III	0	21-30	-	0	0
PROL3	III	4,3	10-20	-	3,3	0
PROL4	II	1,6	>30	-	7	3 *
PROL5	I	0	>30	-	6	9,6
PROL6	III	10,6	10-20	++++	10,3	4,3
THYR1	III	1,3	<10	++++	0	3 *
THYR2	III	0,7	10-20	-	4	0

VEGF-C immunoreactivity was scored as following: - no immunostaining signal, + <10%, ++ 10-30%, +++ 31-60%, ++++ 61-100% of positive cells. In the case of VEGFR-3 and LYVE-1, the raw number is displayed, representing the mean of three counts in three different fields inside the eye-piece grid described before or, where the positive vessels were too few, the total number of

positive vessels counted in the whole tissue. N.d., not determined. * number of LYVE-1 or VEGF-3 positive vessels in whole tissue.

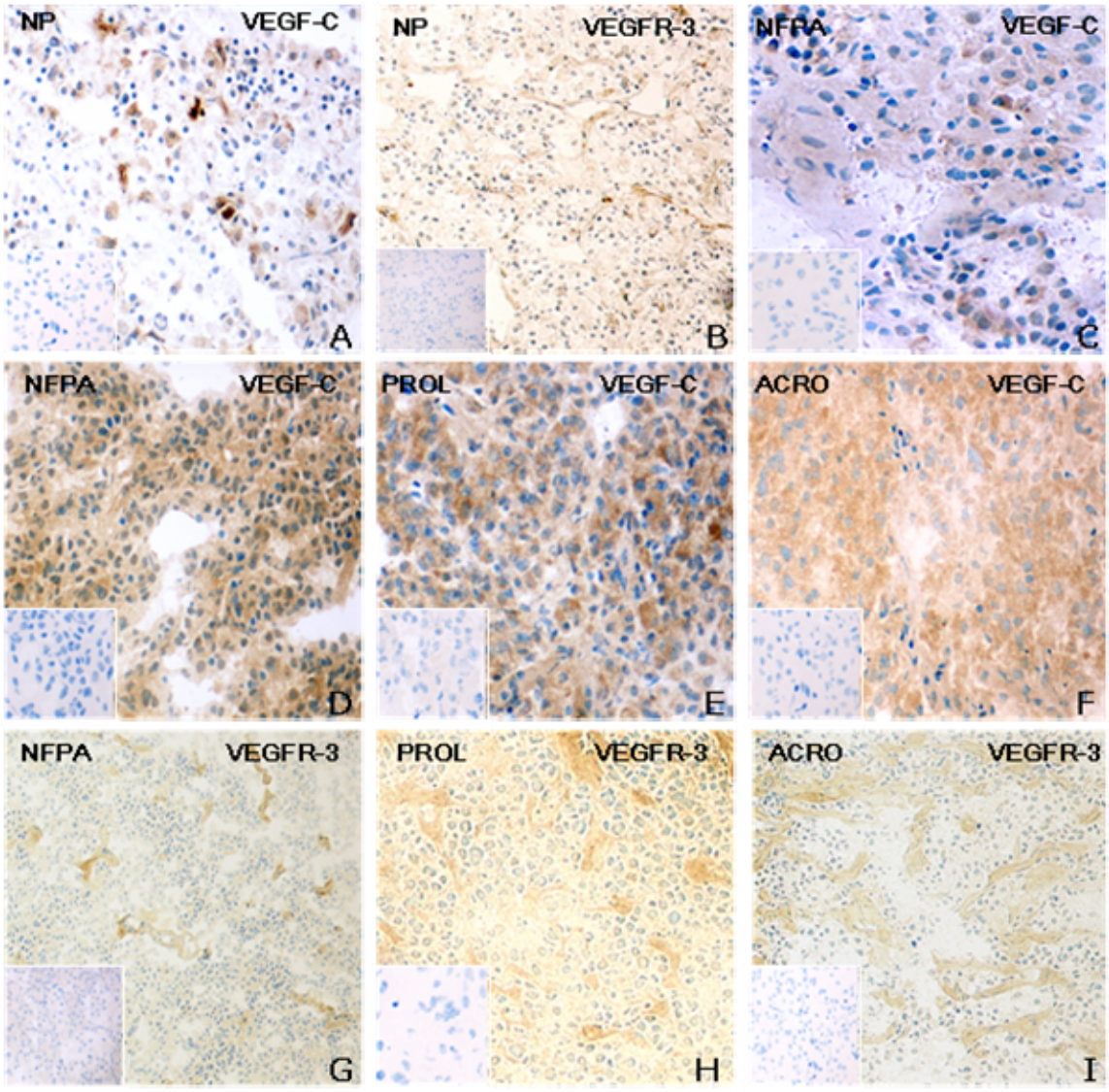


Figure 11. Localization of VEGF-C and VEGFR-3 in human normal and adenomatous pituitary. VEGF-C immunoreactivity in endocrine cells of: normal pituitary (A), NFPA with only some positive cells (C), NFPA with almost all cells are positive (D), PROL (E), ACRO (F). VEGFR-3 immunoreactivity in endothelial cells of: normal pituitary (B), NFPA (G), PROL (H), ACRO (I). Nuclei were counterstained with toluidine blue. Inserts: negative control in which the primary antibody was omitted. Magnification 200X.

4.10 Correlation between VEGF-C, VEGFR-3 expression and clinico-biological parameters of the tumours studied

VEGF-C and VEGFR-3 IHC results were statistically analyzed, with Fisher exact test, for possible correlation with tumour grade, PI, blood and lymphatic

vessels counts. The expression of VEGF-C was also correlated with VEGFR-3 expression.

The statistical analysis showed a significant correlation only between VEGF-C and blood vessels count: surprisingly, the tumours showing low VEGF-C immunoreactivity, displayed high blood vessels count (Tab. 11).

No significant correlation was detected between neither VEGF-C and VEGFR-3 expression and tumour grade, PI and lymphatic vessels counts (Tab. 11) nor between VEGF-C and VEGFR-3 expression (Tab. 12), except the one observed between low expression of VEGF-C and high blood vessel count.

Table 11. Relationship between VEGF-C and VEGFR-3 expression and tumour grade, PI and blood and lymphatic vessels count.

	VEGF-C			**VEGFR-3**		
	Low (-/++)	High (+++/ ++++)	*p*	Low (≤ 5)	High (> 5)	*p*
Grade						
I/II	8	0	0,081	9	4	0,501
III	16	8		14	11	
PI						
≤ 2%	20	5	0,550	18	12	0,698
> 2%	4	3		4	4	
Lymphatic vessels count						
≤ 5	19	8	0,327	20	10	0,134
> 5	4	0		1	4	
Blood vessels count						
≤ 20	9	7	**0,037**	15	6	0,185
> 20	15	1		8	9	

VEGF-C immunoreactivity was classified in two categories: Low (-/++) and High (+++/++++); VEGFR-3 immunoreactivity was classified in two categories: Low (≤ 5 vessels) and High (> 5 vessels). The Fisher exact test was used for statistical analysis and statistical significance was considered at $p< 0,05$. The association between VEGF-C expression and blood vessels count is statistically significant.

Table 12. Association between VEGF-C and VEGFR-3 expression.

	VEGF-C	
	Low (-/++)	High (+++/++++)
VEGFR-3		
Low (≤ 5)	14	4
High (> 5)	9	4

P=0,698 No correlation was found according to Fisher exact test.

4.11 VEGF receptors expression in human and rodent pituitary adenoma cell lines and normal pituitary by RT-PCR

The expression of VEGF receptors mRNA was determined in different human and rodent pituitary tumour cell lines and in normal human and rat pituitary glands by RT-PCR, using the specific primers listed in Tab. 1. From the cell lines analyzed, the rat somatotrophinoma cell line MtT/S expressed VEGFR-1 and neuropilin-1, but not VEGFR-2 mRNA (Fig. 12 A3, B3, C3). VEGFR-3 was expressed only by AtT-20 cell line (Fig. 12 D4), while all the cell lines examined (GH3, TtT/GF, MtT/S, AtT20 and HP75) were positive for neuropilin-1 (Fig. 12C). Normal rat and human pituitary showed an amplification band for all the four VEGF receptors studied (Fig. 12 A5-7, B5-7, C5-7, D5-7).

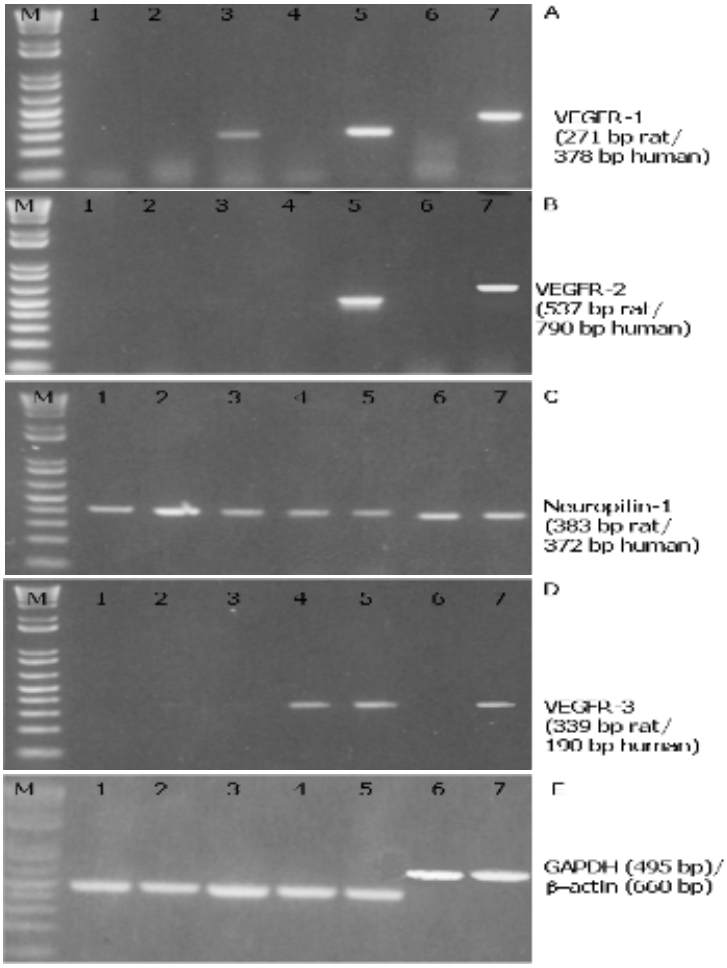


Figure 12. Expression of VEGFR-1 (A), VEGFR-2 (B), neuropilin-1 (C) and VEGFR-3 (D) and GAPDH/β-actin (E) in rat, mouse and human pituitary adenoma cell lines, analyzed by RT-PCR. VEGFR-1 mRNA was detected in somatotrophinoma MtT/S cells, VEGFR-2 was not detected in any cell lines, neuropilin-1 was detected in all the cell lines and VEGFR-3 was

detected in corticotrophinoma AtT-20 cells; normal rat and human pituitaries were both expressing the four VEGF receptors mRNA. 1: rat mammosomatotrophinoma GH3 cells; 2: mouse TtT-GF folliculo-stellate cells; 3: rat somatotrophinoma MtT/S cells; 4: mouse corticotrophinoma AtT-20 cells; 5: rat normal pituitary; 6: human gonadotrophinoma HP75 cell line; 7: normal human pituitary; M: 1 kb Plus DNA Ladder.

4.12 VEGF-A production by MtT/S cells

Constitutive production of VEGF-A from MtT/S cells was observed, in basal condition, with an average secretion of 165, 21 ±22, 25 pg/ml after 24 h.

4.13 VEGF-A effect on rat pituitary and MtT/S cells hormone secretion

The detection of VEGFR-1 mRNA in rat pituitary and in the MtT/S cell line, together with the previous detection of VEGFR-1 protein in human pituitary adenomas, suggested to test the possible effect of VEGF-A on rat pituitary and MtT/S cells hormone secretion. Treatment of rat pituitary cells in primary culture with different concentrations of VEGF-A, increased significantly prolactin secretion in a dose-dependent fashion, after 24 h (Fig. 13A). The treatment with 1 ng/ml VEGF-A induced an increase of 21% (p<0,05) in the secretion of this hormone, but the highest response was obtained after stimulation with 50 ng/ml VEGF-A, which induced an increase of 65% (p<0,005), compared to basal secretion. VEGF-A increased significantly also ACTH secretion by 20% (p<0,005) at 50 ng/ml but not at intermediate doses (Fig. 13B). On the other side, a tendency to enhance GH release, though not significantly, was observed after stimulation with 50 ng/ml VEGF (Fig. 13C). As far as concerned with MtT/S cells, any stimulation in GH secretion was detected, after VEGF-A treatment used at the same concentrations (data not shown).

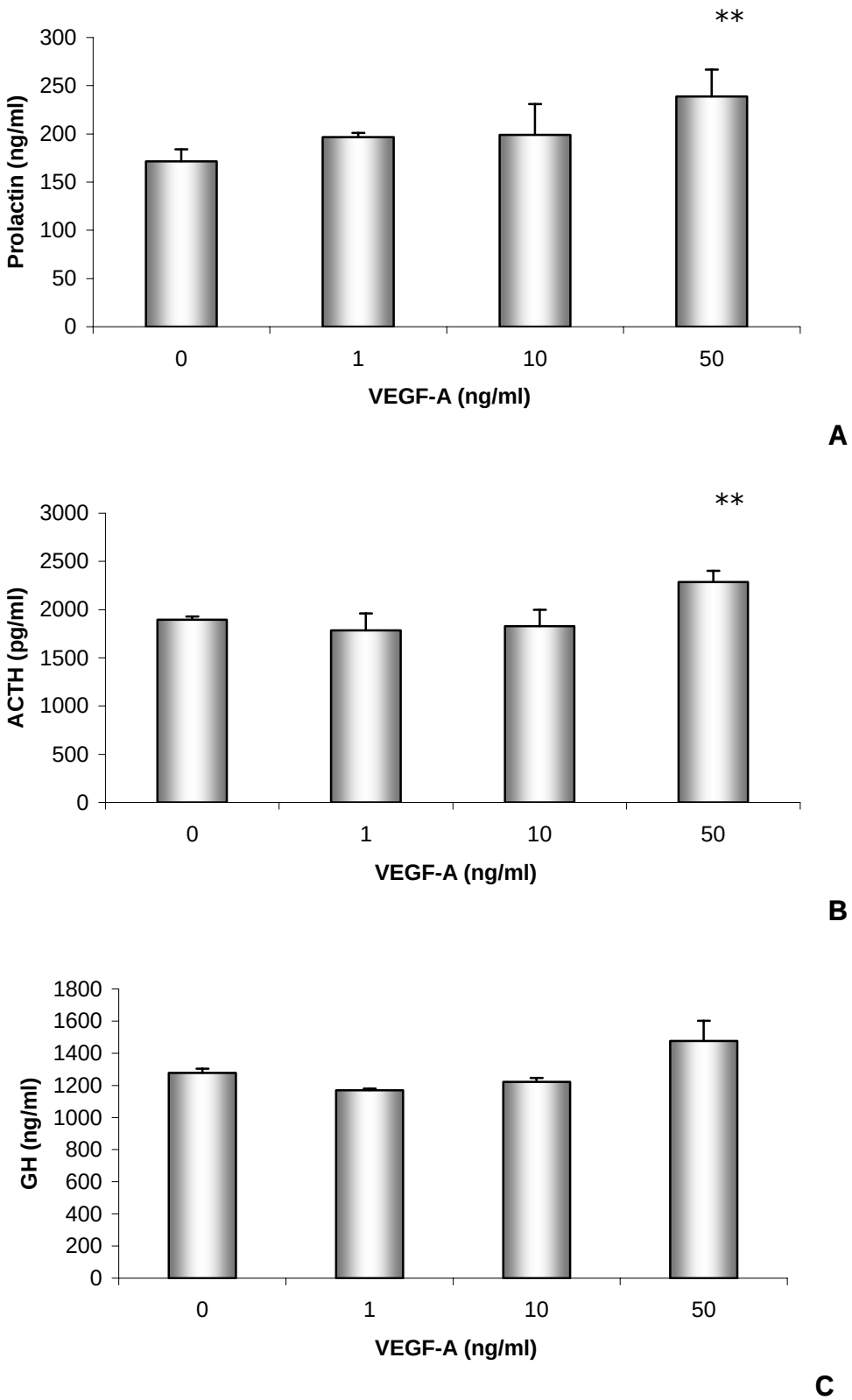


Figure 13. Effect of VEGF-A 24h-treatment on rat anterior pituitary hormone secretion. VEGF-A 50 ng/ml induced a significant secretion of prolactin (A) and ACTH (B). An increase in GH secretion (C) was also detected even if not significant. Values represent the means for 4 replicates. * p< 0,05, ** p<0,005, *** p<0,001.

4.14 VEGFR-1 involvement in MtT/S cell proliferation

The effect of VEGFR-1 ligands on pituitary tumour cell proliferation was also studied. The treatment of MtT/S cells, with VEGF-A for 96 h, induced a significant increase in cell proliferation of 11% and 36% compared to the untreated cells, as determined by [^{3}H]-thymidine incorporation at 50 ng/ml and 100 ng/ml (both p<0,05) (Fig. 14A). Likewise, the treatment of the same cell line with the VEGFR-1 selective ligand PlGF, for 96 h increased significantly cell proliferation, with maximal increases of 24% and 32% (both p<0,001) observed at 50 ng/ml and 100 ng/ml PlGF doses, respectively (Fig. 14B). Stimulation with VEGF-E (that does not bind VEGFR-1), in contrast, was not able to induce any effect on [^{3}H]-thymidine incorporation (Fig. 14C).

The [^{3}H]-thymidine incorporation, induced by PlGF, was significantly inhibited by PI3K specific inhibitor LY294002 (p<0,001) (Fig. 15).

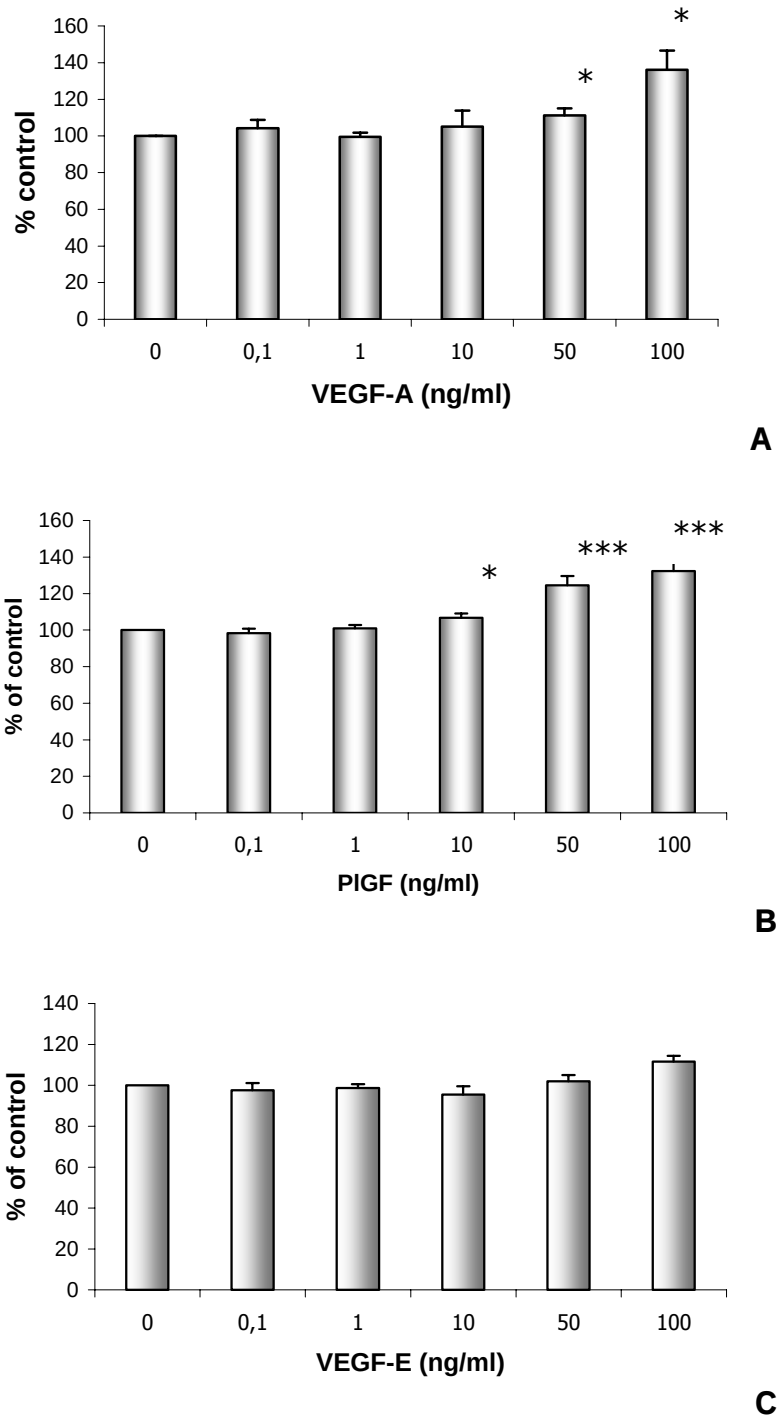


Figure 14. Cell proliferation of MtT/S cells measured by [^{3}H]-thymidine incorporation after VEGF-A, PlGF and VEGF-E stimulation for 96 h. The concentration of 50 ng/ml and 100 ng/ml of VEGF-A (A) and 10 ng/ml, 50 ng/ml and 100 ng/ml of PlGF (B) induced a significant increase of [^{3}H]-thymidine incorporation in comparison to untreated cells, whereas VEGF-E treatment had no effect on [^{3}H]-thymidine incorporation (C). Values represent the means for 4 replicates and results are presented as percentage of control. * p< 0,05, ** p<0,005, *** p<0,001.

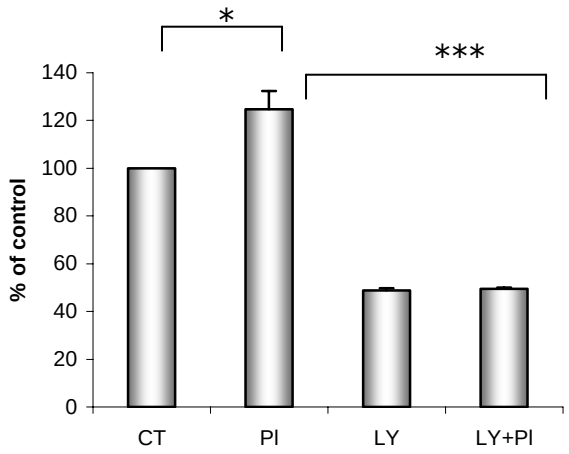


Figure 15. Effect of PI3K specific inhibitor (LY294002) on PlGF induced cell proliferation in MtT/S cells. Cells were treated with LY294002 30 μM (LY) and PlGF 50 ng/ml (Pl) for 96 h and LY294002 inhibited significantly the PlGF induced [^{3}H]-thymidine incorporation in MtT/S cells. Values represent the means for 4 replicates and results are presented as percentage of control. * p< 0,05, ** p<0,005, *** p<0,001.

4.15 VEGFR-1 activates the PI3K/Akt pathway in MtT/S cells

PlGF can induce angiogenesis *in vivo* and stimulate the migration and proliferation of endothelial cells *in vitro* [Ziche et al., 1997]. In order to elucidate whether the same mechanism is responsible for VEGFR-1 proliferative action in pituitary tumour cells, the effect of PlGF on the phosphorylation status of some members of the PI3K/Akt pathway was determined by western blotting. This signalling pathway was investigated, since it was found to be involved in the proliferation of endothelial cells [Yu and Sato, 1999; Thakker et al., 1999] and the p85 PI3K subunit was reported to bind to tyrosine autophosphorylated residues in the intracellular domain of VEGFR-1 [Cunningham et al., 1995; Yu et al., 2001].

Thirty minutes of PlGF treatment (50 ng/ml) increased the phosphorylation levels of PDK-1, Akt (at both phosphorylation sites Thr 308 and Ser 473) and GSK-3β (Fig. 16A). PDK-1 phosphorylation status increased in a time-dependent fashion up to 6 h, while the phosphorylation of Akt at Thr 308 reached maximum levels after 1 h and then slightly decreased. Akt phosphorylation at Ser 473 was maximal after 30 min, but it disappeared completely after 3h of treatment. The

highest levels of GSK-3 β phosphorylation were observed after 3 and 6 h. PlGF treatment did not affect the phosphorylation status of PTEN phosphatase, which is a major negative regulator of the PI3K/Akt signalling pathway (Fig. 16A) [Cantley and Neel, 1999]. Further, MtT/S cells treatment, with PI3K specific inhibitor LY294002 at 30 μM for 1 h, inhibited the PlGF induced phosphorylation of Akt in Thr 308 (Fig. 16B).

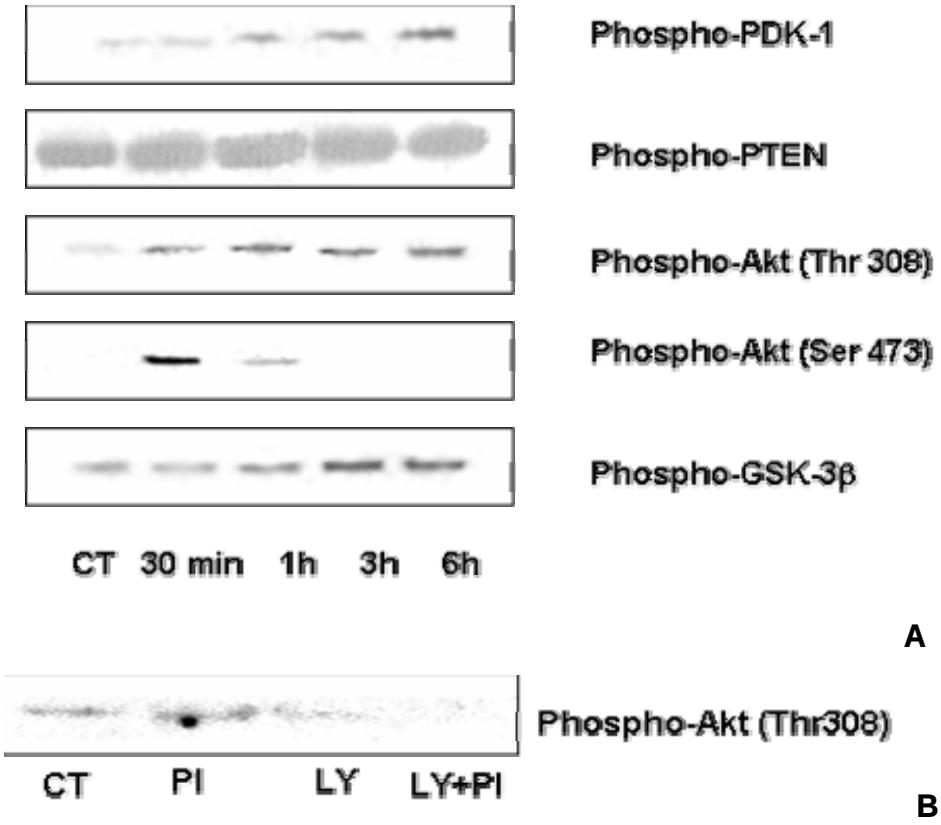


Figure 16. PlGF induced activation of PI3K pathway in MtT/S cells. PlGF is able to induce activation of PI3K/Akt pathway in MtT/S cell line, enhancing an increase in the phosphorylation of PDK-1, Akt (in both Thr 308 and Ser473) and GSK3-β, after 30 min. Whereas the phosphorylation status of the negative regulator PTEN is unchanged (A). MtT/S cell lysates were obtained from cells collected after 30 min, 1h, 3h and 6 h stimulation with PlGF (50 ng/ml), separated on a Tris-Glycine gel and after transfer on a nitrocellulose membrane, immunoblotted with anti pospho-PDK1, anti phospho-PTEN, anti phospho-Akt (Thr 308), anti phospho-Akt (Ser 473) and anti phospho GSK-3β. MtT/S cells treatment with LY294002 30 μM (LY) is able to inhibit PlGF induced Akt phosphorylation at Thr 308 (B). Before collecting cell lysates for western blot analysis with anti phospsho-Akt (Thr 308), cells were treated for 1 h with LY294002 and then stimulated with 50 ng/ml of PlGF for 30 min.

4.16 VEGFR-1 affects components of the cell cycle and apoptotic machinery

GSK3-β was shown to inhibit cell cycle by phosphorylating and therefore marking for degradation, cyclin D1 [Diehl et al., 1998]. In order to see if the phosphorylation leading to inhibition of GSK3- β by PlGF has any effect on cyclin D1, MtT/S cells were treated with 50 ng/ml PlGF for 24, 48, 72 and 96 h. PlGF treatment increased the protein level of cyclin D1 after 24 h. This effect reached a maximum after 48 h and remained for 72 h (Fig. 17A).

Since Akt phosphorylation of CREB protein was reported to induce the transcription of anti-apoptotic genes like Bcl-2 [Pugazhenthi et al., 2000], in order to see if PlGF could affect also Bcl-2 level, MtT/S cells were treated with the same conditions used to study PlGF effect on cyclin D1. An increase in Bcl-2 protein amount was observed, with a maximum after 24 h and lasted for 72 h (Fig. 17A). Furthermore, the treatment with PI3K inhibitor LY294002 at 30 μM inhibited the production of cyclin D1 and Bcl-2 induced by PlGF, after 48 h (Fig. 17B).

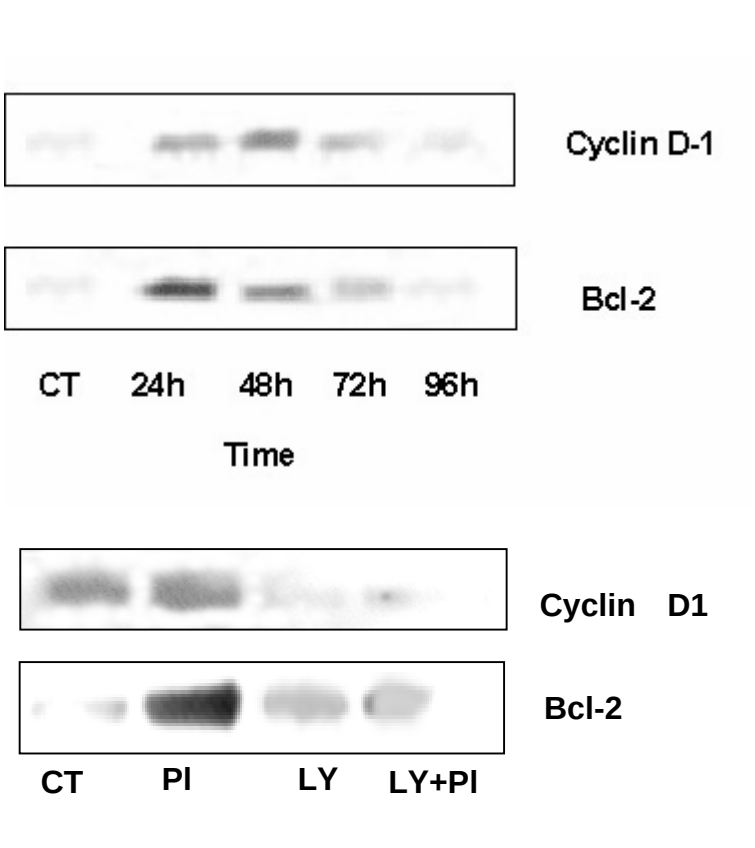


Figure 17. Effect of PlGF on cyclin D1 and Bcl-2 protein amount. Cyclin D1 augmentation is visible after 24 h, reaching a maximum after 48 h and then starting to decrease, whereas Bcl-2 reaches its maximum after 24 and then it starts to decrease (A). MtT/S cell lysates were obtained from cells collected after 24h, 48h, 72h and 96 h stimulation with PlGF (50 ng/ml), separated on a Tris-Glycine gel and after transfer on a nitrocellulose membrane, immunoblotted with anti cyclin D1 and anti Bcl-2; the control consists in MtT/S cell lysate collected at 24h from unstimulated cells. MtT/S cell treatment with LY294002 30 μM (LY) is able to decrease the production of cyclin D1 and Bcl-2 induced by 50 ng/ml of PlGF (Pl) (B). Before collecting cell lysates for western blot analysis with anti cyclin D1 and anti Bcl-2, cells were treated for 1 h with LY294002 and then stimulated with 50 ng/ml of PlGF for 48h.

5 DISCUSSION

To further understand the role of angiogenesis in the development of pituitary adenomas, in the present study, the expression and in particular the localization of one of the most important angiogenic factor, VEGF-A and of its receptors: VEGFR-1, VEGFR-2 and neuropilin-1, was investigated in normal human pituitary and in a series of pituitary adenomas. In addition, the expression and localization of VEGF-C and its receptor VEGFR-3, which are involved in lymphatic vessel development, was investigated in the same type of tissues.

In the present study, it has been shown that VEGF-A is mostly expressed in pituitary adenomas in similar or even greater extent compared to the normal pituitary, therefore confirming previous studies [Lloyd et al., 1999; Ochoa et al., 2000; Viacava et al., 2003]. However, most pituitary tumours are less vascularized than the normal adenohypophysis [Jugenburg et al., 1995; Turner et al., 2000a]. To explain this discrepancy, it was suggested that mechanisms suppressing endothelial cell growth could exist in pituitary tumours. However this hypothesis remains speculative since the expression of corresponding antiangiogenic-acting factors like endostatin, angiostatin and others has not been comprehensively studied in pituitary adenomas so far.

Since it is possible that disturbances and alterations in the VEGF receptors expression are responsible for the reduced vessel formation, the expression of VEGFR-1, -2 and neuropilin-1 was studied in normal and adenomatous pituitary as they regulate the growth and function of the blood vessel endothelium. RT-PCR analysis in normal and tumour tissues, revealed that normal pituitary and most of the tumours synthesize VEGF-A, VEGFR-1, VEGFR-2 and neuropilin-1 transcripts. IHC analysis of a larger number of samples showed expression of all 3 receptors in normal pituitary and in 7 out of 39 pituitary adenomas. Most tumours expressed only one or two receptor types, and 4 cases were negative for all 3 receptors. The discrepancy between RT-PCR and IHC results could be explained considering that receptor functionality is dependent on protein expression, whereas mRNA synthesis is less informative and the presence of a transcript does not assure its transcription into the corresponding protein.

However, there was no correlation between the degree of receptor absence and blood vessel density, suggesting that disturbances in the VEGF receptors expression do not sufficiently explain the poor vascularization of pituitary adenomas.

It should be noted that the 4 tumours negative for all the VEGF receptors were somatotrophinomas and prolactinomas that have been pre-treated with somatostatin analogues and dopamine agonists. The latter drug was formerly reported to down-regulate VEGFR-2 in cerebral endothelial cells by inducing receptor internalization [Basu et al., 2001]. Somatostatin is also considered to act as anti-angiogenic [Woltering et al., 1991; Danesi et al., 1997]. Although pre-medication was suspended at least one week prior to surgery, long-lasting suppressive effects of dopamine agonists and somatostatin analogues on the VEGF/VEGF receptor system can not be excluded.

VEGFR-2 is the most important VEGF receptor for angiogenesis [Waltenberger, 1994]. Its localization was found to be restricted to blood vessel endothelial cells of VEGFR-2-expressing normal and tumoural human pituitaries. This finding confirms previous studies which observed VEGFR-2 expression in blood vessel endothelial cells of estrogen-induced prolactinomas in Fischer-344 rats [Banerjee et al., 1997]. However, in normal rat pituitary, VEGFR-2 was found not only in blood endothelial, but also in epithelial hormone-producing cells. Moreover, mammosomatotrophinoma GH3 rat pituitary tumour cells were reported to express VEGFR-2 [Vidal et al., 2002], but the reason for this discrepancy is not yet clear.

ISH and IHC, in a series of pituitary adenomas, detected no VEGFR-2 mRNA and protein in more than 50% of the tumours analyzed. Furthermore no VEGFR-2 mRNA synthesis was detected in any of the pituitary adenoma cell lines studied. In VEGFR-2 immuno-positive adenomas, the immunoreactivity was present exclusively in blood vessels and there is no evidence of an over-expression in comparison to normal pituitary. No significant correlation between VEGFR-2 expression and vessels number was detected; nonetheless, the lack of VEGFR-2 protein was found to correlate significantly with low PI, probably due to its effect

in increasing vessels permeability and therefore nutrients and oxygen availability for tumour cells.

These results contrast with a previous study, which has shown by quantitative PCR, very high levels of VEGFR-2 mRNA expression in extracts of 121 adenomas compared to normal pituitary [McCabe et al., 2002]. In few of these adenomas studied, a marked over-expression of VEGFR-2 protein level was also observed by Western blotting. Normally, only rapidly expanding, well-vascularized and aggressive types of solid tumours show an over-expression of VEGF and/or VEGF receptors. In the case of pituitary adenomas, only estrogen-induced, rapidly growing prolactinomas of Fischer-344 rats are well vascularized and in fact, VEGF and VEGF receptors were found to be overexpressed in these tumours [Banerjee et al., 1997]. However, the findings of McCabe et al. do not fit very well to extremely slowly growing and poorly vascularized human pituitary adenomas, in contrast to the observations described in the present paper. Although the reason for the discrepancy between the two studies is not clear, it could derive from the different techniques used.

Studies in Fischer rat prolactinomas have shown that not only VEGFR-2 but also neuropilin-1 expression is enhanced [Banerjee et al., 2000]. This is not surprising since the latter was identified as a VEGFR-2 co-receptor that has no intracellular signal transducing domain but improves the action of VEGFR-2 by accelerating the binding of VEGF, in particular the soluble $VEGF_{165}$ isoform [Soker et al., 1998]. Neuropilin-1 was detected in the blood vessels of normal pituitary and in 17 out of 39 adenomas and in 2 cases, its expression was higher in the tumours than in the normal tissue counterpart. Furthermore, neuropilin-1 was co-expressed with VEGFR-2 in only 11 cases, suggesting that in some human pituitary tumours, this receptor might be transiently functionless. Despite these findings, a possible role of neuropilin-1 in enhancing vessel permeability could be hypothesized, since as observed for VEGFR-2, tumours lacking neuropilin-1 expression, showed lower PI values and no influence was seen on blood vessel number. Neuropilin-1 transcripts were detected in all rodent and human endocrine pituitary tumour cell lines analyzed, confirming previous report in mammosomatotrophinoma GH3 cells [Banerjee et al., 2000]. However, the

function of neuropilin-1 in VEGFR-2-negative cells is still not clear; nevertheless different types of metastatic tumours and cell lines were shown to express neuropilin-1, even if it was not associated to any other VEGF receptor expression [Soker et al., 1998; Bachelder et al., 2002; Stephenson et al., 2002]. These findings raise the possibility that neuropilin-1 can also function alone or in concert with other tyrosine kinase-linked receptors to transduce VEGF signalling, at least in metastatic tumours. Moreover $VEGF_{165}$ is able to induce an anti-apoptotic effect in neuropilin-1-expressing breast tumour cell line [Barr et al., 2005]. The detection of this co-receptor in all pituitary adenoma cell lines investigated may be linked to the several transformation steps cell lines undergo, while acquiring a more aggressive phenotype compared to that of the tissue from which they originate.

The fact that VEGF-A is expressed in endocrine cells whereas VEGFR-2 and its co-receptor neuropilin-1 are expressed in blood vessel endothelial cells, suggest the presence of a paracrine loop in the regulation of angiogenesis in pituitary adenomas.

VEGFR-1 was found to be expressed in subsets of all types of endocrine epithelial cells in normal human pituitary, except of TSH-secreting cells, but not in the endothelial cells. VEGF-A treatment of rat pituitary cells in primary culture significantly induced prolactin and ACTH secretion in a dose-dependent way. These findings point for the first time to a direct involvement of VEGF-A and its receptor VEGFR-1 in pituitary hormone secretion, as it was already shown for other growth factors and cytokines, such as, IL-1 [Bernton et al., 1987], TNF-α [Koike et al., 1991], IL-6 [Spangelo et al., 1989] and TGF-β1 [Coya et al., 1999]. Furthermore, the presence of VEGF in the adult anterior pituitary indicates that it may facilitate hormone secretion by increasing vascular permeability, through VEGFR-2-dependent formation of endothelial cells fenestrations. This event would make the transport of the hormones through the capillary walls into the systemic blood stream easier.

VEGFR-1 expression was also demonstrated in the endocrine tumour cells of more than 50% of the adenomas studied. The human HP75 gonadotrophinoma [Horiguchi et al., 2004] and, as shown in the present work, the

somatotrophinoma rat MtT/S pituitary tumour cells also express this receptor. These observations, in addition to the lack of correlation between VEGFR-1 expression and blood vessel count, indicate that VEGFR-1 has little or no impact on the regulation of intrapituitary angiogenesis or vascular permeability, but it may mediate VEGF effects on normal and adenomatous pituitary cells. The hypothesis of a direct action of VEGF on tumour cells proliferation was also suggested by previous reports asserting a positive correlation between VEGF expression and PI in pituitary adenomas [Iuchi et al., 2000] and the lack of correlation between PI and blood vessel count [Turner and Wass, 1999; Vidal et al., 2001]. Therefore it is possible that VEGF plays a direct role in pituitary adenoma cell proliferation, possibly through VEGFR-1. To confirm this hypothesis, the rat somatotrophinoma MtT/S cells were treated with different VEGF family members. VEGF-A and the VEGFR-1 specific ligand PlGF induced a slight but significant increase in cell proliferation, while the VEGFR-2 ligand VEGF-E had no effect. In addition, MtT/S cells were found to secrete VEGF-A indicating that different VEGFs acting through VEGFR-1 may form an autocrine growth regulatory circuit in this kind of endocrine pituitary tumour cells.

While the expression of PlGF has not yet been studied in human pituitary adenomas, we have previously shown that cultured human adenoma cells release 0,05 to 13,5 pg VEGF-A per 1000 cells per 24 h [Lohrer et al., 2001]. Moreover, in the present work, MtT/S cells were found to secrete 1,65 pg VEGF-A per 1000 cells per 24 h. Since cell numbers are much higher in pituitary adenomas and because VEGF-A is secreted into the small volume of intercellular space, effective intratumoural VEGF-A concentrations for growth stimulation may be reached.

The lack of significant correlation between VEGFR-1 and PI values that does not fit very well with this hypothesis, could be also due to the restricted number of samples analyzed, indeed the majority of tumours that did not express VEGFR-1 showed low PI values too, though the difference with other groups was not significant. VEGF-A expression did not correlate with the expression of its receptor VEGFR-1 suggesting that they are not involved in an autocrine loop. Although we do not have any evidence that VEGF-A producing cells are not the ones expressing VEGFR-1 and therefore interacting in a paracrine fashion, this type of interaction could be likely according to this observation. Moreover, the

activation of the VEGF-A/ VEGFR-1 system may be an event occurring in more aggressive pituitary tumours: even though the observations were not statistically significant, we noticed that 10 out of 12 pituitary adenomas graded I/II with a PI value equal to 0 in 6 cases, but never higher than 1,6% in the other cases, did not show VEGFR-1 expression in most of the cases and showed a very weak expression in 2 cases.

On the other hand, VEGF-A did not have any effect on GH secretion from MtT/S cells. However, it has to be considered that these cells have a small number of secretory granules indicating a low secretory capacity and suggesting that MtT/S cells are premature precursors of somatotroph cells [Mogi et al., 2005].

The direct effect of VEGF on tumour cell proliferation was already shown in a variety of tumours. In pancreatic cancer and gastric adenocarcinoma, VEGF treatment was reported to trigger cell growth through VEGFR-2 phosphorylation and MAPK activation [Von Marschall et al., 2000; Tian et al., 2001]; while in bladder tumour cells, VEGF binding to VEGFR-2 induced DNA synthesis through the SPK1-PKC-Ras-MAPK pathway [Wu et al., 2003]. Although these studies highlighted the VEGFR-2 as mediator of VEGF growth effect, VEGFR-1 may also play an important role since treatment with an anti-VEGFR-1 antibody was found to inhibit VEGF induced proliferation of different human tumour cells [Masood et al., 2001]. Moreover, VEGFR-1 expression was observed in breast cancer cells, where it was associated with VEGF-dependent PI3K activation and involved in the reinforcement of their invasive ability [Price et al., 2001]. Recently, VEGFR-1 was also found in human colorectal cancer cells, where it was linked with increased cell mobility and invasiveness and formation of larger and more numerous colonies [Fan et al., 2005].

In the present study, the VEGFR-1-specific ligand PlGF increased MtT/S cell proliferation indicating the involvement of VEGFR-1 in this event. Moreover, the growth inducing effect of PlGF was abolished after co-treatment with the PI3K inhibitor LY294002 [Vlahos et al., 1994], suggesting the activation of the PI3K pathway in this process. The inhibitory effect of LY294002 on MtT/S cell proliferation is much stronger than the increase induced by PlGF, suggesting that LY294002 can inhibit the function of other kinases, as indeed has been shown for

casein kinase 2 [Davies et al., 2000], that is implicated in many cell regulatory processes [Meggio and Pinna, 2003]. PI3K pathway deregulation is widely implicated in carcinogenesis, since this pathway plays a critical role in controlling the balance between cell survival and apoptosis [Vivanco and Sawyers, 2002]. The PI3K activation by tyrosine-kinase receptor determines PDK-1 activation and the consequent phosphorylation of Akt, which after a further phosphorylation becomes completely active [Alessi et al., 1996]. The fully phosphorylated Akt can then exert its action on different target proteins. PlGF treatment in MtT/S cells increased the phosphorylation levels of PDK-1 and Akt, but did not affect the phosphorylation status of the PI3K pathway inhibitor PTEN. Moreover, the activating effect of PlGF on Akt phosphorylation was reversed by the PI3K specific inhibitor LY294002. Since PlGF is a VEGFR-1 specific ligand, the stimulatory effects on the PI3K pathway may be attributed to VEGFR-1. These data are consistent with the previous report in which PI3K could bind tyrosine 1213 of VEGFR-1, in a yeast two-hybrid system [Igarashi et al., 1998]. Moreover, PI3K pathway activation was recently reported in hepatic stellate cells expressing only VEGFR-1, after VEGF treatment [Takahashi et al., 2003].

PlGF determined phosphorylation and therefore de-activation of the Akt target GSK-3β, and this effect was accompanied by increase in the cell cycle promoting protein cyclin D1 levels; this observation is consistent with the lack of cyclin D1 phosphorylation by GSK-3β that prevents its expulsion from the nucleus and the consequent proteosomal degradation [Diehl et al., 1998; Alt et al., 2000]. Treatment with LY294002 inhibited cyclin D1 production induced by PlGF, confirming the involvement of PI3K pathway in this process.

Furthermore, the anti-apoptotic factor Bcl-2 production was also found to be up-regulated after stimulation with PlGF and this effect was reversed by the treatment with PI3K inhibitor LY294002. These findings suggest that VEGFR-1 induces cell cycle progression and cell survival of MtT/S cells, through PI3K signalling pathway activation, highlighting a direct action of VEGF signalling on tumour cells, apart its well-documented role in angiogenesis.

VEGF-C and its receptor VEGFR-3 are mainly expressed in lymphatic endothelial cells and the lymphatic system is known to be involved in the

diffusion of tumour metastases. This growth factor is a potent lymphangiogenic factor, which induces lymphatic endothelial cell proliferation *in vitro* and lymphatic hyperplasia *in vivo* [Makinen et al., 2001]. Even though pituitary adenomas rarely spread into metastases, VEGF-C and its receptor expression was also studied in order to obtain a more complete overview of VEGF receptors expression pattern in normal and tumoural human pituitary. RT-PCR analysis showed VEGFR-3 expression in all human normal pituitaries and in most of the adenomas studied. Immunohistochemical investigation on a wider group of samples confirmed VEGF-C immunoreactivity in all the normal human pituitaries in less than 10% of the endocrine cells, associated with some VEGFR-3 positive vessels and no LYVE-1 (lymphatic vessels specific marker) expressing vessels. These data are in agreement with a previous study reporting VEGF-C expression in prolactin-secreting cells of the human pituitary and VEGFR-3 expression in endothelial cells of fenestrated vessels [Partanen et al., 2000]; while the absence of LYVE-1 expression suggests that normal human pituitary is devoid of lymphatic vessels. The role of VEGF-C and VEGFR-3 in a tissue that does not have lymphatic vessels is rather difficult to be understood, although a possible involvement in the regulation of blood vessels permeability in blood capillaries with fenestrated walls was suggested [Partanen et al., 2000].

The majority of pituitary adenomas investigated did not express VEGF-C, and the few that did, showed VEGF-C immunostaining in more than 80% of their endocrine cells. On the other hand, more than 50% of the tumours showed heterogeneous VEGFR-3 immunoreactivity in vessel endothelial cells. Most of the VEGFR-3-positive tumours did not show any LYVE-1 immunoreactivity, indicating the probable presence of blood vessels positive for VEGFR-3. This is not surprising since VEGFR-3 was found to be expressed by blood vessel endothelial cells in different types of tumours in mice [Kubo et al., 2000]. Moreover VEGFR-3 was detected in the blood vessel endothelial cells of human breast cancer [Valtola et al., 1999], gliomas and colon carcinomas [Witmer et al., 2001]. It is of interest that VEGFR-3 was found to be expressed by blood vessel endothelial cells undergoing active angiogenesis, as observed in blood vessels in the granulation tissue of healing wounds, suggesting a probable role of this receptor not only in lymphangiogenesis but also in angiogenesis.

Testing pituitary adenomas for the presence of lymphatic vessels, it was found that about 30% of the pituitary adenomas investigated were immunopositive for the lymphatic marker LYVE-1. However it has to be noted that the number of lymphatic vessels was much lower compared to the number of CD31-positive blood vessels. This reflects previous observations in murine sarcomas, in which lymphatic vessels were detected in low number and in only few cases. This finding was supposed to be a consequence of the high pressure exerted by growing cancer cells on the other adjacent anatomical structures that could be sustained by blood vessels because of their connection to the high-pressure arterial blood supply, but not by lymphatic vessels which have no comparable high-pressure source [Leu et al., 2000]. The above-outlined picture is quite likely to take place also during pituitary tumour development, as this kind of neoplasm has to grow in a very narrow and confined anatomical space where the achievement of a high mechanical pressure is not surprising.

In spite of the observations that most of pituitary tumours did not bear lymphatic vessels and that anyway in the positive cases, their number was quite low, we also observed that most of these LYVE-1-positive tumours were classified as grade III and one of them showed the highest PI value. This would suggest that the occurrence of lymphatic vessels might be associated with a more aggressive (higher grade and rapidly growing) phenotype, whereas their development is impaired in those tumours in which the intrasellar pressure (due to tumour mass) is very high. Unfortunately we did not have the opportunity to examine pituitary carcinoma cases and anyway this hypothesis remains to be experimentally investigated.

It is nonetheless necessary to remember that the normal pituitary is devoid of lymphatic vessels and it is not clear what their function could be in rarely metastatic and spatially localized tumours like pituitary adenomas.

Concluding, VEGF and VEGF receptors expression in the normal adenohypophysis, as shown in this work, may maintain optimal intrapituitary vascularization and vessel permeability, which is needed for optimal pituitary hormone regulation and release. Moreover, the detection of VEGFR-1 in human normal pituitary endocrine cells combined with the stimulatory action on prolactin

and ACTH secretion, suggest the occurrence of a paracrine loop in which VEGF-A secreted by folliculo-stellate cells act on endocrine cells to regulate hormone secretion. This hypothesis is in agreement with the theory asserting that different cytokines expressed in the pituitary gland can act as paracrine or autocrine factors regulating hormone secretion and pituitary growth and having a physiopathological role in the maintenance of anterior pituitary homeostasis [Arzt et al., 1999].

In the pituitary adenomas, we have observed marked variations in the PI values, the vessel density and the expression of VEGF-A and its receptors, but nearly no correlation between these parameters was found, except the ones mentioned and commented before between the absence of VEGFR-2 and neuropilin-1 and the low PI values, that may suggest a role for VEGFR-2 and neuropilin-1 in the induction of vascular permeability and the consequent higher nutrients and oxygen availability for tumour cells. The lack of correlation between the other parameters (tumour grade, PI, blood vessels count) and the expression of VEGF-A and its receptors could be explained by the fact that pituitary adenomas are slowly growing tumours with a limited metabolic demand. Variations in the PI of the adenomas ranging from 0 to 10% (independently from tumour grade, in the way that there are tumours with grade III but very low PI values) suggest that the tumours or at least the parts of the adenoma studied by IHC undergo periods of quiescence and of variable proliferation activity. If a previously growth arrested pituitary tumour (i.e. grade III that means invading surrounding pituitary structures) would start to proliferate (high PI value), neovascularization would follow with delay (absence or low expression of VEGF-A and its receptors) since a critical amount of newly generated tumour cells is needed to induce hypoxia and subsequent angiogenesis. On the other hand, if the intratumoural proliferation would transiently be terminated (PI value low or zero), angiogenesis may still occur (high VEGF-A and its receptors expression) until sufficient vascularization of the corresponding area within the adenoma is reached. Although this hypothesis needs to be experimentally confirmed, it could explain the discrepancies between PI, tumour grade, vessel density and markers of angiogenesis like VEGF-A and its receptors. However, it has to be pointed that similar to other tumour types, in pituitary adenomas the VEGF-A/VEGF receptors

system is not strictly connected to their angiogenic phenotype. The extent of angiogenesis in a tumour should be determined not by only one factor, but by the net balance between the different positive and negative angiogenic regulators expressed in the tissue under investigation.

As one would expect for tumours that rarely metastasize, lymphangiogenesis does not seem to play a fundamental role in pituitary adenoma development and growth. Moreover, adenomas positive for LYVE-1 have a small number of lymphatic vessels even in cases where the VEGF-C/VEGFR-3 system is highly expressed. This would suggest the presence of an impediment in the development and growth of these tumours due to the confined anatomical space and the probable involvement of this system in other processes connected to tumour angiogenesis rather than to lymphangiogenesis.

Finally, herein is shown the mechanism of the growth promoting action of VEGFR-1 not in endothelial, but in endocrine tumour cells. VEGFR-1 activated the PI3K/Akt pathway with subsequent increase in the synthesis of proteins involved in cell cycle progression (cyclin D1) and cell survival (Bcl-2).

Till recently the focus of interest on VEGF system was limited to its angiogenic action in cancer pathophysiology. The present work constitutes part of an increasing number of studies demonstrating a direct role of VEGF and its receptors on the growth and function of tumour cells. Moreover, these data can shed a light in the obscure process controlling pituitary tumour genesis and provide potential pharmacological targets for the treatment of these tumours.

6 SUMMARY

The role of members of the VEGF family and their receptors in angiogenesis, progression and pathophysiology of pituitary tumours is still poorly understood. In the present work, the expression and localization of the angiogenic factor VEGF-A and the lymphangiogenic factor VEGF-C as well as VEGF receptors (VEGFR-1, VEGFR-2, VEGFR-3 and neuropilin-1) have been comprehensively studied in normal and tumoural pituitary tissue and in transformed pituitary tumour cell lines. In addition, the role and mechanism of action of ligands of VEGFR-1 have been investigated in normal and transformed rat pituitary cells.

Immunohistochemical investigations in 3 normal human adenohypophyses showed expression of VEGF-A and all its receptors (VEGFR-1, VEGFR-2 and neuropilin-1) at protein level. The last two receptors were localized in blood vessel endothelial cells, while the former one was found in endocrine cells. These findings were confirmed at RNA level by *in situ* hybridization analysis. VEGF-A significantly induced ACTH and prolactin secretion in normal rat pituitary cell cultures, indicating a role of VEGF-A and VEGFR-1 in the regulation of the secretion of these pituitary hormones. In contrast, VEGFR-2 and its co-receptor neuropilin-1 may be needed to maintain optimal intrapituitary vascularization and blood vessel permeability. Although no lymphatic vessels were identified in normal adenohypophysis, the lymphangiogenic factor VEGF-C and its receptor VEGFR-3 were detected by immunohistochemistry. Their expression in a low percentage of endocrine cells and in blood vessel endothelial cells, respectively, suggests the involvement of the VEGF-C/VEGFR-3 system, usually implicated in lymphangiogenesis, in the maintenance of blood vessel permeability.

The expression of VEGFR-1, VEGFR-2 and neuropilin-1 in a series of 39 pituitary adenomas reflected the same immunohistochemical localization pattern as observed in the normal adenohypophysis tissue. VEGFR-1 was detected in endocrine tumour cells in 24 cases, whereas VEGFR-2 and neuropilin-1 were both detected in vessel endothelial cells of 18 and 17 cases, respectively. Their expression was highly heterogeneous and mostly no significant correlation with

different parameters, such as: tumour type, tumour grade, proliferation index (PI) and blood vessel number, was noticed. Only the absence of VEGFR-2 and neuropilin-1 correlated with a low PI, suggesting a role of these two receptors in increasing vessel permeability and consequently the availability of nutrients and oxygen for tumour cells. These findings imply that in slowly growing tumours with limited metabolic demand like pituitary adenomas, the development of new blood vessels is not a limiting event for their development and pathogenesis.

Functional studies, with the VEGF-A secreting somatotrophinoma MtT/S rat pituitary cells which express VEGFR-1 and neuropilin-1 but not VEGFR-2, showed that VEGF-A and the VEGFR-1 specific ligand, PlGF, significantly stimulated MtT/S cell proliferation, while the VEGFR-2 specific ligand VEGF-E was ineffective. In the same cell model, PlGF treatment induced the activation of PI3K pathway and the synthesis of Bcl-2 and cyclin D1 responsible for cell survival and cell cycle progression, respectively. These observations would suggest the presence of an autocrine loop between VEGF-A and VEGFR-1 acting directly on pituitary cell tumour growth, rather than on regulation of neovascularization.

The low number of pituitary tumours positive for the lymphatic vessel marker LYVE-1 is not surprising as the lymphatic system has been reported to be implicated in metastasis spread that occurs rarely in pituitary adenomas. Nonetheless, VEGF-C immunostaining was detected in endocrine tumour cells of 10 adenomas and VEGFR-3 immunopositive vessels were found in 22 tumours. This suggests that the VEGF-C/VEGFR-3 system may have a role in the regulation of tumour angiogenesis of pituitary adenomas, rather than in lymphangiogenesis, as already shown in other tumour types.

In conclusion, the results of the present study provide strong evidence that VEGF may not only have a role in regulating pituitary adenoma neovascularization but also, through VEGFR-1, may affect in addition pituitary adenoma pathophysiology by modulating growth, cell cycle progression and survival of the adenoma cells. Whether anti-VEGF treatment strategies will be useful in the therapy of advanced stages of pituitary adenomas or carcinomas, needs to be clarified in future studies.

7 ZUSAMMENFASSUNG

Die Bedeutung von Mitgliedern der VEGF Familie und ihrer Rezeptoren für die Angiogenese, Progression und Pathophysiologie von Hypophysentumoren ist noch weitgehend unklar. In der vorliegenden Arbeit wurde die Expression und Lokalisation des angiogenetischen Faktors VEGF-A und des lymphangiogenetischen Faktors VEGF-C sowie von VEGF-Rezeptoren (VEGFR-1, VEGFR-2, VEGFR-3, und Neuropilin-1) umfassend untersucht, und zwar im normalen Hypophysenvorderlappen, in Hypophysentumoren und in transformierten Hypophysentumorzelllinien. Zusätzlich wurde die Bedeutung und der Wirkmechanismus von Liganden des VEGFR-1 in normalen und transformierten Hypophysenzellen untersucht.

Mittels Immunhistochemie konnte in 3 normalen humanen Hypophysenvorderlappen die Expression von VEGF-A und all seiner Rezeptoren (VEGFR-1, VEGFR-2, und Neuropilin-1) auf Proteinebene nachgewiesen werden. Die beiden letztgenannten Rezeptoren waren in Blutgefäßzellen lokalisiert, während ersterer in endokrinen Zellen gefunden wurde. Diese Befunde wurden auf RNA Ebene mittels *in-situ* Hybridisierung bestätigt. VEGF-A induzierte in Rattenhypophysenzellkulturen die ACTH- und Prolaktin-Sekretion, was darauf hinweist, dass VEGF-A eine Rolle bei der Regulation der Sekretion dieser Hormone spielt. Im Gegensatz dazu dürften VEGFR-2 und sein Co-Rezeptor Neuropilin-1 notwendig sein, um eine optimale intrahypophysäre Vaskularisation und Gefäßpermeabilität aufrecht zu erhalten. Obwohl im Hypophysenvorderlappen keine Lymphgefäße identifiziert wurden, konnte der lymphangiogenetische Faktor VEGF-C und sein Rezeptor VEGFR-3 immunhistochemisch nachgewiesen werden. Ihre Expression in einem geringen Anteil endokriner Zellen und in Blutgefäß-Endothelzellen weist darauf hin, dass das VEGF-C/VEGFR-3 System, das gewöhnlich in der Lymphangiogenese involviert ist, hier am Erhalt der Blutgefäßpermeabilität beteiligt ist.

Die Expression von VEGFR-1, VEGFR-2 und Neuropilin-1 in 39 untersuchten Hypophysenadenomen wies ein ähnliches Lokalisationsmuster wie in der normalen Hypophyse auf. VEGFR-1 wurde in endokrinen Zellen von 24 Tumoren

nachgewiesen während VEGFR-2 und Neuropilin-1 in Blutgefäßzellen von 18 bzw. 17 Hypophysentumoren exprimiert wurde. Die Expression war sehr heterogen und korrelierte meist nicht mit Parametern wie Tumortyp, Tumorgrad, Proliferationsindex und Gefäßdichte. Lediglich eine Korrelation zwischen dem Fehlen von VEGFR-2 und Neuropilin-1 mit einem niedrigen PI ließ sich nachweisen, was auf die Bedeutung dieser Rezeptoren für die Verbesserung der Gefäßpermeabilität und der damit verbesserten Verfügbarkeit von Nährstoffen für die Tumorzellen hinweisen könnte. Die Befunde lassen vermuten, dass in langsam wachsenden Tumoren mit einem geringen Metabolismus, wie in Hypophysenadenomen, die Neovaskularisation nicht der limitierende Faktor für die Entwicklung und Pathogenese ist.

Funktionelle Untersuchungen in VEGF-A sezernierenden somatotropen MtT/S Rattenhypophysentumorzellen, die VEGFR-1 und Neuropilin-1, aber nicht VEGFR-2 exprimieren ergaben, dass VEGF-A und der VEGFR-1 spezifische Ligand PlGF die Proliferation von MtT/S signifikant stimulierten, während der VEGFR-2 spezifische Ligand VEGF-E wirkungslos war. Im gleichen Zellmodell induzierte PlGF den PI3K-Signalweg und die Synthese von Bcl-2 und Cyclin D1, die für das Überleben von Zellen bzw. das Fortschreiten des Zellzyklus verantwortlich sind. Diese Ergebnisse weisen auf einen autokrinen Mechanismus hin, bei dem VEGF-A über VEGFR-1 direkt das Wachstum von Hypophysentumorzellen fördert und weniger die Neovaskularisation.

Dass nur eine geringe Anzahl von Hypophysenadenomen immunopositiv für den Lymphgefäß-Marker LYVE-1 ist, überraschte nicht, da Lymphgefäßexpression mit der Metastasenentwicklung in Beziehung steht, und Hypophysentumoren nur selten metastasieren. Nichtsdestoweniger konnte VEGF-C in endokrinen Zellen von 10 Adenomen und VEGFR-3 in Gefäßzellen von 22 Hypophysentumoren nachgewiesen werden. Wie auch schon in anderen Tumoren beschrieben, weist dies auf eine Rolle des VEGF-C/VEGFR-3 Systems bei der Regulation der Tumorangiogenese und weniger bei der Lymphangiogenese hin.

Schlussfolgernd lässt sich sagen, dass die Ergebnisse der vorliegenden Arbeit stark darauf hinweisen, dass VEGF nicht nur eine Rolle bei der Neovaskularisation von Hypophysenadenomen spielt, sondern über den VEGFR-1 zusätzlich auch die Pathophysiologie dieser Tumoren beeinflusst, indem VEGF das Wachstum, die

Zellzyklusprogression und das Überleben der Adenomzellen moduliert. Ob allerdings anti-VEGF-Behandlungsstrategien bei der Therapie von fortgeschrittenen Hypophysenadenomen oder –karzinomen von Nutzen sein werden, müssen zukünftige Untersuchungen zeigen.

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