Expression profile and protein levels of placental products as indirect measures of placental function in *in vitro*-derived bovine pregnancies

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Abstract

Bovine conceptus development and its association with placental proteins present in maternal, foetal and neonatal plasma and foetal (amniotic and allantoic) fluids were investigated in *in vivo*- and *in vitro*-produced (IVP) concepti and newborn calves. Females were superovulated to obtain control embryos, whereas IVP embryos were derived from established *in vitro* procedures. Pregnant animals were slaughtered on days 90 or 180 of gestation or allowed to develop to term for the assessment of physical traits. Foetal, maternal and neonatal blood and foetal fluids were collected for the determination of bovine placental lactogen (bPL) and bovine pregnancy-specific protein B (bPSPB) concentrations. Placental transcripts for bPL and bPSPB, determined by quantitative RT-PCR, were elevated in IVP placentomes. No major physical differences were observed between groups on day 90, but concentrations of bPL and bPSPB were higher in foetal plasma and allantoic fluid of IVP concepti in day 180 pregnancies, which were correlated with larger uterine and conceptus traits. Maternal concentrations of bPL in IVP pregnancies were lower than controls during the last 8 weeks of gestation, to become similar as parturition approached. Newborn IVP calves and foetal membranes were larger and displayed higher concentrations of plasma bPL than controls (10 and 60 min after birth). Our results indicated that differential patterns of secretion of bPL and bPSPB into the maternal and foetal systems occurred at distinct stages of gestation, and these were associated with altered conceptus development after *in vitro* embryo manipulations, indirectly demonstrating deviations in placental function in IVP pregnancies.

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Introduction

With the onset of the trophoblast apposition to the endometrium during the process of bovine placenta formation and development, large granulated cells, the trophoblast binucleate cells (BCs), migrate from the trophoctoderm to fuse with uterine epithelial cells (Wooding 1992), forming short-lived trinucleate cells. This phenomenon occurs throughout pregnancy in cattle, but the number and period of existence of trinucleate cells decline towards the end of gestation (Wooding 1992, Green *et al.* 2000). Binucleate cells comprise about 20% of the trophoblast cells and are responsible for hormones and bioactive products important for the regulation of foetal growth and development (Anthony *et al.* 1995, Green *et al.* 2000, Schlafer *et al.* 2000). The contents of the membrane-bounded secretory granules of BCs are released by exocytosis towards the maternal capillary bed, reaching the maternal blood system (Wooding 1992). Many proteins are produced by the BCs, including placental lactogen

(PL) and a range of glycoproteins (Wooding 1992), such as pregnancy-specific protein B (PSPB) (Butler *et al.* 1982).

PL lactogen is a member of the growth hormone (GH)/prolactin (PRL) gene family synthesized and stored in secretory granules of the trophoblast BCs (Byatt *et al.* 1992a, Wooding 1992, Gootwine 2004). Although its function and secretory control are not completely understood, *in vivo* and *in vitro* studies have suggested PL to have multiple somatogenic (GH-like) and lactogenic (PRL-like) biological effects, depending on the species, such as placental angiogenesis, maternal and foetal intermediate metabolism, mammary gland development and function, ovarian and placental steroidogenesis, growth rate and luteal function (Patel *et al.* 1996, Pedersen *et al.* 1998, Gregoraszcuk *et al.* 2000, Corbacho *et al.* 2002, Gertler & Djiane 2002, Gootwine 2004). Bovine (b) PL is more structurally similar to PRL than GH and is secreted into both the maternal and foetal systems (Gootwine 2004), with a decrease in foetal concentrations during the course of gestation and a peak in maternal plasma during the last

trimester of pregnancy (Byatt *et al.* 1992a). It has been postulated that bPL recognizes homologous and heterologous somatogenic and lactogenic receptors, acting through components of the insulin-like growth factor system, which may modulate foetal growth (Byatt *et al.* 1992a, Anthony *et al.* 1995).

PSPB was first isolated from bovine placental tissues, as a product of the BCs of the trophoblastic ectoderm (Butler *et al.* 1982). Several isoforms of pregnancy-associated glycoproteins (PAG) from foetal cotyledons showing similarities with PSPB have been isolated (Zoli *et al.* 1991). Currently, PSPB is also known as PAG-1, being inactive members of the aspartic proteinase family (Xie *et al.* 1991, Lynch *et al.* 1992). Gene and cDNA sequencing have shown identical homology between PSPB and PAG-1, with both proteins differing only in glycosylation and sialic acid contents (Xie *et al.* 1991, 1995, Lynch *et al.* 1992). Concentrations of bPSPB in maternal plasma can be detected from early stages of gestation, during early placentation, to increase gradually throughout pregnancy and reach peak values immediately before parturition (Sasser *et al.* 1986, Kiracofe *et al.* 1993). Despite the unknown function and control of PSPB secretion, many potential biological functions have been suggested based on *in vivo* and *in vitro* effects, including prostaglandin release by the endometrium, implantation, immunotolerance of the conceptus as a tissue allograft and uterine remodelling after parturition, among others (Del Vecchio *et al.* 1990, 1995, 1996, Kiracofe *et al.* 1993, Austin *et al.* 1999, Tefera *et al.* 2001). Both PAG-1 and PSPB diagnostic assays have been developed for early plasma diagnosis of pregnancy in cattle and other ruminants (Sasser *et al.* 1986, Zoli *et al.* 1991, Green *et al.* 2000).

The amounts of BC products in maternal circulation appear to be associated with placental mass, foetal number and neonatal birth weight, and have been postulated to be an index for conceptus viability and pregnancy normalcy in cattle (Byatt *et al.* 1992a, Patel *et al.* 1995, 1996, 1997, Vasquez *et al.* 1995, Szenci *et al.* 1998, 2003, Tefera *et al.* 2001, Ravelich *et al.* 2004). Associations between abnormal placental and foetal development after *in vitro* embryo manipulations have been suggested to play a key role in the occurrence of high birth weights. Disturbances in placentation may at least partially affect the pattern of foetal growth (Hill *et al.* 2000, 2001, Bertolini *et al.* 2002a, Hashizume *et al.* 2002, Reik *et al.* 2003). Disruptions in BC function may compromise the viability and growth of the developing conceptus. Since significant spatial and temporal differences in the expression in the more than 20 identified bPAG cDNA isoforms appear to occur during bovine pregnancy (Green *et al.* 2000), distinct isoforms of the PAG-1 group may be more useful for the diagnosis of pregnancy and to predict deviations from normality. This study describes results using the same animals and samples as in previous reports (Bertolini *et al.* 2002a,b, 2004) to further investigate bovine foetal and placental development and their association with placental transcripts

(bPL and bPAG-1) and plasma and foetal fluid protein concentrations (bPL and bPSPB) in *in vivo*-produced and *in vitro*-produced (IVP) concepti and newborn calves for use as potential biological markers for altered conceptus development after *in vitro* embryo manipulations.

Materials and Methods

All chemicals used in the experiments were from Sigma Chemical Co. (St Louis, MO, USA), unless stated otherwise. All animals were from a similar genetic background and origin, and animals in each experiment were maintained under the same environmental, nutritional and general management conditions during the experiments. All procedures conformed to the Guidelines for Care and Use of Laboratory Animals, National Research Council and were approved by the Institutional Animal Care and Use Committee of the University of California, Davis. In this study, the terms bPAG-1 or bPSPB refer specifically to transcripts or protein respectively.

Embryo production, embryo transfer and pregnancy diagnosis

IVP and *in vivo*-produced (controls) *Bos taurus* embryos were generated according to our established procedures, as previously described (Bertolini *et al.* 2002a,b, 2004). We have adopted an IVP system known to induce, at a relatively high frequency, the appearance of symptoms of the Large Calf Syndrome (Behboodi *et al.* 1995, Bertolini *et al.* 2002a, 2004). Bovine cumulus-oocyte complexes from Angus-Hereford cross females were obtained either from a commercial source (Bomed Inc., Madison, WI, USA) or from a regional slaughterhouse. Donor females (Angus or Angus-Hereford crosses) were superovulated for the production of control embryos. Embryos were non-surgically recovered by uterine flushing on day 7 of development (artificial insemination = day 0). The same Angus sires were used for production of both control and IVP embryos. Blastocysts and expanded blastocysts from both embryo production systems (control and IVP) were non-surgically transferred to synchronous (± 12 h) recipients on day 7 of development. Single embryos were transferred to recipients for the group of animals slaughtered on days 90 and 180, whereas one (*in vivo* singleton, *in vitro* singleton) or two (*in vitro* twin) embryos were transferred for the group of females allowed to carry pregnancies to term (Bertolini *et al.* 2002a, 2004). Pregnancy and foetal gender diagnoses were carried out by means of ultrasonography, per rectum, on days 27-30 and 58-60 of gestation respectively (Bertolini *et al.* 2002a, 2004).

Conceptus development and sample collection

Detailed morphometric, morphological and physiological data regarding foetal and placental development during pregnancy and after delivery for the group of recipients

slaughtered on days 90 and 180 or allowed to carry pregnancies to term have been described previously (Bertolini *et al.* 2002a, 2004).

Days 90 and 180 of gestation

A group of pregnant females was slaughtered on days 90 or 180 of gestation ($n = 4$ in control and $n = 5$ IVP pregnancies for each day) for the collection of pregnant uteri and their respective foetuses and placental membranes. Pregnant females were allocated to each harvest day according to foetal gender and foetal viability after ultrasound examinations. In the control group ($n = 8$), animals carrying three females and one male were assigned to each harvest day (90 and 180) and, in the IVP group ($n = 10$), female recipients sustaining pregnancies with four females and one male were harvested on day 90, and recipients carrying three females and two males on day 180 of gestation. Foetal gender diagnoses performed on day 60 of pregnancy were all confirmed after harvest. Upon slaughter, reproductive tracts were excised and immediately weighed. Maternal blood samples were obtained at exsanguination. Following uterine dissection, foetal blood was collected by cardiopuncture, and total allantoic and amniotic fluid volumes were measured and sampled. Maternal and foetal blood, and amniotic and allantoic fluids were centrifuged, and plasma and fluids were frozen and stored at -80°C pending analyses. Individual placentomes were weighed and measured (length and width) for estimation of the total gross surface area (SA). Placentomes were also classified into four categories by type according to gross morphological appearance, as follows: (A) engulfing mushroom-like, (B) sub-engulfing mushroom-like, (C) flattened, non-engulfing and (D) semi-convex placentomes (Fig. 1a). Sample placentomes from the region immediately surrounding the foetuses were excised along their longitudinal axis, and pieces containing both maternal and foetal boundaries were snap-frozen in liquid nitrogen, stored at -80°C and ultimately used for measurement of the relative transcription of genes of placental products (bPL and bPAG-1), as described below.

Pregnancy and postpartum

Birth weights and physical traits were recorded upon delivery; foetal membranes were weighed, dissected and measured, as previously reported (Bertolini *et al.* 2002a). Maternal blood samples were taken from pregnant females on day 30 of gestation and continued to be collected on a weekly basis throughout pregnancy; additional maternal samples were obtained at delivery and 24 h after delivery. Neonatal venous blood samples were collected five times during the first 24 h *ex utero*: within the first 10 min, which reflected intrauterine conditions, and at 1, 6, 12 and 24 h after birth. Blood samples were taken by jugular venipuncture in heparinized tubes. Upon

Figure 1 Morphological characteristics of *Bos taurus* placentomes during gestation. (a) Classification of placentomes by type according to anatomical shapes on day 180 of gestation: (A) engulfing mushroom-like, (B) sub-engulfing mushroom-like, (C) flattened, non-engulfing and (D) semi-convex placentomes (adapted from Vatnick *et al.* (1991), Penninga & Longo (1998) for sheep and from Laven & Peters (2001) and the cotyledon-caruncle anchoring mechanism hypothesis of Eiler & Hopkins (1993) both for cattle); white-dotted line in the sketch of each placentome type represents the external boundary between foetal and maternal tissues. (b) Sonograms of placentomes (within ellipses) immediately surrounding the foetuses (Foet) of *in vivo*- (top row) and *in vitro*-derived (bottom row) pregnancies on day 93 of gestation (scale on left of the screen in each sonogram is in cm). (c) Frequency of distribution (%) of placentomes classified by type between *in vivo*- and *in vitro*-derived *Bos taurus* pregnancies on day 180. * $P < 0.05$, for each placentome type.

collection, blood samples were immediately centrifuged and plasma was frozen at -20°C pending analyses.

bPL and pPSPB measurements in plasma and foetal fluids

Concentrations of bPL and bPSPB were determined by radioimmunoassay, in duplicates of maternal, foetal and

neonatal plasma, and amniotic and allantoic fluids, according to established procedures by Wallace (1993) for bPL and Sasser *et al.* (1986) for bPSPB (BioTracking, Moscow, ID, USA). In this study, intra- and interassay coefficients of variation were 4.9% and 12.0% for bPL and 10.2% and 15.7% for bPSPB respectively.

Relative abundance of bPL and bPAG-1 transcripts in day-90 and -180 placentomes

A real-time TaqMan PCR (Applied Biosystems, Foster City, CA, USA) was optimized to quantify transcripts for bPL and bPAG-1 genes, following procedures described by Bertolini *et al.* (2002b, 2004). The analysis was performed at the Lucy Whittier Molecular Core Facility, School of Veterinary Medicine, University of California, Davis. Quantitative analyses of bovine cDNA from control and IVP day-90 and day-180 placentomes were performed in comparison with the endogenous control (glyceraldehyde-3-phosphate dehydrogenase; GAPDH), and were amplified in an automated laser-based fluorometer (7700 ABI PRISM Sequence Detection System; Applied Biosystems). Final quantification was done using the comparative C_T method (Leutenegger *et al.* 2000). All samples were included in each assay, in single reactions, with one assay per transcript. Sequences of primers and probes for the transcripts are shown in Table 1.

Statistical analyses

Data analyses were done in Proc GLM of SAS (SAS Institute, Cary, NC, USA). Data obtained after slaughter (days 90 and 180) were compared by ANOVA, considering group (*in vivo*, *in vitro*), day (90, 180) and gender (male, female) as main effects, with foetal weight also used as the covariate by ANCOVA. Data obtained in groups of animals that carried pregnancies to term were analysed by the repeated measures procedures of SAS (group and time as main effects with animal nested within group). Pairwise comparisons were performed by least square means (LSM). Data regarding the distribution of placentome size and type were analysed by the Kruskal–Wallis test (Minitab Inc., State College, PA, USA). A Pearson's correlation test was used for analysis of relationships between traits,

also using corrected values for the covariate, as well as the other fixed effects of group, time and animal.

Results

For the group of animals slaughtered on days 90 or 180 of gestation, pregnancy rates on day 27 and pregnancy losses from day 27 of gestation (pregnancy diagnosis) to day 60 (foetal gender diagnosis) for the control and IVP groups were 33% (10/30) and 32% (27/84), and 10% (1/10) and 63% (17/27) respectively. One additional control pregnancy was lost on day 160 of gestation. For the group of animals allowed to carry pregnancies to term, pregnancy rates on day 30 were 43% (10/23), 32% (9/28) and 50% (5/10), and pregnancy losses throughout gestation were 40% (4/10), 33% (3/9) and 40% (2/5), for the *in vivo* singleton (control), *in vitro* singleton and *in vitro* twin groups respectively. No differences were observed between groups in both pregnancy rates (20/53 and 41/122 for the control and IVP groups respectively) and occurrence of losses after day 45 of gestation (2/20 and 4/41 for the control and IVP groups respectively), considering both experiments combined. However, losses were 2.2-fold higher ($P < 0.05$) in the IVP group (18/41) than in controls (4/20) between days 30 and 44 of gestation.

Data regarding the comparison of physical traits between *in vivo*- and *in vitro*-derived concepti, newborn calves and term foetal membranes have been described previously (Bertolini *et al.* 2002a, 2004) and are summarized in Table 2. Placentomes were longer, wider and thinner (Fig. 1b) in the IVP group (Bertolini *et al.* 2004), and distribution of placentome types was also different between groups ($P < 0.05$) on day 180 of pregnancy, with the occurrence of more uncommon types (C and D) in IVP pregnancies (Fig. 1c). The presence of such types, primarily type C, in controls was usually associated with small placentomes located in the non-foetal horn, whereas these placentome types were randomly present in both uterine horns of IVP pregnancies in a wide size range. The difference in individual placentome size in the IVP group on day 90 of gestation, particularly in the foetal horn, was correlated with significantly higher ($P < 0.05$) concentrations of bPL and bPSPB in foetal plasma and allantoic

Table 1 Sequence of PCR primers, TaqMan probes and GenBank accession code numbers for transcripts analysed in control and IVP day-90 and day-180 *Bos taurus* placentomes.

Transcripts	Primers sequences (5'–3') ^{a,b}	Length	Probe sequences (5'–3') ^b	Accession no.
bGAPDH	F-GGGCGTGAACCAACGAGAAGTATAA R-CCCTCCACGATGCCAAAGT	120	ATACCCTCAAGATTGT CAGCAATGCCTCCT	AF022183
bPL	F-GTGGAAATGATACAAAAAAGGTTTCAT R-CATCCTCATCGTCTGCTGTCA	100	AGAACGAGCCCTATCCAGTGTGGTCAGA	M33268
bPAG-1	F-GAGTGCATAGTCAAAATACCTCTAAGGA R-TGGGACAGACTGTAAGCATGCT	110	TGAAGACCATGAGAAATGTCGTCAGTGGA	L27833

^aPrimer orientations: F = forward; R = reverse.

^bExon–exon junctions are italicized.

Table 2 Physical measurements of *Bos taurus* conceptus traits on days 90 and 180 of gestation, and on term foetal membranes and neonates between *in vivo*- and *in vitro* bovine embryo production systems (LSM $\pm$ S.E.M).

Traits	'Day 90' ¹		'Day 180' ¹		Term ²		
	<i>In vivo</i>	<i>In vitro</i>	<i>In vivo</i>	<i>In vitro</i>	<i>In vivo</i> /singlets	<i>In vitro</i> /singlets	<i>In vitro</i> /twins
Body weight (kg)	0.146 $\pm$ 0.013 ^a	0.173 $\pm$ 0.008 ^a	6.462 $\pm$ 0.180 ^b	7.163 $\pm$ 0.399 ^c	32.5 $\pm$ 5.1 ^d	43.2 $\pm$ 7.4 ^c	31.5 $\pm$ 5.4 ^d
Femoral length (mm)	23.3 $\pm$ 1.4 ^a	24.6 $\pm$ 0.3 ^a	100.3 $\pm$ 1.5 ^b	108.0 $\pm$ 1.2 ^c	209.0 $\pm$ 8.0 ^d	233.0 $\pm$ 12.0 ^c	208.0 $\pm$ 12.0 ^d
Weight (kg) ³	0.124 $\pm$ 0.037 ^a	0.085 $\pm$ 0.020 ^a	2.23 $\pm$ 0.03 ^b	2.90 $\pm$ 0.26 ^c	4.30 $\pm$ 1.10 ^d	5.90 $\pm$ 1.90 ^d	8.90 $\pm$ 0.60 ^c
Gross S A (dm) ^{2,4}	2.01 $\pm$ 0.47 ^a	2.92 $\pm$ 0.29 ^a	24.60 $\pm$ 1.29 ^b	32.36 $\pm$ 5.40 ^b	41.0 $\pm$ 10.0 ^c	49.0 $\pm$ 9.0 ^c	51.0 $\pm$ 16.0 ^c

Data in the same row without common superscript letters differ significantly. $P < 0.05$.

^aData adapted from Bertolini *et al.* (2004).

^bData adapted from Bertolini *et al.* (2002a).

^cPlacentome (days 90 and 180) or foetal membrane (term) weight.

^dSA = surface area, referring to placentome (days 90 and 180) or cotyledonary (term) surface area. $P = 0.0636$ for day-180 female concepti.

fluid, and relative abundance of placentome transcripts for bPL and bPAG-1 in IVP concepti (Fig. 2a and b). On day 180, the relative abundance of placental transcripts for bPL and bPAG-1 (Fig. 2a and b) and uterine, foetal and placental physical component weights (Bertolini *et al.* 2004, Table 2) were larger in IVP pregnancies than in controls. Concentrations of bPSPB in the allantoic fluid of IVP pregnancies were 2.9-fold lower than controls (Fig. 2b) but, since day-180 IVP pregnancies had a 2- to 3-fold larger allantoic fluid volume than controls (Bertolini *et al.* 2004), total bPL and bPSPB in the allantois were higher and similar to controls respectively (Fig. 2c).

In general, IVP pregnancies had a higher placental transcriptional activity (bPL and bPAG-1) and foetal plasma concentrations of bPL and bPSPB than controls ($P_{\text{group}} < 0.05$), irrespective of the gestational day (90 or 180), as shown in Fig. 2. Temporally, concentrations of bPL were higher in the uterine compartments (foetal plasma and fluids) than in maternal plasma, apart from the experimental group ($P_{\text{day}} < 0.05$). In addition, maternal concentrations of bPL and bPSPB were higher on day 180 than on day 90 ($P_{\text{day}} < 0.05$), but lower in foetal plasma (bPL and bPSPB) and amniotic fluid (bPL). Positive correlations were observed between bPL and bPAG-1 transcripts ($r = 0.824$, $P < 0.05$), between bPL and bPSPB concentrations in the same fluid type (maternal plasma, $r = 0.715$, foetal plasma, $r = 0.635$, amniotic fluid, $r = 0.505$, $P < 0.05$, except in allantoic fluid), and between maternal concentrations of bPL and bPSPB ($r > 0.850$, $P < 0.05$), and with glucose and fructose accumulations ($r > 0.650$, $P < 0.05$) in the uterine compartments (data not shown, Bertolini *et al.* 2004). Positive correlations were observed also between conceptus component weights, substrate (glucose and fructose, data not shown) accumulations and bPAG-1 transcripts ($r > 0.600$, $P < 0.05$).

Plasma concentrations of bPL throughout gestation in pregnant females carrying *in vivo* singleton, *in vitro* singleton and *in vitro* twin concepti are illustrated in Fig. 3. Three distinct phases can be visualized in the weeks preceding parturition, based on the variation in bPL concentrations among groups (Fig. 3). In phase I (weeks 39 and

24 prepartum), no differences in concentrations of maternal plasma bPL were observed between groups. Then, in phase II (week 23 to week 8-9 prepartum), concentrations of maternal bPL were significantly higher in the *in vitro* twin group ($P < 0.05$). In phase III (week 8-9 to week 1 prepartum), maternal concentrations in the *in vitro* singleton group were lower ($P < 0.05$), but similar to controls and the *in vitro* twin group as parturition approached. At delivery, concentrations of maternal plasma bPL were significantly different between groups (*in vivo* singletons $<$ *in vitro* singletons $<$ *in vitro* twins, $P < 0.05$), to become similar 24 h after parturition.

Concentration profiles for bPSPB between groups followed the same pattern as that seen for bPL, as depicted in Fig. 4, for a steady increase in plasma concentrations from early to late pregnancy, with a decline following parturition. The *in vitro* singleton group had lower bPSPB concentrations than controls and the *in vitro* twin group in the week and the day immediately preceding and following parturition respectively ($P < 0.05$), but no other significant differences were observed between groups at any further stage of gestation examined.

Newborn plasma concentrations of bPL ($P < 0.05$) in IVP calves, either singletons or twins, were 2- to 4-fold higher immediately after birth (10 and 60 min) than controls, to become similar afterwards (Fig. 5a). Plasma concentrations of bPSPB tended to be higher ($P < 0.08$) and became significantly higher ($P < 0.05$) in the *in vitro* singleton group 60 min and 24 h after birth respectively (Fig. 5b). Concentrations of bPL and bPSPB in the first 60 min postpartum were positively correlated with neonatal and foetal membrane physical traits ($r > 0.800$, $P < 0.05$), and correlated with neonatal plasma fructose concentrations (data not shown, Bertolini *et al.* 2004).

Discussion

Changes in placental development and function in IVP pregnancies derived either from *in vitro* fertilization (IVF) or somatic cell nuclear transfer procedures have been associated with the delivery of larger calves and the appearance of aberrant foetal membranes (Hill *et al.* 2000,

Figure 2 Placental relative transcription and plasma and fluid concentrations (ng/ml) for *in vivo*- and *in vitro*-produced bovine pregnancies on days 90 and 180 of gestation (LSM $\pm$ S.E.M.). (a) bPL, (b) bPAG-1 and bPSPB and (c) total bPL and bPSPB in the allantoic fluid. Columns without common superscripts differ ($P < 0.05$).

Figure 3 Maternal plasma concentrations (ng/ml) of bPL for *in vivo*- and *in vitro*-produced bovine pregnancies throughout gestation (LSM $\pm$ S.E.M.); the x axis is downwards, in weeks prepartum.

Figure 4 Maternal plasma concentrations (ng/ml) of bPSPB for *in vivo*- and *in vitro*-produced bovine pregnancies throughout gestation (LSM $\pm$ S.E.M.). Note the distinct scales in the y axis; the x axis is downwards, in weeks prepartum.

2001, Bertolini *et al.* 2002a, 2004, Hashizume *et al.* 2002, Reik *et al.* 2003), but cause-and-effect relationships between morphological and functional changes have been difficult to attain. We have already demonstrated that IVP pregnancies contain larger concepti toward the end of the second trimester of pregnancy. This was associated with disturbances in placental development and function, including substrate transfer and accumulation in the uterine compartments (Bertolini *et al.* 2004), along with differences in the synthetic protein capacity by BCs, as seen in this study, which culminate in the delivery of larger calves (Bertolini *et al.* 2002a). Placental BC products, such as bPL, bPSPB and bPAG-1, have been suggested as useful markers for embryonic or placental health, pregnancy normalcy and embryonic mortality, and for the prediction of abnormal conceptus development and postpartum pathological events (Patel *et al.* 1997, Szenci *et al.* 1998, 2003, Tefera *et al.* 2001). Such BC products or the relative abundances of their placental transcripts have already been used in an attempt to detect or predict conceptus abnormalities after *in vitro* embryo manipulations (Vasquez *et al.*

1995, Schmidt *et al.* 1996, Hill *et al.* 2000, Hashizume *et al.* 2002, Heyman *et al.* 2002, Ishiwata *et al.* 2003, Hofert *et al.* 2004, Patel *et al.* 2004a, Ravelich *et al.* 2004), and results from these studies have increasingly contributed to the understanding of those abnormalities.

In this study, placental and foetal membrane weight and placental and cotyledonary SAs were greater in IVP pregnancies, and both mass and SAs increased as a function of the increase in placentome length and width, but placentomes were thinner at both days 90 and 180 of gestation. Changes in placental morphology and deviations in the morphological distribution of the placentome type (fewer types A and B and increased unusual C and D types) were readily detectable in the first and second trimester of gestation in IVP pregnancies (Fig. 1). Such variations in placentome types may reflect anatomical, histological and functional differences in development, and such changes may ultimately influence placental transport efficiency, function, metabolism and synthetic capacity. Under physiological conditions, the placenta grows faster than the foetus in early pregnancy but slower after mid-gestation, when foetal weight surpasses that of the placenta (Eley *et al.* 1978, Prior & Laster 1979, Reynolds *et al.* 1990). Morphologically, the bovine placenta is considered fully developed at mid- to late gestation, but foetal villous trees continue to develop (branching pattern) and rearrange (spatial relationship between endothelium and trophoblast) in placentomes until near term, increasing the villous SA, which will also accommodate foetal demands for growth towards the end of gestation (Leiser *et al.* 1997). However, the placental tissue holds a great plastic capacity in facing unusual, adverse circumstances. Maternal dietary restriction or hypoxia in early gestation in cattle and sheep can initially impair such a standard pattern of placental growth (Penninga & Longo 1998, Perry *et al.* 1999, Wallace *et al.* 1999, Symonds *et al.* 2001) but, once dietary intake is restored to normal, a placental and foetal growth compensatory enhancement is observed. A coincidental aspect of these findings with our studies is that IVP concepti were significantly smaller during the period of placentation to the early foetal stage (up to day 58 of gestation), which appeared to have preceded a subsequent compensatory growth initiated with the placental tissue and followed by the foetus (Bertolini *et al.* 2002a). A hormonal effect of PSPB has been demonstrated in inducing the *in vitro* release of an α chemokine (granulocyte chemotactic protein-2) by the endometrium, which may be relevant for implantation and placental angiogenesis (Austin *et al.* 1999). Bovine placental PAG-1 and foetal plasma PSPB were elevated in the IVP group at a time at which placental compensatory growth was already detectable (day 90).

The amounts of placental products in maternal circulation appear to be associated with placental and foetal masses, including foetal number and neonatal birth weight, and with pregnancy normalcy (Byatt *et al.* 1992a, Patel *et al.* 1995, 1997, Vasquez *et al.* 1995, Tefera *et al.*

Figure 5 Neonatal plasma concentrations (ng/ml) for *in vivo* singleton, *in vitro* singleton and *in vitro* twin calves in the first 24 h after birth (LSM ± S.E.M.). (a) bPL and (b) bPSPB. * $P < 0.05$, + $P < 0.1$.

2001, Gootwine 2004). In this study, concentrations of maternal and foetal plasma bPL followed similar trends reported by Hossner *et al.* (1997), Holland *et al.* (1997) and Patel *et al.* (1996), but concentrations of bPL and bPSPB in foetal plasma were correlated with conceptus physical traits and with one another in plasma and fluids. Anthony *et al.* (1995) suggested that a relationship exists between ovine PL gene expression and the rate of ovine PL appearance in foetal circulation, an observation that appears to be valid for our findings with both bPL and bPSPB. Increased bPL and bPSPB relative mRNA abundances in day-90 and day-180 placentomes and concentrations in the foetal plasma and allantoic fluid on day 90, along with accumulations of glucose and fructose in foetal plasma and associated fluids (Bertolini *et al.* 2004), were associated with an increase in prenatal growth and foetal number in IVP concepti. As placentomes from IVP pregnancies were distinct from controls in terms of morphology, mass and surface area, the pattern of secretion of BC products may have been affected and explain some of the distinct patterns seen in IVP pregnancies.

The elevation in bPL in the third trimester of pregnancy coincides with the period of faster foetal growth and mammogenesis (Patel *et al.* 1996). In fact, newborn IVP calves coincidentally displayed higher birth weights and plasma bPL and fructose concentrations immediately after birth (Bertolini *et al.* 2002a, 2004). Conversely, maternal concentrations of bPL during phase III of pregnancy (Fig. 3) in the *in vitro* singleton group were lower than *in vitro* twin and *in vivo* singleton groups and, at parturition, maternal bPL concentrations in *in vivo*-produced pregnancies fell

earlier than in those that were *in vitro* derived (Fig. 3). Interestingly, Miles *et al.* (2004) reported a reduction in foetal villi and BC volume densities in day-222 IVF-derived placentomes, which coincides with the period of decrease in maternal bPL levels in females carrying IVP singleton foetuses in this study.

Evidence exists that protein expression in BCs is affected by anatomical location (Wooding *et al.* 1996, Patel *et al.* 2004b), and such differences in synthetic capacity appear to be more under maternal than foetal influence, since both cotyledonary and intercotyledonary tissues are exposed to a similar foetal environment (Wooding *et al.* 1996). The presence of higher concentrations of bPL in IVP calves in the first hour after birth, along with higher concentrations in the allantoic fluid, suggests that a 'leakage' occurs from placental tissue to the foetal circulation, since no or negligible transfer of PL appears to occur from the maternal to the foetal circulation in sheep (Reddy & Watkins 1983). Such findings corroborate the concept that the pattern of secretion of bPL in the foetal circulation may be relevant for foetal growth (Byatt *et al.* 1992a) and disturbances in placental formation may lead to such differential pattern of bPL secretion between the maternal and uterine compartments (Ravelich *et al.* 2004).

In addition to placental mass, parity and nutrition also appear to modulate the secretion of bPL. However, Patel *et al.* (1996) did not find distinctions in maternal bPL concentrations between singleton- and twin-bearing pregnancies, which the authors explained as a reduced placental mass per foetus in twin-bearing pregnancies. For singleton-bearing pregnancies, a relationship exists

between placental and neonatal birth weights and maternal bPL concentrations, which could be used as a marker for conceptus development, viability and predicting abnormal birth weights (Patel *et al.* 1996). The half-life for the recombinant bPL has been reported to be 7.5 min (Byatt *et al.* 1992b), which is shorter than for GH or PRL but, as native bPL is heavily glycosylated (Byatt *et al.* 1992a), a half-life similar to GH and PRL (20 to 30 min) is expected, which is visible in plasma of newborn calves in the first 60 min following birth (Fig. 5a). Conversely, as bPSPB has a rather long half-life, with its plasma levels still detectable 3 months or more after parturition (Sasser *et al.* 1986, Kira-cofe *et al.* 1993), neonatal plasma concentrations remained elevated throughout the period under scrutiny (24 h) following parturition (Fig. 5b). However, differences in plasma clearance perhaps highlight bPL as a more dependable marker for pregnancy normalcy than bPSPB.

Many of the changes in placental development and function in the IVP group in this study were associated with the accelerated pattern of foetal growth, the delivery of larger calves, and the appearance of aberrant foetal membranes at term, and an increase in substrate uptake and protein synthetic capacity in IVP pregnancies (Bertolini *et al.* 2002a, 2004). Concentrations of bPL and bPSPB in foetal plasma were correlated with physical traits and with one another in foetal plasma and associated fluids irrespective of the experimental groups. Differential patterns of secretion of bPL and bPSPB into the maternal and foetal systems occurred at distinct stages of gestation, which were associated with altered conceptus development after *in vitro* embryo manipulations, indirectly demonstrating differences in placental function in IVP pregnancies. However, bPSPB/bPL prenatal measurements in foetal plasma and fluids were useful indicators of abnormal growth and development, but such invasive measurements lack a practical application.

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