Activation and regulation of endogenous retroviral genes in the human pituitary gland and related endocrine tumors

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**Abstract**

**Aims:** Adenohypophysis (AH) hormone producing cells represent the origin of diverse groups of pituitary adenomas (PA). Deregulation of hypothalamic hormone receptors, growth factors and cAMP signaling have been implicated in the etiology of PA. Endogenous retroviruses (ERVs) are derived from past exogenous retroviral infections and represent more than 8% of the human genome. Some ERV genes encode open reading frames and produce functional proteins, e.g. the ERVW-1 envelope gene Syncytin-1, essential for placentogenesis, but also deregulated in human tumors. Data concerning ERV expression in the AH and related endocrine tumors are missing.

**Methods:** Syncytin-1 protein was analysed in normal AH (n=15) and compared to five PA subtypes (n=117) by immunohistochemistry. Absolute gene expression of 20 ERV functional envelope genes and ERVW-5 gag was measured. PA tissues were examined for Syncytin-1 and the cAMP signaling marker phospho-CREB-Ser133 using immunohistochemistry. Isolated primary human PA cells were treated with different hormones. Murine embryonic and adult pituitary gland ERV expressions were compared to human AH.

**Results:** Syncytin-1 protein co-localised with corticotrophic cells of AH. In contrast, all PA demonstrated significant Syncytin-1 protein overexpression, supporting deregulation. All other ERV genes showed significant up-regulations in different PA subtypes. Phospho-CREB-Ser133 and Syncytin-1 co-localized in PA cells. Cultivated primary PA cells with ACTH or CRH induced their respective receptors and ERV genes. Syncytin-A/-B, murine orthologs to human Syncytin-1/-2, localized to embryonic and adult pituitary glands demonstrating functional mammalian conservation.

**Conclusions:** Deregulated ERV genes may contribute to PA development via cAMP signalling.
Introduction

The pituitary is a small gland located at the base of the brain in a bony formation called the sella turcica. In its function as a key regulator of body homeostasis, the gland is connected to the hypothalamus via the infundibular stalk through where hormones and different activators and repressors reach the pituitary fossa. Two different lobes with varying functions and distinct anatomical structures can be distinguished: the adenohypophysis (AH, or frontal pituitary lobe/anterior pituitary) and the neurohypophysis (NH, or posterior pituitary lobe/posterior pituitary). The different types of hormone producing cells in the AH release adrenocorticotropic hormone (ACTH), thyroid-stimulating hormone (TSH), growth hormone (GH also called STH), Prolactin (PRL), the gonadotropins luteinizing hormone (LH), and follicle-stimulating hormone (FSH). These hormone producing cells could be the origin of the vast majority of pituitary tumors so called pituitary adenomas (PA). They constitute about 10 to 15 percent of intracranial neoplasms and represent generally benign lesions [1]. Upon clinical presentation they can be classified as secreting or clinically nonfunctional tumors. One example of hormonal active neoplasms are ACTH-secreting adenomas which cause Cushing’s disease, a severe illness associated with overproduction of cortisol and other adrenocorticoids and is associated with multiple clinical complications including hypercoagulability, insulin resistance, hypertension, bone loss, and immunosuppression [2], [3]. Recently a clinical study involving 343 Cushing’s patients and 34,300 controls from the Danish National Registry and Danish Civil Registration System demonstrated that mortality was doubled in Cushing’s patients {Hazard ratio (HR) 2.3, 95% confidence intervals (CI) 1.8-2.9} compared with controls. Importantly, mortality was due to venous thromboembolism (HR 2.6, 95%CI 1.5-4.7), myocardial infarction (HR 3.7, 95%CI 2.4-5.5), stroke (HR 2.0, 95%CI 1.3-3.2), peptic ulcers (HR 2.0, 95%CI 1.1-3.6), bone fractures (HR 1.4, 95%CI 1.0-1.9), and infections (HR 4.9, 95%CI 3.7-6.4) [3]. Regarding growth hormone (GH) producing PA causing acromegaly, a meta-analysis of sixteen studies demonstrated an increased mortality in acromegalic patients compared with the general population as measured by the weighted average of the standardized mortality ratio (SMR) (1.72 with 95% CI 1.62-1.83) [4].

Concerning the molecular etiology of PA, deregulation of hypothalamic hormone receptors, growth factors, feedback signaling, and especially the cAMP pathway have been implicated. Gain of function mutations in the alpha (α) subunit of the heterotrimeric Gs protein have been observed in 30-40% of GH adenomas, 10% clinically non-functioning (ClNon) adenomas, 5-20% of TSH adenomas and <5% of ACTH adenomas [5]. For example, gene mutations in Gsα, affecting codons 201 and 222, result in constitutive activity due to the inhibition of GTPase [6]. Additionally, aberrant high wild-type Gsα protein levels as
well as unbalanced protein kinase A (PKA) regulatory subunits, overexpression of the cAMP regulatory element binding protein (CREB) gene and elevated phospho (p)CREB-Serine (Ser)133 have been noted in PA [5]. All of the above indicate that multiple alterations of the cAMP pathway represent one molecular signature for PA.

Endogenous retroviruses (ERVs) are derived from exogenous retroviral infections of germ cells and integrated into the genome of mammals between 0.1 and 40 million years ago [7] [8] [9]. More than 8% of the human genome is considered retroviral origin, like retrotransposons, ERVs and elements with ERV origin [10] [11] [12]. ERVs, like exogenous retroviruses, code for group-specific antigen (gag), which is a structural protein forming membrane- and self-associations for virus assembly and budding, as well as binding and packaging of RNA strands. Also coded for is a polymerase (pol) encoding the viral enzyme reverse transcriptase (RT) and the viral envelope (env). Most of the ERV genes are non-functional due to recombination, mutations and deletions; however some gag, pol and env from different ERV encode full-length open reading frames and produce functional proteins [13] [14] [15]. The env protein contains a surface unit (SU) and a transmembrane domain with an immunosuppressive-like peptide similar to exogenous retroviral env proteins [16]. Up to date 19 different fully coding ERV env genes and two ERV env genes with stop codons from 11 different ERV families are known [13, 17]. The env gene of ERVW-1 (chromosome 7q21.2) called Syncytin-1 was found to be expressed in human placentas and plays an essential role in placentogenesis a process that is controlled by an orchestra of different hormones like LH, FSH and PRL [16] [18] [19]. The mouse functional homologs of Syncytin-1 and Syncytin-2 called Syncytin-A and Syncytin-B, respectively, were shown to be essential for placentogenesis using knockout mice [20]. ERV env genes have also been implicated in a variety of malignant tumors, for example, breast, endometrium, ovary and testicles where they were aberrantly expressed [17, 21] [22] [23] [24]. Common to both placental development and tumorigenesis Syncytin-1 was found up-regulated after cAMP-stimulation, either with Forskolin, which induces the adenylate cyclase or directly with a SP-cAMP analog indicating positive protein kinase A regulation [25] [26] [27].

A few studies have demonstrated ERV expression in the human brain. RNA expression of the Erv3-1 and the env gene of ERVE were detected in whole brain tissue, but at lower amounts when compared to placentae [28]. ERVK-102 (chromosome 1q21-q22) and the gag gene of ERVH (chromosome 22q12) were found transcriptionally active in normal brain [29]. Furthermore, ERV expression was related to different brain diseases, especially in amyotrophic lateral sclerosis (ALS), in multiple sclerosis (MS) and in diverse mental disorders. For example, brain tissues from autopsy patients with ALS showed increased ERVK HML-2 and HML-3 pol transcripts [30]. Most studies have investigated expression and
the functional roles of retrovirus-like structures and RT activity defined as the MS associated retrovirus (MSRV) in patients with MS [31][32][33]. A high sequence similarity of MSRV with Syncytin-1 and the env of ERVW-2 (chromosome Xq22.3) were found [34]. Different ERV expression of ERVW, ERVT, ERVFRED, ERVE4-1, ERVH, ERV9 and ERVK-10 were also detected in serum, cell-free cerebrospinal fluids and brains of schizophrenic patients [35][36][37][38].

So far, studies concerning the expression of ERV genes in the human pituitary have not been performed. Additionally, the majority of ERV env coding genes are either not present or not consistently represented on commercially available microarrays. In this present study, we quantified normal AH tissues and five PA subtypes for the expression pattern of Syncytin-1 using immunohistochemistry (IHC) and additionally measured 19 codogenic and 1 non-coding ERV env genes as well as the ERVW-5 gag gene using absolute quantitative real time PCR. Due to the positive correlation of Syncytin-1 induction with cAMP during placentogenesis and tumorigenesis, we established primary human pituitary adenomas cell cultures to test the influence of different cAMP inducing hormones on Syncytin-1 and other ERV env gene expression. We further analyzed for an association of pCREB-Ser133 with Syncytin-1 using IHC and immunofluorescence on the same AH and PA tissues. Lastly, we tested if RNA of Syncytin-A and Syncytin-B, murine orthologs to human Syncytin-1 and Syncytin-2, co-localized to the pituitary glands of embryo day E18.5 and adult mice.

Materials and Methods

Patient subjects and AH adenomas

The following study concept was approved by the Ethical Committee of the University of Erlangen-Nuremberg. Surgical specimens from a total of 187 patients with sellar lesions were retrieved from the archives of the Department of Neuropathology at the University Hospital of Erlangen. None of the PA used in this analysis were obtained from patients with multiple endocrine neoplasia type I (MEN type I). Each tumor specimen was classified and graded according to the currently valid version of the World Health Organisation (WHO) classification system for tumors of endocrine organs using haematoxylin and eosin staining as well as immunohistochemistry (IHC) [1]. Pieces of normal anterior lobe were acquired from patients with sellar exploration, MRI negative microadenomas, as well as patients with Rathke’s cleft cysts served as controls (n=15) (7 men and 8 women, median age 35.5 years). These specimens showed a regular lobulated reticulin fiber network typically observed in the AH and IHC confirmed the regular spectrum of hormone production.
Neuropathological examination and IHC

All biopsy samples were fixed overnight in 4% formalin and routinely processed into liquid paraffin. Sections were cut at 4µm with a microtome (Microm, Heidelberg), stretched in hot water (60°C) and mounted on positively charged slides (Superfrost + Menzel). The slides were air-dried in an incubator at 37°C overnight. The staining procedure with antibodies against the pituitary hormones was performed using a semiautomated benchmark apparatus (Nexes, Ventana, Illkirch, France) and the Ventana DAB staining system following the manufacturer’s recommendations and as described previously [39]. Microwave pre-treatment was used when recommended (0.01 M citrate-buffer pH 6.0, 3 min 900 watt and afterwards 2 x 10 min, 250 watt). All slides were counterstained with hematoxylin. For Syncytin-1 detection, tissue slides were hybridized with the primary antibody overnight at 4°C. Syncytin-1 IHC was performed on five different adenoma subtypes (n=117) which were grouped according to clinical symptoms due to their hormone release: 1) ACTH: ACTH producing adenoma with clinical signs of Cushing disease (n=19; 6 men and 13 women, median age 45.5 yr) 2) CINon: Clinically nonfunctioning adenoma (n=49; 32 men and 17 women, median age 52 yr); 3) PRL: Prolactin producing adenoma (n=13; 8 men and 5 women, median age 43.7 yr) 4) Acro: Adenoma with clinical signs of acromegaly (n=29; 13 men and 16 women, median age 40.7 yr) 5) TSH: TSH producing adenoma (n=7; 2 men and 5 women, median age 38 yr). IHC of all surgical specimens was performed using the following panel of antibodies and respective dilutions: ACTH (1: 2,000, mouse, clone 02A3, DAKO, Glostrup, Denmark), FSH (1:500, mouse, clone AB-3 (FSH03), Thermo Fischer Scientific, Waltham MA, USA), hGH (1:100, mouse, clone 54/9 2A2, Biogenex, Fremont CA, USA), LH (1:2,000, mouse, clone Ab-1 (LH01), Thermo Fischer Scientific, Waltham MA, USA), PRL (1:5,000, mouse, clone AB-2 (PRL02), Thermo Fischer Scientific, Waltham MA, USA), TSH (1:500, mouse, clone 0042, DAKO, Glostrup, Denmark), Syncytin-1 (1:1,000, rabbit polyclonal, Imgenex, San Diego, CA, USA).

Additional antibodies used for IHC experiments were: 1) monoclonal antibodies (1:20 dilution) specific for TGF-β1 (MAB240, R&D) and TGF-β3 (MAB243, R&D) performed with two paraffin tissue sections of PA (CINon and ACTH) and a term human placenta; and 2) pCREB-Ser133 (Millipore, polyclonal antibody, 1: 1,000) with PA (n=6) and AH (n=1).

Evaluation of IHC staining

The immunohistological staining pattern of Syncytin-1 expression was analyzed and scored unaware of any diagnosis concerning the hormonal status of patients. Results were assessed semi-quantitatively as the percent of positive cells and described as the following:

Normal AH biopsies served as controls and were scored according to the number of
Syncytin-1 immunoreactive cells in each sample (0-100%) and the intensity of Syncytin-1 immunoreactivity (graded 0-3); grade 0 = no expression, + = low intensity, ++ = distinct intensity, +++ = vast intensity (like pituicytes in NH). In the same way Syncytin-1 expression scores for every single tumor were calculated according to the number of positive cells and the intensity of immunoreactivity in relation to Syncytin-1 expression in normal AH (graded 0-4, with 0 meaning no stained cells; + = lower intensity than majority of pituitary cells; ++ = similar intensity than majority of pituitary cells; +++ = higher intensity than majority of pituitary cells; ++++ = vast intensity). Thereafter a quotient for every group and marker was calculated, setting 0 = 0, + = 1, ++ = 2, +++ = 3 and ++++ = 4) and divided by the number of samples [40] [41].

IHC of both pCREB-Ser133 and Syncytin-1 were performed using separate tissue sections and then analyzed in the same regions from PA (n=6) and AH (n=1). Four 40μm squared regions were analyzed for the percentage of strong, intermediate, weak and negative pCREB-Ser133 nuclei expression and then compared with the amount of Syncytin-1 positive cells in the same tissue region.

**Double immunofluorescence staining**

Selected tissue sections were incubated over night with primary antibodies directed against Syncytin-1 (Imgenex, rabbit polyclonal 1:100) or Syncytin-1 monoclonal mouse antibody (mab) 14A5 against the SU (1: 350), kind gift from Dr. H. Perron, Geneuro, Geneva Switzerland) in conjunction with either pCREB-Ser133 (1: 1,000); or antibodies against different anterior lobe hormones including ACTH, (mab DAKO, 1: 2,000), FSH (mab Thermo, 1: 500), hGH (mab Sigma/Aldrich, 1: 800), LH (mab Thermo, 1: 2,000), PRL (mab Thermo, 1: 5,000) and TSH (mab DAKO 1: 500) at 4 °C and then subsequently detected with fluorescence labeled secondary anti-rabbit antibodies conjugated with carbocyanine (Cy)2 (diluted 1: 200, Jackson ImmunoResearch/ Dianova, Hamburg, Germany) or anti-mouse Cy3 (diluted 1: 50, Jackson ImmunoResearch/ Dianova, Hamburg, Germany). Cell nuclei staining was performed using Hoechst 33342 (Sigma-Aldrich, Schnelldorf, Germany) for 5 min at room temperature. Slides were analyzed using an Olympus BX-51 fluorescent microscope (Olympus, Hamburg, Germany) equipped with an F-View II CCD camera (Soft Imaging System, Stuttgart). All secondary antibodies were tested separately and showed no specific cellular immunolocalization.

**Confocal Laser Scanning Microscopy**

Confocal microscopy (Leica SP5X) was performed on selected tissue sections as indicated above, which showed co-localization of Syncytin-1 and normal AH hormone producing cells (ACTH). Primary antibodies included Syncytin-1 and ACTH. Secondary antibodies for
Syncytin-1 were a goat anti-rabbit Cy2 detected with an Ar-laser at 488nm and an ACTH goat anti-mouse Cy3 detected with a He-Ne-laser at 543nm. Laser power was set to approximately 1.2 mW in order to avoid extensive photobleaching. To localize Syncytin-1 on primary adenoma cells, following 72 hr of culturing cells on glass cover slips coated with FCS, the cells were fixed with paraformaldehyde 4%, washed with 1x PBS, treated with 5% horse serum and 1% BSA in 1x PBS for 1 hr and then incubated overnight in a humid chamber at 4°C with the Syncytin-1 antibody. Cells were washed with 1x PBS, incubated for 1 hr at RT with a Cy2 antibody then washed with 1x PBS and the nuclei stained with DRAQ5 (Biostatus Lim., UK) according to manufacturer’s instructions. Cy2 was detected with an Argon laser at 488nm and DRAQ5 with a He-Ne laser at 633nm. The Cy2 secondary antibody solely was negative for any immunolocalization. All 3-D imaging processing was performed using the AMIRA (Burlington, MA, USA). For all top view 3-D images, raw images were selected and a volume rendering was performed to visualize the dimensionality of the samples.

**Murine brain preparation**

Pregnant wild-type mice or adult mice of the C57BL/6J and C3H strains were kindly provided from the research group of Dr. Wegner, Institute of Biochemistry of the University Erlangen-Nuremberg. Animal experiments were performed in strict accordance with the protocol, which were approved by the Committee on the Ethics of Animal Experiments of the University of Erlangen-Nuremberg (Permit Number: TS-00/12). Whole pituitary glands from adult brains or total whole mouse brains from E18.5 mice were fixed in 4% PFA for frozen sections for one hour or overnight, respectively. Samples were washed 6 times in 1x PBS, perfused with 30% sucrose overnight, embedded in Tissue-Tek (Sakura, Hartenstein, Germany) and stored at -80°C.

**In situ hybridization (ISH) of murine brain sections.**

Digoxigenin (DIG)-labelled antisense and sense RNA probes were generated for *Syncytin-A* and *Syncytin-B* according to Henke et al. [42]. Plasmids were linearized with restriction enzymes and RNA probes were transcribed and DIG-labelled using a RNA-Labelling Kit (Roche, Germany). Twelve μm tissue pituitary sections were obtained from adult pituitary glands (cross sections) (n=2) and from a whole E18.5 mouse brain (sagittal sections) [43]. Tissue sections were hybridized with *Syncytin-A* and *Syncytin-B* probes using ISH according to Henke et al. [42]. Lastly, ISH tissue sections were counterstained with nuclear fast red (Sigma-Aldrich) and mounted in Mowiol (Roth, Germany).
RNA, cDNA preparation and absolute quantitative real time PCR (qPCR)

Snap frozen human tissue samples were retrieved from our tissue bank (-80°C). Control samples of the human pituitary gland were collected from autopsies (n=8; post mortem interval ranged from 12 – 36 hr). From all tissues, frozen sections were microscopically reviewed to confirm neoplastic growth or absence of tumour growth, respectively. Total RNA was extracted from 30-50 mg of frozen tissues according to Strick et al. [26]. cDNA, ERV env primers and qPCR of 20 ERV env genes {Syncytin-1 (ERVW-1), Syncytin-2 (ERVFRD-1), Syncytin-3 (ERVp), Env3-1-1 (ERVR, ERV3-1), envE4-1 (ERVE4-1), envH1-3 (p62, p60, p59), envV-1 (ERVV-1); envV-2 (ERVV-2), envFc1 (chromosome Xq21.33), envFc2 (ERVFC1-1), envRb (ERVPAUB1-1), envK-1-7 (ERVK-1-7 in addition to envK-1-6 the primer also recognized envK-102, which has only a partial codogenic env) and envW-2 (ERVW-2)} were used according to [17] [44]. We differentiated between Syncytin-1, envW-2 and the env of MSRV by choosing discriminatory primer pairs according to Mameli et al. [45] and Strissel et al. [17]. In addition, ERVV-5 gag (p30) on chromosome 3q26.32 (AF156961) was analyzed quantitatively with the following primer: cloning primers TF (AGATGTATTCTGGAGAATTGGGACCAA) and BR (ATCAATAGCCGTCAGGTATTCTGA) and for real time PCR: TF (AGGCGGAAATGGAGTGAAGTG) and BR (GGACTCTGAGGGCTTCTGT). Regarding primary PA cell cultures described below, RNA was fractionated, purified, DNAseI treated and then cDNA was synthesized according to Strissel et al. [46]. Semi-quantitative real time PCR (Applied BioSystems 7300) using a SYBR green based master mix (Thermo Scientific) was performed with cDNA (40ng/well) and primers for the human CRHR1 (TF: 5’ CACGTCTGAGACCATTCAGTACA; BR: 5’ ACGAAGAACAGCATGTAGGTGAT); CRHR2 (TF: 5’ ACCTACATGCTCTTCTCGTCAAT; BR: 5’ CTGCAGGAAGGAGTTGAAATGAT) and ACTHR (TF: 5’ ATCACACTGACCATCTGCT; BR: 5’ TTCACCTGGAAGAGACATGTAG). Normalized 2−ΔΔCT values for RNA gene expression were calculated according to Strissel et al. [46], where co-amplification of the housekeeping gene 18S-rRNA was used for normalization in semi-quantitative analysis.

Snap frozen mouse adult pituitary glands (n=6) were retrieved from -80°C and then RNA was isolated and processed the same as described above. cDNA, mouse ERV env primers and qPCR of Syncytin-A and Syncytin-B were according to Henke et al. [42].
Primary cell cultures

Three human adenoma primary tissues were obtained from the Institute of Neuropathology, Erlangen, including 1) an atypical densely granulated GH adenoma from a patient with clinical signs of acromegaly (Acro); 2) a corticotropin cell adenoma from a patient suffering from Cushing’s disease (ACTH) and; 3) a gonadotroph cell adenoma with FSH and LH expression (ClNon). A single tissue piece from each PA was frozen at -80°C and used for analyses of protein expression. The remaining tissue sample was used for cell culture involving cAMP induction experiments. Briefly, the adenoma tissue was washed with 1x PBS, cut into small tissue pieces and digested in a solution containing collagenase (1 mg/ml) for 1.5 hr on a rotator at 37°C. Trypsin was added at a final concentration of 0.02% for an additional 30 min with frequent microscopic monitoring. The cell suspension was washed and centrifuged at 179g until the supernatant cleared. Cell pellets were resuspended and plated into T25 tissue culture flasks and maintained in RPMI medium supplemented with 10% FCS, non-essential amino acids, L-glutamine and 1% penicillin/streptomycin. Following 5 days after initial set-up the cellular supernatants were collected and tested for expression of hormones FSH, LH, PRL and hCG using ELISA (Dxl 600; Beckmann Coulter GmbH, Krefeld, Germany) in the Endocrinology Laboratory, Department of Obstetrics and Gynecology, and ACTH and GH at the Neuroendocrinology Laboratory, Department of Neurosurgery both at the University Hospital, Erlangen. For cAMP induction experiments, at day 5 following set-up of initial short term cell cultures the cells were trypsinized and divided into 12-well culture dishes (200,000 cells per well) in RPMI medium with 5% charcoal treated serum (CTS). Sixteen hours later hormones and the known cAMP stimulator Forskolin were added to cell cultures at the concentration: ACTH (2x 10^{-9} M), CRH (0.5x 10^{-9} M) and Forskolin (4x10^{-5} M). The RNA was harvested 48 hr later to analyze ERV gene expression. In addition, primary cells grown on coverslips were treated with either ACTH or Forskolin and then stained with a membrane specific wheat germ agglutinin conjugated with Alexa594 and nuclei specific Hoechst 33342 and analyzed by microscopy according to Ruebner et al. [47]. All primary placental villous trophoblasts were isolated and cultured according to our past publications [44] [47].

Statistical analyses

The non-parametric Mann-Whitney-U test for independent samples was performed using IBM SPSS Statistics 21 (IBM, Germany). For all tests a P value: <0.05 was considered as statistically significant. For each mean value, a standard error of the mean (s.e.m.) was calculated using IBM SPSS Statistics 21.
RESULTS

Human Syncytin-1 protein was expressed in normal AH and significantly overexpressed in PA.

To determine if Syncytin-1 was expressed in human AH, we performed IHC on tissue sections of normal pituitary. Interestingly, Syncytin-1 expression revealed a variable degree of reactivity in epithelial cells throughout the AH. For example, in the pars intermedia a specific Syncytin-1 positive cell population showed a very strong granular cytoplasmic staining pattern with a predominance of basophil cell clusters (Fig. 1). To further characterize this cell population, we performed double immunofluorescence staining with antibodies against Syncytin-1 and the most important hormones produced in the AH (ACTH, FSH, GH, LH, PRL and TSH). Only ACTH and Syncytin-1 antibodies showed a distinct but not exact overlap using confocal microscopy (Fig. 2). This result supports that hormone producing corticotropic cells of the normal AH shows a distinct Syncytin-1 expression.

We then asked the question, if Syncytin-1 protein was also detectable in PA (n=117) and whether there were differences in the expression levels between tumor subgroups and the AH. Therefore, five different hormone producing tumor classes (ACTH, ClNon, PRL, Acro, TSH) comprising 117 different cases were analyzed. Results showed that in contrast to 15 control tissues (AH), Syncytin-1 protein expression was deregulated and associated with all hormone producing neoplastic cells. A calculated overall score of 1.586 +/- 0.06 was determined for the control group (Fig. 1, Table 1), whereas the calculated mean value scores of the PA groups, from the lowest to highest Syncytin-1 expression, were: 1. ClNon = 2.289 +/- 0.04; 2. TSH = 2.314 +/- 0.10; 3. PRL = 2.361 +/- 0.13; 4. Acro = 2.562 +/- 0.06; and 5. ACTH = 2.568 +/- 0.05. Statistical analysis of Syncytin-1 scores revealed a highly significant increase of Syncytin-1 protein expression for each adenoma group when compared to normal AH (Table 1). No correlation between Syncytin-1 expression and patient age and gender was found. In addition, analyzing hematoxylin-eosin stained and Syncytin-1 IHC tissue sections demonstrated no multinuclear cells (>3 nuclei) in the normal AH or any of the hormone producing pituitary adenomas, supporting that Syncytin-1 protein expression did not associate with cell fusions.

ERV env genes and the ERVW-5 gag gene were significantly overexpressed in PA.

Due to our significant findings of Syncytin-1 protein overexpression in PA, we initiated RNA expression analyses of a total of 20 ERV env genes along with the ERVW-5 gag gene using fresh flash frozen adenoma tissues (Fig. 3, suppl. Table 1). Absolute quantification of ERV
env and gag gene expression was performed in an additional adenoma cohort (ACTH=13, CINon=52, Acro=17, PRL=10; total n=92) including matched adenoma tissues from the same paraffin sections as used from our IHC studies (n=22) and control AH tissues (n=8). Regarding control AH we observed three ERV gene expression groups, one over 100 molecules/ ng RNA, one intermediate and one lower than 10 molecules/ ng RNA (Table 1). In contrast to control AH, all tumor subgroups demonstrated significant deregulated gene expression changes, where Acro tumors showed the most significant increase (Fig. 3, suppl. Table 1). For example, 11 ERV genes were either significantly induced {Syncytin-1 (2.7-fold), Syncytin-3 (4.3-fold), Erv3-1 (3.8-fold), envK-1-7 (3.1-fold), envT (3.7-fold) and ERVW-5 gag (1.05-fold)} or reduced in expression {envFc1 (1.9-fold) and envRb (1.8-fold)}. Furthermore, in CINon tumors the highest expressed single env gene was Erv3-1 (669.76 +/- 108.38 molecules /ng RNA) (Fig. 3, suppl. Table 1).

Murine Syncytin-A and Syncytin-B are conserved in gene expression in E18.5 and adult pituitary glands.

Since both human Syncytin-1 and Syncytin-2 and murine Syncytin-A and Syncytin-B are functionally homologous we tested if expression in the pituitary gland was conserved through evolution. Hybridizing E18.5 total murine brain tissue and adult pituitary glands with RNA probes for Syncytin-A and Syncytin-B showed co-localization throughout the cytosol of the pituitary gland (Fig. 4). We noted that Syncytin-A and Syncytin-B gene expression levels were relatively equal in the E18.5. Regarding the adult murine pituitary gland, both Syncytin-A and Syncytin-B expression was the strongest in the intermediate lobe, weaker in the anterior lobe but only Syncytin-A was localized throughout the posterior lobe. In corroboration, Syncytin-A and Syncytin-B gene expression was also confirmed in adult pituitary glands using qPCR (n=6) (Syncytin-A: 43.34 +/-18.12 molecules/ ng RNA; Syncytin-B: 73.86 +/-12.48 molecules/ ng RNA (Fig. 4).

Stimulation of ERV env genes in primary human pituitary adenoma cell cultures following ACTH and CRH hormones and Forskolin treatment.

It is well established that upon CRH binding to its receptor in the anterior pituitary gland cAMP signaling is induced leading to ACTH production in corticotropic cells [5] [6]. The literature also demonstrates a positive correlation of cAMP regulation of Syncytin-1 in human placentae and in endometrial carcinomas, as well as by CRH via its receptor in placentae [48] [26] [49]. Therefore, we asked the question, if Syncytin-1 and other ERV env genes
along with the ERVW-5 gag gene could be functionally induced upon treatment of primary PA short term cell cultures (Acro, ACTH, CINon) with the hormones ACTH, CRH and Forskolin an adenylylate cyclase activator (Fig. 5B,C). Additionally, we tested for the gene expression of ACTHR, CRHR1 and CRHR2. For comparison primary human placental villous trophoblasts were treated with the same substances.

At the time of initial cell culture set-up, all three primary PA tissues showed Syncytin-1 and Erv3-1 protein expression using immunoblotting (Fig. 5A). Similar to normal AH pituitary cell cultures [50] primary PA cells grew initially in suspension with weak attachment points, but following trypsinization the cells began to grow more attached (Fig. 5 D,E). Implementing confocal microscopy and 3D imaging we localized Syncytin-1 to the cellular membrane of a gonadotrophic FSH/LH producing CINon adenoma (Fig. 5D,E). From six individual confocal images Syncytin-1 expression showed membrane specificity. 3D reconstruction simulated the same density distribution of Syncytin-1 protein throughout the cell as seen using 2D confocal microscopy. We determined specific levels of PA hormones in supernatants of primary PA short term culture experiments after five days, which confirmed each endocrine tumor phenotype (Fig. 5B,C). These initial results of PA expressing Syncytin-1 and ERV-3-1 proteins with Syncytin-1 membrane association, which is similar to the placental syncytiotrophoblast, along with PA hormone secretion represented a basis for functional assays involving ERVs.

Following 48 hr incubation with ACTH and CRH hormones or Forskolin five ERV genes were variably increased above control cultures among the tumor types (Fig. 6, suppl. Table 2). Results showed that overall the CINon and ACTH PA had the most significant number of ERV gene inductions with all three substances, especially CRH, which induced all five ERV genes. On the other hand, the Acro PA demonstrated significant induction of either Syncytin-1 or ERVW-5 gag or both genes upon ACTH or CRH treatment, however only ERVW-5 gag was significantly induced after Forskolin. Interestingly, regarding all three PA Syncytin-1 was the highest induced gene following hormone treatment (Fig. 6). Lastly, for all three PA we observed induction of the ACTHR, CRHR1 and CRHR2 upon hormone treatment (suppl. Fig. 1). For example, ACTHR and CRHR1 were induced in PA cell cultures approximately 10-fold in CINon, 5-fold in ACTH and 2-fold in Acro (suppl. Fig. 1).

Compared to cultured primary placental villous trophoblasts, PA cultures showed in part higher ERV gene inductions after ACTH and CRH treatment. Although Forskolin induced comparable significant ERV gene expression levels between PA cells and villous trophoblasts, only a non-significant induction for Syncytin-1 (2.05 +/- 0.36 fold) was observed in Acro primary cells (suppl. Table 2). Furthermore, treatment of primary PA cells with ACTH...
or CRH hormones or Forskolin did not result in any multinuclear cells (>3 nuclei) (data not shown).

**Correlation of Syncytin-1 and pCREB protein expression in AH and PA.** Following our findings supporting a possible co-induction of cAMP signaling with ERV genes in primary PA, we asked the question if the cAMP signaling marker pCREB-Ser133 protein was expressed in the same Syncytin-1 overexpressing PA and AH. Using IHC we demonstrated higher amounts of intermediate and strong positive nuclear pCREB-Ser133 expression in Acro, \((n=3)\) mean=83.6 \%; ACTH \((n=3)\) 85.6 \% and in a single AH \((n=1)\) 81\%. PCREB-Ser133 expression correlated with over 95\% of cells with strong Syncytin-1 expression in the same PA and with 24.6\% strong positive cells in the AH which we deduced were corticotropic cells as shown in Figure 1 (supple. Fig. 2). Importantly, there were pCREB-Ser133 nuclear expression differences comparing PA with AH. For example, mean values of strongly positive nuclei for both ACTH and Acro PAs represented 55.6\% and 53.6\%, whereas for the AH only 32\% was noted. Lastly, it should be noted that all pCREB-Ser133 and Syncytin-1 negative cells represented blood vessels and surrounding stroma (supple. Fig. 2).

In addition to IHC we also performed co-immunofluorescence of pCREB-Ser133 and Syncytin-1 on same PA tissue sections (Fig. 7, suppl. Fig. 2). Results showed that all three Acro and ACTH PAs showed over 80\% co-localization of nuclear pCREB-Ser133 with Syncytin-1 protein expression in the same cells (Fig. 7). These findings corroborate our IHC results above and further support a more direct association between deregulated cAMP signaling and induction of Syncytin-1.

**Discussion**

Pituitary adenomas (PA) are detected in approximately 20\% of the general population and increase with age irrespective of gender. Although they appear histopathologically benign in the vast majority of cases, they can be highly invasive and even lethal due to infiltration of surrounding structures. Severe clinical complications from excess hormone production and metabolic effects are frequently seen [51]. PA progression to a carcinoma is rare, but occurs in about 0.1\%-0.2\% of cases [6]. The molecular etiology of PA is still virtually unknown. Deregulation of signaling pathways mediated by cAMP, growth factors, hormonal stimulation as well as genetic changes have strong implications [5], but there is clearly a need to better understand the molecular basis of PA development. Our investigation points to possible functional roles of ERV genes in both human and mouse AH as well as in PA tumorigenesis associated with regulation of cAMP pathway.
In the normal AH we determined a low to high range of RNA molecules for many codogenic ERV env genes and the ERVW-5 gag, where the highest amounts (>150.00 molecules/ng RNA) were detectable for Syncytin-1, Erv3-1 and envK-1-7. We identified positive RNA localization of Syncytin-A and Syncytin-B to the mouse pituitary gland of E18.5 and adult brains, which indicates conservation through evolution and supports our hypothesis that ERV gene expression in the central nervous system goes along with a normal physiological role. This assumption is supported by other studies where ERV gene expression was detected in brain tissues from normal controls and patients with MS as well as other neurological diseases [52, 53] [54] [55] [56]. Other investigators also showed RNA expression of Syncytin-A in the murine adult brain, the hippocampus as well as total embryonic heads at E11.5 and E15.5. However, these authors did not note the presence of Syncytin-B [20].

ERV genes have also been described in several disorders. Irrespective of the origin of MSRV, a potential causal role for Syncytin-1 in MS brain tissue mediating neuroinflammation and death of oligodendrocytes is known [57] [58]. In this investigation we demonstrated that seven ERV env genes along with ERVW-5 gag increased significantly to aberrant levels in a large cohort of human PA, regardless of hormone expression. This observation leads to the assumption that deregulated ERV env genes in human AH may play a general role in adenoma formation. Several ERV genes have already been strongly implicated with tumorigenesis. The envK gene family has been linked to breast cancer and was recently highlighted as stimulating an antigen immune response [59, 60]. Treatment with envK antibodies of both breast cancer cells in culture and xenograft tumors inhibited growth, supporting a proliferative role of envK in these tumor cells [59]. Similarly, a role for Syncytin-1 in mediating steroid hormone proliferative growth of endometrial carcinoma was also demonstrated [26]. Additionally, envK-1-7 was identified to increase significantly in a step-wise manner from control endometrium to endometrial carcinoma [17]. In line with the above mentioned studies we found a significant 3.1-fold gene overexpression of envK-1-7 in Acro PA samples compared to controls, thus, pointing to a positive role in the etiology. The ERVR env gene (Env3-1) is expressed in many tissues, like testis, skin, thymus, whole brain, placenta and also in a variety of malignomas, like glioma, breast cancer, Wilm’s tumor and endometrial carcinoma [13, 17, 21, 28, 46]. We found that Env3-1 was significantly increased in all four groups of PA under study (GH, CINon, Acro, PRL) compared to controls and represented the highest single expressed env gene with up to 669.76 molecules/ ng RNA in CINon. This is in line with our recently published observations that control human endometrium had low expression of ERV env genes including Env3-1; however, Env3-1 became significantly reactivated in endometrial hyperplasia, polyps and endometrial carcinoma, and correlated significantly with tumor grading to a more undifferentiated state.
It was also shown that following transfection of Env3-1 expression vectors in the choriocarcinoma cell line BeWo resulted in differentiation changes, increased intercellular levels of \( \beta \)-hCG and cAMP [61] [62]. Additionally, over expressing Env3-1 resulted in negatively regulating the cell cycle by inhibiting cyclin B and up-regulating the negative regulator p21 [61] [62]. Thus, similar to other tumors, Env3-1 expression in PA could play a role in controlling differentiation, a speculation that has to be confirmed in further studies.

We especially focused on Syncytin-1 due to its already established relevance noted in MS, carcinogenesis and placentogenesis [32] [16] [57] [55] [26]. A detailed Syncytin-1 analysis was performed especially on PA ranging from IHC, gene expression, primary cell cultures and immunofluorescence including confocal microscopy. Syncytin-1 specifically co-localized to corticotropic cells in the human AH. Normal and aberrant expression of Syncytin-1 in AH and several PA subtypes, respectively, were demonstrated due to primers discriminating between envW-2 and other non-protein coding envW family members locating on chromosomes 5q11.2, 6q21, 14q21.3, 15q21.3 and 17q21. Importantly, Syncytin-1 expression was distinctive to MSRV. It is known that the membrane protein of Syncytin-1 is essential for placentogenesis where it mediates villous cytotrophoblasts cell fusion into a syncytium controlling nutrient and gas exchange between the mother and the foetus [16, 63] [64] [65] [66]. Cell fusion of breast or endometrial cancer cell lines was repressed with antisense RNA or with siRNA against Syncytin-1, respectively [26, 67]. For both placental and cancer cell fusions cAMP-signalling has been strongly implicated [26]. Although, Syncytin-1 showed strong membrane localization using IHC and fluorescent microscopy, including confocal microscopy of primary PA cells, no cell fusions (multinucleated cells) in PA tissue sections or in cell culture experiments upon cAMP signalling were found. This could be explained by the abundance of TGF-\( \beta \)1 and TGF-\( \beta \)3 proteins (suppl. Fig. 3) where it was previously demonstrated that TGF-\( \beta \) negatively regulated cell fusions despite induced Syncytin-1 levels in endometrial carcinoma and placental cell cultures [26]. On the other hand, Syncytin-1 and other fusogenic as well as non-fusogenic ERV env genes may have adapted to use the cAMP pathway for different functional roles depending upon the cell type and differentiation state.

Deregulation of the cAMP pathway is one hallmark among hormone-secreting adenomas, although it is still not known if aberrant cAMP signaling leads to a proliferative advantage of cellular growth [5]. The mitogen activated protein kinase pathway (MAPK) has also been implicated in PA etiology, where cAMP crosstalk’s via ERK1/2 [5]. Interestingly, Forskolin was also shown to induce ERK1/2 activity via cAMP stimulation [5]. In addition to the Forskolin and estrogen receptor regulatory elements in the ERVW-1 promoter we also showed that Syncytin-1 gene expression can be regulated via PPAR\( \gamma \) and RXR\( \alpha \), pointing to
multiple ERVW-1 pathway regulations and different functional outcomes [25] [17] [26] [47]. Importantly, cAMP induced Syncytin-1 expression whereas a PKA inhibitor (H89) repressed expression in vitro, localizing the DNA response region to the U3 part of the ERVW-1 LTR promoter [25]. These experiments indicated a cAMP/PKA/CREB regulation pathway for ERVW-1. Although only half CRE sites were localized to this region, AP1/AP2/Sp1 binding sites were also found [25]. According to several publications, heterodimers of Fos/Jun and ATF/CREB family members can functionally bind to either TRE or CRE [68] [69]. As an example for normal cAMP-dependent PKA regulation in the brain, increased CRH gene expression via CREB occurs in the hypothalamus, results in production of CRH in the paraventricular nucleus, secretion and then binding to the AH CRHR1 with activation of cAMP signaling and ACTH production [70] [5] [6] (Fig. 8). Additionally, the pro-hormone proopiomelanocortin (POMC) peptide induced from AH corticotropic cells is cleaved into ACTH, which then binds the ACTHR in the adrenal gland causing the release of cortisol [71]. The ACTHR signals via G-proteins to activate adenylate cyclase and increases cAMP levels [72]. It is intriguing to note that our findings of both Syncytin-A and Syncytin-B co-localizing to the adult mouse pituitary intermediate lobe corroborates with Mass Spectrometry images of POMC peptides α-MSH and CLIP, supporting a linkage [73]. CRHR, POMC and the ACTHR have also been detected in the placenta [74] [75], where we recently demonstrated a 6-fold Syncytin-1 induction of expression in human primary trophoblasts after 48 hr of CRH treatment [49]. It was also shown that CRH resulted in the induction of CRHR1 and syncytialisation, similar to cAMP alone [76]. Our immunofluorescence and IHC experiments taken together with our primary PA cell culture further corroborates a possible and positive connection between ERV gene expression and cAMP signalling. For example, CRH, ACTH and Forskolin treatments resulted in induction of the corresponding hormone receptors as well as several ERV genes with Syncytin-1 and ERVW-5 gag representing the highest expressed genes. More ERV genes were induced by both hormones and Forskolin in CINon and ACTH PA primary cells compared to Acro PA cells, where only Syncytin-1 and/or ERVW-5 gag increased, perhaps relating to different regulations of cAMP signalling. The correlation of over 80% of pCREB-Ser133 and Syncytin-1 double positive PA cells points to a possible co-regulation of Syncytin-1 expression through cAMP signalling, which could help to explain the deregulation of Syncytin-1 in PA. Therefore, we propose a model where general activation and deregulation of cAMP signaling could be one key for understanding ERV activation in PA (Fig. 8).

Conclusion:

A few studies in the literature have demonstrated ERV gene expression throughout brain tissues, except for the pituitary gland and its associated neoplasms. Our study brings
forth new information about the expression of ERV genes in the normal AH compared to different PA subtypes. Additionally, we demonstrated pituitary gland conservation of ERV env expression between humans and mice. We support the idea that ERV env and gag gene family members are functional in normal brain including the AH, but become highly induced and deregulated in human PA like in other neoplasms. We could demonstrate that Syncytin-1 co-localized exclusively within ACTH producing cells in normal AH, but in contrast to all endocrine tumor cells of PA subtypes. Gene expression of Syncytin-1, other ERV env genes and the ERVVW-5 gag gene were significantly induced in fractionated primary adenoma cells in the presence of ACTH or CRH hormones. Importantly, the cAMP activated marker protein pCREB-Ser133 and Syncytin-1 co-localized in the same cells from various PA. We present a model whereby ERV genes have adapted through evolution to regulate their expression levels via general cAMP signalling thus possibly contributing to aberrant growth or differentiation during tumorigenesis.

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Author contributions
Rolf Buslei – study design, experimental work, acquisition and interpretation of data, data analysis.

Pamela L. Strissel – study design, experimental work, data analysis, drafting manuscript.

Christine Henke – experimental work, critical review.

Regina Schey – experimental work, critical review.

Nadine Lang – confocal and 3D experimental work, critical review.

Matthias Ruebner – experimental work, critical review.

Claus C. Stolt – mouse experimental work, critical review.
Ben Fabry – confocal and 3D experimental work, critical review.

Michael Buchfelder – acquisition and interpretation of patient data, critical review.

Reiner Strick – study design, experimental work, data analysis, final manuscript
Figure Legends:

Figure 1. IHC of Syncytin-1 expression in the normal AH and various PA. Five different PA subtypes are shown; ClNon was an LH producing adenoma. Note the clonal expansion of Syncytin-1 expression throughout each tumor type. Arrows in the ACTH adenoma show examples of strong membrane staining of Syncytin-1. In addition, numbers represent the individual tumor protein expression calculated from IHC (see Table 1). Bars = 20µm.

Figure 2. Syncytin-1 and ACTH expression co-localizes to normal AH corticotropic cells. A: Non-confocal immunofluorescence images of a normal AH paraffin tissue section showing DAPI staining (blue=nuclei) with co-hybridization of primary antibodies specific for Syncytin-1 (green) and ACTH (red) and merged image. Bar = 20µm. B: Confocal microscopy showing immunofluorescence of a normal AH paraffin tissue section co-hybridized with primary antibodies specific for Syncytin-1 and ACTH and then detected using an Ar (488nm) or He-Ne laser (543nm), respectively. Both top and bottom panels from left to right show a green channel with Syncytin-1, red channel with ACTH localization, a merge of both Syncytin-1 and ACTH. Bar = 40µm.

Figure 3. Gene expression of 20 ERV env genes and the ERVV-5 gag in 92 PA and 8 control AH. Top and bottom graphs demonstrate for each PA cohort gene expression (color coded) values of ERV genes in molecules/ng RNA. The non-parametric Mann-Whitney-U test for independent samples was performed using IBM SPSS Statistics 21 with *P<=0.05 significant and ** P<=0.001 highly significant values. Note that in control AH, Syncytin-1, Erv3-1, envK-1-7 and ERVV-5 gag were over 100 molecules/ ng RNA and represented the highest expressed ERV genes, whereas Syncytin-3, env-Rb and envW-2 were <10 molecules / ng RNA (Table 1). From the highest to the lowest fold induction regarding the Acro PA cohort Syncytin-1 (2.7-fold), Syncytin-3 (4.3-fold), Erv3-1 (3.8-fold), envT (3.7-fold), envK-1-7 (3.1-fold), and ERVV-5 gag (1.05-fold) expression were significantly induced compared to the control cohort, whereas envFc1 (1.9-fold) and envRb (1.8-fold) were significantly reduced. The ClNon PA cohort showed significant inductions of Erv3-1 (4.5-fold), envFc2 (4.2-fold), envT (3.5-fold), ERVV-5 gag (2.4-fold) and envRb (1.6-fold). The PRL cohort showed significant inductions of envT (5.1-fold), envFc2 (2.6-fold), envRb (2.6-fold), Erv3-1 (2.1-fold) and ERVV-5 gag (1.5-fold) and the ACTH cohort showed significant inductions of Syncytin-3 (4-fold), envT (3.2-fold), Erv3-1 (2.8-fold) and envFc2 (2.2-fold). In contrast, envE4-1 (3.3-fold) showed significant reduction of expression. Only 3 genes (Erv3-1, envFc2 and envT) were significantly induced in all adenoma cohorts. In contrast to all of...
the above, *Syncytin-2, envH-1-3* and *envW-2* were not significantly deregulated in all PA compared to controls.

**Figure 4. Gene expression of Syncytin-A and Syncytin-B env genes in E18.5 murine and adult pituitary glands.** Top pictures show tissue sections of a whole E18.5 and an adult murine brain stained with Hematoxylin Eosin and a corresponding schematic modified from Sheng and Westphal [77] (PL=posterior pituitary lobe; IL=intermediate pituitary lobe; AL=anterior pituitary lobe). Bottom left and right pictures show magnifications of the pituitary gland from E18.5 murine whole brain sagittal sections and adult pituitary gland cross sections hybridized with *Syncytin-A* or *Syncytin-B* RNA, respectively. Note that the pituitary gland shows the same positive expression for both *Syncytin-A* and *Syncytin-B* in the cytosol (arrows). A graph demonstrating gene expression values of *Syncytin-A* and *Syncytin-B* from six adult murine pituitary glands in molecules/ng RNA is shown at the top right.

**Figure 5. ERV env protein, microscopic and hormonal analyses of PA primary cell cultures.** A) Immunoblot detection of Syncytin-1 and Erv3-1 protein in primary PA from three subtypes at the initial time of collection. Kilodaltons (kDa) are indicated; B) Primary PA cells of ClNon, ACTH and Acro in culture at day 5. C) Graph shows an indicated serum hormone evaluation of primary PA at day 5 of cell culture. The Y axis represents a logarithmic scale for each indicated hormone value signified in mlU/ml or the specific concentration (pg/ml or ng/ml) as indicated to the right (color coded). Above each bar on the X-axis the total amount of hormone (mlU/ml or pg/ml or ng/ml) is shown. Note that the gonadotrophic adenoma (ClNon) demonstrated the highest FSH (640.6 mlU/ml) and LH (627 mlU/ml) levels while the adenoma cultured from a M. Cushing patient (ACTH) secreted the highest amounts of ACTH (111,813 pg/ml) as well as moderate levels of PRL (336.6 mlU/ml). Additionally, an adenoma cultured from a patient diagnosed with clinical signs of acromegaly (Acro) secreted the highest amounts of GH (16,256 ng/ml) and PRL (4,428 mIU/ml) as well as moderate levels of ACTH (2,534 pg/ml). D) Immunolocalization of Syncytin-1 protein expression was analyzed from six different microscopically analyzed 2-D confocal images, each consisting of >25 Z-sections. Pictures show examples of three cells imaging Syncytin-1 on a FSH/LH producing ClNon PA. Syncytin-1 antibody was detected using an Ar-laser (488nm). E) 2-D confocal images constructed into a 3-D image according to top view.

**Figure 6. Activation of cAMP with CRH, ACTH and Forskolin of primary PA cell cultures leads to ERV gene inductions.** Following a 48 hr. treatment of primary PA (5% CTS) with ACTH (2x10^{-9} M), CRH (0.5x10^{-9} M) or Forskolin (4x10^{-5} M) ERV gene expression was determined in molecules/ng RNA. Graph shows the fold increase above untreated control samples. Specific ERV genes are indicated and show the fold increase plus the standard error of the mean above the untreated control sample. For every drug induction a
minimum of least three experiments were performed for each PA. In the case of the ACTH PA ten experiments for each hormone were performed. The non-parametric Mann-Whitney-U test for independent samples was performed using IBM SPSS Statistics 21 with * P=<0.05 significant and ** P=<0.001 highly significant values.

Figure 7. Co-localization of pCREB-Ser133 and Syncytin-1 to the same cells in PA and AH. Non-confocal immunofluorescence images of one ACTH and one Acro PA paraffin tissue section are shown, with DAPI staining (blue= nuclei), co-hybridization of primary antibodies specific for pCREB-Ser133 (red), Syncytin-1 (green) and a merged image. A magnification (zoom) from a part of the merged image (white box) is shown. Note that pink nuclei indicate pCREB-Ser133 positive nuclei (blue plus red), blue nuclei were pCREB-Ser133 negative. Co-localizations of pCREB-Ser133 with Syncytin-1 represent staining of green cytosol/membrane plus pink nuclei. Bar = 20 µm.

Figure 8. Model shows possible ACTH, CRH and GHRH activation of cAMP leading to ERV promoter regulation by pCREB-Ser133 in PA. Following hormone ligand binding to specific receptors, cAMP levels increase via G-protein (Gαs-GTP, Gα) and adenylate cyclase (AC). Forskolin also activates the AC. Cellular increased levels of cAMP lead to activation of protein kinase A (PKA) and subsequently phosphorylation of CREB at Ser (S)133. pCREB-Ser133 binds to its nuclear DNA binding site or CREB responsive element (CRE), which was identified important for placental trophoblast function and the ERV-W1 LTR [78] [47].

Table legends.

Table 1. Results of the Syncytin-1 IHC analyses. Top table shows for each cohort the analyzed mean +/- standard error of the mean for the Syncytin-1 protein expression calculated from IHC. The non-parametric Mann-Whitney-U test for independent samples was performed using IBM SPSS Statistics 21 for calculating the P values. Below is a representative graph showing the mean +/- standard error of the mean of Syncytin-1 expression derived from the IHC analyses.
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Table 1:

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