

cAMP response element-binding (CREB) signalling and ovarian surface epithelial cell survival

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Abstract

cAMP response-element binding (CREB) transcription factors transduce cell survival responses to peptide hormones and growth factors in normal tissues and mutant CREB proteins are implicated in tumorigenesis. Ovarian cancer most frequently arises from the ovarian surface epithelium (OSE), possibly due to repeat inflammation-associated injury-repair episodes that promote neoplasia. We asked if post-receptor signalling involving the CREB family of proteins plays a role in OSE cell survival. In an ovine ovulation model, abundant expression of phospho-CREB/activating transcription factor (ATF) protein was detected immunohistochemically, strongly localised to OSE cells in the proximity of pre-ovulatory follicles. Treatment of primary sheep OSE cell cultures with LH stimulated cAMP accumulation and reduced apoptosis (caspase 3/7 activity) in response to serum withdrawal. When OSE cells were infected with an adenovirus containing a CRE-luciferase

construct, exposure to LH and FSH induced CRE-directed transcription. Finally, when a non-phosphorylatable mutant of CREB (Ad CREB^{S133A}) was adenovirally expressed, apoptosis measured by activation of caspases was increased several fold relative to that caused by transfection with wild-type CREB (Ad CREB^{WT}) or lacZ (Ad lacZ). To test the potential clinical relevance of these findings, we expressed mutant CREB protein in normal human OSE cells from four women and a series of cell lines derived from human ovarian cancers. Infection with Ad CREB^{S133A} markedly increased apoptosis in normal human OSE but had no detectable effect on apoptosis in any of the cancer cell lines. We conclude that CREB/ATF signalling is important for the maintenance of OSE cell survival *in vitro* and is altered in human cell lines derived from ovarian cancers.

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Introduction

Ovarian surface epithelial (OSE) cells are targets for hormones and growth factors that regulate cell proliferation and survival (Murdoch 1995, Auersperg *et al.* 2001). Repeat episodes of OSE destruction and regeneration in the vicinity of ovulating follicles are presumed to explain the high frequency of cancers arising from these cells in human ovaries (Fathalla 1971, Ozols 1991, Salazar *et al.* 1996). Understanding post-receptor signalling pathways through which OSE cell growth and survival are controlled is therefore critical to reproductive health.

Ovulation is caused by luteinising hormone (LH) that triggers target ovarian cells to undergo structural and metabolic changes associated with a natural inflammatory process, leading to follicular rupture (Espey 1980, 1994). Although granulosa and theca cells in pre-ovulatory follicles are primary targets for gonadotrophins, OSE cells also express

functional receptors for LH and FSH (Kuroda *et al.* 2001, Parrott *et al.* 2001, Syed *et al.* 2001, Gubbay *et al.* 2004, Choi *et al.* 2005). A hallmark of post-receptor signalling induced by gonadotrophic hormones in follicular cells is increased intracellular accumulation of cAMP leading to cAMP-dependent protein kinase A (PKA)-mediated phosphorylation of transcription factors, such as cAMP-response element (CRE) binding protein (CREB) and activating transcription factor-1 (ATF1) (Carlone & Richards 1997, Conti 2002). Phosphorylated CREB/ATF1 and related CRE-binding proteins then induce transcription by occupying CREs in the promoter regions of target genes that orchestrate cell proliferation, differentiation and death (Shaywitz & Greenberg 1999, Mayr & Montminy 2001, Impey *et al.* 2004). Based on previous studies of the role of CREB in granulosa cell survival (Johnson *et al.* 2001, Aharoni *et al.* 1995, Somers *et al.* 1999) and evidence that LH is anti-apoptotic in OSE cells (Kuroda *et al.* 2001, Slot *et al.* 2006),

we predicted that gonadotrophic activation of PKA-CREB signalling would be anti-apoptotic in OSE. Here, we use adenoviral expression of a dominant interfering mutant of CREB (Somers *et al.* 1999) to ask if CREB suppresses apoptosis in sheep and human OSE cells and determine if CREB function might be altered in human ovarian cancer.

Materials and Methods

Sheep ovaries

Ovaries for histology and collection of OSE cells were obtained from adult Welsh Mountain ewes. All experiments were conducted in accordance with the UK Home Office Guidelines, the Local Ethics Review Process and the Animals (Scientific Procedure) Act 1986. To obtain ovaries on the verge of ovulation, oestrous cycles were synchronised by withdrawal of progestagen-impregnated sponges (60 mg medroxy-progesterone acetate per sponge; Intervet Laboratories Ltd, Cambridge, UK). Luteolysis was induced on day 10 of the subsequent luteal phase by the administration of a synthetic prostaglandin F2 α analogue (100 mg cloprosterol; Coopers Animal Health, Crewe, Cheshire, UK). Animals were either injected with 50 μ g gonadotrophin-releasing hormone (GnRH), 36 h later (Buserelin; Hoechst, Frankfurt, Germany) to induce a pre-ovulatory LH surge or untreated (no LH surge), killed and ovaries removed for histology 24 h later, when ovulation would normally be imminent (Murdoch 1995, Crawford *et al.* 2000). OSE scrapings were taken from the ovaries of untreated ewes, as described previously (Gubbay *et al.* 2004).

Human OSE cells and ovarian cancer cell lines

Human OSE cells were obtained from the ovaries of premenopausal women aged between 24 and 44 undergoing elective surgery for non-malignant gynaecological conditions with informed consent after the approval of the local ethics committee. The cells were collected during laparotomy by gentle scraping of the ovarian surface with a sterile wooden spatula, which was then rinsed in sterile, warmed culture medium. The primary cell culture system (see below) incorporates a 2–4 week phase of cell propagation assumed to negate any particular influence of age, hormonal status or parity on subsequent cellular function *in vitro* (Hillier *et al.* 1998). Ovarian cancer cell lines were derived from poorly (SKOV-3, PEO-4 and BG-1) and well-differentiated (PEO-14) ovarian adenocarcinomas (Langdon *et al.* 1988) kindly provided by Dr Pascal Pujol, INSERM, Montpellier, France.

Cell culture

Primary ovine and human OSE cell cultures and cancer cell lines were propagated in culture medium consisting of Medium 199:MCDB105 (1:1 v/v) supplemented with fetal calf serum (FCS) (10%, v/v), streptomycin (50 mg/ml),

penicillin (50 IU/ml) and L-glutamine (1 mM). The culture flasks (75 cm²; Corning BV Life Sciences, Schiphol-Rijk, The Netherlands) were incubated at 37 °C in a humidified incubator under an atmosphere of 95% air, 5% CO₂ up to 28 days, with medium renewal every 7 days. Confluent cell monolayers were routinely obtained within 21 days. OSE cell purity was confirmed in selected cases with immunocytochemical staining for cytokeratins 7, 8, 18 and 19 using a commercially available monoclonal anti-human cytokeratin antibody (DAKO Corp, Glostrup, Denmark). Replicate cell cultures for experimental purposes (see below) were established in multi-well plastic culture plates after dispersal of cell monolayers into single cell suspensions by treatment with 0.05% (w/v) trypsin and 0.5 mM EDTA (Invitrogen) at 37 °C for 5 min. Cell number and viability (75–95%) were determined using a haemocytometer and trypan blue dye (Sigma) exclusion. Gonadotrophin treatment was with ovine FSH (NIAMDD-oFSH-16: LH contamination of 0.02 times NIH-LH-S1 Standard) or ovine LH (NIAMDD-oLH-232; <0.5% FSH contamination by weight), generously provided by the Hormone Distribution Program (NIDDK, National Institutes of Health, Bethesda, MD, USA).

Adenoviruses

Adenoviruses expressing a non-phosphorylatable mutant of CREB (Ad CREB^{S133A}) (Gonzalez & Montminy 1989), wild-type CREB (Ad CREB^{WT}), a β -galactosidase (LacZ) reporter gene (Ad LacZ) or CRE-luciferase (Ad CRE-Luc) were obtained as described previously (Somers *et al.* 1999). CREB cDNA was a gift from Dr Richard H Goodman (Oregon Health and Science University, Portland, OR, USA) and the CRE-luciferase construct was from Dr William Walker (University of Pittsburgh School of Medicine, Pittsburgh, PA, USA). Briefly, genes were introduced into the adenoviral shuttle vector pACsk.2CMV (downstream of the CMV promoter) and co-transfected with pJM17 (containing Ad5 genome sequences and an additional prokaryotic DNA fragment inserted into the site of the deleted *E1a* gene) into HEK 293 cells. The additional fragment makes pJM17 too large to package into adenovirus capsids. Recombination between the adenovirus early sequences flanking the expression cassette in pACsk.2CMV and the adenovirus genome sequences in pJM17 resulted in the generation of recombinant adenovirus. The methods for generating and propagating recombinant adenoviruses are described in detail elsewhere (Gerard & Meidell 1994). After transfection, the cells were maintained in DMEM containing 4.5 g/l glucose (Life Technologies) and 10% FCS at 37 °C in 5% CO₂ for 13 days by which time the cells exhibited viral cytopathic effects. The cells and medium were collected, frozen on dry ice and thawed three times, and centrifuged (1000 g, 4 °C, 10 min) to remove cellular debris. The absorbance of the samples was measured at 260 nm and the value obtained was used to calculate virus content using the equation 1.0 absorbance units = 1.1 $\times 10^{12}$ virus particles/ml (Mittereder *et al.* 1996). Adenoviruses were propagated by infecting HEK

293 cells with approximately 10^8 particles/ml in tissue culture medium without serum. The infected cells were incubated until they exhibited a nearly complete cytopathic effect and processed as mentioned above. Virus stocks were prepared to a concentration of 6×10^{12} particles/ml as described above.

Luciferase assay

To demonstrate CRE-mediated gonadotrophin-induced gene transcription, OSE cells in 12-well plates (200 000 cells/well) were exposed to Ad CRE-Luc for 1 h and incubated for 24 h in 0.5% (v/v) FCS medium. Cells were then incubated with and without LH or FSH for a further 6 h. The luciferase activity in cell lysates was measured using the Renilla Luciferase assay system (Promega) and normalised to protein concentration (Bio-Rad Laboratories).

cAMP assay

To assess gonadotrophin-induced cAMP production, OSE cell monolayers in 24-well dishes (100 000 cells/well) were washed twice and pre-incubated for 15 min in serum-free medium supplemented with 0.5 mM isobutylmethylxanthine (Sigma). Triplicate wells were then incubated with 100 ng/ml FSH or 100 ng/ml LH for 30 min after which total cAMP (i.e. cells + medium) was extracted by incubation with 0.1 M HCl for 10 min. Cell extracts were clarified by centrifugation and cAMP determined using a commercial EIA kit (Biomol Research Laboratories Inc., Plymouth Meeting, PA, USA) according to the manufacturer's instructions.

Caspase assay

To test the effects of gonadotrophins and cAMP on apoptosis, OSE cells seeded into 96-well dishes (5000 cells/well) were treated for 48 h in serum-containing medium with or without test substance: 100 ng/ml LH, 100 ng/ml FSH or 2 mM 8-bromo-cAMP (8br-cAMP; Sigma). Serum-containing medium was then replaced with serum-free medium still containing the test substance. In this assay, serum withdrawal is the trigger for apoptosis and the test substance remains throughout. To test the effects of adenovirally expressed proteins on apoptosis, the cells were exposed for 1 h to virus in serum-free medium (50 ml), which was then aspirated and replaced with serum-containing medium for 3 days. Caspase 3/7 activity was determined using the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega), according to the manufacturer's instructions. This assay is based on the fluorescence of rhodamine 110 when excited at a wavelength of 498 nm upon cleavage of a rhodamine 110 containing substrate (Z-DEVD-R110) by caspases 3 and 7.

CREB immunohistochemistry

Sheep ovaries were fixed in Bouin's fluid for 6 h, dehydrated, embedded in paraffin wax, sectioned (5 mm) and processed for

immunohistochemical staining following epitope retrieval by microwave heating for 10 min in 10 mM sodium citrate. The sections were incubated overnight at 4 °C with anti-phospho-CREB(S133)/ATF1(S63) (Cell Signaling, New England Biolabs, Beverly, MA, USA) at a dilution of 1:100 in 20% normal goat serum in PBS containing 5% (w/v) BSA. Negative controls were incubated with a matched concentration of non-specific rabbit IgG diluted in 20% normal goat serum with PBS/5% BSA. Sections were then washed twice for 5 min in PBS containing 0.1% (v/v) Tween-20 and incubated for 30 min at room temperature with biotinylated goat anti-rabbit IgG (Dako Corp., Glostrup, Denmark) diluted 200-fold in 20% normal goat serum in PBS/5% BSA. After a further wash in PBS/0.1% Tween-20, the sections were incubated with pre-balanced avidin and biotin solutions (Vector ABC Elite kit, Vector Laboratories, Burlingame, CA, USA) washed again, and incubated for 2–10 min with diaminobenzidine solution (Dako) for colour development. The sections were counter-stained with haematoxylin and eosin and mounted in xylene before microscopic inspection and photomicrography.

To visualise adenovirally expressed CREB proteins, OSE cells seeded in 8-well chamber slides (9000 cells/well) were exposed for 1 h to Ad CREB^{WT} or Ad CREB^{S133A} diluted in 100 ml serum-free medium. Virus was then removed and incubation continued in serum-containing medium for a further 2–3 days. The washed cell monolayers were fixed in 4% neutral buffered formaldehyde for 10 min and either stained with haematoxylin and eosin or taken for ABC/DAB immunohistochemical staining with polyclonal rabbit anti-CREB anti-serum (Sc-58; Santa Cruz Biotechnology, Santa Cruz, CA, USA; raised against human CREB-1 p43, as recommended for detection of CREB-1 p43 of mouse rat and human tissues).

β -Galactosidase assay

Identification of β -galactosidase-expressing cells in Ad LacZ-infected OSE cell cultures was achieved by histochemistry using X-gal (Bio-Rad Laboratories) as substrate (Mittereder *et al.* 1996). OSE cell monolayers were fixed in 2% glutaraldehyde for 60 min, washed twice in PBS, and incubated in Xgal solution containing 2 mM K₃Fe(CN)₆, 2 mM K₄Fe(CN)₆, 1 mM MgCl₂ and 1 mg/ml Xgal.

Western blotting

OSE cells were homogenised in lysis buffer (150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 10% glycerol, 1% NP40, 10 mg/ml aprotinin, 1 mM PMSF and 1 mM sodium orthovanadate) and cell extracts were prepared by centrifugation for 2 min at 14 000 r.p.m. Protein, 50 mg, was subjected to SDS-PAGE and transferred to PVDF membrane (Millipore, Bedford, MA, USA). Membranes were incubated with antibodies to CREB, actin (Santa Cruz, Biotechnology Inc) and phosphorylated CREB(S133)/ATF-1(S63) (Cell Signaling, New England Biolabs, Inc.). Each antibody was diluted 1000-fold in 2% w/v dried skimmed milk/TBST (20 mM

Tris-HCl pH 7.4, 500 mM NaCl, 0.1% Tween 20). Membranes were washed briefly in TBST and incubated with secondary antibodies conjugated to horseradish peroxidase (Amersham) in 2% w/v milk/TBST. Membranes were again washed in TBST and proteins detected using the ECL Plus Western Blotting Detection kit (Amersham).

Statistics

All experiments were repeated at least three times and the data evaluated by SuperANOVA (Abacus Concepts, Berkeley, CA, USA) followed by paired Student's *t*-test or Tukey-Kramer *post hoc* test, taking $P < 0.05$ to indicate a statistically significant difference.

Results

CREB immunohistochemistry

Twenty-four hours after administration of GnRH to induce ovulation, granulosa-lutein cells lining large pre-ovulatory follicles in sheep ovaries showed intense phospho-CREB/ATF1

immunostaining, which was absent from cells in the expanded cumulus-oocyte complex (Fig. 1A and B). The follicular apex (Fig. 1B) was generally devoid of OSE cells (Fig. 1B), but adjacent ovarian surface regions possessed OSE cells staining positively for phospho-CREB/ATF1 (Fig. 1C). More distally located OSE cells were typically phospho-CREB/ATF1 negative (Fig. 1D). The OSE layer covering anovulatory ovaries was also phospho-CREB/ATF1 negative (data not shown).

cAMP formation and CRE-mediated gene transcription

LH stimulated cAMP accumulation (Fig. 2) and reduced the pro-apoptotic effect of serum withdrawal in sheep OSE cell cultures (Fig. 3). FSH did not significantly stimulate cAMP, but suppressed apoptosis. Both LH and FSH exerted dose-related stimulation of CREB-luciferase activity in sheep OSE cells infected with Ad CRE-Luc (Fig. 4).

Adenovirus-directed CREB protein function

To investigate CREB protein function in sheep OSE cells, they were infected with adenovirus expressing wild type

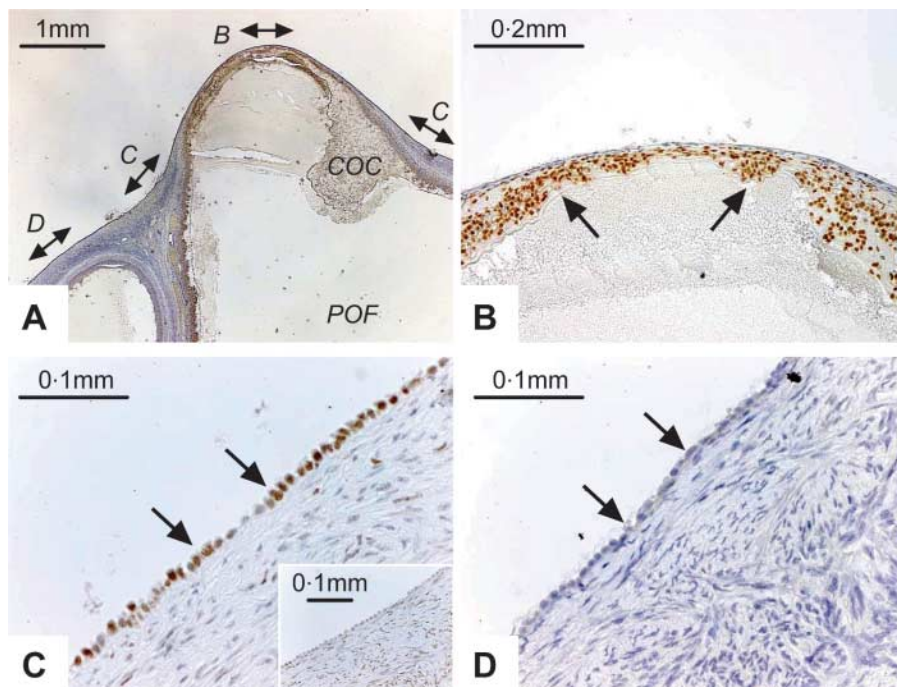


Figure 1 Phospho-CREB/ATF-1 protein expression in sheep ovary. Immunohistochemical staining of sheep ovary containing a pre-ovulatory follicle (POF) with expanded cumulus-oocyte complex (COC), confirming imminence of ovulation 24 h after injection of GnRH. (A) Low-power ($2\times$) photomicrograph orientates ovarian surface regions (double-headed arrows, (B)–(D)). (B) Apex of POF is denuded of OSE cells but contains positively immunostained granulosa-lutein cells (arrows) ($10\times$). (C) OSE cell layer in region proximal to POF shows positive immunostaining (arrows) ($20\times$); insert shows non-immune serum control. (D) OSE cell layer distal to POF shows negative immunostaining (arrows) ($20\times$). Five-micron ovarian sections were processed for double-antibody immunohistochemistry using anti-phospho CREB/ATF-1 as primary antibody (see Materials and Methods).

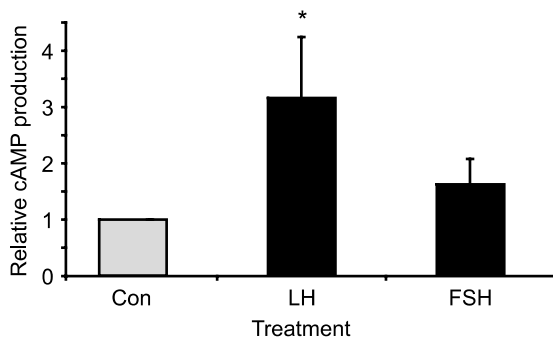


Figure 2 LH stimulates cAMP production by OSE cells. Sheep OSE cell cultures were incubated for 30 min with 100 ng/ml LH or 100 ng/ml FSH and total cAMP (medium plus cells) concentration was determined by enzyme immunoassay. Results (mean \pm S.E.M., $n=5$) are expressed as fold increment relative to control (Con). * $P<0.05$.

(Ad CREB^{WT}) or a dominant interfering mutant of CREB (Ad CREB^{S133A}) with serine to alanine substitution at residue 133. This amino acid substitution prevents phosphorylation of residue 133 and renders the CREB protein unresponsive to factors that normally induce CREB function (Hunter & Karin 1992). Infection of sheep OSE cells with Ad CREB^{WT} or Ad CREB^{S133A} resulted in nuclear expression of immunoreactive CREB proteins at apparently similar amounts, determined by immunocytochemistry (Fig. 5A) and Western blotting (Fig. 5B). To confirm the S133A mutation, cells were infected with Ad CREB^{WT} or Ad CREB^{S133A}, treated with and without 10 mM forskolin for 15 min and CREB phosphorylation examined by immunoblotting. As shown in Fig. 6, CREB^{WT}-infected cells showed a clearly enhanced phospho-CREB signal in response to forskolin, whereas no such response was detected in Ad CREB^{S133A}-infected cells.

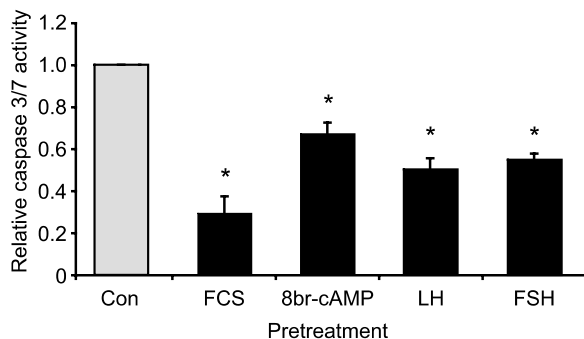


Figure 3 Gonadotrophins and cAMP suppress OSE cell apoptosis. Sheep OSE cells were treated for 48 h in medium containing 10% fetal calf serum with or without (Con) additional 8-bromo-cAMP (8br-cAMP; 2 mM), LH (100 ng/ml) or FSH (100 ng/ml). Serum was then withdrawn (to induce apoptosis) and incubation continued with/without 8br-cAMP, LH or FSH for a further 6 h. Cells treated throughout with serum (FCS) were the positive control. Caspase 3/7 activity was measured as an index of apoptosis and values (mean \pm S.E.M., $n=4$) are presented relative to Con. * $P<0.01$.

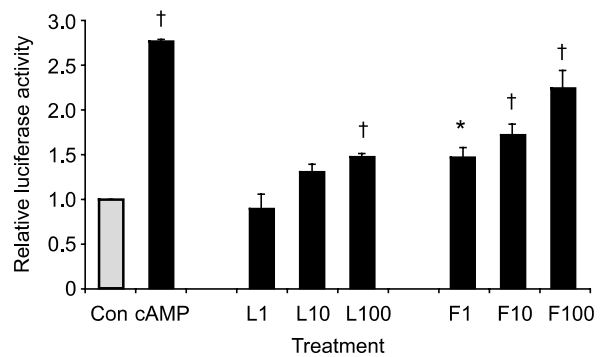


Figure 4 Gonadotrophins induce CRE-mediated gene transcription in OSE cells. Sheep OSE cells were infected with Ad CRE-Luc for 2 days and incubated for 6 h in the absence (Con) or presence of 2 mM 8-bromo-cAMP (cAMP), 1–100 ng/ml LH (L1–L100) or 1–100 ng/ml FSH (F1–F100). Cell lysates were prepared and luciferase activity determined, as described in Materials and Methods. Data (mean \pm S.E.M., $n=3$) are expressed relative to Con. * $P<0.01$ and † $P<0.001$.

CREB protein expression and apoptosis in sheep OSE cells

Infection with Ad CREB^{S133A} to induce expression of mutant CREB caused morphological changes consistent with apoptosis (cell shrinkage and detachment, dense nuclear granulation and membrane blebbing), as illustrated in Fig. 7. The apoptotic response was reflected in cellular caspase 3/7 activity, which was significantly raised (approximately twofold) by low-titre infection (10^9 particles/ml) with Ad CREB^{S133A} relative to Ad LacZ or Ad CREB^{WT} (Fig. 8). High-titre (10^{10} particles/ml) Ad CREB^{WT} infection also increased caspase 3/7 activity, but significantly less than the same dose of Ad CREB^{S133A} (Fig. 8).

Differential effect of CREB protein expression on apoptosis in human OSE cells and human ovarian cancer cell lines

Infection with Ad CREB^{S133A} also stimulated caspase 3/7 activity in normal human OSE cells. However, the mutant CREB had no effect on cell lines derived from poorly differentiated (SKOV-3, PEO-4 and, BG-1) ovarian adenocarcinomas and only marginally increased apoptosis in cells from a well-differentiated (PEO-14) tumour (Fig. 9). Ad CREB^{WT} infection was also weakly pro-apoptotic in normal human OSE cells but had no effect on any of the ovarian cancer cell lines studied.

Discussion

Our results shed light on the cell survival mechanisms of a poorly understood ovarian cell type intimately involved in ovulation and the development of major ovarian disease. Their novelty concerns gonadotrophic activation of OSE cell

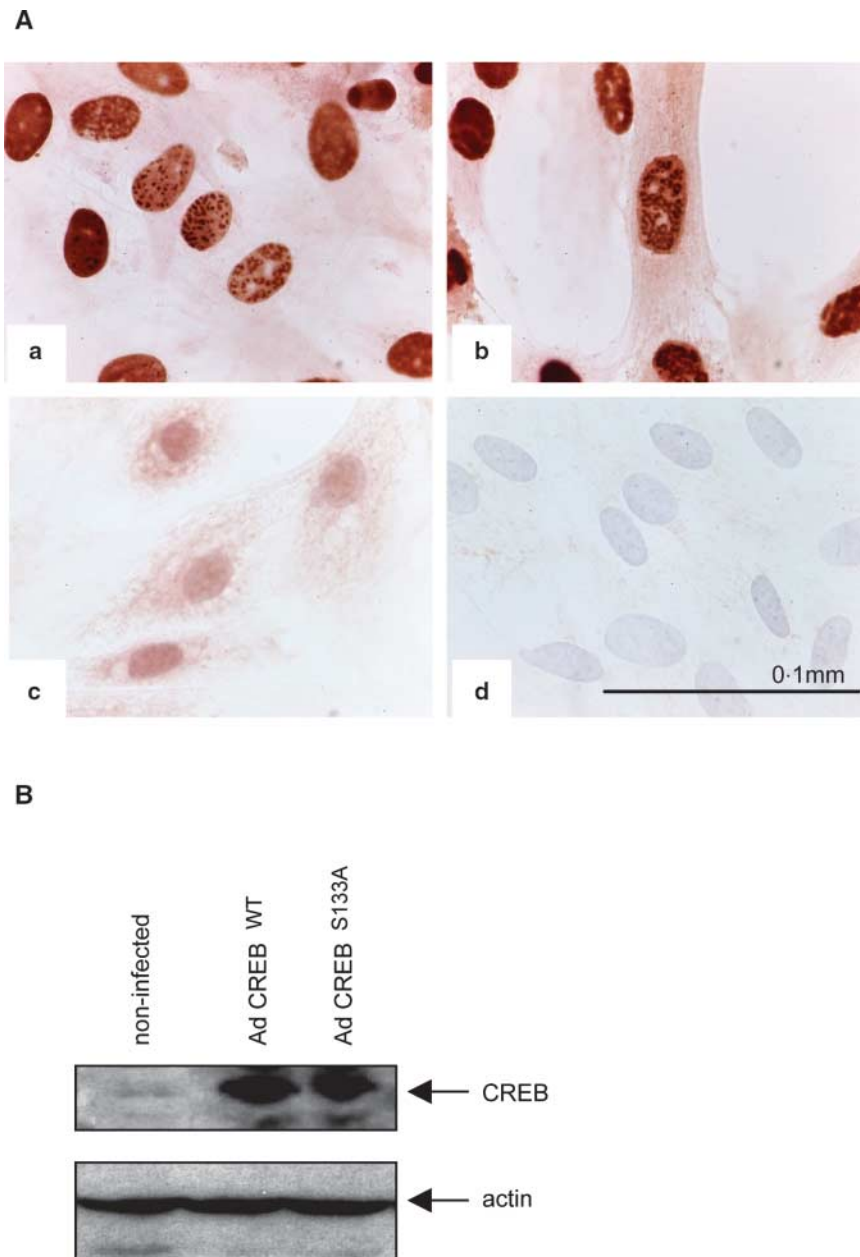


Figure 5 Adenovirus-directed CREB protein expression in sheep OSE cells. Cultured OSE cells were infected with 10^{10} viral particles/ml Ad CREB^{WT} or Ad CREB^{S133A} and 48 h later CREB protein expression was assessed by immunocytochemistry or western analysis. (A) Immunocytochemistry, OSE cells infected with: (a and d) Ad CREB^{WT}, (b) Ad CREB^{S133A} or (c) mock-infected; immunostaining with: (a–c) anti-CREB serum or (d) non-immune serum, as described in Materials and Methods. Light microscope (100 \times). (B) Western analysis: OSE cells infected with Ad CREB^{WT} or Ad CREB^{S133A} were lysed and submitted to western analysis with anti-CREB or anti-actin antibody, as described in Materials and Methods.

CREB, involvement of CREB in OSE cell survival and links between ovulation and ovarian cancer.

First, CREB and/or ATF1 are activated in a subset of sheep OSE cells close to the time of ovulation *in vivo* and similar activation can be induced by gonadotrophin exposure *in vitro*.

Since gonadotrophins (cAMP) suppress apoptosis in sheep OSE cells and a dominant interfering mutant of CREB potently enhances apoptosis in both sheep and human OSE cells, our results indicate that activation of CREB is a determinant of OSE cell survival through stimulation of

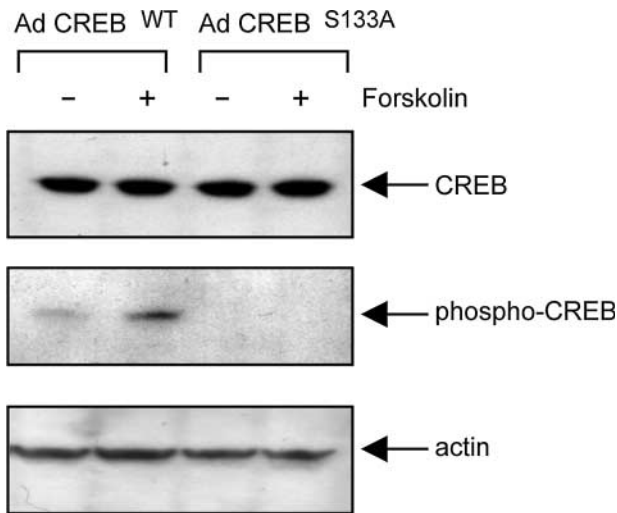


Figure 6 Non-phosphorylation of CREB^{S133A} protein in sheep OSE cells. Cultured OSE cells were exposed to 10^{10} viral particles/ml of Ad CREB^{WT} or Ad CREB^{S133A} and 48 h later treated with and without 10 mM forskolin for 15 min. Immunoblotting was performed using antibodies to CREB, phospho-CREB/ATF and actin (as gel-loading control) as described in Materials and Methods.

proliferation and protection against apoptosis (Dworet & Meinkoth 2006, Slot *et al.* 2006). OSE cells overlying periovarian follicles are normally lost through apoptosis (Murdoch 1995). Here, we noted such loss in association with

survival of an adjacent zone of phospho-CREB expressing OSE cells, presumed to be a source of cells required for subsequent post-ovulatory epithelial restitution. We interpret the acute loss of OSE overlying ovulatory follicles as the response to multiple gonadotrophin-induced cues (e.g. prostaglandins, cytokines, free oxygen radicals, proteases, mechanical forces, etc.) leading to follicle rupture. This is distinct from any potential cytoproliferative (anti-apoptotic) effects of gonadotrophins and/or other growth/differentiation factors that might be required for the preservation and regeneration of OSE cells residing adjacent to the ovulatory wound.

Inhibition of sheep OSE cell apoptosis by cAMP agrees with previous evidence for cytoproliferative or anti-apoptotic effects of gonadotrophins on OSE cells *in vitro* (Kuroda *et al.* 2001, Parrott *et al.* 2001, Syed *et al.* 2001, Edmondson *et al.* 2006, Slot *et al.* 2006). However, pro-apoptotic effects of FSH have also been observed (Pon *et al.* 2005) and gonadotrophic effects are variable in OSE cells from different women (Edmondson *et al.* 2006). Crucially, we demonstrate ovulation-associated activation of CREB/ATF1 in ovarian surface regions proximal to expected sites of follicular rupture. Activation of CREB/ATF1 proteins was also observed in the granulosa cells of healthy follicles at various stages of follicular development, including large pre-ovulatory follicles, consistent with previous studies demonstrating expression and function of CREB proteins in rat granulosa cells (Pei *et al.* 1991, Somers *et al.* 1995, 1999, Carlone & Richards 1997,

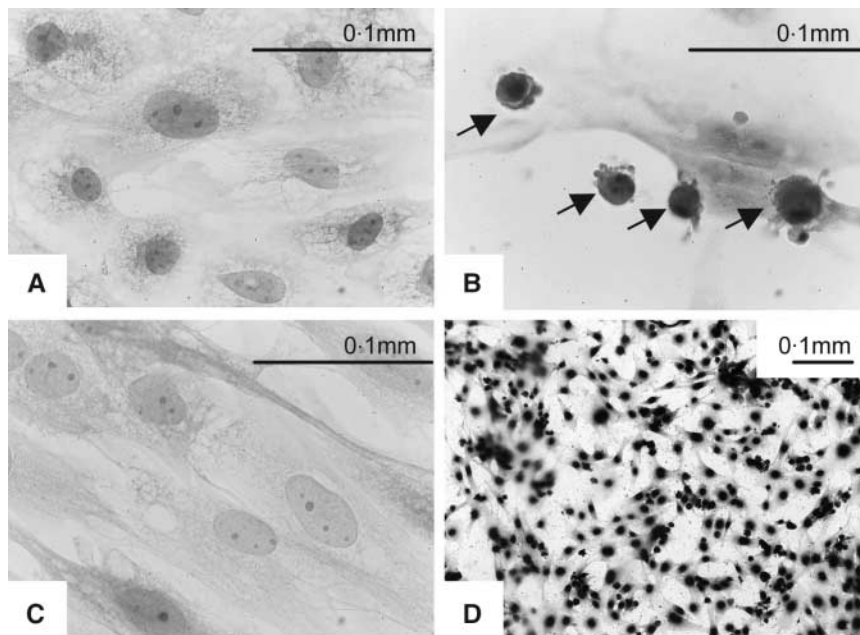


Figure 7 Adenoviral expression of CREB S133A protein causes OSE cell apoptosis. Cultured sheep OSE cells were (A) non-infected or infected (10^{10} viral particles/ml) with (B) Ad CREB^{S133A} or (C and D) Ad LacZ. Cell monolayers were (A–C 100 \times) stained with haematoxylin and eosin or (D 20 \times) stained for β -galactosidase activity and examined by light microscopy. Arrows identify cells with apoptotic features such as shrinkage, dense granular nuclei and membrane blebbing.

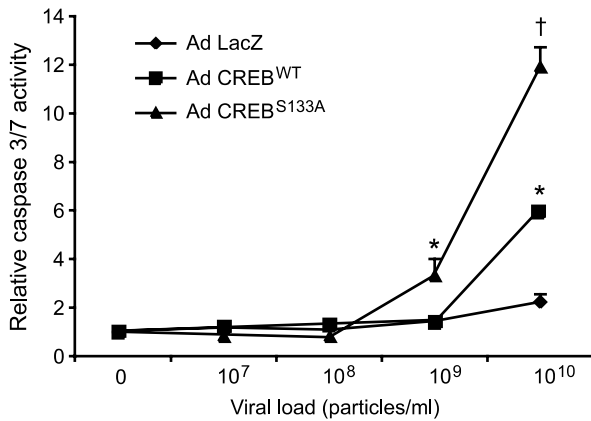


Figure 8 Adenovirus-directed expression of CREB S133A protein enhances caspase 3/7 activity in OSE cells. Cultured sheep OSE cells were infected with Ad CREB^{S133A}, Ad CREB^{WT} or Ad LacZ at the viral loads indicated. Caspase 3/7 activity was measured 3 days later as an index of apoptosis. Data (mean \pm s.e.m., $n=6$) are expressed relative to the non-infected (0) control. Asterisks denote significant difference from corresponding Ad LacZ infection: * $P < 0.05$, † $P < 0.001$.

Mukherjee *et al.* 1998). The adverse effect of dominant interfering CREB protein expression on OSE cells reported here is also similar to that reported previously for granulosa cells (Somers *et al.* 1999). This is consistent with the common histogenetic lineage of OSE and granulosa cells (Juengel *et al.* 2002) and suggests that CREB-associated signalling may be important to maintain the OSE as well as follicular development.

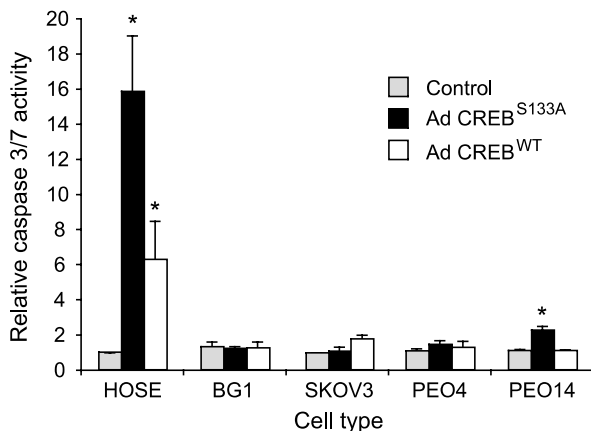


Figure 9 Pro-apoptotic effect of adenovirus-directed expression of CREB S133A protein in normal human OSE cells is lost in human ovarian cancer cell lines. Cultured human OSE (HOSE) cells from women undergoing surgery for non-malignant gynaecological conditions and four human ovarian cancer cell lines (PEO4, PEO14, SKOV3 and BG1) were infected with 10^{10} particles/ml Ad CREB^{S133A} or Ad CREB^{WT}. Caspase 3/7 activity was measured 3 days later as an index of apoptosis. Data (mean \pm s.e.m., $n=3$) are expressed relative to non-infected HOSE cells. Asterisk denotes significant difference from matched non-infected cells ($P < 0.05$).

When dominant interfering CREBS133A protein is expressed in sheep and human OSE cells, they undergo increased apoptosis *in vitro*. Absence of a pro-apoptotic response to infection with Ad LacZ rules out non-specific effects due to the viral vector. Expression of mutant CREB protein would be expected to blockade target CRE-binding sites throughout the genome and thereby derange transcriptional responses to multiple CREB/ATF1 family members (Impey *et al.* 2004). Apoptosis due to interference with CREB involves aberrant cell-cycle progression and check-point activation in thyroid cells (Dworetz & Meinkoth 2006). In hepatocytes, loss of CREB function correlates with increased expression of FAS death receptor and p53 upregulate modulator of apoptosis (PUMA) and decreased expression of cFLIP caspase (Qiao *et al.* 2003). Further work is required to determine if CREB affects these or related death pathway signals in OSE cells.

Secondly, we provide new evidence for biphasic involvement of CREB in OSE cell survival. At first sight, increased apoptosis in response to over-expression of wild-type CREB in normal OSE cells might seem inconsistent with the hypothesis that CREB/ATF1 proteins promote cell survival. However, we previously reported a biphasic cytoproliferative response of human granulosa-lutein cells to increasing concentrations of exogenous (dibutyl) cAMP *in vitro*, with low concentrations causing stimulation and higher concentrations being inhibitory (Yong *et al.* 1992). The present results imply a similar relationship between CREB and apoptosis. Infection with wild-type CREB at a viral dilution of 10^9 particles had no effect on apoptosis. However, a tenfold higher load of Ad CREB^{WT} increased apoptosis albeit to a far lesser extent than the same load of Ad CREB^{S133A}. Equivalent amounts of CREB protein were expressed by both the wild-type and mutant virus (Fig. 5B), eliminating the possibility that dissimilar protein levels might have artefactually accounted for the differences in apoptosis caused by the two CREBs. Induction of apoptosis in cells experimentally induced to over-express CREB has been reported before (Saeki *et al.* 1999). The simplest explanation is that occupancy of CRE elements on promoters by non-phosphorylated CREB would block transcription. Additionally, since PKA activity may limit CREB phosphorylation (Hagiwara *et al.* 1993) over-expression of CREB protein would be expected to cause a disproportionately enlarged fraction of non-phosphorylated CREB protein capable of sequestering and inhibiting the actions of endogenous CRE-binding proteins that negatively regulate CRE-dependent transcription, such as ATF4 (Persengiev & Green 2003). In this regard, we find that apoptosis induced by wild-type CREB over-expression in sheep OSE cells is not significantly reduced by treatments that induce PKA activity, such as forskolin or gonadotrophin, suggesting that PKA activity might indeed be limiting in these cells (O Gubbay, unpublished observations). We also note both pro- and anti-apoptotic functions have been assigned to CREB/ATF family members. For example, ATF2 (Ivanov & Ronai 1999),

ATF3 (Yan *et al.* 2005) and ATF5 (Wei *et al.* 2006) are pro-apoptotic, whereas ATFx stimulates apoptosis by disrupting post-receptor signalling from activated death receptors to initiator caspases, hence promoting cell survival (Persengiev & Green 2003). Alternatively, supraphysiological levels of CREB protein may simply 'overload' and disrupt essential components of the CRE-responsive transcriptional machinery. Either way, the cardinal point is that treatment with mutant CREB more strongly (by an order of magnitude) promotes apoptosis than the equivalent treatment with wild-type CREB.

Finally, the epidemiological link between ovulation and ovarian cancer was established (Ness & Cottréau 1999). During ovulation, OSE cells are exposed to inflammatory mediators capable of inducing genetic changes that predispose to neoplasia (Murdoch *et al.* 1999, Rae & Hillier 2005) and the elimination of OSE cells overlying the ovulatory follicle via apoptosis is probably an oncoprotective mechanism brought into play before post-ovulatory re-epithelialisation (Murdoch 1995, Ghahremani *et al.* 1999). However, we found persistence of CREB-expressing OSE cells bordering the denuded follicular apex at sites of pending follicular rupture in sheep ovaries. Presumably, these cells would normally elude apoptosis subsequently in order to proliferate and recolonise the locally denuded ovarian surface post-ovulation. Our data suggest that this process could be driven, at least in part, by gonadotrophins acting via CREB to suppress apoptosis, which could potentially promote neoplasia. Additionally, we provide preliminary evidence that neoplastically transformed OSE cells lose their ability to undergo CREB-mediated apoptosis. Thus, expression of dominant negative CREB generally did not stimulate apoptosis in human ovarian cancer cell lines, in contrast to normal sheep or human OSE cells. Caveats are that these comparisons were made under non-gonadotrophin-simulated conditions and we did not determine basal phospho-CREB level/activity in human OSE relative to ovarian cancer-derived cell lines. However, the results clearly distinguish healthy and diseased OSE cells, and implicate differential CREB signalling as a cause or consequence of the cancer cell phenotype.

The significance of this finding is that CREB is known to modulate the transcription of multiple genes involved in cell growth, angiogenesis and resistance to apoptosis in tumour progression (Abramovitch *et al.* 2004) and aberrant CREB family signalling has been noted in diverse endocrine tumours (Rosenberg *et al.* 2002). Interestingly, the single human ovarian cancer cell line (PEO-14) in which mutant CREB over-expression increased apoptosis here was derived from a more differentiated tumour than the other cell lines studied (Langdon *et al.* 1988). Moreover, this same cell line has previously been shown to display more similarities to normal human OSE cells than the other cancer cell lines with respect to basal and interleukin-1-induced inflammatory gene expression *in vitro* (Gubbay *et al.* 2005). The overall reduced pro-apoptotic response to the expression of mutant CREB in ovarian cancer cell lines remains to be explained. Others have noted compensatory interactions among CREB/ATF family proteins

associated with loss of CREB expression in the human adrenocortical cancer cell line H295R, which may be linked to cellular transformation (Groussin *et al.* 2000). Further work is needed to determine if such compensatory interactions between CREB/ATF proteins occur in ovarian cancer cell lines and, crucially, whether primary ovarian cancers behave similarly.

In conclusion, these experiments suggest that gonadotrophins are capable of signalling via post-receptor generation of cAMP to orchestrate targeted cell survival signals in OSE cells adjacent to sites of ovarian follicle rupture. Through stimulating cytoproliferation and suppressing apoptosis, gonadotrophins potentially limit collateral damage to ovarian surface cells remaining after follicle rupture and ovulation and thereby contribute to the post-ovulatory ovarian healing. Our results suggest that activation of CREB/ATF1 transcription factors is a key to this process, since dominant interfering CREB promotes OSE cell apoptosis in sheep and human OSE cells. The observed loss of a pro-apoptotic response to interference with CREB in human ovarian cancer cell lines highlights the potential importance of CREB family protein signalling in ovarian cancer. Against the backdrop of an established epidemiological link between ovulation and ovarian epithelial cancer, a challenge for the future is further to define specific roles of CREB/ATF proteins in normal and abnormal OSE function with the expectation that they will suggest novel therapeutic strategies for managing ovulation associated ovarian disease.

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