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Early pregnancy modulates survival and apoptosis pathways in the corpus luteum in sheep

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Abstract

The corpus luteum (CL) is a transient endocrine gland. Functional and structural demise of the CL allows a new estrous cycle. On the other hand, survival of CL and its secretion of progesterone are required for the establishment of pregnancy. Survival or apoptosis of the luteal cells is precisely controlled by interactions between survival and apoptosis pathways. Regulation of these cell signaling components during natural luteolysis and establishment of pregnancy is largely unknown in ruminants. The objective of the present study was to determine the regulation of survival and apoptosis signaling protein machinery in the CL on days 12, 14, and 16 of the estrous cycle and pregnancy in sheep. Results indicate that: i) expressions of p-ERK1/2, p-AKT, β-catenin, NFκB -p65, -p50, -p52, p-Src, p-β -arrestin, p-GSK3β, X-linked inhibitor of apoptosis protein (XIAP), and p-CREB proteins are suppressed during natural luteolysis; in contrast, their expressions are sustained or increased during establishment of pregnancy; ii) expressions of cleaved caspase-3, apoptosis inducing factor (AIF), c-Fos, c-Jun, and EGR-1 proteins are increased during natural luteolysis; in contrast, their expressions are decreased during establishment of pregnancy; and iii) expressions of Bcl-2, Bcl-XL, Bad, and Bax proteins are not modulated during natural luteolysis while expressions of Bcl2 and Bcl-XL proteins are increased during establishment of pregnancy in sheep. These proteomic changes are evident in both large and small luteal cells. These results together indicate that regression of the CL during natural luteolysis or survival of the CL during establishment of pregnancy is precisely controlled by distinct programmed suppression or activation of intraluteal cell survival and apoptosis pathways in sheep/ruminants.

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Introduction

In ruminants, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is the luteolytic hormone (McCracken *et al*. 1999). During the process of luteolysis, $PGF_{2\alpha}$ is synthesized and released from the endometrial luminal epithelial cells in a pulsatile pattern which causes luteolysis. In sheep, continuous exposure of endometrium to progesterone (P_4) for 8–10 days down-regulates expression of nuclear P_4 receptor in luminal epithelial cells between days 11 and 13, thereby allowing a rapid increase in expression of nuclear E_2 receptor α (ESR-1) after day 13, followed by an increase in expression of membrane oxytocin receptor (OXTR) after day 14 of the estrous cycle (McCracken *et al*. 1999, Spencer *et al*. 2004). Pulsatile releases of oxytocin from the posterior pituitary and corpus luteum (CL) after days 13–14 of the estrous cycle act via OXTR to induce release of luteolytic pulses of $PGF_{2\alpha}$ from luminal epithelial cells between days 14 and 16 of the estrous cycle (McCracken *et al*. 1999). Endometrial $PGF_{2\alpha}$ is transported into each adjacent uterine vein which joins the adjacent ovarian vein to form the utero-ovarian vein. Luteolytic $PGF_{2\alpha}$ pulses are transported from the utero-ovarian vein into the ovarian artery locally through a complex vascular structure called the utero-ovarian plexus (UOP) (Lee *et al*. 2010). A minimum of five 1-h long pulses of $PGF_{2\alpha}$ over a period of 48 h is required to cause complete luteolysis consistently in sheep (McCracken *et al*. 2012).

At the time of establishment of pregnancy, interferon tau secreted by the trophoblast of the conceptus inhibits endometrial pulsatile release of $PGF_{2\alpha}$ and prevents luteolysis (Spencer *et al*. 2004). Experiments involving anastomosis of uterine vein or ovarian artery from pregnant to nonpregnant uterine horn indicate that both luteolytic and luteoprotective mediators need to be transported from the utero-ovarian vein to the ovarian artery via the UOP in sheep and cattle (Mapletoft & Ginther 1975, Mapletoft *et al*. 1975, 1976*a*,*b*, Ginther 1981). Embryo/conceptus transfer and hysterectomy experiments indicate that the luteolytic and luteoprotective mechanisms are locally mediated between the uterus and the CL of the ipsilateral ovary and do not act systemically in sheep (Moor & Rowson 1966*a*, Moor

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et al. 1969, 1970). Early studies indicate that during the establishment of pregnancy in sheep, a factor(s) from the conceptus or gravid uterus reaches the ovary locally through the UOP and protects the CL from luteolysis (Moor & Rowson 1966*a*,*b*, Moor *et al*. 1969, Mapletoft & Ginther 1975, Mapletoft *et al*. 1975, 1976*a*, Silvia & Niswender 1986).

In ruminants, the CL of early pregnancy is more resistant to the luteolytic action of $PGF_{2\alpha}$ (Inskeep *et al*. 1975, Pratt *et al*. 1977, Nancarrow *et al*. 1982, Silvia & Niswender 1984, 1986) on days 12–16, and the resistance is even greater when multiple embryos are present (Nancarrow *et al*. 1982). Injection of $PGF_{2\alpha}$ into an ovarian artery or follicles of early pregnant sheep causes luteolysis in 28% or 17% of animals compared with 78% or 83% in nonpregnant sheep respectively (Inskeep *et al*. 1975, Pratt *et al*. 1977). Exogenous estradiol at doses causing premature luteolysis in cyclic sheep is less effective in pregnant sheep (Kittok & Britt 1977). Infusions of IFNT directly into the uterine vein maintained a functional CL in 80% of sheep for up to 32 days through yet unidentified mechanisms (Oliveira *et al*. 2008, Bott *et al*. 2010). Intraovarian administration of PGE_2 dose dependently counteracts the luteolytic actions of $PGF_{2\alpha}$ (Henderson *et al*. 1977). Intrauterine or intraovarian infusions of PGE_2 in nonpregnant ewes extend the inter-estrus interval and reduce luteal sensitivity to both endogenously secreted and exogenously administered $PGF_{2\alpha}$ (Henderson *et al*. 1977, Pratt *et al*. 1977, 1979, Magness *et al*. 1981, Reynolds *et al*. 1981, Weems *et al*. 2006). Our resent study (Lee *et al*. 2012) shows that during establishment of pregnancy, a large proportion of PGE_2 is transported from the uterus to the ovary through the UOP. Luteal PG biosynthesis is selectively directed towards $PGF_{2\alpha}$ at the time of luteolysis; by contrast, towards PGE_2 during establishment of pregnancy.

Our current understanding is that regression of CL occurs in two phases: i) functional luteolysis and ii) structural luteolysis. Functional luteolysis is defined as decrease in P_4 secretion whereas structural luteolysis is defined as loss of luteal cells and volume (McCracken *et al*. 1999). It is well accepted that functional luteolysis precedes structural luteolysis. A recent study indicates that inhibition of luteal PG production by indomethacin at mid cycle decreases P_4 levels and induces functional luteolysis, however; it does not decrease size and weight of the CL or induce structural luteolysis in sheep (Niswender *et al*. 2007). It suggests that existence of distinct or separate mechanism that governs the functional vs structural luteolysis in sheep. P_4 secreted by the CL is required for establishment of pregnancy. Secretion of P_4 depends on the survival and healthy status of luteal cells which is precisely controlled by interactions between cell survival and apoptosis pathways. ERK1/2, AKT, β-Catenin and NFκB pathways are the important intracellular pathways determine survival of cells (Datta *et al*. 1997, Bonni *et al*. 1999, Kumar *et al*. 2004, Grigoryan *et al*. 2008). By contrast, interactions between pro-apoptotic and anti-apoptotic signaling pathways and activation of caspases-3 dependent or independent intrinsic apoptosis pathways determine the death of cells (Adams & Cory 1998, Jiang & Wang 2004). Previous studies have shown that administration $PGF_{2\alpha}$ regulates genes or protein associated with cell survival and apoptosis in cows (Davis & Rueda 2002, Hou *et al*. 2008, Arvisais *et al*. 2010, Atli *et al*. 2012), sheep (Romero *et al*. 2013), pigs (Diaz *et al*. 2013), rodents (Carambula *et al*. 2002, Slot *et al*. 2006), and primates (Peluffo *et al*. 2005, Yadav *et al*. 2005) during induced luteolysis *in vivo* and *in vitro* models. Although there is a large body of information available on induced luteolysis in various species, temporal regulations of cell survival and apoptosis signaling protein machinery in the CL during natural luteolysis and establishment of pregnancy in ruminants are largely unknown. The objective of the present study was to determine the effects of early pregnancy on regulation of survival and apoptosis signaling protein machinery in the CL on days 12, 14, and 16 in sheep, using as a ruminant model.

Materials and methods

Materials

Prestained protein markers, Bio–Rad assay reagents and standards (Bio–RAD Laboratories); protran BA83 Nitrocellulose membrane (Whatman, Inc., Sanford, ME, USA); pierce ECL (Pierce, Rockford, IL, USA); protease inhibitor (Roche Applied Biosciences); Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA); Progesterone RIA kits (Diagnostic Systems Laboratories, Webster, TX, USA); Blue X-ray film (Phoenix Research Products, Hayward, CA, USA) were purchased. Details of antibodies used in this study are given in Table 1. The other chemicals used were molecular biological grade from Fisher (Pittsburgh, PA, USA) or Sigma–Aldrich.

Animal husbandry

All experiments were in accordance with the Guide for Care and Use of Agricultural Animals and approved by Texas A&M University's Laboratory Animal Care and Use Committee. Mature crossbred Suffolk ewes (Ovis aries) were observed daily for estrus in the presence of vasectomized rams. Ewes that had exhibited at least two estrous cycles of normal duration (17–18 days) were used in this study. At estrus (day 0), the ewes were bred to either an intact or a vasectomized ram. The ewes ($n=4$ per day) were necropsied on days 12, 14, or 16 of the estrous cycle or pregnancy as described previously (Banu *et al*. 2008*a*, Simmons *et al*. 2010). The uterus was flushed with 20 ml physiological saline and pregnancy was confirmed on each day by the presence of a normal conceptus in the uterine lumen flushing as described previously (Simmons *et al*. 2010). The ovaries were collected and the CL isolated. Longitudinal

Table 1 Details of antibodies used.

Peptide/ protein target	Antigen sequence (if known)	Name of antibody	Manufacturer	Catalog no., and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used WB	Dilution used IHC
pEGFR	Purchased	p-EGFR (Tyr1173)	Santa Cruz	sc-12351	Rabbit polyclonal	1:500	1:100
tEGFR	Purchased	EGFR (1005)	Santa Cruz	sc-03	Rabbit polyclonal	1:500	
pERK1/2	Purchased	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody	Cell Signaling	9101	Rabbit polyclonal	1:1000	
pERK1/2	Purchased	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP rabbit mAb	Cell Signaling	4370	Rabbit monoclonal		1:100
tERK1/2	Purchased	p44/42 MAPK (Erk1/2) antibody	Cell Signaling	9102	Rabbit polyclonal	1:1000	
pAKT	Purchased	Phospho-Akt (Ser473) antibody	Cell Signaling	9271	Rabbit polyclonal	1:1000	
pAKT	Purchased	Phospho-Akt (Ser473) (736E11) rabbit mAb	Cell Signaling	3787	Rabbit monoclonal		1:100
tAKT	Purchased	Akt antibody	Cell Signaling	9272	Rabbit polyclonal	1:1000	
pSRC416	Purchased	Phospho-Src family (Tyr416) antibody	Cell Signaling	2101	Rabbit polyclonal	1:1000	1:50
t-SRC	Purchased	Src (36D10) rabbit mAb	Cell Signaling	2109	Rabbit monoclonal	1:1000	1:50
p-β-arrestin	Purchased	Phospho-β-arrestin 1 (Ser412) (6-24) mouse mAb	Cell Signaling	2416	Mouse monoclonal	1:1000	1:50
β-cathenin	Purchased	β-cathenin	Cell Signaling	9562	Rabbit polyclonal	1:1000	1:100
β-actin	Purchased	Monoclonal anti-β-actin antibody produced in mouse	Sigma	A2228	Mouse monoclonal	1:8000	
Bcl-xL	Purchased	Bcl-xL antibody	Cell Signaling	2762	Rabbit polyclonal	1:1000	1:25
Bcl-2	Purchased	Bcl-2 antibody	Cell Signaling	2872	Rabbit polyclonal	1:1000	
Bcl-2	Purchased	Bcl-2 (DC 21): sc-783	Santa Cruz	sc-783	Rabbit polyclonal		1:25
t-caspase-3	Purchased	Caspase-3 antibody	Cell Signaling	9662	Rabbit polyclonal	1:1000	
Cl-caspase-3	Purchased	Cleaved caspase-3 (Asp175) antibody	Cell Signaling	9661	Rabbit polyclonal	1:1000	1:100
XIAP	Purchased	XIAP antibody	Cell Signaling	2042	Rabbit polyclonal	1:1000	1:100
AIF	Purchased	AIF antibody	Cell Signaling	4642	Rabbit polyclonal	1:1000	1:50
Survivin	Purchased	Survivin (D-8)	Santa Cruz	sc-17779	Mouse monoclonal	1:500	
c-Fos	Purchased	c-Fos antibody	Cell Signaling	4384	Rabbit polyclonal	1:1000	
c-Jun	Purchased	c-Jun antibody	Cell Signaling	9162	Rabbit polyclonal	1:1000	1:50
EGR-1	Purchased	EGR1 antibody	Cell Signaling	4152	Rabbit polyclonal	1:1000	1:100
p-CREB	Purchased	Phospho-CREB (Ser133) antibody	Cell Signaling	9191	Rabbit polyclonal	1:1000	1:100
NFκB p50	Purchased	NFκB p105/p50 antibody	Cell Signaling	3035	Rabbit polyclonal	1:1000	1:100
NFκB p52	Purchased	NFκB2 p100/p52 antibody	Cell Signaling	4882	Rabbit polyclonal	1:1000	
NFκB p65	Purchased	NFκB-p65 antibody	Cell Signaling	3034	Rabbit polyclonal	1:1000	1:100
Ras	Purchased	Ras antibody	Cell Signaling	3965	Rabbit polyclonal	1:1000	1:100
p-c-Raf	Purchased	Phospho-c-Raf (Ser338) (56A6) rabbit mAb	Cell Signaling	9427	Rabbit monoclonal	1:1000	1:100
p-GSK	Purchased	Phospho-GSK-3α/β (Ser21/9) antibody	Cell Signaling	9331	Rabbit polyclonal	1:1000	1:75
Bad	Purchased	Bad antibody	Cell Signaling	9292	Rabbit polyclonal	1:1000	
Bax	Purchased	Bax antibody	Cell Signaling	2774	Rabbit polyclonal	1:1000	
β-actin	Purchased	Monoclonal anti-β-actin antibody produced in mouse	Sigma	A2228	Mouse monoclonal	1:8000	
Goat anti-rabbit IgG	Purchased	Anti-rabbit IgG (H+L) antibody, peroxidase labelled	Kirkegaard & Perry Laboratories	474-1506	Goat polyclonal	1:10000	
Goat anti-mouse IgG	Purchased	Anti-mouse IgG (H+L) antibody, human serum adsorbed and peroxidase labeled	Kirkegaard & Perry Laboratories	474-1806	Goat polyclonal	1:10000	
Rabbit anti-goat IgG	Purchased	Anti-goat IgG (H+L) antibody, peroxidase labeled	Kirkegaard & Perry Laboratories	14-13-06	Rabbit polyclonal	1:5000	

cross sections were cut in the middle of each CL and fixed in fresh 4% buffered paraformaldehyde, and processed for immunohistochemistry using standard procedures. The remaining CL tissue was cut into small pieces, snap-frozen in liquid nitrogen, and stored at −80 °C for further analysis.

Protein extraction

Total protein was isolated from CL tissues as we described previously (Arosh *et al*. 2003, Banu *et al*. 2008*b*). Briefly, tissues were homogenized in TED buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1 mM diethyldithiocarbamic acid-DEDTC, and 0.1% Tween-20) and centrifuged at 30 000 ***g*** for 1 h at 4 °C. The homogenized tissue pellets were sonicated in TED sonication buffer (20 mM Tris, pH 8.0, 0.5 mM EDTA, 0.1 mM DEDTC, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail tablets, complete EDTA-free 1 tablet/50 ml and PhosStop 1 tablet/10 ml, and 1.0% Tween-20) using a Microson ultrasonic cell disruptor (Microsonix Incorporated, Farmingdale, NY, USA) and centrifuged at 15 000 ***g*** for 15 min at 4 °C and the supernatants (total protein) were stored at −80 °C until analyzed. Total protein concentrations were determined using the Bradford method (Bradford 1976) and a Bio–Rad Protein Assay kit.

Western blot

Total protein samples (75 µg) were resolved using 7.5%, 10%, or 12.5% SDS–PAGE and western blot was performed as we described previously (Arosh *et al*. 2003, Banu *et al*. 2008*b*). The blots were incubated with primary antibody for overnight at 4 °C (Table 1). Then, the blots were washed and incubated with goat anti-rabbit or anti-mouse IgG conjugated with HRP secondary antibody for 1 h at room temperature. Chemiluminescent substrate was applied according to the manufacturer's instructions (Pierce Biotechnology). The blots were exposed to Blue X-Ray film and densitometry of autoradiograms was performed using an Alpha Imager (Alpha Innotech Corporation, San Leandro, CA, USA).

Immunohistochemistry

Paraffin sections (5 µm) were used for immunohistochemical localization of the proteins using a Vectastain Elite ABC kit (Vector Laboratories, Inc.) according to the manufacturer's protocols, and as we described previously (Arosh *et al*. 2003, Banu *et al*. 2009, 2010*a*). Endogenous peroxidase activity was removed by fixing sections in 0.3% hydrogen peroxide in methanol. Tissue sections were blocked in 10% goat serum for 1 h at room temperature, and then incubated with primary antibody for overnight at 4 °C (Table 1). The tissue sections were further incubated with the secondary antibody (goat anti-rabbit IgG biotinilated) for 45 min at room temperature. For the negative control, serum or IgG from respective species with reference to the primary antibody at the respective dilution was used.

Digital images were captured using a Zeiss Axioplan 2 Research Microscope (Carl Zeiss, Thornwood, NY, USA) with an Axiocam HR digital color camera. The intensity of staining for each protein was quantified using Image-Pro Plus 6.3 image processing and analysis software according to the manufacturer's instructions (Media Cybernetics, Inc., Bethesda, MD, USA). The detailed methods for quantification are given in the instruction guide: 'The Image-Pro Plus (Media Cybernetics, Inc.): the proven solution for image analysis'. In brief: a minimum of three images of at ×400 magnification were captured randomly without hot-spot bias in each tissue section per animal. Integrated optical intensity (IOD) of immunostaining was quantified under RGB mode as we published recently (Lee *et al*. 2012). Numerical data were expressed as least square mean ± S.E.M. This technique is more quantitative than conventional blind scoring systems and the validity of the quantification was reported previously by our group (Arosh *et al*. 2003, Banu *et al*. 2010*b*, Lee *et al*. 2012).

Progesterone assay

Jugular venous blood samples were collected in tubes treated with EDTA 10.8 mg at the time of necropsy and plasma was separated immediately. Concentrations of progesterone in plasma were determined using DSL-3900 ACTIVE Progesterone Coated-Tube RIA kit according to the manufacturer's instructions (Diagnostic Systems Laboratories). The RIA used rabbit anti-progesterone immunoglobulin-coated tubes and iodinated progesterone. The primary antiserum cross-reacts 6.0, 2.5, 1.2, 0.8, 0.48, and 0.1% with 5α-pregnane-3,20-dione, 11-deoxycorticosterone, 17α-hydroxyprogesterone, 5β-pregnane-3,20-dione, 11-deoxycortisol, and 20β-dihydroprogesterone respectively. The progesterone standard curve (0–10.57 ng/ml) was provided in the assay kit. The sensitivity or minimum detection limit of this assay is ∼0.12 ng/ml. The intra-assay variation was 8.8%.

Statistical analyses

Statistical analyses were performed using general linear models of Statistical Analysis System (SAS, Cary, NC, USA). Data were checked for normality or homogeneity of variance before analyzing the data statistically. Day (12, 14, or 16) and status (estrous cycle vs pregnancy) interactions on expression of various proteins were tested using repeated measures for multivariate analysis of variance. Comparison of means was tested by Wilks' Lambda or Orthogonal contrast tests. Effects of day 16 of the estrous cycle or pregnancy on cell-specific expression of various proteins in luteal cells were analyzed using one-way ANOVA. Comparison of means was performed by Tukey HSD test. Numerical data are presented as least squares means with standard errors. Statistical significance was considered as $P<0.05$. The statistical model accounted for sources of variation including treatments, replicates, and ewes as appropriate.

Results

Functional and structural luteolysis

We determined the concentration of P_4 in plasma and luteal tissue weight/volume (Fig. 1). The plasma

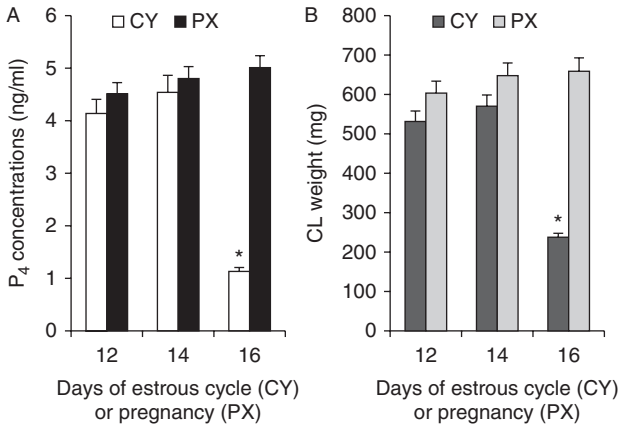

Figure 1 Progesterone concentration and luteal weight/volume. (A) P_4 concentration. (B) Luteal weight/volume. Jugular venous blood samples were collected on days 12, 14, and 16 of the CY or PX. The CL was collected on days 12, 14, and 16 at necropsy. *CY vs PX, $P<0.05$. The numerical data were expressed in least square S.E.M., $n=4$ for each day. More details are given in the Materials and methods section.

concentration of P_4 was ~4.1 ng/ml on day 12 and decreased ($P<0.05$) to ~1.1 ng/ml on day 16 of the estrous cycle (CY) whereas the P_4 concentration was maintained ($P<0.05$) at 4.3–4.9 ng/ml between days 12 and 16 of pregnancy (PX). The mean weight of the CL decreased from day 14 (~600–625 mg) to 16 of the CY (~200–225 mg). In contrast, it was maintained at 600–700 mg ($P<0.05$) on days 12, 14, and 16 of PX. These results together indicted that the CL entered into functional as well as structural luteolytic processes on day 16 of the CY; by contrast, the CL was rescued from both functional and structural luteolysis at the time of establishment of pregnancy.

Regulation of cell survival signaling protein machinery in the CL at luteolysis and establishment of pregnancy

We determined temporal regulation of important cell survival signaling proteins in the CL on days 12, 14, and 16 of the CY and PX (Fig. 2).

Expression of p-EGFR protein was unchanged on days 12–16 of the CY and PX. Expression of p-ERK1/2 protein was decreased ($P<0.05$) on days 14–16 compared with day 12 of the CY. In contrast, it was abundantly expressed without significant modulation on days 12–16 of PX. CY–PX interaction indicated that expression of p-ERK1/2 protein was sustained ($P<0.05$) on days 14–16 of PX compared with that of the CY.

Expression of p-AKT473 protein was decreased ($P<0.05$) on day 16 compared with days 12–14 of the CY. In contrast, it was abundantly expressed without significant modulation on days 12–16 of PX. CY–PX interaction indicated that expression of p-AKT473 protein was sustained ($P<0.05$) on day 16 of PX compared with that of the CY.

β-catenin protein was constantly expressed at low level on days 12–16 of the CY. In contrast, it was constantly expressed at high level on days 12–16 in PX. CY–PX interaction indicated that expression of β-catenin protein was increased ($P<0.05$) on days 12–16 of PX compared with that of the CY.

p-GSK3β protein was expressed at very low levels on days 12–16 of the CY. In contrast, it was abundantly expressed on days 12–14 of PX. Notably, its expression was decreased ($P<0.05$) on day 16 compared with days 12–14 of PX. CY–PX interaction indicated that expression p-GSK3β protein was increased ($P<0.05$) on days 12–16 of PX compared with that of the CY.

Expression of NFκB-p50 protein was very low on days 12–16 of the CY. In contrast, it was abundantly expressed on days 12–14 of PX. Notably, its expression was decreased ($P<0.05$) on day 16 compared with days 12–14 of PX. CY–PX interaction indicated that expression NFκB-p50 protein was increased ($P<0.05$) on days 12–16 of PX compared with that of the CY.

Expression of NFκB-p52 protein was temporally decreased ($P<0.05$) on days 14–16 compared with day 12 of the CY. In contrast, it was abundantly expressed without significant modulation on days 12–16 of PX. CY–PX interaction indicated that expression of NFκB-p52 protein was sustained ($P<0.05$) on days 14–16 of PX compared with that of the CY.

Expression of NFκB-p65 protein was decreased ($P<0.05$) on days 14–16 compared with day 12 of the CY. In contrast, it was abundantly expressed without significant modulation on days 12–16 of PX. CY–PX interaction indicated that expression of NFκB-p65 protein was increased ($P<0.05$) on days 14–16 of PX compared with that of the CY.

Expression of Ras protein was decreased ($P<0.05$) on day 16 compared with days 12–14 of the CY. In contrast, it was abundantly expressed without modulation on days 12–16 of PX. CY–PX interaction indicated that expression of Ras protein was sustained ($P<0.05$) on days 16 of the PX compared with that of the CY.

Expression of p-cRaf protein was decreased ($P<0.05$) temporally on days 14–16 compared with day 12 of the CY. In contrast, it was abundantly expressed without significant modulation on days 12–16 of PX. CY–PX interaction indicated that expression of p-cRaf protein was increased ($P<0.05$) on days 14–16 of PX compared with that of the CY.

Expression of p-Src416 protein was temporally decreased ($P<0.05$) on days 14–16 compared with day 12 of the CY. In contrast, it was highly expressed without modulation on days 12–16 of PX. CY–PX interaction indicated that expression of p-Src416 protein was increased ($P<0.05$) on days 14–16 of PX compared with that of the CY.

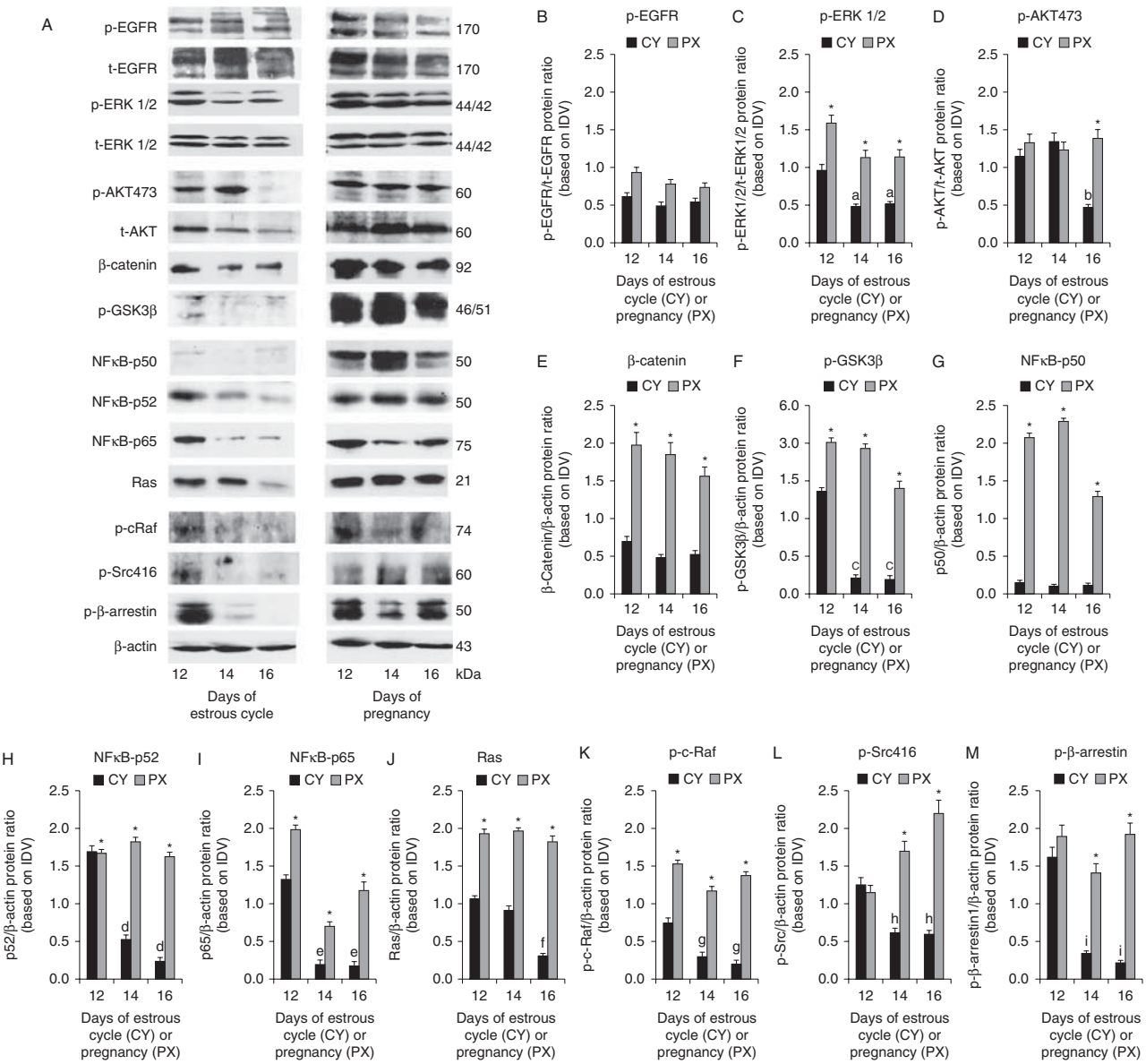

Figure 2 Temporal expression of cell survival signaling proteins in the CL on days 12, 14, and 16 of the CY and early PX in sheep. (A) Western blot analysis. β-actin protein was measured as an internal control. (B, C, D, E, F, G, H, I, J, K, L and M) Densitometry. *Denotes comparisons of expression pattern of respective protein on days 12, 14, or 16 of the CY vs PX, $P<0.05$. Letters 'a–i' denote changes in expression pattern of respective protein on days 12,14, or 16 of the CY, $P<0.05$. a: p-ERK1/2, 14–16 vs 12. b: p-AKT473, 16 vs 12–14. c: p-GSK3β, 14–16 vs 12. d: NFκB-p52, 14–16 vs 12. e: NFκB-p65, 14–16 vs 12. f: c-Ras, 16 vs 12–14. g: p-c-Raf, 16 vs 12–14. h: p-Src416, 14–16 vs 12. i: p-β-arrestin, 14–16 vs 12. Densitometry was performed using Alpha Imager and expressed at integrated density value (IDV). The numerical data are expressed in least square S.E.M., $n=4$ for each day.

Expression of β-arrestin protein was temporally decreased ($P<0.05$) on days 14–16 compared with day 12 of the CY. β-arrestin protein was abundantly expressed without significant modulation on days 12–16 of PX. CY–PX interaction indicated that expression of β-arrestin protein was sustained ($P<0.05$) on days 14–16 of PX compared with that of the CY.

Spatial expressions of p-ERK1/2, p-AKT473, β-catenin, NFκB-p50, NFκB-p52, and NFκB-p65, and p-Src416 proteins in LLC and SLC were increased ($P<0.05$) on day 16 of PX compared with that of CY (Fig. 3).

Regulation of intrinsic apoptotic pathway proteins in the CL at luteolysis and establishment of pregnancy

We determined temporal regulation of important intrinsic apoptotic pathway proteins in the CL on days 12, 14, and 16 of the CY and PX (Fig. 4).

Bcl-XL protein was expressed at very low level on days 12–16 of the CY. In contrast, it was abundantly expressed without significant modulation on days 12–16 of PX. CY–PX interaction indicated that Bcl-XL protein was increased ($P<0.05$) on days 12–16 of PX compared with that of the CY.

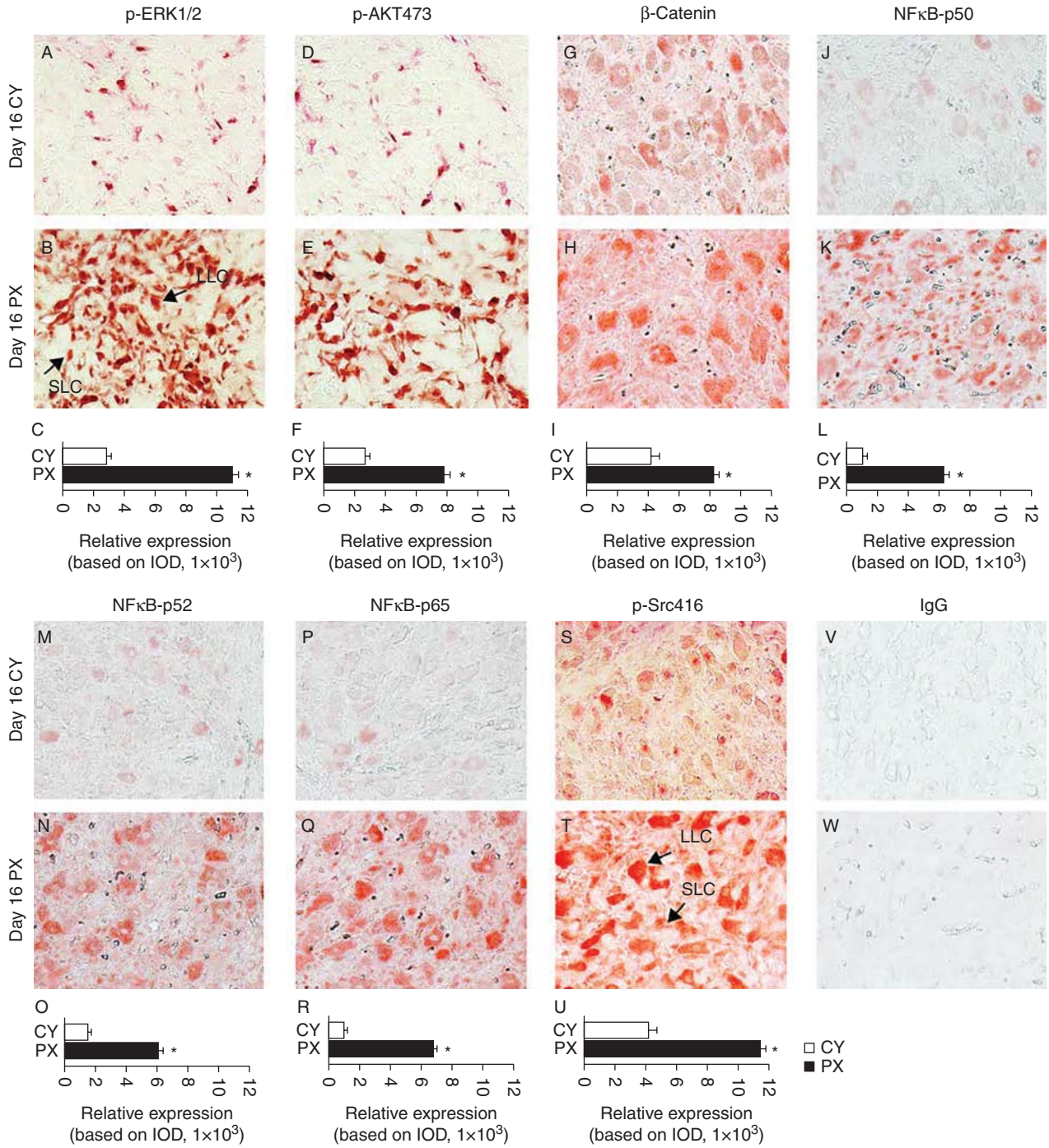

Figure 3 Cellular localization of cell survival signaling proteins in the CL on day 16 of the CY and early PX in sheep. (A, B and C) p-ERK1/2, (D, E and F) p-AKT473, (G, H and I) β-catenin, (J, K and L) NFκB-p50, (M, N and O) NFκB-p52, (P, Q and R) NFκB-p65, (S, T and U) p-Src416, and (V and W) IgG. LLC, large luteal cells; SLC, small luteal cells. (C, F, I, L, O, R and U) Relative expression: *Expression of each protein on day 16 of the CY vs PX, $P<0.05$. Immunohistochemistry was performed using Vectastain Elite ABC kit (Vector Laboratories, Inc.) and representative photomicrographs at 400× magnification are shown. Densitometry of relative spatial expression of each protein in both LLC and SLC was quantified using Image-Pro Plus (Media Cybernetics, Inc.) and expressed as IOD. The numerical data are expressed in least square S.E.M. Statistical significance was $P<0.05$. Please see text in Materials and methods for more details.

Bcl-2 protein was expressed at a very low level on day 12 and further decreased ($P<0.05$) on days 14–16 of the CY. In contrast, it was abundantly expressed without significant modulation on days 12–16 of PX. CY–PX interaction indicated that Bcl-2 protein was increased ($P<0.05$) on days 12–16 of PX compared with that of the CY.

Expression of Bad protein was not significantly modulated on days 12–16 of the CY. Bad protein was

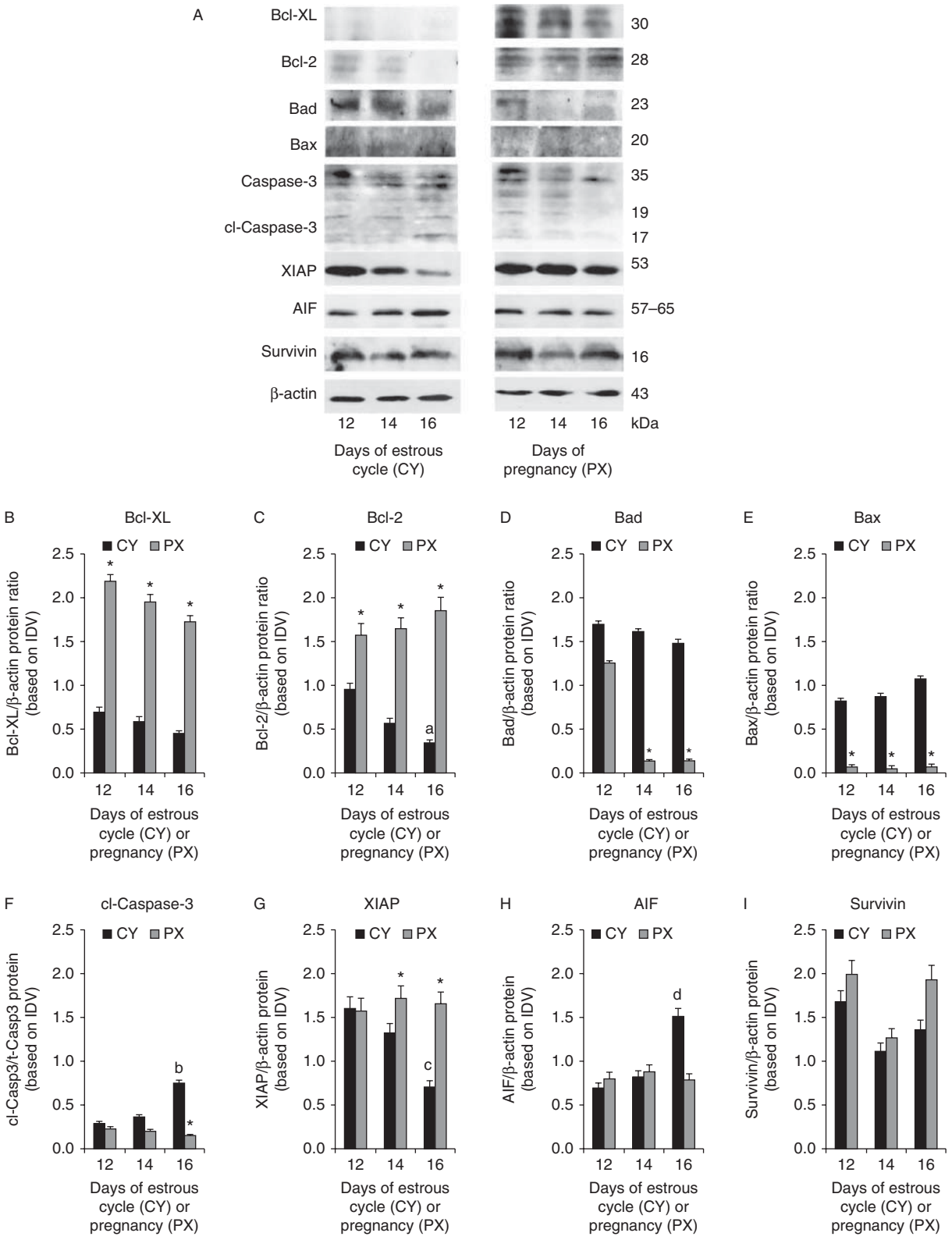

Figure 4 Temporal expression of intrinsic apoptosis signaling proteins in the CL on days 12, 14 and 16 of the CY and early PX in sheep. (A) Western blot analysis. β-actin protein was measured as an internal control. (B, C, D, E, F, G, H and I) Densitometry. *Denotes comparisons of expression pattern of respective protein on days 12, 14, or 16 of the CY vs PX, $P<0.05$. Letters 'a–d' denote changes in expression pattern of respective protein on days 12,14, or 16 of the CY, $P<0.05$. a: Bcl2, 16 vs 12–14. b: cl-caspase-3, 16 vs 12–14. c: XIAP, 16 vs 14–12. d: AIF, 16 vs 14–12. Densitometry was performed using Alpha Imager and expressed at IDV. The numerical data are expressed in least square S.E.M., $n=4$ for each day.

expressed on day 12 and its expression was decreased ($P<0.05$) on days 14–16 of PX. CY–PX interaction indicated that Bad protein was decreased ($P<0.05$) on days 14–16 of PX compared with that of the CY.

Bax protein was minimally expressed at constant level on days 12–16 of the CY; while, it was expressed at very low level or not detectable on days 12–16 of PX. CY–PX interaction indicated that Bax protein was ($P<0.05$) decreased on days 12–16 of PX compared with that of the CY.

Caspase-3 protein was detected at 35, 19, and 17 kDa. The cleaved protein detected at 17 kDa was an active form. Caspase-3 protein was cleaved at 17 kDa ($P<0.05$) on day 16 compared with that of days 12–14 of the CY. In contrast, caspase-3 protein was not cleaved at 17 kDa on days 12–16 of PX. CY–PX interaction indicated that caspase-3 protein was not cleaved at 17 kDa ($P<0.05$) on day 16 of PX compared with that of the CY.

Expression of X-linked inhibitor of apoptosis protein (XIAP) protein was temporally decreased ($P<0.05$) from days 12–16 and suppressed on day 16 of the CY. In contrast, it was ($P<0.05$) expressed at steady state levels on days 12–16 of PX. CY–PX interaction indicated that XIAP protein was highly expressed on days 14–16 of PX compared with that of the CY.

Expression of apoptosis inducing factor (AIF) protein was increased ($P<0.05$) on day 16 compared with 12–14 of the CY. In contrast, it was not regulated on days 14–16 of PX. CY–PX interaction indicated that AIF protein was decreased on day 16 of PX compared with that of the CY. Expression of survivin protein was not modulated in the CL on days 12–16 of the CY or PX.

Spatial expression of Bcl2, Bcl-XL, and XIAP proteins were increased ($P<0.05$) and expression of cl-capspe-3 and AIF proteins were decreased ($P<0.05$) in LLC and SLC on day 16 of PX compared with that of the CY (Fig. 5).

Regulation of important transcriptional factor proteins in the CL at luteolysis and establishment of pregnancy

We determined temporal regulation of important transcription factor proteins involved in cell survival and apoptosis pathways in the CL on days 12, 14, and 16 of the CY and PX (Fig. 6).

c-Fos protein was ($P<0.05$) expressed on days 14–16 compared with day 12 of the CY. Expression of c-Fos protein was decreased ($P<0.05$) on days 14–16 compared with day 12 of PX. CY–PX interaction indicated that expression of c-Fos protein was decreased on days 14–16 of PX compared with that of the CY.

Expression of c-Jun protein was temporally increased ($P<0.05$) on days 12–16 and highly expressed on day 16 of the CY. c-Jun protein was constantly expressed on day 12–16 of PX. CY–PX interaction indicated that expression of c-Jun protein was decreased on days 14–16 of PX compared with that of the CY.

Expression of EGR-1 protein was increased ($P<0.05$) on day 16 compared with that of 12–14 of the CY; while, it was barely detectable on days 12–16 of PX. CY–PX interaction indicated that EGR-1 protein was highly expressed on day 16 of PX compared with that of the CY.

Expression of p-CREB protein was temporally decreased ($P<0.05$) from days 12–16 and highly suppressed on day 16 of the CY. By contrast, p-CREB protein was temporally increased ($P<0.05$) from days 12–16 and highly induced on day 16 of PX. CY–PX interaction indicated that p-CREB protein was highly expressed on day 14–16 of PX compared with that of the CY.

Spatial expression of c-Jun and EGR-1 proteins were decreased ($P<0.05$) and expression of p-CREB protein was increased ($P<0.05$) in LLC and SLC on day 16 of PX compared with that of the CY (Fig. 7).

Discussion

The CL is a transient endocrine gland. Functional and structural demise of the CL allows a new estrous cycle. On the other hand, maintenance of the CL and its secretion of P_4 are required for establishment of pregnancy. Secretion of P_4 depends on the survival of luteal cells which is precisely controlled by interactions between cell survival and apoptosis pathways (McCracken *et al.* 1999).

In the mammalian cells, ERK1/2, AKT, NFκB, and β-catenin signaling are well characterized as prosurvival pathways. It is well known that activation of EGFR in turn triggers Ras-Raf-ERK1/2 and PI3K-AKT signaling modules (Zandi *et al.* 2007). Heterodimer complex of p50/p65 or p52/p65 is the most common active form of NFκB signaling in the majority of cells. In the absence of NFκB stimuli, p50, p52, and p65 proteins are sequestered in the cytoplasm with their inhibitory protein IκBα and form p50/p65/IκB or p52/p65/IκB inactive protein complex. In response to cytokines TNFα, IL1-β or other stimuli, IκBα protein is phosphorylated and targeted for protein degradation. It allows formation of active p50/p65 or p52/p65 heterodimer and translocation of these protein complexes into the nucleus (Kumar *et al.* 2004). β-catenin protein is the active component of Wnt signaling. In the absence of Wnt or other Wnt-related signaling, β-catenin protein is sequestered in the cytosol by scaffold protein complex consists of glycogen synthase kinase 3β (GSK3β, axin, and adenomatosis polyposis coli, and targeted for protein degradation). In response to stimuli, β-catenin is released from this destruction complex and translocates into nucleus (Grigoryan *et al.* 2008). Importantly, recent studies indicate that GPCR signaling intracellularly transactivates: i) EGFR through a c-Src/β-arrestin 1 complex which in turn activates ERK1/2 and PI3K-AKT

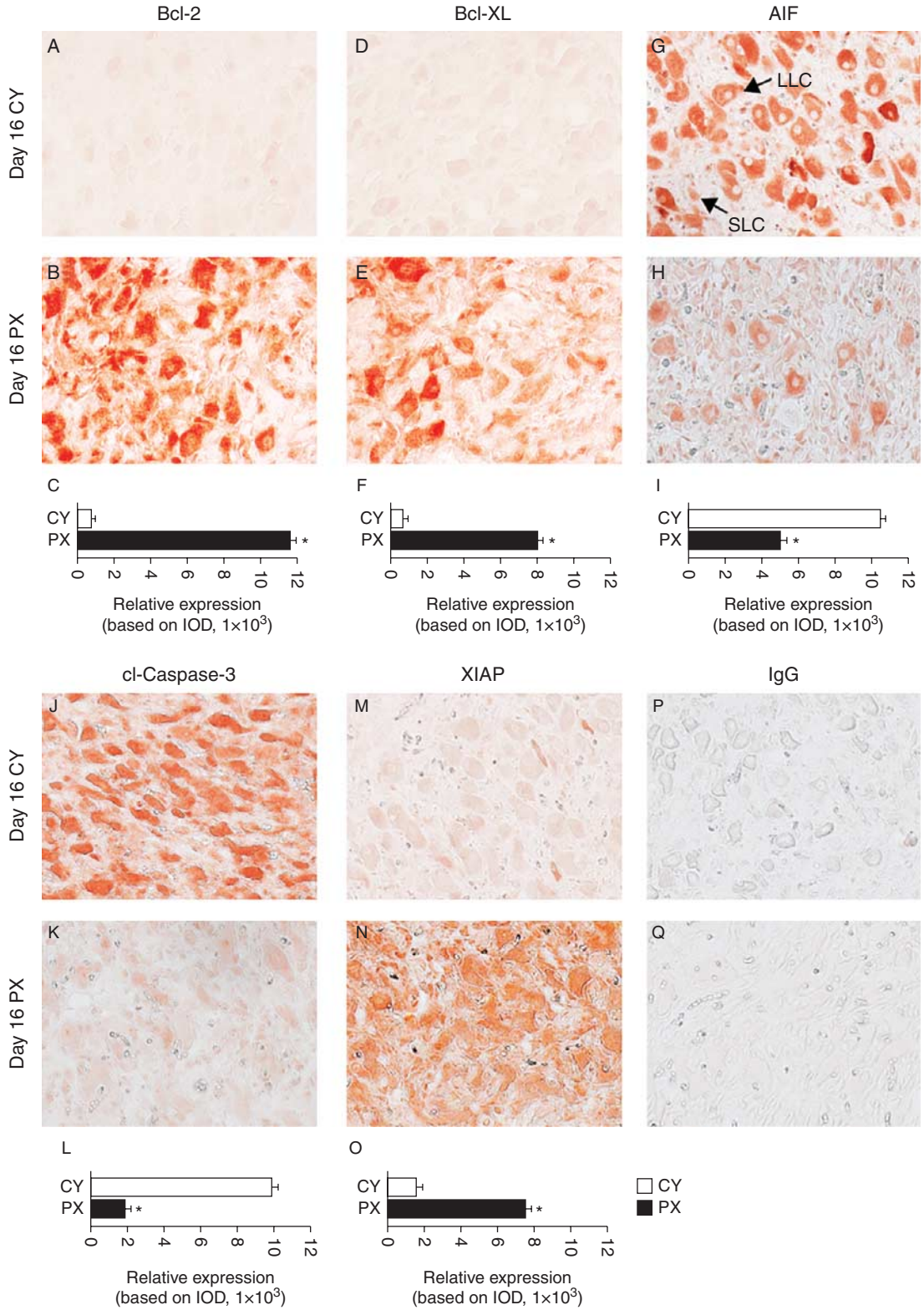

Figure 5 Cellular localization of cell survival signaling proteins in the CL on day 16 of the CY and early PX in sheep. (A, B and C) Bcl-2, (D, E and F) Bcl-XL, (G, H and I) AIF, (J, K and L) cl-caspase-3, (M, N and O) XIAP, (P and Q) IgG. LLC, large luteal cells; SLC, small luteal cells. (C, F, I, L and O) Relative expression: *Expression of each protein on day 16 CY vs PX, $P<0.05$. Immunohistochemistry was performed using Vectastain Elite ABC kit (Vector Laboratories, Inc.) and representative photomicrographs at 400× magnification are shown. Densitometry of relative spatial expression of each protein in both LLC and SLC was quantified using Image-Pro Plus (Media Cybernetics, Inc.) and expressed as IOD. The numerical data are expressed in least square S.E.M. Statistical significance was $P<0.05$. Please see text in Materials and methods for more details.

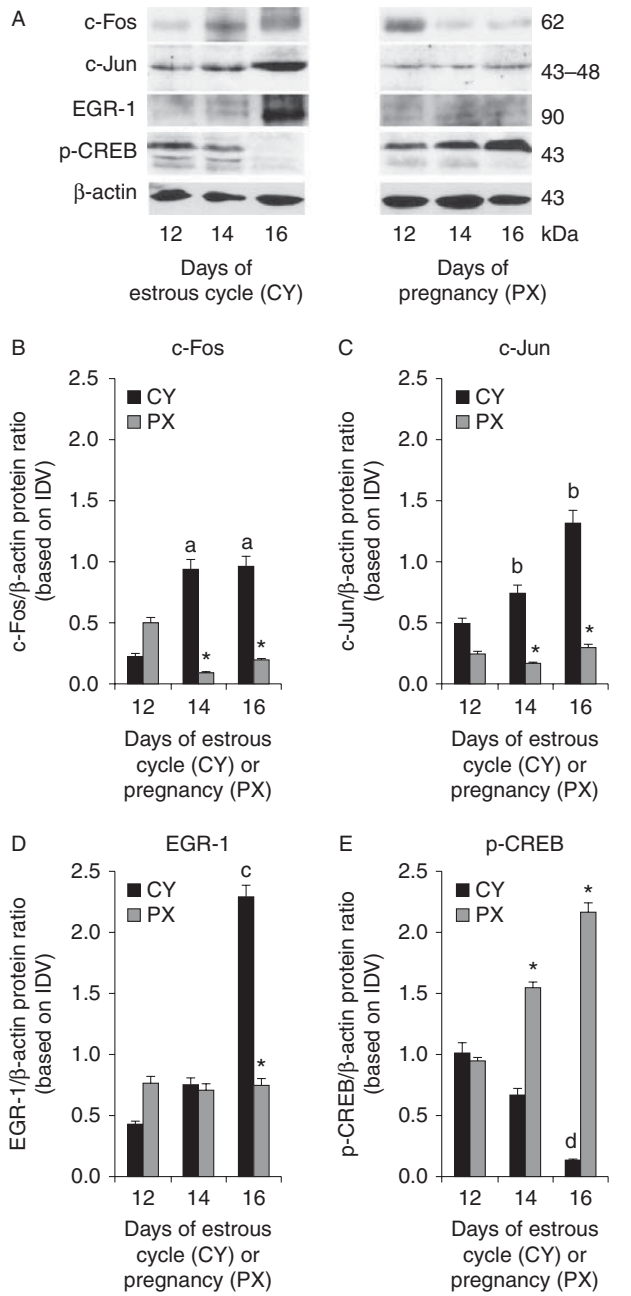

Figure 6 Temporal expression of transcriptional factors associated with cell survival and intrinsic apoptosis signaling proteins in the CL on days 12, 14 and 16 of the CY and early PX in sheep. (A) Western blot analysis. (B, C, D and E) Densitometry. *Denotes comparisons of expression pattern of respective protein on days 12, 14, or 16 of the CY vs PX, $P<0.05$. Letters 'a–d' denote changes in expression pattern of respective protein on days 12, 14 or 16 of the CY, $P<0.05$. a: c-Fos, 14–16 vs 12. b: c-Jun, 14–16 vs 12. c: EGR-1, 16 vs 14–12. d: p-CREB, 16 vs 14–12. Densitometry was performed using Alpha Imager and expressed at IDV. The numerical data are expressed in least square S.E.M., $n=4$ for each day.

pathways (Pai *et al*. 2002, Regan 2003, Jabbour & Sales 2004, Cha & DuBois 2007); ii) β-catenin signaling pathways through axin protein and/or AKT-mediated phosphorylation/inactivation of GSK3β protein (Castellone *et al*. 2005, Buchanan *et al*. 2006); and NFκB pathways (Cha & DuBois 2007) through phosphorylation of IκBα protein. In the present study, we determined the regulation of proteins associated with these important intracellular survival pathways in the CL at the time of natural luteolysis and establishment of pregnancy in sheep.

Results indicate that ERK1/2, AKT, β-catenin, NFκB, Src, β-arrestin, and GSK3 β signaling proteins are temporally suppressed in the CL from days 14–16 of the estrous cycle. It is important to note that ERK1/2, β-catenin, NFκB, Src, and β-arrestin signaling pathways are suppressed on day 14 whereas AKT pathway is suppressed on day 16 of the estrous cycle. By contrast at time of establishment of pregnancy, expression/activation of ERK1/2, AKT, β-catenin, NFκB, Src, β-arrestin, and GSK3β proteins are sustained or increased on days 14–16 to maintain the function and structure of the CL. Immunohistochemistry data demonstrate that most of these proteomic changes are obvious in both LLC and SLC on day 16 of the estrous cycle or pregnancy. These results together indicate that programmed suppression of ERK1/2, AKT, β-catenin, NFκB pathways in LLC and SLC is required for natural luteolysis. In contrast, programmed activation of these pathways is required to maintain the survival of the CL during establishment of pregnancy in sheep. One of the interesting findings is that NFκB-p50, NFκB-p52, and NFκB-p65 proteins are highly expressed on days 12–16 of PX compared with that of the CY. It is well known that NFκB is an important downstream mediator of inflammatory cytokines such as TNFα, IL-1β, and IL-6, and these cytokines play essential roles in induced luteolysis (Davis & Rueda 2002). On the other hand, roles for these proinflammatory cytokines are well established in survival of tumor cells (Kumar *et al*. 2004). Dual role for NFκB signaling in luteolysis as well as luteal maintenance in ruminants warrants further studies.

The interactions between antiapoptotic proteins (Bcl-2 and Bcl-XL) and proapoptotic proteins (Bad and Bax) determine the survival or apoptosis of cells (Adams & Cory 1998). ERK1/2, AKT, β-catenin, and NFκB pathways interact with proapoptotic and antiapoptotic proteins (Datta *et al*. 1997, Bonni *et al*. 1999, Kumar *et al*. 2004, Grigoryan *et al*. 2008). In the absence of apoptotic stimuli, Bax and Bad proteins are phosphorylated at serine 112 and 136 by ERK1/2 and AKT pathways and sequestered in the cytosol with 14-3-3 proteins (Zha *et al*. 1996). In response to apoptotic stimuli, Bad and Bax proteins dissociate from 14-3-3 proteins, translocate from the cytosol to the mitochondria, dimerize with Bcl-XL and/or Bcl-2 proteins, and thereby facilitate the release of cytochrome C from the mitochondria to the cytosol (Zha *et al*. 1996, Adams & Cory 1998, Jiang & Wang 2004). Activation of caspase-3 is the important terminal event which executes apoptosis of cells (Jiang & Wang 2004).

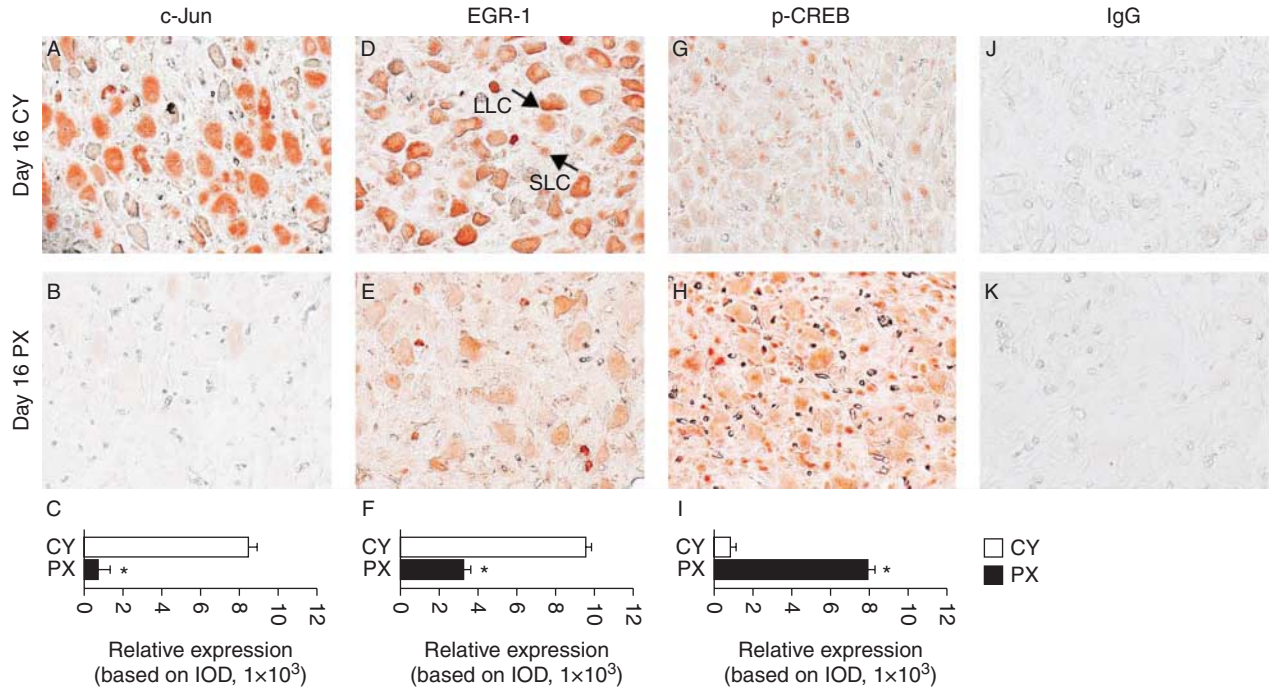

Figure 7 Cellular localization of cell survival signaling proteins in the CL on day 16 of the CY and early PX in sheep. (A, B and C) c-Jun, (D, E and F) EGR-1, (G, H and I) p-CREB, and (J and K) IgG. LLC: large luteal cells. SLC: small luteal cells. (C, F and I) Relative expression: *Expression of each protein on day 16 CY vs PX, $P<0.05$. Immunohistochemistry was performed using Vectastain Elite ABC kit (Vector Laboratories, Inc.) and representative photomicrographs at 400× magnification are shown. Densitometry of relative spatial expression of each protein in LLC and SLC was quantified using Image-Pro Plus (Media Cybernetics, Inc.) and expressed as IOD. The numerical data are expressed in least square S.E.M. Statistical significance was $P<0.05$. Please see text in Materials and methods for more details.

Activation of caspase-3 is regulated by multiple mechanisms which include cytochrome C, survivin, and XIAP pathways (Berthelet & Dubrez 2013). In addition, apoptosis can be induced by AIF (Hangen *et al.* 2010) independent of caspase-3 pathway. In the present study, we determined the regulation of proteins involved in caspase-3 dependent as well as independent apoptotic pathways in the CL during natural luteolysis and establishment of pregnancy in sheep.

Results indicate that expressions of antiapoptotic proteins Bcl-XL and Bcl-2 are increased; while, expressions of proapoptotic proteins Bad and Bax are decreased on days 14–16 pregnancy compared with that of the estrous cycle. These results suggest that expression of proapoptotic proteins need to be suppressed; whereas, expression of antiapoptotic proteins need to be sustained to rescue the CL from luteolysis at the time of establishment of pregnancy. Caspase-3 protein is cleaved or activated on day 16 of the estrous cycle; in contrast, its activation is inhibited on day 16 of pregnancy. It suggests an important role for caspase-3 in natural luteolysis in sheep. Expression of XIAP protein is decreased on day 16 of the estrous cycle; whereas, its expression is sustained on day 16 of pregnancy. It suggests that decreased expression of XIAP protein may reverse its inhibitory effect and activates caspase-3 protein independent of cytochrome C pathway in luteal cells during natural luteolysis in sheep. Expression of AIF protein is temporally increased on days 14–16 of the estrous cycle; while, its expression is temporally decreased on days 14–16 of pregnancy. It suggests that AIF may induce apoptosis of luteal cells independent of caspase-3 during natural luteolysis in sheep. Immunohistochemistry data demonstrate that most of these proteomic changes are evident in both LLC and SLC on day 16 of the estrous cycle or pregnancy. Previous studies clearly indicate a role for caspase-3 in luteal cell apoptosis in various species (Carambula *et al.* 2002, Davis & Rueda 2002, Peluffo *et al.* 2005, Yadav *et al.* 2005, Slot *et al.* 2006). Together, present results along with previous findings suggest that activation of caspase-3 dependent as well as independent apoptosis pathways are required for natural luteolysis; whereas, these pathways need to be inhibited or suppressed to maintain the survival of the CL during establishment of pregnancy in sheep.

Studies using various animal models have shown that transcription factors c-Jun (Diaz *et al.* 2013), EGR-1 (Hou *et al.* 2008) and CREB (Zeleznik & Somers 1999, Niswender 2002, Xu *et al.* 2005) play roles in luteal functions. Studies using various cell lines have shown that EGR-1 can induce growth proliferation, mutagenesis, proapoptosis or tissue remodeling depends on the cell context (Liu *et al.* 1998). AP-1 family transcription factors (c-fos, c-Jun) regulate a wide range of

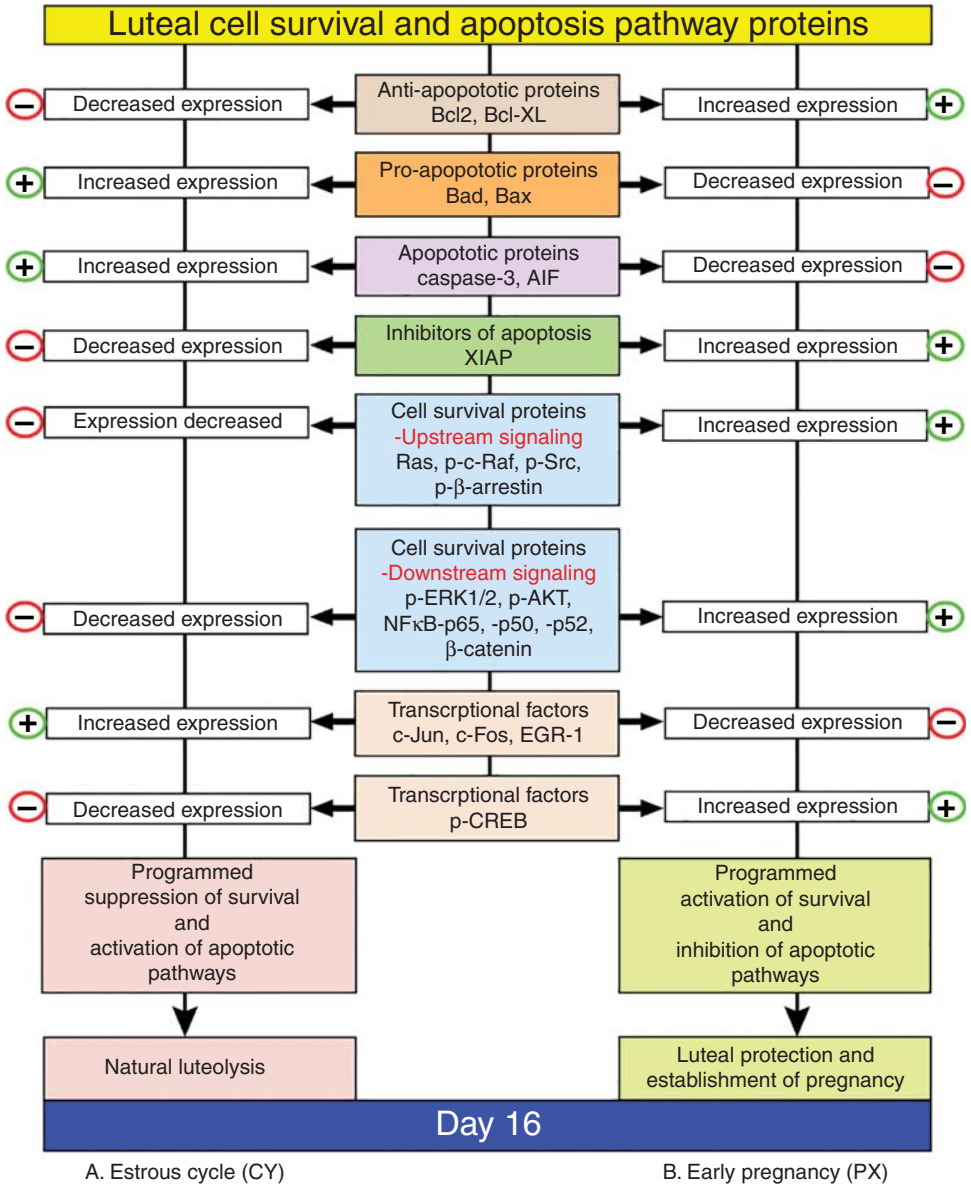

Figure 8 (A) Estrous cycle: at the time of natural luteolysis (day 16), anti-apoptotic and cell survival pathways are suppressed while pro-apoptotic and apoptotic pathways are increased. (B) Early pregnancy: in contrast at the time of establishment of pregnancy (day 16), anti-apoptotic and cell survival pathways are sustained or increased while pro-apoptotic and apoptotic pathways are inhibited. Regression of the CL during natural luteolysis vs maintenance of CL during establishment of pregnancy is precisely controlled by programmed intraluteal cell survival and apoptotic pathways. More details are provided in the Results and Discussion sections.

pathophysiological responses such as cell death, inflammation, and proliferation (Shaulian & Karin 2002). The Jun family proteins homodimerize with other Jun proteins or heterodimerize with Fos proteins and form active transcription complexes (Shaulian & Karin 2002). The classical adenylyl cyclase/cAMP/PKA is the primary hormonal signaling module control synthesis of P_4 by the CL (Zeleznik & Somers 1999, Niswender 2002, Xu *et al.* 2005). CREB is one of the final transcription factors mediates cAMP-mediated signaling cascades in variety of cell types (Zeleznik & Somers 1999, Niswender 2002, Xu *et al.* 2005). In the present study, given the strategic roles for AP-1, EGR-1, and CREB transcription factors in the CL function, we determined their regulation in the CL at the time of natural luteolysis and establishment of pregnancy in sheep.

Results indicate that expressions of c-Fos, c-Jun, and EGR-1 proteins are increased during luteolysis; in contrast, expressions of these proteins are suppressed during of establishment of pregnancy. Interestingly, p-CREB protein is decreased during luteolysis; in contrast, its expression is increased during establishment of pregnancy. Immunohistochemistry data demonstrate that most of these proteomic changes are evident in both

LLC and SLC on day 16 of the estrous cycle or pregnancy. EGR-1 and c-Fos have been shown as important key players of luteolytic acquisition in pig and cows (Chen *et al*. 2001, Hou *et al*. 2008, Atli *et al*. 2012, Diaz *et al*. 2013). $PGF_{2\alpha}$–FP interaction increases intracellular Ca^{2+} which activates multiple cell signaling pathways and mediates the acquisition of luteolytic sensitivity to $PGF_{2\alpha}$ in the bovine luteal cells (Goravanahally *et al*. 2009, Wright *et al*. 2014). Interactions among $PGF_{2\alpha}$, Ca^{2+}, PKC, ERK1/2, c-Fos, and c-JUN have been shown in luteal cells in cows (Chen *et al*. 2001, Yadav *et al*. 2005). Our present results along with previous findings together indicate c-Fos, c-Jun, EGR-1, and CREB are important transcription factors which determine the apoptosis of luteal cells during natural luteolysis or survival of luteal cells during establishment of pregnancy. At present, down-stream signaling of these transcription factors is poorly understood.

The new findings of the present study is that c-Fos, c-Jun, EGR-1, CREB, caspase-3, and XIAP proteins are differentially expressed in luteal cells during natural luteolysis and establishment of pregnancy in sheep. These proteins work together as key players of acquisition of luteal cell apoptosis during natural luteolysis or acquisition of luteal cell survival during establishment of pregnancy in sheep. The other important finding is that β-catenin, NFκB-p65, Bcl-2, and BCl-XL proteins are highly expressed on days 12–14 of PX compared with that of CY. These results suggest that these early proteomic changes might drive the survival pathways and protect the CL from luteolysis during establishment of pregnancy. It is possible that IFNT produced by the conceptus or PGE2 produced by the conceptus and/or endometrium may activate these signaling pathways in the luteal cells as early on day 12 of pregnancy. Unraveling upstream and downstream signaling pathways associated with regulation of these proteins is expected to provide additional new molecular information on luteal function in sheep or other ruminants.

In conclusion, results of the present study together (Fig. 8) indicate that: i) during natural luteolysis, apoptosis of luteal cells may be orchestrated by suppression of multiple intracellular cell survival pathways ERK1/2, AKT, β-catenin, NFκB, and activation of intrinsic apoptosis pathways through dependent and independent mechanisms of caspase-3; ii) during establishment of pregnancy, survival of luteal cells may be precisely controlled by sustained ERK1/2, AKT, β-catenin, NFκB pathways and suppressed intrinsic apoptotic pathways; and iii) regression of the CL during natural luteolysis vs maintenance of CL during establishment of pregnancy is governed by multiple intraluteal cell signaling mechanisms in sheep. Functional studies are required to identify the factors or mediators transported from the gravid uterus to the CL to initiate these intraluteal signaling at the time of establishment of pregnancy in sheep/ruminants.

Summary

In sheep, multiple luteal cell survival pathways are suppressed and intrinsic apoptosis pathways are induced during natural luteolysis; whereas, these luteal cell survival pathways are sustained or increased and intrinsic apoptosis pathways are suppressed during the establishment of pregnancy.

Declaration of interest

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

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