

RSUME is implicated in HIF-1-induced VEGF-A production in pituitary tumour cells

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Abstract

The recently cloned small RWD-domain containing protein RSUME was shown to increase protein levels of hypoxia-inducible factor-1 α (HIF-1 α). The latter is the oxygen-regulated subunit of HIF-1, the most important transcription factor of the cellular adaptive processes to hypoxic conditions. It is also a major regulator of vascular endothelial growth factor-A (VEGF-A), which is critically involved in the complex process of tumour neovascularisation. In this study, the expression and role of RSUME in pituitary tumours was studied. We found that RSUME mRNA was up-regulated in pituitary adenomas and significantly correlated with HIF-1 α mRNA levels. Hypoxia (1% O₂) or treatment with hypoxia-mimicking CoCl₂ enhanced RSUME and HIF-1 α expression, induced translocation of HIF-1 α to the nuclei and stimulated VEGF-A production both in pituitary tumour cell lines and primary human pituitary adenoma cell cultures. When RSUME expression was specifically down-regulated by siRNA, the CoCl₂-induced increase VEGF-A secretion was strongly reduced which was shown to be a consequence of the RSUME knockdown-associated reduction of HIF-1 α synthesis. Thus, RSUME plays an important role in initiating pituitary tumour neovascularisation through regulating HIF-1 α levels and subsequent VEGF-A production and may therefore be critically involved in pituitary adenoma progression.

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Introduction

RSUME is a small RWD-domain containing protein that was recently cloned from lactosomatotroph GH3 rat pituitary tumour cells overexpressing the cytokine signal transducing protein gp130 (Carbia-Nagashima *et al.* 2007). These cells form rapidly growing and densely vascularised tumours when injected subcutaneously in nude mice, and show the opposite phenotype of gp130-depleted GH3-derived tumours, which are small and poorly vascularised (Castro *et al.* 2003). It was shown that RSUME enhances sumoylation, is induced by hypoxia, and increases the level of hypoxia-inducible factor-1 α (HIF-1 α) protein (Carbia-Nagashima *et al.* 2007), the oxygen-regulated subunit of HIF-1.

HIF-1 is a heterodimeric transcription factor and represents the most important regulator of cellular

adaptive processes to hypoxic conditions (Wang *et al.* 1995, Wei & Yu 2007). It is composed of the constitutively expressed HIF-1 β subunit, and the oxygen-regulated HIF-1 α subunit. Although HIF-1 α protein is continuously synthesised, it is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions (Jaakkola *et al.* 2001, Appelhoff *et al.* 2004, Wei & Yu 2007, Webb *et al.* 2009). Thus, little or no heterodimeric HIF-1 protein is available under normoxia. However, when the oxygen availability declines, HIF-1 α degradation is rapidly inhibited and thus intracellular HIF-1 protein levels strongly increase (Wei & Yu 2007, Webb *et al.* 2009). HIF-1 then induces genes involved in multi-faceted actions in tissues that have impaired oxygen supply due to ischaemia of different origin (Hickey & Simon 2006). Moreover, HIF-1 α is not only regulated by

O₂-dependent mechanisms but also stabilised by intracellular signalling components (MAP kinases, mTOR and others) of growth factors that affect angiogenesis (e.g. EGF, PDGF or IGF1; Mylonis et al. 2006, Flugel et al. 2007, Land & Tee 2007, Wei & Yu 2007). In contrast to these physiological functions, HIF-1 also plays a significant role in pathological vascularisation, among them tumour neovascularisation (Harris 2002, Carmeliet 2003). In growing solid tumours, oxygen and nutrient supply through diffusion is only possible to a tumour diameter of 1–2 mm. Further expanding tumours show cellular hypoxia which leads to a rise in HIF-1 protein expression leading to the production of angiogenic factors, such as vascular endothelial growth factor-A (VEGF-A), which is involved in the sprouting of new vessels into the growing tumour (Carmeliet 2003). Indeed, VEGF-A is one key factor in this process acting through different receptors to induce blood and lymph vessel formation (Ferrara 2004).

As in many kinds of tumours, neovascularisation through angiogenesis is essential for pituitary adenoma expansion (Turner et al. 2000, Onofri et al. 2006). Since pituitary tumours grow very slowly in most cases, the vessel density of pituitary adenomas is mostly lower than that of the densely vascularised normal pituitary (Vidal et al. 2001, Onofri et al. 2006). However, VEGF-A is slightly overexpressed in the majority of pituitary adenomas and interestingly, VEGF-A is acting not only as an angiogenic but also as a growth factor on pituitary adenomas through differently expressed VEGF-A receptors in tumour and vessel cells of pituitary adenomas (Onofri et al. 2006).

By organotypic expression analysis, the anterior pituitary had been identified as one of the organs with the strongest basal RSUME expression suggesting a specific and important but not yet identified physiological and pathophysiological role of this protein in normal and tumoural pituitary (Carbia-Nagashima et al. 2007). In this study, we demonstrate RSUME expression in pituitary adenomas and its involvement in the regulation of HIF-1 α and VEGF-A production in these tumours, suggesting an important role of RSUME in the process of pituitary adenoma neovascularisation.

Materials and methods

Materials

Cell culture materials and most reagents were obtained from Life Technologies, Falcon (Heidelberg, Germany), Nunc (Wiesbaden, Germany), Seromed (Berlin, Germany) and Sigma.

Human pituitary and pituitary adenoma tissues

This study was performed after approval of the local ethics committee (Ethics Grant No. 141-07) and informed written consent was received from each patient whose pituitary adenoma tissue was used in the study. Normal human pituitaries ($n=3$) obtained from autopsies, and a part of the tumour tissue received after transsphenoidal surgery from patients with 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 (10–20 min after surgery), stored at -80°C and later used for mRNA isolation for RT-PCR assays. If additional tumour tissue was left, it was used for primary cell culture (see below). In patients pre-treated with somatostatin analogues or dopamine agonists, medication was stopped at least 1 week before surgery.

Cell culture

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

For primary human pituitary tumour cell culture, adenoma tissue was dispersed and the obtained pituitary tumour cells were cultivated as described previously (Renner et al. 1998). The human adenoma cells attached to the plastic wells within 48 h and were then used for stimulation experiments. Depending on the amount of tumour tissue obtained, limited numbers of human pituitary adenoma cells were available after dispersion for primary cell culture. Therefore, it was not possible to perform all HIF-1 α and VEGF-A stimulation experiments in all adenomas in parallel.

Treatment of cells under hypoxia or CoCl₂

Before hypoxia exposure in a hypoxic chamber or CoCl₂ (Sigma) treatment (=hypoxia-mimicking condition), cell lines or primary cell cultures were incubated in medium containing 1% FCS overnight and all subsequent treatments were performed in the same medium. For exposure to hypoxia, 300 000 AtT20 or 150 000 TtT/GF cells were cultured in a six-well plate and incubated at normoxia or hypoxia (1% O₂) for the indicated time periods. For CoCl₂ treatment, cell lines or primary cell cultures were treated with different concentrations (range: 31.2–500 μM) of CoCl₂ for different time periods as indicated, and untreated cells were used as a control in

each experiment. Because in control experiments no difference was found between incubation in the hypoxic chamber and the treatment with hypoxia-mimicking CoCl_2 , the majority of the experiments with the cell lines and all studies with primary pituitary adenoma cell cultures were performed under hypoxia-mimicking conditions due to easier handling. Even after 72 h exposure to the highest concentration of CoCl_2 , no effect on the viability of the treated pituitary tumour cells was observed excluding unspecific, putative toxic effects of CoCl_2 .

Transfection of pituitary cell lines with siRNA against mouse RSUME and mouse HIF-1 α

The siRNA against mouse RSUME (5'-GGA CTT GTG GGT GAG GAT G-3'; *Carbia-Nagashima et al. 2007*) and siRNA against mouse HIF-1 α (5'-CAC CAT GAT ATG TTT ACT A-3'; *Chen et al. 2011*) were transfected into cells with lipofectamine 2000 (Invitrogen) to suppress endogenous RSUME and HIF-1 α expression. Scrambled siRNA (100 nM; Scramble II, MWG Biotech, Ebersberg, Germany) was used as a control. Cells were used 24 or 48 h post-transfection for experiments to study the influence of siRNA on VEGF-A mRNA synthesis (RT-PCR) and cellular protein expression (western blot, immunocytochemistry) as well as on VEGF-A secretion (ELISA).

Transfection of primary human pituitary tumour cells with siRNA against human RSUME

Whereas transfection of pituitary tumour cell lines with oligonucleotides is well established, we developed in this study for the first time a method enabling transfection of siRNA into primary human pituitary adenoma cells. BLOCK-iT Alexa Fluor red fluorescent oligo (Invitrogen Corp.) was used for determination of transfection efficiency. The BLOCK-iT Alexa Fluor red fluorescent oligo is Alexa Fluor 555-labelled, double-stranded RNA (dsRNA) oligomer. It is designed for use in RNAi analysis to facilitate assessment and optimisation of dsRNA oligonucleotides delivery into mammalian cells using cationic lipids (such as lipofectamine 2000). In this study, 10, 20, 50 or 100 nM fluorescent oligo was transfected with lipofectamine 2000 into primary human pituitary cells. Twenty-four 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 stained with red fluorescent in 100 cells.

The optimised 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 tumour medium. After overnight incubation, for each well, 100 μl Opti-MEM I reduced serum medium containing 20 or 50 nM siRNA against human RSUME (5'-GGA TTT ATG GAT GCG GAT A-3') and 1 μl lipofectamine 2000 was added to the cells to suppress endogenous RSUME expression. Scrambled siRNA (100 nM; Scramble II, MWG Biotech) was used as a control. Stimulation experiments were performed 4–5 days post-transfection.

RNA isolation and RT-PCR

RNA was extracted from human pituitary tissues, primary human pituitary tumour cells, TtT/GF and AtT20 cells using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed using random hexanucleotides under restrictive conditions as described previously (*Pagotto et al. 2000*).

For semi-quantitative RT-PCR, 35 amplification cycles were performed with specific primers for mouse RSUME (sense: 5'-GAG AGC GAG GAC TAA ATA TGT C-3'; anti-sense: 5'-CAT TTT CTC TTT GCA TTT CTT TC-3'), mouse VEGF (sense: 5'-TCT ACC AGC GAA GCT ACT GCC-3'; anti-sense: 5'-TTA CAC GTC TGC GGA TCT TG-3'), mouse β -actin (sense: 5'-AGT ATC CAT GAA ATA AGT GGT TAC AGG-3'; anti-sense: 5'-CAC TTT TAT TGG TCT CAA GTC AVT GTA-3'), human RSUME (sense: 5'-TAC CTG GTA TCT CGA TTA ACT CTG AAC-3'; anti-sense: 5'-TCA GTA TTA TTT TAC CCA TGA ACA TCA-3') and human β -actin (sense: 5'-ACG GGG TCA CCC ACA CTG TGC-3'; anti-sense: 5'-CTA GAA GCA TTT GCG GTG GAC GAT G-3') at cycle conditions of 94 $^{\circ}\text{C}$ for 30 s, 58 $^{\circ}\text{C}$ for 30 s and 72 $^{\circ}\text{C}$ for 60 s.

Quantitative real-time RT-PCR was performed with cDNA samples of TtT/GF, AtT20 and human pituitary tumour cells as templates. The amplification reactions of 35 cycles were carried out with specific primers for human HIF-1 α (sense: 5'-CAT AGA ACA GAC AGA AAA ATC TCA TCC-3'; anti-sense: 5'-TTA ACT TGA TCC AAA GCT CTG AGT AAT-3'), human VEGF-A (sense: 5'-CAG ATT ATG CGG ATC AAA CCT-3'; anti-sense: 5'-CAA ATG CTT TCT CCG CTC TGA-3') and mouse HIF-1 α (sense: 5'-TAC TGA GTT GAT GGG TTA TGA-3'; anti-sense: 5'-AAG GCA GCT TGT ATC CTC-3'). The amplifications for human RSUME, human β -actin, mouse RSUME,

mouse VEGF and mouse β -actin were performed by the primers shown above. Absolute Blue QPCR SYBR Green Mix (Thermo Scientific, Dreieich, Germany) was used following the manufacturer's instructions. PCR amplifications were performed in a MiniOpticon Real-time PCR Detection System (Bio-Rad), and the data were analysed with CFX Manager Software for MiniOpticon (version 1.5, Bio-Rad). For each sample, the values were normalised by the amount of β -actin. All experiments were carried out in triplicates.

Western blot analysis

After CoCl_2 stimulation for different time periods as indicated, cell lysates were harvested in RIPA lysis buffer supplemented with protease inhibitor cocktail (Sigma). The protein concentrations of the cell lysates were determined by Bradford dye assay (Bradford 1976). Each sample (30 μg) was separated by a precast Tris-glycine gel (Anamed, Darmstadt, Germany) in an electrophoresis apparatus (Invitrogen), and then transferred on a nitrocellulose membrane (Hybond ECL) with Novex Semi-Dry Blotter (Invitrogen). Membranes were blocked with 5% non-fat milk solution (dissolved in TBS/0.1% Tween) and were then incubated overnight at 4 °C with mouse monoclonal antibody against HIF-1 α (R&D Systems, Wiesbaden, Germany) diluted 1:500 in 2.5% non-fat milk solution. After washing three times with TBS/0.1% Tween, the membranes were incubated with HRP-conjugated anti-mouse antibody (Cell Signalling, Frankfurt, Germany) diluted 1:2000 in 2.5% non-fat milk solution. The ECL system and hyperfilm (GE Healthcare, Munich, Germany) were used for membrane visualisation.

Immunofluorescence assay

Twenty-four hours after transfection with siRNA against RSUME and scramble siRNA in six-well plates, cells were split onto Falcon culture slides (BD Biosciences, Heidelberg, Germany) and stimulated with 250 μM CoCl_2 for 3 h the next day. After treatment, cells were fixed in 4% paraformaldehyde for 5 min on ice, and then blocked in 5% goat serum with 0.1% (v/v) triton X-100 for 30 min at room temperature. Slides were incubated with mouse monoclonal anti-HIF-1 α (1:100, Novus, Littleton, CO, USA) or rabbit polyclonal anti-VEGF-A (1:100, Abcam, Cambridge, UK) overnight at 4 °C, and then washed and incubated with Alexa Fluor 594 goat anti-mouse antibody or Alexa Fluor 594 goat anti-rabbit antibody (both from Invitrogen) for 45 min at room temperature. ProLong Gold antifade reagent with DAPI (Invitrogen) was used for visualisation of the

cell nucleus. Images were obtained using a confocal microscope (FluoView FV1000, Olympus, Munich, Germany). Images were obtained using 20 \times or 60 \times objectives.

ELISA

Measurement of VEGF-A secretion in cell culture supernatant was carried out with mouse- and human-specific VEGF-A ELISA kits (R&D Systems) following the manufacturer's instruction. All experiments were carried out in quadruplicates.

Statistical analysis

Results are expressed as mean \pm s.d. One-way ANOVA was used to compare variables, and $P < 0.05$ was considered as significant. A contribution ratio (square of coefficient of determination value $R^2 > 0.6$) was considered significant in linear regression analysis. The statistic analyses were performed with SigmaStat 2.0 (SPSS, Inc., Ehningen, Germany).

Results

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

Total RNA was extracted from three human normal pituitaries and 31 pituitary adenomas, and then quantitative real-time RT-PCR was performed to detect mRNA levels of HIF-1 α , VEGF-A and RSUME (Fig. 1A–C). Although there were no statistically significant differences among the mRNA levels of HIF-1 α , RSUME and VEGF-A when one-way ANOVA statistic analysis was used, these results showed a clear tendency in which mRNA levels of HIF-1 α , RSUME and VEGF-A are higher in human pituitary adenomas compared with normal pituitaries (data not shown). The mRNA levels of HIF-1 α , RSUME and VEGF-A were not significantly different among various types of adenomas we examined. Simple linear regression analysis showed that in the pituitary adenomas studied, VEGF-A and RSUME mRNA levels significantly correlated with HIF-1 α mRNA levels ($R^2 = 0.6610$ and $R^2 = 0.7296$ respectively; Fig. 1D).

Effect of hypoxia and hypoxia-mimicking CoCl_2 treatment on RSUME mRNA expression

To test whether RSUME is induced by hypoxia in pituitary tumour cells, RSUME mRNA expression was investigated by quantitative real-time PCR in cells exposed to hypoxia or CoCl_2 . RSUME mRNA levels in

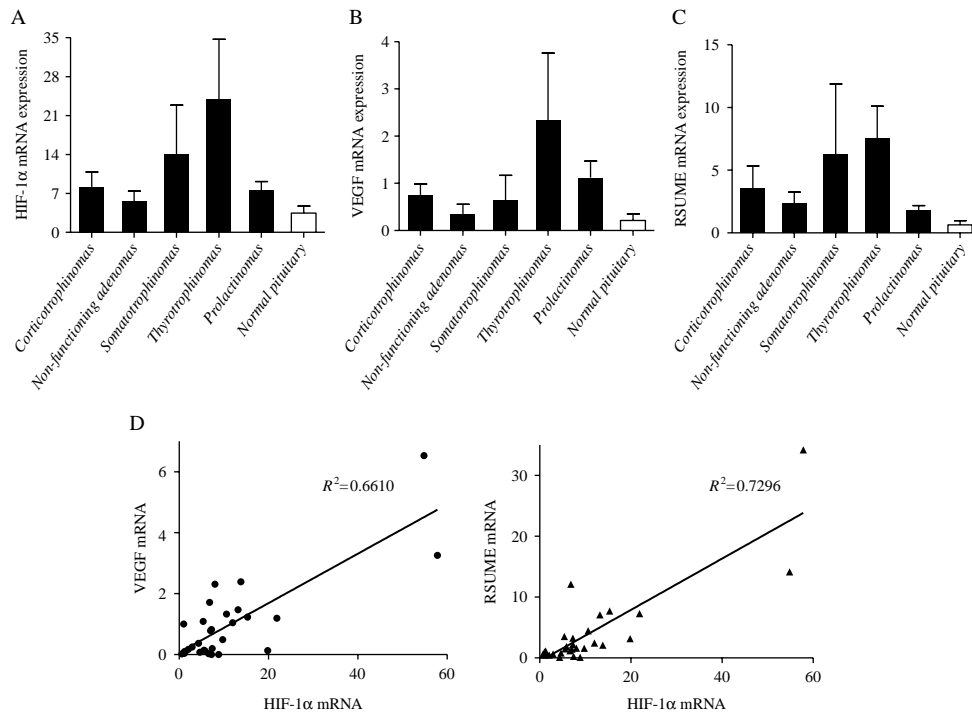


Figure 1 mRNA expression levels of HIF-1 α , RSUME and VEGF-A in human normal pituitaries and pituitary adenomas. Quantitative real-time RT-PCR was performed with 31 human pituitary adenomas and 3 normal pituitaries to detect the mRNA levels of HIF-1 α (A), VEGF-A (B) and RSUME (C). Values are given as mean \pm s.d. after normalisation to the amount of β -actin. (D) Simple linear regression analysis showed that VEGF-A and RSUME mRNA levels were significantly correlated with HIF-1 α mRNA levels in pituitary adenomas we tested ($R^2=0.6610$ or $R^2=0.7296$ respectively).

AtT20 and TtT/GF cells were significantly increased after CoCl₂ treatment and reached a peak at 30 min (Fig. 2A). RSUME mRNA levels also increased after exposure of the cells to hypoxic conditions (Fig. 2B). RSUME mRNA was also quantified in primary cell cultures of one somatotroph and two non-functioning adenomas. RSUME mRNA levels were also significantly increased under CoCl₂ treatment in all the tested primary tumour cells (Fig. 2C shows the results of one non-functioning adenoma and one somatotroph adenoma).

Effect of CoCl₂ on HIF-1 α protein expression in pituitary tumour cells

To investigate whether HIF-1 α protein is expressed after hypoxia conditions in pituitary tumour cells, TtT/GF and AtT20 cells were stimulated with CoCl₂ at different doses and time periods, and then HIF-1 α levels were determined in cell lysates by western blot. CoCl₂ dose- and time-dependently increased HIF-1 α protein levels, and the maximum HIF-1 α protein levels were reached after 2 and 3 h in TtT/GF and AtT20 cells respectively (Fig. 3A). CoCl₂ did not only stimulate expression of HIF-1 α but also its translocation into the

nucleus as shown by confocal immunofluorescence microscopy (Fig. 3B). Neither toxic effects nor changes in cell viability were observed compared with non-stimulated cells.

Primary cell cultures of eight human pituitary adenomas (five non-functioning adenomas, two somatotroph adenomas and one lactotroph adenoma) were also used to find out whether HIF-1 α expression was regulated by CoCl₂ treatment as in murine pituitary tumour cell lines. HIF-1 α protein expression was time- and dose-dependently increased after CoCl₂ treatment in all primary cell cultures tested (Fig. 3C shows the representative findings from two tumour cell cultures).

Effect of CoCl₂ on VEGF-A secretion

VEGF-A is a well-characterised HIF-1 target gene and a key factor in angiogenesis. To explore whether CoCl₂ not only induces HIF-1 α protein expression but also stimulates VEGF-A secretion in pituitary tumour cells, TtT/GF, AtT20 and primary cell cultures of 17 human pituitary adenomas (11 non-functioning adenomas, 3 somatotroph adenomas, 2 lactotroph adenomas and 1 corticotroph adenoma) were stimulated with CoCl₂ and VEGF-A was measured by ELISA in the cell

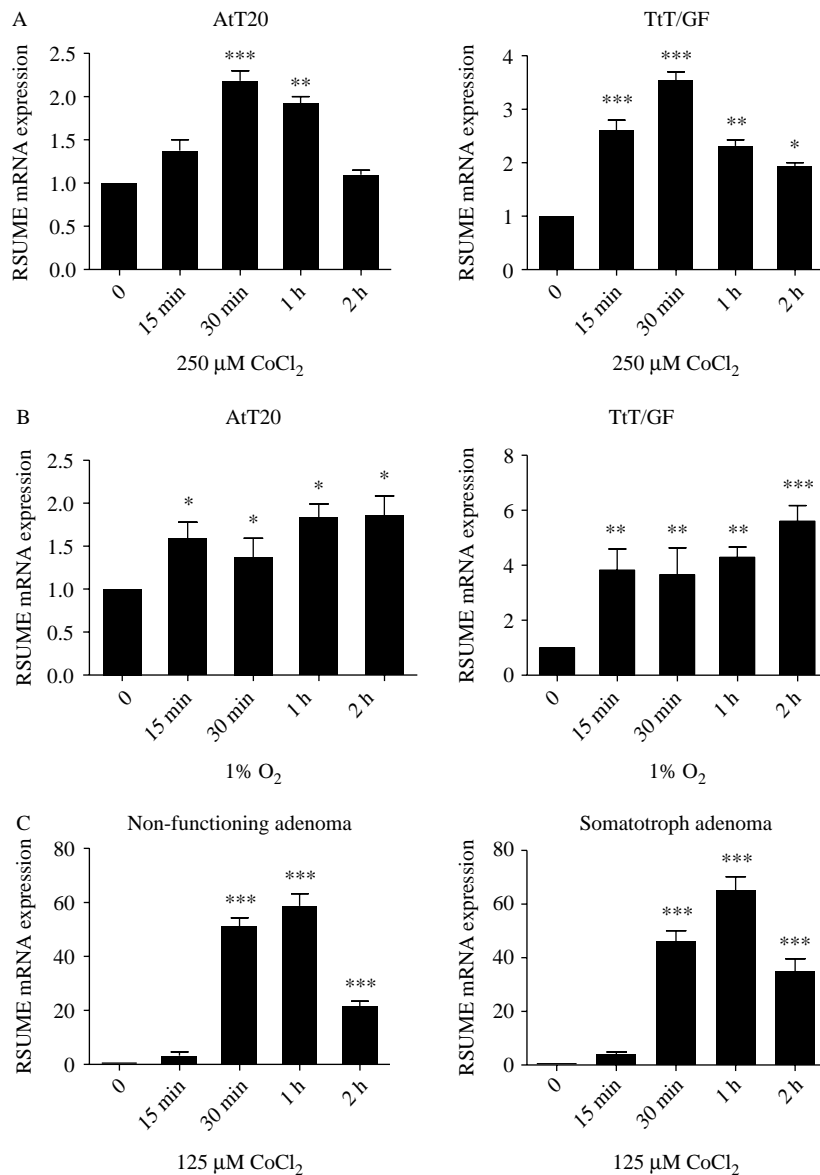


Figure 2 RSUME mRNA levels in TtT/GF, AtT20 and primary human pituitary adenomas under hypoxia (1% O₂) and CoCl₂ stimulation conditions. RSUME mRNA level was analysed by quantitative real-time RT-PCR in triplicates, and the values are given as mean ± s.d. after normalisation to β-actin. (A) RSUME mRNA level time-dependently increased under treatment with 250 μM CoCl₂ in both TtT/GF cells and AtT20 cells. (B) Time-dependent increase of RSUME mRNA in TtT/GF and AtT20 cells kept under hypoxic conditions (1% O₂) for different time periods. (C) RSUME mRNA level also increased in time-dependent manner under 125 μM CoCl₂ treatments in primary human pituitary adenoma cells as shown in two representative cases. **P*<0.05, ***P*<0.01 and ****P*<0.001 vs untreated cells.

supernatants. VEGF-A was significantly increased under CoCl₂ treatment, following a time- and concentration-dependent manner, in the TtT/GF cells and all primary cell cultures tested (Fig. 4A–E shows the representative findings). In contrast to TtT/GF cells and all the primary cell cultures tested, CoCl₂ treatment did not significantly stimulate the increase of VEGF-A secretion in AtT20 cells (data not shown).

Effect of RSUME knockdown on HIF-1α and VEGF-A production in mouse pituitary tumour cell lines

The siRNA against mouse RSUME and scrambled siRNA were transfected into AtT20 and TtT/GF cells to study the role of RSUME in the hypoxia-induced increase of HIF-1α expression in pituitary tumour cells. Twenty-four hours after transfection, the total

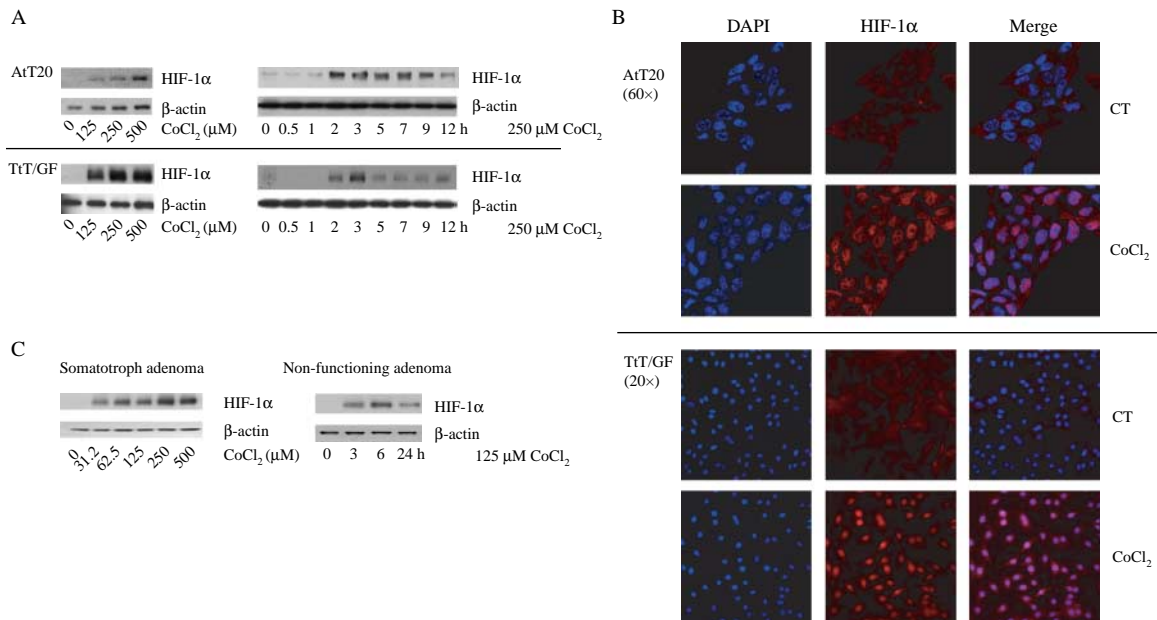


Figure 3 HIF-1 α protein levels in AtT20, TtT/GF and primary human pituitary adenomas under CoCl₂ stimulation. (A) Western immunoblotting showed that the HIF-1 α protein level was increased under CoCl₂ stimulation in a concentration- and time-dependent manner in both AtT20 and TtT/GF cells. Each image is representative of three experiments with similar results. (B) Immunofluorescence assays showed HIF-1 α nuclear accumulation after CoCl₂ treatment in both AtT20 and TtT/GF cells. Red colour corresponds to HIF-1 α , and blue colour corresponds to nucleus. The cells were stimulated with 250 μ M CoCl₂ for 3 h. The images were taken from confocal microscope using 60 \times and 20 \times objectives for AtT20 and TtT/GF cells respectively. The parameters were fixed when taking images by confocal microscopy, and one representative image of five observations from two independent experiments with similar results is shown. (C) HIF-1 α was increased in the primary human pituitary tumour cell cultures after CoCl₂ treatment, following a time- and concentration-dependent manner. Western blot images from primary human somatotroph and non-functioning adenoma cells were shown as representatives.

RNA was extracted and RT-PCR for mouse RSUME was performed. As expected, RSUME mRNA levels were decreased in both cell lines when transfected with RSUME siRNA compared with non-transfected or scrambled siRNA-transfected cells (Fig. 5A). AtT20 and TtT/GF cells previously transfected with RSUME or scrambled siRNA were stimulated with 250 μ M CoCl₂, and then HIF-1 α protein levels were determined in cell lysates by western blot. CoCl₂-stimulated HIF-1 α expression was significantly suppressed in RSUME siRNA-transfected but not in non-transfected or scrambled siRNA-transfected AtT20 and TtT/GF cells (Fig. 5B). Moreover, as a consequence of the inhibition of HIF-1 α expression, confocal immunofluorescence microscopy studies showed that CoCl₂-induced nuclear translocation of HIF-1 α was impaired in AtT20 and TtT/GF cells in which RSUME had been knocked down (Fig. 5C and D). To find out whether RSUME knockdown not only suppresses HIF-1 α protein expression but also reduces VEGF-A secretion, VEGF-A ELISA was performed in AtT20 and TtT/GF cells after transfection with siRNA against RSUME. Forty-eight hours after transfection of RSUME siRNA, TtT/GF cells were

treated with 250 μ M CoCl₂ for 24 h, and then the culture supernatant was collected for VEGF-A ELISA and RNA was extracted for RT-PCR assays. It was found that RSUME knockdown significantly decreased CoCl₂-stimulated VEGF-A expression while the reduction of the basal VEGF-A secretion was less pronounced (Fig. 6A). As shown by RT-PCR, VEGF-A mRNA expression was increased by CoCl₂ stimulation, and was significantly suppressed by RSUME knockdown compared with non-transfected or scrambled siRNA-transfected control (Fig. 6B).

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-A secretion in this cell line (see previous paragraph). To this end, 48 h after transfection of RSUME siRNA, the culture supernatant of AtT20 cells was collected for VEGF-A ELISA, RNA from a part of cells was extracted for RT-PCR, and cells were also fixed to perform immunofluorescence studies. From VEGF-A ELISA and RT-PCR, it was found that basal VEGF-A expression was suppressed at both protein and mRNA levels by RSUME knockdown compared with non-transfected and scrambled siRNA-transfected

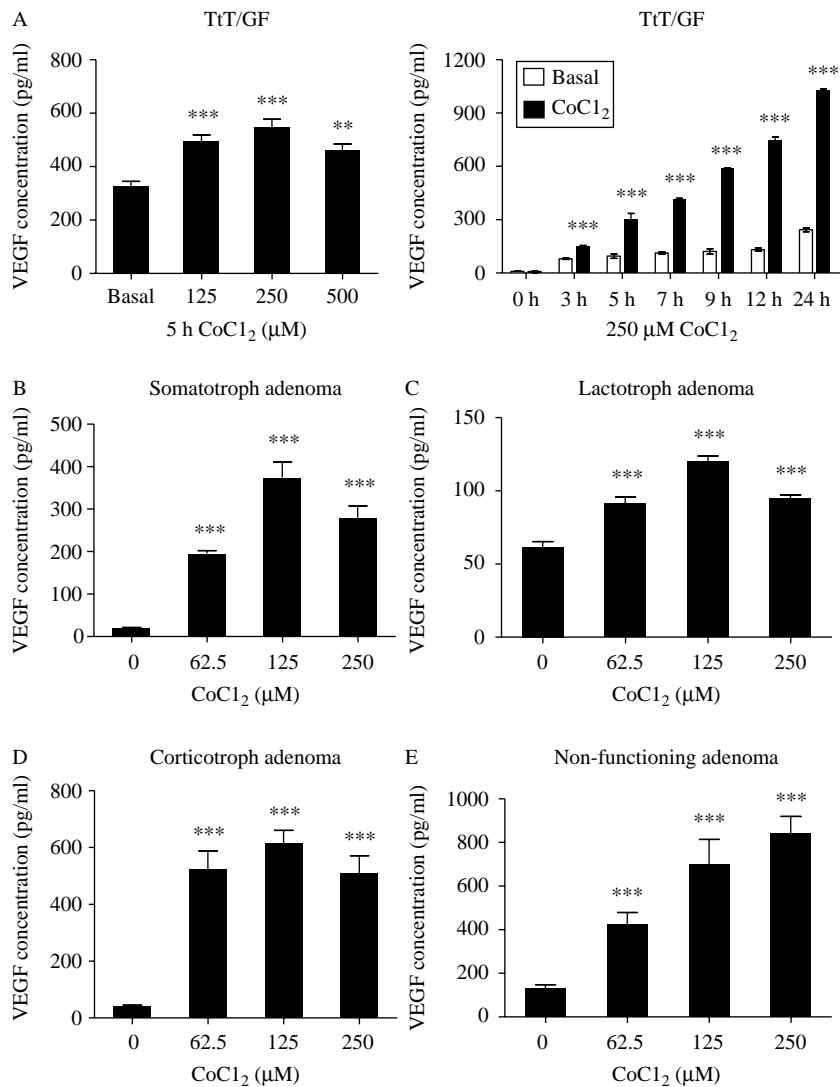


Figure 4 VEGF-A secretion in TtT/GF and primary human pituitary adenomas under CoCl₂ stimulation. (A) VEGF-A secretion was increased after CoCl₂ treatment in TtT/GF cells, following a dose- and time-dependent manner. B, C, D and E representatively show that VEGF-A secretion was increased after CoCl₂ treatment in primary pituitary tumour cells. The similar effects were found in the other primary human pituitary adenomas we tested (data not shown). ***P*<0.01 and ****P*<0.001 vs untreated cells.

control (Fig. 6C and D). Immunofluorescence studies showed a weaker VEGF-A staining in cells with RSUME knockdown in comparison to non-transfected and scrambled siRNA-transfected control, confirming that RSUME knockdown resulted in a significant reduction of basal VEGF-A expression in AtT20 cells (Fig. 6E).

Effect of HIF-1 α knockdown on CoCl₂-induced VEGF-A production in TtT/GF cells

TtT/GF cells were representatively used to confirm that hypoxia-induced RSUME up-regulation leads to increased VEGF-A secretion via HIF-1. To this end, HIF-1 α was knocked down by transfecting TtT/GF

with siRNA against mouse HIF-1 α ; scrambled siRNA was used as control. Experiments were performed or started 24 h after transfection. Quantitative real-time RT-PCR with mRNA extracted from transfected TtT/GF cells showed a dose-dependent suppression of mouse HIF-1 α mRNA synthesis (Fig. 7A). When HIF-1 α siRNA-transfected TtT/GF cells were stimulated with 250 μ M CoCl₂ for 3 h a dose-dependent reduction of CoCl₂-induced HIF-1 α was observed by western blotting (Fig. 7B). However, the knockdown of HIF-1 α levels in TtT/GF cells had no effect on the stimulation of RSUME mRNA synthesis induced by 30 min treatment with CoCl₂ (Fig. 7C, upper panel).

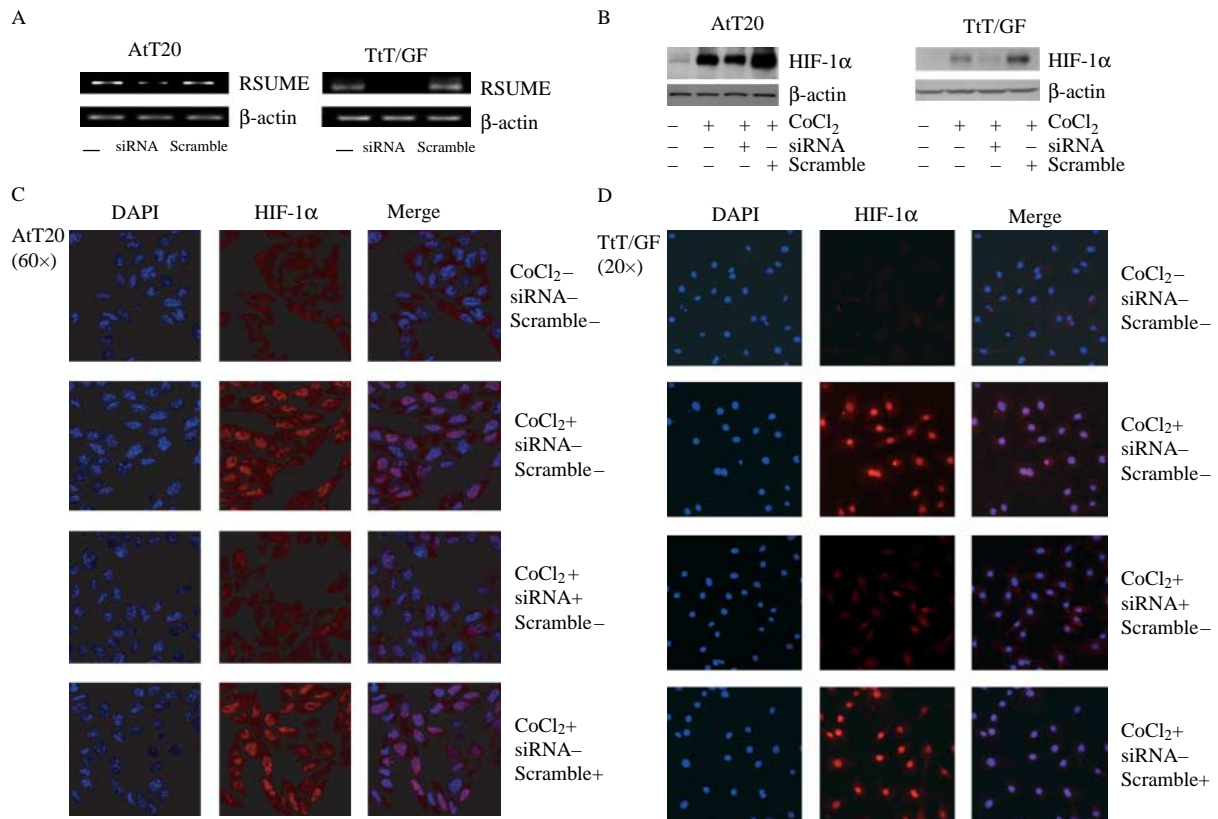


Figure 5 Effect of RSUME knockdown on HIF-1 α expression in TtT/GF and AtT20 cells. Controls correspond to non-transfected cells (–) and cells transfected with scrambled siRNA (scramble). (A) As shown by RT-PCR, RSUME mRNA expression was decreased after 24 h transfection of siRNA against mouse RSUME into AtT20 and TtT/GF cells compared with controls. (B) By western blot, HIF-1 α expression was suppressed by RSUME knockdown in both AtT20 and TtT/GF cells. Each image is representative of three experiments with similar results. (C and D) By immunofluorescence assays, HIF-1 α was suppressed in both AtT20 and TtT/GF cells with RSUME knockdown. Red colour corresponds to HIF-1 α and blue colour corresponds to nucleus. The images were taken from confocal microscope using 60 \times and 20 \times objectives for AtT20 and TtT/GF cells respectively. The parameters were fixed when taking images by confocal microscopy, and one representative image of five observations from two independent experiments with similar results is shown.

In contrast, the stimulatory effects of 24 h CoCl₂ stimulation on VEGF-A mRNA expression (determined by quantitative real-time RT-PCR; Fig. 7C, middle panel) and VEGF-A secretion (measured by ELISA; Fig. 7C, lower panel) were dose-dependently reduced in TtT/GF cells with knocked down HIF-1 α . These findings suggest that CoCl₂-induced RSUME mRNA expression up-regulates VEGF production via HIF-1 α and in addition indicate that the short-term hypoxia-induced RSUME production is not regulated by HIF-1 α .

Effect of RSUME knockdown on HIF-1 α and VEGF-A production in primary human tumour cell cultures

RSUME knockdown was also applied in primary human pituitary tumour cell cultures to clarify whether

RSUME is implicated in HIF-1 α expression as in mouse pituitary cell lines. Transfection of human pituitary tumour cells with siRNA was performed in primary cell cultures of six human pituitary non-functioning adenomas, one corticotroph adenoma and one somatotroph adenoma. Using the BLOCK-iT Alexa Fluor method a transfection efficiency of about 50% was found 24 h after transfection (Fig. 8A). RT-PCR experiments with RNA extracted from the adenoma cells 24 h post-transfection confirmed that siRNA against human RSUME suppressed RSUME mRNA levels in primary tumour cells (Fig. 8B). Four to five days after transfection of RSUME siRNA, the tumour cells were either stimulated with CoCl₂ for 3 h to study HIF-1 α protein expression in cell lysates or were treated with CoCl₂ for 24 h to investigate the effect of on VEGF-A secretion. In all eight human tumour cell cultures studied, RSUME knockdown by

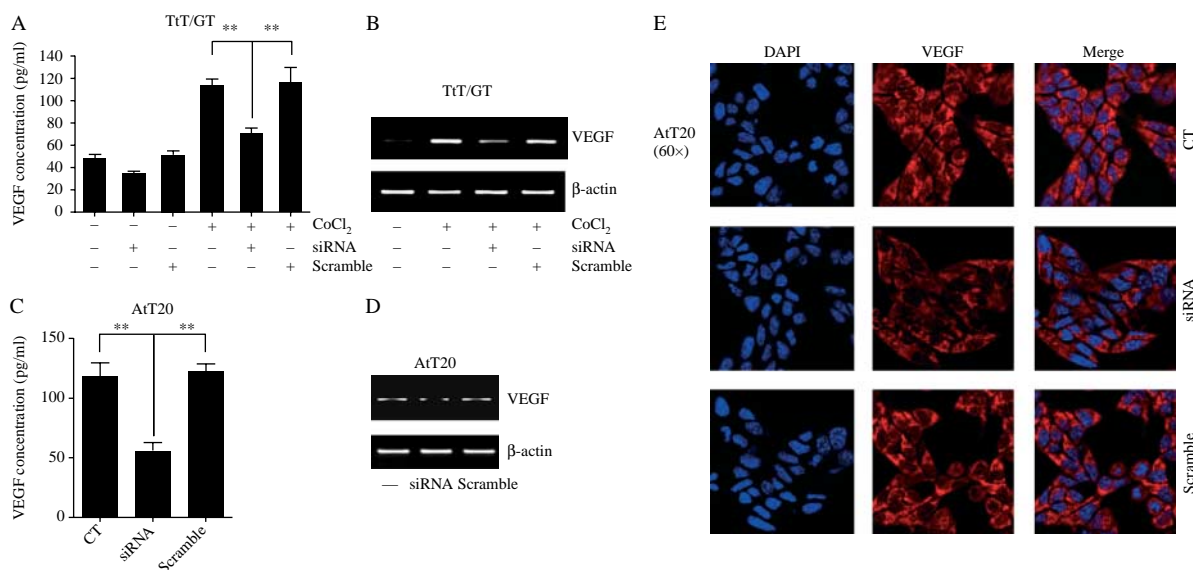


Figure 6 Effect of RSUME knockdown on VEGF-A expression in TtT/GF and AtT20 cells. Images of ethidium bromide agarose gel are representatives of three experiments with similar results. (A and B) CoCl₂-stimulated VEGF-A secretion and mRNA expression were suppressed by RSUME knockdown in TtT/GF cells as shown by VEGF ELISA and RT-PCR. (C–E) Basal VEGF-A protein and mRNA expression were suppressed by RSUME knockdown in AtT20 cells as shown by VEGF ELISA, RT-PCR and immunofluorescence assay. Red colour corresponds to VEGF and blue colour corresponds to nucleus. The images were taken from confocal microscope using 60× objectives. The parameters were fixed when taking images by confocal microscopy, and one representative image of five observations from two independent experiments with similar results is shown. ***P*<0.01.

transfection of RSUME siRNA led to a significant suppression of HIF-1α protein synthesis and VEGF-A secretion compared with scrambled siRNA-transfected controls (Fig. 8C and D shows representative results).

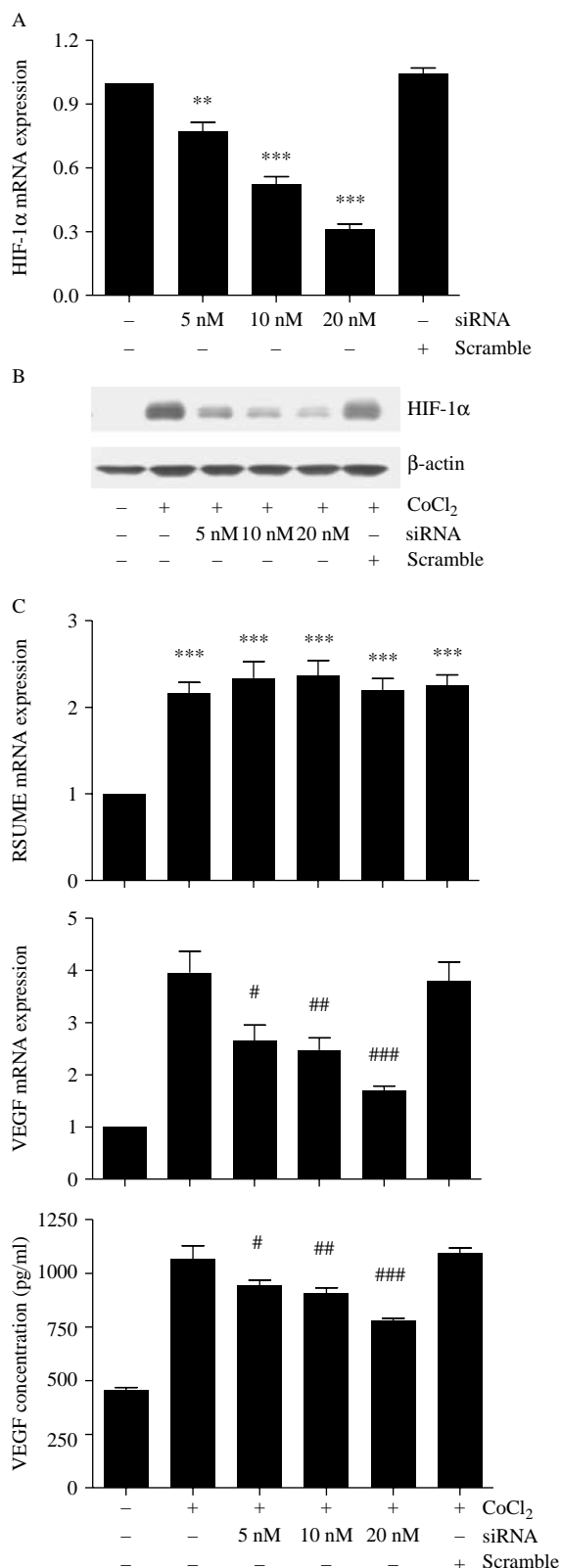
Discussion

In this study, the role of RSUME in VEGF-A production of pituitary adenomas and cell lines was comprehensively studied for the first time. In particular, its co-expression with HIF-1α and VEGF-A in pituitary adenomas as well as its implication in the regulation of these two potent angiogenesis regulating factors in pituitary tumour cells was investigated. It was demonstrated that RSUME critically regulates HIF-1α and VEGF-A production and thus might play an important role in pituitary tumour neovascularisation.

An essential step in the development of solid tumours is the neovascularisation of the expanding tumour mass by angiogenesis to supply the tumour cell with enough nutrients and oxygen (Carmeliet 2003). The expansion of the tumour mass causes intratumoural cellular hypoxia (Harris 2002), which induces the formation of the heterodimeric transcription factor HIF-1 by stabilising its oxygen-regulated subunit HIF-1α. Activated HIF-1 triggers the expression of VEGF-A protein, which is a key mediator of

angiogenesis (Carmeliet 2005). A large number of studies have demonstrated higher vascularisation and HIF-1α and VEGF-A overexpression in the majority of human cancers in comparison to their corresponding normal tissues (Zhong et al. 1999, Ferrara 2004). However, so far little is known about HIF-1α and VEGF-A regulation in pituitary adenomas due to limited and controversial information from few immunohistochemistry studies (Lloyd et al. 1999, Vidal et al. 2003a, Kim et al. 2005, Yoshida et al. 2005).

Herein the mRNA expression of RSUME as well as HIF-1α and VEGF-A was studied in parallel in a series of 31 pituitary adenomas by quantitative RT-PCR and the results were compared with the findings from three normal human pituitaries. Expression analysis at mRNA level had been chosen because still no appropriate RSUME antibodies are available to study RSUME protein expression by immunohistochemistry. RSUME, HIF-1α and VEGF-A mRNA levels were all slightly but not significantly enhanced in pituitary tumours in comparison to normal pituitaries. Based on previous observations that RSUME increases HIF-1α and VEGF-A expression in COS7 cells (Carbia-Nagashima et al. 2007), correlation analyses among RSUME, HIF-1α and VEGF-A mRNA levels were performed in pituitary tumours. HIF-1α mRNA significantly and positively correlated with RSUME



and VEGF-A mRNA expression in pituitary tumours suggesting an interrelationship among these factors.

In contrast to studies in other types of solid tumours, it has been shown that pituitary adenomas appear less vascularised than non-tumourous anterior pituitary tissues (Schechter 1972, Jugenburg *et al.* 1995, Turner *et al.* 2000). The extremely dense vascular network within the anterior pituitary is needed for the rapid release or suppression of pituitary hormones induced by blood-delivered stimulators or inhibitors respectively. The maintenance of the intrapituitary blood vessel system and the microvessel permeability seems to be regulated by VEGF-A derived by FS cells, which represent the major source of this factor in the normal pituitary (Vidal *et al.* 2001, Onofri *et al.* 2006). Inside pituitary adenomas FS cells are rare and the tumour cells produce VEGF-A by themselves (Vidal *et al.* 2001, Onofri *et al.* 2006). We found higher RSUME, HIF-1 α and VEGF-A expression in adenomatous tissues compared with more densely vascularised normal pituitary gland. This is consistent with some studies, which have demonstrated that there is no correlation between vessel density and HIF-1 α as well as VEGF-A (Giatromanolaki *et al.* 2001, Vidal *et al.* 2001, 2003a, Viacava *et al.* 2003). 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 (Yu *et al.* 2001, Vidal *et al.* 2003a). 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 tumours. This conclusion indicates that the interactions between vascular supply and pituitary tumour cell behaviour may not be fully explained only by microvessel density (Vidal *et al.* 2003a,b). Another explanation could be that HIF-1 α can induce growth factors such as IGF1 and TGF- α to activate signal transduction pathways that lead to cell proliferation

Figure 7 Effect of HIF-1 α knockdown on CoCl₂-induced VEGF-A production. (A) As shown by quantitative real-time RT-PCR, HIF-1 α mRNA expression was decreased after 24 h transfection of siRNA against mouse HIF-1 α in TtT/GF cells compared with non-transfected and scramble-transfected controls. (B) HIF-1 α expression was suppressed by HIF-1 α knockdown under CoCl₂ stimulation as shown by western blot. (C) HIF-1 α knockdown had no effect RSUME mRNA expression induced by 30 min of treatment with CoCl₂ (upper panel) but dose-dependently reduced VEGF-A mRNA synthesis and VEGF-A secretion induced by 24 h treatment with CoCl₂. ** P <0.01, and *** P <0.001 vs untreated cells. # P <0.05, ## P <0.01 and ### P <0.001 vs CoCl₂-treated cells.

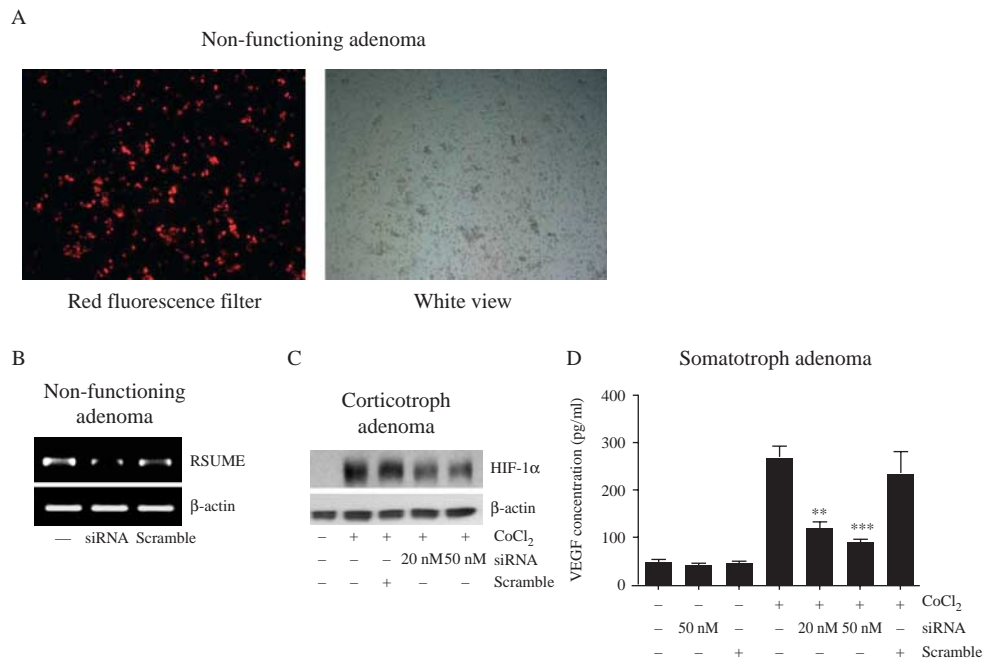


Figure 8 Effect of RSUME knockdown on HIF-1 α and VEGF-A expression in primary human pituitary tumour cells. (A) Evaluation of siRNA transfection efficiency in primary human pituitary tumour cells. The red fluorescent oligo was transfected into primary cells. The images were taken from fluorescence microscope using 10 \times objectives. (B) RSUME mRNA expression was suppressed by transfection of siRNA against human RSUME in primary human pituitary tumour cells. (C and D) CoCl₂-stimulated HIF-1 α synthesis and VEGF-A secretion were suppressed by RSUME knockdown in primary human pituitary adenoma cells. Representative results of HIF-1 α and VEGF-A suppression are shown for a human corticotroph (C) and somatotroph (D) tumour cell culture respectively. ** $P < 0.01$ and *** $P < 0.001$ vs CoCl₂-treated cells.

and survival under hypoxia (Feldser *et al.* 1999, Krishnamachary *et al.* 2003). Moreover, VEGF-A may not only play a role in angiogenesis but may also participate in proliferation and survival of pituitary tumour cells through VEGF receptor-1 (VEGFR-1) as shown in our previous study (Onofri *et al.* 2006).

In this study, in most experiment cells were treated with CoCl₂ as in control experiments the effects induced by this well-established hypoxia-mimicking substance (Ebert & Bunn 1999, Webb *et al.* 2009) were very similar to those of cells incubated at 1% oxygen in a hypoxic chamber. No effect on the viability of the treated pituitary tumour cells was observed excluding unspecific, putative toxic effects of CoCl₂. Co²⁺ is thought to mimic the hypoxia by binding instead of Fe²⁺ to the heme molecules which are regarded as oxygen sensors in both mammalian and bacteria, causing decreased oxygen affinity due to a conformational change (Goldberg *et al.* 1988, Ebert & Bunn 1999). In addition, it has been shown that Co²⁺ is able to stabilise HIF-1 α in normoxia by substituting Fe²⁺ from the Fe²⁺-binding site of prolyl hydroxylases (PHDs), leading to inhibition of PHDs activity (Masson & Ratcliffe 2003). Moreover, it has been shown that Co²⁺ inhibits the interaction between

HIF-1 α and its specific E3 ubiquitin ligase VHL, by direct binding to HIF-1 α (Yuan *et al.* 2003).

When studying the effect of hypoxia-mimicking CoCl₂ or 1% oxygen on RSUME synthesis we confirmed previous results (Carbia-Nagashima *et al.* 2007) that this factor is induced by hypoxia in rodent pituitary tumour cell lines and human pituitary adenoma cells. Interestingly, time course studies showed that RSUME mRNA synthesis is induced by hypoxia within 15–30 min. However, we observed a rise in HIF-1 α protein production only after 2 h of hypoxia and HIF-1 α knockdown in Tt/GF cells did not affect hypoxia-induced RSUME stimulation. This would indicate that, in these cells and experimental conditions, the remaining small amounts of HIF-1 are sufficient for the maximal induction of RSUME, or that the initial rapid induction of RSUME mRNA is independent of the transcriptional activity of HIF-1 and mediated by a novel hypoxia-dependent mechanism that has to be explored in future studies.

The CoCl₂-mediated induction of HIF-1 α and VEGF-A in mouse pituitary cell lines and in primary cell cultures of human pituitary adenomas was similar as shown in other cell types (Shima *et al.* 1995, Okada *et al.* 1998, Liu *et al.* 1999, Steinbrech *et al.* 2000,

Webb *et al.* 2009), CoCl₂ treatment time- and dose-dependently stimulated HIF-1 α protein expression and nuclear accumulation in AtT20, TtT/GF cell lines and all the tested human pituitary adenoma cultures. The increase in HIF-1 α is then followed by a significant, time- and dose-dependent rise in VEGF-A release induced by CoCl₂ in TtT/GF cells and all human pituitary adenoma cell cultures. In summary, hypoxia induces similar changes in pituitary tumour cells as already described in many other types of tumour cells. However, although CoCl₂-induced RSUME and HIF-1 α production in AtT20 cells, it did not stimulate VEGF-A secretion. It is known that AtT20 cells basally secrete the highest level of VEGF-A in comparison to other pituitary tumour cell lines (Lohrer *et al.* 2001) and it may be possible that this constitutively high VEGF-A production cannot further be stimulated. In some types of tumour cells hypoxia-independent mechanisms such as activated β -catenin/TCF signalling have been suggested to be responsible for constitutively high VEGF-A production (Easwaran *et al.* 2003, Yamamoto *et al.* 2003). Whether this is the case also in AtT20 cells need to be clarified in future studies.

When RSUME was down-regulated by specific siRNAs to further clarify the involvement of RSUME in the regulation of HIF-1 α and VEGF-A in pituitary adenomas, HIF-1 α expression in both mouse pituitary cell lines and primary human pituitary tumour cells was strongly suppressed. In addition, 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-A secretion in TtT/GF cells and primary human pituitary tumour cells. The CoCl₂-induced RSUME mRNA expression failed to up-regulate VEGF-A production when HIF-1 α expression was knocked down by specific siRNA, confirming that induction of RSUME leads to increased VEGF-A via HIF-1. These findings suggest that RSUME is required in HIF-1 activation in pituitary tumour cells under hypoxia conditions pointing to an essential role of RSUME in the induction of angiogenic processes in pituitary adenomas, and probably other types of solid tumours.

In summary it has been shown that the novel RWD-domain containing protein RSUME is a new and important player in pituitary tumour pathogenesis. It is overexpressed in human pituitary adenomas and critically regulates HIF-1 α expression and VEGF-A production under hypoxia. Through supporting pituitary tumour neovascularisation, RSUME might be critically involved in pituitary adenoma progression. Therefore, it represents a new putative therapeutic

target for the development of novel anti-angiogenic treatment concepts of human pituitary tumours.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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