INTEGRATION OF STIMULATORY AND INHIBITORY SIGNALS IN PITUITARY TUMOUR CELLS THROUGH THE PI3K PATHWAY

by

Tânia Cristina Antunes Colaço

Faculdade de Medicina da Universidade do Porto

MESTRADO EM MEDICINA E ONCOLOGIA MOLECULAR
MASTER DEGREE IN MOLECULAR AND ONCOLOGY MEDICINE

Porto 2009

ABSTRACT

Pituitary adenomas produce multiple growth factors, which in concert strongly stimulate pituitary tumour cell growth. Cell proliferation is associated *in vitro* with increased expression of the main angiogenic factor, VEGF-A. Although the combined stimulation of growth and angiogenesis would indicate a rapid progression of pituitary adenomas, most pituitary tumour types expand extremely slowly *in vivo*, suggesting the presence of extra- and intra-tumoural inhibitory factors and mechanisms, which counteract the stimulatory signals.

So far, little is known about the mechanisms that integrate stimulatory and inhibitory signals in pituitary tumour cells at molecular level. MtT-S cells express the two PDGF receptors (α and β) and the SSTR2, that apparently are involved in pituitary tumour cell lines PI3K signalling control, thus emerging as a good model to study the integration of stimulatory (PDGF) and inhibitory (octreotide) signals in growth and VEGF-A production, through the PI3K pathway.

In the present work it was demonstrated that PDGF-AB significantly stimulated MtT-S proliferation and, surprisingly, octreotide had no effect on MtT-S at low concentrations and even stimulated cell growth at high dosages. It was also verified that, although both PDGF-AB and octreotide stimulated the proliferation of MtT-S cells, the combined application of these substances inhibited the action of each other on cellular proliferation.

In addition it was shown that PDGF-AB stimulation of MtT-S cells resulted in a strong dose-dependent enhancement of basal VEGF-A secretion. The induction of VEGF-A production by PDGF-AB was dose-dependently blocked by octreotide whereas the basal VEGF-A release was not affected. The PDGF-AB-induced phosphorylation of key components of the PI3K pathway such as PDK-1, Akt and mTOR was inhibited in the presence of octreotide, strongly supporting the involvement of this signal pathway in both stimulation and inhibition of VEGF-A release. The PDGF-AB-induced VEGF-A

secretion was also suppressed by the mTOR inhibitors rapamycine and RAD001. The PI3K inhibitor LY294002, another suppressor of this pathway, also dose-dependently reduced the production of VEGF-A.

Taken together, the results presented in this study strongly suggest that MtT-S cells might represent a good model for studying mechanisms responsible for the resistance to the growth inhibitory action of octreotide. Moreover, it was shown for the first time a way through which somatostatin and its analogues probably act as anti-angiogenic factors in pituitary adenomas, namely by suppressing the PDGF-induced production of the most important angiogenic factor, VEGF-A. Inhibitors of the PI3K-mTOR pathway, which seems to be the major pathway involved in PDGF-stimulated VEGF-A release, also suppressed the production of this angiogenic factor. Although the present findings need to be confirmed in human pituitary adenomas, PI3K-mTOR pathway inhibitors may provide an alternative and a new pharmacological tool for the treatment of pituitary adenomas.

PREAMBLE

This work was performed in the Neuroendocrinology group of the Max Planck Institute of Psychiatry (Munich, Germany), under the local supervision of Prof. Dr. Günter Stalla and co-supervision of Prof. Delminda Magalhães (Medical Faculty of the University of Porto).

Acknowledgments

I would like to thank Prof. Dr. Günter K. Stalla for offering me the opportunity to join his Clinical Neuroendocrinology group of Max Planck Institute of Psychiatry.

A special word of gratitude to Dr. Ulrich Renner for his friendly and continuous support that kept me going on with the research project and the manuscript writing. Without his encouragement and scientific advices this thesis would not have been possible.

To Prof. Delminda Magalhães, who was of great help to finish this work, a particular thank you for her believing on my capacities.

To Eliane Corrêa-de-Santana who taught me how to give the first steps in the laboratory and to Chiara Onofri who constantly clarified all my doubts, an unforgettable acknowledgement.

A warm thank you to Dr.ª Ana Sardinha for her silent respect for other's happiness.

I thank also all my friends, for their patience and encouragement during these last years of intensive work.

I want to express my full gratitude to my parents and brother whose love and unconditional support accompanies me through all my life.

The meaning of acknowledgment fades away when we have someone who is a part of us and whose existence overcomes all geographical distances…

TABLE OF CONTENTS

Page

LIST OF FIGURES

FIGURES Page

LIST OF GRAPHICS

GRAPHIC Page

LIST OF TABLES

TABLE Page

ABBREVIATIONS

cAMP	Cyclic adenosine monophosphate
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
GH	Growth hormone
mTOR	Mammalian target of rapamycin
PBS	Sterile phosphate based buffer
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGRF	Platelet-derived growth factor receptor
PDK-1	Phosphoinositide-dependent kinase-1
PI3K	Phosphatidylinositol-3-kinase
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PTEN	Phosphatase and tensin homolog
RT-PCR	Reverse transcriptase- Polymerase chain reaction
SHP	Src-homology 2 domain-containing protein tyrosine phosphatase
SSTR	Somatostatin-type receptor
VEGF	Vascular endothelial growth factor

1. INTRODUCTION

1.1 Pituitary adenomas

Pituitary adenomas are epithelial tumours that arise from adenohypophysis cells. They account for ~10% of all intracranial neoplasms. Nevertheless, the prevalence of incipient pathologies is rather higher, since, up to a quarter of all pituitary glands analysed at autopsy harbour an unsuspected microadenoma. Similarly, pituitary imaging detects small lesions in at least 10% of normal people [Melmed, 2008].

In effect, pituitary adenomas are the most common cause of pituitary hormone hypersecretion and hyposecretion syndromes in adults. Besides the symptoms directly related with hormone secretion, all tumours can cause local pressure effects, including visual disturbances, cranial nerve palsy, and headache [Melmed, 2008].

The classification of pituitary neoplasms has undergone a variety of modifications since its conception. Although classical designations of tumours were based on the morphological characteristics of cell cytoplasm after cytological staining (acidophilic, basophilic and chromophobic tumours), pituitary adenomas are now classified based primarily on size and also on the hormones they produce. Thus, microadenomas present a maximum diameter below 10mm and macroadenomas higher than that. About one-third of tumours are endocrinologically "silent" (non-functioning pituitary adenomas), which grow insidiously for many years and usually present as an expanding pituitary mass. Otherwise, pituitary tumours are hormonally active and classified depending on the cell type from which they are derived: lactotrope (prolactin), somatotrope (growth hormone - GH), corticotrope (adrenocorticotrophic hormone), thyrotrope (thyroid stimulating hormone) or gonadotrope (luteinizing hormone, follicle stimulating hormone). Hormonal production does not always correlate with tumour size [Asa & Ezzat, 2009]. Additionally, in very rare cases (less than 0.1% of all pituitary tumours), aggressive and metastases forming pituitary carcinomas develop from formerly benign adenomas [Asa & Ezzat, 2002; Cai et al., 1994; Pei et al., 1995].

1.1.1 Pituitary adenomas treatment

A comprehensive strategy for treating patients with pituitary tumours aims to normalize excess pituitary hormone secretion, alleviate symptoms and signs of hormonal hypersecretion syndromes, shrink or remove the pituitary mass and preserve residual normal pituitary function [Shimon & Melmed, 1998].

Pituitary tumours may be treated medically, surgically, radiotherapeutically or with any of these approaches in combination. The choice of each of these modalities of therapy depends on the type of tumour and the availability of expertise. Microadenomas are mainly treated with transsphenoidal microsurgery with success rates reaching about 90%. On the other hand, macroadenomas are treated with surgery and, if appropriate, with radiation therapy after incomplete removal of the adenoma. Medical treatment often includes dopamine agonists such as cabergoline and bromocriptine for GH and prolactin-secreting tumours, and the somatostatin analogue octreotide for GH secreting adenomas [Melmed, 2008].

Increased understanding regarding the etiopathogenesis of pituitary tumours (*vide* 1.1.2) has identified new proteins and pathways that may lead to novel therapies. In addition, it is being exploited the applications in oncology of some of the already known treatments, namely octreotide. Beyond its normal endocrine actions, octreotide can be helpful in cancer treatment via direct somatostatin-type receptor (SSTR) signalling, autocrine/paracrine effects or cell labelling [Grimberg, 2004]. In pituitary lactosomatotroph GH3 cells, it has been already shown that the antiproliferative effects of octreotide are mediated trough SSTR2 and suppression of the phosphatidylinositol-3-kinase (PI3K) pathway [Theodoropoulou et al., 2006]. Unfortunately, the exact molecular mechanisms of pituitary adenoma proliferation are still poorly understood, but there is a general believe that once they are better defined, clinical decision making will be facilitated.

1.1.2 Pituitary tumourigenesis

Studies in pituitary tumourigenesis have shown that adenomas are monoclonal in origin, and that neoplastic progression depends on oncogenes activation/over-expression, or, conversely on inactivation/loss of tumour suppressor genes [Alexander et al., 1990; Asa & Ezzat, 2002]. Hitherto, the only initiating mutation described is a single amino acid mutation in the alpha-chain of the guanosine triphosphate-binding protein, Gs, that provokes a constitutive activation of the cyclic adenosine monophosphate (cAMP) pathway, observed in 10% to 40% of GH secreting tumours. This results in elevated cAMP formation and subsequent GH hypersecretion and adenoma proliferation [Lyons et al., 1990; Spada & Vallar, 1992].

Several evidences have demonstrated the involvement of multiple factors in pituitary tumourigenesis. Thus, oncogenes linked to adenoma progression include: cAMP-responsive nuclear transcription factor, CREB, which is also thought to be promoted by the over-expression of Gsα [Lania et al., 1998]; ras oncogene mutations, which have been detected in aggressive prolactinomas [Cai et al., 1994]; and pituitary tumour transforming gene, PTTG, which is a putative new marker for invasiveness in secretory adenomas [Zhang X et al., 1999; Heaney et al., 1999]. Tumour suppressor genes lost or deregulated in pituitary tumours are: *Rb* [Pei et al., 1995], *menin* [Theodoropoulou et al., 2004], p27 [Lidhar et al., 1999], p16 [Woloschak et al., 1997] and Zac [Pagotto et al., 2000]. Whereas altered expression or mutations of oncogenes and tumour suppressors play a causative role in pituitary tumour arising, hormones, neuropeptides, growth factors, cytokines and angiogenic factors support pituitary tumour progression and expansion [Asa & Ezzat, 2009]. In fact, it has been already shown altered expression or function of different factors and their specific receptors, in pituitary tumours [Renner et al., 2004]. In the following, the role of platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) in pituitary tumour development is summarised.

1.1.2.1 PDGF and pituitary tumours

The platelet-derived growth factor family consists of 5 isoforms, three of which (PDGF-AA, -AB, -BB) are essential angiogenic factors and important mitogens for connective tissue cells and other cell types such as smooth muscle cells and oligodendrocytes. The other isoforms (PDGF-CC and –DD) have been detected later and remain poorly characterised [Fredriksson et al., 2004]. All PDGF isoforms engage two receptor tyrosine kinases, PDGF receptors α and β (PDGFR-α, PDGFR-β) which homo- or heterodimerize (PFGFR-αα, PDGFR-ββ, PDGFR-αβ) after ligand binding, leading to the activation of the intrinsic tyrosine domain. Subsequently to receptor autophosphorylation, different signalling systems (Src, PLCg, Ras) are activated, among them the PI3K pathway [Tallquist & Kazlauskas, 2004].

PDGF receptors activation controls important physiological functions such as development of lung, kidney and brain in mice, regulation of intestinal tissue pressure and vessel remodelling during wound healing [Yeh et al., 1993; Heldin & Westermark, 1999; Betsholtz, 2004]. In addition, pathogenetic and pathophysiological roles for the PDGF/PDGF receptor system were previously described as mutational over-expression of the system in distinct types of cancer, auto/paracrine-stimulation of tumour cell growth, and stimulation of tumour neovascularisation through angiogenesis [Smits et al., 1992; Simon et al., 1997; Maher et al., 2001; Steer & Cross 2002; Heinrich et al., 2003; Distler et al., 2003; Ostman, 2004]. Regarding the latter, PDGF-BB has been shown to promote tumour vascularisation mainly through induction of pericyte proliferation and recruitment to newly formed vessels [Guo et al., 2003; Abramsson et al., 2003]. PDGF isoforms, such as PDGF-AA and PDGF-BB, also stimulate the expression of VEGF (described in detail below), the main angiogenic factor involved in tumour neovascularisation, as recently reported [Shikada et al., 2005; Matei et al., 2007]. Pituitary tumours secrete numerous growth factors, which together modulate pituitary adenoma progression and pathophysiology [Ray & Melmed, 1997; Renner et al., 2004]. However, so far, little is known about the expression, localisation and function of PDGF

and PDGF receptors in pituitary adenomas [Sasahara et al., 1995; Leon et al., 1994; Sullivan & Tashjian, 1983]. Messenger RNA expression of PDGF-A and –B chains has been found by Leon et al. in normal human anterior pituitary and in the totality of the 34 adenomas studied. Normal pituitary also expresses both PDGF receptors, while PDGFR-α and PDGFR-β have been found in 44% and 94% of pituitary adenomas, respectively [Leon et al., 1994]. No correlation between mRNA expression of PDGF isoforms or receptors and tumour volume could be found [Leon et al., 1994]. Taking in account the co-existence of ligand and receptors in the same tissue, it has been speculated, though never proven, that PDGF might be an auto/paracrine-acting growth factor for pituitary tumour function and growth.

Regarding functional aspects, PDGF has been reported to stimulate GH secretion in somatotroph GC pituitary cells and to inhibit prolactin release of lactosomatotroph GH4C1 and GH3 cells [Sullivan & Tashjian, 1983]. However, the latter remains unclear, since, in a very recent study, no PDGF receptors have been detected in GH3 cells [Kowarik et al., 2009]. In the same report, it has been demonstrated that the expression of PDGF-A and –B chains and of the 2 receptors is found exclusively in folliculostellate TtT-GF pituitary cells. Conversely, somatotroph MtT-S cells expressed PDGF-B and both PDGF receptors and corticotroph AtT20 pituitary cells synthesised only PDGF-B and PDGFR-α. Gonadotroph aT3-1 and lactosomatotroph GH3 cells were negative for PDGF-A and –B and the two PDGF receptors [Kowarilk et al., 2009]. Functional studies evidenced that PDGF-BB and PDGF-AB stimulate VEGF-A production in TtT-GF cells through the PI3K signalling pathway [Kowarik et al., 2009].

1.1.2.2 VEGF and pituitary tumours

VEGF is a homodimeric glycosylated protein produced by numerous cell types, that plays a key role in both physiological (embryogenesis, skeletal growth and reproductive functions) and pathological (tumour growth, intraocular neovascular disorders and other

diseases) angiogenesis. The VEGF protein family consists of several growth factors among which one of the most prevalent forms is VEGF-A, also referred as VEGF. The human VEGF-A gene is localised to 6p21.3, and alternative exon splicing generates four different isoforms in the human, designated according to the numbers of amino acids VEGF121, VEGF165, VEGF189 and VEGF206 [Ferrara & Gerber, 2003]. The secreted and soluble VEGF189 protein is the most prominent stimulator of angiogenesis among the different VEGF-A variants. Regulation of VEGF-A gene expression and protein production occurs via different mechanisms including VEGF-A induction by hypoxia and/or by growth factors and cytokines [Ferrara, 2000].

Although pituitary adenomas are normally less vascularised than normal anterior pituitary, there is no doubt that VEGF-A plays an essential role in neovascularisation which supports pituitary tumour formation and growth [Turner et al., 2003]. In normal anterior pituitary VEGF-A expression is exclusive of folliculostellate cells, whereas after tumoural transformation hormone-producing cells seem to acquire the ability to produce VEGF [Ferrara & Henzel, 1989]. Therefore, VEGF-A is detectable in variable amounts, both in primary cell cultures pituitary tumour cell lines and in human pituitary adenoma cells. In addition, VEGF production is strongly suppressed by glucocorticoids and is differently regulated by cytokines, growth factors and neuropeptides depending on the tumour cell type [Lohrer et al., 2001; Onofri et al, 2004; Borg et al., 2005; Renner et al., 2002; Gloddek et al., 1999]. As already mentioned, PDGF isoforms act as an up-regulator of the production of VEGF-A in folliculostellate TtT-GF pituitary tumour cells [Kowarik et al., 2009].

An interesting study has evidenced that VEGF-A, trough VEGF receptor-1 interaction, directly modulates growth, cell cycle progression and survival of the adenoma cells in pituitary adenoma, concomitantly with neovascularisation induction [Onofri et al., 2006].

1.2 Phosphatidylinositol-3-kinase (PI3K) pathway

PI3K-Akt signalling pathway seems to be deregulated in a wide spectrum of human cancers. It is activated by multiple cellular stimuli or toxic insults and regulates fundamental cellular functions such as transcription, translation, proliferation, growth and survival [Vivanco & Sawyers, 2002].

PI3K is a heterodimer composed of a catalytic subunit (P110) and an adapter/regulatory subunit (P85), which is activated after binding to receptors with protein tyrosine kinase activity or G protein-coupled receptors. In fact, following its recruitment to these receptors in the plasma membrane, activated PI3K phosphorylates phosphatidylinositol-4,5-biphosphate (PIP2) on the 3-OH group generating phosphatidylinositol-3,4,5-triphosphate (PIP3), a potent second-messenger. This conversion occurs in seconds. Intracellular PIP3 levels are tightly regulated by the action of phosphatases such as phosphatase and tensin homolog (PTEN) and Src-homology 2 domain-containing protein tyrosine phosphatase (SHP) [Nicholson & Anderson, 2002; Osaki et al., 2004].

After its synthesis, PIP3 does not seem to activate Akt directly; instead, it helps the recruitment of Akt to the plasma membrane and promotes conformational changes exposing its two main phosphorylation sites: Thr308 in the kinase domain and Ser473 in the C-terminal regulatory domain. Subsequently phosphoinositide-dependent kinase-1 (PDK-1), which is thought to be activated constitutively, phosphorylates Akt at Thr308, leading to stabilisation of the active conformation. Full activation of Akt requires Ser473 phosphorylation, but this mechanism remains controversial [Nicholson & Anderson, 2002; Osaki et al., 2004].

Finally, activated Akt is translocated to the cytoplasm and nucleus where many of its substrates are located, which include: apoptotic factor Bad, procaspase-9, inhibitor of nuclear factor kappa B kinase, cAMP-response-element-binding protein, the forkhead family of transcription factors, glycogen synthase kinase-3, mammalian target of rapamycin (mTOR), p21 and p27 [Vivanco & Sawyers, 2002].

1.2.1 PI3K pathway and pituitary tumours

The over-expression and over-phosphorylation of Akt in human pituitary tumour cells has been already demonstrated. This increased activation does not seem to be secondary to mutations of PTEN gene, which encodes a protein that negatively regulates intracellular levels of PIP3, but rather due to a change at a more proximate part of the cell-signalling pathway [Musat et al., 2005].

Several growth factors activate PI3K pathway and, specifically in pituitary, it has been demonstrated that PDGF-AB enhances VEGF-A secretion in TtG-GF cells, in response to activation of this pathway [Kowarik et al., 2009]. Oppositely, octreotide (a somatostatin analogue) presents inhibitory effects on lactosomatotroph GH3 cells proliferation, mediated trough SSTR2 and suppression of PI3K pathway [Theodoropoulou et al., 2006].

1.3 Pituitary cell lines

Immortalised cell lines constitute suitable *in vitro* models of endocrine and non-endocrine cell types to study pituitary adenomas, namely for the study of VEGF expression. Recent studies in pituitary adenoma VEGF-A production have been done in TtT-GF cells [Kowarik et al., 2009], which is a cell line derived from non-endocrine folliculostellate cells of the anterior pituitary gland [Inoue et al., 1992]. Folliculostellate cells are rarely found in adenomas and intra-tumoural endothelial cells represent a target but not a source of VEGF within solid tumours [Ferrara et al., 2003]. Therefore, pituitary adenoma cells seem to be responsible for VEGF production in pituitary tumour cell cultures, and cell lines of pituitary adenomas constitute a particularly good model to study this growth-factor expression. MtT-S is a rat somatrotoph cell line, established from estrogen-induced mammotropic pituitary [Inoue et al., 1990]. MtT-S cells express the two PDGF receptors (α and β) [Kowarik et al., 2009] and the SSTR2 [Morishita et al., 2003; Theodoropoulou et al., 2006], thus emerging as a good model to study the integration of stimulatory (PDGF) and inhibitory (octreotide) signals in growth and VEGF production, through the PI3K pathway.

2. AIM OF THE STUDY

Pituitary adenomas produce multiple growth factors, which in concert strongly stimulate pituitary tumour cell growth, that is associated *in vitro* with increased expression of the main angiogenic factor, VEGF-A. Although the combined stimulation of growth and angiogenesis would indicate a rapid progression of pituitary adenomas, most pituitary tumour types expand extremely slowly *in vivo*, suggesting the presence of extra- and intra-tumoural inhibitory factors and mechanisms, which counteract the stimulatory signals.

So far, little is known about the mechanisms that integrate stimulatory and inhibitory signals in pituitary tumour cells at molecular level. It has recently been shown that inhibitory effects of the somatostatin analogue octreotide in lactosomatotroph GH3 pituitary tumour cells are mediated through the somatostatin type-2 receptor and the suppression of the PI3K signalling pathway [Theodoropoulou et al., 2006]. In contrast, PDGF-AB enhanced VEGF-A secretion in the folliculostellate TtT-GF pituitary tumour cell line by stimulating PI3K signalling [Kowarik et al., 2009].

In the present work, effects of PDGF-AB and octreotide on proliferation and VEGF-A secretion were studied for the first time in the somatotroph MtT-S pituitary tumour cell line. Based on findings from other types of tumours, it was expected that PDGF-AB should stimulate growth and VEGF-A production of MtT-S cells, whereas octreotide should inhibit these effects. Thus, the aim of the present study was to investigate how the stimulatory effects of PDGF-AB and the inhibitory actions of octreotide are integrated in MtT-S cells and whether the PI3K signalling pathway was involved in this process.

3. MATERIAL AND METHODS

3.1 Reagents

Standard cell culture materials were purchased from Life Technologies (Karlsruhe, Germany), Nunc (Wiesbaden, Germany) and Sigma (St Louis MO, USA) unless specified.

3.2 Solutions

Platelet-derived growth factor AB (PDGF-AB), from R&D Systems (Minneapolis, MN, USA), was dissolved in sterile phosphate based buffer (PBS) (Gibco/Invitrogen, Carlsbad, USA), with 0.1% bovine serum albumin (Invitrogen Corp, Paisley, UK), and stored at -20ºC in aliquots of 100µl (concentration 10µg/ml). Octreotide, provided by the American Peptide Company, was dissolved in PBS to a concentration of 1mM, and was stored at -20ºC. Rapamycin (1mg/ml) from Sigma Chemicals, LY294002 (30µM) from Calbiochem (Bad Soden, Germany) and RAD001 (10mM) from Novartis Pharma AG were dissolved in Dimethyl sulfoxide (DMSO) (Sigma, St Louis MO, USA).

3.3 Cell culture

Somatotroph MtT-S rat pituitary tumour cells (Riken BioResource Center, Tsukuba, Japan) were routinely grown in Dulbecco's Modified Eagle's Medium (Invitrogen Corp, Paisley, UK), supplemented with 10% foetal calf serum (Gibco, Karlsruhe, Germany), 2mmol/l L-glutamine (Biochrom AG, Berlin, Germany), 2.5mg/l partricin (Biochrom AG, Berlin, Germany) and 10^5U/l penicillin/streptomycin (Biochrom AG, Berlin, Germany). Cells were incubated at 37^{o}C in 5% CO_2 (Incubator 1/004; Thermo Scientific, Whaltam, MA, USA) until became confluent, and then were used for proliferation studies, RNA and protein molecular analysis and supernatant collection.

3.4 Cell Proliferation assay

The proliferation of MtT-S cells in response to PDGF-AB and octreotide treatments was measured by WST-1 assay (Roche Molecular Biochemicals, Manheim, Germany). The cells were plated in a 96-well plate (5000 cells in a final volume of 100µl per well) and incubated for 24h at 37^{o}C in 5% CO_2. Subsequently, cells were under overnight serum deprivation and then were treated with PDGF-AB (1, 5, 10, 50 and 100 ng/ml) alone and with octreotide (10µM, 10nM, 10^{-12}M and 10^{-15}M) alone or conjugated with PDGF 50ng/ml, for 24h. Cells grown in the absence of treatments were used as negative controls. Treatment was stopped by addition of 10µl of WST-1 cell proliferation reagent to each well, and absorbance was measured at 450 nm in a spectrophotometer (SmartSpec Plus; Bio-Rad, Hercules, CA, USA) after incubation for 30 min.

3.5 RNA isolation

The cells were plated in a 6-wells plate (3.0 x 10^5 cells in a final volume of 1ml per well) and incubated for 24h at 37^{o}C in 5% CO_2. Subsequently, cells were under overnight serum deprivation and then were treated with PDGF-AB 50ng/ml alone or associated to octreotide at various concentrations (10^{-6} and 10^{-12}M), for 24h. Cells grown without stimulation were used as negative controls

The cells were lysed directly by addition of 1ml of TRIzol to each well and passing the cell lysate several times through a pipette. The resulting homogenised samples were incubated for 5min at room temperature, to permit the complete dissociation of nucleoprotein complexes. Subsequently, it was added 200µl of chloroform (Sigma, St Louis MO, USA) to each tube, which were vigorously shaken by hand for 15s and then incubated at room temperature for 3min. Following incubation, the samples were centrifuged (Centrifuge 5415R; Eppendorf; Hamburg, Germany) at 12052g for 15min at 4ºC. The aqueous phase was transferred to a fresh tube and the RNA was precipitated with 500µl of isopropyl alcohol (Sigma, St Louis MO, USA). The samples were

incubated for 10min at room temperature and centrifuged at 12052g for 15min at 4ºC. The supernatant was discarded and the RNA pellet was washed with 1ml of 70% ethanol (Sigma, St Louis MO, USA). Each sample was mixed by vortexing, centrifuged at 12052g for 10min at 4ºC and, after supernatant rejection the pellet was left to dry in a thermo-dry-block (Thermo Dry Block DB-2A; Techne Inc, Burlington, USA) at a maximum temperature of 65ºC and then dissolved in an appropriate amount of DEPC-treated water (Sigma, St Louis MO, USA). The RNA was left for 1h at 60ºC in the thermo-dry-block.

The RNA concentration of each sample was calculated after absorbance measurement with a spectrophotometer (SmartSpec Plus; Bio-Rad, Hercules, CA, USA), according to the following formula: RNA (µg/µl) = (A_{260}x40x60)/1000, where A_{260} is the sample absorbance at 260nm, 40 is the concentration in µg/µl of RNA giving A_{260} value equal to 1 in the standard curve, and 60 is the dilution factor used to measure the sample concentration (1µl RNA + 59µl DEPC water).

All RNA samples were submitted to a Polymerase Chain Reaction (PCR) for GAPDH (housekeeping gene) to exclude genomic DNA contamination. In the absence of DNA, no band is visible after loading the PCR product on an ethidium bromide gel (as described below).

3.6 Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

The cDNA production from RNA isolated from samples was performed after incubating for 1h at 45ºC (Incubator 1/004; Thermo Scientific, Whaltam, MA, USA) the following mixture: 1µg of RNA, 1µl of dNTP mix 2mM (MBI Fermentas, Vilnius, Lithuania), 2µl of 62,5 U/ml random primers (Hexanucleotide mix – Roche, Mannheim, Germany), 2µl of 10 nM dithiothreitol (DTT – Sigma, St Louis MO, USA) and 1µl of 200U reverse transcriptase (SuperScript II – Invitrogen, Carlsbad, USA), 4µl of 5x first strand buffer

(Invitrogen, Carlsbad, USA) and DEPC-water to get a final volume of 20µl. Reaction was stopped by boiling the samples for 5min at 95ºC.

Each produced cDNA was amplified by PCR, incubating 1µl of cDNA sample with 1,5µl of 10x PCR buffer (MBI Fermentas, Vilnius Lithuania), 0,9µl of 25mM $MgCl_2$ (MBI Fermentas, Vilnius Lithuania), 1,5µl dNTP mix 2mM (MBI Fermentas, Vilnius Lithuania), 0,5µl of amplification VEGF primer sense 10pmol/µl (MWG Biotech), 0,5µl of amplification VEGF primer anti-sense 10pmol/µl (MWG Biotech), 0,15µl of *Thermus aquaticus* (Taq) DNA polymerase (MBI Fermentas, Vilnius Lithuania) and 8,95µl of autoclaved distilled water. The PCR reaction included 35 cycles of the following program (T3 Thermocycler; Biometra, Göttingen, Germany): denaturation at 94ºC for 1min, annealing at 60ºC for 1min and elongation at 72ºC for 1min.

Meanwhile, a 1-1,5% agarose (Life Technologies, Paisley, UK) gel, according to the expected size of the amplification product, was prepared, supplemented with ethidium bromide (Sigma, St Louis MO, USA). After amplification, the DNA molecules were separated by electrophoresis (Electrophoresis chamber Mini-Protean 3 and Power Supply PowerPac 1000; Bio-Rad, Hercules, CA, USA) in 1 x Tris Borate EDTA buffer for 15-20min at 80V, and the bands were visualised under UV light. Amplification products molecular weight was determined by comparison with fragments of known size (1kb Plus DNA ladder marker - Life Technologies, Paisley, UK).

Table I. Primer sequences and cDNA fragment size

Primers		Sequence (5'-3')	Ta (ºC)	Amplified fragment (bp)
GAPDH rat/mouse	sense	ATGGTGAAGGTCGGTGTGAACG	60	495
	anti-sense	GTTGTCATGGATGACCTTGGC	60	
VEGF rat/mouse	sense	TCTGCTCTCTTGGGTGCACTG	61.8	232
	anti-sense	CATTAGGGGCACACAGGACGG	63.7	

Ta – annealing temperature; bp – base pairs

PCR primers (Table I) were selected from sequences previously employed by colleagues with success. Each sequence was checked with the NCBI BLAST program in order to exclude eventual annealing with other genes different from the ones studied.

3.7 Western immunoblotting

Protein analysis was carried out on confluent cells grown in Petri dishes, after overnight serum deprivation, followed by treatment with PDGF-AB (50 ng/ml) alone or together with octreotide 10^{-15}M, for different periods (5, 10, 15 and 30min). Each stimulation condition was carried out in an independent dish; in the negative control the cells were not stimulated.

After the treatment period, all the procedures were performed on ice. The cells were washed with 2ml of cold PBS (that was discarded), and removed from the dish with a plastic scraper and 1ml of PBS. After 3min centrifugation at 10392g (Centrifuge 5415R; Eppendorf; Hamburg, Germany) the cell membranes were mechanically disrupted by pipeting up and down through an insulin syringe, in PBS supplemented with 1:100 proteases inhibitor cocktail (Sigma, St Louis MO, USA). The volume of the proteases inhibitor cocktail solution varied according to the dimension of the membranes pellet obtained after centrifugation. Total protein concentration in samples was determined with Bradford dye colorimetric assay [Bradford, 1976].

One hundred and fifty µg of the extracted protein mixture obtained for each sample was separated by SDS-PAGE, in a pre-cast Tris-glycine 10% gel (Anamed, Darmstadt, Germany) in an Invitrogen electrophoresis apparatus (Invitrogen Corp, Paisley, UK), during 30min at 200V. This procedure separates the proteins according to their size, which is compared to the protein marker (Bio-rad, Hercules, CA, USA).

The separated proteins were subsequently transferred to a nitrocellulose membrane (Hybond ECL) by Western blotting, during 2h at 30V. In this procedure the gel was on the negative electrode (cathode) of the apparatus and the nitrocellulose membrane on the

positive one (anode), so the negative-charged proteins are driven from the gel to the positive-charged membrane, in an equivalent position.

The nitrocellulose membrane was quickly stained with Ponceau S, and the protein standards distribution marked with a pen, and then blocked at room temperature for 2h, in 1xTBS containing 0.1% Tween solution (TBS-T) and 5% nonfat milk powder, with gentle shaking. After blocking, the membrane was incubated overnight with the primary antibody (Table II) in TBS-T with 2,5% nonfat milk powder, at 4ºC with gentle shaking. Afterwards, the membrane was washed 4 times with TBS-T (5 + 5 + 10 +10min) and was incubated for 1.5h with Horseradish Peroxidase-conjugated secondary antibody (dilution 1:1000) in 2.5% nonfat milk powder TBS-T. The washing procedure was then repeated as previously described and the protein bands were visualised using a commercially available chemiluminescence kit (Roche, Mannheim, Germany) according to manufacturer's instructions.

Table II. Antibodies for the Western immunoblotting

Antigens	**Primary antibodies**	**Secondary antibodies**
pPDK1	Rabbit anti-rat (1:1000)	Donkey anti-rabbit
pPTEN-Ser^{380}	Rabbit anti-rat (1:1000)	Donkey anti-rabbit
pAkt-Thr^{308}	Rabbit anti-rat (1:1000)	Donkey anti-rabbit
pmTOR	Rabbit anti-rat (1:1000)	Donkey anti-rabbit

Primary antibodies: phosphorylated phosphoinositide-dependent kinase 1 (pPDK1 (Ser ^{241})), phosphorylated phosphatase and tensin homologue (pPTEN-Ser^{380}), phosphorylated Akt (pAkt-Thr^{308}), phosphorylated mammalian target of rapamycin (pmTOR (Ser^{2448})), all from Cell Signalling Technologies (Beverly, MA).
Secondary antibody: donkey anti-rabbit, horseradish peroxidase conjugated, from Amersham Biosciences (Bucks, UK).

3.8 VEGF Enzyme-linked immunosorbent assay (ELISA)

MtT-S cells were seeded in 48-well plates at 3 x 10^4 cells/well, serum deprived overnight and then stimulated with PDGF-AB alone at different concentrations (1, 5, 10, 50 or 100ng/ml). For the experiment with LY294002, the cells were stimulated with PDGF-AB 10ng/ml alone or conjugated with LY294002 1, 10, and 20µM. When testing rapamycin and RAD001 effects, the stimulation was done also with PDGF-AB 10ng/ml alone or conjugated with rapamycin 1nM or RAD001 10^{-8}M. It was also performed an experiment with PDGF-AB 50ng/ml and PDGF-AB plus several concentrations of octreotide (10^{-6}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}, 10^{-13}, 10^{-14} and 10^{-15}M). In all these experiments the vehicle solution in which substances were dissolved was used individually as control (ex: DMSO for LY294002, rapamycin and RAD001). Supernatant was collected and analysed always 24h after stimulation.

VEGF protein concentration in MtT-S cells supernatants was measured using a Quantikine ELISA kit specific for rat VEGF (R&D Systems, Minneapolis, MN, USA). All reagents were reconstituted and stored following the manufacturer's instructions. The PBS solution was freshly prepared to obtain a final concentration in distilled water of 137nM NaCl, 2,7mM KCl, 8,1mM Na_2HPO_4, 1,5mM KH_2PO_4, at a pH between 7,2 - 7,4, and was filtered using a 0,2µm filter.

Each well of the 96-well plate was coated with 100µl of the Capture Antibody diluted to 1,0µg/ml in PBS immediately before. Then the plate was sealed and incubated overnight at room temperature. The following washing process consisted of three cycles of aspiration and wash with 400µl of Wash Buffer per well, using a multi-channel pipette. The plate was blocked with 300µl of Reagent Diluent per well and incubated at room temperature for 1h, followed by the already described washing process.

Standard dilutions in Reagent Diluent and samples were pipetted into the wells (100µl per well), the plate was covered with an adhesive strip and incubated at room temperature for 2h. The wells were washed as previously described and 100µl of the Detection Antibody, at a working concentration of 100ng/ml, was added to each well.

After incubation during 2h at room temperature the washing process was repeated. Streptavidin-HRP was diluted in Reagent Diluent at a working concentration 1:200, and 100µl was added to each well. The plate was covered and incubated for 20min at room temperature, avoiding direct light exposure. The Substrate Solution was prepared as a 1:1 mixture of Reagent A and Reagent B, and 100µl were added to each well. After an incubation period of 20min at room temperature, avoiding direct light exposure, the reaction was stopped by adding 50µl of Stop Solution to each well. The optical density of each sample was determined immediately using a spectrophotometer (SmartSpec Plus; Bio-Rad, Hercules, CA, USA) set at 450nm.

3.9 Statistical analysis

Cell proliferation experiments and VEGF-A secretion were all performed in quintuplicate and triplicate wells respectively. The results are expressed as mean ± standard error.

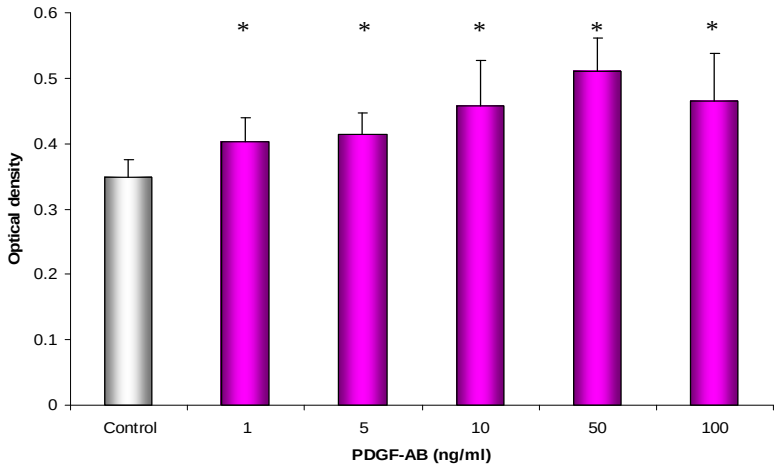
For statistical analysis of stimulation experiments, the mean values were compared using the t-student test (SPSS® 11.0 - SPSS Inc, Chicago, USA). Statistical significance was considered at $p<0,05$.

4. RESULTS

4.1 PDGF-AB increases MtT-S cells proliferation

The influence of PDGF-AB and octreotide on MtT-S cell proliferation was evaluated employing cell proliferation reagent WST-1, which is designed to be used for non-radioactive, spectrophotometric quantification of cell growth and viability in proliferation and chemosensitivity assays. It can therefore be used as a rapid, convenient and automated method for the measurement of cell proliferation in response to growth factors, cytokines, mitogens and nutrients.

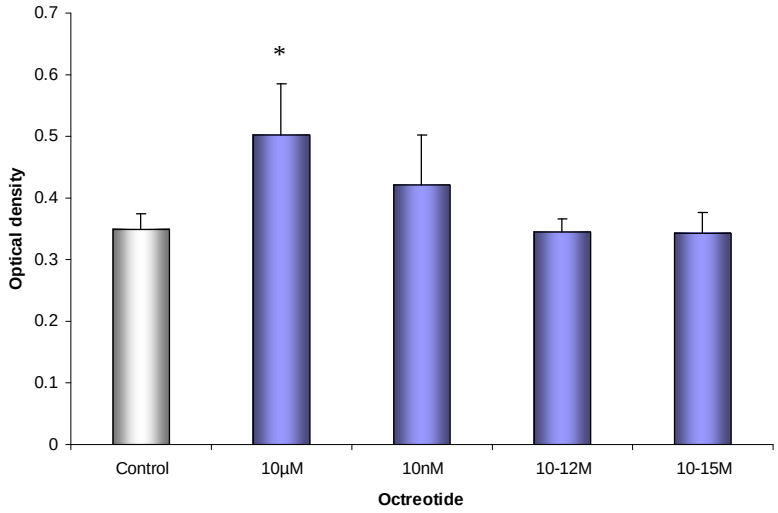
It was observed that the treatment of MtT-S cells during 24h with different concentrations of PDGF-AB increased cell proliferation in a dose-dependent way. This effect was statistically significant and reached the saturation at PDGF-AB concentration of 50ng/ml (Graphic 1).



Graphic 1. Effect of PDGF-AB treatment on MtT-S cell proliferation.
MtT-S cells were treated with PDGF-AB (1, 5, 10, 50 and 100 ng/ml) for 24h. Cells grown in the absence of treatments were used as negative controls. Treatment was stopped by addition of 10µl of WST-1 cell proliferation reagent to each well. After 30min incubation, absorbance was measured at 450 nm spectrophotometer. Values are a mean of 5 replicates. *$p<0,05$ when compared to Control.

4.2 Octreotide increases MtT-S cells proliferation

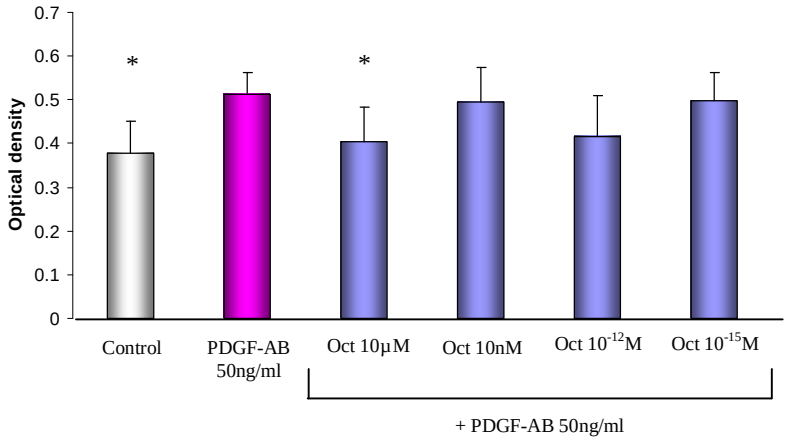
WST-1 assay was also performed after treatment of MtT-S cells during 24h with different concentrations of octreotide. The present results demonstrated that octreotide increased cell proliferation in a statistically significant way at 10µM, whereas lower doses had no effect (Graphic 2).



Graphic 2. Effect of octreotide treatment on MtT-S cells proliferation.
MtT-S cells were treated with octreotide (10µM, 10nM, 10^{-12}M and 10^{-15}M) for 24h. Cells grown in the absence of treatments were used as negative controls. The treatment was stopped by addition of 10µl of WST-1 cell proliferation reagent to each well. After 30min incubation, absorbance was measured at 450 nm spectrophotometer. Values are a mean of 5 replicates. *p<0,05 when compared with Control.

4.3 PDGF-AB and octreotide co-stimulation have no synergistic effect on MtT-S cells proliferation

The effect of stimulation induced by PDGF-AB alone and PGDF-AB plus different doses of octreotide on proliferation of MtT-S cells, was also evaluated. Contrarily to the expected result, these two substances inhibited each other when combined (Graphic 3).

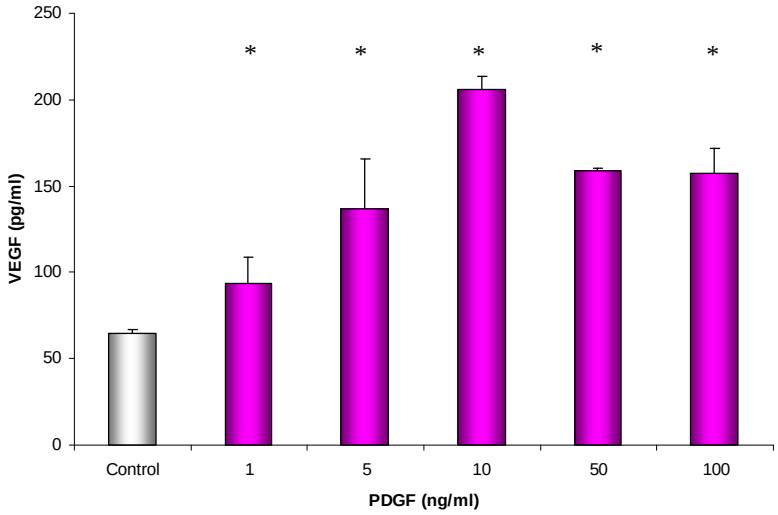


Graphic 3. Effect of octreotide plus PDGF 24h-treatment on MtT-S cells proliferation.
MtT-S cells were treated for 24h with PDGF 50ng/ml or PDGF-AB plus octreotide (10µM, 10nM, 10^{-12}M and 10^{-15}M). Cells grown in the absence of treatments were used as negative controls. Treatment was stopped by addition of 10µl of WST-1 cell proliferation reagent to each well. After 30min incubation, absorbance was measured at 450 nm spectrophotometer. Values are a mean of 5 replicates. *p<0,05 when compared with PDGF-AB 50ng/ml.

4.4 PDGF-AB induces VEGF-A secretion by MtT-S cells

The effect of PDGF-AB treatment on VEGF-A secretion by MtT-S cells was assayed, after a 24h stimulation period employing different concentrations of PDGF-AB. VEGF-A concentration was measured in the supernatant using a Quantikine ELISA kit, that is a powerful method in estimating low concentration substances in solution (up to 1 pg/ml).

Indeed, it was verified that PDGF-AB induced a 2 to 3-fold increase in VEGF-A secretion, which was dose-dependent until saturation was reached, at PDGF-AB 10ng/ml concentration (Graphic 4). This increase was statistically significant.



Graphic 4. Effect of PDGF-AB on VEGF-A secretion.

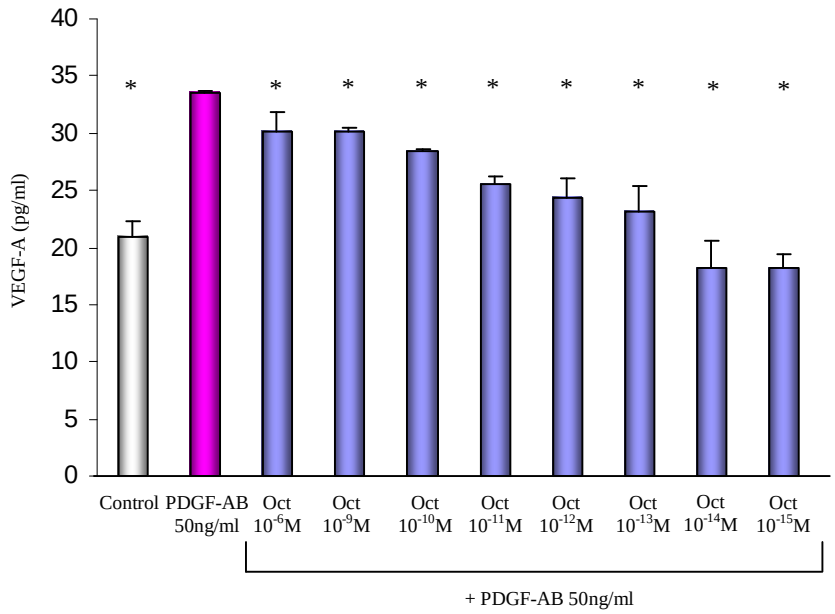
MtT-S cells were stimulated with PDGF-AB alone at different concentrations (1, 5, 10, 50 or 100ng/ml), during 24h. VEGF protein concentration was measured in the supernatant using a Quantikine ELISA kit specific for rat VEGF. Optical density of each sample was determined using a spectrophotometer set at 450nm. Values are a mean of 3 replicates. *p<0,05 when compared with Control.

4.5 PDGF-induced VEGF-A production is inhibited by octreotide

The effect of the somatostatin analogue octreotide in PDGF-AB induced VEGF secretion was also studied. After a 24h stimulation period, the VEGF-A secretion was measured in the supernatant by ELISA.

It was observed that the PDGF-AB-induced increase in VEGF-A secretion was inhibited by octreotide in a statistically significant way (Graphic 5). This effect was inversely dependent on the applied dose, with the lowest concentration of octreotide used producing the highest inhibitory effect. However, at concentrations lower than $1x\ 10^{-12}$, the inhibitory effects seems to stabilize.

.



Graphic 5. Effect of octreotide on PDGF-AB induced VEGF-A secretion.

MtT-S cells were stimulated with PDGF-AB 50ng/ml alone and PDGF-AB plus different octreotide concentrations (10^{-6}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}, 10^{-13}, 10^{-14} and 10^{-15}M), during 24h. VEGF protein concentration was measured in the supernatant using a Quantikine ELISA kit specific for rat VEGF. Optical density of each sample was determined using a spectrophotometer set at 450nm. Values are a mean of 3 replicates. *p<0,05 when compared with PDGF-AB 50ng/ml.

The expression of VEGF-A mRNA by MtT-S cells under different stimulus was assayed by RT-PCR (Figure 1) and it was verified that the results obtained corroborate previous measurements of the secreted peptide on culture medium (Graphics 4 and 5). The RT-PCR technique implies that extracted RNA molecules were primarily reversed transcribed into its complementary DNA (cDNA), which was then amplified by PCR. The GAPDH mRNA expression was evaluated for all experimental conditions. Indeed, a band with near 500bp was observed for every analysed sample. VEGF-A mRNA expression levels were evaluated by the intensity of the band that was observed between 200 and 300bp of the molecular weight marker (nearer the 200). A marked decrease in the VEGF-A band was observed for the octreotide treated cells, which contrasts with the

increased expression induced by PDGF treatment when compared with control conditions (Figure 1). Only octreotide representative concentrations (10^{-6}M, 10^{-12}M) in terms of VEGF secretion inhibitory effect were shown.

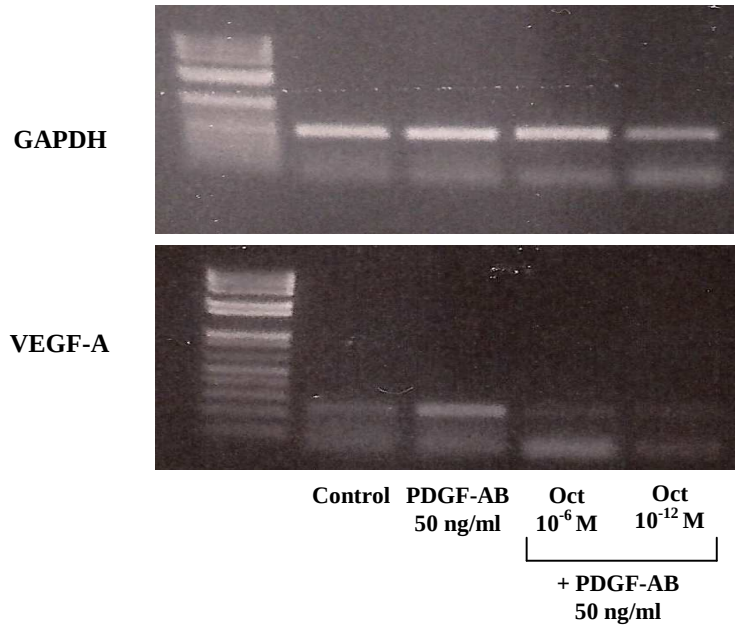


Figure 1. PDGF-AB 24h-treatment effects on VEGF-A mRNA expression.
Photographs of the ethidium bromide-stained PCR amplification products separated in an agarose gel electrophoresis. cDNA of GAPDH and VEGF-A was amplified using specific primers. Cells were previously treated during 24h with medium, PDGF-AB 50ng/ml or PDGF-AB 50ng/ml plus octreotide at representative concentrations (10^{-6}M, 10^{-12}M) in terms of VEGF secretion inhibitory effect.

4.6 PDGF-induced activation of PI3K-Akt-mTOR pathway is inhibited by octreotide

In order to study the involvement of the PI3K-Akt-mTOR pathway in the increase of VEGF-A secretion induced by PDGF-AB and to elucidate whether octreotide inhibits PDGF-AB induced VEGF-A secretion via this pathway, it was assayed the expression of

pPDK1, pAkt-Thr ^{308}, pmTOR and pPTEN-Ser^{380} by Western immunoblotting, a technique that allows for the identification of proteins.

As expected, PDGF-AB increased the phosphorylation levels of Akt and mTOR after 5 minutes of treatment (Figure 2). The same effect was observed for PDK-1, however the maximum levels were reached at 10 minutes of treatment. It was also verified that octreotide decreased the PDGF-induced phosphorylation levels of Akt, mTOR and PDK-1, which increased with the time of exposition.

Interestingly, the phosphorylation status of PTEN phosphatase, the major negative regulator of PI3K signalling pathway, has not changed in any of the studied samples.

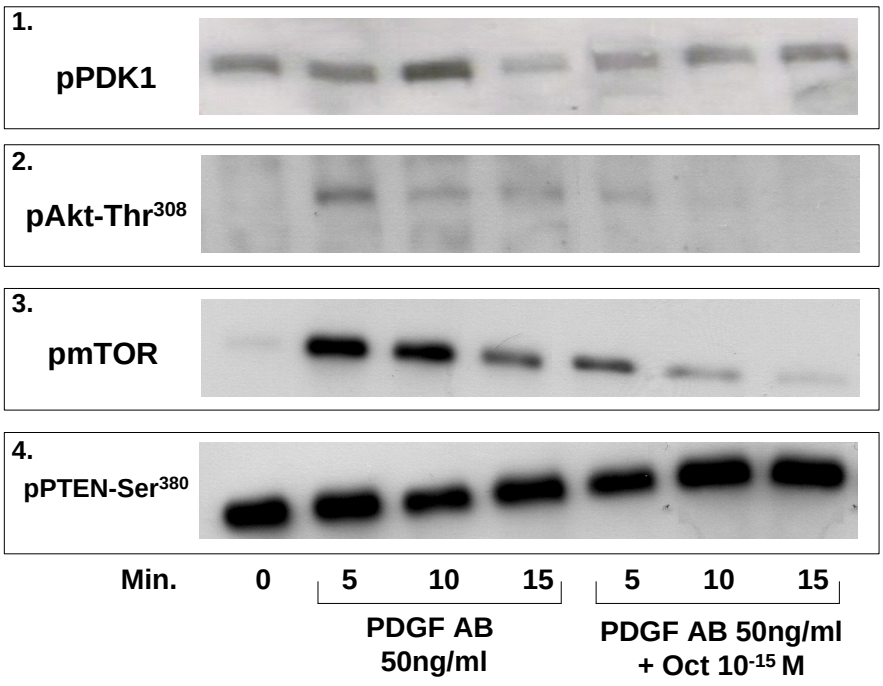


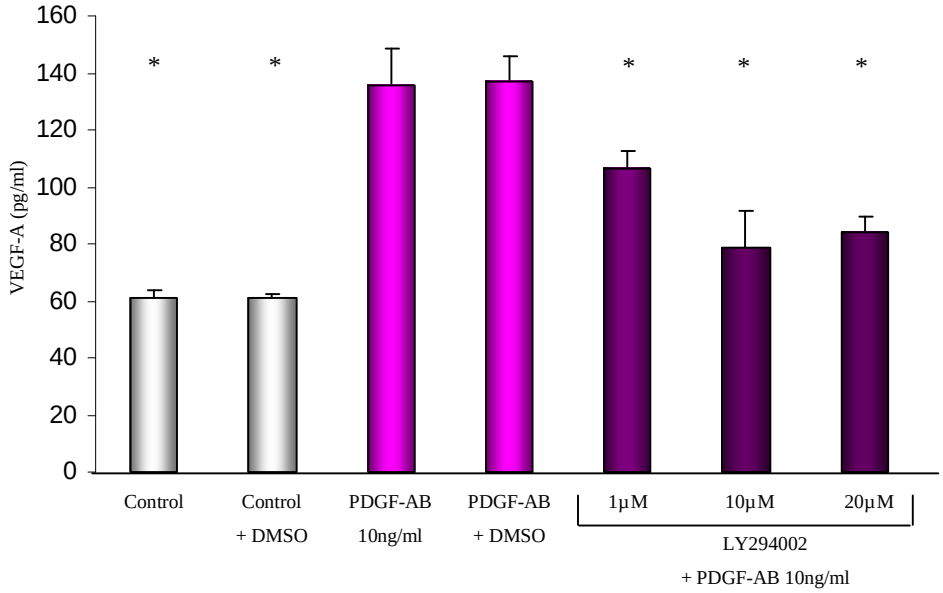
Figure 2. PDGF-AB-induced activation of PI3K-Akt-mTOR pathway in MtT-S cells, and octreotide inhibitory effect.

Detection of pPDK-1, pAkt-Thr^{308}, pmTOR and pPTEN-Ser^{380} by Western immunoblotting on MtT-S cell lysates, treated with PDGF-AB 50ng/ml or PDGF-AB 50ng/ml plus octreotide 10^{-15}M for 0, 5, 10 and 15min.

4.7 PDGF-induced VEGF-A production is inhibited by PI3K-mTOR pathway inhibitors

In order to evaluate if PI3K-mTOR pathway inhibitors influence the increase of VEGF-A secretion induced by PDGF-AB, three studies were performed using the following substances: LY294002 (a specific inhibitor of PI3K), rapamycin (a macrolide antibiotic that inhibits mTOR) and RAD001 (a derivative of rapamycin that is also a mTOR inhibitor). VEGF-A levels in the supernatant were quantified by ELISA, after a 24h stimulation period.

LY294002 markedly inhibited VEGF-A secretion already at the dose of 1µg/ml, nevertheless, the higher effect on PDGF-AB induced VEGF-A secretion was verified for 10µg/ml, reducing VEGF-A towards basal levels (Graphic 6). These differences were statistically significant. DMSO, the vehicle substance of the inhibitor, had no influence on VEGF-A secretion.

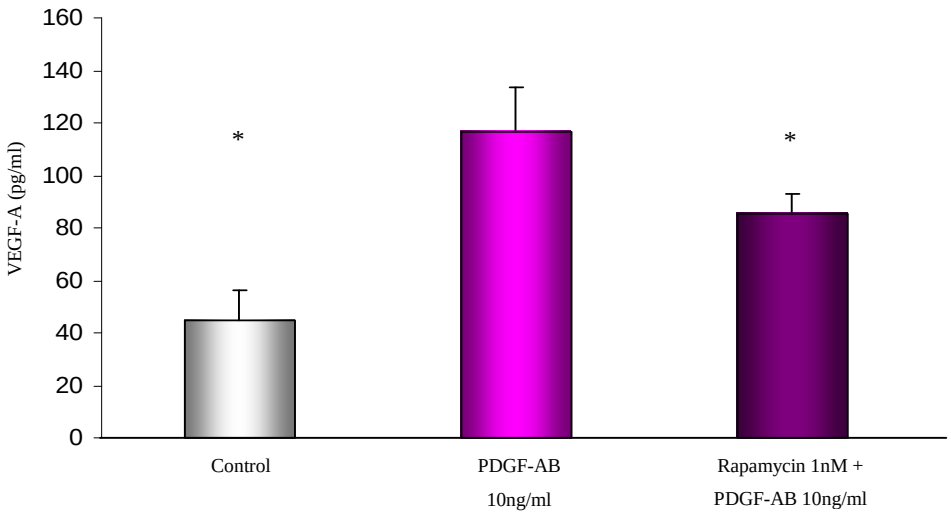


Graphic 6. Effect of LY294002 on PDGF-AB induced VEGF-A secretion.

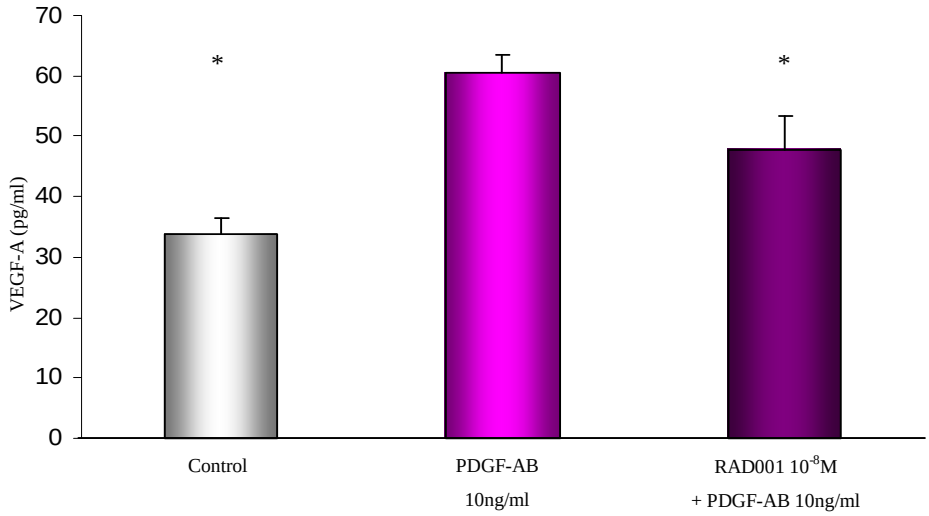
MtT-S cells were stimulated with PDGF-AB 10ng/ml alone and PDGF-AB plus different LY294002 concentrations (1, 10 and 20µM), during 24h. LY294002 vehicle solution (DMSO) alone was used as control. VEGF protein concentration was measured in the supernatant using a

Quantikine ELISA kit specific for rat VEGF. Optical density of each sample was determined using a spectrophotometer set at 450nm. Values are a mean of 3 replicates. *p<0,05 when compared with PDGF-AB + DMSO.

Similar effects were observed for rapamycin at 1nM (Graphic 7) and RAD001 at 10^{-8}M treatments (Graphic 8), that also inhibited VEGF-A secretion induced by PDGF-AB, in a statistically significant way.



Graphic 7. Effect of rapamycin on PDGF-AB induced VEGF-A secretion.
MtT-S cells were stimulated with PDGF-AB 10ng/ml alone and PDGF-AB plus rapamycin 1nM, during 24h. VEGF protein concentration was measured in the supernatant using a Quantikine ELISA kit specific for rat VEGF. Optical density of each sample was determined using a spectrophotometer set at 450nm. Values are a mean of 3 replicates. *p<0,05 when compared to PDGF-AB 10ng/ml.



Graphic 8. Effect of RAD001 on PDGF-AB induced VEGF-A secretion.

MtT-S cells were stimulated with PDGF-AB 10ng/ml alone and PDGF-AB plus RAD001 10^{-8}M, during 24h. VEGF protein concentration was measured in the supernatant using a Quantikine ELISA kit specific for rat VEGF. Optical density of each sample was determined using a spectrophotometer set at 450nm. Values are a mean of 3 replicates. *$p<0,05$ when compared with PDGF-AB 10ng/ml.

5. DISCUSSION

It is well established that pituitary tumoural cells produce a variety of growth factors, which potently stimulate tumour growth and strongly contribute to pituitary adenoma progression. In effect, the growth factors expressed by tumour cells in part determine adenoma pathophysiology by enhancing hormone secretion of the adenoma cells [Renner et al., 2004]. Moreover, pituitary tumour cells express the most important angiogenic factor, VEGF-A, which may be critically involved in neovascularisation of pituitary adenomas [Lohrer et al., 2001; Onofri et al., 2004; Onofri et al., 2006]. Despite pituitary tumours relatively high production of growth and angiogenic factors, which potentially support a rapid expansion of these tumours, most pituitary adenoma types expand extremely slowly *in vivo* [Asa & Ezzat, 2002]. This evidence indicates that extra- and intra-tumoural inhibitory factors and mechanisms must be continuously activated in pituitary adenomas for counteracting the stimulatory signals.

In the present work, PDGF-AB was selected as a stimulatory factor and the somatostatin analogue octreotide as an inhibitory agent. The aim of the study was to clarify how these two factors interact in the regulation of a pituitary tumoural cell line, specifically to evaluate whether octreotide blocks the tumour supportive actions exerted by PDGF, and through which molecular mechanisms these opposite effects are mediated. The study was performed in MtT-S rat pituitary tumour cells, which represent a well established *in vitro* model of a GH-producing somatotroph tumour cell [Inoue et al., 1990]. Although the low GH secretion rates of MtT-S cells suggest that these cells are immature somatotroph tumour cells, they express all characteristic receptors of this cell type such as growth hormone releasing hormone and insulin-like growth factor-1 receptors. MtT-S cells also express all the 5 subtypes of somatostatin receptors (SSTR1 to SSTR5) [Morishita et al., 2003; Nogami et al., 2006] which are particularly important for the study of the action of octreotide as here reported. Moreover, in a previous screening of different types of pituitary tumour cell lines, it was demonstrated that MtT-S cells expressed both α and β subunits of the PDGF receptor [Kowarik et al. 2009]. This

indicates that MtT-S cells are able to express homodimeric PDGF-αα and –ββ receptors as well as the heterodimeric PDGF-αβ receptor. Thus, the requirements for the action of PDGF-AB, which binds predominantly to PDGF-αα and PDGF-αβ receptors, are given [Tallquist and Kazlauskas, 2004], and MtT-S cell line emerges as an adequate choice.

PDGF, whose action depends on engagement with its receptors, is already known to be involved in the development of different types of tumours (e.g. gliomas, soft tissue sarcomas) through autocrine growth stimulation of tumour cells, stimulation of angiogenesis and recruitment of tumour fibroblasts [Maher et al., 2001; Smits et al., 1992]. Therefore, PDGF receptor tyrosine kinase inhibitor Gleevec (Imatinib) has been tested in clinical studies for different types of cancer, presenting beneficial effects on tumours treatment [Ren et al., 2009]. In the present work it was shown that PDGF-AB significantly stimulates MtT-S proliferation, although the observed effect was lower than the initially expected. However, this result agrees with what is known about the growth of human pituitary adenomas *in vivo*. Whereas in a reduced number of solid tumours the expression levels of PDGF and PDGF receptors closely correlates with the growth rate of the tumours [Varela et al., 2004; Carvalho et al., 2005; Nakamura et al., 2008], human pituitary adenomas often expand extremely slowly [Asa & Ezzat, 2002] despite abundant expression of the PDGF/PDGF receptor system [Leon et al., 1994; Sasahara et al., 1995]. Thus, we can conclude from the present results that PDGF exerts a growth stimulatory effect in this pituitary tumour cell line suggesting that cells with similar characteristics may contribute to tumour progression *in vivo*. Moreover, the slow but significant growth stimulatory potential of PDGF seems to explain in part why most human pituitary adenomas, that abundantly express both PDGF and its receptors, expand only very slowly.

It has recently been shown that the growth of lactosomatotroph GH3 rat pituitary tumour cells, which express only SSTR1 and SSTR2 [Garcia & Myers, 1994], was significantly inhibited by octreotide [Theodoropoulou et al., 2006]. In the present work, the effect of octreotide on the proliferation of MtT-S cells, which express all the 5 SSTRs [Morishita

et al., 2003], was studied for the first time. It was expected that octreotide would exert an inhibitory effect on MtT-S cell growth, similarly to that described for GH3 cells. Surprisingly, octreotide had no effect on MtT-S cell proliferation at low concentrations and even stimulated cell growth at higher dosages. This result strongly suggest that MtT-S cells would belong to a tumour cell type that – in terms of growth - is not responsive or is even adversely regulated by octreotide. In fact, it was already described that octreotide-based therapeutics present variable effectiveness for human pituitary tumours treatment [Chanson & Salenave, 2008]. Today, patients with somatotroph pituitary tumours often receive somatostatin analogue therapy as first line treatment. However, whereas in the majority of patients octreotide efficiently reduces GH secretion and often induces tumour shrinkage, about 30 to 40% of the cases are resistant to somatostatin analogue treatment and, in rare situations, octreotide worsens tumour growth and GH secretion [Chanson & Salenave, 2008]. Additionally, evidence from other tumour types demonstrated that octreotide therapies present unpredictable and even opposite effects on growth. For instance, somatostatin analogue therapy is effective in suppressing tumour expansion of SSTR-expressing neuroendocrine tumours of the gastroentero-pancreatic system [Grozinsky-Glasberg et al., 2008; Moreno et al., 2008], whereas octreotide stimulates tumour progression of SSTR-expressing meningiomas [Koper et al., 1992; Lamberts et al., 1995]. These reports evidence that there are obvious tumour-specific differences in the response to octreotide, as well as individual differences in the responsiveness in the same type of tumours, such as somatotroph pituitary adenomas. Regarding the latter, different explanations have been proposed to justify the differential action of octreotide. Most likely, the expression pattern of the different subtypes of somatostatin receptors may influence the different responses to octreotide since each receptor subtype activates specific intracellular pathways for proliferation control. Thus, the signalling effect that prevails at the end, in a given cell, will depend on the cell specific distribution of SSTR subtypes and signalling elements, as well as on SSTR internalisation, desensitisation and/or receptor crosstalk [Lahlou et al., 2004; Schonbrunn, 2008]. Finally, intracellular proteins involved in intracellular trafficking,

can selectively affect the expression pattern of SSTR subtypes and their activity in response to an agonist treatment [Tulipano & Schultz, 2007]. In fact, all these evidences support the cell-specific effects observed for octreotide treatments. Human pituitary tumours express all types of somatostatin receptors (SSTR) and, although there are some differences between pituitary adenomas subtypes, SSTR2A and SSTR5 always predominate [Thodou et al., 2006; Pawlikowski et al., 2008]. It has recently been shown that SSTR2 is particularly important for the inhibitory action of octreotide in somatotroph tumours, since octreotide non-responders present low SSTR2 expression levels and experimental re-expression of SSTR2 induces responsiveness to octreotide treatment [Acunzo et al., 2008]. At present, the quantitative expression pattern of SSTRs in MtT-S cells is not known and studies on this subject would have been beyond the time scope of the present thesis. Nevertheless, the present results strongly suggest that MtT-S cells might represent a good model for studying mechanisms responsible for the resistance to the growth inhibitory action of octreotide.

Interestingly, it was also verified that, although both PDGF-AB and octreotide stimulated the proliferation of MtT-S cells, the combined application of these substances did not induce synergistic or even additive effects. Conversely, PDGF-AB and octreotide when applied together seem to inhibit the action of each other on cellular proliferation. This further underlines the complex interaction of these two substances, probably through interference at the level of intracellular signalling, but future studies are necessary to clarify this.

After finding that the regulation of growth of MtT-S cells was not unequivocally stimulated or inhibited by PDGF-AB and octreotide, respectively, it was tested whether the regulation of VEGF-A production in MtT-S cells was differently affected by PDGF and octreotide.

PDGF and VEGF represent key factors in the induction of tumour neovascularisation through angiogenesis [Carmeliet, 2003]. The major trigger for the expression of both angiogenic factors is cellular hypoxia, which emerges with the increase of the tumour

size [Harris, 2002]. The decline of oxygen availability in the developing tumour induces the production of hypoxia-inducible factor-1 (HIF-1), a transcription factor that controls the synthesis of multiple angiogenic factors, among which VEGF and PDGF are the most important [Harris, 2002; Carmeliet, 2003]. In fact, VEGF and PDGF isoforms act in concert with each other and, additionally, with other angiogenic factors to induce the sprouting of new vessels into the developing tumour [Carmeliet, 2003]. Whereas VEGF-A stimulates mainly endothelial cell proliferation and survival, increasing vessel permeability [Tammela et al., 2005], PDGF isoforms predominantly stimulate growth and migration of pericytes, which are needed for the stabilisation of newly formed vessels [Ostman, 2004]. In addition to hypoxia, growth factors like insulin-like growth factor-1 or EGF can stimulate, even agonistically, the production of angiogenic factors as demonstrated for some tumour types, such as colon or breast cancers [Fukuda et al., 2002; Peng et al., 2006]. Interestingly, it has been demonstrated that PDGF specifically enhances the synthesis and release of VEGF-A in different tumours [Shikada et al., 2005; Matei et al., 2007], inclusively in the folliculostellate TtT-GF pituitary cell line [Kowarik et al., 2009]. In the present work it was demonstrated that PDGF-AB could enhance the mRNA synthesis and secretion of VEGF-A in MtT-S cells. A previous study suggested that, in pituitary adenomas, VEGF-A might not only influence neovascularisation, through its angiogenic action, but also stimulate pituitary tumour cells proliferation [Onofri et al., 2006]. Taking into account this evidence, we can speculate that, in addition to its direct effects on tumour cells proliferation, the PDGF/PDGF receptor system expressed in human pituitary tumours indirectly affects tumour growth, vessel permeability and angiogenesis in pituitary adenomas through the stimulation of VEGF-A expression. However, further studies on human tumour cell lines will be necessary to approve this.

The role of somatostatin and its analogues in the regulation of angiogenesis and particularly of VEGF-A mediated mechanisms is still a matter of debate and, only recently, increasing attention has been paid to this subject. It has been demonstrated that SSTR expressing human umbilical vein endothelial cells proliferation could be directly

inhibited by octreotide [Adams et al., 2005] but so far, no reports about the expression of somatostatin receptors in vessels of normal or tumoural pituitary exist. More likely, somatostatin and its analogues may exert anti-angiogenic effects by suppressing the production of angiogenic factors like VEGF-A. In fact, this hypothesis is supported by an immunohistochemical analysis of VEGF expression in somatotroph tumours surgically removed from patients that received, or alternatively did not, octreotide therapy before surgery. In the tumours of the patients that had received octreotide, the VEGF expression was significantly reduced in comparison to untreated patients [Kurosaki et al., 2008]. Moreover, it has been demonstrated that both somatostatin and pasireotide (SOM230) - a somatostatin analogue acting through SSTR1, 2, 3 and 5 - suppress VEGF secretion in a subset of non-functioning pituitary tumours [Zatelli et al., 2007]. In contrast, Lohrer et al. could not detect reduction of basal VEGF-A release by octreotide treatment in different types of human pituitary tumours, as well as in corticotroph AtT20, lactosomatotroph GH3 and gonadotroph aT3-1 pituitary tumour cell lines; in folliculostellate TtT-GF pituitary cells octreotide even enhanced VEGF-A secretion [Lohrer et al., 2001]. The different action of octreotide and pasireotide on VEGF secretion apparently depends on the differential SSTRs expression. In effect, the specific mechanism of each somatostatin analogue needs to be clarified in further studies.

In the present work, the basal secretion of VEGF-A by MtT-S cells was not affected by octreotide (not shown); however, octreotide completely abolished the PDGF-AB-induced VEGF-A release. This result was strongly supported by RT-PCR assay that evidenced a marked reduction in VEGF-A mRNA expression in octreotide treated cells. Interestingly, the maximum suppressive effect of octreotide on PDGF-AB-induced VEGF-A secretion was obtained with very low concentrations of octreotide, whereas high dosages of the somatostatin analogue were less effective or had no effect on VEGF-A production. These results corroborate those found in experiments with human retinal pigment epithelial cells [Sall et al., 2004]. The reason that makes low octreotide concentration more efficient on VEGF-A production inhibition is not yet clarified;

nevertheless, differences in the expression patterns of the receptors through which octreotide might influence different stimulatory and/or inhibitory signalling cascades may explain that. The results of the present work suggest that basal VEGF production is independent of somatostatinergic control, both in normal and tumoural cells, and that only growth factor/hypoxia-induced VEGF-A secretion is blocked by somatostatin analogues by interfering with commonly used intracellular signalling pathways, most likely the PI3K-Akt-mTOR cascade. In fact, this signalling cascade, which is critically involved in growth stimulation, apoptosis and angiogenesis, is particularly deregulated in several tumours [Shaw & Cantley, 2006; Memmott & Dennis, 2008; Yuan and Cantley, 2008]. At present, components of this pathway constitute important therapeutic targets for developing novel anti-tumourigenic treatment strategies [Maira et al., 2009; Höpfner et al., 2008; Franke, 2008]. As other receptor tyrosine kinases, PDGF receptors mediate their effects through PI3K signalling pathway (figure 3) and, according to Zhang H et al., mTOR, a downstream component of this signalling cascade, negatively regulates PDGF receptor expression suggesting an intrinsic mechanism for preventing over-stimulation. Thus, the stimulatory action of PDGF isoforms would decline during extended action due to receptor down-regulation. In addition, the negative feedback regulation of mTOR on PDGF receptor expression apparently reduces the growth potential of PDGF and may be responsible for the benign characteristics of some PDGF/PDGF receptor expressing tumours like tuberous sclerosis complex (TSC) tumours [Zhang H et al., 2007]. In other solid tumours, in which the degree of PDGF/PDGF receptor expression strongly correlates with tumour aggressiveness and poor prognosis, the intrinsic mTOR signalling feedback mechanisms seem to be deregulated [Varela et al., 2004; Carvalho et al., 2005; Nakamura et al., 2008]. In the present work, it was clearly evidenced that PDGF-AB induced phosphorylation of PDK1, Akt and mTOR whereas the phosphorylation status of the tumour suppressor PTEN was not affected. Taken together, these findings strongly suggest a PDGF-induced intracellular mTOR feedback mechanism. Whether this explains the moderate effects of

PDGF on MtT-S cell growth and VEGF-A production, needs to be clarified in future studies.

Octreotide has been shown to suppress the PI3K-Akt-mTOR pathway in lactosomatotroph GH3 cells, which finally leads to activation of ZAC1, a tumour suppressor that has been shown to mediate the anti-proliferative effects of octreotide in GH3 cells [Theodoropoulou et al., 2006]. This effect of octreotide seems to be mediated through SSTR2, since SSTR1, the only other somatostatin receptor expressed in GH3 cells [Garcia & Myers, 1994], is not a target of this somatostatin analogue. It was also demonstrated that octreotide suppresses the PI3K signalling pathway. The proposed mechanism implies activation of the phosphotyrosine phosphorylase SHP-1 that directly decreased the tyrosine phosphorylation levels of the PI3K regulatory subunit p85, induced dephosphorylation of PDK1 and Akt, and activated glycogen synthase kinase 3β [Theodoropoulou et al., 2006]. In the present work, it has not been studied whether in MtT-S cells octreotide initiated its inhibitory action through SHP-1 activation and p85 dephosphorylation, as described in GH3 cells, but in the following steps of the signalling cascade it was observed that octreotide inhibited PDGF-induced phosphorylation of PDK1, Akt and its downstream target mTOR. These actions, opposite to those induced by PDGF, strongly suggest that the inhibitory effect of octreotide on PDGF-induced VEGF secretion is achieved by reversion of the phosphorylation of the components of the PI3K signalling cascade. No effect of octreotide on PTEN phosphorylation was observed, suggesting no interaction with this intrinsic suppressor of the PI3K pathway. PDK-1 presented a 5 minutes delay in the peak of phosphorylation, compared with the other cascade components, which can presumably be justified by its involvement in other pathways independent of Akt. PDK-1 phosphorylates and activates other kinases, namely protein kinase A, serum and glucocorticoid-induced protein kinase and S6 kinase 1 [Vanhaesebroek & Alessi, 2000]. Although the PI3K-Akt-mTOR cascade has traditionally been viewed as linear, considerable feedback regulation exists within this pathway as well as crosstalk with other intracellular signalling cascades [Henessy et al., 2005; Granville et al., 2006]. Since MtT-S cells express not only SSTR2, but also the

other targets of octreotide, SSTR3 and SSTR 5, it is not clear whether SSTR2 alone is responsible for the suppressive effects of octreotide on PDGF-induced VEGF-A production or whether the 3 somatostatin receptors act in concert.

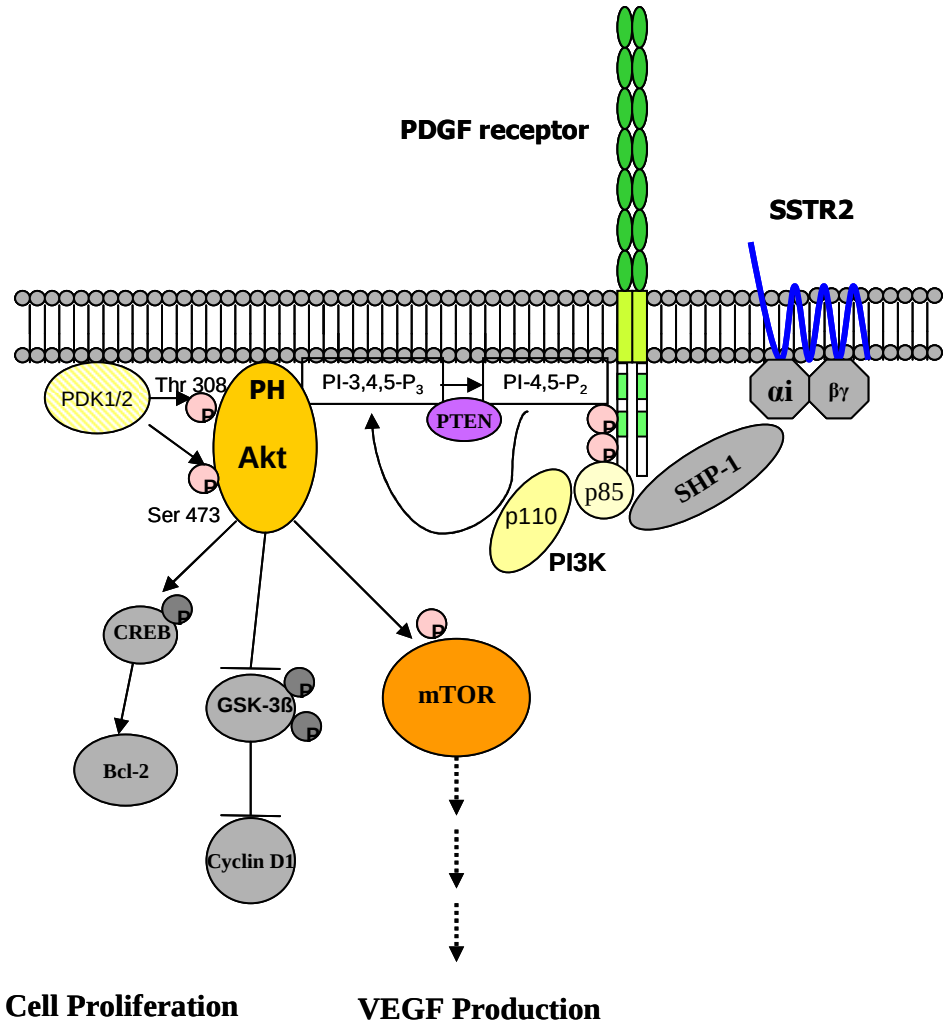


Figure 3. Scheme of the SSTR2-mediated interaction of PDGF and octreotide on VEGF production through the PI3K-Akt-mTOR signalling cascade.

The results presented in this study clearly demonstrate the relevance of the PI3K-Akt-mTOR signalling cascade for VEGF-A production in MtT-S cells. In order to validate this finding, it was studied whether a pharmacological blockade of this signalling pathway could also suppress VEGF-A release in MtT-S cells. To this end, the suppressive effects of the PI3K inhibitor LY294002 and of the mTOR blockers rapamycin and RAD001 were independently investigated. In fact, it was verified that the PDGF-AB-induced VEGF-A secretion was inhibited by the PI3K inhibitor LY294002, a compound that targets the p110 catalytic subunit of PI3K. Despite LY294002 recognised anti-tumour efficacy, poor solubility, short half-life and high toxicity strongly limited its clinical applications. However, other isoform-specific inhibitors have been developed in an attempt to achieve a better pharmacological profile [Henessy et al., 2005; Granville et al., 2006; Maira et al., 2009]. Some of these new promissory PI3K inhibitors are already in clinical trials targeting different types of cancer, and soon will also be available for the treatment of pituitary tumours [Maira et al., 2009]. Corroborating the hypothesis that PI3K-Akt-mTOR signalling cascade is involved in PDGF-induced VEGF expression, it was also verified that both the mTOR inhibitor Rapamycin and its derivative RAD001 are capable to inhibit PDGF-AB induced VEGF-A secretion. Actually, inhibitors of mTOR are the most developed class of inhibitors of PI3K-Akt-mTOR pathway [Franke, 2008]. Preclinical studies with these compounds have shown potent anti-tumoural activity as single agents or in combination with cytotoxic chemotherapy and radiation. Further studies taking in attention the complex role of mTOR in this pathway are however needed, because of feedback loops that can compromise the success of a single therapeutic target [Henessy et al., 2005; Granville et al., 2006; Franke, 2008].

The results herein suggest that a combined treatment of octreotide and inhibitors of the PI3K-Akt-mTOR signalling pathway may have additive or even synergistic inhibitory effects. However, in SSTR-expressing tumour cell lines of the gastroenteropancreatic system, either in cell culture or in experimental tumours in nude mice, the combined application of octreotide and mTOR inhibitors had no or only small increased inhibitory effects in comparison to treatment with the single drugs [Grozinsky-Glasberg et al.,

2008; Moreno et al., 2008]. Because this may probably be the consequence of specific SSTR expression profiles in these tumours in which octreotide in general is often not very efficient, future studies on this subject should be performed in pituitary tumours in which somatostatin analogues are more effective. Moreover, it should also be tested whether inhibitors of the PI3K-Akt-mTOR pathway could overcome somatostatin analogue resistance in a subpopulation of pituitary adenomas and thus provide a future tool for the treatment of these types of tumours.

6. CONCLUSIONS

In summary, the results presented in this study strongly suggest that MtT-S cells might represent a good model for studying mechanisms responsible for the resistance to the growth inhibitory action of octreotide. Moreover, it is shown for the first time a way through which somatostatin and its analogues probably act as anti-angiogenic factors in pituitary adenomas, namely by suppressing the PDGF-induced production of the most important angiogenic factor, VEGF-A. Inhibitors of the PI3K-mTOR pathway, which seems to be the major pathway to be involved in PDGF-stimulated VEGF-A release, also suppressed the production of this angiogenic factor. Although the present findings need to be confirmed in human pituitary adenomas, PI3K-mTOR pathway inhibitors may provide an alternative and new pharmacological tool for the treatment of pituitary adenomas.

7. REFERENCES

Abramsson A, Lindblom P, Betsholtz C. Endothelial and non-endothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors. J Clin Invest 2003; 112: 1142-51.

Acunzo J et al. Somatostatin receptor sst2 decreases cell viability and hormonal hypersecretion and reverses octreotide resistance of human pituitary adenomas. Cancer Res 2008; 68: 10163-70.

Adams RL, Adams IP, Lindow SW, Zhong W, Atkin SL. Somatostatin receptors 2 and 5 are preferentially expressed in proliferating endothelium. British J Cancer 2005; 92: 1493-98.

Alexander JM, Biller BM, Bikkal H, Zervas NT, Arnold A, Klibanski A. Clinically nonfunctioning pituitary tumors are monoclonal in origin. J Clin Invest 1990; 86: 336-40.

Asa SL, Ezzat S. The pathogenesis of pituitary tumors. Nat Rev Cancer 2002; 2: 836-49.

Asa SL, Ezzat S. The pathogenesis of pituitary tumors. Annu Rev Pathol 2009; 4: 97-126.

Betsholtz C. Insight into the physiological functions of PDGF through genetic studies in mice. Cytokine Growth Factor Rev 2004; 15: 215-28.

Borg SA, Kerry KE, Royds JA, Battersby RD, Jones TH. Correlation of VEGF production with IL1α and IL6 secretion by human pituitary adenoma cells. European Journal of Endocrinology 2005; 152: 293-300.

Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-54.

Cai WY et al. Ras mutations in human prolactinomas and pituitary carcinomas. J Clin Endocrinol Metab 1994; 78: 89-93.

Carmeliet P. Angiogenesis in health and disease. Nat Med 2003; 9: 653-60.

Carvalho I, Milanezi F, Martins A, Reis RM, Schmitt F. Over-expression of platelet-derived growth factor receptor a in breast cancer is associated with tumour progression. Breast Cancer Res 2005; 7: R788-95.

Chanson P, Salenave S. Acromegaly. Orph J Rare Disord 2008; 3: 17.

Distler JH, Hirth A, Kurowska-Stolarska M, Gay RE, Gay S, Distler O. Angiogenic and angiostatic factors in the molecular control of angiogenesis. Q J Nucl Med 2003; 47: 149-61.

Ferrara N. Vascular endothelial growth factor and the regulation of angiogenesis. Recent Prog Horm Res 2000; 55: 1-36.

Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Comm 1989; 161: 851-58.

Ferrara N, Gerber H-P, LeCouter J. The biology of VEGF and its receptors. Nature Med 2003; 9(6): 669-76.

Franke TF. PI3K/Akt: getting it right matters. Oncogene 2008; 6473-88.

Fredriksson L, Li H, Eriksson U. The PDGF family: four gene products form five dimeric isoforms. Cytokine Growth Factor Rev 2004; 15: 197-204.

Fukuda R, Hirota K, Fan F, Jung YD, Ellis LM, Semenza GL. Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. J Biol Chem 2002; 277: 38205-11.

Garcia PD, Myers RM. Pituitary cell line GH3 expresses two somatostatin receptor subtypes that inhibit adenylyl cyclase: functional expression of rat somatostatin receptor subtypes 1 and 2 in human embryonic kidney 293 cells. Mol Pharmacol 1994; 45: 402-9.

Gloddek J, Pagotto U,Paez Pereda M, Arzt E, Stalla GK. Pituitary adenylate cyclase-activating polypeptide, interleukin-6 and glucocorticoids regulate the release of vascular endothelial growth factor in pituitary folluculostellate cells. Journal of Endocrinology 1999; 160: 483-90.

Granville CA, Memmott RM, Gills JJ, Dennis PA. Handicapping the race to develop inhibitors of the phosphoinositide 3-kinase/Akt/mammalian target of rapamycin pathway. Clin Cancer Res 2006; 12(3): 679-89.

Grimberg A. Somatostatin and Cancer. Applying Endocrinology to Oncology. Cancer Biology & Therapy 2004; 3(8): 731-33.

Grozinsky-Glasberg S et al. Octreotide and the mTOR inhibitor RAD001 (Everolimus) block proliferation and interact with the Akt-mTOR-p70S6K pathway in a neuroendocrine tumour cell line. Neuroendocrinology 2008; 87: 168-81.

Guo P et al. Platelet-derived growth factor-B enhances glioma angiogenesis by stimulating vascular endothelial growth factor expression in tumor endothelia and by promoting pericyte recruitment. Am J Pathol 2003; 162: 1083-93.

Harris AL. Hypoxia – a key regulatory factor in tumour growth. Nat Rev Cancer 2002; 2: 38-47.

Heaney AP et al. Early involvement of estrogen-induced pituitary tumor transforming gene (PTTG) and fibroblast growth factor expression in prolactinoma pathogenesis. Nature Med 1999; 5: 1317-21.

Heinrich MC et al. PDGFRA activating mutations in gastrointestinal stromal tumors. Science 2003; 299: 708-10.

Heldin CH, Westermark B. Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 1999; 79: 1283-1316.

Henessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/Akt pathway for cancer drug discovery. Nature Rev 2005; 4: 988-1004.

Höpfner M, Schuppan D, Scherübl H. Growth factor receptors and related signaling pathways as targets for novel treatment strategies of hepatocellular cancer. World J Gastroenterol 2008; 14: 1-14.

Inoue K, Hattori M, Sakai T, Inukai S, Fujimoto N, Ito A. Establishment of a series of pituitary cell lines differing in morphology, hormone secretion, and response to estrogen. Endocrinology 1990; 126(5): 2313-20.

Inoue K, Matsumoto H, Koyama C, Shibata K, Nakazato Y, Ito A. Establishment of a foliculo-stellate-like cell line from a murine thyrotropic pituitary tumor. Endocinology 1992; 131(6): 3110-16.

Koper JW, Markstein R, Kohler C, Kwekkeboom DJ, Avezaat CJ, Lamberts SW, Reubi JC. Somatostatin inhibits the activity of adenylate cyclase in cultured human

meningioma cells and stimulates their growth. J Clin Endocrinol Metab 1992; 74: 543-47.

Kowarik M, Onofri C, Colaco T, Stalla GK, Renner U. Platelet-derived growth factor (PDGF) and PDGF receptor expression and function in folliculostellate pituitary cells. Exp Clin Endocrinol Diabetes 2009; Epub ahead of print.

Kurosaki M, Saegert W, Abe T, Lüdecke DK. Expression of vascular endothelial growth factor in growth hormone-secreting pituitary adenomas: special reference to the octreotide treatment. Neurol Res 2008; 518-22.

Lahlou H et al. Molecular signalling of somatostatin receptors. Annals of New York Academy of Sciences 2004; 1014: 121-31.

Lamberts SW, de Herder WW, van Koetsveld PM, Koper JW, van der Lely AJ, Visser-Wisselaar HA, Hofland LJ. Somatostatin receptors: clinical implications for endocrinology and oncology. Ciba Found Symp 1995; 190:222-36.

Lania A et al. Constitutively active Gs alpha is associated with an increased phosphodiesterase activity in human growth hormone-secreting adenomas. J Clin Endocrinol Metab 1998; 83: 1624-28.

Leon SP, Carroll RS, Dashner K, Glowacka D, Black PM. Messenger ribonucleic acid expression of platelet-derived growth factor subunits and receptors in pituitary adenomas. J Clin Endocrinol Metab 1994; 79: 51-55.

Lidhar K et al. Low expression of the cell cycle inhibitor p27Kip1 in normal corticotroph cells, corticotroph tumors, and malignant pituitary tumors. J Clin Endocrinol Metab 1999; 84: 3823-30.

Lohrer P et al. Vascular endothelial growth factor production and regulation in rodent and human pituitary tumor cells in vitro. Neuroendocrinology 2001; 74: 95-105.

Lyons J et al. Two G protein oncogenes in human endocrine tumors. Science 1990; 249: 655-59.

Maher EA, Furnari FB, Bachoo RM, Rowitch DH, Louis DN, Cavenee WK, DePinho RA. Malignant glioma: genetics and biology of a grave matter. Genes Dev 2001; 15: 1311-33.

Maira S-M, Stauffer F, Schnell C, Garcia-Echeverria C. PI3K inhibitors for cancer treatment: where do we stand? Biochem Soc Trans 2009; 37: 265-72.

Matei D, Kelich S, Cao L, Menning N, Emerson RE, Rao J, Jeng MH, Sledge GW. PDGF BB induces VEGF secretion in ovarian cancer. Cancer Biol Ther 2007; 6: 1951-59.

Melmed S. Update in pituitary disease. J Clin Endocrinol Metab 2008; 93: 331-38.

Memmott RM, Dennis PA. Akt-dependent and –independent mechanisms of mTOR regulation in cancer. Cell Signal 2009; 21: 656-64.

Moreno A, Akcakanat A, Munsell MF, Soni A, Yao JC, Meric-Bernstam F. Antitumor activity of rapamycin and octreotide as single agents or in combination in neuroendocrine tumors. Endocr-Rel Cancer 2008; 15: 257-66.

Morishita M et al. The effects of GH-releasing hormone/somatostatin on the 5'-promoter activity of the GH gene *in vitro*. J Mol Endocrinol 2003;31: 441-48.

Musat M et al. Enhanced protein kinase B/Akt signalling in pituitary tumours. Endocrine-Related Cancer 2005; 12: 423-33.

Nakamura Y, Tanaka F, Yoshikawa Y, Mimori K, Inoue H, Yanaga K, Mori M. PDGF-BB is a novel prognostic factor in colorectal cancer. Ann Surg Oncol 2008; 15: 2129-36.

Nicholson KM, Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. Cellular Signalling 2002; 14: 381-95.

Nogami H, Hiraoka Y, Inoue K, Aiso S, Hisano S. Regulation of 5´-promoter activity of the rat growth hormone and growth hormone-releasing hormone receptor genes in the MtT/S and MtT/E cells. Neuroendocrinology 2006; 84: 31-41.

Onofri C et al. Estradiol stimulates vascular endothelial growth factor and interleukin-6 in human lactotroph and lactosomatotroph pituitary adenomas. Exp Clin Endocrinol Diabetes 2004; 112: 18-23.

Onofri C et al. Localization of vascular endothelial growth factor (VEGF) receptors in normal and adenomatous pituitaries: detection of a non-endothelial function of VEGF in pituitary tumours. Journal of Endocrinology 2006; 191: 1-14.

Osaki M, Oshimura M, Ito H. PI3K-Akt pathway: Its functions and alterations in human cancer. Apoptosis 2004; 9(6): 667-76.

Ostman A. PDGF receptors – mediators of autocrine tumor growth and regulators of tumor vasculature and stroma. Cytokine Growth Factor Rev 2004; 15: 275-86.

Pagotto U et al. The expression of the antiproliferative gene ZAC is lost or highly reduced in nonfunctioning pituitary adenomas. Cancer Res 2000; 60: 6794-99.

Pawlikowski M, Pisarek H, Kunert-Radek J, Radek M. Somatostatin receptors in GH-secreting pituitary adenomas – their relationship to the response to octreotide. Polish Journal of Endocrinology 2008; 59(3): 196-99.

Pei L, Melmed S, Scheithauer B, Kovacs K, Benedict WF, Prager D. Frequent loss of heterozygosity at the retinoblastoma susceptibility gene (RB) locus in aggressive pituitary tumors: evidence for a chromosome 13 tumor suppressor gene other than RB. Cancer Res 1995; 55: 1613-16.

Peng XH, Karna P, Cao Z, Jiang BH, Zhou M, Yang L. Cross-talk between epidermal growth factor receptor and hypoxia-inducible factor-1alpha signal pathways increases resistance to apoptosis by up-regulating surviving gene expression. J Biol Chem 2006; 281: 25903-14.

Ray D, Melmed S. Pituitary cytokine and growth factor expression and action. Endocr Rev 1997; 18: 206-28.

Ren H, Tan X, Dong Y, Glese A, Chou TC, Rainov N, Yang B. Differential effect of Imatinib and synergism of combination treatment with chemotherapeutic agents in malignant glioma cells. Basic Clin Pharmacol Toxicol 2009; 104: 241-52.

Renner U et al. Transforming growth factor-β stimulates vascular endothelial growth factor production by folliculostellate pituitary cells. Endocrinology 2002; 143(10):3759-65.

Renner U et al. Growth Factors and Cytokines: Function and Molecular Regulation in Pituitary Adenomas. Front Horm Res 2004; 32: 96-109.

Sall JW at al. Somatostatin inhibits IGF-1 mediated induction of VEGF in human retinal pigment epithelial cells. Experimental Eye Research 2004; 79: 465-76.

Sasahara M et al. Expression of platelet-derived growth factor B-chain in the mature rat brain and pituitary gland. Brain Res Mol Brain Res 1995; 32: 63-74.

Schonbrunn A. Selective agonism in somatostatin receptor signalling and regulation. Molecular and Cellular Endocrinology 2008; 286: 35-9.

Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signaling controls tumour cell growth. Nature 2006; 441: 424-30.

Shikada Y et al. Platelet-derived growth factor-AA is an essential and autocrine regulator of vascular endothelial growth factor expression in non-small cell lung carcinomas. Cancer Res 2005; 65: 7241-48.

Shimon I, Melmed S. Management of pituitary tumors. Ann Intern Med 1998; 129: 472-83.

Simon MP et al. Deregulation of the platelet-derived growth factor B-chain gene via fusion with collagen gene COL1A1 in dermatofibrosarcoma protuberans and giant-cell fibroblastoma. Nat Genet 1997; 15: 95-98.

Smits A et al. Expression of platelet-derived growth factor and its receptors in proliferative disorders of fibroblastic origin. Am J Pathol 1992; 140: 639-48.

Spada A, Vallar L. G-protein oncogenes in acromegaly. Horm Res 1992; 38: 90-93.

Steer EJ, Cross NC. Myeloproliferative disorders with translocations of chromosome 5q31-35: role of the platelet-derived growth factor receptor beta. Acta Haematol 2002; 107: 113-22.

Sullivan NJ, Tahjian AH Jr. Platelet-derived growth factor selectively decreases prolactin production in pituitary cells in culture. Endocrinology 1983; 113: 639-45.

Tallquist M, Kazlauskas A. PDGF signalling in cells and mouse. Cytokine & Growth Factor Reviews 2004; 15: 205-13.

Tammela T, Enholm B, Alitalo K, Paavonen K. The biology of vascular endothelial growth factors. Cardiovasc Res 2005; 65: 550-63.

Theodoropoulou M et al. Differential expression of menin in sporadic pituitary adenomas. Endocr Relat Cancer 2004; 11: 333-44.

Theodoropoulou M et al. Octreotide, a somatostatin analogue, mediates its antiproliferative action in pituitary tumor cells by altering phosphatidylinositol 3-kinase signalling and inducing Zac1 expression. Cancer Res 2006; 66(3): 1576-82.

Thodou E, Kontogeorgos G, Theodossiou D, Pateraki M. Mapping of somatostatin receptor typesin Gh or/and PRL producing pituitary adenomas. J Clin Pathol 2006; 59: 274-79.

Tulipano G, Schultz S. Novel insights in somatostatin receptor physiology. European Journal of Endocrinology 2007; 156: S3-11.

Turner HE et al. Angiogenesis in Endocrine Tumors. Endocrine Reviews 2003; 24(5): 600-32.

Vanhaesebroeck B, Alessi DR. The PI3K –PDK1 connection: more than just a road to PKB. Biohem J 2000; 346: 561-76.

Varela M et al. EGF-R and PDGF-R, but not bcl-2, over-expression predict overall survival in patients with low-grade astrocytomas. J Surg Oncol 2004; 86: 34-40.

Vivanco I, Sawyers CL. The phosphatidilinositol 3-kinase-akt pathway in human cancer. Nature Rev 2002; 2: 489-501.

Woloschak M, Yu A, Post KD. Frequent inactivation of the p16 gene in human pituitary tumors by gene methylation. Mol Carcinogen 1997; 19: 221-24.

Yeh HJ, Silos-Santiago I, Wang YX, George RJ, Snider WD, Deuel TF. Developmental expression of the platelet-derived growth factor alpha-receptor gene in mammalian central nervous system. Proc Natl Acad Sci USA 1993; 90: 1952-56.

Yuan TL, Cantley LC. PI3K pathway alterations in cancer: variations on a theme. Oncogene 2008; 27: 5497-510.

Zatelli MC et al. Pasireotide, a multiple somatostatin receptor subtypes ligand, reduces cell viability in non-functioning pituitary adenomas by inhibiting vascular endothelial growth factor secretion. Endocr Rel Cancer 2007; 14: 91-102.

Zhang H et al. PDGFRs are critical for PI3K/Akt activation and negatively regulated by mTOR. J Clin Invest 2007; 117: 730-38.

Zhang X et al. Pituitary tumor transforming gene (PTTG) expression in pituitary adenomas. J Clin Endocrinol Metab 1999; 38: 761-67.

CONTACT INFORMATION

Name: Tânia Cristina Antunes Colaço

Email Address: tcacolaco@hotmail.com