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Short title: steroidogenesis postnatal fetal Leydig cells

Steroidogenesis and steroidogenic gene expression in postnatal fetal rat Leydig cells

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Keywords: postnatal fetal Leydig cells, steroidogenesis, steroidogenic gene expression

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Abstract

We studied steroidogenesis and the regulation of Leydig cell-specific gene expression in primary cultures of highly purified postnatal fetal Leydig cells (PFLCs). PFLCs activated by hCG and (Bu)₂cAMP demonstrated transient capacity to produce testosterone (T) *in vitro*. A time dependent decline in T production by (Bu)₂cAMP –stimulated PFLCs was observed and associated with the accumulation of progesterone in the culture media and complete suppression of P450c17 expression at the translational but not transcriptional level. PFLCs was found to lose their capacity to express Leydig cell-related genes (e.g. 3βHSD, P450c17, Insl3), which was restored by treatment with (Bu)₂cAMP. It was also found that PDGFα alone and in combination with (Bu)₂cAMP significantly stimulated proliferation of the isolated PFLCs *in vitro*. Our data indicate that cAMP-activated signaling pathway(s) play an important role in the regulation of PFLCs differentiation and function.

Keywords: postnatal fetal Leydig cells, steroidogenesis, steroidogenic gene expression

1. Introduction

Fetal Leydig cells (FLCs) are a distinct population of Leydig cells that originate in the embryo. These cells secrete androgens that are critical for the normal development of male reproductive organs as well as insulin-like factor 3 (InsI3) required for the abdominal phase of the scrotal descent of the testis (Huhtaniemi and Pelliniemi, 1992; Ivell *et al.*, 1997). In rats, FLCs start to differentiate and produce testosterone at gestational age of 14 days before they undergo functional regression from fetal day 18.5 onward (Habert and Picon, 1984; Kuopio *et al.*, 1989). In humans, the initiation of differentiation of these cells must be independent of LH, since the onset of testicular androgen production precedes the secretion of LH by the pituitary (Habert *et al.*, 2001). This finding strongly support the idea that neither hCG secreted by the placenta nor LH are involved in regulating the initial phase of human fetal Leydig cell differentiation (Habert *et al.*, 2001). However, LH does stimulate testicular testosterone production after the appearance of this hormone and its receptor, indicating that hCG/LH is absolutely required for the maintenance of testicular androgen production at later stages (Rabinovici and Jaffe, 1990). In rodents, fetal Leydig cell function is normal in the absence of LH or its receptor (O'Shaughnessy *et al.*, 1998; Zhang *et al.*, 2001) and LH does not regulate steroidogenic enzyme expression (Majdic *et al.*, 1998).

Desert hedgehog (*Dhh*) and platelet-derived growth factor A (*Pdrga*), two Sertoli cell-derived signaling molecules, play an important role in differentiation of fetal Leydig cells (Pierucci-Alves *et al.*, 2001; Yao *et al.*, 2002; Brennan *et al.*, 2003). Secreted by the Sertoli cell, *Dhh* acts in a paracrine fashion to induce the differentiation of both the fetal Leydig cells and of the peritubular myoid cells, surrounding the testicular cords (Yao *et al.*, 2002). *Dhh* activated signaling was suggested to trigger fetal Leydig cell differentiation by up-regulating SF-1 and cytochrome P450 side-chain cleavage (P450_{scc}) expression in precursor cells located outside

testis cords (Yao *et al.*, 2002). Similarly, mutations in the gene encoding PDGF-A were found to attenuate the expression of P450_{scc} and thereby disrupt early Leydig cell differentiation in mice (Gnessi *et al.*, 2000).

There are several suggestions concerning the fate of FLCs after birth. Postnatal FLCs were found to decrease in number and to disappear from the interstitium probably due to cell death (Roosen-Runge and Anderson, 1959), while later reports demonstrated that the total number of FLCs in the testis does not change significantly after birth (Mendis-Handagama *et al.*, 1987; Kuopio *et al.*, 1989). Kuopio *et al.* (1989) observed an increase in total number of FLCs between postnatal days 3 and 11, a cellular process associated with elevated levels of LH. However, during and after the second postnatal week FLCs showed signs of regression associated with a decreased steroid content per cell (Kuopio *et al.*, 1989). FLCs were shown to persist in the adult testis and do not undergo early postnatal degeneration (Kerr and Knell, 1988). All these findings suggest that postnatal FLCs (PFLCs) may differ from adult Leydig cell lineage in their biology and steroidogenesis.

At present, virtually nothing is known about the function of PFLCs in the neonatal testis. Thus, the aim of this study was to characterize phenotype and steroidogenesis of highly purified PFLCs *in vitro*. We also investigated the role of cAMP-PKA-dependent signalling in the regulation of the expression of Leydig cell-specific genes in PFLCs and explored paracrine factors controlling their proliferation.

2. Material and Methods

2.1 Materials

Dulbecco's Modified Eagle's Medium (DMEM)-Ham's nutrient mixture F-12, Modified Eagle's Medium (MEM), fetal calf serum (FCS), bovine serum albumin (BSA) and antibiotics were obtained from Gibco/BRL (Life Technologies, Paisley, Scotland). Percoll, HEPES, hCG (14000 U/mg), (Bu)₂cAMP and collagenase type I were purchased from Sigma (Sigma Chemical Co., St. Louis, USA). The following antibodies were purchased from Santa Cruz Biotechnology: rabbit polyclonal antiserum against LHR (catalog no. sc-25828); rabbit polyclonal antiserum against Insl3 (catalog no. sc-134587), rabbit polyclonal antiserum against P450c17 (catalog no. sc-66850) and rabbit polyclonal antiserum against WT1 (catalog no. sc-192). Other antibodies were: 3 β HSD (kindly provided by J.I.Mason, University of Edinburg, UK) and StAR (kindly provided by D. Stocco, Texas Tech University, USA).

2.2 Animals

Testes from 8-day old Sprague-Dawley rats (B&K Laboratories, Sollentuna, Sweden and Charles River, Germany) were used for the preparation of PFLCs. These experiments were approved by the Northern Stockholm Animal Ethics Committee (registration no. N319/08).

2.3 Preparation of testicular cell suspension

The testes were excised and decapsulated. Testicular tissue was minced using fine scissors and transferred into MEM containing collagenase type I (0,25 mg/ml). Digestion was performed at 37⁰C for 20 min in a shaking water bath operating at 100 cycles/min.

Interstitial cells were separated from tubuli mechanically and filtrated via 70- μ m nylon mesh. The obtained filtrate was centrifuged at 300xg for 7 minutes and after resuspension in HBSS

containing 0.1% BSA the cells were filtrated via 40- μm nylon mesh and counted using phase-contrast microscope.

2.4 Magnetic labeling and isolation of fetal Leydig cells

Aliquots of cell preparations with a final concentration of 10^7 cells/100 μl were labeled using an indirect labeling technique with the magnetic beads attached to a secondary antibody. The separation procedure was carried out according to the manufacturer's instructions (Miltenyi Biotech). In brief, the cells were incubated with polyclonal rabbit anti-LHR IgGs (20 μl /10⁷ cells) for 20 min at 4⁰C. After two washes the cells were labeled with 30 μl /10⁷ cells goat anti-rabbit IgG microbeads (Miltenyi; order no. 130-048-602) diluted in 70 μl /10⁷ cells HBSS (containing EDTA and BSA as described above). The cells were incubated for 20 min at 4⁰C. For the subsequent magnetic separation, ice-cold degassed HBSS buffer containing EDTA and BSA was utilized. The cell suspension was resuspended in degassed buffer (500 μl /10⁷ cells) and poured into through a MS separation column (Miltenyi: order no. 130-042-201). LHR-positive and thus magnetically labeled cells were retained within the magnetized column matrix, whereas nonlabeled cells passed through and were collected as the nonmagnetic fraction. To increase the purity of the magnetic fraction, the column was washed three times. In order to retrieve the magnetic fraction, the column was removed from the separator and the cells were twice flushed out of the column in 1ml of degassed buffer. Purified fetal Leydig cells were resuspended in DMEM-F12 supplemented with 365mg/L glutamine, 2% FCS, 100 IU/ml penicillin and 100 μg /ml streptomycin. The yield of PFLCs isolated from 2-3x10⁷ testicular cells that had been isolated from the testes of 10 pups was 1% (2-3x10⁵ cells, on average 12500 cells per testis). 97-98% of the cells obtained were 3 β HSD-positive after immunohistochemical staining for 3 β HSD

(Fig. 1C). To exclude contamination with Sertoli cells, which are abundantly present in the neonatal testis, primary cultures of PFLC were stained for WT1, a marker for Sertoli cells. No expression of WT1 was detected in the primary cultures of PFLC (data not shown). For culturing, 100 μ l of a suspension containing 1×10^5 cells/ml was plated into each well of a 96-well Falcon plate (Falcon, USA) and treated with hCG (10 ng/ml) and $(\text{Bu})_2\text{cAMP}$ (1 mM) for different time-points at 34°C .

2.5 Immunohistochemistry

The immunohistochemical detection of PFLCs was performed as described earlier (Renlund *et al.*, 2006). Cells were cultured for 48 hours on cover glasses placed in 12-well plates followed by fixation with 4% paraformaldehyde at room temperature for 20 min and permeabilization with 0.3% Triton X-100 for 5 min. After washing, they were incubated in blocking buffer (10% donkey serum in PBS and 0.1% BSA) for 20 min at room temperature followed by incubation with primary rabbit anti-StAR, Ins13, LHR, and $3\beta\text{HSD}$ polyclonal antibody at 1:100 dilution for 2 h at room temperature. The cover slips were then washed 3 times with PBS before incubation with secondary Cy3-conjugated donkey anti-rabbit antibodies (Jackson Immuno Research Lab Inc.) in a dilution of 1:300 for 1 h at 37°C and mounted with Vectashield (HardSet, mounting medium with DAPI, Vector lab Inc, Burlingame, CA). The cells were viewed with a Nikon fluorescent microscope (Nikon, Bergstrom Inst., Solna, Sweden). Negative controls were performed using rabbit IgGs in a dilution of 1:100.

2.6 Assay of steroidogenic enzymes activity

Measurement of P450_{scc}, 3 β HSD, P450_{c17}, 17 β HSD and 17KSR activities was performed by incubating overnight pre-cultured cells (2×10^4 cells/well) with appropriate labeled substrate (e.g., ^3H -25-OH-cholesterol, ^3H -pregnenolone, ^3H -progesterone and ^3H -androstenedione (10^5 cpm/well)) and 1 μM of corresponding unlabeled steroids for 2 h at 34 $^{\circ}$ C. Control samples of culture medium without cells were incubated and processed in parallel with each enzyme assay and provided blank values that were subtracted from the respective experimental samples. The reaction was stopped by the addition of 5 μl 5 N NaOH and the steroids present then extracted once by ice-cold ethyl acetate and organic phases combined and evaporated to complete dryness. The activity of P450_{scc} was determined by measuring the conversion of 25-[26,27- ^3H] hydroxycholesterol to pregnenolone (PREGN), progesterone (PROG), 17 α -hydroxyprogesterone (17 α -OH-PROG), androstenedione (DIONE) and testosterone (T). The activity of 3 β HSD was assayed by measuring the conversion of PREGN to PROG, 17 α -OH-PROG, DIONE and T. The activity of P450_{c17} was determined by measuring conversion of PROG to 17 α -OH-PROG, DIONE and T. The activities of 17KSR and 17 β HSD were assayed by measuring the conversion of DIONE to T and T to DIONE, respectively.

The steroids were separated on 60 F₂₅₄ TLC plates (Merk, Darmstadt, Germany) in chloroform-ethyl acetate (4:1, v:v) as the mobile phase. The 25-OH-cholesterol, PREGN, PROG, 17 α -OH-PROG, DIONE and T were detected under UV light and by exposure to iodine vapor. The amounts of substrate remaining and products formed were quantified by cutting out the appropriate sections of the silica gel and subjecting these to scintillation counting.

2.7 Steroid assays

Culture medium samples were stored at -20°C prior to analysis of estradiol, testosterone, androstenedione and progesterone. Estradiol, testosterone, androstenedione and progesterone were quantified employing the Coat-a-Count RIA kit (Diagnostic Products Corp., Los Angeles, CA), according to the manufacturer's instructions. Intraassay and interassay coefficients for estradiol, testosterone and androstenedione RIA were 7.0, 6.4, 5.0% and 7.4, 3.2 and 5.6 %, respectively. The same parameters for progesterone RIA were 4 and 3.9%.

2.8 Isolating RNA and producing cDNA

Total RNA was extracted from control and $(\text{Bu})_2\text{cAMP}$ -treated fetal Leydig cells by RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the protocol provided by the manufacturer. The amount of total RNA was measured by photometry (BioPhotometer, Hamburg, Germany). The RNA was kept at -80°C until further analysis.

Total RNA was further processed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) as proposed in the manufacturer's protocol.

2.9 Gene expression analysis by RT-PCR

The Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany) and dNTP set (GE Healthcare, Germany) were used to prepare the PCR mix (Primer: $0,25\mu\text{M}$ each, dNTP: $0,2\text{mM}$, MgCl_2 $1,5\text{mM}$, enzyme: $0,9\text{U}$). The conditions for amplification were 5 min at 96°C , followed by 28–35 cycles of denaturation for 30 s at 96°C , annealing for 30–60 s at 52 – 58°C and extension for 1 min at 72°C , and finally extension for 5 min at 72°C and cooling down to 4°C (Applied Biosystems, 2720 Thermal Cycler). For specific conditions for each gene see Table

1. Expression of the house-keeping gene β -actin was also analysed for each testis sample. Primer pairs specific for rat Leydig cell-specific gene cDNAs were designed and then produced by Sigma (Table 1). DNA contamination was excluded by running PCR without reverse transcriptase.

Reaction products were mixed with loading buffer and analysed by electrophoresis through 2% agarose gel (SeaKem, LE Agarose, Lonza, Rockland, ME) containing gel red (Nucleic Acid Gel Stain, 10000x, Biotium, Hayward, CA). Sample size was controlled by using DNA molecular weight Marker (Roche Diagnostics, Germany). Gels were scanned using a Canon PowerShot A640 and AlphaProView 1.0 software (Alpha Innotech Corporation, San Leandro, CA).

2.10 Gene expression analysis by qPCR

The samples for qPCR were prepared using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and the PCR cycles were run at 95 °C for 10s, 60 °C for 45s, 95 °C for 60s and 55 °C for 60s followed by a melting curve from 55-95°C in steps of 0.5 °C and then held at 4 °C (iCycler iQ, Bio-Rad Laboratories, Hercules, CA) after having estimated the best reaction conditions by running a temperature gradient. cDNA samples from 8 day old rats were used as positive control. The gene expression of P450c17 and the house keeping gene β -actin were analysed using the same primers as for RT-PCR (Table1).

2.11 Western blot analysis

The effect of (Bu)₂cAMP on the level of expression of cytochrome P450c17 by PFLCs was examined by PAGE/Western blotting as described earlier (Svechnikov *et al.*, 2003; Svechnikov *et al.*, 2005). Briefly, PFLCs stimulated with or without (Bu)₂cAMP for 4 days were washed

twice with PBS and then lysed and sonicated in a buffer containing 62.5 mM Tris-Cl (pH 6.8), 2% SDS, 50 mM dithiothreitol, and 10% glycerol. Subsequently, the fraction thus solubilised was separated from debris by centrifugation at 10,000xg for 6 min and the proteins present in the resulting supernatants (30 µg protein from each sample) separated by electrophoresis on 10% SDS/polyacrylamide gels and thereafter transferred electrophoretically to Hybond-P polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK), using 25 mM Tris-Cl, 185 mM glycine, pH 8.3, containing 20% methanol. Polyclonal cytochrome P450c17 antiserum (rabbit polyclonal IgG, Santa Cruz Biotech, USA) was used for incubation at 1:500 in TBS containing 5% non-fat dry milk overnight at 4 °C. Antibody against β-actin (mouse monoclonal IgG, 1:5000; Sigma Chemical Co., St. Louis, USA) was used as an internal standard. After washing, membranes were incubated with donkey anti-rabbit IgG secondary antibodies conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, UK) and the bands detected by incubation with ECL Plus™ Western blotting agent (Amersham Pharmacia Biotech, UK) and subsequent exposure to Hyperfilm ECL (Amersham Pharmacia Biotech, UK).

2.12 Assays of PFLCs proliferation

Isolated cells were cultured for 72 hours with or without (Bu)₂cAMP, PDGF α , LIF and their combinations followed by labelling with ³H-thymidine (Amersham Pharmacia Biotech, UK) at concentration of 1 µCi per well for the last 24 hours. The cells were washed and then lysed with NaOH (5 N) and incorporated radioactivity was measured in a Beckman liquid scintillation counter.

2.13 Statistical analysis

The differences between various values were analysed for statistical significance by one-way analysis of variance (ANOVA) followed by the Dunnett t-test, using the SigmaStat (v 3.00) package (SPSS, Inc, Chicago, IL). $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Phenotype of postnatal fetal Leydig cells

Most (97-98%) of isolated cells were 3β HSD-positive (Fig. 1C). As shown in Figure 1, PFLCs expressed various Leydig cell-specific proteins such as *Ins13* (Fig.1A), *LHR* (Fig.1B), 3β HSD (Fig.1C) and *StAR* (Fig.1D), indicating that this population of Leydig cells was fully differentiated.

3.2 Basal and hCG-stimulated steroidogenesis in PFLCs

PFLCs showed low basal production of progesterone and testosterone but their treatment with hCG resulted in a significant (17- and 11-fold, $P < 0.05$, respectively) increase in steroid biosynthesis during first 24 hours of stimulation, a process that was significantly attenuated after prolongation of stimulation with the stimulator for the next 48 hours (Fig.2A,B). This finding indicates that LHR-coated microbeads did not affect LHR-coupled signaling in PFLCs.

We also measured the basal activity of steroidogenic enzymes in PFLCs. The order of the activity of the steroidogenic enzymes in PFLCs was found to be 3β HSD > P450_{scc} > P450_{c17} > 17KSR > 17 β HSD (Fig. 3), suggesting attenuation of progesterone conversion into androstenedione and testosterone.

3.3 Time-dependent steroid production by (Bu)₂cAMP -activated PFLCs

We further explored the profile of steroids production by (Bu)₂cAMP -activated PFLCs during different time-points of culture. The biosynthesis of testosterone and androstenedione by (Bu)₂cAMP –stimulated cells was observed to be maximal after 24 hours of culturing and was then significantly declined in time-dependent manner (Fig 4 A,B). In contrast, (Bu)₂cAMP time-dependently activated progesterone production by PFLCs during 72 hours of incubation (Fig. 4C), suggesting that the expression of the cytochrome P450c17 was significantly suppressed in those cells.

3.4 Effect of long-term stimulation with (Bu)₂cAMP on Leydig cell-related gene expression in PFLCs

Since (Bu)₂cAMP is a well-known activator of protein kinase A (PKA) signaling, which up-regulates the expression of certain steroidogenic enzymes in adult Leydig cell lineage (Payne and Hales, 2004), we investigated whether treatment with (Bu)₂cAMP influenced the expression of Leydig cell-specific genes in PFLCs. We observed that after 4 days of culturing, PFLCs lost their capacity to express some Leydig cell-derived genes, excluding 17βHSD (Fig.5). However, treatment with (Bu)₂cAMP up-regulated the expression of all studied Leydig cell-specific genes, excluding LHR, suggesting that cAMP-dependent signaling is required to support PFLCs to stay in a differentiated state. Moreover, we observed that (Bu)₂cAMP slightly but clearly stimulated P450c17 expression, the cellular process associated with accumulation of progesterone in the culture media from PFLCs cells. This apparent contradiction let us to suggest that (Bu)₂cAMP up-regulated P450c17 expression at the transcriptional but not translational level.

To confirm that (Bu)₂cAMP stimulates P450c17 gene expression, a quantitative real-time RT-PCR analysis was performed. We demonstrated that treatment with (Bu)₂cAMP dramatically (100-fold over control, P<0.01) activated the expression of P450c17 (Fig. 6).

3.5 Effect of (Bu)₂cAMP treatment on P450c17 expression at the protein level

Indeed, western blot analysis of the P450c17 expression in (Bu)₂cAMP activated PFLCs did not show the cytochrome expression at the protein level, while this steroidogenic enzyme was clearly detected in immature Leydig cells served as positive control (Fig. 7). To test the hypothesis that hCG/(Bu)₂cAMP-stimulated PFLC may undergo desensitization via an estradiol-mediated mechanism that suppressed P450c17 function, we measured the levels of estradiol in culture media from same cells activated with (Bu)₂cAMP. We observed that (Bu)₂cAMP was not able to stimulate estradiol production by PFLC during 96 hours of stimulation, suggesting lack of activation of aromatase expression in those cells (data not shown).

3.6 Effect of (Bu)₂cAMP and paracrine factors on PFLC proliferation

We further explored the role of cAMP-dependent signaling as well as PDGF α and LIF, the paracrine factors that control Leydig cell development (Gnessi *et al.*, 2000; Ge *et al.*, 2006), in the regulation of PFLC proliferation. We observed that PDGF α significantly (1.8-fold over control, P<0.05) stimulated the proliferation of PFLCs. Similarly, this paracrine factor in combination with (Bu)₂cAMP enhanced markedly the mitotic activity of PFLCs, while (Bu)₂cAMP and LIF alone had no significant effect on PFLC proliferation (Fig.8).

4. Discussion

In the present study we isolated and explored the regulation of steroidogenesis and function of LHR-positive steroidogenic cells from 8-day old neonatal testis designated as PFLCs. Those purified native PFLCs showed a transient (within 24 hours) capacity to produce testosterone in response to exposure to hCG and (Bu)₂cAMP in primary cultures. However, long-term stimulation with (Bu)₂cAMP resulted in accumulation of progesterone in the culture media of PFLCs, suggesting that the function of P450c17 is significantly suppressed in those cells. Further experiments demonstrated that (Bu)₂cAMP up-regulated P450c17 gene expression in PFLCs at the transcriptional level but its translation into functional protein was completely suppressed. Our study has also demonstrated that during long-term culturing PFLCs lost their capacity to express the major Leydig cell-related genes (e.g., 3βHSD, P450c17 and Insl3), the cellular events that were restored by treatment with (Bu)₂cAMP. Moreover, we have observed that PDGFα alone and in combination with (Bu)₂cAMP significantly stimulated proliferation of isolated PFLCs in vitro, suggesting that PDGFRα-activated signaling play an important role in controlling the mitotic activity of PFLCs and cAMP-PKA-dependent pathway may promote the proliferative effects of PDGFRα on those cells.

It was previously reported that FLCs decline in number after birth, a cellular process suggested to be associated with: (a) cell death (Kuopio *et al.*, 1989), (b) dedifferentiation to fibroblastic-type cells (Gondos *et al.*, 1974) or (c) transformation to adult-type Leydig cell population (Mendis-Handagama *et al.*, 1998). However, several studies suggested that PFLCs do not all enter apoptosis and degenerate but can persist in later puberty (Kerr and Knell, 1988; Ariyaratne and Chamindrani Mendis-Handagama, 2000; Ivell *et al.*, 2003). Thus, the fate, function and the regulation of PFLCs after birth are still unknown, while testosterone production by PFLCs

during neonatal development is thought to require for neonatal masculinization (Ariyaratne and Chamindrani Mendis-Handagama, 2000; Scott *et al.*, 2009).

The present study has demonstrated a role for the cAMP-PKA transduction pathway in the regulation of Leydig-cell specific genes in PFLCs. In the absence of cAMP-activated signaling, PFLCs lost their capacity to express Leydig cell-specific genes such as LHR, 3 β HSD, P450c17 and Ins13 and to produce steroids. However, bypassing stimulation of PFLCs with (Bu)₂cAMP beyond LHR supported the expression of 3 β HSD, P450c17 and Ins13 but not LHR, indicating that the persistent activation of cAMP-dependent signaling is a requisite condition for supporting steroidogenic gene expression in PFLCs. This observation agrees well with the earlier studies demonstrated that the cAMP-PKA transduction pathway plays an important role in supporting expression and function of 3 β HSD and P450c17 in mouse Leydig cells (Anakwe and Payne, 1987; Keeney and Mason, 1992). Moreover, our data support the idea that expression of P450c17 is highly dependent on cAMP stimulation not only in adult Leydig cells but also in fetal Leydig cells (Scott *et al.*, 2009). However, the present study demonstrated that stimulation of PFLCs with (Bu)₂cAMP up-regulates P450c17 gene expression at the transcriptional but not translational level. The lack of functional protein was reflected by an accumulation of progesterone in the culture medium. The mechanism behind this observation is not clear but appears not to be associated with desensitization induced by overstimulation with hCG/(Bu)₂cAMP, a cellular event associated with suppression of P450c17 expression in adult Leydig cells via an estrogen-dependent mechanism (Tsai-Morris *et al.*, 1985). A previous study has demonstrated that the inability of the FLC to be desensitized by LH/hCG treatment was associated with low levels of aromatase activity and undetectable estradiol production (Tsai-

Morris *et al.*, 1986). This observation is in line with our finding showing lack of estradiol production by cultures of PFLCs stimulated with (Bu)₂cAMP, suggesting that aromatase expression is not regulated by cAMP-dependent signaling pathway(s) in those cells.

Our finding that PFLCs express *Insl3*, a factor of importance for the first phase of testicular descent (Ivell *et al.*, 1997) is agreed well with previous studies demonstrating high levels of *Insl3* expression in FLCs (Balvers *et al.*, 1998; McKinnell *et al.*, 2005). We demonstrated that PFLCs lost their capacity to express *Insl3* after long-term culturing and that (Bu)₂cAMP prevented this down-regulation, indicating that *Insl3* gene expression in PFLCs might be controlled by cAMP-dependent signaling pathway(s). We hypothesize that under basal unstimulated conditions *in vitro* PFLCs undergo dedifferentiation, a process accompanied by significant attenuation and/or cessation of the expression of key Leydig cell-derived genes including *Insl3*. These findings are supported by several studies in which dedifferentiation of the Leydig cells from the testis of short-day exposed hamsters was associated with losing their steroidogenic capacity (Lerchl *et al.*, 1993) and ability to express *Insl3* (Ivell *et al.*, 2003). Moreover, the expression of *Insl3* was found to be markedly reduced in dedifferentiated hyperplastic or neoplastic human Leydig cells (Klonisch *et al.*, 1999). However, our present *in vitro* observations on purified native PFLCs need to be verified under *in vivo* conditions.

The present study also demonstrated a role of PDGF α for the regulation of PFLC proliferation. We have found that PDGF α alone and in combination with (Bu)₂cAMP significantly stimulated proliferation of PFLCs, suggesting that the cAMP-PKA-dependent pathway and PDGFR α -activated signaling may converge and influence the same target proteins that control mitotic activity of PFLCs. An important role of PDGF α in controlling FLCs development was demonstrated in recent studies, showing that PDGF α -dependent signaling acts in fetal Leydig

cells (Schmahl *et al.*, 2008) and PDGFR α null males have reduced or absent fetal Leydig cells due to attenuated proliferation of SF-1 positive precursor fetal Leydig cells (Brennan *et al.*, 2003).

In summary, the present study show for the first time that purified native PFLCs possess a transient capacity to produce testosterone due to attenuation of P450c17 translation into the functional protein. Long-term culturing of PFLCs induces a significant suppression of the expression of a number of Leydig cell-derived genes (e.g., 3 β HSD, P450c17 and Insl3), a process that was rescued by (Bu)₂cAMP. These findings indicate that cAMP-dependent signaling may play an important role in supporting PFLC differentiation and function. Moreover, the present study demonstrated that PDGF α and cAMP-PKA signaling operate in concert to stimulate the proliferation of PFLCs. Taken together, the present results may contribute to better understanding of mechanism(s) behind disorders of sex development including cryptorchidism in boys.

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Figure Legends

Table 1. Primer sequences and PCR conditions for Leydig cell-specific genes

Fig.1 Expression of Leydig cell specific proteins by PFLCs. PFLCs positively stained for InsI3 (A), LHR (B), 3 β HSD (C) and StAR (D). Negative controls for the corresponding proteins are also showed (E-H).

Fig.2 Time-dependent changes in testosterone (A) and progesterone (B) production by hCG (10ng/ml)- stimulated PFLCs and unstimulated cells. Purified PFLCs were incubated with or without hCG (10 ng/ml) for 24, 48 and 72 h. Testosterone and progesterone in the culture medium were then measured by RIA and the results expressed as ng/10⁵cells per 24h. Mean values \pm SE for three independent PFLCs preparations are presented. * p<0,05 compared to unstimulated control.

Fig.3 Levels of steroidogenic enzyme activities in PFLCs. Cells were cultured overnight and the enzyme activities measured as described in *Materials and Methods*. Mean values \pm SE for three independent PFLCs preparations are presented.

Fig.4 The profile of steroids production by (Bu)₂cAMP -activated PFLCs during different time-points of culture. Purified PFLCs were incubated with or without (Bu)₂cAMP (1mM) for 24, 48, 72 and 96h. Testosterone (Fig.4A), androstenedione (Fig.4B) and progesterone (Fig.4C) in the culture medium were then measured by RIA and the results expressed as ng/10⁵cells per 24h. Mean values \pm SE for four independent PFLCs preparations are presented. * p<0,05; *** p<0,001 compared to unstimulated control.

Fig.5 Effect of (Bu)₂cAMP on the expression of Leydig cell-derived genes in PFLCs. Cells were cultured with or without (Bu)₂cAMP for 4 days, followed by investigation of gene expression by RT-PCR as described in *Materials and Methods*. Each experiment was repeated twice with the similar result.

Fig. 6

Effect of (Bu)₂cAMP treatment on the expression of P450c17 mRNA in PFLCs. Cells were treated with or without (Bu)₂cAMP for 4 days, followed by quantitative real-time RT-PCR analysis as described in *Materials and Methods*. P450c17 mRNA levels were determined by quantitative real-time RT-PCR using β -actin as housekeeping gene. Each experiment was performed independently three times with similar results. ** p<0,01 compared with unstimulated control.

Fig. 7

Effect of (Bu)₂cAMP treatment on the level of P450c17 expression in PFLCs. The cultures of PFLCs were treated with or without (Bu)₂cAMP for 4 days. Thereafter, the cells were lysed and the level of P450c17 in aliquots of these whole cell lysates (30 μ g/protein) analyzed by PAGE/Western blotting as described in *Materials and Methods*. PC-positive control (protein extract from 40-day old Leydig cells). Each experiment was repeated twice with the similar result.

Fig.8 Effect of paracrine factors and activators of steroidogenesis on proliferation of PFLCs.

Cells were cultured with or without LIF, PDGF α and (Bu)₂cAMP for 72 hours and labeled with ³H-thymidine for 24h, followed by cell lysis. Incorporated radioactivity was determined as described in *Materials and Methods*. Data were expressed as incorporation of ³H-

thymidine per number of cells during the last 24 h of culture. Each experiment was performed independently three times with similar results. * $p < 0,05$ compared with unstimulated control.

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Name	Sequence	Size	Cycles; annealing temperature
LHR	5'-CTGCGCCTTCAGGAATTTGCC-3' 5'-AATCATAATCCCAGCCACTGAGTTCATTCT-3'	161bp	30, 56°C
Insl3	5'-ACGCAGCCTGTGGACACC-3' 5'-CAATCCGGGGGTGTTTCATT-3'	313bp	28, 58°C
3 β -HSD	5'-ATGCCCAGTACCTGAGGAGA-3' 5'-TTGAGGGCCGCAAGTATCA-3'	427bp	30, 58°C
P450c17	5'-ACCTAGAGGCCACAACATAACATCC-3' 5'-GAGGCACTGGGACTAGCACCT-3'	80bp	28, 54°C
17 β -HSD	5'-AATGTGCTTTCCATTTGCAAGGT-3' 5'-ATGCCACTGGCAGAGGAGATG-3'	80- 150bp	30, 56°C
β -actin	5'-TGAAGATCAAGATCATTGCTCC-3' 5'-ACTCATCGTACTCCTGCTTGC-3'	120bp	30, 56°C

Fig 1

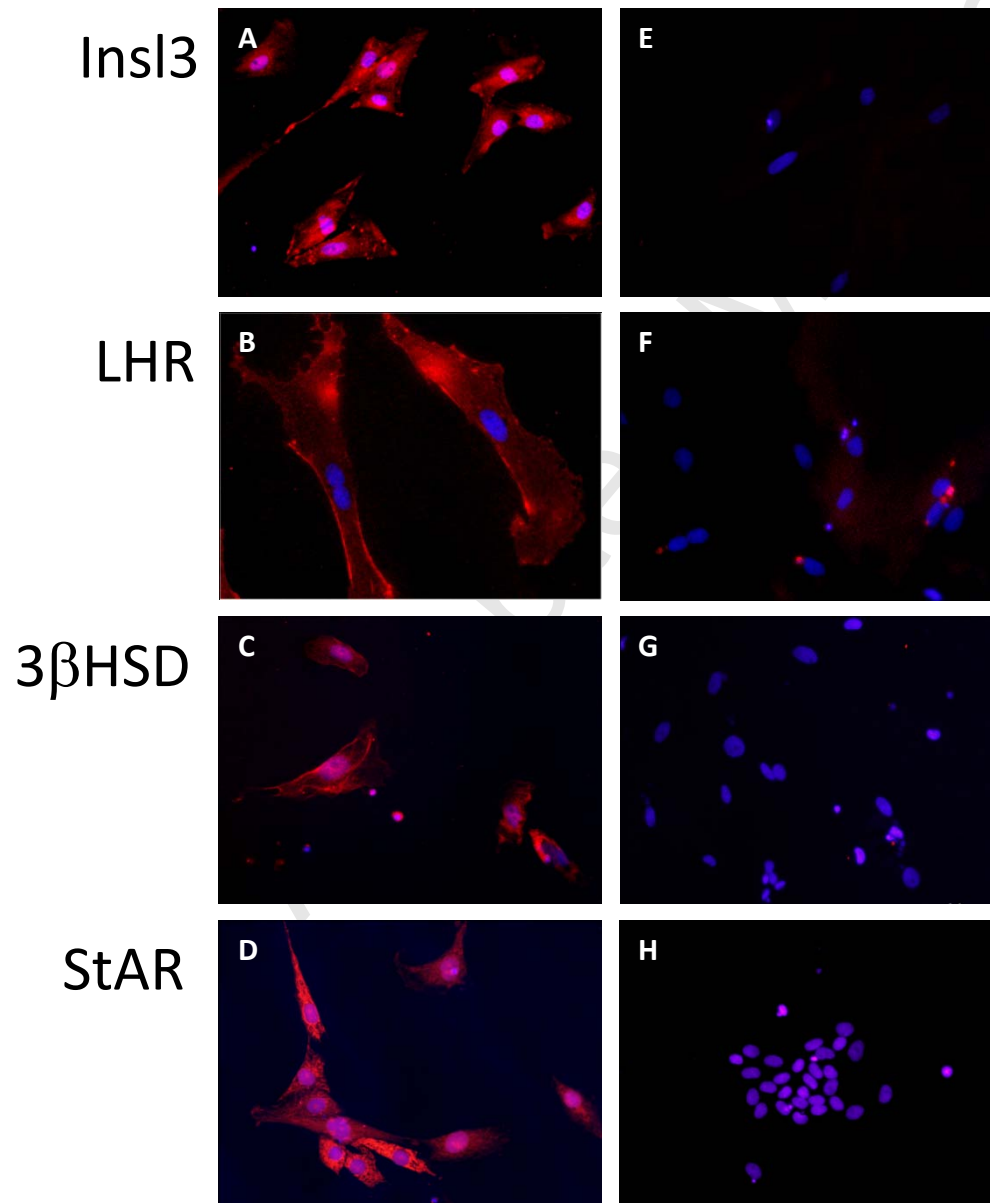
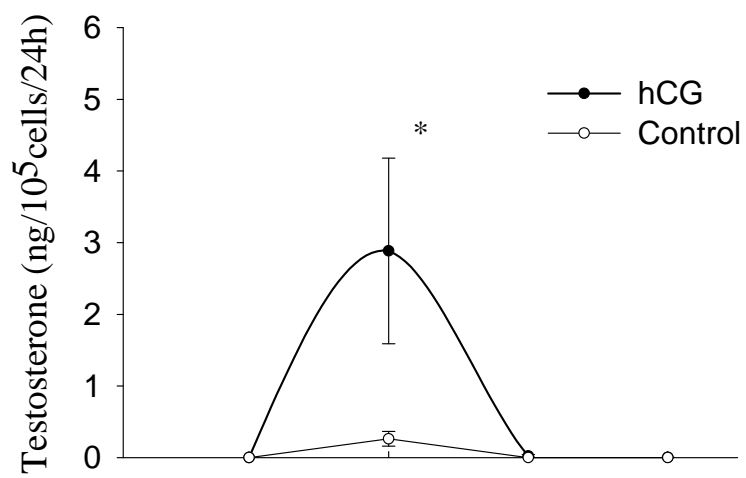


Fig 2

A



B

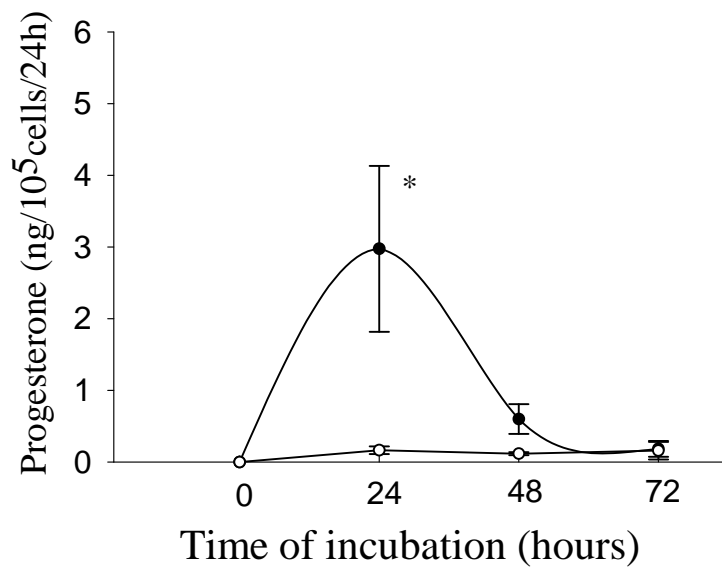


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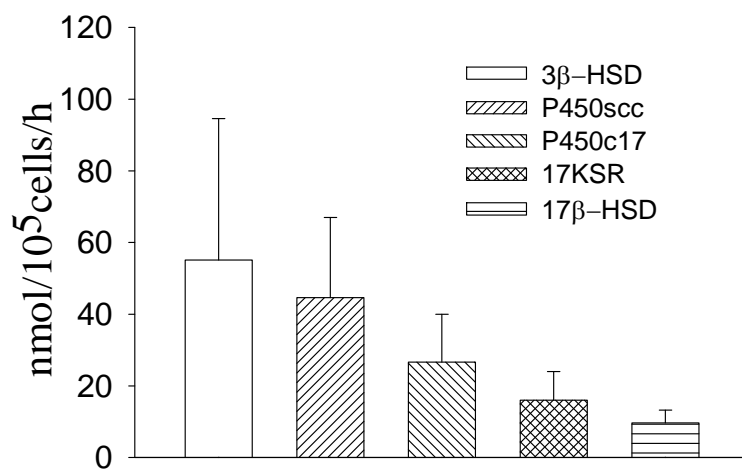


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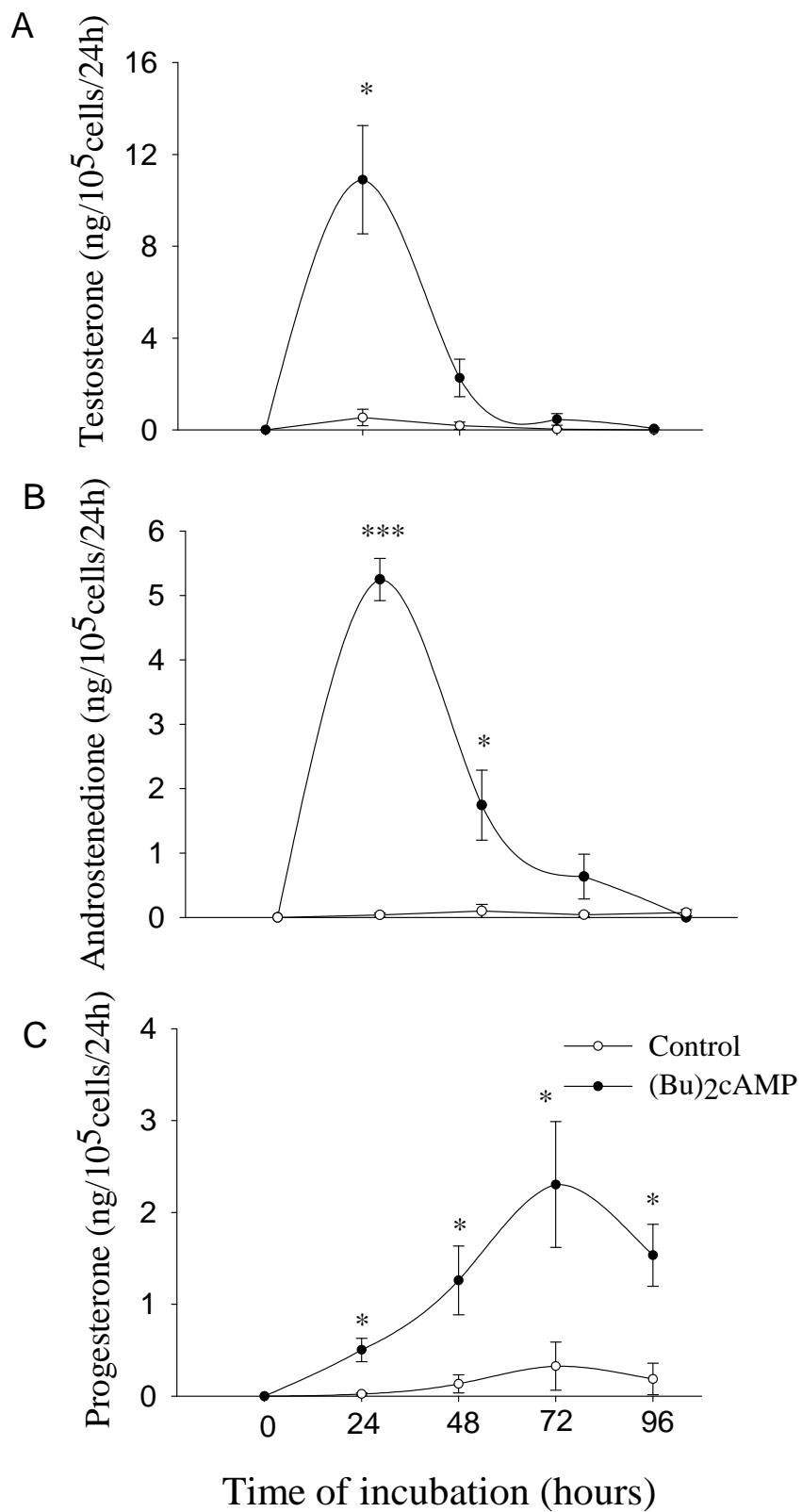


Fig 5

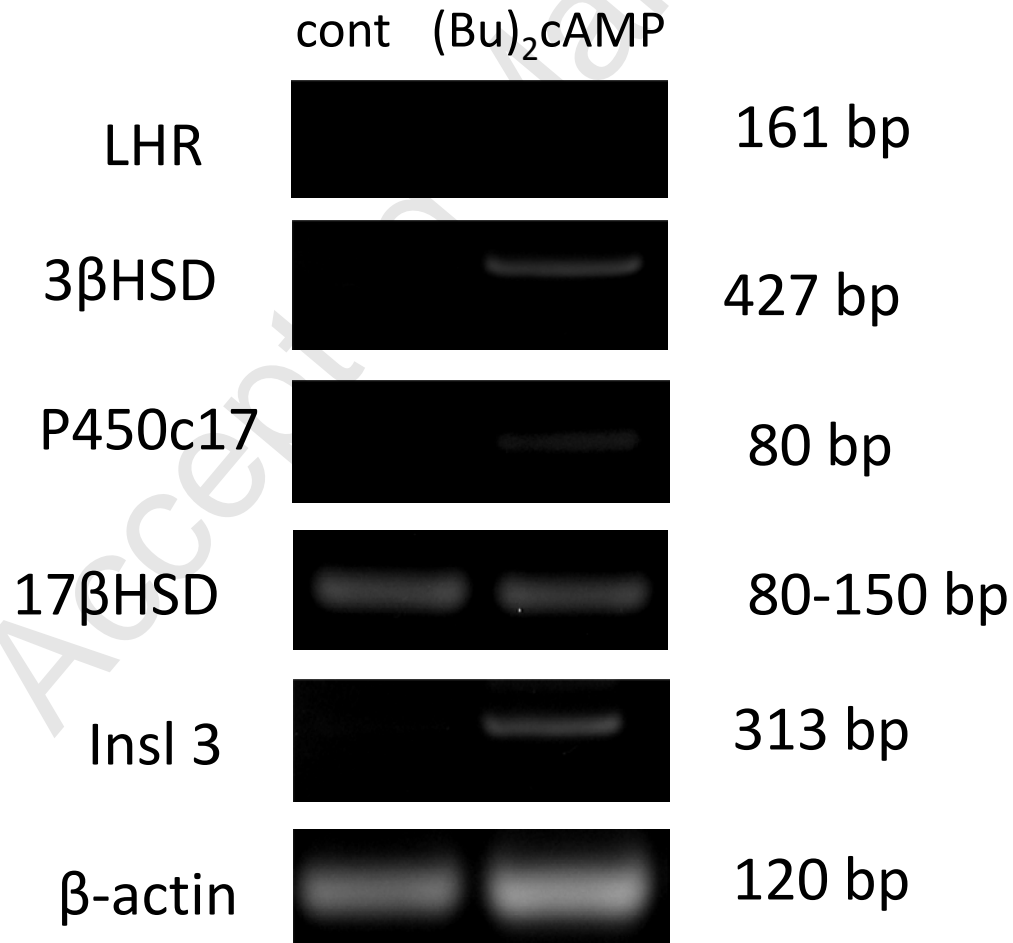


Fig 6

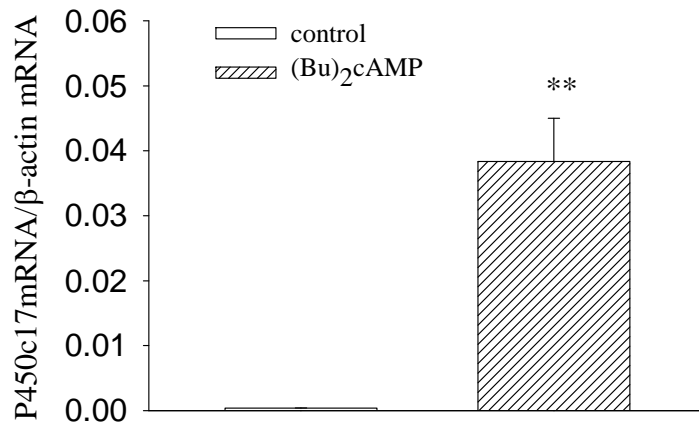


Fig 7

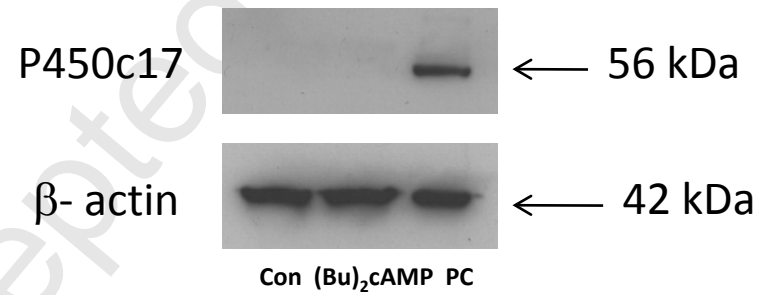


Fig 8

