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The novel sumoylation enhancer RSUME is implicated in pituitary tumor pathogenesis



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1 Introduction

1.1 Pituitary gland

1.1.1 Anatomy of Pituitary gland

The pituitary gland is a small (pea-sized in humans) endocrine gland, lies in close proximity to the hypothalamus at the base of brain, and is protected by a bony cavity structure of the sphenoid bone called sella turcica (also known as Turkish saddle) (Figure 1). The pituitary gland is regulated by the central nervous system to control normal homeostasis through neuroendocrine pathways involving the hypothalamus. By feedback effects from peripheral target gland hormones, the hypothalamus secretes stimulatory and inhibitory hormones to regulate pituitary gland function. Under control of hypothalamus, the pituitary gland is a central regulator, responsible for the producing trophic hormones, and these hormones affect peripheral endocrine glands which are essential for growth, reproduction, metabolism, development, adaptation to external environmental changes, and stress.

The pituitary gland is composed of three compartments which are anterior (adenohypophysis), posterior (neurohypophysis) and intermediate lobe. The anterior pituitary arises from a depression in the dorsal wall of the pharynx (stomodial part) known as Rathke's pouch (Kelberman and Dattani 2007). It consists of two anatomical regions known as the pars tuberalis and pars distalis. The pars distalis represents the majority of the anterior pituitary and is where the pituitary hormone production occurs. The function of the anterior pituitary is to synthesize and secrete important endocrine hormones, such as adrenocorticotrophic hormone (ACTH), thyroid-stimulating hormone (TSH), prolactin (PRL), growth hormone (GH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH). These hormones are produced by specific types of endocrine cells, and work on target glands or tissues, regulating corresponding release of hormones and several processes of human physiology (Table 1). The anterior pituitary itself is regulated by hypothalamic hormones which are transported from hypothalamus by a system of blood vessels called hypothalamo-hypophyseal portal system.

Table 1. Anterior pituitary hormones and their control (Nussey and Whitehead 2001)

| Hormone | Target organs | Effect | Secretory cell types (% of pituitary cell population) | Hypothalamic hormone | Predominant hypothalamic nucleus of synthesis |
|--|-----------------------|--|---|------------------------------|---|
| Adrenocorticotrophic hormone (ACTH) | Adrenal gland | Stimulates glucocorticoids; induces stress response | Corticotroph (15%-20%) | CRH (+) | Paraventricular, supraoptic |
| Growth hormone (GH) | Liver; adipose tissue | Stimulates IGF-1; promotes bone and muscle growth; lipid and carbohydrate metabolism | Somatotroph (40%-50%) | GHRH (+) Somatostatin (-) | Arcuate, anterior periventricular |
| Prolactin (PRL) | Ovary, mammary gland | Stimulates estrogens/progesterone; milk production | Lactotroph (10%-25%) | TRH (+) Dopamine (-) | Arcuate, paraventricular |
| Luteinizing hormone (LH) Follicle-stimulating hormone (FSH) | Gonads | Stimulates sex hormones; regulates the development, growth, pubertal maturation of the reproductive system | Gonadotroph (10-15%) | GnRH (+) | Arcuate |
| Thyroid-stimulating hormone (TSH) | Thyroid gland | Stimulates thyroid hormone; regulates thyroid function | Thyrotroph (3%-5%) | TRH (+) Somatostatin (-) | Paraventricular, anterior periventricular |

CRH, Corticotropin-releasing hormone; GHRH, growth-hormone-releasing hormone; TRH, Thyrotropin-releasing hormone; GnRH, Gonadotropin-releasing hormone. (+), stimulatory; (-), inhibitory.

Among the endocrine cells scattered pluripotent progenitor cells, stem cells, and folliculostellate cells (FS cells) (Landolt, et al. 2006). FS cells are of neuro-ectodermal origin, and represent 5% of anterior pituitary cell population (Landolt, et al. 2006). They are immunopositive for S-100 protein and glial fibrillary acidic protein (GFAP), and are characterized by their star-like morphology and their ability of forming follicles (Allaerts and Vankelecom 2005; Jin, et al. 2001; Kagayama 1965; Rinehart and Farquhar 1953). They not only act as supporting cells for the endocrine cells, but also offer an intrapituitary communication system through gap junctions and their long cytoplasmic processes surround the neighboring endocrine cells (Acosta, et al. 2010; Fauquier, et al. 2001; Inoue, et al. 1999; Morand, et al. 1996). FS cells do not produce hormones but express interleukin-6 (IL-6), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), transforming growth factor-beta (TGF- β), platelet-derived growth factor-A (PDGF-A), PDGF-B and some cytokine and hormone receptors, suggesting that they are implicated in regulation of hormone secretion, angiogenesis promotion, formation and permeability of blood vessels by auto- and paracrine manner (Inoue, et al. 1999; Kowarik, et al. 2010; Lohrer, et al. 2001; Lohrer, et al. 2000; Renner, et al. 2009; Renner, et al. 2002; Vlotides, et al. 2009).

The posterior pituitary gland develops by the evagination of neural tissue from the floor of the third ventricle (Mehta and Dattani 2008). It is mainly composed of the terminals of the distal axons which extend from the magnocellular neurons of supraoptic and paraventricular nuclei in hypothalamus. Besides axons, the posterior pituitary also contains glial cells called pituicytes which surround the axon projections. Two hormones named oxytocin and arginine vasopressin (AVP) are produced by the hypothalamus and these hormones are transported through extended axons to the posterior pituitary where they are stored and released as required under hypothalamic control (di Iorgi, et al. 2009). Pituicytes were reported to regulate the release of these two hormones by morphological changes (Rosso and Mienville 2009; Theodosis 2002). Oxytocin increases the uterine contraction during labor and stimulates lactation, and recently, some studies have shown the implication of oxytocin in social memory and attachment, sexual and maternal behavior, human bonding and trust, and aggression (Lee, et al. 2009). AVP is involved in regulating body's retention of water. Additionally, AVP has primarily been implicated in male-typical social behaviors, including aggression and pair-bond formation, and in stress-responsiveness (Heinrichs, et al. 2009).

The intermediate lobe, which is the part between anterior pituitary and posterior pituitary, is derived from oral ectoderm together with the anterior pituitary (Mehta and Dattani 2008). It mainly contains melanotroph cells, which produce pro-opiomelanocortin (POMC) and release processed POMC products such as β -endorphin and α -melanocyte stimulating hormone (α -MSH) (Bicknell 2008; Saland 2001). In humans, in contrast to the mouse, the intermediate lobe largely disappears during embryogenesis, and fuses with the anterior lobe soon after birth, thought to be represented in the adult by cysts at the junction of anterior and posterior lobes (Kelberman, et al. 2009; McNicol 1986; Rasmussen 1930). The function of the intermediate lobe as defined in animals has no discrete anatomical location within human pituitary gland (McNicol 1986).

The pituitary stalk is the bridge between hypothalamus and pituitary gland, carrying neural and vascular connections, responsible for delivering hypothalamic hormones to the anterior pituitary and neural tracts from the hypothalamic nuclei to the posterior pituitary (Kelberman, et al. 2009; Mehta and Dattani 2008).

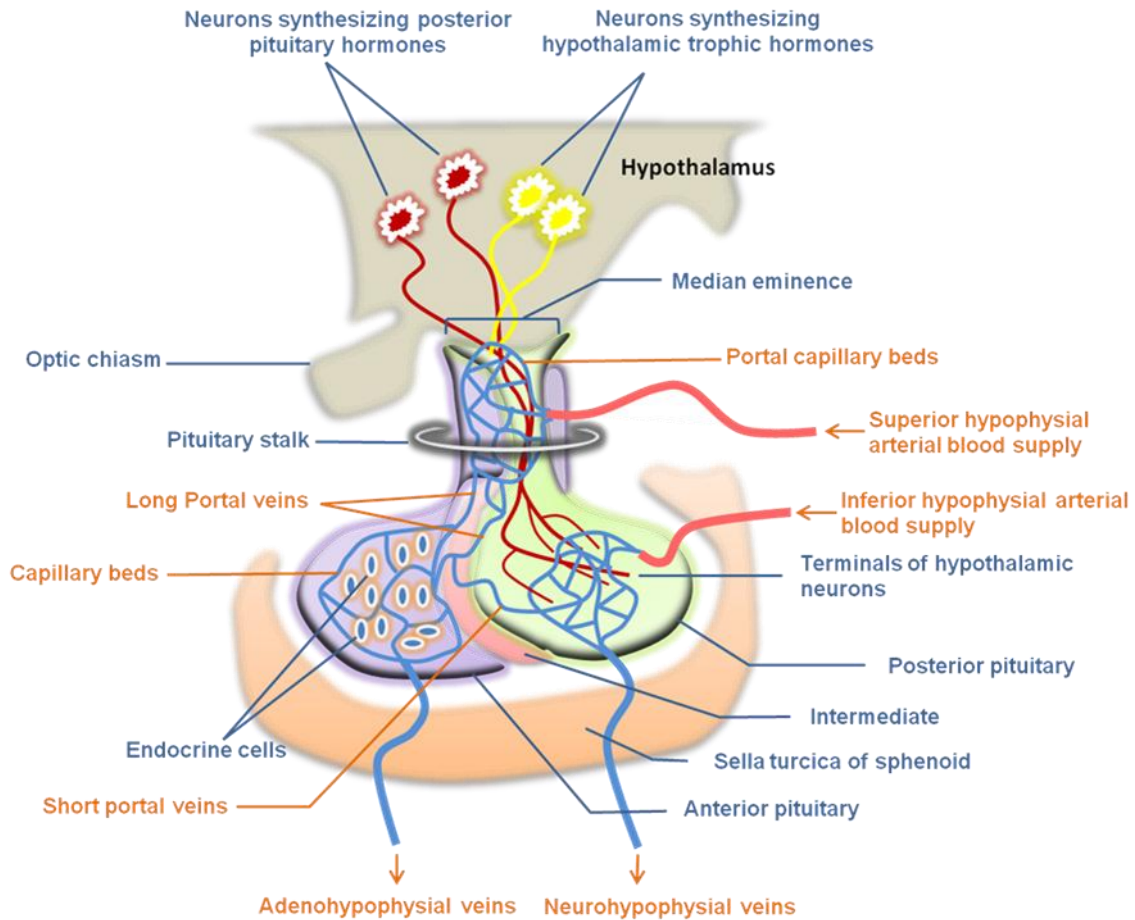


Figure 1. Anatomy and blood supply of pituitary gland. The pituitary gland is located below the hypothalamus, sitting in the sella turcica of sphenoid bone. It contains three parts: anterior, posterior and intermediate lobe. The hypothalamic trophic hormones which target endocrine cells in the anterior pituitary are produced by neurosecretory cells in the hypothalamus. The terminals of these neurosecretory cell axons reach to the median eminence capillaries, where the hormones are transported to and released into the anterior pituitary by portal blood system. The posterior hormones are produced by the magnocellular neurons in the hypothalamus and are released into the posterior pituitary through the extending magnocellular neuron axons. The secreted pituitary hormones diffuse into the systemic blood circulation through capillaries network in the anterior and posterior pituitary.

1.1.2 Blood supply and vascularization of pituitary gland

The pituitary gland is highly vascularized and gains blood supply through the hypothalamo-hypophyseal portal system (Figure 1). The capillaries of the hypothalamo-hypophyseal portal system are characterized by fenestrated endothelia, improving the delivery and diffusion of hormones (Nussey and Whitehead 2001). A separate branch of the internal carotid artery called superior hypophysial artery, which ascends from cavernous sinus, delivers the arterial

blood into portal capillary beds in the median eminence, and then reaches the pars distalis of anterior pituitary through the long portal veins that descend along the pituitary stalk, and interconnect the portal capillary beds and capillary beds inside the pars distalis (Leclercq and Grisoli 1983). In addition, approximately 30% of the total blood supply to the anterior pituitary is from the venous blood draining from the posterior pituitary through the short portal veins (Gross, et al. 1993). The pars distalis of anterior pituitary receives little or no arterial blood supply from the internal carotid artery (Bergland and Page 1978; Leclercq and Grisoli 1983). Rapidly enhanced magnetic resonance images (dynamic MRI) showed in man that the perfusion sequence of arterial blood first reached the posterior pituitary and the median eminence, followed by venous drainage to the anterior pituitary (Yuh, et al. 1994). The venous blood drainage from the anterior pituitary flows into systemic circulation through the adenohypophysial veins (Bergland and Page 1978). This blood flow pattern allows anterior pituitary to collect both hypothalamic factors and posterior information. The posterior pituitary is fed by the inferior hypophyseal artery branches distributing in the cavernous sinus. The venous drainage from the posterior pituitary goes into the neurohypophysial veins besides the short portal veins, and then enters the venous circulation.

Some studies about activators contributing to the angiogenesis and vascularization of pituitary gland have been performed. VEGF, which is a well known angiogenic factor, has been localized mainly in corticotroph, somatotroph and folliculostellate cells (Lloyd, et al. 1999; Vidal, et al. 1999; Yamamoto, et al. 1999). A study in rat using FITC-labeled gelatin injection, immunohistochemistry and in-situ RT-PCR showed that VEGF is involved in development of primary capillaries in median eminence and vascularization of pars distalis (Nakakura, et al. 2006). Pituitary autotransplantation demonstrated that VEGF expression in pituitary is not critical under hypothalamic control and hypoxia may play a crucial role in the induction of VEGF expression in the non-tumorous pituitary (Lombardero, et al. 2006). Leptin, which was reported to have a significant role in induction of neovascularization, is also expressed in pituitary gland, suggesting that it could play a role in pituitary angiogenesis (Cao, et al. 2001; Lloyd, et al. 2001; Ribatti, et al. 2007a). The mouse pituitaries with Prop1 (Prophet of pituitary transcription factor 1) gene mutations are poorly vascularized and dysmorphic, with a striking elevation in apoptosis, indicating that Prop 1 is implicated in pituitary vascularization during the pituitary development (Ward, et al. 2006). bFGF, a stimulator of angiogenesis, has been shown to be localized in gonadotroph cells, FS cells and endothelial cells in the anterior pituitary, and is released by cellular disruption during pituitary vascular development in rats (Schechter, et al. 1996; Schechter, et al. 1993).

1.2 Pituitary adenomas

Pituitary adenomas, arising from adenohypophysial cells, are the most common tumors in the sella region. The majority of pituitary adenomas grow slowly, and are defined as benign because of the absence of metastases, but some exhibit a faster growth pace and can be highly invasive (Kovacs, et al. 2001). About 15–25% of cases demonstrate tumor invasion into the hypophysis or cavernous sinus at initial diagnosis (Yoshida and Teramoto 2007). Pituitary adenomas may cause serious health problems due to either compression of neighboring tissues (i.e. visual nerve) or major changes in endocrine homeostasis resulted from the hormone excess syndromes they cause. It is difficult to determine the true incidence of the pituitary adenomas, because they are often asymptomatic (Swearingen and Biller 2008b). They are present in approximately 16.9% of the general population, and represent up to 25% of all intracranial tumors (Ezzat, et al. 2004; Oberholtzer 1999). There are no significant differences in the frequency of pituitary adenomas between men and women, if the types are ignored and both surgically and autopsy obtained tissues are combined (Kovacs, et al. 2001). Although rarely diagnosed in prepubertal age, pituitary adenomas can be found in every age group, from infancy to senescence (Kovacs, et al. 2001). The prevalence, which increases with advancing age, is about 14% and 22% in postmortem and imaging studies, respectively (Swearingen and Biller 2008b).

Pituitary adenomas can be classified in various ways, such as functional, anatomic/radiologic, histologic, immunohistochemical, ultrastructural, and clinicopathologic (Asa and Ezzat 2009). The functional classification, based on hormonal activity, divides pituitary adenomas into six groups: ACTH-producing adenomas, linked to Cushing disease and Nelson's syndrome; GH-producing adenomas, linked to acromegaly and/or gigantism; PRL-producing adenomas, linked to hyperprolactinemia, TSH-producing tumors linked to thyroid dysfunction; rare clinically detectable gonadotroph adenomas, linked to hypogonadism due to paradoxical down-regulation of function; non-functioning adenomas, clinically endocrinologically inactive adenomas, unaccompanied with detectable hormone activity (Asa and Ezzat 2009; Kovacs, et al. 2001). The anatomic/radiologic classification is based on size and degree of local invasion. On the basis of their size, pituitary adenomas can be separated into macroadenomas with more than 10 mm in their largest diameter and microadenomas with less volume (Kovacs, et al. 2001). The histologic classification is based on the tinctorial characteristics of the cell cytoplasm, and separates pituitary adenomas into acidophilic, basophilic and chromophobic tumors (Asa and Ezzat 2009; Kovacs, et al. 2001). This classification has limited value, since it fails to take into consideration hormone production, cellular derivation and structure–function relationship, and has been largely abandoned (Kovacs, et al. 2001). The immunohistochemical classification is based on the detection of antigens in tissue, providing

the information primarily about hormone content, additionally about transcription factors and keratins (Asa and Ezzat 2009; Kovacs, et al. 2001). The ultrastructural classification with application of electron microscopy is based on subcellular features of cell differentiation. The electron microscopy provides conclusive information on the cellular composition and secretory activity of the tumor, and it's a very valuable tool for research. However, the ultrastructural investigation is time consuming, expensive and has difficulties in correct diagnosis (Asa and Ezzat 2009; Kovacs, et al. 2001). Clinicopathologic classification, based on both morphological and clinical features, is most effective and practical (Table 2).

Table 2. Clinicopathological classification of pituitary adenomas (Asa and Ezzat 2009)

| Functioning adenomas | Non-functioning adenomas |
|---|--|
| GH-PRL-TSH family | |
| Adenomas causing GH excess | |
| Densely granulated somatotroph adenomas | |
| Sparsely granulated somatotroph adenomas | Silent somatotroph adenomas |
| Mammotroph adenomas | |
| Adenomas causing hyperprolactinemia | |
| Lactotroph adenomas | Silent lactotroph adenomas |
| Acidophil stem cell adenomas | |
| Adenomas causing TSH excess | |
| Thyrotroph adenomas | Silent thyrotroph adenomas |
| ACTH family | |
| Adenomas causing ACTH excess | |
| Densely granulated corticotroph adenomas | Silent corticotroph adenomas (Type I) |
| Sparsely granulated corticotroph adenomas | Silent corticotroph adenomas (Type II) |
| Gonadotroph family | |
| Adenomas causing gonadotropin excess | |
| Gonadotroph adenomas | Silent gonadotroph adenomas (null-cell adenomas, oncocyomas) |
| Unclassified adenomas | |
| Unusual plurihormonal adenomas | Immunonegative adenomas |

ACTH, adrenocorticotropin; GH, growth hormone; PRL, prolactin; TSH, thyrotropin-stimulating hormone.

1.2.1 Prolactinomas

Prolactinomas (also known as lactotroph adenomas) are the most common type of pituitary adenomas. It arises from lactotroph cells of anterior pituitary, secreting prolactin (PRL). They comprise 45% of the clinical pituitary tumor cases with an incidence of 6 to 10 per million per year and a prevalence of 60 to 100 cases per million in clinical series (Ciccarelli, et al. 2005). Prolactinomas are the most frequent cause of PRL excess, and usually the most relevant clinical manifestations are infertility, gonadal and sexual dysfunction in both sexes (Gillam, et al. 2006; Schlechte 2003). The number of these tumors in surgical series is very low, due to the success of dopamine agonist therapy.

There are three variants of prolactinomas: sparsely granulated and densely granulated lactotroph adenomas and the rare, but aggressive, acidophil stem cell tumor (Asa and Ezzat 2009). The most common variant is sparsely granulated lactotroph adenomas which consist of chromophobic tumor cells (Asa and Ezzat 2009). Immunohistochemistry shows strong immunostaining for PRL with paranuclear globular pattern corresponding to the Golgi area (Asa and Ezzat 2009). Densely granulated lactotroph adenomas are much less common than the sparsely granulated variant (Asa and Ezzat 2009). Differing from the sparsely granulated adenomas, these tumors cells are acidophilic and show diffuse cytoplasmic positivity for PRL (Asa and Ezzat 2009). The acidophil stem cell adenomas are composed of oncocytic cells with large cytoplasmic vacuoles corresponding to giant mitochondria (Asa and Ezzat 2009). These tumors may have an unusual bihormonal profile with diffuse immunoreactivity for PRL and scant immunoreactivity for GH (Asa and Ezzat 2009).

1.2.2 Somatotroph adenomas

Somatotroph adenomas, which arise from GH-producing cells, account for 10~15% of pituitary adenomas (Asa and Ezzat 2009). GH hypersecreting tumors cause gigantism in young patients and acromegaly in adults (Asa and Ezzat 2009). The incidence of acromegaly is about 2 to 4 per million with a mean age at presentation of 40–50 years (Ciccarelli, et al. 2005). Morphologically, the majority of these tumors are macroadenomas and frequently have suprasellar growth and expansion to the lateral sellar wall (Swearingen and Biller 2008b).

The subtypes of these adenomas include densely granulated, sparsely granulated somatotroph adenomas and mammosomatotroph adenomas (Swearingen and Biller 2008b). Densely granulated somatotroph adenomas are the most common finding in adult acromegaly, and mammosomatotroph adenomas that produce both GH and PRL are the most frequent findings in childhood-onset gigantism and in young patients with acromegaly (Asa and Ezzat 2009). Both of these two types of adenomas express α -subunit, and resemble normal somatotroph and mammosomatotroph cells (Asa and Ezzat 2009). They are characterized by acidophilic and densely granulated cytoplasm, strong and diffuse cytoplasmic immunoreactivity for GH, and strong nuclear immunoreactivity for Pit-1 (pituitary transcription factor-1) (Asa and Ezzat 2009; Kovacs, et al. 2001). The immunohistochemistry shows that the low molecular weight cytokeratins (CAM 5.2) in densely granulated somatotroph adenomas cells are of diffuse cytoplasmic pattern (Asa and Ezzat 2009; Kovacs, et al. 2001; Swearingen and Biller 2008b). In contrast, the sparsely granulated somatotroph adenomas with chromophobic and less granular cytoplasm have weak and focal GH immunoactivity and the staining for α -subunit is not identified (Asa and Ezzat 2009; Swearingen and Biller 2008b). The immunostaining for CAM 5.2 shows dot-like juxtannuclear aggresome corresponding to the

fibrous body (Asa and Ezzat 2009; Swearingen and Biller 2008b). However, they are usually nuclear immunopositive for Pit-1 like densely somatotroph adenomas (Asa and Ezzat 2009).

1.2.3 Corticotroph adenomas

The corticotroph adenomas which arise from ACTH-producing corticotroph cells comprise about 10%~15% of clinical cases of pituitary adenomas (Asa and Ezzat 2009). ACTH is overproduced by corticotroph adenomas which cause an excessive release of adrenal glucocorticoids leading to the different symptoms of Cushing's disease. The majority of corticotroph adenomas are microadenomas, softer and paler than the normal gland (Swearingen and Biller 2008b). Three variants of corticotroph adenomas are known: densely granulated corticotroph adenomas, sparsely granulated corticotroph adenomas and Crooke's cell adenomas (Swearingen and Biller 2008b). Cushing's disease is mostly caused by densely granulated corticotroph adenomas, which have densely granulated basophilic cytoplasm apparent with the classical hematoxylin and eosin stain (Asa and Ezzat 2009; Swearingen and Biller 2008b). The cytoplasm is also strongly PAS (Periodic acid-Schiff stain)-positive (Swearingen and Biller 2008b). The macroadenomas with lower florid hormone excess in patients are less common, and have sparsely granulated chromophobic cytoplasm and are less PAS-positive (Asa and Ezzat 2009; Swearingen and Biller 2008b). They may be associated with Nelson's syndrome in patients who have undergone bilateral adrenalectomy as treatment for pituitary Cushing's disease without initial identification of a discrete lesion (Asa and Ezzat 2009). The Crooke's cell adenomas are rare. The normal corticotroph cells respond to high levels of glucocorticoids by developing Crooke's hyalinization corresponding to intracytoplasmic accumulation of cytokeratins 7 and 8, which are intermediate filaments on electron microscopy (Asa and Ezzat 2009; Swearingen and Biller 2008b). Usually the corticotroph adenomas don't have this marker of feedback suppression by glucocorticoids; however, the Crooke's adenomas have the same change as nontumorous corticotroph cells (Asa and Ezzat 2009; Kovacs and Horvath 1986; Swearingen and Biller 2008b). They can be associated with Cushing's disease as well (Asa and Ezzat 2009).

1.2.4 Thyrotroph adenomas

The thyrotroph adenomas are rare and make up to 1% of pituitary adenomas (Asa and Ezzat 2009). They can be associated with hyperthyroidism or hypothyroidism, but the majority are clinically nonfunctioning (Asa and Ezzat 2009; Swearingen and Biller 2008b). The thyrotroph adenomas in general are macroadenomas and invasive, and mass effects with visual-field disturbances are common (Asa and Ezzat 2009). The tumor cells are chromophobic and can have small cytoplasmic granules with PAS stain (Swearingen and Biller 2008b).

Immunohistochemistry shows positivity for TSH and variable staining for α -subunit; some also express GH and PRL (Swearingen and Biller 2008b). Characteristically, the tumor cells with significant nuclear pleomorphism have a polygonal or elongated shape and resemble nontumorous thyrotroph cells (Asa and Ezzat 2009).

1.2.5 Gonadotroph adenomas

The gonadotroph adenomas comprise the majority of clinically nonfunctioning adenomas (Swearingen and Biller 2008b), and clinical manifestations of gonadotropin excess are rare. The elevated serum FSH level induced by the gonadotroph adenomas led to ovarian enlargement in a 10-year old girl (Tashiro, et al. 1999). A case of clearly high circulating immunoreactive FSH due to a functioning FSH-secreting gonadotroph adenoma in a man with the MEN 1 syndrome was also reported (Sztal-Mazer, et al. 2008). The tumor cells exhibit strong nuclear staining with SF-1 (steroidogenic factor-1), but variable and often only focal positive reaction for α -subunit, β -FSH, and β -LH, and show variable degrees of differentiation (Asa and Ezzat 2009). Well-differentiated tumor cells are elongated, with the nucleus occupying one pole and secretory granules accumulating at the opposite pole (Asa and Ezzat 2009). In contrast, poorly differentiated cells are generally ovoid or polygonal and lack polarity (Asa and Ezzat 2009).

1.2.6 Non-functioning adenomas

The non-functioning adenomas lack detectable hormone activity, and cause no signs or symptoms secondary to hormonal hypersecretion by the tumor (Greenman and Stern 2009). They typically tend to be present with signs of mass effects such as headaches, visual field defects and hypopituitarism, when the tumor has reached the stage of a macroadenoma, and are usually large at the time of diagnosis (Greenman and Stern 2009; Snyder 1985). Non-functioning adenomas comprise roughly 50% of pituitary tumors in surgical series (Saeger, et al. 2007). The vast majority of these lesions are actually silent gonadotroph adenomas, accounting for 85% of non-functioning adenomas, and they are morphologically and pathogenetically identical to functioning gonadotroph adenomas (Asa and Ezzat 2009; Greenman and Stern 2009). They are positively stained with FSH, LH and their subunits (Greenman and Stern 2009). In a minority of patients, intact gonadotropins, mainly FSH, may be detected on basal conditions in vivo, but hormone-related symptoms are rare (Greenman and Stern 2009). Based on hormone and transcription factor staining, the non-functioning adenomas also include silent somatotroph adenomas, silent prolactinomas, silent thyrotroph adenomas and silent corticotroph adenomas (Table 3) (Greenman and Stern 2009). They may express hormones but do not secrete them, and their lack of clinical manifestation may be

attributable to reduced hormonal activity or to synthesis of nonfunctional hormone products (Asa and Ezzat 2009; Greenman and Stern 2009). The majority are the silent corticotroph adenomas which express ACTH (Greenman and Stern 2009). The silent type 3 adenomas may express several pituitary hormones and transcription factors (Greenman and Stern 2009). Poorly differentiated gonadotroph adenomas cells are often classified as null-cell or undifferentiated adenomas because of focal or weak immunoreactivity, but they are positive stained with SF-1 and can secrete gonadotropins *in vitro*, indicating their gonadotroph origin (Asa and Ezzat 2009; Greenman and Stern 2009). The truly null cell adenomas stain negatively both to pituitary hormones and transcription factors (Greenman and Stern 2009). Additionally, most oncocytomas represent silent gonadotroph adenomas with extensive oncocytic change (Asa and Ezzat 2009).

Table 3. Clinicopathological classification of non-functioning adenomas (Greenman and Stern 2009)

| Tumor Type | Transcription factors | Hormone staining |
|------------------------------|-----------------------|--|
| Silent gonadotroph adenomas | SF-1, GATA-2, ER | β -FSH, β -LH, α -subunit |
| Silent somatotroph adenomas | Pit-1 | GH |
| Silent prolactinomas | Pit-1, ER | PRL |
| Silent thyrotroph adenomas | Pit-1, TEF, GATA-2 | β -TSH, α -subunit |
| Silent corticotroph adenomas | Tpit | ACTH |
| Null cell adenomas | None | None |
| Silent subtype 3 adenomas | | Multiple |

SF-1, steroidogenic factor-1; ER, estrogen receptor; Pit-1, pituitary transcription factor-1; TEF, thyrotropin embryonic factor; GATA-2, GATA binding protein-2; Tpit-1, T-box transcription factor.

1.2.7 Plurihormonal adenomas

Plurihormonal pituitary adenomas are capable of producing more than one hormone (Kovacs, et al. 2001). Morphologically, they can be divided into monomorphous and plurimorphous tumors (Kovacs, et al. 2001). Monomorphous plurihormonal adenomas consist of one cell population which produces two or more hormones, and they can not be explained by a common cellular origin (Kovacs, et al. 2001; Swearingen and Biller 2008b). Plurimorphous adenomas, representing mixed adenomas, are composed of two or more distinct cell types, each producing one hormone (Kovacs, et al. 2001). The 2004 WHO classification includes only monomorphous adenomas in the category of plurihormonal adenomas, which excludes adenomas with any combination of GH, PRL and TSH, or FSH and LH. By this definition these tumors are rare (Swearingen and Biller 2008b).

1.2.8 Pituitary carcinomas

Pituitary carcinomas are defined based on the presence of metastasis (Swearingen and Biller 2008b). In contrast to the high frequency of local invasion, pituitary adenomas only rarely metastasize to distant locations in the central nervous system, lymphnodes, and liver (Asa and Ezzat 2009). The pituitary carcinomas represent approximately only 0.2% of operated pituitary neoplasms (Swearingen and Biller 2008b). Microscopically, the tumors have different degrees of high cell density, pleomorphism, necrosis, and invasion, but all of these characteristics can also be found in benign pituitary adenomas (Swearingen and Biller 2008b).

1.3 Pathogenesis of pituitary adenomas

The pathogenesis of pituitary adenomas has been intensively studied, but the exact mechanisms involved in pituitary cell transformation and tumorigenesis is still unclear. X-chromosomal inactivation analysis demonstrated that pituitary adenomas arise from the replication of a single transformed pituitary cell, indicating that they are monoclonal neoplasms (Alexander, et al. 1990; Gicquel, et al. 1992; Herman, et al. 1990; Schulte, et al. 1991). This observation also suggests that the tumor formation results from the growth advantage characteristics of the monoclonal transformed pituitary cell with either activation of proto-oncogenes or inactivation of tumor-suppressor genes.

A lot of etiologic factors involved in pituitary tumor formation have been extensively studied, including genetic abnormalities in pituitary tumors, over-expression of proto-oncogenes and proliferative signals, down-regulation of tumor suppressor genes and inhibitory signals, and hormonal dysregulation.

1.3.1 Genetic abnormalities of proto-oncogenes and anti-proliferative signals

Many common genetic defects in proto-oncogenes which occur frequently in human malignancies are not identified in most of pituitary adenomas (i.e. abnormalities of the Ras-family).

The mutations in the gene encoding the α subunit of Gs (GNAS1) frequently appear in about 30–40% GH-secretion adenomas, leading to constitutively active cAMP-dependent pathway (Landis, et al. 1989; Vallar, et al. 1987). However, these mutations infrequently occur in other tumors, i.e., in about 10% of non-functioning pituitary adenomas and less than 5% of ACTH-secreting adenomas (Tordjman, et al. 1993; Williamson, et al. 1995).

The aberrant expression of an N-terminally truncated variant of fibroblast growth factor (FGF) receptor-4 (ptd-FGFR4) has been detected in 40% of pituitary adenomas, and ptd-FGFR4 is constitutively phosphorylated in the absence of ligand and transformation *in vitro* and *in vivo* (Ezzat, et al. 2002).

Germline mutations of the tumor suppressor gene *MEN1* with LOH (loss of heterozygosity) inactivate the normal allele in tumors, resulting in familial pituitary adenomas associated with multiple endocrine type 1 (MEN1) (Chandrasekharappa, et al. 1997). However, *MEN1* mutations rarely occur in sporadic pituitary adenomas (Prezant, et al. 1998; Wenbin, et al. 1999; Zhuang, et al. 1997), although LOH have been found in the region of *MEN1* gene in 10–30% of sporadic pituitary adenomas and the expression of Menin is down-regulated in high percentage of cases with or without LOH. Therefore, allelic loss may be responsible for the lower menin levels in some, but not all, cases with low menin expression, and post-transcriptional mechanisms may be involved (Tanaka, et al. 1998; Theodoropoulou, et al. 2004).

Germline Inactivating mutations in the aryl hydrocarbon receptor (AHR) interacting protein (AIP) gene with LOH of the normal allele in familial presentation of somatotroph adenomas and prolactinomas have been reported (Vierimaa, et al. 2006). However, *AIP* mutations have not been identified in sporadic pituitary adenomas (DiGiovanni, et al. 2007).

Germline mutations in protein kinase-A (PKA) regulatory subunit 1 gene *PRKAR1* are responsible for Carney's complex, an autosomal dominant disorder associated with somatotroph adenomas and other endocrine tumors (Kirschner, et al. 2000a; Kirschner, et al. 2000b). However, no mutations of this gene were found in sporadic pituitary adenomas, although significantly reduced protein expression of this gene was shown in non-functioning and somatotroph adenomas (Kaltsas, et al. 2002; Lania, et al. 2004; Mantovani, et al. 2005; Sandrini, et al. 2002; Yamasaki, et al. 2003).

1.3.2 Over-expression of proto-oncogenes and proliferative signals

Cyclin proteins are cell cycle regulators, which promote cell progression by activating cyclin-dependent kinases (CDKs). It has been shown that cyclin proteins, especially cyclin D1, are over-expressed in aggressive non-functioning adenomas without cyclin D1 gene (*CCND1*) amplification, indicating that additional mechanisms are involved in the deregulating of cyclin D1 expression in human pituitary tumorigenesis. Moreover, cyclin E is preferentially present in corticotroph adenomas (Hibberts, et al. 1999; Jordan, et al. 2000).

The pituitary tumor transforming gene (*PTTG*) which is an estrogen-inducible gene responsible for chromosomal separation and mitosis during the cell division is mostly over-expressed in adenomas with a more than 50% increase (up to a 10-fold increase in some cases) in non-functioning, GH-, PRL-, and ACTH-producing adenomas compared with normal pituitaries, suggesting that *PTTG* is indeed involved in some manner in pituitary tumorigenesis (Zhang, et al. 1999); however, specific mutation of *PTTG* has not been shown in sporadic pituitary adenomas (Dworakowska and Grossman 2009). *PTTG* mediates the estrogen-induced up-regulation of growth factors with potent mitogenic and angiogenic activity, such as

fibroblast growth factor-2 (FGF-2) (Zhang, et al. 1999). FGF-2 (also known as basic or bFGF) is over-expressed in pituitary tumor cells (Ezzat, et al. 1995).

Akt, the best-characterized phosphorylation target of phosphatidylinositol 3-kinase (PI3K), which enhances cell proliferation by activating the serine-threonine kinase mTOR (mammalian target of rapamycin) is over-expressed (at both mRNA and protein levels) as well as over-activated (through phosphorylation) in all pituitary tumors, especially non-functioning pituitary adenomas (Musat, et al. 2005; Volarevic and Thomas 2001).

1.3.3 Down-regulation of tumor suppressors and inhibitory signals

The retinoblastoma protein (pRb), coded by *Rb1* gene, is a main inhibitor of cell cycle progression between G1 and S phases. The phosphorylation of the pRb by cyclin/CDK complex leads to inactive pRb and thus allowing a cell cycle transit from G1 to S phase (Musat, et al. 2004). Additionally, CDK inhibitors (CDKI) including Ink4 family (p15, p16, p18, for CDK4 and 6) and Kip/cip family (p21, p27, p57 for CDK2) are involved in regulation of cell cycle (Musat, et al. 2004). The vast majority of pituitary adenomas (90%) display alterations of the pRb pathway. In particular, promoter hypermethylation (resulting in transcription silencing) of the p15 (INK4b), p16 (INK4a) and pRb genes was detected in 32~36%, 59~71% and 29~35% of pituitary tumors, respectively (Ogino, et al. 2005; Yoshino, et al. 2007). It has been demonstrated that p27 (Kip1) protein expression level is lower in all types of pituitary adenomas without changes of the mRNA levels compared with normal pituitaries, especially in corticotroph adenomas and pituitary carcinomas, suggesting enhanced p27 protein degradation (Lidhar, et al. 1999; Yoshino, et al. 2007).

The pituitary tumor apoptosis gene (PTAG) which is a differentially methylated chromosome 22 CpG island-associated gene (C22orf3) has been recently identified (Bahar, et al. 2004). It has been shown that 80% of the pituitary adenomas failed to express this gene compared with normal pituitaries, and in this group approximately 20% showed methylation of the CpG islands of the PTAG promoter (Bahar, et al. 2004). The methylation of the promoter was invariably associated with loss of transcript expression (Bahar, et al. 2004).

ZAC, a zinc-finger protein that induces apoptosis and cell cycle, is highly expressed in the anterior pituitary gland, and is strongly down-regulated at both mRNA and protein expression level in non-functioning adenomas (Pagotto, et al. 2000).

1.3.4 Endocrine specific mechanisms

The vast majority of sporadic pituitary adenomas do not show hyperplasia in the surrounding tissue, suggesting that hormonal stimulation is not a primary etiologic mechanism in pituitary adenomas; however, hormone dysregulation and impaired feedback inhibition may be involved in development of the pituitary adenomas, concerning that the differentiation,

proliferation and hormone release of the adenohypophysial cells are under the strict control of hypothalamic neurohormones and regulated by the feedback mechanisms (Asa and Ezzat 2009; Swearingen and Biller 2008a).

High levels of CRH and vasopressin V3 receptors have been detected in ACTH-secreting adenomas without identified mutational changes in the genes (de Keyzer, et al. 1998). Glucocorticoid hormones regulate corticotroph function through specific negative feedback loop, and loss of glucocorticoid feedback might cause Cushing's disease (Asa and Ezzat 2009). A germline mutation of glucocorticoid receptor (GR) which results in glucocorticoid resistance by diminished ligand binding has been implicated as the cause of pituitary Cushing's disease in a small number of patients (Karl, et al. 1996a). However, rare GR mutations have been found in sporadic pituitary corticotroph tumors (Karl, et al. 1996b).

GH auto-regulation and Insulin-like growth factor-1 (IGF-1) regulate GH secretion through negative feedback inhibition (Dworakowska and Grossman 2009). The decreased expression of the GH receptors and IGF receptors in 18 somatotroph tumors (both at the mRNA and protein level) may be the cause of the continuous secretion of GH from the tumor despite high circulation levels of IGF-1 and GH; However, no somatic mutations of GH receptor mRNA was detected in that group, indicating that decreased feedback inhibition of GH due to somatic mutations of the coding region of the GH receptor is unlikely to be a common factor in the pathogenesis of GH-producing adenomas (Kola, et al. 2003).

Dopamine inhibits prolactin secretion through dopamine 2 receptor (D2R). D2R-deficient mice develop lactotroph adenomas, suggesting that inactivating mutations of this receptor might contribute to the development of lactotroph adenomas (Asa, et al. 1999). However, no mutations in the D2R gene have been found in human prolactinomas, including those dopaminergic drug resistant prolactinomas which frequently show a reduction of D2R transcript (Caccavelli, et al. 1994; Friedman, et al. 1994). Inactivation of the nerve growth factor (NGF) receptor (p75) gene results in D2R loss in dopamine-responsive cells and prevents D2R expression by NGF in dopamine-nonresponsive cells, which may account for some dopamine resistant prolactinomas (Asa and Ezzat 2009; Fiorentini, et al. 2002).

The mutations in the thyroid hormone receptor β isoform (TR β) causing diminished T3 binding resulted in reduced inhibition of TSH secretion in two TSH-secreting adenomas (Ando, et al. 2001).

1.4 General overview of angiogenesis

Angiogenesis is the process of new capillary formation by sprouting or by splitting from pre-existing blood vessels (Risau 1997). Angiogenesis by vessel sprouting is a multistep process. Firstly, after a stimulatory signal, the basement membrane is degraded by the proteolytic

enzymes (such as matrix metalloproteinases (MMPs) and plasminogen activators) released by activated endothelial cells (ECs), which leads to the formation of tiny sprouts penetrating the perivascular stroma. Next, the ECs at the sprout tip migrate toward the angiogenic stimulus and proliferate, whereby the ECs form a tubular structure by cell adhesion molecules which establish the polarity, luminal versus abluminal, followed by the formation of capillary loops, which leads to the development of functioning circulatory network. Finally, the new vessels are stabilized, and this process requires the recruitment of pericytes and vascular smooth muscle cells (VSMCs), which is regulated by platelet-derived growth factor (PDGF) (Ribatti, et al. 2007a; Risau 1997). The non-sprouting angiogenesis is the process of splitting pre-existing vessels by transcapillary pillars or posts of extracellular matrix, first described in the embryonic lung (Patan, et al. 1996; Risau 1997).

Angiogenesis is controlled by the balance of proangiogenic and inhibitory stimuli (Ribatti, et al. 2007b). The quiescence of endothelial cells in the healthy adult organism is maintained by the dominant influence of endogenous angiogenesis inhibitors over angiogenic stimuli (Ribatti, et al. 2007a). Negative regulators may be particularly important in endocrine organs that are very vascular, and down-regulation of inhibitor production may be required to activate the angiogenic switch (Hanahan and Folkman 1996). Angiogenesis involves a large number of endogenous proangiogenic factors including VEGF, FGF-2, IL-8, TGF- β , PDGF, angiopoietins, matrix metalloproteinases, which play important roles in endothelial cell proliferation, vascular stabilization, recruitment of inflammatory cells releasing angiogenic factors, or extracellular matrix degradation (Li, et al. 2003; Ribatti, et al. 2007a). In addition to metabolic factors such as hypoxia, the process of angiogenesis is also regulated by some hormones such as estrogen, GH, IGH-1 and TSH through promoting endothelial cell proliferation or increasing VEGF production (Hellstrom, et al. 1999; Hyder, et al. 2000; Smith, et al. 1999). Gene mutations or down-regulation of tumor suppressor genes such as *p53* and *VHL* facilitate tumor angiogenesis by inactivating angiogenesis inhibitors or enhancing angiogenic factors (Dameron, et al. 1994; Levine 1997; Teodoro, et al. 2007; Turner, et al. 2002). The endogenous inhibitors of angiogenesis include thrombospondin-1, angiostatin, endostatin and so on, which inhibit endothelial cell proliferation or matrix metalloproteinases (Ribatti, et al. 2007a). In pathological situations angiogenesis may be triggered not only by the overproduction of proangiogenic factors, but also by the down-regulation of inhibitory factors (Ribatti, et al. 2007a). Pathogenic angiogenesis may occur in several diseases such as atherosclerosis (O'Brien, et al. 1994), rheumatoid arthritis (Colville-Nash and Scott 1992), diabetic retinopathy (Sharp 1995) and psoriasis (Nickoloff, et al. 1994).

A large number of studies have firmly demonstrated that angiogenesis plays an essential role in tumorigenesis, tumor development and progression, besides in physiological processes such as embryogenesis, wound healing and the normal menstrual cycle. It is well known that

tumors require neovascularization to supply themselves with nutrients, oxygen and also give the metabolites access to blood circulation if they are to grow beyond 2 mm³ (Jugenburg, et al. 1997). It is proposed that increased tumor diameter require a corresponding increase in vascularization (Folkman 1972). Tumor invasion of surrounding structures and tumor metastasis may occur due to the degradation of extracellular matrix during angiogenesis, and the new blood vessels provide a way for metastatic tumor cells to enter the systemic circulation (Folkman 1990; Gasparini and Harris 1995). The acquisition of angiogenic capability can be regarded as a milestone of progression from neoplastic transformation to tumor growth and metastasis (Ribatti, et al. 2007a). Many studies have shown the relationship between angiogenesis and tumor behavior; for example, increased angiogenesis has been shown to be associated with the development of metastases, poor prognosis, and reduced survival in breast, prostate and stomach malignancies (Horak, et al. 1992; Maeda, et al. 1995; Turner, et al. 2003; Weidner, et al. 1993; Weidner, et al. 1992; Weidner, et al. 1991). Angiogenesis is measured as microvessel density using antibodies against different endothelial markers on both frozen and paraffin-embedded sections. Antibodies that are most commonly used are against the endothelial antigens factor eight-related antigen (F8), CD31 (platelet endothelial cell adhesion molecule), CD34, and the lectin ulex europaeus agglutinin 1 (UEA1) (Turner, et al. 2003).

1.5 Angiogenesis in pituitary adenomas

The pituitary, as a central endocrine organ, is highly-vascularized, and in contrast to other tumor types such as prostate and breast tumors, which are more vascularized than respective normal tissue, pituitary adenomas have been shown of less vascular density compared with normal pituitary specimens as measured by different endothelial markers (Horak, et al. 1992; Jugenburg, et al. 1995; Schechter 1972; Turner, et al. 2000b; Weidner, et al. 1991). It is not simply the benign nature of pituitary adenomas that explains their relatively low vascular density, because benign or precancerous breast or cervical lesions are more vascular than corresponding normal tissues (Brem, et al. 1978; Dobbs, et al. 1997; Turner, et al. 2003). This low vascular density or inhibition of angiogenesis may partly explain why pituitary adenomas have relatively low growing speed besides another possible explanation that these relatively slow-growing tumors have low metabolic demands that do not require increased vascularization (Turner, et al. 2003).

The neuroradiological and morphological studies suggest that pituitary adenomas receive a direct systemic arterial blood supply in contrast to the normal anterior pituitary gland, and tumors without arterial supply were described as small (Gorczyca and Hardy 1988; Powell, et al. 1974; Schechter, et al. 1988; Yuh, et al. 1994). A potential explanation for the relatively low

vascular density of pituitary adenomas is that the indeed increased angiogenesis has led to in-growth of blood vessels from the systemic circulation and the escape from the hypothalamic controlling factors found in the portal circulation (Turner, et al. 2003). In addition, it has been shown that the majority of pituitary tumors have capillaries with reduced or even absent fenestrations and complete absence or fragmentation of parenchymatous basement membrane, and the endothelial cells of the capillaries are swelling and blebbing due to the compression of the long portal veins and ischemia along with tumor growth (Farnoud, et al. 1992; Kovacs and Horvath 1973; Tuffnell, et al. 1991).

Many activators of angiogenesis, including VEGF and its receptors, FGF and its receptors, hypoxia-inducible factor-1 (HIF-1), angiogenin, angiopoietin and Tie2 tyrosine kinase receptors, matrix metalloproteinases (MMPs), pituitary tumor transforming gene (PTTG), and estrogens, have shown to be associated with pituitary tumor angiogenesis (Lloyd, et al. 2003; Turner, et al. 2003). Some studies about therapeutic application of angiogenesis inhibitors have been performed. The fumagillin analog TNP-470 and a synthetic MMPs inhibitor batimastat have been demonstrated to reduce neovascularization and lactotroph proliferation in Fischer 344 rat estrogen-induced prolactinoma model (Mucha, et al. 2007; Stepien, et al. 1996; Takechi, et al. 1994).

1.6 Sumoylation and RSUME

Sumoylation is a form of post-translational modification by SUMO (small ubiquitin-related modifier), regulating protein function (Johnson 2004). SUMO, which has four isoforms (SUMO-1, SUMO-2, SUMO-3, and SUMO-4) belongs to the ubiquitin-like protein family (Wilkinson and Henley 2010). SUMO share only ~18% sequence identity with ubiquitin, but the folded structure of SUMO is similar with ubiquitin (Bayer, et al. 1998). During the process of sumoylation, SUMO proteins are firstly activated by a thioester linkage with enzyme E1, a heterodimer of SAE1 (SUMO-activating enzyme E1) and SAE2 in mammals (Gong, et al. 1999). Then, SUMO is passed to conjugating enzyme Ubc9 (ubiquitin-conjugating 9), again via a thioester linkage (Gong, et al. 1997; Johnson and Blobel 1997; Schwarz, et al. 1998). SUMO E3 ligase acts as scaffolds bringing SUMO-loaded Ubc9 into contact with the substrate protein or holding the SUMO-Ubc9 thioester in a conformation facilitating SUMO transfer (Figure 2) (Alarcon-Vargas and Ronai 2002; Wilkinson and Henley 2010).

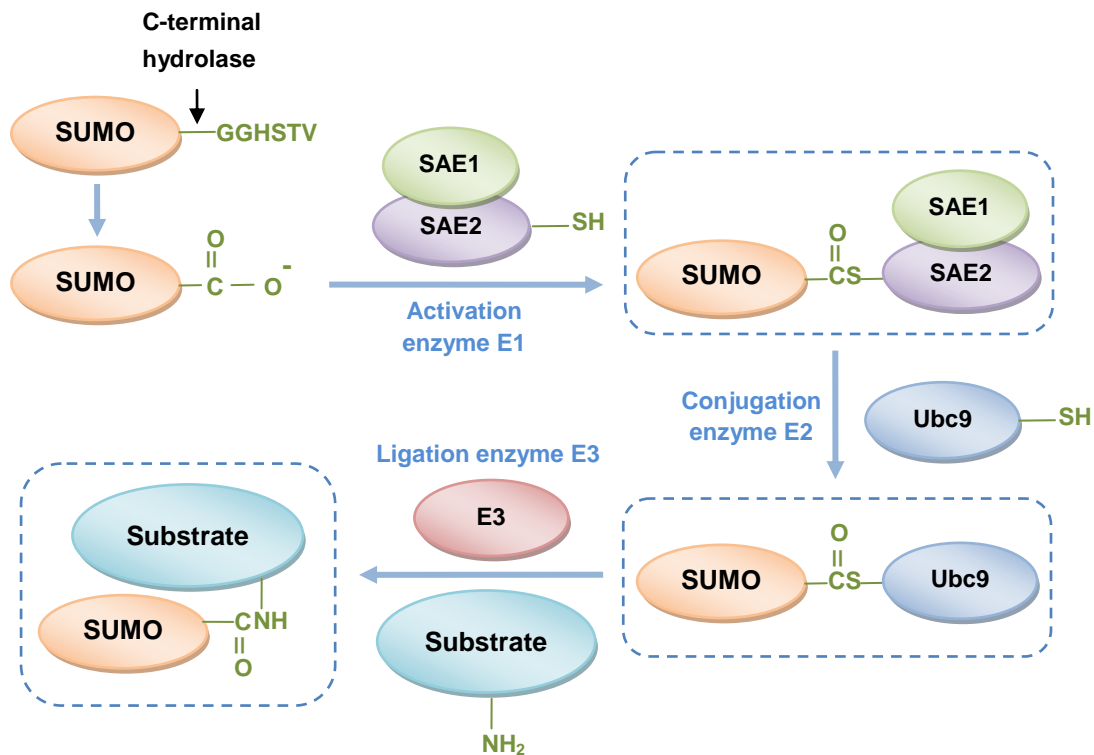


Figure 2. The SUMO conjugation pathway. SUMO is synthesized as a precursor and processed by hydrolases to make the carboxy-terminal double-glycine motif available for conjugation. It is subsequently conjugated to proteins by means of E1 activation, E2 conjugation and E3 ligation enzymes. The E3 enzymes might serve to increase the affinity between Ubc9 (E2) and the substrates by bringing them into close proximity in catalytically favorable orientations, allowing sumoylation to occur at a maximal rate. (Alarcon-Vargas and Ronai 2002)

Most sumoylation occurs in the nucleus, and has the effects on modulating activity of transcription factors negatively or positively, participates in protein nuclear transport, and also affects DNA repair and chromosome organization and function (Figure 3) (Alarcon-Vargas and Ronai 2002; Johnson 2004). In contrast to ubiquitination that leads to proteasomal degradation of proteins, the functional consequences of sumoylation vary greatly from substrate to substrate, and in many cases are not understood at molecular level (Johnson 2004). An increasing number of studies show that sumoylation positively or negatively regulates the substrate proteins which are involved in human cancers, such as PML (a nuclear body-associated phosphoprotein), I κ B α , Mdm2 and c-Jun (Verger, et al. 2003), suggesting that this process may be important in tumorigenesis and development.

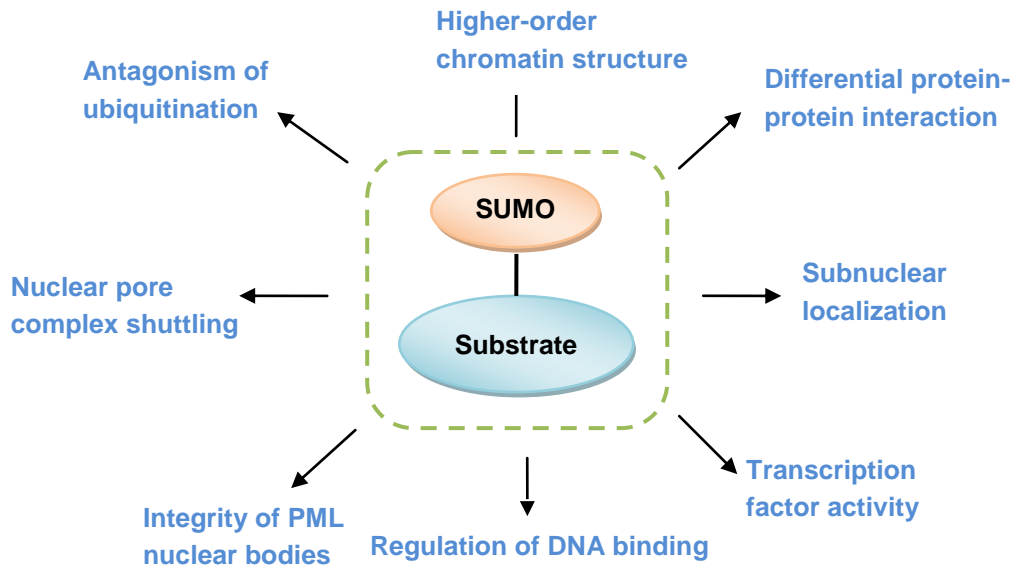


Figure 3. Functions of sumoylation. Some of the known functions of sumoylation are indicated. PML, promyelocytic leukaemia protein. (Alarcon-Vargas and Ronai 2002)

Frequently sumoylation alters protein function by influencing the interactions of substrates with other proteins or with DNA, and this may be achieved by blocking the interaction site, creating a new binding face on the substrate to recruit other binding partners, or change in conformation of the substrate protein to alter its activity or revealing previously masked binding sites (Johnson 2004; Wilkinson and Henley 2010). In addition, sumoylation can block ubiquitin binding sites by competing with ubiquitination on the same lysine residue to increase protein stabilization (Desterro, et al. 1998).

RSUME (RWD-containing sumoylation enhancer) has been recently identified in rat pituitary lactosomatotroph tumor cell line GH3 (Carbia-Nagashima, et al. 2007). As an E3 enzyme, it increases noncovalent binding of SUMO to Ubc9, enhances Ub9-SUMO thioester formation and SUMO polymerization, and therefore enhances sumoylation (Carbia-Nagashima, et al. 2007; Huang, et al. 2007). RSUME is expressed in various tissues, with higher expression in cerebellum, pituitary, heart, kidney, liver, stomach, pancreas, prostate, and spleen (Carbia-Nagashima, et al. 2007). It has been shown that RSUME is induced by hypoxia, and RSUME expression is in the necrotic inner zone rather than in the peripheral zone of two gliomas (Carbia-Nagashima, et al. 2007). By enhancing sumoylation, RSUME increases I κ B levels and stabilize HIF-1 α in monkey kidney cell line COS-7, leading to inhibition of NF- κ B transcriptional activity, and increased HIF-1 α transcriptional activity and VEGF expression (Figure 4) (Carbia-Nagashima, et al. 2007).

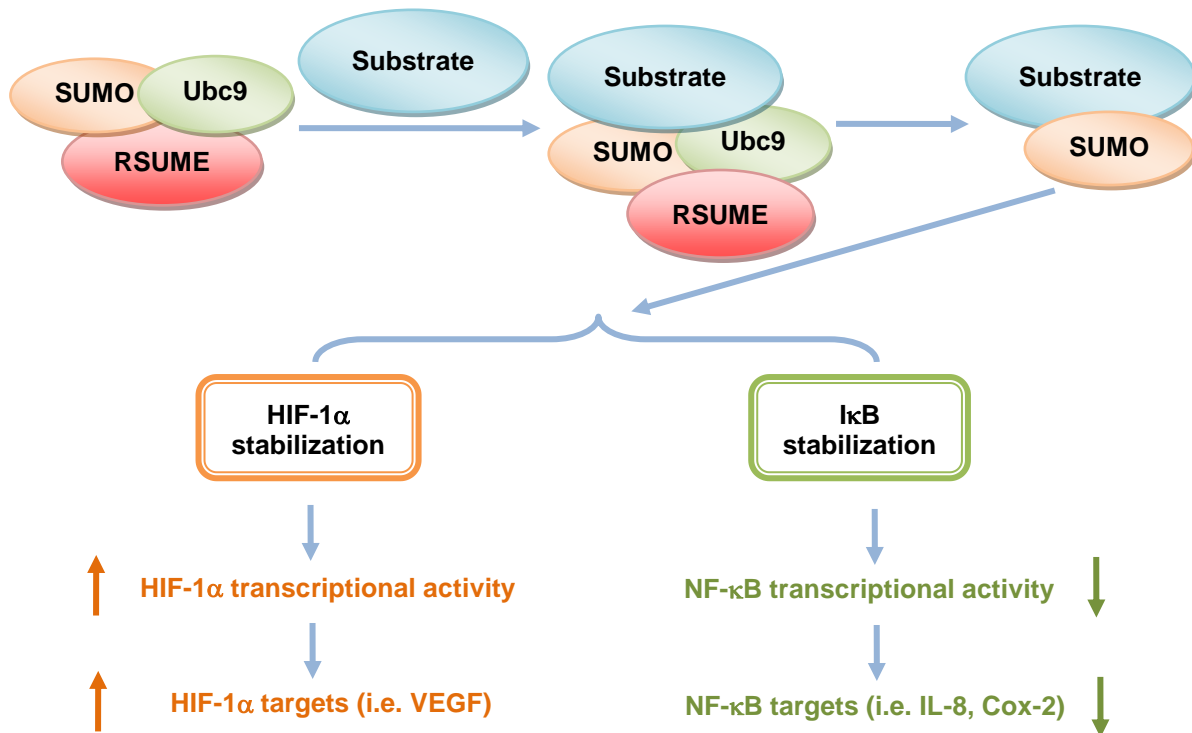


Figure 4. Actions of RSUME on the sumoylation process. RSUME enhances Ubc9-SUMO thioester formation and facilitates the progression of the SUMO transfer cascade. By enhancing sumoylation, RSUME inhibits NF- κ B activity through the stabilization of I κ B, which leads to the inhibition of its targets, and activates HIF-1 α and consequently its targets. (Carbia-Nagashima, et al. 2007)

1.7 Hypoxia-inducible factor 1 (HIF-1) α

HIF-1 plays an essential role in cellular and systemic oxygen homeostasis by adopted oxygen sensing mechanism in higher eukaryotes (Lee, et al. 2004). HIF-1 is a heterodimeric transcription factor containing the α subunit and β subunit (also known as aryl hydrocarbon receptor nuclear translocator (ARNT)), and both belong to bHLH (basic helix-loop-helix)-PAS (Per/ARNT/Sim) family (Wang, et al. 1995). The α subunit is stabilized and activated by hypoxic conditions and maintained at low levels under normoxia conditions; whereas the β subunit is constitutively expressed and its activity is not affected by hypoxia (Li, et al. 1996). HIF-1 α dimerizes with HIF-1 β through bHLH and PAS motifs and binds to the hypoxia response element (HRE) of the DNA sequence, which leads to transcription activation (Jiang, et al. 1996). Two transactivation domains (TAD), N-terminal (N-TAD) and C-terminal (C-TAD) are located in the C-terminal half of the HIF-1 α (Figure 5) (Ke and Costa 2006; Ruas, et al. 2002). The N-TAD overlaps with the oxygen-dependent degradation domain (ODDD), and it is continuous with protein stability (Cockman, et al. 2000). The C-TAD which is required for full

HIF-1 activity has been shown to interact with coactivators such as CBP/p300 to activate gene transcription independent of protein stability (Lando, et al. 2002b).

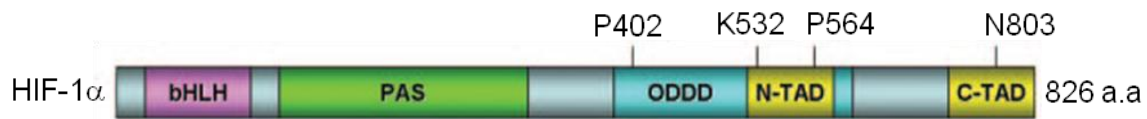


Figure 5. Domain structure of human HIF-1 α . HIF-1 α belongs to the bHLH and PAS protein family. It contains an ODDD that mediates oxygen-regulated stability through the hydroxylation of two proline (P) residues and the acetylation of a lysine (K). HIF-1 α also contains two transcription domains (C-TAD and N-TAD). The total number of amino acids of HIF-1 α is marked at the end of the domain structure. (Ke and Costa 2006)

Under normoxia, two proline residues Pro402 and Pro564 within ODD domain of HIF-1 α are hydroxylated by prolyl hydroxylase (PHD) to promote the interaction of HIF-1 α with pVHL (von Hippel-lindau tumor suppressor) which is a part of ubiquitin ligase complex to tag HIF-1 α with ubiquitin, leading to HIF-1 α degradation by 26S proteasome (Masson and Ratcliffe 2003; Masson, et al. 2001; Srinivas, et al. 1999). The lysine residue 532 (Lys532) located in the ODDD domain of HIF-1 α is acetylated by an acetyl-transferase called arrest-defective-1 (ARD1), which facilitates the binding of HIF-1 α with pVHL, and thus destabilizes HIF-1 α (Jeong, et al. 2002). The hydroxylation of an asparagine residue N803 in the C-TAD by the factor inhibiting HIF-1 (FIH-1) prevents the association of HIF-1 α with CBP/p300 which results in transcriptional inactivity (Hewitson, et al. 2002; Lando, et al. 2002b; Sang, et al. 2002). The PHD and FIH-1, regarded as oxygen sensors, are 2-oxoglutarate (2-OG)-dependent dioxygenases requiring oxygen for hydroxylation as well as Fe²⁺ and ascorbate as cofactors. Their activity to impair HIF-1 α stabilization and transcriptional activity, respectively, is known to depend on the oxygen concentration (Epstein, et al. 2001; Jewell, et al. 2001; Lando, et al. 2002a; Lando, et al. 2002b; Schofield and Zhang 1999). The ARD1 is not influenced by oxygen, but the mRNA and protein levels of ARD1 were higher in normoxia than in hypoxia, causing more acetylated HIF-1 α in normoxia (Jeong, et al. 2002). Under hypoxia, PHD and FIH-1 lose the activity of mediating hydroxylation of HIF-1 α due to lack of oxygen, thus non-hydroxylated HIF-1 α is stabilized without pVHL binding and CBP/p300 recruitment is allowed. Then, stabilized HIF-1 α translocates into the nucleus and binds to HIF-1 β , resulting in gene transcription (Figure 6) (Ke and Costa 2006). In addition, recently sumoylation of HIF-1 α has been identified as another posttranslational modification to stabilize HIF-1 α against degradation (Bae, et al. 2004; Carbia-Nagashima, et al. 2007; Shao, et al. 2004).

Besides hypoxia, HIF-1 is also regulated by cytokines, growth factors, environmental stimuli, and other signaling molecules under normoxia (Feldser, et al. 1999; Gorlach, et al. 2001;

Haddad and Land 2001; Hellwig-Burgel, et al. 1999; Ke and Costa 2006; Li, et al. 2004; Richard, et al. 2000; Salnikow, et al. 2000; Stiehl, et al. 2002). HIF-1 α transactivation or synthesis is also shown to be induced by activation of mitogen-activated protein kinase (MAPK) or the phosphatidylinositol 3-kinase (PI3K) signaling pathways (Li, et al. 2004; Zelzer, et al. 1998).

HIF-1 α , inducing a large number of target gene transcriptions, plays a general role in multiple physiological responses to hypoxia, such as erythropoiesis and glycolysis, which quickly counteract oxygen deficiency, and angiogenesis, which provides a long-term solution (Ke and Costa 2006; Semenza 1998). Tumor growth leads to progressively hypoxic condition in the inner area as its size increases until enough blood vessels are built by tumors. Over-expression of HIF-1 α has been found in various human cancers such as breast, colon, pancreas, prostate, bladder, ovary, liver, kidney and brain, probably as a consequence of intratumoral hypoxia or genetic alteration, resulting in the subsequent induction of proangiogenic genes such as VEGF which is one the major target genes of HIF-1 (An, et al. 2000; Berra, et al. 2000; Conway, et al. 2001; Harris 2000; Josko, et al. 2000; Talks, et al. 2000; Zhong, et al. 1999). HIF-1 also induces growth factors such as insulin-like growth factor-2 (IGF-2) and transforming growth factor- α (TGF- α), leading to cell proliferation and survival in hypoxia (Feldser, et al. 1999; Krishnamachary, et al. 2003). Paradoxically, HIF-1 α has been shown to promote apoptosis induced by hypoxia (Carmeliet, et al. 1998). For example, proapoptotic protein Bcl-2/adenovirusE1B19-KDa interacting protein 3 (BNip3) and its homolog Nip3-like protein X (NIX) have shown to be up-regulated in a HIF-dependent manner (Bruick 2000).

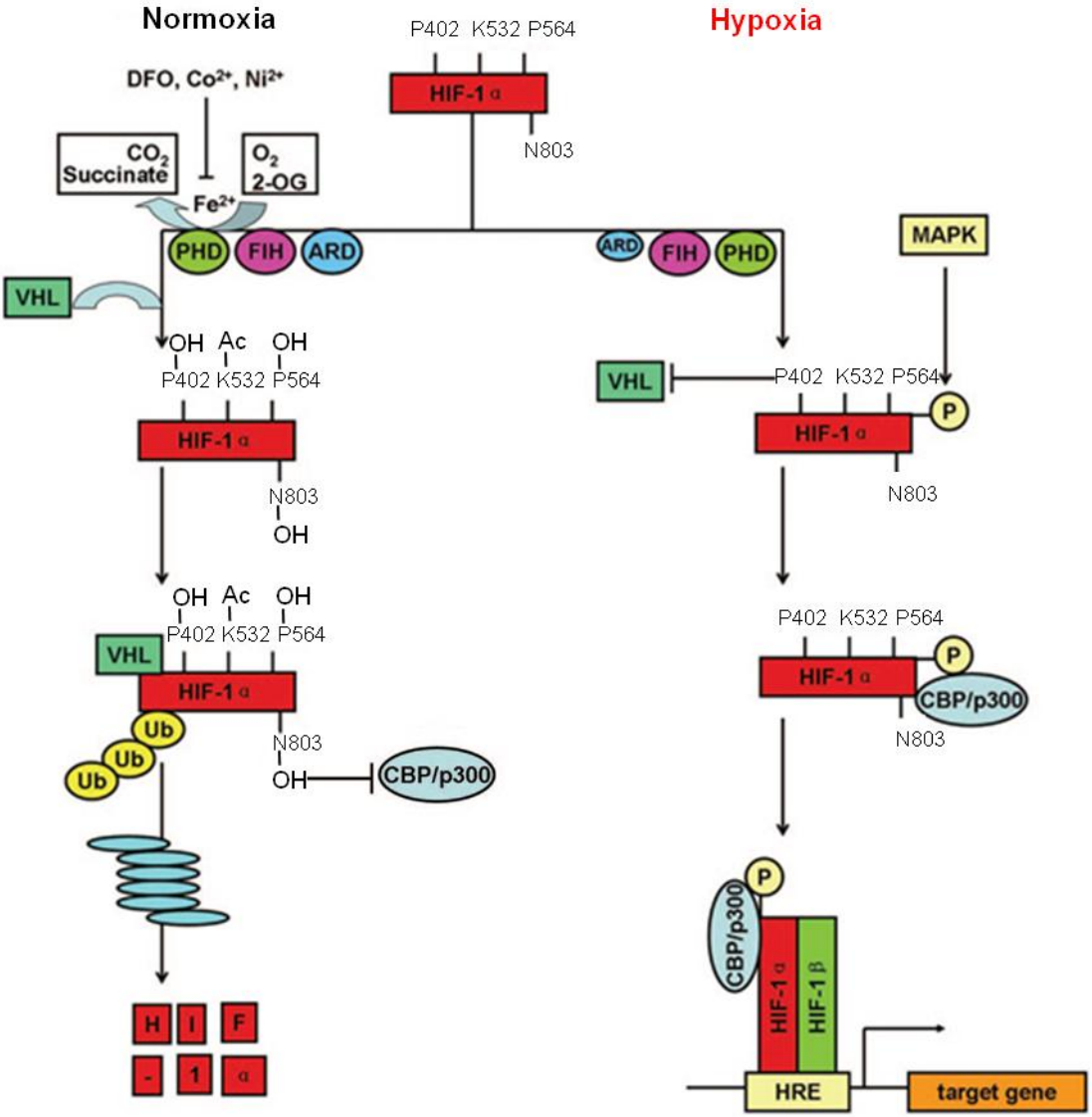


Figure 6. Oxygen-dependent regulation of HIF-1 stabilization and transactivation. In normoxia (left), two proline residues of HIF-1 α (P402 and P564) and asparagine (N803) are hydroxylated by PHDs and FIH-1, respectively, in an O₂, 2-OG, and Fe²⁺-dependent manner. Hydroxylated HIF-1 α proteins bind to the E3 ubiquitin ligase VHL complex, leading to its degradation by the proteasome. Acetylation of lysine (K532) by ARD1 favors the interaction of HIF-1 α with VHL. Hydroxylated N803 blocks the recruitment of transcriptional coactivator CBP/p300. In hypoxia (right), the activities of PHDs and FIH-1 are inhibited due to lack of O₂, resulted in no proline and asparagine hydroxylation. Therefore, there is no VHL binding and HIF-1 α is stabilized. Stabilized HIF-1 α proteins translocate to the nucleus and bind to HIF-1 β . HIF-1 β may bind preferentially to the MAPK-induced phosphorylated form of HIF-1 α . Nonhydroxylated N803 of HIF-1 α allows CBP/p300 recruitment to the target genes, resulting in gene transcription. In addition, the expression of ARD1 is decreased under hypoxia, causing less acetylated HIF-1 α . (Ke and Costa 2006)

1.8 VEGF

VEGF (also known as VEGF-A) belongs to the VEGF family which also includes VEGF-B, C, D, E, and placental growth factor (PlGF) (Achen, et al. 1998; Joukov, et al. 1996; Lee, et al. 1996; Lyttle, et al. 1994; Maglione, et al. 1991; Maglione, et al. 1993; Orlandini, et al. 1996). VEGF is a key regulator of angiogenesis, while VEGF-B is required for normal heart function in adults but is not required for cardiovascular development or for angiogenesis, and VEGF-C and VEGF-D participate in embryonic lymphangiogenesis (Ferrara 2004; Karkkainen, et al. 2004; Roskoski 2007). VEGF-E is encoded by the Orf parapoxvirus, supporting the angiogenesis associated with parapoxvirus-infected lesion (Meyer, et al. 1999). PlGF enhances VEGF signaling (Carmeliet, et al. 2001).

Alternative exon splicing of *VEGF* gene generates four different isoforms of VEGF: VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆, having 121, 165, 189, and 206 amino acids, respectively, after signal sequence cleavage (Houck, et al. 1991; Tischer, et al. 1991). VEGF₁₆₅, which is the predominant isoform, is a heparin-binding homodimeric glycoprotein of 45 KD, and it is secreted but a significant fraction remains bound to the cell surface and extracellular matrix (Ferrara and Henzel 1989; Houck, et al. 1992; Park, et al. 1993). VEGF₁₂₁, which does not bind heparin, is a freely diffusible protein (Houck, et al. 1992; Park, et al. 1993). VEGF₁₈₃ and VEGF₂₀₆ have high affinity with heparin, and they are almost completely sequestered in the ECM (extracellular matrix), but may be released in a diffusible form by heparin or heparinase, which displaces them from their binding to heparin-like moieties, or by plasmin cleavage at the COOH terminus, which generates a bioactive fragment containing the first 110 NH₂-terminal amino acids (Houck, et al. 1992; Park, et al. 1993). VEGF interacts with two highly related receptor tyrosine kinases (RTKs), VEGFR-1 and VEGFR-2; while the co-receptor neuropilin 1 (NP1) enhances the binding of VEGF₁₆₅ to VEGFR-2 and VEGF₁₆₅-mediated chemotaxis (de Vries, et al. 1992; Soker, et al. 1998; Terman, et al. 1992).

VEGF has the ability to promote growth and survival of vascular endothelial cells (ECs) derived from arteries, veins and lymph vessels (Ferrara and Davis-Smyth 1997), and also has mitogenic effects on certain non-EC types such as retinal pigment epithelial cells, pancreatic duct cells and Schwann cells (Compornolle, et al. 2002; Guerrin, et al. 1995; Oberg-Welsh, et al. 1997; Sondell, et al. 1999). VEGF also has effects on the bone marrow-derived cells and hematopoiesis. It promotes monocyte chemotaxis and controls hematopoietic stem cell survival during hematopoietic repopulation (Clauss, et al. 1990; Gerber, et al. 2002). The potential role of VEGF as a neuronal protective factor has been demonstrated by recent studies in amyotrophic lateral sclerosis (Lambrechts, et al. 2003). Moreover, VEGF induces vascular permeability, underlining its important role in inflammation (Dvorak, et al. 1995; Senger, et al. 1983).

VEGF mRNA transcription is induced by hypoxia in various pathophysiological circumstances (Dor, et al. 2001). The *VEGF* gene has a single hypoxia response element (HRE) in the promoter, which is the HIF-1 binding site (Madan and Curtin 1993). Besides oxygen tension, several major growth factors and inflammatory cytokines, including epidermal growth factor, TGF- α , TGF- β , IGF-1, FGF, keratinocyte growth factor, IL-1 α and IL-6, also up-regulate VEGF expression, indicating that VEGF release is under regulation of the autocrine or paracrine factors together with local hypoxia in the microenvironment (Ferrara and Davis-Smyth 1997; Neufeld, et al. 1999). Oncogenic mutations or amplification of *ras* proto-oncogene over-activate the Raf-Mek-Erk MAP kinase cascade, leading to increased VEGF expression (Grugel, et al. 1995; Okada, et al. 1998).

VEGF plays an essential role in both physiological angiogenesis (such as angiogenesis during embryonic and postnatal development, skeletal growth, endochondral bone formation, and angiogenesis in endocrine glands) and pathological angiogenesis (such as angiogenesis in solid tumors, haematological malignancies, intraocular neovascular syndromes, inflammatory disorders and brain edema, and diseases of the female reproductive system) (Ferrara 2004).

As mentioned above, tumor growth has the requirement for angiogenesis, and therefore, VEGF is identified as an essential factor for tumor angiogenesis. It has been shown by *in situ* hybridization studies that VEGF mRNA is significantly up-regulated in the vast majority of human tumors, such as carcinoma of the lung, breast, gastrointestinal tract, kidney, bladder, ovary, endometrium, and several intracranial tumors including glioblastoma multiforme and sporadic, as well as VHL syndrome-associated, capillary hemangioblastomas (Ferrara 2004). VEGFR-1 and VEGFR-2 are expressed in endothelial cells, indicating that VEGF released by tumor cells may influence the endothelial cells nearby in a paracrine manner (Roskoski 2007). VEGF is also expressed in pituitary adenomas, although it has been shown to be generally less intense than in normal pituitary by immunohistochemistry (Lloyd, et al. 1999). Pituitary carcinomas show relatively increased VEGF expression compared with adenomas, suggesting an up-regulation of VEGF expression during pituitary tumor progression (Lloyd, et al. 1999). Interestingly, VEGF-A is not only acting as an angiogenic but also as a growth factor on pituitary adenomas through differently expressed VEGF receptors in tumor and vessel cells of pituitary tumors (Onofri, et al. 2006). Monoclonal anti-VEGF antibodies suppress growth of various transplanted tumor cell lines in nude mice (Kim, et al. 1993). Therapeutic trials using anti-VEGF antibodies, genetically engineered VEGF-binding proteins and VEGF receptor protein-tyrosine kinase inhibitors have been performed, and VEGF pathway inhibitors are now being explored in combination with chemotherapy for many types of solid tumor (Roskoski 2007).

2 Aim of this study

RSUME, a novel protein, was shown to enhance HIF-1 α stabilization and transcriptional activity in monkey kidney cell line COS-7, and a large number of studies have demonstrated that HIF-1 α and its target VEGF play essential roles in growth and progression of a wide variety of solid tumors by promoting tumor angiogenesis. However, only few reports focus on the role of HIF-1 α and VEGF in slowly growing and poorly vascularized pituitary adenomas, and whether RSUME is implicated in pituitary adenoma angiogenesis and development is completely unclear.

The aim of this study is to investigate the expression and regulation of HIF-1 α , VEGF and RSUME in pituitary adenomas and also to explore novel additional functions of RSUME in pathogenesis of pituitary adenomas.

In order to achieve these goals, experiments were carried out with human pituitary adenomas and mouse pituitary cell lines. HIF-1 α , VEGF and RSUME were examined at RNA level by quantitative real-time PCR in human pituitary adenoma tissues. HIF-1 α , VEGF protein expression and RSUME mRNA expression were measured by western blot, ELISA and quantitative real-time PCR respectively after hypoxia-mimicking stimulation in mouse pituitary adenoma cell lines and primary human pituitary adenoma cell cultures. HIF-1 α translocation after hypoxia mimicking treatment was demonstrated by immunofluorescence studies. RSUME knockdown was performed by siRNA technology to clarify its relationship with HIF-1 α and VEGF in pituitary cell lines and primary cell cultures. The role of RSUME in pituitary cell proliferation and survival was noticed in the experiments with RSUME knockdown and analyzed by WST-1 assay, cell number counting, cell death ELISA, and immunofluorescence studies on pro-apoptotic protein cleaved caspase-3.

3 Materials and methods

3.1 Regents

| Product | Company |
|--|---|
| Acridine orange | Sigma (St. Louis. MO, USA) |
| Ammonium persulfate | Sigma (St. Louis. MO, USA) |
| Ampuwa water | Fresenius (Germany) |
| Alexa Fluor 594 | Invitrogen (Carlsbad, CA, USA) |
| Beta-mercaptoethanol | MERCK (Darmstadt, Germany) |
| Biomax MR films | Kodak (Stuttgart, Germany) |
| BLOCK-iT™ Alexa Fluor® red fluorescent oligo | Invitrogen Corp. (Paisley, UK) |
| 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) |
| Culture slides | BD biosciences (California, USA) |
| Developer solution | Kodak (Stuttgart, Germany) |
| Diethyl-pyrocabonate (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, Lithuania) |
| Dulbecco's modified Eagle medium (DMEM) | Invitrogen Corp (Paisley, UK) |
| Ethylenediaminetetraacetic acid (EDTA) | MERCK (Darmstadt, Germany) |
| Ethidium bromide | Sigma (St. Louis. MO, USA) |
| Fetal calf serum | Gibco (Karlsruhe, Germany) |
| Fixer solution | Kodak (Stuttgart, Germany) |
| Guanidine thiocyanate | Fluka Chemie AG (Buchs, Switzerland) |
| Hexanucleotide Mix | Roche (Mannheim, Germany) |

| | |
|--|--|
| Hydrochloric acid | MERCK (Darmstadt, Germany) |
| Hyperfilm ECL | Amersham Biosciences (Uppsala, Sweden) |
| Isopropanol | Sigma (St. Louis, MO, USA) |
| KCl | MERCK (Darmstadt, Germany) |
| KH ₂ PO ₄ | MERCK (Darmstadt, Germany) |
| Lipofectamine™ 2000 | Invitrogen Corp (Paisley, UK) |
| L-Glutamine | Biochrom AG (Berlin, Germany) |
| Loading Buffer 4x | Roth (Karlsruhe, Germany) |
| Lumi-Light Western Blotting Substrate | Roche (Mannheim, Germany) |
| 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) |
| Partricin | Biochrom (Berlin, Germany) |
| PBS | Gibco/invitrogen (Carlsbad, CA, USA) |
| Penicillin+Streptavidine mix | Biochrom AG (Berlin, Germany) |
| Cultrex® Poly-L-Lysine | R&D Systems (Wiesbaden, Germany) |
| Ponceau S solution | Sigma (St. Louis, Mo, USA) |
| Phenol | Roth (Karlsruhe, Germany) |
| Phosphate based buffer PBS | Life Technologies (Paisley, UK) |
| Polyacrylamide | Invitrogen Corp (Paisley, UK) |
| ProLong gold anti-fade reagent with DAPI | Invitrogen (Carlsbad, CA, USA) |
| Goat serum | Sigma (St. Louis, Mo, USA) |
| RNAsin (RNase inhibitor) | Promega Corp. (Madison, WI, USA) |
| Rneasy Mini kit | QIAGEN (Hilden, Germany) |
| Reverse transcriptase (SuperScript II™) | Invitrogen (Carlsbad, CA, USA) |
| Semi-dry blotter | Invitrogen (Carlsbad, CA, USA) |

| | |
|--|---------------------------------------|
| siRNA against RSUME | MWG Biotech (Ebersberg, Germany) |
| 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 ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) | MERCK (Darmstadt, Germany) |
| Sodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) | MERCK (Darmstadt, Germany) |
| Sodium peroxyde (NaOH) | MERCK (Darmstadt, Germany) |
| Taq DNA polymerase | MBI Fermentas |
| TEMED | Sigma (St. Louis, Mo, USA) |
| Transferrin | 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) |
| Trizol | Invitrogen (Carlsbad, CA, USA) |
| Trypsin | Sigma (St. Louis, Mo, USA) |
| Tween 20 | Sigma (St. Louis, Mo, USA) |
| WST-1 reagent | Roche (Mannheim, Germany) |

3.2 Solutions

| | |
|---|--|
| Acridin orange/ ethidium bromide solution | Acridinorange: 50 μl 200 mg/l Ethidiumbromid: 50 μl 10 mg/l PBS: 900 μl |
| 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 |
| HDB buffer | Hepes: 5,95 g/l NaCl : 8 g/l KCl: 0,37 g/l Na ₂ HPO ₄ .H ₂ O: 0,12 g/l Glucose: 1,982 g/l Amphotericine B 25µg/ml: 10 ml Penicillin/Streptomycin 105U/l : 10 ml Adjust pH to 7,3 with NaOH Store at +4°C |
| 4% Paraformaldehyde (PFA) | 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) | NaCl: 8 g/l KCl: 0.2 g/l Na ₂ HPO ₄ .2H ₂ O: 1.44 g/l KH ₂ PO ₄ : 0.2 g/l Adjust to pH 7.4 |
| RIPA buffer | 50 mM Tris pH 8.0 150 mM NaCl 1% NP40 0.5% Sodium Deoxicholate 0.1% SDS 1% Triton X-100 |
| 10 x Running buffer for protein electrophoresis | Tris-base: 30.3 g/L Glycine: 144.2 g/L SDS: 10 g/L Adjust pH to 8.3 |
| 2M Sodium acetate | 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 |
| Solution D | 4 M Guanidium thiocyanate: 250 g/337 ml 0.75 M sodium citrate pH 7.0 : 17.6 ml/337 ml 10% Sarcosyl: 26.4 ml/337 ml dissolve in 293 ml DEPC To complete the medium add: 180 µl beta-mercaptoethanol/25 ml solution just |

| | |
|---------------------------------------|---|
| | before use |
| Transfer buffer for semi-dry blotting | 39 mM Glycine 48 mM Tris-Cl 0.037% SDS 10% Methanol |
| 10x Tris borate EDTA buffer (TBE) | Boric acid (H ₃ BO ₃): 61.83 g/l EDTA: 37.2 g/l Tris pure: 30.03 g/l Adjust to pH 8.0 |
| Tris-based buffer (TBS) | Tris pure: 2.42 g/l NaCl: 8 g/l Adjust to pH 7.6 |

3.3 Human pituitary and pituitary adenoma tissues

This study was performed after approval of the local ethics committee (Ethics Grant No. 141-07) and in the case of pituitary adenomas, informed written consent was received from each patient.

3.3.1 Tissues used for investigation of HIF-1 α , VEGF and RSUME mRNA expression

Normal human pituitaries (n=3) were obtained from autopsies, performed within 12 h after accidentally occurred death of 3 healthy persons: 2 males (age 67 and 47) and 1 female (age 37). The tumor tissues received after transsphenoidal surgery from 31 patients: 13 males and 18 females with 47.9 \pm 17.8 average age (range 26-80 years), classified according to clinical presentation in somatotrophinomas (n=6), corticotrophinomas (n=6), non-functioning adenomas (n=8), prolactinomas (n=7) and thyrotrophinomas (n=4) were shock frozen as soon as possible, stored at -80°C and later used for RT-PCR. All the tumors were benign and tumor 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: 1 grade I, 7 grade II, 18 grade III cases were identified, and 5 cases couldn't be identified from clinical data offered by neurosurgeons (Table 4). In patients pre-treated with somatostatin analogues or dopamine agonists, medication was stopped at least 1 week prior to surgery. All the tumor tissues were received within 12~16 hours after surgery.

Table 4. The clinical characteristics of the normal and adenomatous pituitary tissues included in this study.

| Tissue | Gender | Age | Grade |
|--------|--------|-----|-------|
| NP1 | M | 67 | - |
| NP2 | M | 46 | - |
| NP3 | F | 37 | - |
| CUSH1 | F | 46 | II |
| CUSH2 | F | 67 | - |
| CUSH3 | M | 37 | III |
| CUSH4 | F | 27 | III |
| CUSH5 | F | 64 | - |
| CUSH6 | F | 45 | I |
| NFPA1 | M | 65 | II |
| NFPA2 | M | 75 | II |
| NFPA3 | F | 40 | III |
| NFPA4 | F | 77 | III |
| NFPA5 | F | 70 | II |
| NFPA6 | F | 30 | - |
| NFPA7 | M | 54 | III |
| NFPA8 | F | 66 | III |
| ACRO1 | F | 34 | II |
| ACRO2 | F | 80 | II |
| ACRO3 | F | 27 | III |
| ACRO4 | F | 41 | III |
| ACRO5 | M | 36 | III |
| ACRO6 | F | 77 | - |
| TSH1 | F | 29 | III |
| TSH2 | F | 55 | II |
| TSH3 | F | 32 | III |
| TSH4 | M | 53 | III |
| PROL1 | M | 40 | - |
| PROL2 | M | 31 | III |
| PROL3 | M | 64 | III |
| PROL4 | F | 28 | III |
| PROL5 | M | 30 | III |
| PROL6 | M | 40 | III |
| PROL7 | M | 26 | III |

NP, normal pituitary; CUSH, corticotrophinomas; NFPA, non-functioning adenomas; ACRO, somatotrophinomas; TSH, thyrotrophinomas; PROL, prolactinomas; “-“: no data.

3.3.2 Tissues used for primary cell culture

Human pituitary adenomas obtained from 36 patients were used for primary cell culture. For investigation of HIF-1 α expression under hypoxia-mimicking conditions, 8 human pituitary adenomas (5 non-functioning, 2 somatotroph, and 1 lactotroph) were used. For investigation

of VEGF expression under hypoxia-mimicking conditions, 17 human pituitary adenomas (11 non-functioning, 3 somatotroph, 2 lactotroph, and 1 corticotroph) were used. For investigation of RSUME mRNA expression under hypoxia-mimicking conditions, 3 human pituitary adenomas (2 non-functioning and 1 somatotroph) were used. For investigation of effect on RSUME knockdown, 8 human pituitary adenomas (6 non-functioning, 1 somatotroph, and 1 corticotroph) were used. All the tumor tissues were received within 12~16 hours after surgery and were cultivated as soon as possible. Due to the limited amount of human pituitary tumor tissue available, not all the experiments could be done in parallel in primary human pituitary tumor cells.

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 μ l of solution D supplemented with β -mercaptoethanol, using the Ultra-TURRAX T8 (IKA Labortechnik, Germany) tissue homogenizer.

Guanidium isothiocyanate and β -mercaptoethanol inhibit the RNase action activated by cell disruption, preventing in this way RNA degradation. 80 μ l of sodium acetate 2 M pH 4.0 were added afterwards to precipitate RNA, followed by 800 μ l of saturated phenol and 160 μ l of a chloroform-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 minutes at 4°C, the supernatant was discarded and the pellet was washed with ice-cold ethanol 70%. After 10 minutes 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. RNA extraction from pituitary adenoma cell lines (TtT/GF and AtT-20) and primary human pituitary adenoma cell cultures was performed with TRIzol[®] Reagent according to the manufacturer's instruction.

The samples absorbance was measured with a photometer. The lack of DNA contamination was assessed performing a PCR reaction for a housekeeping gene 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). If DNA contamination was detected, RNA purification was performed with DNase I.

3.5 Reverse Transcriptase- Polymerase Chain Reaction

Reverse transcription was performed by incubating 1 µg of total 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, 1 µl of 200 U reverse transcriptase (SuperScript II), 4 µl of 5x 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.

For semi-quantitative RT-PCR, 1 µl of cDNA samples obtained from TtT/GF and AtT20 cells were used for PCR reaction with primers specific for mouse RSUME, mouse VEGF, mouse β -Actin, human RSUME and human β -Actin (Table 5). The cDNA samples were incubated with 1.5 µl 10x PCR buffer, 0.9 µl MgCl₂ 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 58° C for 30 sec 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 minutes at 80 V and then visualized under UV light. The 1 kb Plus DNA Ladder marker was used to determine the fragments size.

Quantitative real time RT-PCR was performed with cDNA samples of TtT/GF, AtT-20 and primary human pituitary tumor cells as templates. The amplification reactions of 35 cycles were carried out with specific primers for human HIF-1 α , human VEGF, human RSUME, human β -Actin, mouse RSUME, and mouse β -Actin (Table 5). Absolute Blue QPCR SYBR Green Mix (Thermo Scientific) was used following the manufacturer's instructions. PCR amplifications were performed in a MiniOpticon Real-Time PCR Detection System (Bio-Rad, Munich, Germany), and the data were analyzed with CFX Manager Software for MiniOpticon (version 1.5, Bio-Rad). For each sample, the gene copy number was normalized by the amount of β -Actin. All experiments were carried out in triplicates.

Each sequence of the primers 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.

Table 5. Primers used for RT-PCR

| Target | Sequence (5'-3') | T _m (° C) | Fragment (bp) |
|---------------------------------------|---|----------------------|---------------|
| Human HIF-1α | Sense: CAT AGA ACA GAC AGA AAA ATC TCA TCC Anti-sense: TTA ACT TGA TCC AAA GCT CTG AGT AAT | 58 | 450 |
| Human VEGF | Sense: CAG ATT ATG CGG ATC AAA CCT Anti-sense: CAA ATG CTT TCT CCG CTC TGA | 58 | 148 |
| Human RSUME | Sense: TAC CTG GTA TCT CGA TTA ACT CTG AAC Anti-sense: TCA GTA TTA TTT TAC CCA TGA ACA TCA | 58 | 300 |
| Human β-Actin | Sense: ACG GGG TCA CCC ACA CTG TGC Anti-sense: CTA GAA GCA TTT GCG GTG GAC GAT G | 58 | 660 |
| Mouse VEGF | Sense: TCT ACC AGC GAA GCT ACT GCC Anti-sense: TTA CAC GTC TGC GGA TCT TG | 58 | 349 |
| Mouse RSUME | Sense: GAC TCA AGT GGA AAG AAA TGC AA Anti-sense: GGA AAT CAA AAC CAG GCT GT | 58 | 199 |
| Mouse β-Actin | Sense: AGT ATC CAT GAA ATA AGT GGT TAC AGG Anti-sense: CAC TTT TAT TGG TCT CAA GTC AVT GTA | 58 | 300 |

3.6 Cell culture and stimulation experiments

Folliculostellate TtT/GF and corticotroph AtT20 mouse pituitary tumor cells were grown in DMEM supplemented with 10% FCS, 2 nmol/l glutamine, 0.5 mg/l partricin, and 10⁵ U/l penicillin-streptomycin at 37°C and 5% CO₂.

For primary human pituitary tumor cell culture, adenoma tissue was cultivated as below. 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 tumor medium (DMEM supplemented with 2 mM essential vitamins, 40U/l insulin, 20 ng/l natrium selenate, 5 mg/l transferrin, 30 pM triiodothyronine (T₃), 10% fetal calf serum, 2 mmol/l L-glutamine, 0.5 mg/l partricin and 10⁵ 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). Cells were distributed in 48-well plates or 24-well plates and incubated at 37°C under 5% CO₂. The human adenoma cells attached to the plastic wells within 24 h and were then used for stimulation experiments.

CoCl₂ was dissolved in distilled water as a stock solution of 200 mM, and was diluted in cell culture medium to stimulate pituitary tumor cells for various time periods and at different concentrations as indicated.

3.7 Cell counting

In this study, cell number was counted with the Neubauer chamber before seeding plates or petri dishes. After trypsinizing and washing the cells, the cells were resuspended according to the size of pellet in 5ml, 10ml or 20 ml Medium. 50 µl cell suspension and 50 µl color solution (mixture of 50 µl 200mg/l Acridinorange, 50 µl 10 mg/l Ethidiumbromid and 900 µl PBS) were mixed. The coverslip was placed over the counting chambers and the diluted cell suspension was loaded into the counting chambers. Approximately 10 µl was required. The entire volume of the chamber was filled, but not overfilled. The cells were viewed under a microscope at 100x magnification. The cells were visible above the grid of the counting chamber (Figure 7). The number of viable cells (cells with green color under UV light) was determined overlying 4 x 1 mm² areas of the counting chamber (labeled A-D in Figure 7). The cell concentration was calculated: firstly, the total cells counted in 4 mm² was divided by 4; secondly, the average of cell number in 1 mm² area was divided by the dilution factor 1/2; thirdly, the cell number of 1 mm² area was divided by 10⁻⁴ ml, the volume of 1 mm² area; finally, the cell number of 1 ml medium was obtained.

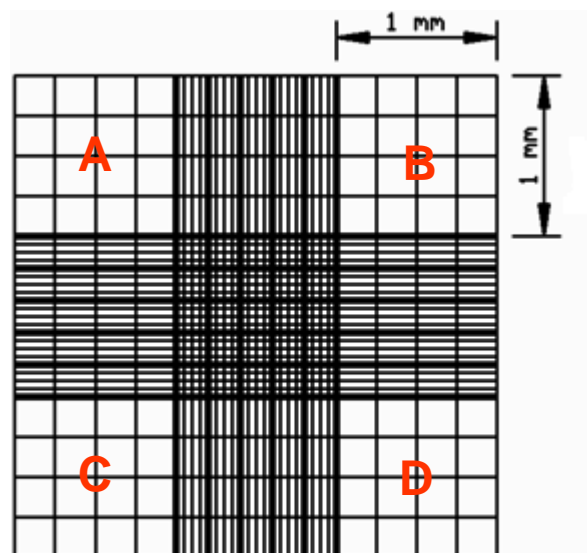


Figure 7. The Neubauer chamber for cell counting.

3.8 Transfection of siRNA against RSUME

RNA interference (RNAi) is a gene silencing system in eukaryotic cells, by which double-stranded RNA (dsRNA) triggers the destruction of mRNA with sequence complementarity.

RNAi is initiated by the conversion of dsRNA into 21~23 nt fragments known as small interfering RNAs (siRNAs) by the multidomain RNase III enzyme (Dicer). These siRNAs are then incorporated into a second enzyme complex, the RNA-induced silencing complex (RISC), in an ATP-dependent step or series of steps during which the siRNA duplex is unwound into single strands. The resulting single stranded siRNA guides the RISC to recognize and cleave the target RNA complementary to the siRNA sequence (Figure 8) (Schwarz, et al. 2002).

Nowadays, siRNA is applied in biology research. Designed siRNA is transfected into mammalian cells to suppress expression of specific genes, which has become a useful tool to explore the function of target genes.

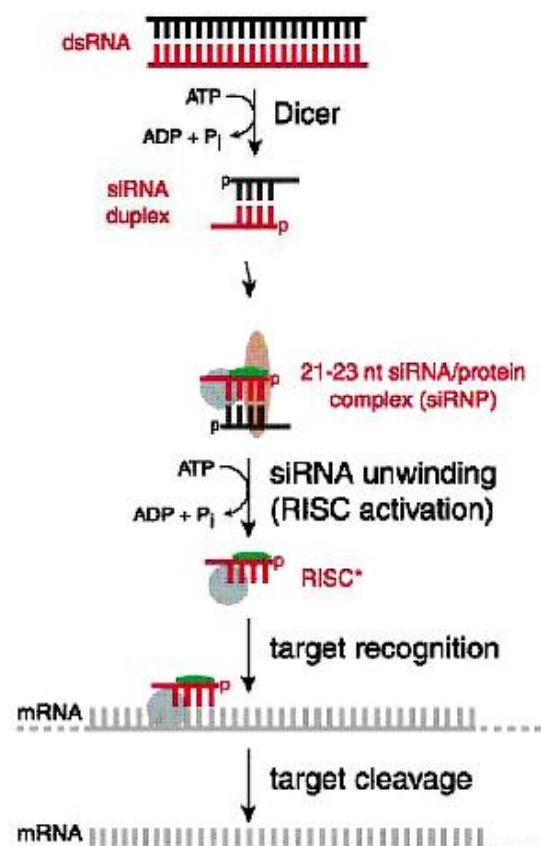


Figure 8. The siRNA pathway. Long double-stranded RNA (dsRNA) is digested by Dicer in an ATP-dependant way. Then siRNAs are uptaken by RISC. ATPs are hydrolysed to help unwind of siRNA but the incorporation is ATP independent. The single-stranded antisense strand help RISC to find the target mRNA, and the mRNA is cleaved. (Schwarz, et al. 2002)

3.8.1 Transfection of siRNA against mouse RSUME in pituitary cell lines

TtT/GF and AtT20 cells were seeded in plates with antibiotics-free culture medium. After overnight growing, 100 nM siRNA against mouse RSUME (5'- GGA CTT GTG GGT GAG GAT G-3', supplied by Prof. Arzt, FCEN-Universidad de Buenos Aires, Argentina) was transfected

into cells with lipofectamineTM 2000 (Invitrogen, Karlsruhe, Germany) to suppress endogenous RSUME expression. 100 nM scrambled siRNA (Scramble II, MWG Biotech, Ebersberg, Germany) was used as a control. The time periods of transfection are described in results.

3.8.2 Transfection of siRNA against human RSUME in primary human pituitary adenoma cells

Transfection of siRNA in primary human pituitary adenoma cells was established in 6 primary cell cultures of human non-functioning pituitary adenomas in this study. BLOCK-iTTM Alexa Fluor[®] red fluorescent oligo (Invitrogen Corp, Paisley, UK) was used for determination of transfection efficiency. The BLOCK-iTTM Alexa Fluor[®] red fluorescent oligo is Alexa Fluor 555-labeled, double-stranded RNA (dsRNA) oligomer. It is designed for use in RNAi analysis to facilitate assessment and optimization of dsRNA oligonucleotides delivery into mammalian cells using cationic lipids (such as lipofectamineTM 2000). In this study, 10 nM, 20 nM, 50 nM or 100 nM fluorescent oligo was transfected with lipofectamineTM 2000 into primary human pituitary cells. 24 hours after transfection, primary cells were observed under fluorescence microscope with standard filter set for detection of Texas red (Carl Zeiss MicroImaging, Munich, Germany). Transfection efficiency was determined by counting the number of cells with red color in 100 cells.

The optimized transfection conditions are described as follows. After dispersion of human pituitary adenomas, 200,000~250,000 adenoma cells/well were cultivated in poly-L-Lysine coated 24-well plates with 500 µl antibiotics-free tumor medium. After overnight incubation, for each well, 100 µl Opti-MEM I reduced serum medium containing 20 nM or 50 nM siRNA against human RSUME (5'- GGA TTT ATG GAT GCG GAT A-3', supplied by Prof. Arzt, FCEN-Universidad de Buenos Aires, Argentina) and 1 µl lipofectamineTM 2000 was added to the cells to suppress endogenous RSUME expression. 100 nM scrambled siRNA (Scramble II, MWG Biotech, Ebersberg, Germany) was used as a control. The time periods of transfection are described in the results.

3.9 Western blot analysis

The western blot is a kind of immunoblot technique for detection of specific proteins in various samples, such as tissue homogenate or extract of cell cultures. The native or denatured proteins are firstly separated by molecular weight or 3D-structure, respectively, using gel electrophoresis, and then the proteins are transferred onto nitrocellulose or polyvinylidene fluoride membranes. For the two-step method, the primary antibody is applied to probe the target protein after blocking the membrane typically with bovine serum albumin (BSA) or non-fat milk solution to avoid unspecific binding. Afterwards, the membrane is incubated with the

secondary antibody which binds to a species-specific binding site of the primary antibody. The secondary antibody is usually conjugated with a reporter enzyme such as horseradish peroxidase (HRP), which produces luminescence visualized on the film in proportion to the amount of target protein after reaction with chemiluminescent agent.

In this study, after CoCl_2 stimulation, 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 pipetting up and down through a very small (insulin) syringe, in proteases inhibitor cocktail diluted 1:100 in RIPA buffer, working always on ice.

The protein samples concentration was determined with Bradford dye assay (Bradford 1976). In the acidic environment of the reagent, protein binds to the coomassie dye. This results in a spectral shift from the reddish/brown form of the dye (absorbance maximum at 465 nm) to the blue form of the dye (absorbance maximum at 610 nm). The difference between the two forms of the dye is greatest at 595 nm, which therefore is the optimal wavelength to measure the blue color from the coomassie dye-protein complex. The linear standard curve was established by a serial concentration (0, 1, 2, 4, 6, 10, 15 $\mu\text{g/ml}$) of BSA/coomassie dye solution. The absorbance was measured by a spectrophotometer (Bio-rad, SmartSpecTMPlus). This standard curve is valid when the square of coefficient of determination value $R^2 \geq 0.991$; otherwise, the assay should be repeated. To determine the protein concentration of a test sample from absorbance, the standard curve was used to find the concentration of standard that would have the same absorbance as the sample.

30 μg of each sample was separated by a pre-cast Tris-glycine gel (Anamed, Darmstadt, Germany) in an electrophoresis apparatus (Invitrogen). Then, the separated proteins were transferred on a nitrocellulose membrane (Hybond ECL) with Novex[®] Semi-Dry Blotter (Invitrogen) through a 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 membrane was then blocked in 5% non-fat milk solution (dissolved in TBS/0.1% tween) for 2 h. The blocked membrane was incubated with mouse monoclonal antibody against HIF-1 α (diluted in 2.5% non-fat milk solution, 1:500, R&D Systems, Germany) or β -Actin (dilute in 2.5% non-fat milk solution, 1:10000, Milipore, USA) overnight at 4 °C. After washing three times with TBS/0.1% tween, the membrane was incubated with HRP-conjugated anti-mouse antibody (diluted in 2.5% non-fat milk solution, 1:2000, Cell Signaling, USA) for 1 h. ECL system and hyperfilm (GE Health, Germany) were used for membrane visualization.

3.10 Immunofluorescence assay

Immunocytochemistry was applied in cultured cells in this study. It is a common technique to assess the localization, distribution and expression level of target protein in situ, using a specific antibody, and this antibody-antigen interaction can be visualized by many methods under the conventional microscope, fluorescence microscope or confocal microscope. Immunofluorescence is a method widely used in immunocytochemistry to visualize the target proteins with fluorescent dye. As for the indirect immunofluorescence assay, the secondary antibody labeled with fluorescent dye is used to bind with the primary antibody which is against the antigen being probed for (Figure 9). The Alexa Fluor fluorescent dyes conjugated secondary antibodies are typically used, because the Alexa Fluor family has more robust fluorophores which are less prone to photobleaching.

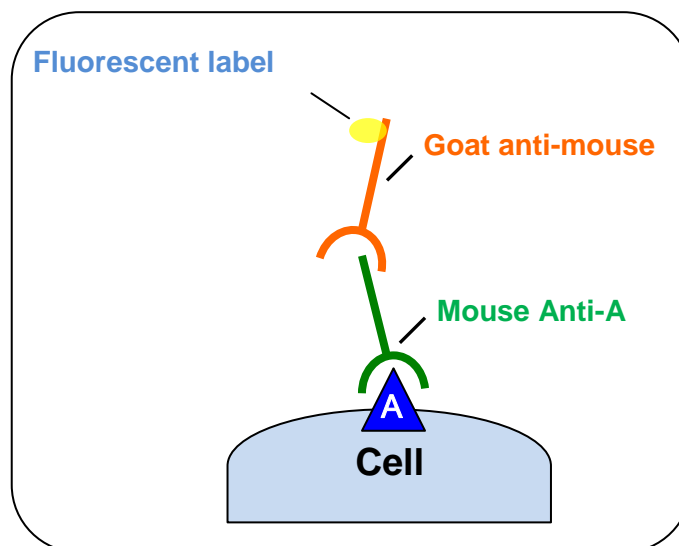


Figure 9. The principle of indirect fluorescence assay. The target antigen is recognized by the specific primary antibody which is recognized by the fluorescent labeled secondary antibody. The fluorescence is observed under fluorescence microscope or confocal microscope.

The confocal microscope is popularly applied in life sciences, and it uses a kind of optical imaging technique which can increase micrograph contrast and reconstruct three dimensional images. With conventional microscopes, the fluorescence of the entire of specimen excited in the optical path is detected as background signal. In contrast, the confocal microscope uses point illumination and an aperture in front of fluorescence detector to eliminate out-of-focus signal, so only fluorescence very closed to the focal plane and in the sample depth direction can be detected, which results in higher image resolution than that of conventional fluorescence microscopes. However, scanning over a regular raster (i.e. a rectangular pattern

of parallel scanning lines) in the specimen is required in 2D or 3D imaging, because only one point in the sample is illuminated at a time (Figure 10).

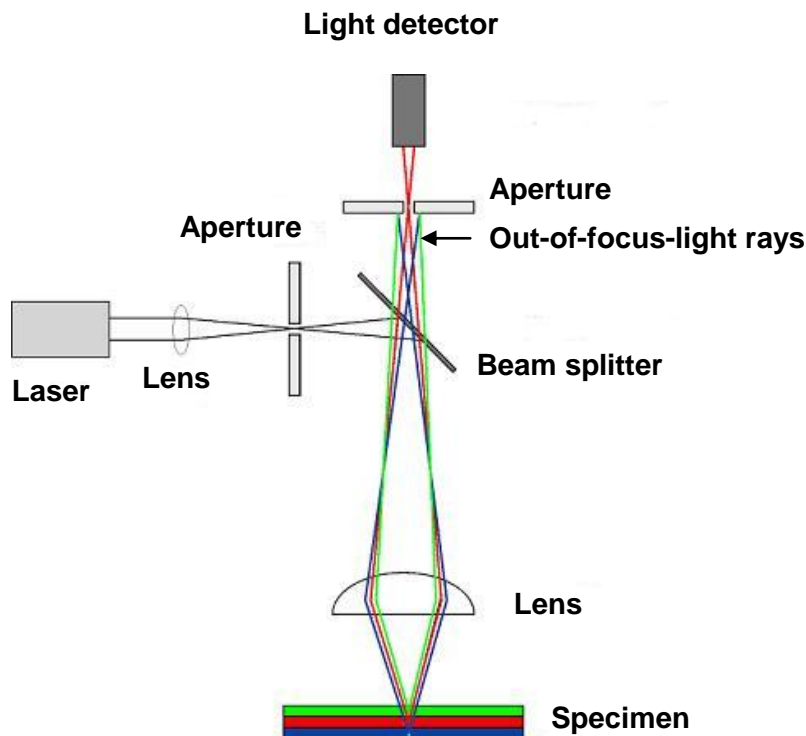


Figure 10. The principal light pathways in confocal microscope. A laser provides the excitation light, and the laser light reflects off a beam splitter to get across the specimen. Afterwards, the fluorescence dyes in the specimen is emitted, then the emitted light passes through the beam splitter and is focused onto the aperture. The light passing through the aperture is measured by the fluorescence detector.

In this study, 24 hours after transfection with siRNA against RSUME and scramble siRNA in 6-well plates, cells were split onto Falcon culture slides (BD biosciences), and stimulated with 250 μM CoCl_2 for 3 hours the next day. After treatment, cells were fixed in 4% paraformaldehyde for 5 minutes on ice, and then blocked in 5% goat serum with 0.1% (v/v) triton X-100 for 30 minutes at room temperature. Slides were incubated with mouse monoclonal anti-HIF-1 α (1: 100, Novus Biologicals, USA), rabbit polyclonal anti-cleaved-caspase-3 (1:200, Cell Signaling, USA) or rabbit polyclonal anti-VEGF (1:100, Abcam, USA) overnight at 4 $^{\circ}\text{C}$, and then washed and incubated with Alexa Fluor[®] 594 goat anti-mouse antibody ((Invitrogen, Paisley, UK) or Alexa Fluor[®] 594 goat anti-rabbit antibody (Invitrogen, Paisley, UK) for 45 minutes at room temperature. ProLong[®] Gold antifade reagent with DAPI (Invitrogen, Paisley, UK) was used for visualization of the cell nucleus. Images were obtained using a confocal microscope (FluoView[™] FV1000, Olympus, Munich, Germany). The proportion of cells immunopositive for cleaved caspase-3 was determined by counting the

number of positive cells out of 100 cells in five different areas of each slice. Images were obtained using 10x, 20x or 60x objectives.

3.11 Enzyme-Linked Immunosorbent Assay (ELISA)

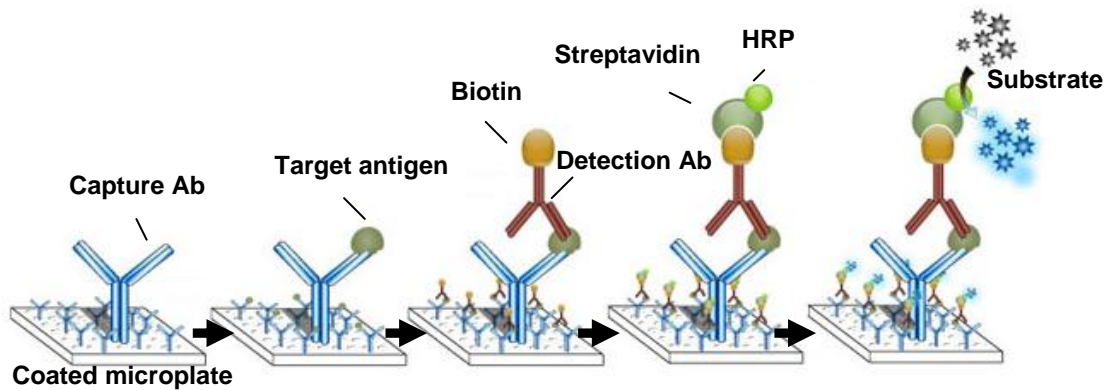


Figure 11. The procedure of sandwich ELISA.

ELISA is a biochemical technique to detect and quantify target antigen from ng/ml to pg/ml in samples, such as blood, urine and cell culture supernatant. The sandwich ELISA which measures the amount of antigen between two layers of antibodies (i.e. capture and detection antibody) is more sensitive and specific compared with other ELISA methods.

The general steps are as follows (Figure 11): 1) a known quantity of capture antibody is coated in a 96-well polystyrene plate; 2) non specific binding sites of the coated plate are blocked with blocking buffer (i.e. 5% BSA solution); 3) the appropriately diluted sample is applied and incubated for 2 hours at room temperature; 4) unbound antigen is washed away from the plate; 5) the biotinylated detection antibody that binds specifically to the antigen is applied and incubated for 2 hours at room temperature; 6) unbound biotinylated detection antibody is washed away from the plate; 7) the horseradish-peroxidase (HRP)-conjugated streptavidin which binds to the detection antibody is applied and incubate the plate for 30 minutes; 8) unbound streptavidin conjugates are washed away from the plate; 9) the substrate (1:1 mixture of H_2O_2 and tetramethylbenzidine), which is converted by HRP into a color, is applied and incubated for 15~30 minutes; 10) the absorbance of each well is measured using a multiwell spectrophotometer set to 450 nm to determine the presence and quantity of antigen.

In this study, cells were seeded in 48-well plates. After stimulation, the supernatant of cell culture was collected. The measurement of VEGF secreted into the cell culture supernatant

was carried out with VEGF ELISA kit (R&D Systems, Wiesbaden, Germany) following the manufacturer's instruction. All experiments were carried out in quadruplicates.

3.12 Proliferation assay

Cell proliferation was assessed using the non-radioactive colorimetric WST-1 assay. The Cell Proliferation Reagent WST-1 is a clear, slightly red ready-to-use solution, containing tetrazolium salt WST-1 and an electron coupling reagent, diluted in sterile phosphate buffered saline. This assay is based on the cleavage of WST-1 into formazan dye by mitochondrial dehydrogenases in viable cells, and provides a non-radioactive alternative to the [³H]-thymidine incorporation assay. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates to the number of metabolically active cells in the culture. The absorbance of the dye solution is measured by a multiwell spectrophotometer (DYNATECH, MR5000) at 420-480 nm.

In this study, 6×10^3 cells/well TtT/GF cells or 15×10^3 cells/well AtT20 cells were seeded into 96-well plates, and 24 hours after transfection of siRNA against RSUME, the cell culture medium was changed to DMEM containing 2% FCS and incubated the cells for indicated time periods. Then, 10 μ l of WST-1 solution was added into the cell culture medium of each well and the plate was incubated at 37°C and 5% CO₂ for 30 minutes. The absorbance of samples was measured by a multiwell spectrophotometer set to 450 nm. All experiments were carried out in quadruplicates.

The effect of RSUME knockdown on cell growth was confirmed by direct cell counting using a cell size-adapted coulter counter (Beckman, Z1™ Series COULTER COUNTER®). The cell counting was carried out in triplicates.

3.13 Apoptosis detection

Apoptosis is characterized by membrane blebbing (zeiosis), condensation of cytoplasm, and the activation of an endogenous endonuclease. This nuclease cleaves double-stranded DNA at the most accessible internucleosomal linker region, generating mono- and oligonucleosomes. In contrast, the DNA of the nucleosomes is tightly complexed with the core histones H2A, H2B, H3, and H4, and is thus protected from cleavage by the endonuclease. The yielded DNA fragments are discrete multiples of a 180-bp subunit, detected as a "DNA ladder" on agarose gels after extraction and separation of the fragmented DNA. The enrichment of mono- and oligonucleosomes in the cytoplasm of the apoptotic cell is due to the fact that DNA degradation occurs several hours before plasma membrane breakdown.

DNA fragmentation as an indicator of apoptosis was measured by a cell death ELISA kit (Roche Molecular Biochemicals, Germany) according to the manufacturer's instruction. All experiments were carried out in triplicates. This assay is based on the quantitative "sandwich enzyme immunoassay" principle using mouse monoclonal antibodies directed against DNA and histones. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. The samples are placed into a streptavidin-coated microplate and incubated with a mixture of anti-histone-biotin and anti-DNA-peroxidase. During the incubation interval, nucleosomes will be captured via their histone component by the anti-histone-biotin antibody, while binding to the streptavidin-coated microplate. Simultaneously, anti-DNA-peroxidase binds to the DNA part of the nucleosomes. After removal of the unbound antibodies, the amount of peroxidase retained in the immunocomplex is photometrically determined with ABTS as the substrate (Figure 12, Roche Applied science).

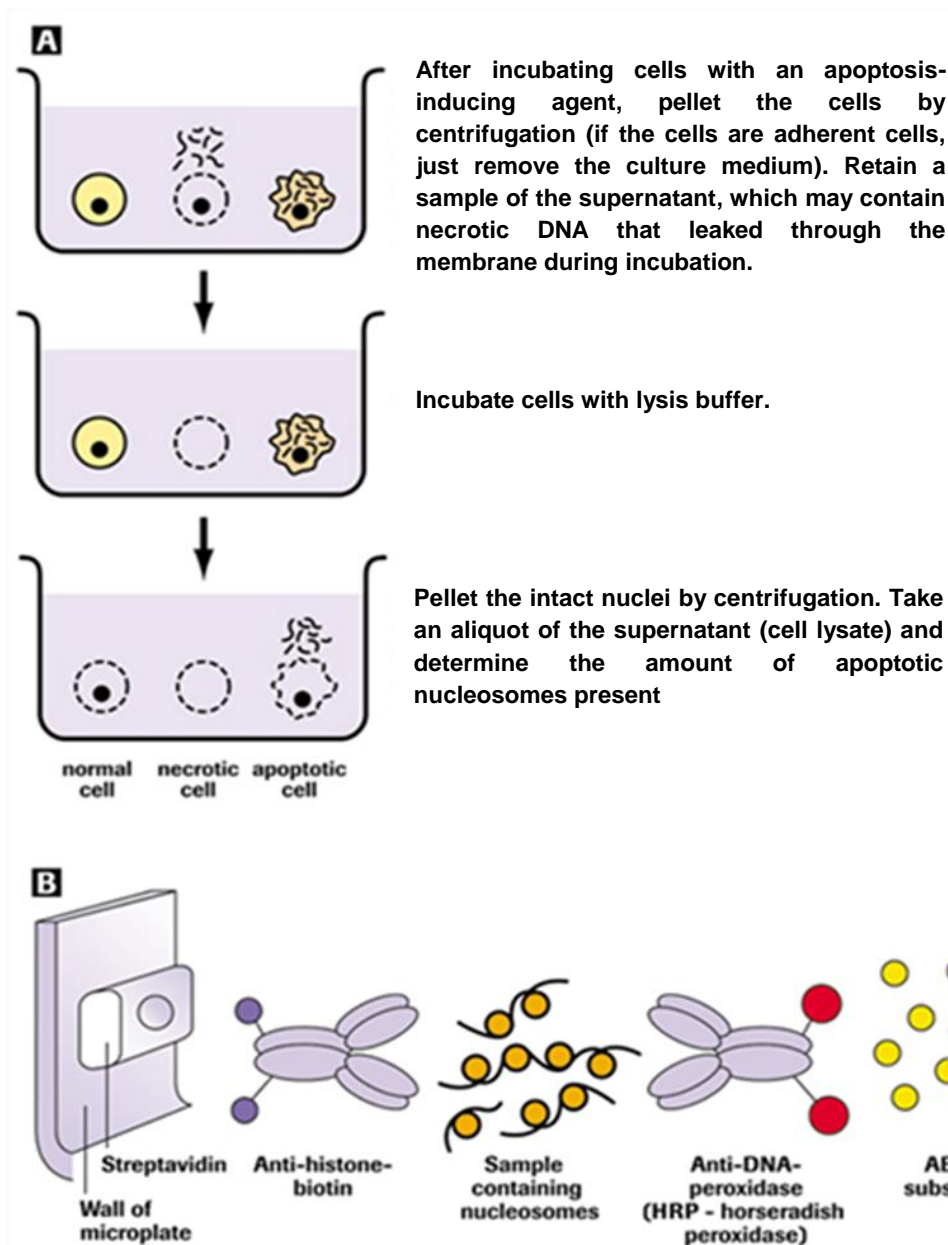


Figure 12. The principle of the Cell Death Detection ELISA^{PLUS}. Panel A: sample preparation; Panel B: ELISA. (Roche Applied Science).

3.14 Statistics

Results are expressed as mean \pm SD. The correlation analysis of HIF-1 α , VEGF and RSUME mRNA expression with the tumor grade, age and gender of patients was performed with the Fisher exact test, and statistical significance was considered at $P < 0.05$. The simple linear regression analysis was used to examine the correlation of HIF-1 α , VEGF and RSUME mRNA expression between each other, and a contribution ratio (square of coefficient of determination value R^2) >0.6 were considered significant. One-way ANOVA was used to compare variables in stimulation experiments and HIF-1 α , VEGF and RSUME mRNA expression in different

tumor types and normal pituitary glands, and $P < 0.05$ was considered as significant. The statistic analyses were performed with SigmaStat 2.0 (SPSS Inc).

4 Results

4.1 HIF-1 α , VEGF and RSUME mRNA expression in human normal pituitaries and pituitary adenomas

RNA were extracted from 3 human normal pituitaries and 31 pituitary adenomas, and then quantitative real time RT-PCR was performed to evaluate HIF-1 α , VEGF and RSUME mRNA levels with normalization to the amount of house keeping gene β -Actin (Figure 13 A-C).

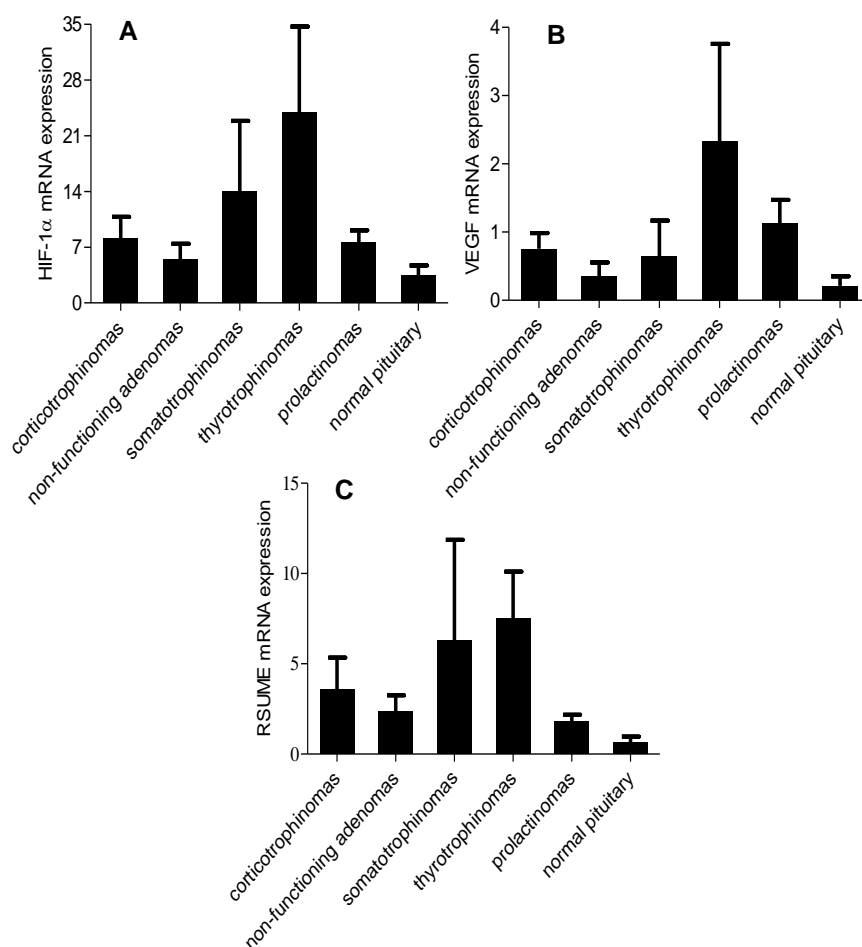


Figure 13. mRNA expression levels of HIF-1 α , RSUME and VEGF in human normal pituitaries and pituitary adenomas. Quantitative real time PCR was performed with 31 human pituitary adenomas and 3 normal pituitaries to detect the mRNA levels of HIF-1 α (A), VEGF (B) and RSUME (C). Values are given as mean \pm SD (standard deviation) after normalization to the amount of β -Actin. While the mRNA expression levels of HIF-1 α , RSUME and VEGF in human pituitary adenomas tended to be higher compared with normal pituitaries, the difference was not statistically significant, and the difference among the various types of adenomas examined was not statistically significant as well.

One-way ANOVA statistical analysis showed that the mRNA expression levels of HIF-1 α , RSUME and VEGF in human pituitary adenomas tended to be higher compared with normal pituitaries, the difference was not statistically significant, and the difference among the various types of adenomas examined was not statistically significant as well, although thyrotroph adenomas appeared to produce highest HIF-1 α , VEGF and RSUME levels. Lowest level of HIF-1 α and VEGF mRNA were found in non-functioning adenomas and lowest level of RSUME was found in prolactinomas.

Simple linear regression analyses showed that in the pituitary adenomas studied, VEGF and RSUME mRNA levels were significantly correlated with HIF-1 α mRNA levels ($R^2=0.6610$ or $R^2=0.7296$, respectively) (Figure 14 A, B). No significant correlation was found between HIF-1 α , VEGF, RSUME mRNA expression and patient age, gender, or tumor grade.

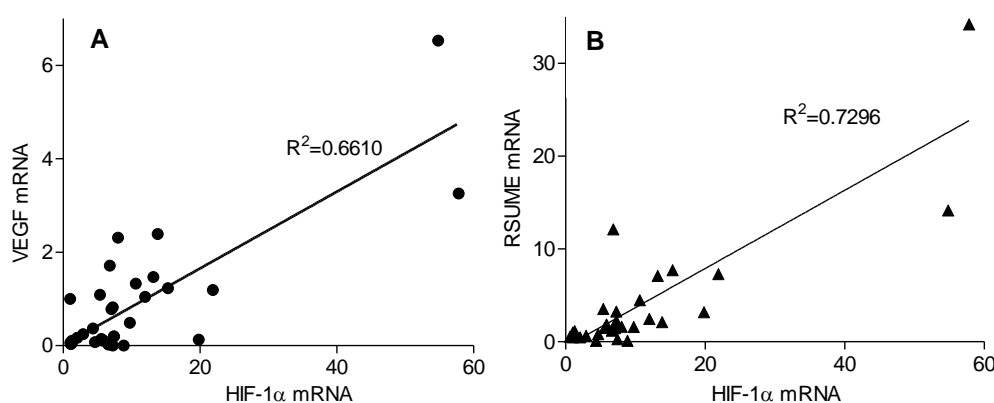


Figure 14. Correlations of mRNA expression levels among HIF-1 α , RSUME and VEGF in human normal pituitaries and pituitary adenomas. Values are given as mean \pm SD (standard deviation) after normalization to the amount of β -Actin. A, B: Simple linear regression analyses showed that VEGF and RSUME mRNA levels were significantly correlated with HIF-1 α mRNA levels in the tested pituitary adenomas ($R^2=0.6610$ or $R^2=0.7296$, respectively).

4.2 Effect of hypoxia mimicking conditions on RSUME mRNA expression

In order to test whether RSUME responds to hypoxia in pituitary tumor cells, RSUME mRNA expression under hypoxia mimicking conditions was investigated.

Before stimulation with hypoxia-mimicking agent CoCl₂, AtT20 and TtT/GF cells were exposed to medium containing only 1% FCS overnight, and then stimulated with 250 μ M CoCl₂ in the same medium. After CoCl₂ treatment, RSUME mRNA expression was measured with quantitative real time PCR. RSUME mRNA levels in AtT20 and TtT/GF cells were significantly increased shortly after CoCl₂ treatment and reached the peak at 30 minutes. Maximal

stimulations of 3.22 ± 0.58 and 2.65 ± 0.69 fold versus untreated cells were achieved in AtT20 and TtT/GF cells, respectively (Figure 15).

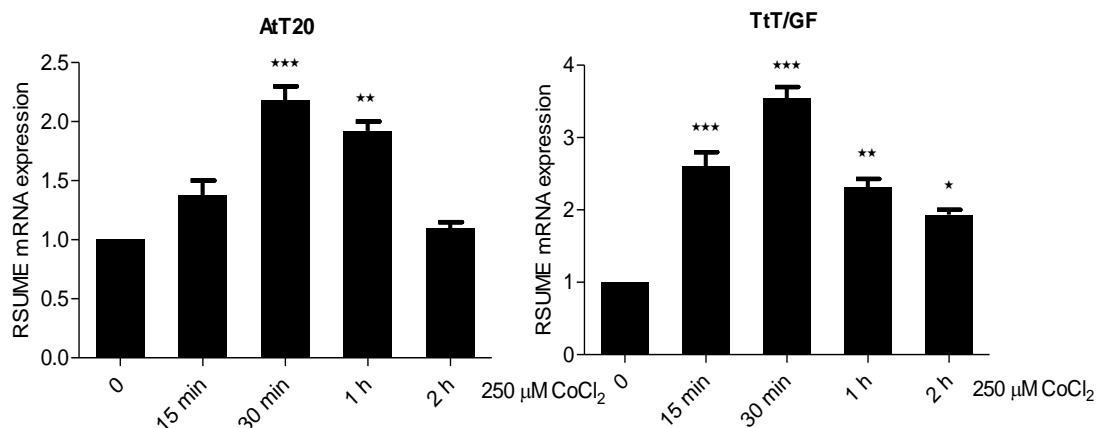


Figure 15. RSUME mRNA expression were increased under CoCl_2 stimulation in AtT20 and TtT/GF cells. AtT20 and TtT/GF cells were stimulated with $250 \mu\text{M CoCl}_2$. RSUME mRNA level was analyzed by quantitative real time PCR in triplicates, and the values are given as mean \pm SD after normalization to β -Actin. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. untreated cells.

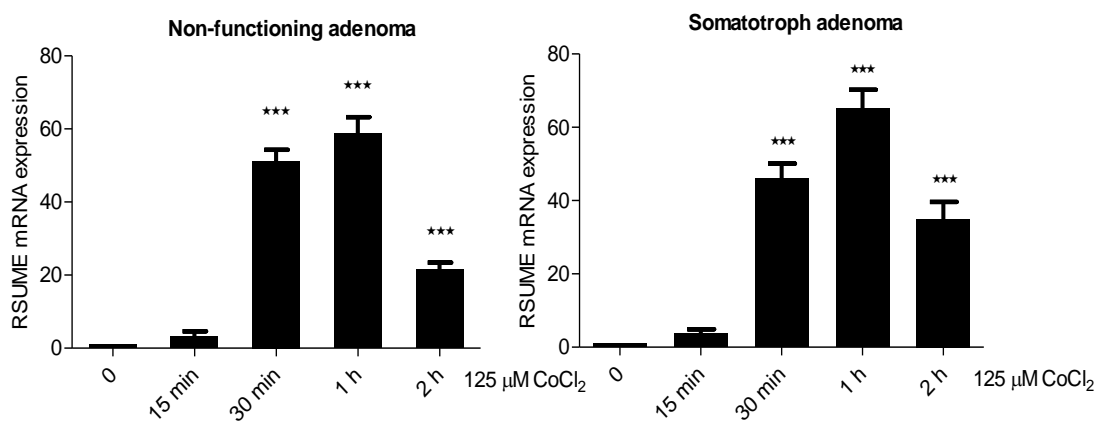


Figure 16. RSUME mRNA expression were increased under CoCl_2 stimulation in primary human pituitary tumor cells. Primary human pituitary tumor cells were stimulated with $125 \mu\text{M CoCl}_2$. RSUME mRNA level was analyzed by quantitative real time PCR in triplicates, and the values are given as mean \pm SD after normalization to β -Actin. ***, $P < 0.001$ vs. untreated cells. The similar effect was found in another primary non-functioning adenoma cells (data not shown).

The primary cell cultures of 2 non-functioning adenomas and 1 somatotroph adenoma were also tested. Before stimulation, primary human pituitary tumor cells were exposed to tumor

medium containing 2% FCS over night, and stimulated with 125 μM CoCl_2 in the same medium. After CoCl_2 treatment, RSUME mRNA expression was measured with quantitative real time PCR. RSUME mRNA levels were also significantly increased under CoCl_2 treatment in all the tested tumor cells (Figure 16 shows the results of 1 non-functioning adenoma and 1 somatotroph adenoma). Maximal stimulations of 58.80 ± 7.64 and 65.10 ± 8.84 fold versus untreated cells were achieved in the non-functioning adenoma and somatotroph adenoma shown above, respectively. RSUME mRNA expression reached the peak at 1 hour after stimulation. The similar result was found in another non-functioning adenoma (data not shown).

4.3 Effect of hypoxia mimicking conditions on HIF-1 α protein expression

In order to investigate whether HIF-1 α can be activated after hypoxia in pituitary tumor cells, HIF-1 α protein levels under hypoxia mimicking conditions were determined by western blot. Before stimulation, TtT/GF and AtT20 cells were exposed to medium containing only 1% FCS overnight, and then stimulated in the same medium with CoCl_2 of different doses and for different time periods. After stimulation, HIF-1 α was determined in cell lysates by western blot. CoCl_2 dose-dependently increased HIF-1 α protein levels in both cell lines (Figure 17). With stimulation of 5 hours, the maximal HIF-1 α expression was achieved at 500 μM CoCl_2 . The stimulatory effect was time-dependent and with the stimulation of 250 μM CoCl_2 the maximum HIF-1 α protein levels were reached after 2 h and 3 h in AtT20 and TtT/GF cell lines, respectively (Figure 17). During CoCl_2 stimulation, compared with untreated cells, the toxic effects of CoCl_2 and the changes in cell viability were not observed.

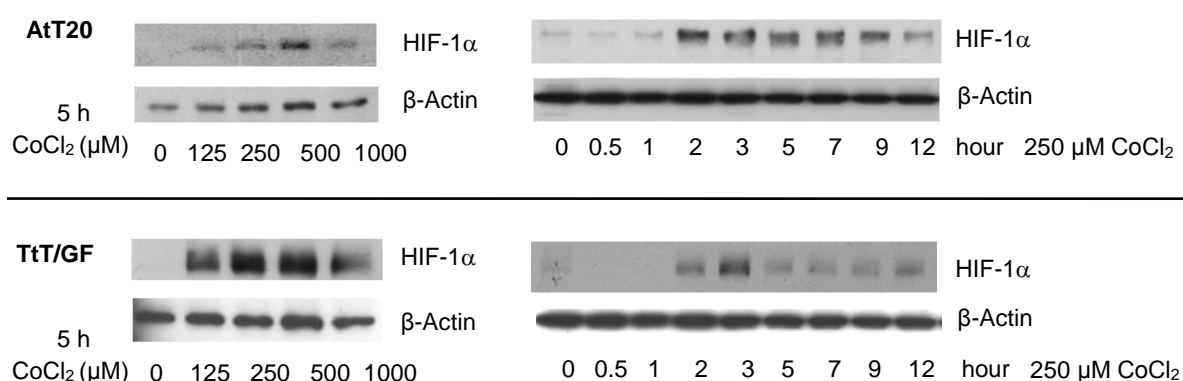


Figure 17. HIF-1 α protein level was increased under CoCl_2 stimulation in a concentration- and time-dependent manner in AtT20 and TtT/GF cells. AtT20 and TtT/GF cells were stimulated with indicated concentrations of CoCl_2 and time periods. Each image is representative of 3 experiments with similar results.

For immunofluorescence studies, after stimulation of 250 μM CoCl_2 for 3 hours, AtT20 and TtT/GF cells were fixed and immunofluorescence assay for HIF-1 α was performed. By confocal immunofluorescence microscopy, CoCl_2 did not only stimulate expression of HIF-1 α but also its translocation into the nucleus as shown in both cell lines (Figure 18).

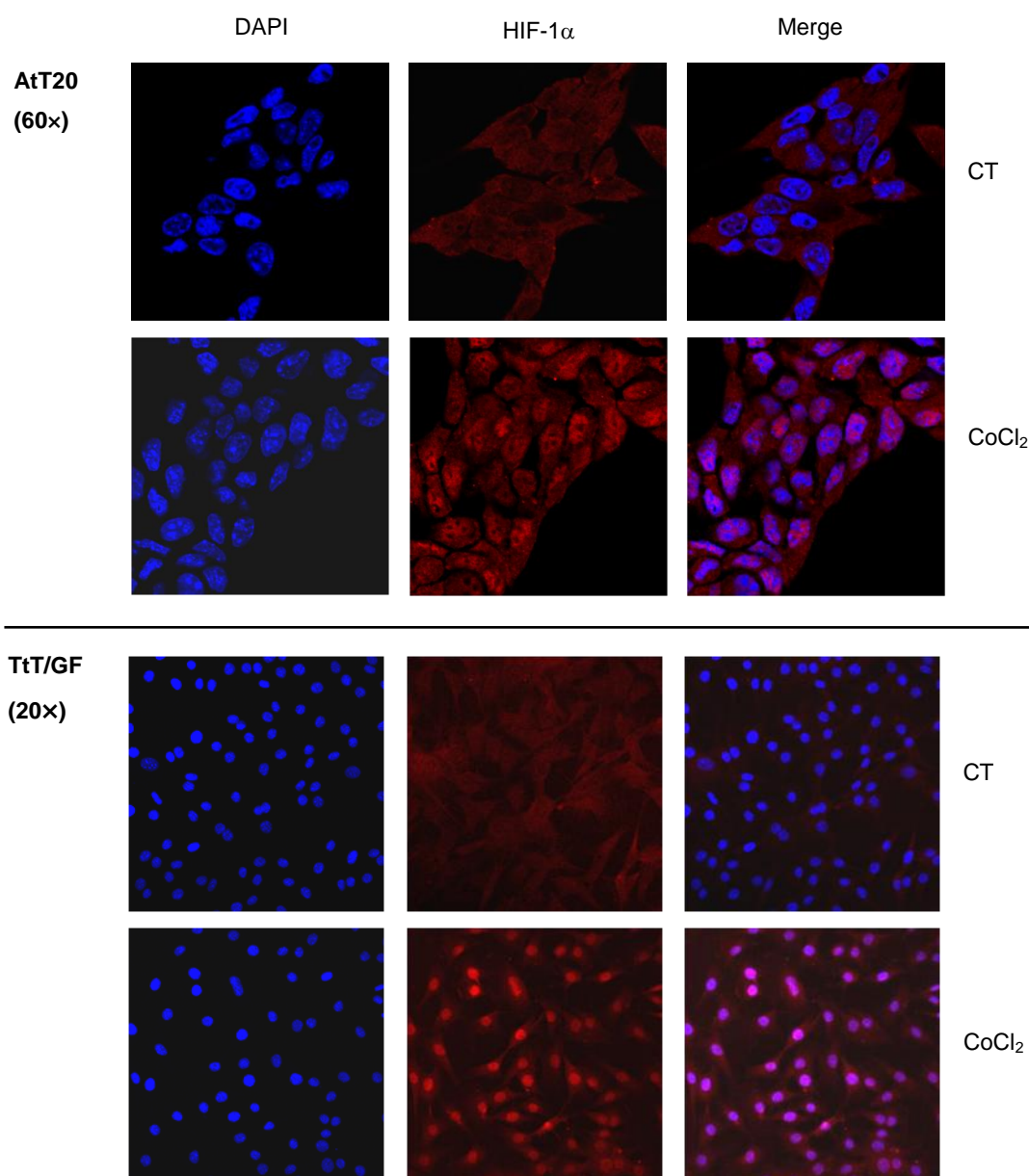


Figure 18. HIF-1 α is translocated and accumulated in nucleus after CoCl_2 treatment in AtT20 and TtT/GF cells. AtT20 and TtT/GF cells were stimulated with 250 μM CoCl_2 for 3 hours. Red color corresponds to HIF-1 α , and blue color corresponds to nucleus. The immunofluorescence images were taken from confocal microscope using 60x and 20x objectives for AtT20 and TtT/GF cells, respectively. The parameters were fixed when taking images by confocal microscopy, and one representative image of 5 observations from two independent experiments with similar results is shown.

Primary cell cultures of 8 human pituitary adenomas (5 non-functioning, 2 somatotroph adenomas, 1 lactotroph adenomas) were also treated with CoCl_2 in order to find out whether HIF-1 α can be activated as in mouse pituitary tumor cell lines. Before stimulation, primary tumor cells were exposed to tumor medium containing only 2% FCS overnight, and then stimulated in the same medium with CoCl_2 of different doses and for different time periods. After stimulation, HIF-1 α was determined in cell lysates by western blot. HIF-1 α protein expression was time- and dose-dependently increased after CoCl_2 treatment in all the tested primary cell cultures (Figure 19 shows the representative findings from two tumor cell cultures). Due to limited amount of primary tumor cells, not all the stimulation experiments of different doses and time periods could be done in parallel.

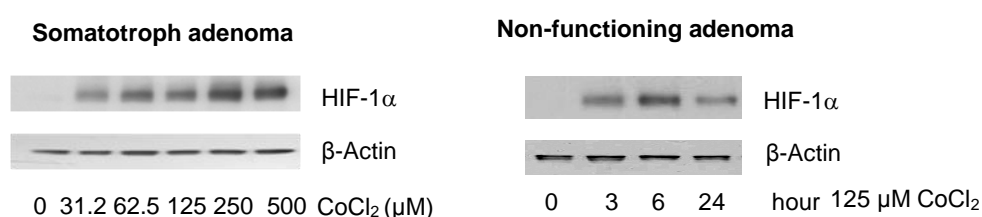


Figure 19. HIF-1 α expression was increased under CoCl_2 stimulation in a concentration- and time-dependent manner in primary human pituitary tumor cells.

Primary somatotroph adenoma cells were stimulated with different concentrations of CoCl_2 for 3 h, and primary non-functioning adenoma cells were stimulated with 125 μM CoCl_2 for different time periods. Each image is representative of 3 experiments with similar results. HIF-1 α was increased in all the tested primary human pituitary tumor cell cultures after CoCl_2 treatment, following a time- and concentration-dependent manner. Western blots from primary human somatotroph adenoma cells and non-functioning adenoma cells were shown as representatives.

4.4 Effect of hypoxia mimicking conditions on VEGF expression

VEGF is a well-characterized HIF-1 α target and a key factor in angiogenesis. In order to find out whether CoCl_2 not only induces HIF-1 α protein expression but also stimulates VEGF secretion, VEGF secretion after CoCl_2 stimulation was investigated by VEGF ELISA.

Before stimulation, TtT/GF and AtT20 cells were exposed to medium containing only 1% FCS overnight, and then stimulated in the same medium with CoCl_2 of different doses and for different time periods. After stimulation, the supernatant of the cell culture was collected and VEGF secretion was determined by ELISA. VEGF was significantly increased under CoCl_2 treatment, following a time- and concentration-dependent manner, in TtT/GF cells (Figure 20). However, CoCl_2 treatment did not significantly stimulate the increase of VEGF secretion in

AtT20 cells. From the previous study, it is shown that AtT20 cells have a very high basal VEGF production rate (Lohrer, et al. 2001). It may be that the VEGF output by AtT20 cells is already saturated and can not be further enhanced under hypoxia mimicking conditions.

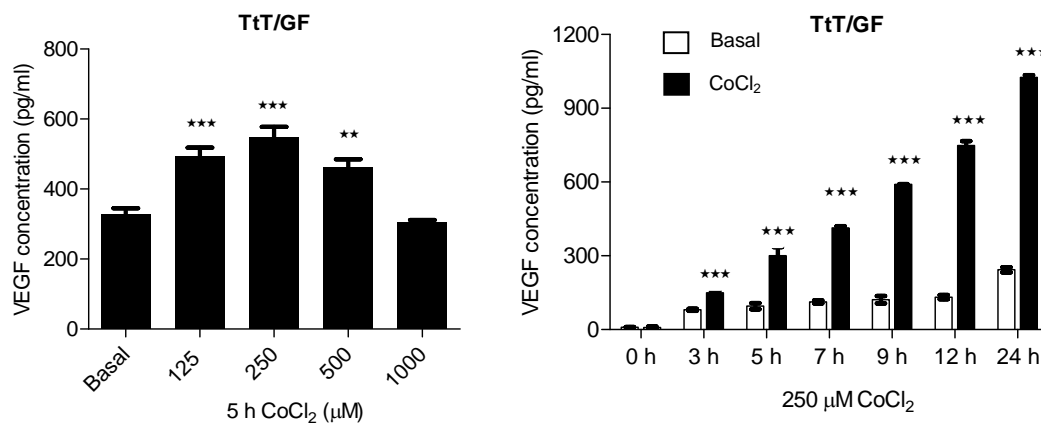


Figure 20. VEGF expression was increased under CoCl₂ stimulation in a concentration- and time-dependent manner in TtT/GF. TtT/GF cells were stimulated with indicated concentrations of CoCl₂ and time periods. Values were from ELISA assays, and given as mean±SD. **, $P < 0.01$; ***, $P < 0.001$ vs. untreated cells. All experiments were carried out in quadruplicates.

VEGF secretion under CoCl₂ stimulation in primary cell cultures of 17 human pituitary adenomas (11 non-functioning, 3 somatotroph, 2 lactotroph, 1 corticotroph) was also tested. Before stimulation, primary tumor cells were exposed to tumor medium containing only 2% FCS overnight, and then stimulated in the same medium with different doses of CoCl₂ and for different time periods. After stimulation, VEGF secretion in the supernatant of culture medium was determined by ELISA. CoCl₂ dose-dependently increased VEGF secretion in all the tested primary tumor cells (Figure 21 A-D show the representative findings). Due to limited amount of primary tumor cells, not all the stimulation experiments of different doses and time periods could be done in parallel.

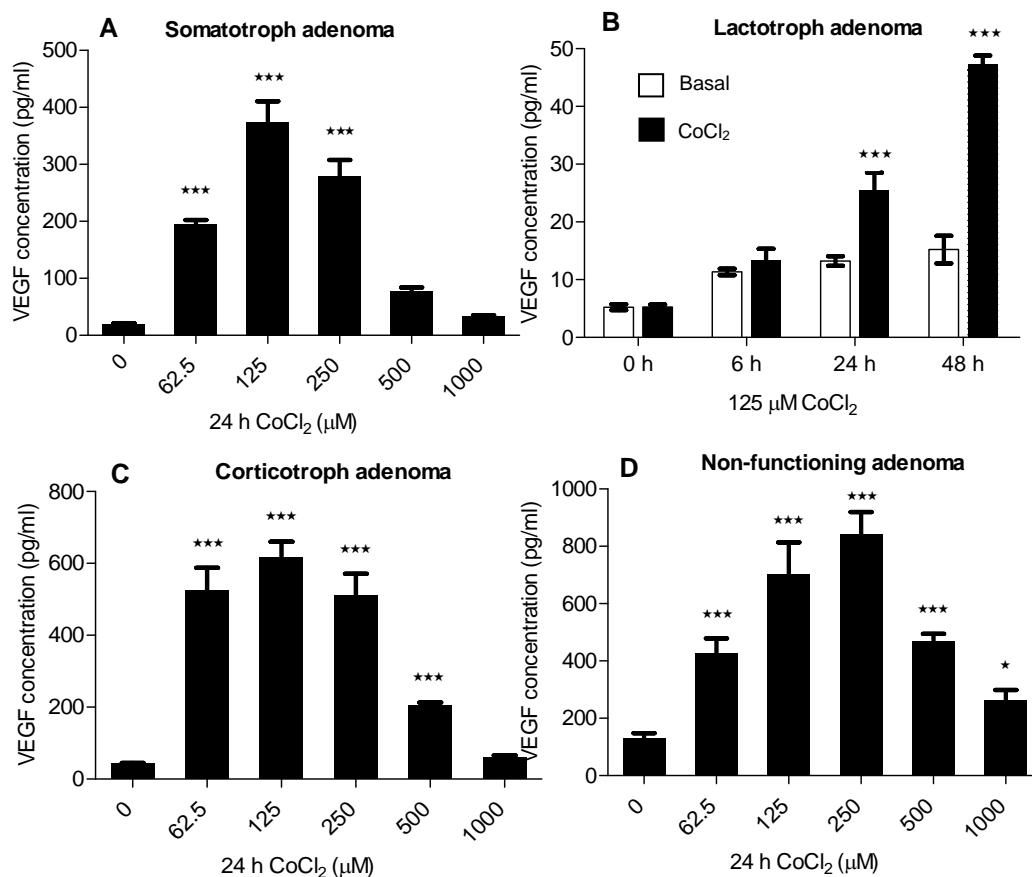


Figure 21. VEGF expression was increased under CoCl₂ stimulation in a concentration- and time-dependent manner in primary human pituitary tumor cells. Primary human tumor cells were stimulated with indicated concentrations of CoCl₂ and time periods. Values were from ELISA assays, and given as mean±SD. **, $P < 0.01$; ***, $P < 0.001$ vs. untreated cells. All experiments were carried out in quadruplicates.

4.5 Effect of RSUME knockdown on HIF-1 α and VEGF production

4.5.1 In mouse pituitary cell lines

In order to investigate whether RSUME plays a role in HIF-1 α increase after hypoxia in pituitary tumor cells, siRNA against mouse RSUME and scrambled siRNA were transfected into AtT20 and TtT/GF cells. 24 hours after transfection, the total RNA was extracted and RT-PCR for mouse RSUME was performed. RSUME mRNA was obviously decreased in both cell lines 24 hours after RSUME siRNA transfection compared with un-transfected and scrambled siRNA transfected control (Figure 22). 48 hours after transfection of RUSME siRNA and scrambled siRNA, AtT20 and TtT/GF cells were stimulated with 250 μ M CoCl₂ in medium containing 1% FCS for 3 hours. After stimulation, HIF-1 α was determined in cell lysates by western blot. CoCl₂-stimulated HIF-1 α expression was significantly suppressed in RSUME

siRNA transfected AtT20 cells and TtT/GF compared with un-transfected and scrambled siRNA transfected control (Figure 23).

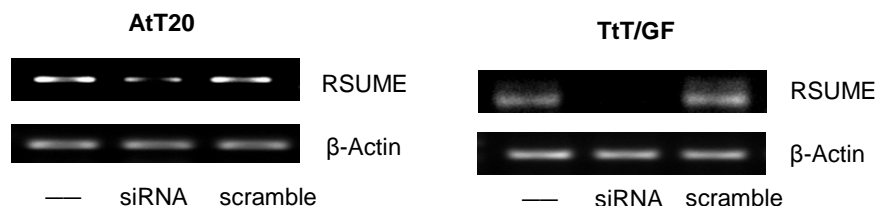


Figure 22. RSUME mRNA expression was suppressed by transfection of siRNA against mouse RSUME in AtT20 and TtT/GF cells. AtT20 and TtT/GF cells were transfected with siRNA against mouse RSUME, and RT-PCR was performed 24 hours after transfection.

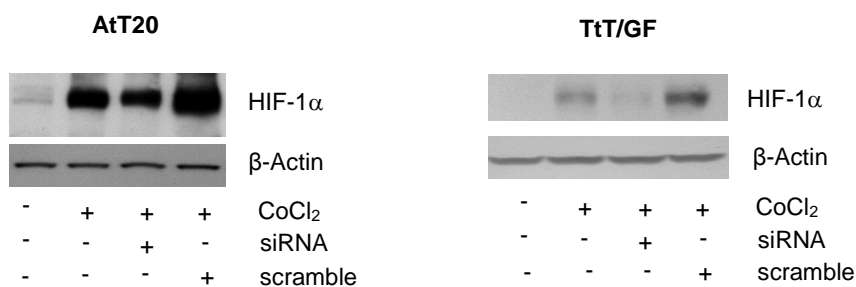


Figure 23. RSUME knockdown suppressed HIF-1 α in both TtT/GF and AtT20 cells. AtT20 and TtT/GF cells were transfected with mouse RSUME siRNA for 48 h, and then treated with 250 μ M CoCl₂ for 3 h. Each image is representative of 3 experiments with similar results.

For immunofluorescence studies, 48 hours after transfection of RSUME siRNA and scrambled siRNA, AtT20 and TtT/GF cells were stimulated with 250 μ M CoCl₂ in medium containing 1% FCS for 3 hours. After stimulation, AtT20 and TtT/GF cells were fixed and immunofluorescence assay for HIF-1 α was performed. Confocal immunofluorescence microscopy showed that the translocation of HIF-1 α in response to CoCl₂ treatment was impaired in AtT20 and TtT/GF cells in which RSUME had been knocked down (Figure 24 and Figure 25).

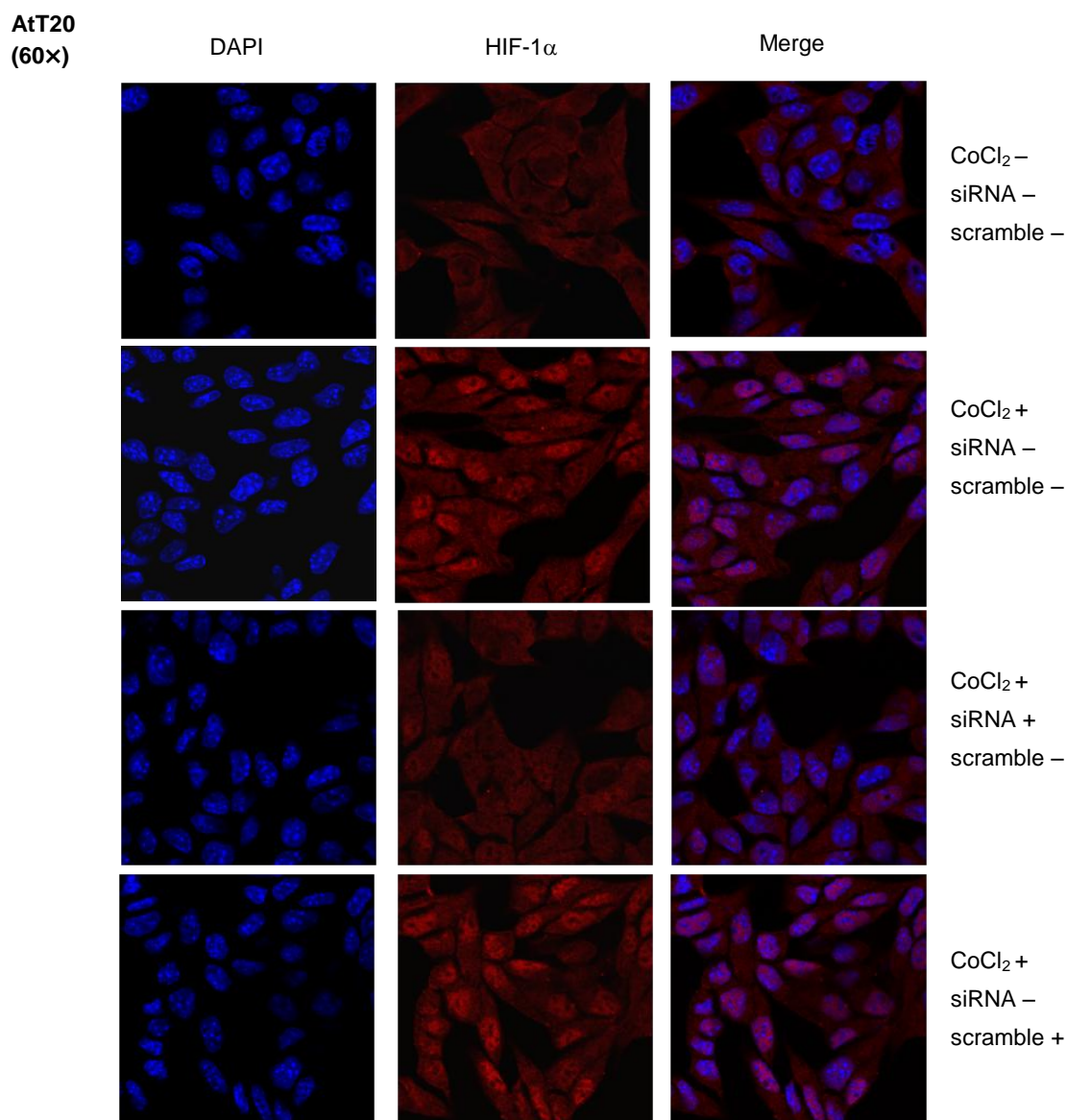


Figure 24. HIF-1 α was suppressed by RSUME knockdown in AtT20 cells as shown by immunofluorescence assay. 48 hours after transfection of RUSME siRNA and scrambled siRNA, AtT20 cells were stimulated with 250 μ M CoCl₂ for 3 hours. Red color corresponds to HIF-1 α , and blue color corresponds to nucleus. The immunofluorescence images were taken from confocal microscope using 60x objectives. The parameters were fixed when taking images by confocal microscopy, and one representative image of 5 observations from two independent experiments with similar results is shown.

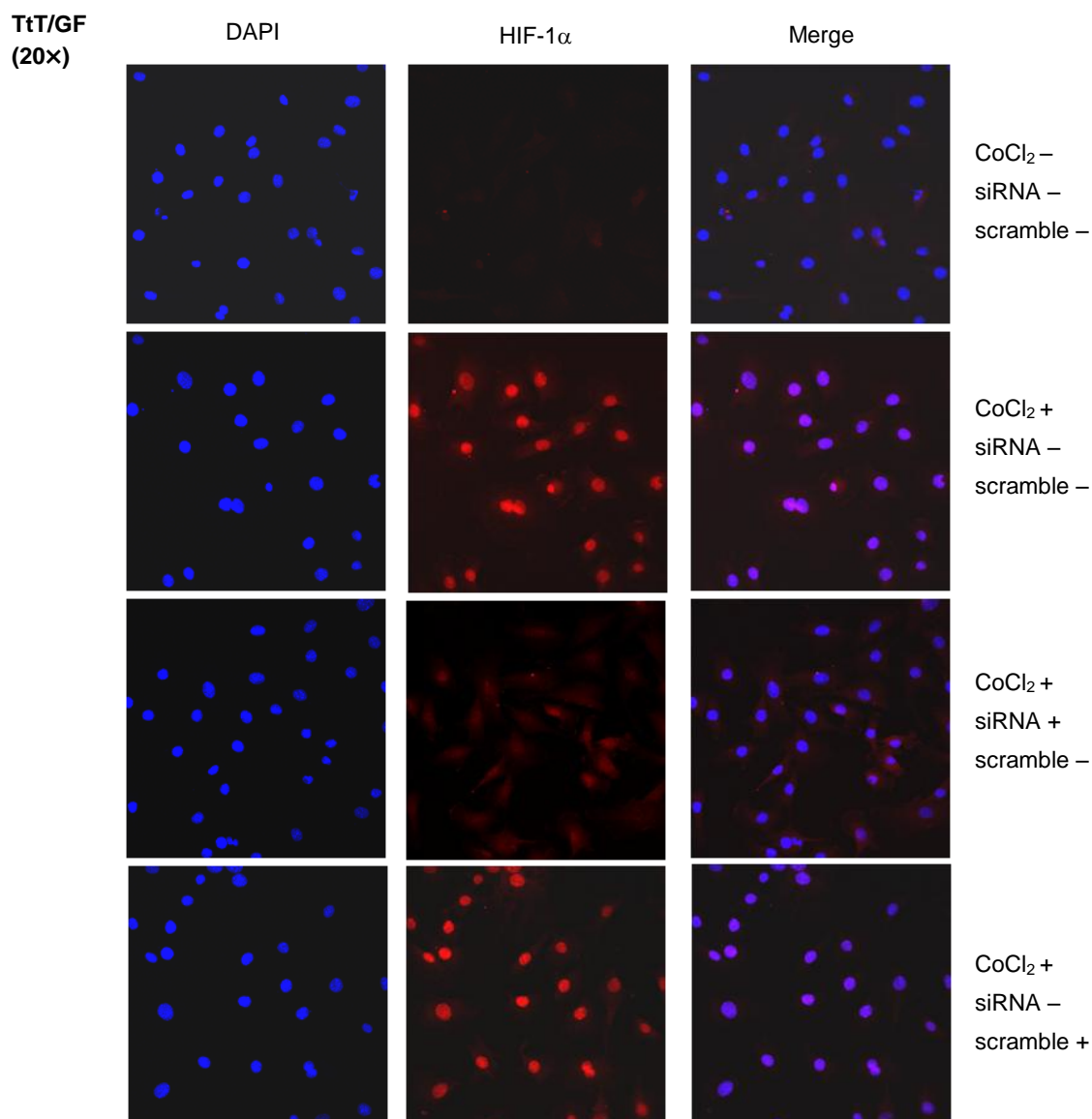


Figure 25. HIF-1 α was suppressed by RSUME knockdown in TtT/GF cells as shown by immunofluorescence assay. 48 hours after transfection of RUSME siRNA and scrambled siRNA, TtT/GF cells were stimulated with 250 μ M CoCl₂ for 3 hours. Red color corresponds to HIF-1 α , and blue color corresponds to nucleus. The immunofluorescence images were taken from confocal microscope using 20x objectives. The parameters were fixed when taking images by confocal microscopy, and one representative image of 5 observations from two independent experiments with similar results is shown.

In order to find out whether RSUME knockdown not only suppresses HIF-1 α protein expression but also reduces VEGF secretion, VEGF ELISA was performed to determine VEGF secretion in AtT20 and TtT/GF cells after transfection of siRNA against RSUME.

For TtT/GF cells, 48 hours after transfection of RSUME siRNA, cells were treated with 250 μ M CoCl₂ for 24 h, and then the culture supernatant was collected for VEGF ELISA and RNA was extracted for RT-PCR. From VEGF ELISA, it was found that RSUME knockdown did not significantly reduce the basal VEGF secretion, but significantly decreased CoCl₂-stimulated VEGF expression. From RT-PCR, VEGF mRNA expression was obviously increased by CoCl₂ stimulation, and was significantly suppressed by RSUME knockdown compared with un-transfected and scrambled siRNA transfected control (Figure 26)

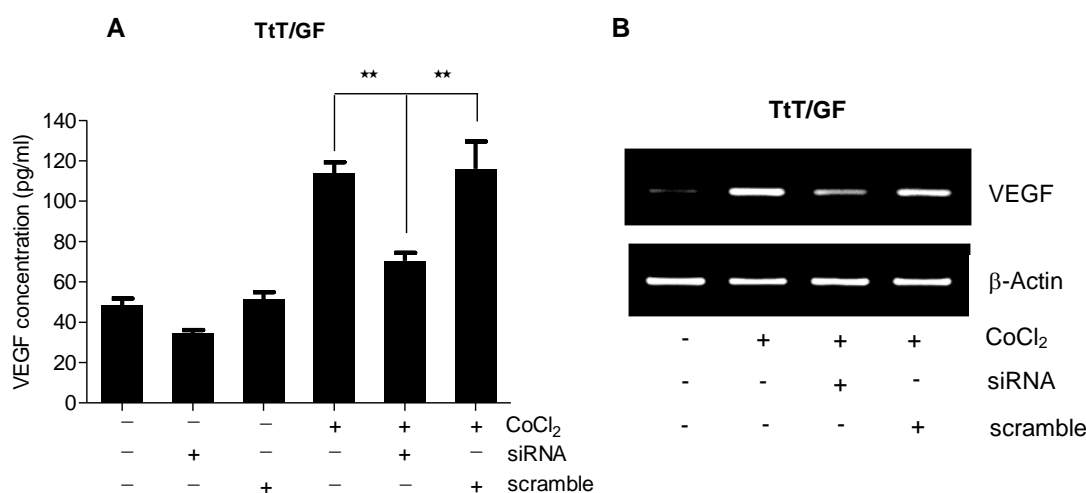


Figure 26. CoCl₂-stimulated VEGF expression was suppressed by RSUME knockdown at both protein and mRNA levels in TtT/GF cells. After 48 h transfection of RSUME siRNA, TtT/GF cells were treated with 250 μ M CoCl₂ for 24 h, and then VEGF ELISA (A) and RT-PCR (B) were performed to determine VEGF protein and mRNA expression. ELISA assay was carried out in quadruplicates and values were given as mean \pm SD. **, $P < 0.01$. Images of ethidium bromide agarose gel are representatives of 3 experiments with similar results.

For AtT20 cells, the effect of RSUME silencing was only tested at basal conditions, as it was shown that CoCl₂ treatment did not significantly enhance VEGF secretion in this cell line. To this end, 48 hours after transfection of RSUME siRNA, the culture supernatant of AtT20 cells was collected for VEGF ELISA, RNA from a part of cells was extracted for RT-PCR, and cells were also fixed to perform immunofluorescence studies. From VEGF ELISA and RT-PCR, it was found that basal VEGF expression was suppressed at both protein and mRNA levels by RSUME knockdown compared with un-transfected and scrambled siRNA transfected control

(Figure 27). From Immunofluorescence studies, VEGF staining was obviously weaker in cells with RSUME knockdown compared with un-transfected and scrambled siRNA transfected control, confirming that RSUME knockdown resulted in a significant reduction of basal VEGF expression in AtT20 cells (Figure 28).

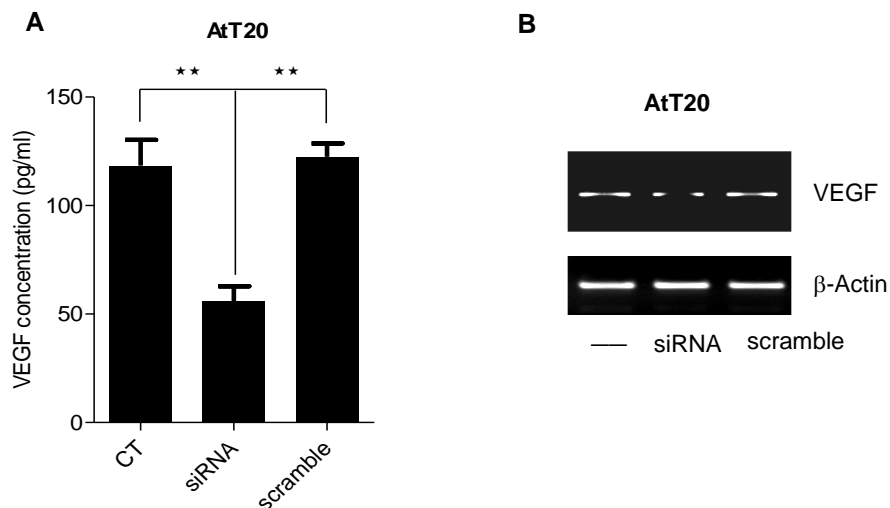


Figure 27. Basal VEGF expression was suppressed by RSUME knockdown at both protein and mRNA levels in AtT20 cells. After 48 h transfection of RSUME siRNA, VEGF ELISA (A) and RT-PCR (B) were performed to determine VEGF protein and mRNA expression. ELISA assay was carried out in quadruplicates and values were given as mean \pm SD. **, $P < 0.01$. Images of ethidium bromide agarose gel are representatives of 3 experiments with similar results.

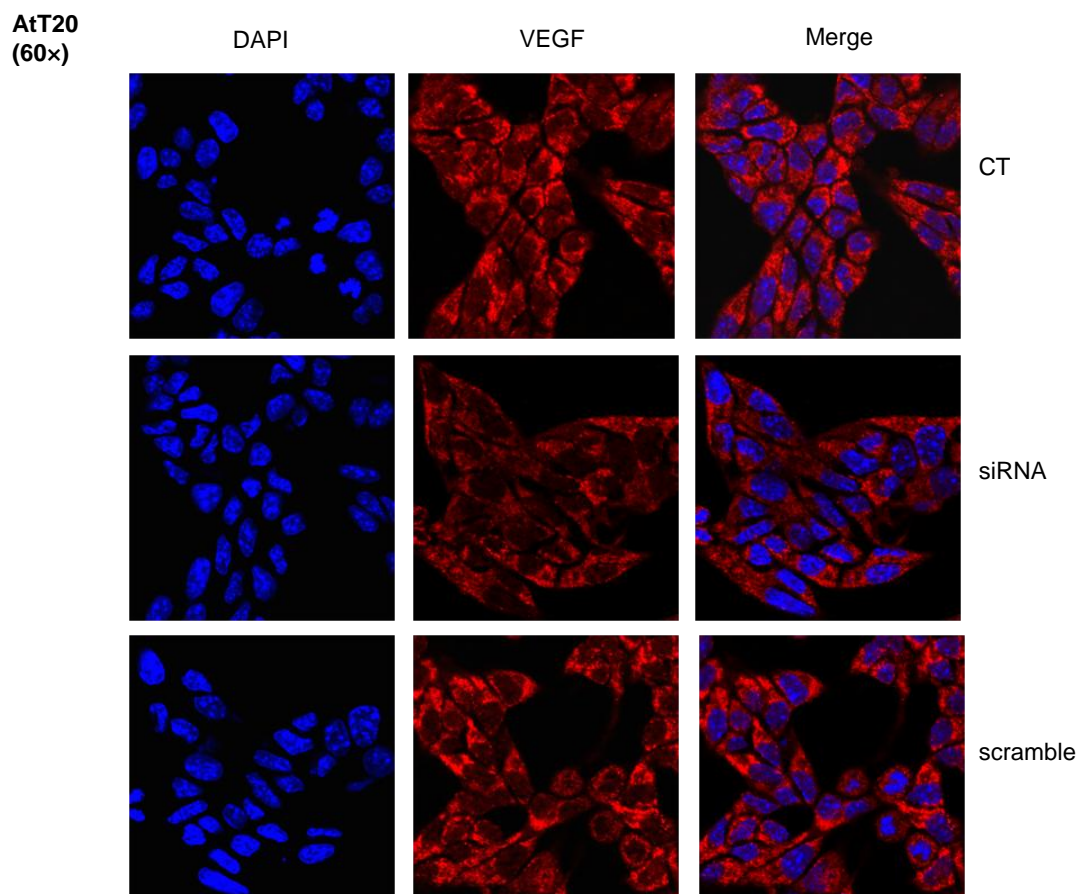


Figure 28. Basal VEGF production was suppressed by RSUME knockdown in AtT20 cells as shown by immunofluorescence assay. After 48 h transfection of RSUME siRNA, immunofluorescence was performed to stain VEGF. Red color corresponds to VEGF, and blue color corresponds to nucleus. The parameters were fixed when taking images by confocal microscopy using 60x objectives, and one representative image of 5 observations from two independent experiments with similar results is shown.

4.5.2 In primary human pituitary tumor cell cultures

RSUME knockdown was applied in primary human pituitary tumor cell cultures to find out whether RSUME is implicated in HIF-1 α activation as in mouse pituitary cell lines. Due to limited amount of primary tumor cells, the experiments on HIF-1 α and VEGF expression after RSUME knockdown could not be done in parallel.

Transfection of siRNA in primary human pituitary tumor cells was established for the first time in 6 primary cell cultures of human pituitary non-functioning adenomas, and BLOCK-iT™ Alexa Fluor® red fluorescent oligo was used to estimate transfection efficiency. With optimized transfection conditions, transfection efficiency was determined to be about 50% by counting red fluorescence stained cells in 100 cells 24 hours after transfection of fluorescent oligo (Figure 29). RNA was extracted for RT-PCR 24 hours after transfection of RSUME siRNA.

From RT-PCR, it was confirmed that 50 nM siRNA against human RSUME obviously suppressed RSUME mRNA in primary tumor cells (Figure 30).

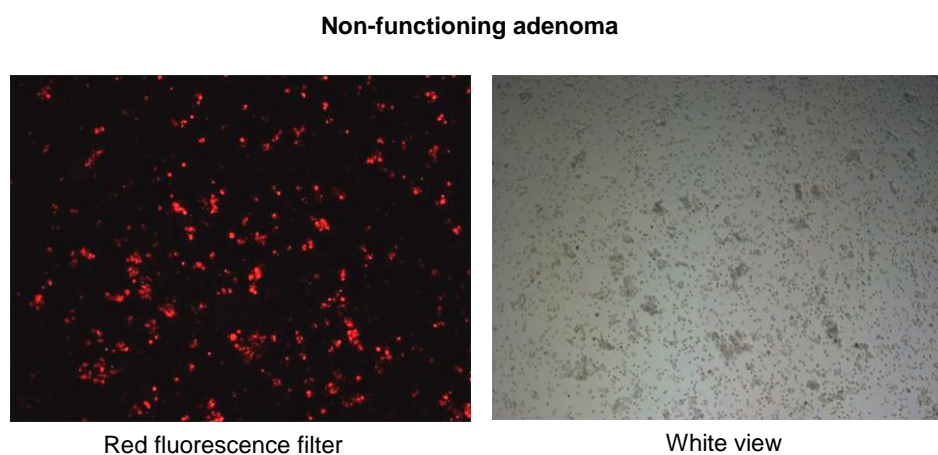


Figure 29. Evaluation of siRNA transfection efficiency in primary human pituitary tumor cells. The red fluorescent oligo was transfected into primary cells. 24 hours after transfection, the cells were observed under fluorescence microscope, and the number of cells with red color was counted in 100 cells to determine the transfection efficiency. The images were taken from fluorescence microscope using 10x objectives.

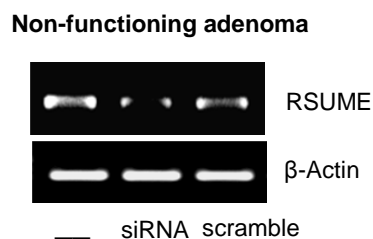


Figure 30. RSUME mRNA expression was suppressed by transfection of siRNA against human RSUME in primary human pituitary tumor cells. The primary human non-functioning tumor cells were transfected with siRNA against human RSUME, and RT-PCR was performed 24 hours after transfection.

With optimized transfection conditions, RSUME knockdown was also performed in the primary human cell cultures of 1 corticotroph adenoma and 1 somatotroph adenoma. 5 days after transfection of 20 nM or 50 nM RSUME siRNA, the corticotroph tumor cells were stimulated with 125 μ M CoCl₂ for 3 hours, and then HIF-1 α expression in cell lysates was determined by western blot. For the primary somatotroph tumor cells, 4 days after transfection of RSUME siRNA, cells were stimulated with 125 μ M CoCl₂ for 24 hours, and then the supernatant of cell culture was collected for VEGF ELISA. CoCl₂-stimulated HIF-1 α protein expression and VEGF secretion was significantly suppressed in that corticotroph adenoma and somatotroph

adenoma, respectively, compared with scrambled siRNA transfected controls (Figure 31 A, B). The suppression of HIF-1 α and VEGF expression by RSUME knockdown was not found until 5 days after transfection of siRNA.

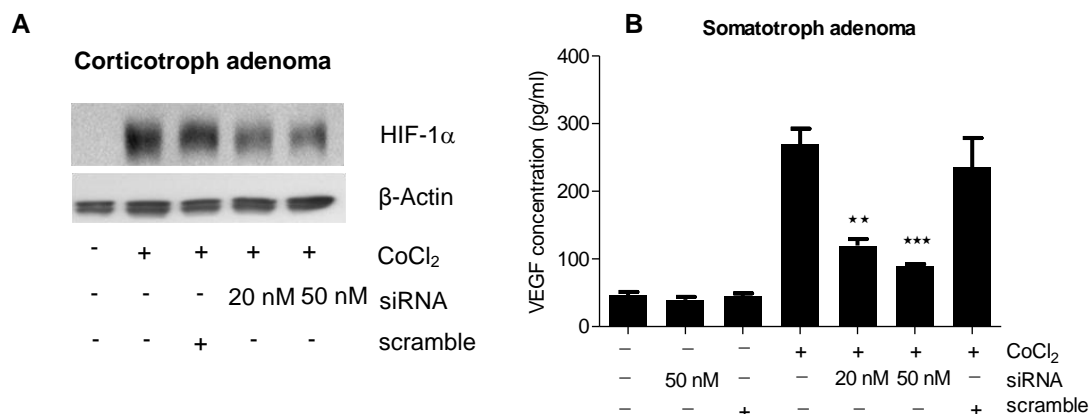


Figure 31. CoCl₂-stimulated HIF-1 α and VEGF secretion were suppressed by RSUME knockdown in primary human pituitary adenoma cells. The primary human corticotroph and somatotroph tumor cells were stimulated with CoCl₂ 5 days and 4 days after transfection of RSUME siRNA, respectively. HIF-1 α and VEGF expression were determined by western blot in corticotroph tumor cells (A) and by VEGF ELISA in somatotroph tumor cells (B), respectively. **, $P < 0.01$; ***, $P < 0.001$.

4.6 Effect of RSUME knockdown on proliferation and apoptosis in mouse pituitary cell lines

During the application of RSUME silencing, it was noticed that a part of cells appeared dead. Therefore, the effect of RSUME knockdown on proliferation and apoptosis in pituitary tumor cells was studied in AtT20 and TtT/GF cells.

AtT20 and TtT/GF cells were transfected with RSUME siRNA in 6-well plates. 24 hours after transfection, the cells were split into 96-well plates and cultured in medium containing 2% FCS for 48 hours. Afterwards, WST-1 assays were performed and the absorbance of samples was measured by a multiwell spectrophotometer set to 450 nm. Meanwhile, the cell number was also counted using a cell size-adapted coulter counter. By decreased absorbance values from WST-1 assay and the decreased cell number, RSUME knockdown significantly suppressed cell proliferation in both AtT20 and TtT/GF cells compared with un-transfected and scrambled siRNA transfected control (Figure 32 A, B).

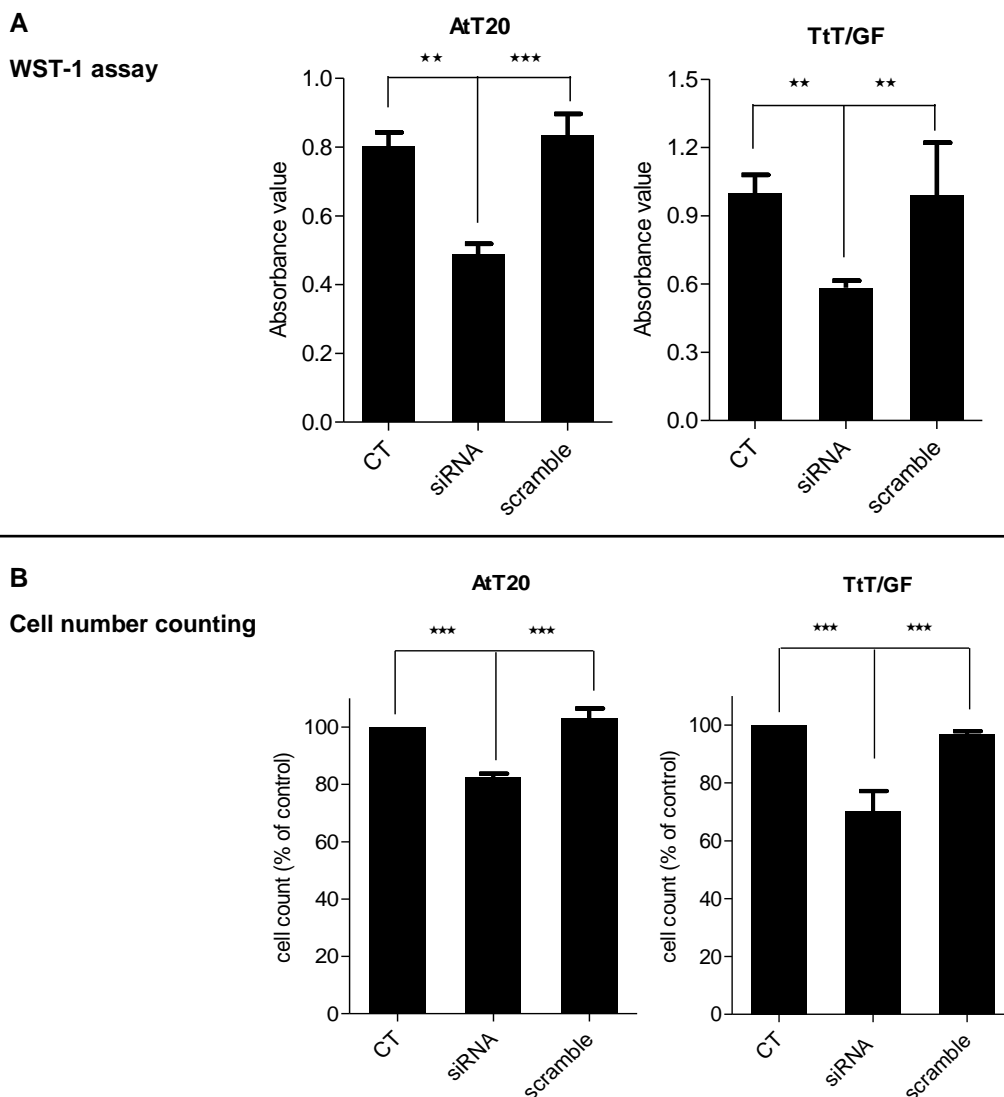


Figure 32. RSUME knockdown suppressed proliferation in both AtT20 and TtT/GF cells. AtT20 and TtT/GF cells were transfected with RSUME siRNA for 72 h, and then cell proliferation was determined by WST-1 assay (A) and cell number counting (B). **, $P < 0.01$; ***, $P < 0.001$. The WST-1 assay was carried out in quadruplicates and cell number counting was carried out in triplicates.

RSUME knockdown could be involved in induction of apoptosis besides in the suppression of cell proliferation in pituitary tumor cells. Therefore, studies on apoptosis in AtT20 and TtT/GF cells were carried out. AtT20 and TtT/GF cells were transfected with RSUME siRNA in 6-well plates. 24 hours after transfection, the cells were split into 96-well plates or slides and cultured in medium containing 2% FCS for 48 hours. Afterwards, apoptosis-associated DNA fragmentation was measured with a specific ELISA in 96-well plates, and the cells on slides were fixed to perform immunofluorescence studies on cleaved caspase-3. From cell death ELISA, a significant increase of apoptosis-associated DNA fragmentation by RSUME knockdown was observed in both AtT20 and TtT/GF cells compared with un-transfected and

scrambled siRNA transfected control (Figure 33). From immunofluorescence studies on cleaved caspase-3, the active fragment of a well-known pro-apoptotic protein caspase-3, it was found that the number of cells with immunoreaction of cleaved caspase-3 strongly increased by RSUME knockdown compared with un-transfected and scrambled siRNA transfected control in both AtT20 cells (Figure 34) and TtT/GF cells (data not shown). This finding indicates that the RSUME knockdown induces pro-apoptotic caspase-3, and thus confirms the involvement of RSUME in apoptosis of pituitary tumor cells.

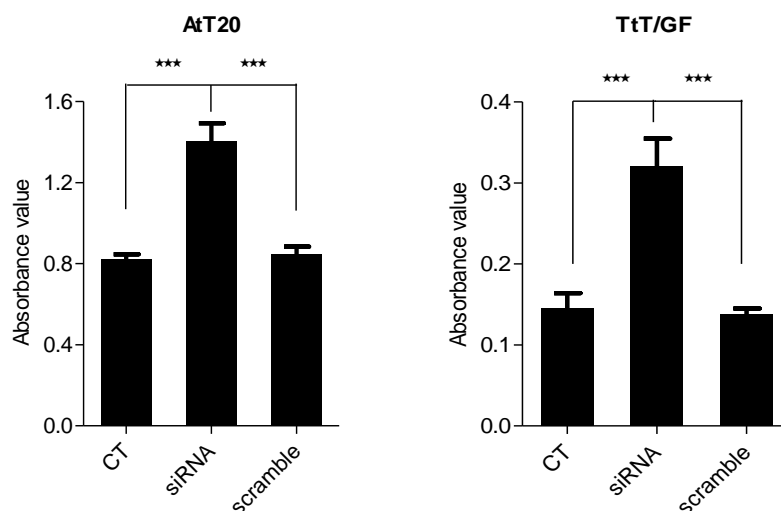


Figure 33. RSUME knockdown induced apoptosis in AtT20 and TtT/GF cells by cell death ELISA. AtT20 and TtT/GF cells were transfected with RSUME siRNA for 72 h, and then cell death ELISA was performed to detect apoptosis-associated DNA fragmentation. ***, $P < 0.001$. All the experiments were carried out in triplicates.

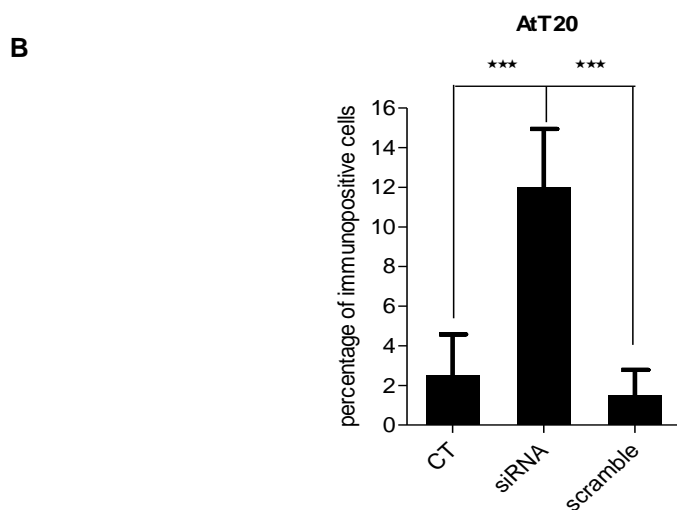
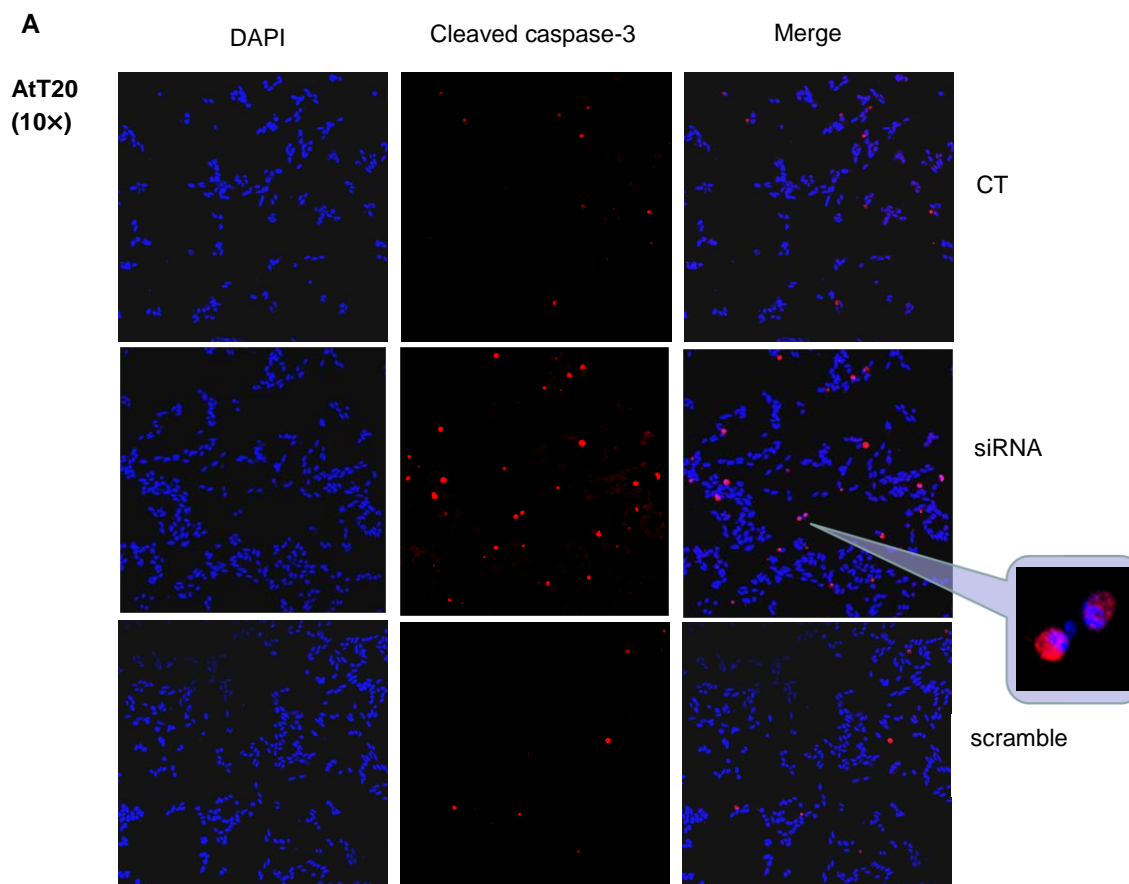


Figure 34. RSUME knockdown increased cleaved caspase-3 in AtT20 cells. AtT20 cells were transfected with RSUME siRNA for 72 h, and then immunofluorescence was performed to detect cleaved caspase-3. Red color corresponds to cleaved caspase-3, and blue color corresponds to nucleus. The images were taken by confocal microscopy using 10x objectives, and one representative image of 5 observations from two independent experiments with similar results is shown (A). The proportion of cells immunopositive for cleaved caspase-3 was determined by counting the number of positive cells out of 100 cells in five different areas of each slice (B). ***, $P < 0.001$.

5 Discussion

In the present study, the role of the novel sumoylation enhancer RSUME in pituitary tumorigenesis was comprehensively studied for the first time. In particular, its co-expression with HIF-1 α and VEGF in pituitary adenomas as well as its implication in the regulation of these two potent angiogenesis regulating factors in pituitary tumor cells was investigated. It could be demonstrated that RSUME critically regulates HIF-1 α and VEGF production and thus might play an important role in pituitary tumor neovascularization. Moreover, novel functions of RSUME on proliferation and survival of pituitary tumor cells have been identified suggesting that RSUME is of considerable importance for pituitary tumor development and progression and therefore, represents an interesting candidate for development of novel targeted therapies for the treatment of pituitary tumors.

An essential step in the development of any kind of solid tumor is the neovascularization of the expanding tumor mass by angiogenesis to supply the tumor cell with enough nutrients and oxygen (Carmeliet 2003). The expansion of the tumor mass causes intratumoral cellular hypoxia (Harris 2002), which induces the formation of the dimeric transcription factor HIF-1 by stabilizing its regulated subunit HIF-1 α . Activated HIF-1 triggers the expression of VEGF protein, which is a key mediator of angiogenesis (Carmeliet 2005). A large number of studies have demonstrated that vascularization is more advanced and HIF-1 α and VEGF are over-expressed in the majority of human cancers in comparison to their corresponding normal tissues (Ferrara 2004; Zhong, et al. 1999). However, so far little is known about HIF-1 α and VEGF regulation in pituitary adenomas due to limited and controversial information from few immunohistochemistry studies.

Herein the mRNA expression of RSUME as well as HIF-1 α and VEGF was studied in parallel in a series of 31 pituitary adenomas by quantitative RT-PCR and the results were compared with the findings from 3 normal human pituitaries. Expression analysis at mRNA level had been chosen since for comparative analysis at protein level, still no appropriate RSUME antibodies are available for immunohistochemistry. RSUME, HIF-1 α and VEGF mRNA levels were all slightly but not significantly enhanced in pituitary tumors in comparison to normal pituitaries.

This is the first time to show RSUME expression in pituitary adenomas, indicating its involvement in pituitary adenoma pathogenesis. Inspired by its function of enhancing HIF-1 α stabilization (Carbia-Nagashima, et al. 2007), if the appropriated RSUME antibodies are available, it would be interesting to explore RSUME protein expression in pituitary adenomas and its relationship with tumor microvessel density by immunohistochemistry, which could offer more evidences about the role of RSUME in angiogenesis of pituitary adenomas.

The present study showed HIF-1 α mRNA expression in both normal and adenomatous pituitaries, whereas one immunohistochemistry study showed no HIF-1 α staining in normal pituitary gland but distributed HIF-1 α staining in pituitary adenomas (Vidal, et al. 2003a). This could be explained by previous conclusions that the transcription and synthesis of HIF-1 α is constitutive but this protein is degraded rapidly in normoxia and stabilized in hypoxia (Ke and Costa 2006). The slight increase of HIF-1 α mRNA expression in pituitary adenomas compared with normal pituitaries shown here underlines that activated HIF-1 up-regulates target genes such as PDGF and triggers MAPK and PI3K pathways that contribute to up-regulating transcription of HIF-1 α (Semenza 2003; Zhang, et al. 2003). Such autocrine-signaling pathways are crucial for tumor progression.

Previous studies from ISH and immunohistochemistry have shown higher VEGF expression in the non-tumorous pituitary which is adjacent to tumor tissue compared with pituitary adenomas (Lloyd, et al. 1999). In contrast, a RT-PCR study using autopsy-derived pituitary as a normal control like in the present study showed the same result as obtained here that VEGF expression is higher in pituitary adenomas compared with normal pituitaries (McCabe, et al. 2002). The differing results between these studies may be caused by different controls. The non-tumorous pituitary tissue surrounding the tumor may theoretically be subject to subtle changes due to the presence of a tumor, whereas degradation and time to fixation may lead to loss of detectable antigens in autopsy tissue (Turner, et al. 2003).

In contrast to studies in other types of solid tumors, it has been shown that pituitary adenomas appear less vascularized than non-tumorous anterior pituitary tissues (Jugenburg, et al. 1995; Schechter 1972; Turner, et al. 2000b). However, higher HIF-1 α and VEGF expression is shown here in adenomatous tissues compared with normal pituitary gland. One of the explanations could be that HIF-1 α can induce growth factors such as IGF-1 and TGF- α to activate signal transduction pathways that lead to cell proliferation and survival under hypoxia (Feldser, et al. 1999; Krishnamachary, et al. 2003). Moreover, VEGF may not only play a role in angiogenesis but may also participate in proliferation and survival of pituitary tumor cells through VEGFR-1 as shown in our previous study (Onofri, et al. 2006). Additionally, non-tumorous pituitary tissue which is adjacent to tumor tissue may differ in its characteristics of vascularization from normal pituitary gland. Therefore, using normal tissue besides the tumor to address vascularization of normal pituitary is not convincing.

In this study, RSUME, HIF-1 α and VEGF mRNA expression is not significantly different among the various types of pituitary adenomas examined, which is consistent with previous studies. Despite this, there was a tendency that all of them appeared highest level in thyrotroph adenomas, and lowest level of HIF-1 α and VEGF were found in non-functioning adenomas and lowest level of RSUME was found in prolactinomas. One

immunohistochemistry study showed strongest staining of HIF-1 α in prolactinomas and weak staining in corticotroph adenomas (Yoshida, et al. 2005), whereas another immunohistochemistry study showed strongest HIF-1 α staining in GH-producing adenomas and lowest in corticotroph as well (Vidal, et al. 2003a). Additionally, previous immunohistochemistry study with a large cohorts of human pituitary adenomas showed strongest VEGF staining in GH, corticotroph, silent corticotroph, silent subtype3, and non-oncocytic null cell adenomas and relatively weak staining in PRL, gonadotroph and thyrotroph adenomas (Lloyd, et al. 1999), whereas another study by RT-PCR showed strongest VEGF staining in thyrotroph adenomas and lowest in GH-producing adenomas (Kim, et al. 2005). The present study could not confirm the results from the study mentioned above, and this is probably due to different investigating technology and limited sample number, and different classification of non-functioning adenomas. These controversial previous reports together with present study indicate that HIF-1 α and VEGF expression are not associated with pituitary adenoma histotypes. Whether RSUME expression has significant relationship with types of pituitary adenomas need to be clarified with more samples.

An immunohistochemistry study on angiogenesis by measurement of microvessel density (MVD) in pituitary adenomas showed highest and lowest MVD in thyrotroph adenomas and prolactinomas, respectively (Niveiro, et al. 2005). In the present study, RSUME mRNA expression level is highest in thyrotroph adenomas and lowest in prolactinomas, which is probably correlated with the MVD reported in the study mentioned above; however, relatively low level of RSUME mRNA expression did not correlate with relatively high MVD in non-functioning adenomas (Niveiro, et al. 2005). Although in the present study HIF-1 α and VEGF mRNA expression were highest in thyrotroph adenomas as well, they showed lowest level in non-functioning adenomas which have high MVD reported in some studies (Niveiro, et al. 2005; Turner, et al. 2000b). It was concluded that the present study could not correlate the HIF-1 α , VEGF mRNA expression with the microvessel density of pituitary adenomas described in previous study. This conclusion is consistent with other studies. One study using RT-PCR showed higher levels of HIF-1 α expression in pituitary carcinomas and GH-producing adenomas (Vidal, et al. 2003a), the most and least vascularized pituitary tumors, respectively, which was shown by another study (Vidal, et al. 2001). And a high rate of HIF-1 α has been shown in cases of non-small cell lung cancer with both high and low MVD (Giatromanolaki, et al. 2001). This could be explained by the hypothesis that HIF-1 α is required not for vessel formation but for the regular distribution of the vascular network, and disordered vasculature causes microenvironmental hypoxia which induces HIF-1 α activity (Vidal, et al. 2003a; Yu, et al. 2001). A study based on microvessel structural entropy has shown that rather than microvessel density, regular and less chaotic microvascular geometry contributes to increased

cell proliferation activity in PRL-producing tumors. This conclusion indicates that the interactions between vascular supply and pituitary tumor cell behavior may not be fully explained only by microvessel density (Vidal, et al. 2003a; Vidal, et al. 2003b). On the other hand, most of patients with prolactinomas or somatotroph adenomas have the history of dopamine agonist or somatostatin treatment that show anti-angiogenic effects (Gomez, et al. 2006; Novella-Maestre, et al. 2009), which probably leads to alteration in HIF-1 α , VEGF and RSUME expression. Therefore, it is necessary to perform separate studies for treated and untreated adenomas, and otherwise a comparison may not be valid.

Consistent with previous study, no correlation was found in the present study between HIF-1 α , VEGF expression and tumor grade, age and gender of patients (Viacava, et al. 2003; Vidal, et al. 2003a), and RSUME mRNA expression also did not correlate with these parameters. A relationship between tumor size and MVD in prolactinomas has been shown, and microprolactinomas were less vascular than macroprolactinomas; in contrast, there was no such a difference between vascular densities of microadenomas and macroadenomas producing GH (Turner, et al. 2000b). No significant difference was found in MVD between invasive and non-invasive pituitary adenomas (Jugenburg, et al. 1995; Vallar, et al. 1987), whereas another study showed significant higher MVD in invasive than non-invasive PRL-producing adenomas but not in GH and ACTH-producing adenomas (Vallar, et al. 1987). Therefore, it seems that HIF-1 α , VEGF and RSUME expression and even MVD are not significantly implicated in pituitary adenoma behavior. However, HIF-1 α , VEGF and MVD are significantly higher in pituitary carcinoma than adenomas (Jugenburg, et al. 1995; Lloyd, et al. 1999; Turner, et al. 2000a; Vidal, et al. 2003a), indicating the up-regulation of HIF-1 α and VEGF during pituitary tumor progression and also a relationship between aggressive pituitary behavior and angiogenesis.

Based on previous observations that RSUME acts through enhancing sumoylation on HIF-1 α expression (Carbia-Nagashima, et al. 2007) and thus probably on angiogenesis-regulating VEGF, correlation analyses among RSUME, HIF-1 α and VEGF mRNA expression were performed in pituitary tumors. HIF-1 α mRNA significantly and positively correlated with RSUME and VEGF mRNA expression in pituitary tumors indicating an interrelationship among these factors in pituitary adenomas.

Regulation of VEGF expression has been studied previously in pituitary adenomas, and it has been shown that several stimuli such as IL-6, estradiol and pituitary adenylate cyclase activating peptide (PACAP) up-regulate VEGF expression (Banerjee, et al. 1997; Gloddek, et al. 1999), whereas glucocorticoids inhibit VEGF (Lohrer, et al. 2001). However, whether VEGF can be induced in pituitary adenomas by hypoxia through HIF-1 signaling pathway involving RSUME has not yet been studied.

In the present study, CoCl_2 was chosen to simulate hypoxia condition. CoCl_2 is a well established hypoxia mimicking substance (Ebert and Bunn 1999; Webb, et al. 2009). Co^{2+} are thought to mimic the hypoxia by binding instead of Fe^{2+} to the heme molecules which are regarded as oxygen sensors in both mammalian and bacteria, causing decreased oxygen affinity due to a conformational change (Ebert and Bunn 1999; Goldberg, et al. 1988). Additionally, It has been shown that sharing similar mechanism in hypoxia Co^{2+} are able to stabilize HIF-1 α in normoxia by substituting Fe^{2+} from the Fe^{2+} -binding site of PHDs, leading to inhibition of PHDs activity (Masson and Ratcliffe 2003), and Co^{2+} inhibits the Interaction between HIF-1 α and VHL Protein by direct binding to HIF-1 α (Yuan, et al. 2003). Moreover, like in hypoxia, Co^{2+} activates mRNA transcription of hypoxia response genes such as erythropoietin, glycolytic enzymes and VEGF by stimulating reactive oxygen species (ROS) generation, although mitochondrial-dependent ROS generation was found in hypoxia but not found after Co^{2+} exposure (Chandel, et al. 1998).

In order to test whether RSUME responds to hypoxia in pituitary tumor cells, stimulation with CoCl_2 was performed. RSUME mRNA expression was shown to increase within 30 minutes after CoCl_2 stimulation and to go down within 2 hours in mouse pituitary cell lines and primary human pituitary adenoma cell cultures. This is not paradoxical in view of the results obtained for HIF-1 α which reached its accumulation peak 2 hours after the onset of CoCl_2 stimulation. As shown in the previous study, RUSME enhances HIF-1 α stabilization by facilitating SUMO transfer (Carbia-Nagashima, et al. 2007), therefore RSUME mRNA expression is probably down-regulated after SUMO chain formation. Due to lack of proper antibody of RSUME, determination of RSUME at protein level could not be performed.

It was next tested whether CoCl_2 induces HIF-1 α and VEGF in mouse pituitary cell lines and as well as in primary cell cultures of human pituitary adenomas. As shown in other cell types (Liu, et al. 1999; Okada, et al. 1998; Shima, et al. 1995; Steinbrech, et al. 2000; Webb, et al. 2009), CoCl_2 treatment time- and dose-dependently stimulated HIF-1 α protein expression in AtT-20, TtT/GF cell lines and all the tested human pituitary adenoma cultures, and HIF-1 α accumulation in nucleus was shown in both AtT20 and TtT/GF cells. A significant rise in VEGF release by CoCl_2 stimulation following a time- and dose- dependent manner was found in TtT/GF cells and all the human pituitary adenoma cell cultures, indicating that HIF-1 α can be induced and activated in hypoxia-mimicking conditions in pituitary adenoma cells.

Since neither RSUME inhibitors nor neutralizing antibodies are actually available, RSUME was down regulated by siRNA technology to further clarify the involvement of RSUME in regulation of HIF-1 α and VEGF in pituitary adenomas. Transfection of siRNA in primary human pituitary tumor cells was established for the first time. siRNA against RSUME strongly down-regulated RSUME expression examined by RT-PCR and significantly reduced the HIF-

1 α expression in both mouse pituitary cell lines and primary human pituitary tumor cells. Additionally, it was shown that the RSUME knockdown abolished nucleus accumulation of HIF-1 α in both AtT20 and TtT/GF cell lines, and also decreased CoCl₂-stimulated VEGF secretion in TtT/GF cells and primary human pituitary tumor cells. These findings suggest that RSUME is required in HIF-1 activation in pituitary tumor cells under hypoxia-mimicking conditions. Although all these experiments with CoCl₂ need to be confirmed under real hypoxic conditions at 1% oxygen, the results shown here already point to an essential role of RSUME in the induction of angiogenic processes in pituitary adenomas, and probably other types of solid tumors.

In the present study, a significant rise in VEGF release by CoCl₂ stimulation was not found in AtT20 cells, but RSUME knockdown significantly decreased basal secretion of VEGF in AtT20 cells. It is known that AtT20 cells basally secrete the highest level of VEGF compared with other pituitary tumor cell lines such as TtT/GF, GH3 and α T3-1 (Lohrer, et al. 2001). From previous studies, VEGF is also regulated by other mechanisms besides hypoxia. It has been demonstrated that the gene promoter of VEGF contains consensus binding sites for transcription factor β -catenin/TCF complex, and VEGF is up-regulated by transfection of normal colon epithelial cells with activated β -catenin (Easwaran, et al. 2003). The transcriptional activity of β -catenin/TCF complex is reduced when TCF-4 lacks SUMO attachment sites, suggesting that sumoylation activates β -catenin/TCF (Yamamoto, et al. 2003). Activated β -catenin/TCF signaling may be responsible for the constitutively high secretion of VEGF by AtT20 cells, and CoCl₂ induced HIF-1 accumulation may not be able further stimulate already saturated VEGF production. Additionally, RSUME knockdown in AtT20 cells probably leads to lack of sumoylation of TCF-4, and thus abolishes the activity of β -catenin/TCF, which results in decreased VEGF expression.

Since RSUME has been detected only recently, little is known about other functions beyond its action on HIF-1 α stabilization and thus, angiogenesis. Interestingly, it was found that a part of cells were floating after RSUME knockdown, whereas the control cells without RSUME silencing had no changes. Therefore, the putative role of RSUME is implicated in the regulation of proliferation and survival was investigated in the present study for the first time. RSUME knockdown by siRNA in pituitary tumor cell lines led to a significant suppression of proliferation as shown by WST-1 assay and direct cell number counting. Cell death ELISA and detection of cleaved caspase-3 proved that apoptosis was induced by RSUME knockdown. This would mean that in pituitary adenomas in which RSUME is present and probably over-expressed, this factor would stimulate pituitary adenoma cell proliferation and would prevent intratumoral apoptosis and thus in total would support pituitary adenoma progression.

Some apoptosis key regulators involved in human cancers which have been shown to be regulated by sumoylation may be affected also in pituitary tumor cells by the sumoylation enhancer RSUME (Alarcon-Vargas and Ronai 2002; Kim and Baek 2006). For instance, it has already been demonstrated, that RSUME can block the transcriptional activity of nuclear factor- κ B (NF- κ B) by enhancing the sumoylation of I κ B (Carbia-Nagashima *et al.* 2007). Sumoylated I κ B escapes from ubiquitin-mediated degradation and acts as the inhibitor of NF- κ B. NF- κ B generally acts as a tumor promoter through up-regulating anti-apoptotic proteins; however it is sometimes pro-apoptotic depending on cell types, stimuli as well as the subunit involved (Radhakrishnan and Kamalakaran 2006). It has been shown that inhibition of NF- κ B activity combined with expression of oncogenic Ras in epidermal keratinocytes leads to invasive neoplasia with features similar to those of squamous cell carcinoma (Dajee, *et al.* 2003; Dutta, *et al.* 2006; Kim and Baek 2006). From our previous study, NF- κ B is expressed in normal anterior pituitary and in pituitary adenomas and facilitates the inhibitory effect of interferon- γ on *POMC* gene transcription in AtT20 cells (Labeur, *et al.* 2008). It is speculated that NF- κ B activation by RSUME knockdown could be responsible for the here observed apoptosis induction in pituitary tumor cells.

The sumoylation of the important tumor suppressor p53 is well studied. It has been demonstrated that PIAS1 (protein inhibitor of activated STAT-1), which is an E3-like ligase, promotes sumoylation of p53 and in this way represses its transcriptional activity, suggesting that sumoylation of p53 restricts its ability to control cell death and/or growth (Alarcon-Vargas and Ronai 2002; Schmidt and Muller 2002). Furthermore, Mdm2 which mediates ubiquitination and degradation of p53 is also SUMO-modified (Alarcon-Vargas and Ronai 2002; Buschmann, *et al.* 2000). Sumoylation of Mdm2 enhances its stabilization and increases its activity of mediating degradation of p53 through ubiquitination (Buschmann, *et al.* 2000). Therefore, it was speculated that the activation of p53 by RSUME silencing through abolished sumoylation of p53 and Mdm2 contributes to apoptosis induction in pituitary tumor cells.

Alternatively, the pro-apoptotic effects of RSUME knockdown on pituitary adenoma cell proliferation may be explained by the down-regulation of HIF-1 α and VEGF, since HIF-1 α and VEGF not only promote angiogenesis but also facilitate cell proliferation and survival (Feldser, et al. 1999; Krishnamachary, et al. 2003; Lee, et al. 2004; Onofri, et al. 2006). Through supporting pituitary tumor neovascularization and growth in parallel, RSUME might enhance pituitary adenoma progression and therefore represents a putative therapeutic target for the development of novel concepts for the treatment of human pituitary tumors (Figure 35).

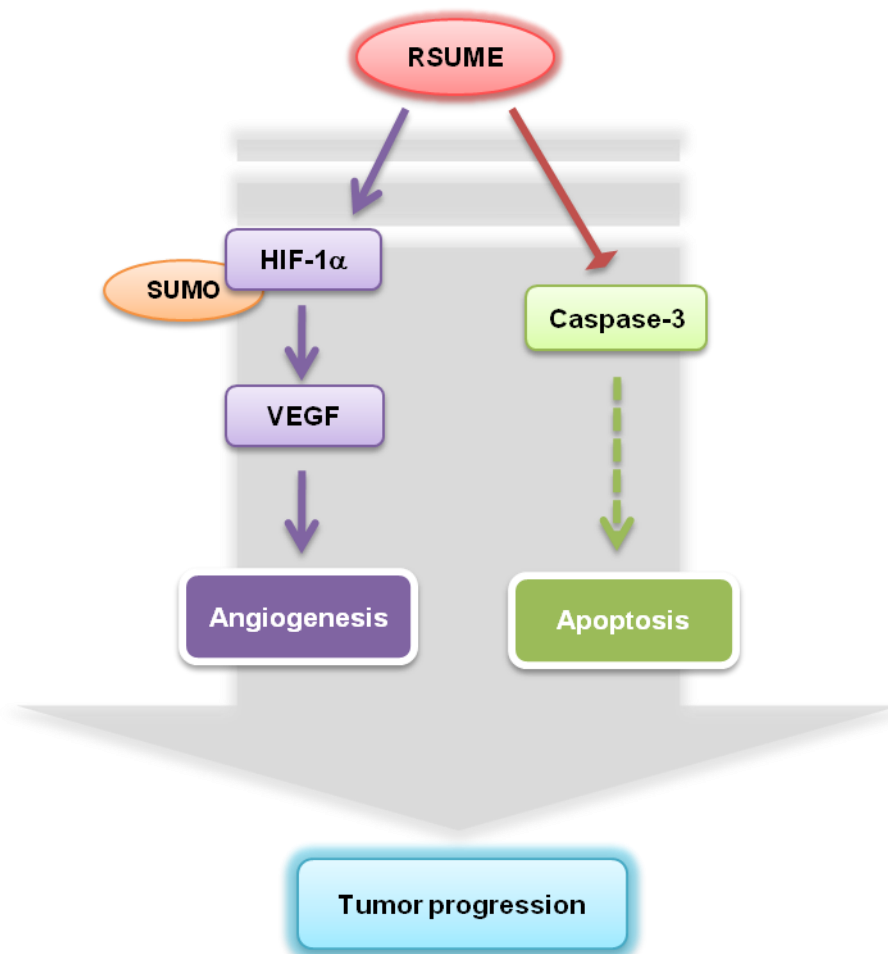


Figure 35. Schematic model for the role of RSUME on pituitary tumor pathogenesis.

It is proposed that RSUME enhances pituitary tumor progression through supporting tumor angiogenesis and growth as well as inhibiting tumor cell apoptosis.

Sumoylation consequences are variable with different substrates and different cell types, and therefore sumoylation may promote or inhibit tumor progression. In Wnt signaling pathway, sumoylation of TCF-4 enhances its transcriptional activity, thus enhances its target gene expression, including some proto-oncogenes such as c-Myc, c-jun and cyclin-D1 (He, et al. 1998; Mann, et al. 1999; Tetsu and McCormick 1999; Yamamoto, et al. 2003). SUMO-

conjugating enzyme Ubc9 is over-expressed in several human tumors such as lung adenocarcinoma, ovarian carcinoma and melanoma, and several approaches to target Ubc9 functions are in experimental phases to explore the therapeutic application (Hoeller and Dikic 2009). However, sumoylation may reversely suppress tumor progression. It has been shown that sumoylation negatively regulates c-jun activity, thereby limits its oncogenic capacity (Muller, et al. 2000). Additionally, arsenic trioxide, which stimulates degradation of oncogenic fusion protein PML-RAR- α by enhancing its sumoylation, is currently used in clinics as an efficient therapeutic agent for the treatment of patients with APL (acute promyelocytic leukemia) (Hoeller and Dikic 2009). As a sumoylation enhancer, RSUME could adopt both tumor-promoting and tumor-suppressing roles, and it would be meaningful to perform more extensive studies on the exploration of RSUME actions and putative therapeutic strategies not only in human pituitary tumors but also in other types of human cancers.

6 Summary

Despite considerable progress, neither the processes involved in the genesis nor the mechanisms triggering the progression of pituitary adenomas are well understood. A large number of studies have shown that HIF-1 α and VEGF play essential roles in growth and progression of a wide variety of solid tumors by promoting tumor neovascularization. However, so far only few reports focus on the role of these two most powerful, hypoxia-induced angiogenic factors in slowly growing and poorly vascularized pituitary adenomas. RSUME was recently isolated from experimentally induced pituitary tumors in nude mice, and was shown to increase stability and transcriptional activity of HIF-1 α in monkey kidney cell line COS-7 by acting as a sumoylation enhancer. These findings suggest a putative role of RSUME in pituitary tumor formation and progression.

In the present study, the pathological role of RSUME in angiogenesis and progression of pituitary adenomas was studied for the first time in mouse pituitary cell lines AtT20 and TtT/GF as well as in human pituitary adenoma cells.

By real-time quantitative PCR, it was demonstrated that RSUME, HIF-1 α and VEGF mRNA expression tended to be over-expressed in human pituitary adenomas compared with the expression levels in normal pituitaries. However, no significant differences among adenoma types, tumor grade, age or gender of patients were observed. Nevertheless, correlation analyses showed that HIF-1 α mRNA level was significantly correlated with VEGF and RSUME mRNA levels in the examined pituitary adenomas, giving the first evidence for the interference of RSUME with the two major angiogenic factors and thus, with pituitary adenoma neovascularization.

To further explore the angiogenic role of RSUME, the hypoxia-mimicking substance CoCl₂ was applied in mouse pituitary cell lines and primary human pituitary tumor cell cultures to investigate the regulatory role of RSUME on HIF-1 α and VEGF. Under CoCl₂ treatment, RSUME mRNA expression was significantly enhanced and HIF-1 α with translocation into nucleus was remarkably increased at protein level. The increased transcriptional activity of HIF-1 α was demonstrated by a significant elevation of the VEGF production in pituitary tumor cells. In order to test whether RSUME was implicated in up-regulation of HIF-1 α and VEGF under hypoxia-mimicking conditions, the corresponding experiments were performed after RSUME knockdown using the siRNA technology. This approach was not only performed in easily transfectable pituitary tumor cell lines but also for the first time in primary cell cultures of human pituitary adenomas. RSUME knockdown significantly suppressed HIF-1 α and VEGF expression in mouse pituitary tumor cell lines and primary cell cultures of human pituitary adenomas, indicating that RSUME is critically involved in pituitary tumor angiogenesis through regulating HIF-1 α and VEGF production.

As changes in pituitary tumor cell viability were noticed after RSUME silencing, this factor may have additional functions in pituitary tumors. Indeed, RSUME knockdown was shown to inhibit pituitary tumor cell proliferation and to enhance the apoptosis in these cells. Therefore, in addition to its pro-angiogenic action, RSUME has proliferative and anti-apoptotic activities in pituitary tumor cells.

In conclusion, the findings of the present study suggest that, the novel protein RSUME is an important player in pituitary tumor pathogenesis by supporting intratumoral angiogenesis, stimulating pituitary tumor cell proliferation and inhibiting tumor cell apoptosis. Therefore, this factor could represent a novel future target for the development of new concepts for the treatment of pituitary tumors.

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8 Abbreviations

| | |
|---------------|---|
| 2-OG | 2-oxoglutarate |
| α -MSH | Alpha-melanocyte stimulating hormone |
| ACTH | Adrenocorticotropic hormone |
| AHR | Aryl hydrocarbon receptor |
| AIP | Aryl hydrocarbon receptor interacting protein |
| APL | Acute promyelocytic leukemia |
| ARD1 | Arrest-defective-1 |
| AVP | Arginine vasopressin |
| ARNT | Aryl hydrocarbon receptor nuclear translocator |
| bFGF | Basic fibroblast growth factor |
| bHLH | Basic helix-loop-helix |
| CD31 | Platelet endothelial cell adhesion molecule |
| CDK | Cyclin-dependent kinase |
| CDKI | Cyclin-dependent kinase inhibitor |
| CRH | Corticotropin-releasing hormone |
| D2R | Dopamine 2 receptor |
| EC | Endothelial cell |
| ECM | Extracellular matrix |
| ER | Estrogen receptor |
| F8 | Endothelial antigens factor eight-related antigen |
| FIH | Factor inhibiting HIF-1 |
| FS | Folliculostellate cell |
| FSH | Follicle-stimulating hormone |
| GFPA | Glial fibrillary acidic protein |
| GH | Growth hormone |
| GHRH | Growth-hormone-releasing hormone |
| GnRH | Gonadotropin-releasing hormone |
| GR | Glucocorticoid receptor |
| HIF-1 | Hypoxia inducible factor-1 |
| HRE | Hypoxia response element |

| | |
|----------------|---|
| IGF-1 | Insulin-like growth factor-1 |
| IL-6 | Interleukin-6 |
| LH | Luteinizing hormone |
| LOH | Loss of heterozygosity |
| MAPK | Mitogen-activated protein kinase |
| MEN1 | Multiple endocrine type 1 |
| MMP | Matrix metalloproteinase |
| mTOR | Mammalian target of rapamycin |
| MVD | Microvessel density |
| NF- κ B | Nuclear factor- κ B |
| NGF | Nerve growth factor |
| ODDD | Oxygen-dependent degradation domain |
| PAS domain | Per-ARNT-Sim domain |
| PAS stain | Periodic acid-Schiff stain |
| PDGF | Platelet-derived growth factor |
| PHD | Prolyl hydroxylase |
| PI3K | Phosphatidylinositol 3-kinase |
| PIAS1 | Protein inhibitor of activated STAT-1 |
| Pit-1 | Pituitary transcription factor 1 |
| PKA | Protein kinase-A |
| PML | Promyelocytic leukemia protein |
| POMC | Pro-opiomelanocortin |
| pRb | Retinoblastoma protein |
| PRL | Prolactin |
| Prop1 | Prophet of pituitary transcription factor 1 |
| PTAG | Pituitary tumor apoptosis gene |
| PTTG | Pituitary tumor transforming gene |
| RAR | Retinoic acid receptor |
| SAE | SUMO-activating enzyme |
| SF-1 | Steroidogenic factor-1 |
| STAT | Signal transduction and transcription protein |
| SUMO | Small ubiquitin-related modifier |

| | |
|--------------|------------------------------------|
| TAD | Transactivation domain |
| TGF- β | Transforming growth factor-beta |
| Tpit-1 | T-box transcription factor |
| TR β | Thyroid hormone receptor β |
| TRH | Thyrotropin-releasing hormone |
| TSH | Thyroid-stimulating hormone |
| Ubc9 | Ubiquitin-conjugating 9 |
| UEA1 | Ulex europaeus agglutinin 1 |
| VEGF | Vascular endothelial growth factor |
| VHL | Von Hippel-lindau |
| VSMC | Vascular smooth muscle cell |

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