GENE EXPRESSION IN THE PREATTACHMENT BOVINE EMBRYO- REGULATION BY RETINOIC ACID AND IN VITRO CULTURE SYSTEM

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Graduate College of the
Oklahoma State University
In partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
August, 2003

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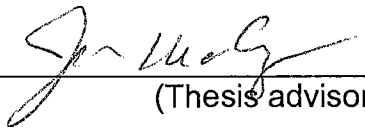

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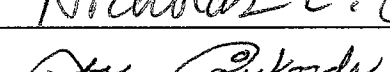

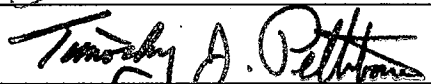

Dean of the Graduate College

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my mentor, Dr. Jerry Malayer, for his constructive guidance, supervision, encouragement and inspiration during my entire graduate program. I greatly appreciate his willingness to let me pursue whatever research objectives I wanted and as a result I feel very confident working as an independent investigator in the near future. I am also thankful to him for nominating me for the 2003 graduate student phoenix award. I would also like to extend my appreciation to my graduate committee members, Dr. Rodney Geisert, Dr. Nicholas Cross, Dr. Lee Rickords, and Dr. Reed Holyoak for their support, insights and willingness to serve. I would also like to thank the Department of Physiological Sciences for providing me the teaching associateship and Dr. Charlotte Ownby and Dr. Nicholas Cross for supporting my appointment as a teaching associate.

I would like to express my deep gratitude to Vivenne Woods for her generous assistance in the lab, her sincere friendship, understanding and encouragement during the early days of my PhD program; to David Goad for his excellent invaluable technical assistance, guidance and encouragement; to Jason Ross, Amy Hurst, Misti Spatz, Leanne Wier, Akhilesh Ramachandran, Nagaraja Thirumalapura for their constant support and feedback. I am very grateful to Betty Handlin, Amy Hurst, Leanne Wier for helping me make posters

for presentation of select chapters of this dissertation at scientific meetings. I also want to express my sincere thanks to Joe Donnenhoefer (Roche Diagnostics Corporation),Khurshid Gandhi, Randa (Clontech BD Biosciences), and Abigail Farfan (Promega Corporation) for their excellent technical support. Special thanks also go to Quality Meats Inc., Wellington, Kansas and Mikkelson's Beef Inc., Oklahoma City, Oklahoma for their generous donation of biological material without which this project would have been difficult to pursue.

Finally, I would like to dedicate this thesis work to my Dad, Mom and Sister for their constant love, understanding and support all through my life.

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Abbreviations

ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
All-*trans* RA	All-trans retinoic acid
AP-1	Activator protein-1
ATPase	Adeonsine triphosphatase
BO	Brackett and Oliphant
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CDS primer	cDNA synthesis primer
CK2	Casein kinase 2
COC	Cumulus oocyte complex
CRABP I & II	Cellular retinoic acid binding proteins I and II
CRABPs	Cellular retinoic acid binding proteins
CRBP I & II	Cellular retinol binding proteins I and II
CRBPs	Cellular retinol binding proteins
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DD-RT-PCR	Differential display Reverse transcription polymerase chain reaction
dGTP	Deoxyguannosine triphosphate
DIG	Digoxigenin
DPBS	Dulbeco's phosphate buffered saline
dTTP	Deoxythymidine triphosphate
ECS	Estrus cow serum
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
EST	Expressed sequence tag
FCS	Fetal calf serum
FBS	Fetal bovine serum
FSH	Follcile stimulating factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HBNF	Heparin binding neurite factor
HPLC	High performance liquid chromatography
ICM	Inner cell mass
IGF I & II	Insulin-like growth factor I and II
IVC	In vitro culture
IVF	In vitro fertilization

IVM	In vitro maturation
IVP	In vitro embryo production
kFGF	Keratinocyte fibroblast growth factor
LH	Lutenizing hormone
LIF	Leukemia inhibitory factor
LSD	Least significance difference
MK	Midkine
mRNA	messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween-20
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDGF-α	Platelet derived growth factor alpha
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGK	Phosphoglycerate kinase
PPARα	Peroxisome proliferators activated receptor alpha
PPARβ	Peroxisome proliferators activated receptor beta
PPARγ	Peroxisome proliferators activated receptor gamma
PPARs	Peroxisome proliferators activated receptors
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenase
RARs	Retinoic acid receptors
RARα	Retinoic acid receptor alpha
RARβ	Retinoic acid receptor beta
RARγ	Retinoic acid receptor gamma
RARE	Retinoic acid response element
RBP	Retinol binding protein
RBPr	Retinol binding protein receptor
RI-HB	Retinoic acid inducible heparin binding growth factor
RNAse	Ribonuclease
RT-PCR	Reverse transcription polymerase chain reaction
RXRα	Retinoic X receptor alpha
RXRβ	Retinoic X receptor beta
RXRγ	Retinoic X receptor gamma
RXRs	Retinoic X receptors
SDS	Sodium dodecyl sulphate
SMART	Switching mechanism at the 5' end of RNA transcripts
SSC	Saline sodium citrate

SSCT	Saline sodium citrate with Tween 20
SSH	Suppression subtractive hybridization
TAE	Tris acetate EDTA
TBP1	TAT binding protein 1
TCM-199	Tissue culture medium 199
TE buffer	Tris EDTA buffer
TE	Trophectoderm
TGF-β	Transfromaing growth factor beta
TNE	Tris sodium chloride EDTA
ZO-1	Zonula occludens
9-cis RA	9-*cis* retinoic acid

FOREWORD

This thesis comprises the following three peer-reviewed scientific publications (Chapters III, IV & VI) and are presented in the published journal format .

1. Mohan M, Malayer JR, Geisert RD, Morgan GL. Expression of retinol-binding protein messenger RNA and retinoic acid receptors in preattachment bovine embryos. Mol Reprod Dev 2001; 60:289-296.
2. Mohan M, Malayer JR, Geisert RD, Morgan GL. Expression patterns of retinoid X receptors, retinaldehyde dehydrogenase, and peroxisome proliferator activated receptor gamma in bovine preattachment embryos. Biol Reprod 2002; 66:692-700.
3. Mohan M, Ryder S, Claypool PL, Geisert RD, Malayer JR. Analysis of gene expression in the bovine blastocyst produced in vitro using suppression-subtractive hybridization. Biol Reprod 2002; 67:447-453.

Chapters V and VII will be submitted for publication soon and have been written in the format prescribed for *Molecular Reproduction and Development.*

Chapter I

Introduction

Vitamin A (retinol) and its metabolic derivatives, collectively called retinoids, are essential for normal embryo development and epithelial differentiation. From studies conducted as early as in the 1940s, it was known that both deficiency and excess of vitamin A could seriously impair embryogenesis. At this stage, however, the mode of action of retinoic acid at the molecular level was not known. Approximately fifteen years ago two independent laboratories identified the first nuclear receptor, retinoic acid receptor alpha (RARα), that was responsible for transducing the downstream effects of retinoic acid. Since then a second set of receptors, retinoid X receptor (RXRs), along with their isoforms, subisoforms, enzymes involved in the synthesis and metabolism of retinoic acid (RA), retinoid binding proteins and several other RA-related metabolites have been identified.

Retinol is oxidized to retinaldehyde by a group of enzymes called alcohol dehydrogenases (ADH). Retinoic acid, the active metabolite, is then generated through oxidation by a second group of enzymes called aldehyde dehydrogenases (ALDH) or retinaldehyde dehydrogenases (RALDH-2). It is now clear that the RA generated *in situ* exerts its molecular action by binding to two sets of receptors, namely, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). The RA-RAR complex can then heterodimerize with RXRs and bind to

specific regulatory elements (retinoic acid response elements) found in the promoter regions of target genes and can either up or down regulate the expression of that gene. In addition to binding RARs, RXRs can also heterodimerize with thyroid hormome receptor, vitamin D receptor, peroxisome proliferator activated receptor (PPAR). Several approaches have been undertaken to study the biological significance of the vitamin A signaling pathway and the majority of these studies have been performed in mice.

Deficiency of vitamin A in pregnant rats leads to decreased ovarian steroid production and fetal resorption (Ganguly et al., 1971). In cycling animals, deficiency may lead to decreased ovarian size, decreased ovarian steroid production, and reproductive senescence (Juneja et al., 1966; Jayaram et al., 1973). Increased germ cell number and germ cell meiosis resulted following treatment of in vitro cultured fetal mouse ovaries with RA (Livera et al., 2000). Treatment of pregnant rats with RA led to an elevation in the total number of primordial oocytes in the ovary of female offspring (Morita and Tilly, 1999). Retinol administration to swine before breeding advanced meiotic resumption, altered follicular hormone concentrations, and increased litter size. In the ewe, administration of retinol in combination with superovulation was associated with increased developmental potential of embryos in vitro (Whaley et al., 2000). Similarly, in cattle and sheep, retinol administered simultaneously during superovulation improved the quality and development of the resulting embryos (Shaw et al., 1995; Eberhardt et al., 1999). These studies indicated that retinoids could regulate oogenesis and survival of the oocyte in the ovary of mouse

fetuses and improve embryo survival in farm species, possibly, by positively influencing oocyte maturation in vivo. The evidence provided above is based on work in which vitamin A was administered in the maternal diet or by injection. In addition, at the beginning of this project no evidence was available to show that vitamin A or its derivatives has a direct effect on the preattachment embryo or whether vitamin A added to embryos in culture affects development. Therefore, in order to elucidate the mode of action of retinol and its metabolites it was necessary to investigate if the different components of the retinoid signaling pathway existed in the early embryo. Therefore, I have attempted to test the hypothesis that retinol and its metabolites exert a direct effect upon the preattachment bovine embryo in vitro. The following objectives were pursued in order to test the above hypothesis.

1. Determine the capability for retinoic acid synthesis by characterizing the expression of transcripts for the enzymes ADH, ALDH and RALDH-2 in the preattachment bovine embryo between the 2-cell and hatched blastocyst stages to metabolize retinol to retinoic acid in vitro.
2. Further characterize patterns of expression of transcripts for RARs, RXRs, RBPs, PPAR and RALDH-2 in the preattachment embryo.

Recently, following upon our findings on the expression of the different components of the retinoic acid signaling pathway in the preattachment bovine embryo, Duque et al. (2002) reported that addition of of 5 nM 9-*cis* retinoic acid during prematuration of bovine oocytes in the presence of roscovitine improved

cytoplasmic granular migration, embryonic development, cryopreservation tolerance, total cell numbers and, as a consequence, embryonic quality. More recently, the same laboratory also showed that addition of 9-*cis* retinoic acid during prematuration improved early embryonic development and pregnancies in cattle (Hidalgo et al., 2003). Therefore, my second hypothesis is that addition of 5 nm 9-*cis* retinoic acid during prematuration enhanced early embryonic development and pregnancy rates by interacting directly with the cumulus-oocyte complex (COC's). Since cumulus-granulosa cells play an important role during oocyte maturation I am hypothesizing that retinoic acid had a positive effect on events associated with oocyte maturation in vitro. In order to test this hypothesis the following objectives were pursued.

1. Determine the capability of COC's to metabolize retinol to retinoic acid in vitro by characterizing the expression of transcripts for the enzymes ADH, ALDH and RALDH-2.
2. Determine the expression of transcripts for RARs, RXRs, RBPs, PPAR and RALDH-2 in COC's.
3. Determine the capacity for a receptor-mediated response to retinoic acid in COC's using transfected reporter gene constructs in-vitro.

Studying differential gene expression with the available molecular techniques requires a certain minimum concentration of total RNA (~2-4 μg). However, it is extremely difficult to obtain such concentrations of total RNA from preattachment embryos. Therefore, these constraints present a great challenge to the

successful implementation of a study of this nature. Therefore, our third hypothesis is that it is possible to perform a global transcriptome analysis using small amounts of total RNA such as obtainable from preattachment embryos: The following objective was pursued:

To identify differentially expressed genes in in vitro generated preattachment intact and hatched blastocysts using SMART PCR, SSH and Real-time PCR.

In vitro produced bovine embryos offers an attractive alternative to generate blastocysts and stage specific preattachment embryos useful for commercial purposes and basic research. Although oocytes aspirated from follicles can undergo spontaneous maturation, their development to the preattachment stages following in vitro fertilization is less successful than oocytes matured in vivo. Several differences have been documented between in vitro and in vivo derived bovine embryos. In vitro produced bovine embryos exhibit aberrations in the expression of developmentally important genes during the first 7-8 days of in vitro culture. Therefore, we hypothesize that, in addition to the existing information, differences in relative abundance exist for several hitherto unidentified developmentally important genes.

My final objective is to identify changes in gene expression between in vitro produced and in vivo derived day 7 blastocysts using SMART PCR, SSH and Real-time PCR.

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Chapter II

Literature Review

Introduction

Vitamin A and its physiological metabolites, collectively known as retinoids, have profound effects on embryonic morphogenesis, cell growth and differentiation, vision and reproduction (Hofmann and Eichele, 1994; Eskild and Hansson, 1994; Deluca, 1991). It has been known for many years that an adequate level of vitamin A in the maternal diet is crucial for the normal development of the embryo (Kalter and Warkany, 1961). Both deficiency and excess of vitamin A during pregnancy results in fetal death or congenital anomalies. The biologically active form of vitamin A, retinoic acid, is now recognized as an important signaling molecule. Retinoic acid can regulate cell division and differentiation in tissues of ectodermal, endodermal and mesodermal origin (Brockes 1989, Roberts and Sporn, 1984, Wolf, 1984) by causing changes in the expression of homeobox genes, growth factors and their receptors. These effects of retinoids have been shown through exogenous administration of retinoic acid. During the last two decades it has become clear that vitamin A exerts the majority of its effects via a complex signal transduction pathway. This pathway involves specific retinol binding proteins, retinoid receptors located in target cell nuclei, and biologically active metabolites of vitamin A, which function as ligands for these receptor proteins.

Metabolism of Retinoids

Retinol Dehydrogenases

Retinol (vitamin A), through a series of oxidation reactions, can give rise to several physiologically active compounds (Napoli et al., 1993) inside the cell. A specific protein called retinol-binding protein (RBP) is involved in the systemic and intercellular transport of retinol. Intracellular transport of retinol is accomplished by cellular retinol-binding protein (CRBP). CRBP accumulates retinol, stimulates the mobilization of retinol from retinol esters, and transfers retinol to alcohol dehydrogenase (ADH) which catalyzes the first step involving the reversible oxidation of retinol to retinal (Chen et al., 1995). Two types of alcohol dehydrogenase have been identified that perform this function, namely, medium chain cytoplasmic alcohol dehydrogenases and the short-chain membrane bound alcohol dehdyrogenases. Very recently, another enzyme called 9-*cis* retinol dehydrogenase that can oxidize 9-*cis* retinol to 9-*cis* retinoic acid was identified in mouse embryonic tissues (Romert et al., 1998).

Medium-Chain alcohol dehydrogenase family

This family of alcohol dehydrogenases is known to comprise about four classes of zinc-dependant cytosolic enzymes, namely, classes I, II, III and IV (ADH1, -2, -3, and -4). Two of these, ADH1 and ADH4 dehydrogenases, can oxidize retinol to retinaldehyde. Among the two, only ADH1 is inhibited by

ethanol. The deer mouse which is negative for alcohol dehydrogenase does not have any ethanol dehydrogenase activity and still can oxidize retinol to retinaldehyde. This would indicate that the ethanol sensitive form of alcohol dehydrogenase is not an absolute requirement during embryogenesis. ADH1 has been localized in few places such as the developing kidneys (Rossant et al., 1991; Vonesch et al., 1994) and is entirely absent from the central nervous system (Vonesch et al., 1994). Another enzyme, ADH4, can also oxidize retinol to retinaldehyde and among the alcohol dehydrogenases in the mouse, ADH4 has been identified to have a high catalytic efficiency for retinol oxidation (Connor and Smit, 1987; Boleda et al., 1993; Yang et al., 1994; Kedishvili et al., 1995). In the mouse, using whole mount in situ hybridization, ADH4 mRNA has been detected at low levels in the primitive streak mesoderm by E7.5 (7.5 days post-coitum) (Ang et al., 1997). By E8.5, much higher expression levels were detected in the posterior mesoderm and cranial mesenchyme (Ang et al., 1996). Its expression has also been detected in the central nervous system, in tissues around the developing eye, otic vesicles and migrating neural crest (Haselbeck and Duester, 1998). Interestingly, ADH4 is not present in the embryonic retina and is totally lost from the embryo by E10.5 (Ang et al., 1996). There certainly exists a redundancy in retinol dehydrogenases since null mutations for ADH4 and ADH1 develop normally (Deltour et al., 1999).

Short-chain dehydrogenases/reductases and 9-cis-RA synthesis

In the adult organism, the expression of about five different microsomal retinol dehydrogenase/reductase is known (Su et al., 1998). However, the distribution of all five dehydrogenases in the embryo is not known. Recently, two short chain dehydrogenase/reductases specific for *cis*-retinol isomers were identified (Mertz et al., 1997; Romert et al., 1998). One of them, 9-*cis* retinol dehydrogenase, is widely expressed in the central nervous system, eye, ear and somites (Romert et al., 1998). The second candidate was found to be identical to 11-*cis* retinol dehydrogenase, an enzyme required for the regeneration of the visual chromophore in the retinal pigment epithelium (Driessen et al., 1998; Simon et al., 1995). The activity of 9-*cis* retinol dehdyrogenase is important for two reasons. First, the existence of a metabolizable pool of 9-*cis* retinol is known, at least in the adult (Labrecque et al., 1995). Secondly, 9-*cis* retinoic acid, the ligand responsible for activating RXRs can be generated from 9-*cis* retinol rather than isomerized from all-*trans* retinoic acid. To date, 9-*cis* retinoic acid has only been identified in the *Xenopus* embryo (Kraft et al., 1994). However, lower levels of this isomer may be present in other species.

Short chain dehydrogenase/reductases may also be involved in the reduction of retinaldehyde to retinol through a specific retinaldehyde oxidase activity. In this way, retinol can be regenerated from retinaldehyde. The evidence for this activity comes from the fact that the type II microsomal dehydrogenase was found to be associated with the cytochrome P450/CTP2D1 oxidase which

when combined with NADPH-P450 reductase can promote retinaldehyde oxidase acitivity.

Retinaldehyde dehydrogenases

Members of the cytosolic class I aldehyde dehydrogenases (ALDH), catalyze the irreversible oxidation of retinaldehyde to all-*trans* retinoic acid in the embryo (Chen et al., 1995). Two enzymes, ALDH2 and V1, are responsible for retinoic acid synthesis from retinaldehyde in the dorsal and ventral retina, respectively (McCafferey et al., 1992). In addition, three cytosolic retinaldehyde dehydrogenases (RALDH-1, RALDH-2 and RALDH-3) can function as efficient retinal dehydrogenases (Zhao et al., 1996; Wang et al., 1996; Yoshida et al., 1998; Niederreither et al., 1999; Grun et al., 2000). A similar expression pattern has been detected for ALDH-I (Ang and Duester, 1997). Zymographic assays have demonstrated the presence of RALDH-2 activity in mouse embryos as early as E8.0 (McCaffery et al., 1997). In situ hybridization studies have shown the expression of RALDH-2 to be initially similar to ALDH-I, but unlike ALDH-I is not expressed in the cranial mesenchyme by E8.5 (Niederreither et al., 1997). Further studies performed using a tissue explant bioassay have shown that retinoic acid is detectable as early as E7.5 during primitive streak formation (Ang et al., 1996). Recently, the distribution of mRNA for all three RALDHs during fetal development and organ differentiation in the mouse was examined (Niederreither et al., 2002). Similarly, expression of a *LacZ* transgene linked to a retinoic acid response element introduced into mouse embryos was observed at E7.5 in the

posterior half of the embryo (Rossant et al., 1991). In the presence of labeled retinol, retinoic acid production was primarily in posterior tissues of E7.75 mouse embryos (Hogan et al., 1992). RALDH-2 knock out mice do not survive beyond day 10 of gestation demonstrating the importance of RALDH-2 mediated retinoic acid synthesis during embryogenesis (Niederreither et al., 1999). These embryos exhibit shortened trunks and open neural tubes.

Retinoic acid metabolizing enzymes, Cyp26A1 and Cyp26B1

Several cytochrome P450 enzymes have the ability to oxidize embryonic all-*trans* RA. Among these, two enzymes, Cyp26A1 and Cyp26B1, have received a lot of attention. The expression of Cyp26A1 (Fujii et al., 1997; De Roos et al., 1999) and Cyp26B1 (MacLean et al., 2001) during embryogenesis in the mouse has been described. Interestingly, regions in the embryo expressing RALDH-2 have high concentrations of retinoic acid and the boundaries of these regions express Cyp26A1 meaning that excess retinoic acid needs to be metabolized or neutralized to prevent any embryo toxicity. Similar to RALDH-2 knock out mice, Cyp26A1 is absolutely essential for normal embryogenesis (Abu-Abed et al., 2001; Sakai et al., 2001). Cyp26A1 null mutant embryos show defects similar to those observed in embryos that have been subjected to RA toxicity during embryogenesis (Abu-Abed et al., 2001; Sakai et al., 2001). Differential expression patterns of both Cyp26A1 and Cyp26B1 during mouse embryogenesis was recently described (Abu-Abed et al., 2002.).

Retinoid Transport Proteins

Retinoids being lipophilic molecules depend on several retinoid binding proteins for their cytoplasmic transport. A specific protein called retinol-binding protein (RBP) is involved in the systemic and intercellular transport of retinol. Several other retinoid binding proteins such as cellular retinol binding protein (CRBP-I & II) for retinol and cellular retinoic acid binding protein (CRABP I & II) for retinoic acid have been identified and their expression patterns in the developing embryo characterized (Ruberte et al., 1992). Transcripts for CRBP have been detected in the primitive streak region and allantois of the late presomite stage mouse embryo as early as day 7.5 post-coitum (Ruberte et al., 1991). As mentioned earlier, it is assumed that CRBP can bind retinol and present it to retinol dehydrogenases and promote RA synthesis. The formed retinaldehyde can again bind CRBP-1 which is then made available to RALDH for oxidation to RA (Ottonello et al., 1993).

A second binding protein called cellular retinoic acid binding protein (CRABP) is involved in intracellular retinoic acid homeostasis (Napoli, 1996). CRABP has been suggested to serve as a transport protein delivering retinoic acid to the nucleus (Donovan et al., 1995) and also as a sequestering agent limiting the transcriptional potential of retinoic acid (Napoli, 1993). CRABP transcripts appear in the decidual tissue surrounding the early conceptus even before they appear in the embryo (Sapin et al., 1997). The yolk sac membranes express CRBP transcripts and not CRABP, suggesting the possible need to

function to protect the early embryo from any free retinoic acid in the maternal blood passing through the sinuses close to the embryo. CRABP-1 may also play a role in the breakdown of RA as catabolic enzymes like Cyp26A1 have more affinity for holo-CRABP-1 as a substrate (Fiorella and Napoli, 1994). RBP is dispensable for embryo development, as homozygous RBP null mutant mice are viable and fertile. However, the ability of these RBP null mutant mice to maintain adequate liver retinol stores is impaired (Quadro et al., 1999). Interestingly, in the total absence of both CRBP-1 and CRABP, development continues uninterrupted (Lampron et al., 1995).

Nuclear Retinoic acid Receptors

Retinoic acid exerts its effect by interacting with two separate subfamilies of nuclear receptors. Retinoic acid receptors (RAR) bind the ligands all-*trans* retinoic acid and 9-*cis* retinoic acid, while retinoic X receptors (RXR) bind only 9-*cis* retinoic acid (Allenby et al., 1993; Levin et al., 1992; Mangelsdorf et al., 1990). Three subtypes for RARs (RARα, β, γ) and RXRs (RXRα, β, γ) have been identified by cDNA cloning in both human and mouse. Most of these subtypes can generate different isotypes by alternate splicing and/or alternative promoter usage (Giguere et al., 1990; Leroy et al., 1991a,b; Zelent et al., 1991). To date seven different isotypes have been identified for both RARα (Leroy et al., 1991a) and γ (Kastner et al., 1990) and at least four isotypes for RARβ. Unlike RARs, among all three RXR isotypes, an additional isotype has been identified for only RXRγ (Liu and Linney, 1993). In situ hybridization studies have shown specific

spatio-temporal patterns of distribution of all three RAR transcripts at all stages of embryogenesis in the mouse (Ruberte et al., 1990a; Ruberte et al., 1991; Dolle et al., 1989; Dolle et al., 1990). While RARα showed a generalized pattern of expression, RARs β and γ showed a more restricted pattern of expression. The appearance of precartilage condensation and keratinizing squamous epithelia has been shown to correlate with the expression of either RARγ (Ruberte et al., 1990b) or complementary expression of both RARβ and RARγ (Dolle et al., 1989; Dolle et al., 1990). RARβ expression has been localized to sites undergoing programmed cell death and also in nervous structures (Dolle et al., 1989; Dolle et al., 1990). Northern blot analysis has revealed the expression of specific isoforms of RARs at various stages of mouse development (Leroy et al., 1991a; Zelent et al., 1991; Kastner et al., 1990). Transgenic mouse lines bearing a *Lac* Z reporter gene under the control of a RARβ P2 promoter have been used to reveal the distribution pattern of various RAR transcripts (Mendelsohn et al., 1991; Reynolds et al., 1991). The chick embryo expresses RARβ (Smith and Eichele, 1991; Rowe et al., 1991) and RARγ is expressed in the *Xenopus* embryo (Ellinger-Ziegelbauer and Dreyer, 1991). Transcripts for all three RARs have been detected in the early developing porcine conceptus on days 10-12 of gestation (Yelich et al., 1997). Among the RXRs, RXRβ shows a generalized pattern of expression, however, RXRα and RXRγ exhibit more restricted patterns of expression (Mangelsdorf et al., 1992; Dolle et al., 1994). For the RXRs, transcripts for RXRα and γ have been detected in *Xenopus* eggs and during early development (Blumberg et al., 1992). The expression of all three RXRs has been

detected using northern analysis at all stages of mouse gestation from day 10.5 to birth (Mangelsdorf et al., 1992). In the chick, expression of RXRγ was detected in the spinal ganglia and nerves as well as neural crest cells (Rowe et al., 1991). These studies to a certain extent show that the expression of the various RAR subtypes is conserved across higher vertebrates.

RARs can heterodimerize with RXRs. In contrast, RXRs can form homodimers with other RXR molecules and heterodimers not only with RARs (Giguere, 1994; Petkovich; 1992; Zhang et al., 1992) but also with other homologous nuclear receptors. These include the thyroid hormone receptors (TRα and TRβ) (Giguere, 1994), vitamin D receptor (VDR) (Giguere, 1994), peroxisome proliferator-activated receptor α (PPAR) (Palmer et al., 1994) and several orphan receptors (Apfel et al., 1994; Kliewer et al., 1992). The RAR or RXR dimers then regulate gene expression by binding to specific DNA sequence elements (retinoic acid response elements; RAREs) found in or near retinoid-responsive gene promoters. The DNA-bound receptor then recruits other nuclear proteins to the target promoter site. This complex serves to modulate the stability of the basal transcription complex and thereby control the rate of formation of the target gene transcript. Each retinoic acid receptor is believed to regulate an entirely different subset of genes.

The physiological functions of retinoid receptors have been investigated by creating homozygous mutant mice for either a particular subtype or an isotype of a subtype using homologous recombination. Interestingly, mice homozygous for isoforms such as RARα1 (Lufkin et al., 1993), RARβ2 or γ2 (Lohnes et al.,

1993) are viable and do not exhibit any recognizable defects or malformations. The failure to observe any developmental defects in these single mutant mice can be partially explained by the redundancy between RARs, meaning that the other RAR subtypes/isoforms of each subtype expressed in a given cell can compensate for a particular missing subtype. Knocking out the entire RARα gene showed testicular degeneration and early post-natal mortality (Lufkin et al., 1993). RARγ mutant mice on the other hand showed several abnormalities such as homeotic transformations of the cervical vertebra and occipital region of the skull, fused first and second ribs, and irregularities of the tracheal rings (Lohnes et al., 1993). However, subsequent studies performed along these lines employing double mutants for both RARα and RARγ saw all of the congenital defects that have been previously reported for embryos from vitamin A deficient mothers (Lohnes et al., 1994). RXRβ mutant mice were morphologically normal with the exception that males were sterile (Kastner et al., 1996). RXRα mutant mice died between 13.5 to 16.5 days post coitum. In addition, these mutant mice also exhibited hypoplastic development of the ventricular septation, abnormalities of the eye (Sucov et al., 1994). RAR double or compound mutants were lost either in utero or immediately after birth (Lohnes et al., 1994; Luo et al., 1996; Mendelsohn et al., 1994). Majority of the abnormalities in RAR mutant mice including multiple abnormalities reiterated those described earlier for vitamin A deficiency mice. Noticeably, $RXR\beta^{-/-}/RXR\gamma^{-/-}$ double and $RXR\alpha^{+/-}$/ $RXR\beta^{-/-}/RXR\gamma^{-/-}$ triple mutant mice survived and showed no congenital or postnatal abnormalities. However, these double and triple mutant mice exhibited marked growth

deficiency and sterility in males due to non-functional RXRβ (Krezel et al., 1996). This would mean that a single copy of RXRα is sufficient to fulfill most functions of RXRs. The importance of RXRα is further clear from the fact that synergy exists only between RXRα and RAR mutations and not when RXRβ or RXRγ mutations were combined with RARs (Kastner et al., 1997). Embryos carrying null mutations of both RXRα and β die between 9.5 and 10.5 days of gestation (Wendling et al., 1999). Lack of formation of the labyrinthine zone of the chorioallantoic placenta appeared to be the cause. These authors concluded that heterodimerization of RXRs with RARs and PPARγ was essential for postimplantation development and formation of the chorioallantoic placenta, respectively.

Retinoids and Growth Factor Synthesis

An important role attributed to retinoids is in regulating and controlling the expression of the genes for many growth factors and their receptors. Development and differentiation events occurring in the fertilized oocyte and continuing through the spherical blastocyst stage begin during transit through the fallopian tube (Kaye, 1997). During this journey, it is believed that diffusible factors can serve as signals to optimize events in preimplantation development. These events include synthesis of DNA, RNA, and protein, cell proliferation, blastocoel expansion and blastocyst hatching from the zona pellucida (Wood and Kaye, 1989; Harvey and Kaye; 1989, 1991, 1992; Gardner and Kaye, 1991;

Rappolee et al., 1992; Zhang et al., 1994). These diffusible factors may include products secreted by the embryo (i.e., insulin-like growth factor), the reproductive tract (i.e., tumor necrosis factor-α) or those, like insulin, originating from distant organs.

Retinoids have been shown to have an influence on the expression of peptide growth factors TGF-β, IGF-I and II, EGF, and their corresponding receptors. Retinoids are potent inhibitors of cell growth and inducers of cell differentiation. Retinoic acid induced secretion of bioactive TGF-β2 when added to primary murine keratinocyte cultures (Glick et al., 1989), human lung carcinoma A-549 cells, and normal rat kidney NRK fibroblasts (Danielpour et al., 1991). In the presence of retinoic acid, F9 and PC13 teratocarcinoma cells differentiated and, at the same time, expressed TGF-β receptor, which is normally absent in these cells (Rizzino, 1987). Suboptimal concentrations of retinoic acid required to induce differentiation in cells when added to HL-60 promyelocytic leukemia cells induced expression of both TGF-β and its receptor. These observations suggest that retinoic acid can directly regulate the expression of TGF-β ligand and its receptor and that the growth-inhibitory effects of retinoic acid on these cell types are mediated through a negative autocrine effect of TGF-β during the process of differentiation (Falk et al., 1991). A single oral dose of 100μg of retinoic acid when given to vitamin A deficient rats caused tissue specific alterations in the expression of the TGF-β isoforms. Vitamin A deficient rats had low expression levels of TGF-β1 in epidermal, tracheobronchial and intestinal mucosal epithelium than rats treated with retinoic acid (Glick et al.,

1991). Retinoic acid when administered at teratogenic doses to mice on day 8 of pregnancy slightly decreased the expression of TGFβ1, had very little effect on expression on TGFβ2 and completely abolished the expression of TGFβ3 in several fetal tissues, including mesenchyme of the head and epithelial structures of the heart (Mahmood et al., 1992). Considering the fact that the craniofacial and cardiac malformations resulting from excessive dosing with retinoic acid are localized to tissues known to express high levels of TGF-β, it appears that a peptide like TGF-β may have a possible role in mediating certain actions of retinoids. On the whole, data from the vitamin A deficient and overdosed rats suggests that patterns of TGF-β isoform expression in epithelial tissues during normal development is likely to be under the regulatory control of retinoids.

Epidermal growth factor (EGF) and Transforming growth factor-α (TGF-α) are potent mitogens and retinoic acid is involved in the control of both receptor and ligand expresion (Gudas et al., 1994). Retinoids can either enhance epidermal growth factor receptor (EGF-R) expression (Oberg et al., 1988; Junquero et al., 1990; Kinoshita et al., 1992), or suppress EGF-R expression (Oikarinen et al., 1989; Steck et al., 1990) and modulate cellular responses to EGF with no major effects on EGF-R number or affinity (Yung et al., 1989; Harper et al., 1988). TGF-β also has effects on EGF-R expression similar to retinoic acid in several cell lines (van Zoelen at al., 1986; Assoian, 1985). This raises the question whether the effects of retinoic acid are mediated via TGF-β, which is known to be induced by retinoic acid treatment of the same cells (Danielpour et al., 1991). On the other hand, retinoic acid has been shown to

suppress the expression of TGF-α (Miller et al., 1990). Furthermore, retinoid induced suppression of EGF-R synthesis correlated well with the inhibitory effects of retinoic acid on cell growth (Steck et al., 1990). In the same study cells that became resistant to the growth-inhibitory effects of retinoic acid were unresponsive to regulation of EGF-R by retinoids (Steck et al., 1990). These data suggests that regulation of cell growth/differentiation by retinoids might involve increasing the activity of negative growth factors like TGF-β and decreasing the activity of positive growth factors like EGF and TGF- α.

Retinoic acid has also been reported to regulate the expression of yet another diverse set of growth factors called heparin-binding growth factors which includes platelet-derived growth factor, the family of fibroblast growth factors, and a newly described class of retinoid-inducible heparin-binding growth factor which includes heparin- binding neurite promoting factor (HBNF) and midkine (MK). Retinoids exert effects on the expression of both acidic and basic FGF but their expression during embryogenesis depended on the stage of cell differentiation (Hebert et al., 1990). However, another member of the FGF family called kFGF or hst-1/HSTF1, even though expressed, is restricted to undifferentiated embryonal carcinoma and embryonic stem-cell lines. Under the influence of retinoic acid these cells differentiate with a concomitant downregulation of the kFGF expression (Hebert et al., 1990; Schoorlemmer and Kruijer, 1991). Similarly, retinoic acid treatment of cultured chicken embryo fibroblasts and myotubes induces the expression of the chicken homolog of MK, RI-HB (Retinoic

acid inducible heparin binding growth factor) (Urios et al., 1991; Raulais et al., 1991).

Platelet derived growth factor (PDGF) consists of two chains, A and B, their corresponding receptors are termed α and β. PDGF A chain is expressed abundantly in undifferentiated F9 mouse embryonal carcinoma cells (Mercola et al., 1990). mRNA levels are low for the B chain and their receptors, however (Mercola et al., 1990). Following retinoic acid induced differentiation of these cells, mRNA as well as the mitogenic activity of the A chain start to decline (Rizzino and Bowen-Pope, 1985; Mercola et al., 1990). At the same time the expression of both α and β receptors begin to increase during the differentiation process (Mercola et al., 1990).

Insulin-like growth factors (IGF-I and IGF-II) are potent mitogens which play an important role in cell growth and differentiation. IGF-I is believed to mediate the actions of growth hormone (GH) in regulating normal growth and development. On the other hand, IGF-II is expressed in the fetus. In rat glioma C6 cells, retinoic acid has been shown to reduce the expression of IGF-I mRNA, thereby suppressing the growth of these cells (Lowe et al., 1992). In human neuroblastoma cell line, retinoic acid stimulated the expression of IGF-II mRNA and protein (Matsumoto et al., 1992). Both these effects of retinoic acid required de novo protein synthesis indicating that the effects were probably indirect. In MCF-7 human breast cancer cells and in rat hepatocytes retinoic acid was shown to regulate the activity of IGFs by indirectly stimulating the production of IGF binding proteins suggesting that this could in yet another way contribute to the

growth suppressing effects of retinoic acid (Fontana et al., 1991; Schmid et al., 1992).

Leukemia inhibitory factor (LIF) is a multifunctional cytokine and depending on the specific cell type can either stimulate or inhibit the differentiation of cells. LIF has been to shown to maintain pluripotent embryonic stem cells in their undifferentiated state (Williams et al., 1988). In nullipotent embryonal carcinoma cell lines, effects of LIF are cell specific. While LIF has no effect on the differentiation of F9 cells induced by retinoic acid, it blocked retinoic acid-induced differentiation of an F9 derivative, OTF9 cells. This shows that LIF was exerting its effect at a point downstream to the steps requiring retinoic acid (Brown et al., 1992). In summary, it is clear that retinoids suppress the growth of epithelial cells by regulating the activity of growth factors. As far as negative growth factors like TGF-β are concerned, retinoic acid upregulates the expression of both the ligand and its receptor. In the case of positive growth factors, retinoic acid downregulates the expression of TGF-α ligand and its receptor, EGF-R. Retinoic acid also inhibits the expression of IGFs and stimulates the expression of IGF- binding proteins. Interestingly, the early bovine embryo expresses transcripts for TGF-β2, TGF-α, PDGF-α, IGF-I, IGF-II, PDGF-α receptor, insulin receptor, IGF-I and IGF-II receptor (Watson et al., 1992), LIF (Eckert and Niemann, 1998) and IGF binding proteins (Winger et al., 1997). Collectively, a possible regulator of gene expression like retinoic acid during blastocyst formation can be envisioned since this stage represents the first differentiation event in the mammalian preattachment embryo.

Retinoids and Placental Function

Transplacental transfer of retinol has been studied in humans, mice, rats and monkeys (Creech Kraft et al., 1987; 1988; 1989a,b; Kochhar et al., 1988; Ward and Morriss-kay, 1995; Tzimas et al., 1996). Retinoic acid and its metabolites accumulate in the embryo rapidly, with maximum concentrations appearing 2-4 h following maternal administration, thereby suggesting that a specific uptake pathway exists for the transfer of vitamin A from the mother to the embryo. Maternal RBP-bound retinol interacts with a cell surface receptor for RBP (RBPr) and is actively accumulated at the maternal face of the yolk-sac placenta which is mainly composed of the visceral endoderm, a highly endocytotic tissue. Developing rodent embryos therefore acquire retinol from neighbouring maternal blood sinuses via interaction of maternal RBP-retinol with an RBPr in the visceral yolk sac endoderm. The visceral endodermal cells synthesize RBP and serve to transfer retinol from the yolk sac vasculature to the embryo. Several aspects of placental function such as control of estradiol production (Piao et al., 1997) and regulation of production of chorionic gonadatropin and placental lactogen (Chou, 1992; Stephanou and Hanwerger, 1995) are dependent on retinoid activity. During placental development, retinoic acid through moderating the production of metalloproteinase inhibitors or the spatiotemporal expression pattern of connexins might aid in regulating the trophoblastic invasion of the uterus (Winterhager et al., 1996). Human

trophoblast cells express CRBPs and some nuclear retinoid receptors lending support to the involvement of retinoids in chorioallantoic placental function (Roulier et al., 1994; Stephanou et al., 1994). In addition, the level of expression of some retinoic acid receptors varies during the differentiation of cultured cytotrophoblasts into syntiotrophoblasts (Stephanou et al., 1994).

Retinoic acid acting via RARs can stimulate gene expression and secretion of laminin β1 (Marotti et al., 1985; Ross et al., 1994) and β1 integrin expression (Ross et al., 1994) in murine F9 teratocarcinoma stem cells. Treatment of expanding mouse blastocyst with retinoic acid results in increased transcription and translation of laminin β1 (Kang et al., 1990). Gene expression and the active protein of β1 integrin has been detected in the pig (Yelich et al., 1997) and bovine conceptuses (Maclaren et al., 1995). In the pig, gene expression for laminin has been detected during the transition from tubular to filamentous morphology (Cunningham et al., 1996). While laminin is required for the assembly of the extracellular matrix, changes in cell shape and in cell migration (Hakamori et al., 1984), integrins serve as receptors for the laminins (Ruoslahti and Pierschbacher, 1987). Dynamic cellular interactions that occur when integrins bind their ligands influence the actin cytoskeleton and modify cell shape and function (Tamkun et al. 1986; Adam and Watt, 1993). In the pig, retinoic acid can indirectly regulate the breakdown of extracellular matrix by influencing the production of proteinase urokinase-type plasminogen activator and its inhibitor (Fazleabas et al., 1983). Retinoids play a very important role in the development and growth of the pig placenta (Dantzer and Winther, 2001).

Both transcripts and the mature protein for RBP have been detected in the pig placenta and uterus (Harney et al., 1990, 1994a,b; Trout et al., 1992, Schweigert et al., 1999). Recently, immunohistochemical localization of RBP, CRBP-I and CRABP-I within the interareolar region and the areolar gland complex was reported in the pig suggesting that vitamin A was most likely transported to the conceptus through the areolar gland complex (Schweigert et al., 2002). Retinoic acid has been implicated in the formation of giant cells during placentation in the mouse (Yan et al., 2001). Retinoic acid treatment of trophoblastic stem cells in vitro prevented further proliferation and induced differentiation into giant cells. Similarly, retinoic acid administered to pregnant mice suppressed spongiotrophoblast formation and accelerated their differentiation into giant cells. The expression of retinoic acid inducible Stra genes such as ephrinB1 receptor tyrosine kinase ligand, Meis2 homeobox gene, AP-2 related gene AP-2 gamma in the murine placenta was recently reported (Sapin et al., 2000). This is the first report on the identification of specific retinoic acid induced target genes during placentation indicating that retinoids are likely to regulate several signal transduction pathways in various placental cell types.

Retinoids and Early Embryo Development

As mentioned earlier, embryonic development is a complex process that is extremely sensitive to vitamin A as both deficiency and excess can lead to abortion and embryonic malformation. In the human, important vitamin A

dependant events are likely to begin at the time of gastrulation and neurulation, sometime during the first 2-3 weeks of gestation (Zile, 1998). In the quail embryo, an absolute requirement for vitamin A during the very early stages of life has been demonstrated, absence of which leads to gross abnormalities and early embryonic death (Zile, 1998).

Administration of retinol or β-carotene tremendously enhanced the survivability of embryos in mice (Chew and Archer, 1983) and rabbits (Besenfelder et al., 1993). In swine, retinol palmitate administered as a single injection increased litter size when given 5 days before observed estrus (Brief and Chew, 1985; Coffey and Britt, 1993; Britt et al., 1992). In cattle, measurement of retinol concentrations during follicular development has shown higher concentrations to be present in healthy follicles and correlated well with estradiol concentrations (Schweigert and Zucker, 1988; Schweigert et al., 1988; Brown et al., 2003). However, very low concentrations were detected in atretic follicles. Accordingly, these authors used retinol concentrations in follicular fluid as a measure of follicular health (Schweigert and Zucker, 1988; Schweigert et al., 1988).

A single injection of retinol palmitate given 5-7 days before induced estrus in combination with FSH did not increase ovulation rates or total yield of embryos on day 7 (Shaw et al., 1995). Surprisingly, retinol-treated cows yielded more blastocysts evaluated to be of high quality than untreated controls (Shaw et al., 1995). More recently, adminstration of retinol to heifers four days prior to ovum pick improved the yield of cumulus oocyte complexes (Hidalgo et al., 2002). At

this time it was not known if these effects were attributable to retinol or its metabolite, retinoic acid. Again the nature of these effects, whether direct on the embryo or indirect through the production of embryotrophic factors from reproductive tract, was not clear. RBP has been recorded to be synthesized in several reproductive tissues such as the ovine ovary (Eberhardt et al., 1996), ovine oviduct (Eberhardt et al., 1999a) and bovine endometrium (Lui and Godkin, 1992; Mackenzie et al., 1997). Expression of retinol-binding protein and its mRNA has been detected in the extraembryonic membranes (Day 45), expanding blastocysts but not in spherical blastocysts (Liu et al., 1993). More recently, the mature protein for RBP was localized to the theca and granulosa cells of both antral and preantral follicles in the bovine ovary (Brown et al., 2003). CRBP immunostaining was, however, stronger in the granulosa cells of preantral follicles than antral follicles (Brown et al., 2003). Therefore, it appears that the follicular cells are well equipped to take up retinol that is either naturally available or administered exogenously. Similarly, treatment of ewes during a superovulation regimen with retinol but not retinoic acid improved the developmental competence of embryos in vitro. More morulae recovered on day 6 developed to blastocyst and hatched in retinol treated than retinoic acid treated ewes (Eberhardt et al., 1999b). This meant that a specific transport protein for retinol and not retinoic acid may have been existent in the blood and, possibly, in ovine preimplantation embryos too. Treatment of gilts with vitamin A altered oocyte and embryo development by advancing meiotic resumption and as a result fewer oocytes recovered were in the germinal vesicle stage compared to

control gilts (Whaley et al., 2000). In addition, vitamin A treated gilts also had higher concentrations of progesterone, IGF-1, and $PGF_{2\alpha}$ than control untreated gilts (Whaley et al., 2000). More recently, Duque et al. (2002) treated bovine oocytes with 5nM 9-*cis* retinoic acid during prematuration (under meiosis inhibitory conditions with 25 μM of roscovitine). These authors reported improved cytoplasmic cortical granular migration, embryonic development, tolerance to cryopreservation and increased total cell numbers in blastocysts. These studies indicate that retinoids have the potential to enhance embryonic survival by positively enhancing oocyte development/maturation before ovulation. Therefore, it may be concluded that retinol can have embryotrophic effects when administered in vivo and retinoic acid on the other hand can exert similar effects in vitro.

Using high peformance liquid chromatography, it was recently shown that the porcine blastocyst, in particular the trophoblast region is capable of synthesizing retinoic acid (Parrow et al., 1998). Retinoids in blastocyst explants and TE1, a continuous trophectoderm cell line, induced gene expression from a retinoic acid responsive enhancer element in an in vitro assay system (Parrow et al., 1998). Therefore, it appears that the porcine conceptus does not solely rely on the circulating retinoic acid from the uterine environment to influence embryonic differentiation, morphogenesis and development.

In the studies of Shaw et al. (1995) and Eberhardt et al. (1999b) involving cattle and sheep, respectively, the mechanism by which retinol influenced subsequent embryonic development is not clear. As suggested by Eberhardt et

al. (1999b), retinol administration might have through some unknown ways first affected the oocyte and later facilitated the transition from maternal to embryonic genome control, thereby, preventing the 8-16 cell block. The differentiation events in the early preimplantation embryo begin during the transition from morulae to the blastocyst involving structural and functional alterations (Pedersen and Burdsal, 1994). These events include the processes of compaction, formation of the blastocoel and differentiation of the trophectoderm and inner cell mass (Pedersen and Burdsal, 1994). The last two events are important for the development of the placenta and the fetus itself. It thus appears that the influence of retinol and its metabolites on early embryo development may begin during the growth of the oocyte inside the follicle. This influence is likely to continue during the development of the preattachment embryo via direct or indirect means. No direct evidence, however, is available to support these statements in the preattachment bovine embryo.

Retinoic acid has been shown to induce tight junction structures and regulate the expression of several tight junction-associated molecules, such as ZO-1, occludin, claudin-6, and claudin-7, as well as a barrier function in the genetically engineered cell line F9 murine embryonal carcinoma cells (Kubota et al., 2001). During preattachment embryogenesis, formation of tight junctions between trophectodermal cells is critical for the formation of the blastocoel and subsequently for blastocyst formation. In this context, retinoic acid treatment of human hepatoma Hep G2 cells increased the amounts and phosphorylation of connexins (Ara et al., 2002). Retinoic acid treatment also stabilized connexin

molecules in plasma membrane plaques and enhanced gap junctional intercellular communication (Ara et al., 2002). Establishment of tight junctions and expression of intercellular communication proteins such as connexins are critical determinants of blastocyst formation (Watson et al., 2001). Diez et al., 2002, recently reported that the addition of 5 nM of 9-*cis* RA during in vitro maturation could alter inner cell mass/trophectoderm cell ratio. Based on these observations, it is likely that retinoic acid may regulate an important developmental event such as blasotycst formation during preattachment embryogenesis.

Evidence is also available to show that the postimplantation development requires the mammalian embryo to metabolize retinol into retinoic acid. Targeted disruption of the retinaldehyde dehydrogenase-2 (RALDH-2) enzyme in mice resulted in embryonic mortality around midgestation (Niederreither et al., 1999). RALDH-$2^{-/-}$ embryos died around midgestation without undergoing axial rotation, shortened along the anterioposterior axis, and did not form limb buds (Niederreither et al., 1999). These embryos presented with anomalous hearts, truncated frontonasal region and severely reduced otocysts (Niederreither et al., 1999).

Genes Regulated by Retinoic Acid during Embryonic Development

In an attempt to identify retinoic acid regulated genes during embryonic development, Chen et al., (2002) created retinoic acid deficient embryos

employing retinol-binding protein antisense oligodeoxynucleotide treatment in contrast to the classic retinoic acid receptor knock out mice. Using differential-display RT-PCR these authors identified genes in the Wnt signaling pathway, components of the ubiquitin-dependant proteasome degradation pathway, and enzymes involved in oxidative phosphorylation pathway in the mitochondria. More recently, genes specifically regulated by a single retinoic acid receptor isoform, RARβ2, in F9 teratocarcinoma cells were isolated (Zhuang et al., 2003). The identified genes encoded various transcription factors, cell surface signal transduction molecules, and metabolic enzymes. Some of these genes included c-myc, FOG1, GATA6, glutamate dehydrogenase, glutathione-S-transferase homologue (p28), Foxq1, Hic5, Meis1a, Dab2, midkine, and the PDGF-α receptor.

Thus, it is of interest to study the interactions between the early preattachment bovine embryo and the environment in the reproductive tract and the possible involvement of retinoids during this critical stage. Much information has been generated concerning the effects of retinoids on cellular differentiation and embryonic development in rodents. However, with very little information known in the bovine, it is necessary to examine the possible role retinoic acid may have during the early development and the expression of genes they regulate that may play critical roles during development and in preparing the early conceptus for the process of attachment. Furthermore, there is little insight into either the synthesis or metabolism of retinoids in the bovine embryo or receptors that might transduce their morphogenetic signals. Therefore, we

assumed that the molecular and genetic dissection of the retinoid signaling pathway using in-vitro produced bovine embryos would provide us greater insight into understanding the influence of retinoids on embryo development.

The long term objective of this thesis research is to identify and characterize downstream target genes regulated by both retinoic acid and the in vitro culture system. Microarrays would be the technique of choice for studying differential gene expression irrespective of the tissue and species of choice. The bovine genme is not yet sequenced and therefore, it may not be possible to construct an array specific to the bovine. In addition, at least 5-10 μg of RNA is necessary to label the target before probing the slide containing the different target cDNAs. At present using SMART PCR it may be possible to generate 5-10 μg of cDNA for labeling. Due to the above mentioned constraints other techniques such as DD-RT-PCR, RT-AFLP have been used extensively to study differential gene expression in preattachment bovine embryos. DD-RT-PCR has a few disadvantages such as it is biased towards amplifying high abundance transcripts and hence is not sensitive enough to identify differentially expressed genes with low abundance. In addition, DD-RT-PCR requires the use of radioisotopes and large sequencing gels which can impose serious health hazards and make the whole process cumbersome (Diatchenko et al., 1996). SSH on the other hand, is very sensitive and can pick both low and high abundance transcripts (Diatchenko et al., 1996). No prior knowledge of gene expression is required and SSH enables subtraction of common sequences and enriches only the differentially expressed genes. Basically, this technique

involves comparing gene expression patterns between two different tissue samples. One of them, say, the tissue in which we are interested in identifying the differentially expressed genes serves as the tester. The cDNA is first digested with a restriction enzyme to obtain fragments with an average size of about 600 bp. The restriction enzyme digested products are then ligated with two different adaptors in independent ligation reactions. cDNA from the second tissue sample, which is called the driver, is added in several fold excess and allowed to hybridize so that majority of the common gene sequences between the two samples form hybrids and are eliminated. This step results in enrichment of genes that are mostly unique to the tissue in which we are interested in identifying differentially expressed genes. The tissue samples are switched around and the procedure is once again repeated. The differentially expressed products are cloned into plasmids and a differential screening analysis is performed to identify true differentially expressed gene products.

The SSH protocol, however, involves digesting the cDNA with restriction enzymes such as *Rsa*I, a four base cutter, to generate smaller cDNA fragments with an average size of about 600 bp. These smaller sized fragments hybridize more efficiently since larger fragments tend to form complex secondary structures which may interfere with the hybridization process. This step makes it impossible to obtain full length cDNAs representing differentially expressed genes. SSH also requires at least 2-4 μg of cDNA to begin with and this amount can be easily generated using SMART PCR. The final products of SSH are in the form of small fragments ranging in size from 250 bp to 1.5 kb called

expressed sequence tags (ESTs). These ESTs can be used to discover novel genes, characterize gene function, quantify the expression patterns of a particular gene of interest.

It is always advisable to reconfirm the data obtained with SSH using an independent technique such as a northern blot or competitive PCR or using the more recently developed real-time quantitative RT-PCR. In majority of the cases, the SSH data should agree with the real-time quantitative RT-PCR data.

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Chapter III

DETECTION OF TRANSCRIPTS FOR RETINOL-BINDING PROTEIN AND RETINOIC ACID RECEPTORS IN BOVINE PREIMPLANTATION EMBRYOS

Abstract

In cattle, retinoic acid (RA) has been indirectly associated with developmental potential of the embryo. RA is transported by retinol-binding protein (RBP) and actions of RA are mediated by several subtypes of nuclear retinoic acid receptors (RAR). Bovine embryos, produced in vitro from oocytes harvested from ovaries collected at a local abattoir, were frozen in liquid nitrogen at the oocyte, 2-, 4-, 8-, 16 to 20-cell, morula, blastocyst and hatched blastocyst stages. Employing reverse transcription polymerase chain reaction (RT-PCR) we investigated mRNA expression for RBP, RARα, RARβ, RARγ, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Total RNA was extracted from 25 pooled embryos at each stage and RT-PCR analysis was repeated thrice. GAPDH transcript was detected in all stages. Transcripts for RBP, RARα and RARγ were also detected in all stages from the oocyte through to the hatched blastocyst. Expression of RARβ was not detected at any stage. Whole-mount immunohistochemistry was performed with intact and hatched blastocysts using polyclonal antibodies against RARα and RARγ2 to investigate if these

embryonic mRNAs were translated to the mature protein. Strong immunostaining was observed for both RARα and RARγ2 in the inner cell mass and trophectoderm of intact and hatched blastocysts. Expression of mRNA for RBP, RARα, RARγ and of the RARα and RARγ2 receptor proteins in the bovine embryo suggests that RA is likely to directly regulate gene expression during preimplantation development.

Introduction

Vitamin A and its physiological metabolites, collectively known as retinoids have profound effects on embryonic morphogenesis, cell growth and differentiation, vision and reproduction (Deluca, 1991; Hofmann and Eichele, 1994; Eskild and Hansson, 1994). It has been known for a long time that an adequate level of vitamin A in the maternal diet is crucial for the normal development of the embryo (Kalter and Warkany, 1959). Retinol (vitamin A) is metabolized in the cell, giving rise to several physiologically active compounds (Napoli et al., 1993). Retinoids are believed to induce cell differentiation in vitro by causing changes in the expression of homeobox genes, growth factors and their receptors.

A specific protein called retinol-binding protein (RBP) is involved in the systemic and intercellular transport of retinol. Intracellular sequestration of retinol is accomplished by cellular retinol-binding protein (CRBP). CRBP accumulates

retinol, stimulates mobilization of retinol from retinol esters and transfers retinol to retinol dehydrogenase for conversion to retinal and finally all-*trans* retinoic acid.

Retinoic acid exerts its effect by interacting with two separate subgroups of nuclear receptors, retinoic acid receptors (RARα, RARβ, RARγ) and retinoid X receptors (RXRα, RXRβ, RXRγ). Ligand-receptor complexes can then either activate or repress specific target genes by associating with specific response elements found near the promoter region.

Embryonic development is a complex process that is extremely sensitive to vitamin A as both deficiency and excess can lead to abortion and embryonic malformation. In the human, important vitamin A-dependent events occur at the time of gastrulation and neurulation, sometime during the first 2-3 weeks of gestation (Zile, 1998). In the quail embryo, an absolute requirement for vitamin A during the very early stages of life has been demonstrated, absence of which leads to gross abnormalities and early embryonic death (Zile, 1998). In polytoccus species like mice (Chew and Archer, 1983), rabbits (Besenfelder et al., 1993) and swine (Brief and Chew, 1983; Britt et al., 1992; Coffee and Britt, 1993) retinol supplementation has been shown to promote embryo survival. In cattle, retinol administered at the time of superovulation increased the number of transferrable embryos (Shaw et al., 1995). Although the importance of retinoids to embryonic development is well known in rodents, amphibians and quail, little is known about its role during preimplantation development in cattle. The objectives of this study were to detect specific components of the vitamin A signaling pathway in bovine preimplantation embryos.

Materials And Methods

In vitro Embryo Production

Oocyte Maturation

Ovaries were collected from cows at a local abattoir and transported to the laboratory in physiological saline supplemented with penicillin-G (100 IU/ ml) and streptomycin sulfate (0.2 μg/ml) at 26-30°C within 5 hrs. Oocytes were aspirated from follicles ranging in diameter from 2 to 5 mm using an 18-gauge needle into modified-PBS solution. The oocytes were then washed twice using maturation medium (TCM-199; Sigma Chemical Co., St. Louis, MO, USA) supplemented with estrus cow serum (ECS), 0.01 mg/ml follicle stimulating hormone (FSH; Folltropin, Vetrepharm, Canada), and 50 μg/ml gentamycin (Sigma Chemical Co.). Cumulus-oocyte complexes (100-200) were introduced into a 35 x 10 mm polystyrene petri dish containing 2.5 ml of the maturation medium covered with mineral oil and cultured for 24 h at 38.5°C under 5% CO_2 in air.

In vitro fertilization

Frozen spermatozoa were thawed in a water bath (37°C) and washed twice in Brackett and Oliphant's medium supplemented with caffeine (2.5 mM) (Caff-BO) by centrifugation at 500*g* for 5 min. Spermatozoa were then

resuspended in Caff-BO supplemented with bovine serum albumin (1%) (BSA, Sigma Chemical Co.) and heparin (20 μg/ml) (Sigma Chemical Co.) to give a final sperm concentration of 5 x 10^6/ml. A droplet of the sperm preparation (100 μl) covered with mineral oil was preincubated for 10 min under the same conditions described earlier for in vitro maturation. In vitro matured oocytes numbering 15-20 were transferred into a sperm drop for insemination. Approximately 18-20 h post insemination, the oocytes were washed and transferred to the culture medium contained in a polystyrene dish (4-well multidish; Nunclon; Roskilde, Denmark) covered with 0.5 ml mineral oil for further development. The culture medium consisted of TCM-199 supplemented with 5% ECS, insulin (5 μg/ml) (Sigma Chemical Co.) and gentamycin (50 μg/ml) (Sigma Chemical Co.). The cumulus cells surrounding the embryos were removed at 48 h post in vitro fertilization (IVF) by repeated pipetting. The cumulus cells were allowed to form a monolayer on which the embryos were cultured until day 9. The culture medium was replaced every 48-72 h.

RNA extraction

Immature bovine oocytes and embryos at the 2-, 4-, 8-, 16 to 20-cell, morula and blastocyst stages were frozen in 250 μl of denaturing solution [guanidium isothiocyanate (4 M), sodium citrate (25 mM; pH 7.0), sarcosyl (0.5%), 2-β mercaptoethanol (0.1 M)]. Total RNA was extracted from a pool of 25 embryos at each stage in 500 μl of denaturing solution plus 70 μl sodium acetate (2 M; pH 4.0), 500 μl phenol, 140 μl chloroform/iso-amyl alcohol (49:1 fresh

dilution). All tubes were vortexed, cooled on ice (15 min), and centrifuged for 20 min (21,000 rpm) at 4°C. The aqueous phase (600 μl) was transferred to a fresh, sterile microcentrifuge tube (1.5 ml) and 500 μl of chloroform was added. Tubes were vortexed and centrifuged at 3000 rpm for 10 min at 4°C. The aqueous phase (500 μl) was removed and transferred into a fresh sterile microcentrifuge tube and the chloroform extraction repeated. The aqueous phase (400 μl) was recovered and an equal volume of RNA binding salt was added (RNaid Matrix; BIO 101, LaJolla, CA, USA). A glass bead suspension matrix (RNaid Matrix; BIO 101) was added and the sample vortexed. The suspension was vortexed (15 min) at room temperature, transferred to a fresh tube and centrifuged (3,000 rpm) for 2 min. The aqueous phase was discarded, and the remaining pellet was washed with 500 μl of a 50% RNA wash solution (RNaid Matrix; BIO 101) and 50% ethanol solution, vortexed, and centrifuged (10,000 rpm) for 2 min. The pellet was washed three times, dried at room temperature (10 min), and resuspended in 50 μl of diethyl pyrocarbonate-treated water. The solution was subsequently heated for 4 min (56°C) to elute the bound RNA and centrifuged (14,000 rpm) for 2 min. Approximately, 40 μl of the aqueous phase was transferred to a sterile microcentrifuge tube.

Reverse transcription-polymerase chain reaction

In order to maximize the sensitivity of detection, reverse transcription-polymerase chain reaction (RT-PCR) was the method of choice for investigating gene expression from the small quantities of RNA obtained from bovine embryos.

Two micrograms of total RNA was denatured by heating to 70°C and reverse transcribed in the presence of random hexamers (pdN_6; 100 pmole; Pharmacia, Piscataway, NJ, USA), dATP, dTTP, dCTP and dGTP (dNTPs, Pharmacia), $MgCl_2$, RNase inhibitor (20 U/reaction; Promega, Madison, WI) and reverse transcriptase (Superscript™, 200 U/reaction; Gibco-BRL, Gaithersburg, MD, USA) at 42°C for 1 hr. The RT reaction was terminated by heating to 70°C.

One to five microliter aliquots of reverse transcribed cDNA was denatured by heating to 95°C and subjected to PCR in the presence of pmole quantities of specific primers, $MgCl_2$, dNTPs, and Amplitaq™ DNA polymerase (0.5 U/reaction; Perkin-Elmer, Foster City, CA). Specific primers and the PCR conditions used to generate target cDNA fragments are shown in Tables 1 and 2. Products of RT-PCR were resolved on 1.5% agarose-TAE [*Tris*-acetate (40 mM), EDTA (1 mM)] gels and visualized on an UV-transilluminator following ethidium bromide staining. Representative RT-PCR products from each primer were excised from agarose gels, subcloned and subjected to dideoxy chain termination sequencing (Applied Biosystems, Model 373A Automated Sequencer, OSU Recombinant DNA/Protein Resource Facility). The identity of each product was confirmed in a sequence homology analysis using the Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990).

Whole mount immunohistochemistry

In vitro produced bovine blastocysts and hatched blastocysts in a polystyrene dish (4-well multidish; Nunclon) were washed in PBS and fixed in 4%

paraformaldehyde overnight at 4°C. Fixed embryos were washed thrice with PBS and permeabilized in PBS containing 0.1% Triton X-100 (PBST) at room temperature for 30 min. Embryos were then incubated in blocking solution [PBST containing BSA (10 mg/ml)] for 1 hr. Embryos were incubated with the polyclonal primary antibody (either RARα or RARγ2) (Affinity Bioreagents®, Golden, CO) at a 1:500 dilution in blocking solution overnight at 4°C. Embryos were washed 5 times in PBST with the last wash done overnight at 4°C. Detection of RARα and RARγ primary antibody was performed using a goat anti-rabbit IgG conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratory, Gaithersburg, MD), diluted at 1:500 in blocking solution, and incubated overnight at 4°C. Embryos were again washed 5 times in PBST with the last wash done overnight at 4°C. Embryos were then transferred to a dish containing diaminobenzidine tablets (Sigma Chemical Co) dissolved in 15 ml of water. Embryos were frequently observed for the appearance of a reddish brown color. The color reaction was stopped by moving the embryos to water.

Results

Bovine in vitro embryo production

In vitro fertilization and production of bovine embryos has been conducted in our laboratory on a weekly basis since October 1998. Our cleavage rates have ranged from 60-70% of all fertilized oocytes. Blastocyst production rates have

ranged between 35 and 40% of all presumptive zygotes. Our first in vitro fertilized embryo transfer resulted in the birth of a normal, healthy calf in August 1999.

Presence of Transcripts for RBP and RARs in the Early Bovine Embryo

Primer sequences and PCR conditions used to amplify RBP, RARα, RARγ and GAPDH are given in Tables 1 and 2. Products of the predicted size (Table 1) were detected for each target cDNA (Figs 1-4). The identity of the PCR products was verified by sequence analysis. The isolated bovine cDNA sequences exhibited a very high (90-96%) homology to published mouse and human RBP, RARα and RARγ cDNA sequences.

Transcript for GAPDH was detected at all stages of embryo development examined (Fig. 1) suggesting that RNA populations suitable for RT-PCR amplification of specific cDNAs were produced. Although methods employed in this study are not quantitative, under similar conditions, the level of the message was apparently lower at the 16 to 20-cell stage than at other times examined, and was elevated again at the morula stage. Disappearance of the message between the 8 to 16-cell and morula stages suggests that utilization and/or degradation of all maternally-derived transcripts had occurred followed by reappearance at the initiation of transcription from the embryonic genome. This is in agreement with the model for transition from maternal to embryonic genome control in the bovine occurring around the 8 to 16-cell stage (Telford et al., 1990).

Transcripts for RARα and RARγ (Fig. 2) were also detected in all stages from the 2-cell embryo through to the hatched blastocyst. On the other hand,

expression of RARβ was not detected at any stage examined (data not shown). Using these same primers, we have previously shown that RARβ message to be present in bovine tissue (Malayer and Woods, 1998). As in the case of GAPDH expression, the level of the message was apparently lower at the 16 to 20-cell stage than at other times examined, and elevated again at the morula stage. While expression of RARα was apparently consistent across stages examined, expression of RARγ was low from the 2-cell through blastocyst stages and increased in the hatched blastocyst. Expression of mRNAs for RARα, RARγ suggests that RA is likely to be able to regulate gene expression during preattachment development in the bovine through nuclear receptor-mediated pathways.

Transcript for RBP (Fig. 3) was also detected in all stages from the 2-cell embryo through to the hatched blastocyst. Again, the level of the message was apparently lower at the 16 to 20-cell stage than at other times examined, and elevated again at the morula stage. Expression of the RBP gene in the preattachment embryo suggests the capacity for intercellular binding and transport of retinol. Transcripts for RARα, RARγ, and RBP were also identified in immature oocytes (Fig. 4).

Presence of RAR proteins in the Early Bovine Embryo

Presence of the transcript does not necessary result in protein expression. Employing whole mount immunohistochemistry, we were able to detect immunoreactive RARα and RARγ2 proteins in the trophectoderm and the inner

cell mass of blastocysts, and hatched blastocysts (Figs. 5 and 6). Immunostaining for RARα appeared heavier in the inner cell mass and lighter in trophectoderm of intact blastocysts (Fig. 5). In the hatched blastocysts, staining of RARα immunoreactive protein appeared similar in the inner cell mass and trophectoderm. Immunostaining for RARγ2 appeared similar in the inner cell mass and trophectoderm of intact blastocysts as well as hatched blastocysts (Fig. 6). In the mouse, RARγ2 is the isoform expressed during early embryogenesis (Kastner et al., 1990). These results suggest that, at least in the blastocyst stages, expression of transcripts for these receptor molecules translates into protein expression. Further, RA may exert effects at the level of the nuclear RARs in both inner cell mass and trophectoderm target cells.

Discussion

It has been shown that vitamin A administered to the dam improved pregnancy rates and/or embryo quality. Retinol administered to cattle on the first and last day during a five day superovulation regimen increased the number of high quality embryos without any significant increase in ovulation rates in comparison to control animals (Shaw et al., 1995). Similar increases in embryo survival rate have been demonstrated in mice (Chew and Archer, 1983), rabbits (Besenfelder et al., 1993), swine (Brief and chew, 1983; Britt et al., 1992; Coffey and Britt, 1993) and sheep (Eberhardt et al., 1999) following administration of retinol or β-carotene. It remains unclear whether these effects were the result of

either a direct effect on the embryo or an indirect effect through stimulation of the secretion of embryotrophic factors by the oviduct and/or uterine endometria. Even though the importance of vitamin A to developmental biology has been known for decades, its mechanism of action came to light only in 1987. In the same year, two laboratories (Giguere et al., 1987; Petkovich et al., 1987) independently identified a nuclear retinoid receptor, RARα1. Later, two more receptors, RARβ and RARγ were identified and cloned. Three years later, another family of nuclear retinoid receptors, the RXRs, were identified (Mangelsdorf, 1990).

The retinoid signalling pathway has been very extensively researched in the mouse with emphasis on its role after gastrulation (Gudas, 1994). However, there is not much information in other vertebrate lineages, especially the domestic animals. The one exception to this is perhaps the pig (Harney et al., 1994; Yelich et al., 1997, Parrow et al., 1998). Further, most of the available information is concentrated towards advanced stages of embryonic development with very little known concerning the preimplantation stages. Given that the trophoblast is the first differentiated tissue in the preimplantation embryo (Pederson, 1986) and RA being a potent differentiating agent, possibilities exist for RA to perform a crucial role in cellular differentiation processes during early stages of development. Keeping this in mind, in the present study we have addressed the importance of vitamin A in preattachment embryo development more specifically by hypothesizing the existence of the binding protein and nuclear receptors that constitute the retinoid signalling pathway.

Retinol is believed to serve as a potent morphogen during early embryonic development in vertebrates (Kraft et al., 1994; Horton and Maden, 1995); however excess can lead to embryonic anomalies (Horton and Maden, 1995). A specific protein, RBP, is believed to bind retinol and serve as a transport system for retinol from the plasma to its target cell (Blomhoff, 1990). Following entry into the cell, retinol is first oxidized to retinaldehyde by alcohol dehydrogenases, then again oxidized to the active form, retinoic acid, by aldehyde dehydrogenases (Gregg Duester, 1997). Retinoic acid is then believed to bind with nuclear RARs and, along with other specific proteins, form a complex that binds to specific retinoic acid response elements on the DNA (Langston et al., 1997). This transcriptional control has been linked to several important homeobox genes, growth factor genes, and other genes important in developmental processes (De Luca et al., 1991; Malcolm Maden, 1994). In the present study, mRNA for RBP was detected in all stages of bovine preimplantation embryos produced in vitro beginning from the oocyte all the way through hatched blastocysts (Figs. 3 and 4). Previously, in situ hybridization and immunohistochemistry studies have revealed the presence of the transcript for RBP and immunoreactive protein, respectively, in day 13 tubular conceptuses (Liu et al., 1993). In the same study, day 13 spherical conceptuses apparently did not synthesize RBP. RT-PCR is a very sensitive technique and is capable of detecting low abundance transcripts that may be beyond the detection power of in situ hybridization depending on the signal to noise ratio in the in situ assay. Similarly, RBP has been shown to be actively secreted by the day 13 elongated sheep conceptus (Liu et al., 1992) and

the elongated pig conceptus (Harney et al., 1990). Transcripts for RBP have been localized to the inner cell mass and trophectoderm of the elongating pig conceptus (Trout et al., 1991). In cattle, measurement of retinol concentrations during follicular development has shown higher concentrations to be present in healthy follicles and correlated with estradiol concentrations (Schweigert and Zucker, 1988; Schweigert et al., 1988). However, very low concentrations of retinol were detected in atretic follicles. Accordingly, these authors used retinol concentrations in follicular fluid as a measure of follicular health (Schweigert and Zucker, 1988; Schweigert et al., 1988). This finding would indicate that the immature bovine oocyte does encounter retinol under physiologically normal conditions even before fertilization. The detection of transcripts for RBP in the immature oocyte (Fig. 4) in the present study further supports these early findings. The avian oocyte takes up retinol by endocytosis (Vieira and Schneider, 1993). Retinol bound to RBP and TTR (transthyretin) along with other retinoid metabolites derived from retinol are stored in the oocytic yolk and is believed to be important during avian embryonic development (Bermudez et al., 1993; Dong and Zile, 1995).

Messenger RNA transcripts encoding two of the retinoid receptors, RARα and RARγ were detected in preimplantation bovine embryos produced in vitro from the oocyte through to the hatched blastocyst stage (Fig. 2). The presence of the transcripts encoding the different RA receptor subtypes suggests the existence of a specific retinoid signaling system for retinol/retinoic acid in bovine preimplantation embryos. This also suggests that these mRNAs are synthesized

during oogenesis and persist in the cleaving embryo up to the 16 to 20-cell stage when they are completely utilized and/or degraded and reappear after the embryonic genome becomes activated. Using immunocytochemistry we were able to identify the presence of the RARα and RARγ2 subtype receptor proteins in bovine blastocysts. These receptors are present in both the inner cell mass and trophectoderm. In the mouse, extensive in situ hybridization studies have revealed the presence and the specific spatiotemporal patterns of distribution of mRNA for RARα, RARβ, RARγ at all stages of embryogenesis (Ruberte et al., 1990; 1991; Dolle et al., 1989, 1990). Similarly, there are a few reports on the expression of RARβ in the chick embryo (Noji et al., 1991; Rowe et al., 1991; Smith et al., 1991). In the pig embryo, transcripts for RARα, RARβ, RARγ have been detected in both spherical and tubular conceptuses (Yelich et al., 1997). Unfertilized *Xenopus laevis* eggs have been shown to contain functionally active RARα and RARγ (Blumberg et al., 1992). Collectively, based on the information available in different species it is likely that expression of RARs is conserved across higher vertebrates. Further, homology searches revealed greater than 90% similarity of bovine RAR sequences with human and rat homologs.

The physiological functions of these RARs have been investigated by creating homozygous mutant mice for either a particular subtype or an isotype of a subtype using homologous recombination. Interestingly, homozygous mice deficient in either RARα1 (Lufkin et al., 1993), RARβ2 or γ2 (Lohnes et al., 1993) are viable and do not exhibit any recognizable defects or malformations. The failure to observe any developmental defects in these single mutant mice can be

partially explained by the redundancy between RARs, meaning that the other RAR subtypes expressed in a given cell can compensate for a particular missing subtype. However, subsequent studies employing double mutants for both RARα and RARγ generated all of the congenital defects that have been previously reported for embryos from vitamin A deficient mothers (Lohnes et al., 1994).

Taken together, the demonstration of the presence of RBP and two RAR subtypes in the bovine preattachment embryo suggests that improved embryo quality following retinol administration resulted in part from direct action on the embryo. This effect may result from events as early as the oocyte, and is likely to result at least in part from events at the blastocyst stage, day 7 or 8 post-fertilization, if not before.

Acknowledgements

The authors thank David Goad and Thea Pratt for excellent technical assistance. We also thank the staff of Wellington Quality Meats, Wellington, KS for their assistance and generous donation of materials used in the study. The authors acknowledge the Oklahoma State University Recombinant DNA/Protein Resource Facility for the synthesis of synthetic oligonucleotides and the sequencing of cloned cDNA. This study was supported by the Oklahoma Agricultural Experiment Station.

Table 1. Sequences of primers used in RT-PCR assay

mRNA Species	Primer Nucleotide Sequence	Product Size (bp)
RBP	5'-TTCCGAGTCAAAGAGAACTTCG- 3' 5'-TCATAGTCCGTGTCGATGATCC-3'	311
RARα	5'-GCATCCAGAAGAACATGGTGT- 3' 5'-CTGCTTGGCGAACTCCACAGT-3'	392
RARβ	5'-GCAGGAATGCACAGAGAGCTAT- 3' 5'-GAAGGCCTGTTTCTGTGTCAT-3'	373
RARγ	5'-GGCATGTCCAAGGAAGCTGT- 3' 5'-GTTCTCCAGCATCTCTCGGAT-3'	795
GAPDH	5'-CCTTCATTGACCTTCACTACATGGTCTA-3' 5'-GCTGTAGCCAAATTCATTGTCGTTACCA-3'	800

Table 2. PCR conditions for amplification of GAPDH, RBP, RARα, RARβ, RARγ

mRNA Species	$MgCl_2$ (mM)	dNTPs (μM)	Primer (μM)	Annealing Temperature (°C)	PCR Cycles
GAPDH	1.5	0.1	1	60	40
RBP	1.5	0.2	0.6	60	25
RARα	1.5	0.2	1	60	25
RARβ	1.5	1.5	1.5	60	25
RARγ	2.5	0.1	1	60	25

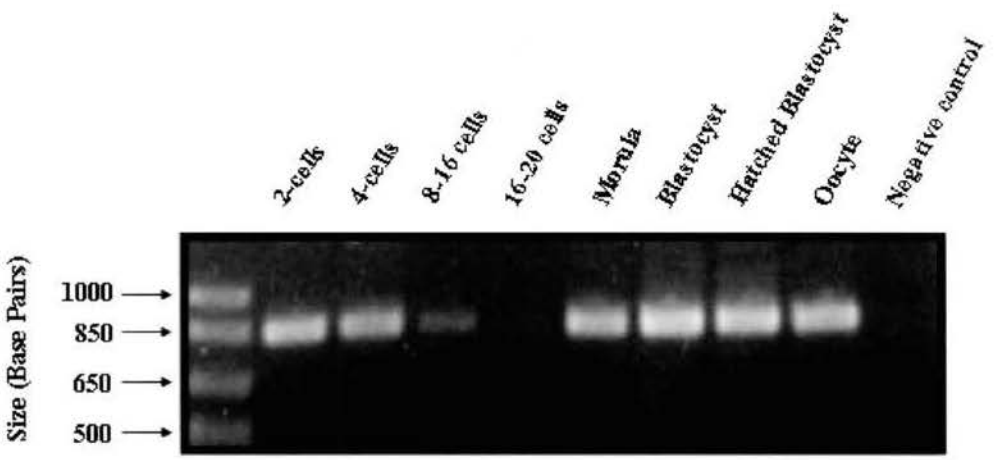

Figure 1. Expression of transcript for GAPDH (800 base pairs) using RT-PCR in 25 pooled embryos at the oocyte, 2-, 4-, 8-, 16 to 20-cell, morula, blastocyst, and hatched blastocyst stages. Negative control lane contains the product of RT-PCR under identical conditions in the absence of RNA template. Products of RT-PCR were resolved in 1.5% TAE-agarose gels and visualized by ethidium bromide staining.

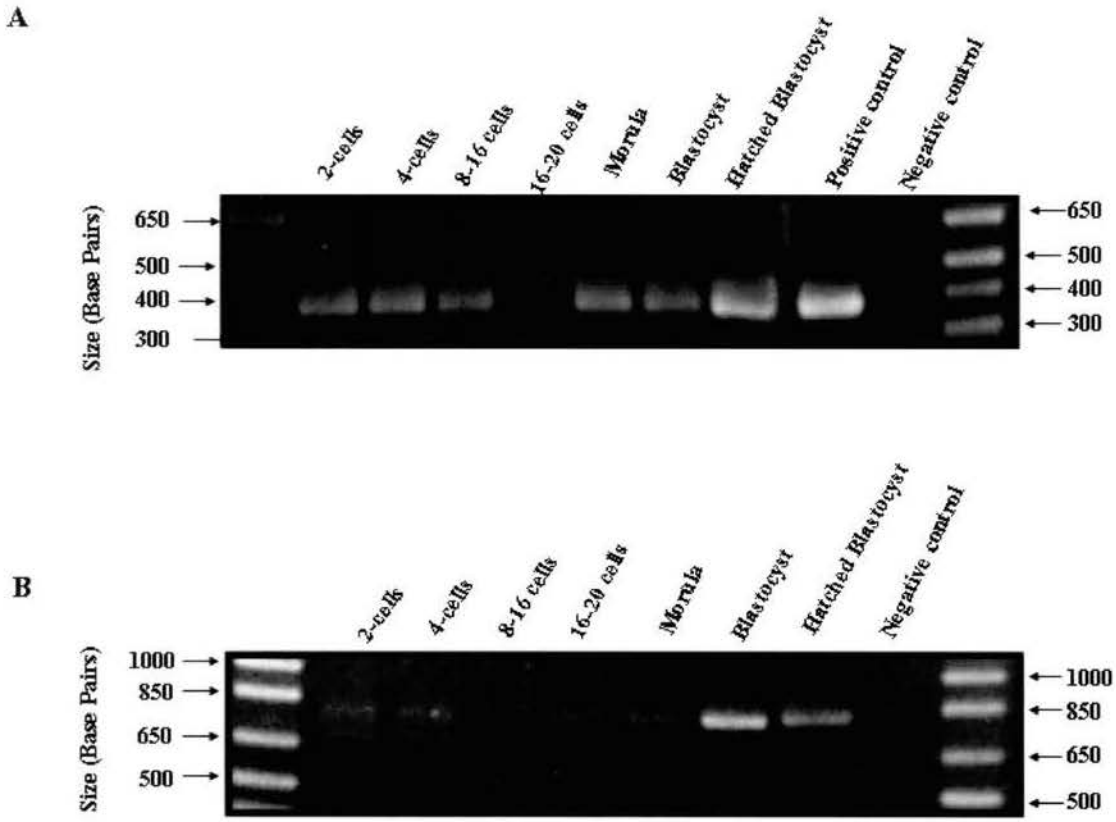

Figure 2. Expression of transcript for RAR α (392 base pairs) **(A)** and RARγ (795 base pairs) **(B)** using RT-PCR in 25 pooled embryos at the 2-, 4-, 8-, 16 to 20-cell, morula, blastocyst, and hatched blastocyst stages. Negative control lane contains the product of RT-PCR under identical conditions in the absence of RNA template. Positive control lane contains the product of RT-PCR using RNA from hatched blastocysts. Products of RT-PCR were resolved in 1.5% TAE-agarose gels and visualized by ethidium bromide staining.

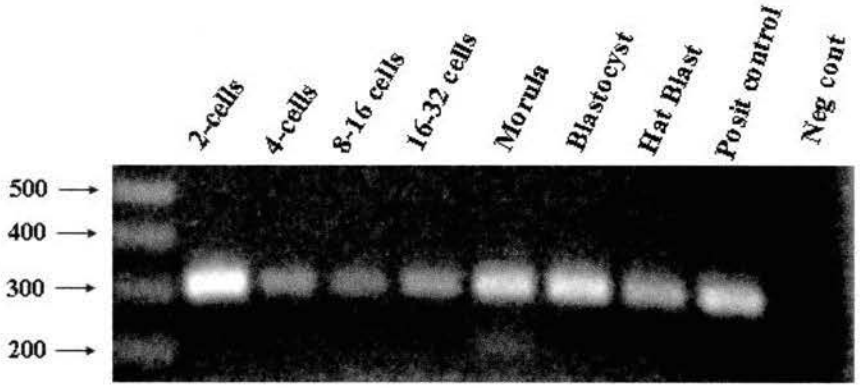

Figure 3. Expression of transcript for RBP (311 base pairs) using RT-PCR in 25 pooled embryos at the 2-, 4-, 8-, 16 to 20-cell, morula, blastocyst, and hatched blastocyst stages. Negative control lane (not shown here) contained the product of RT-PCR under identical conditions in the absence of RNA template. Positive control lane contains the product of RT-PCR using RNA from bovine uterine endometrium. Products of RT-PCR were resolved in 1.5% TAE-agarose gels and visualized by ethidium bromide staining.

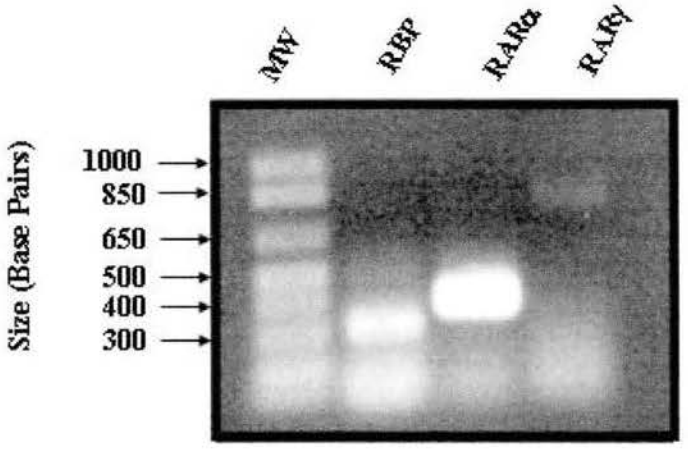

Figure 4. Expression of transcript for RBP (311 base pairs), RARα (392 base pairs) and RARγ (795 base pairs) using RT-PCR in unfertilized bovine oocytes collected at the time of in vitro fertilization from abattoir ovaries. Negative control lane (not shown here) contained the product of RT-PCR under identical conditions in the absence of RNA template. Products of RT-PCR were resolved in 1.5% TAE-agarose gels and visualized by ethidium bromide staining.

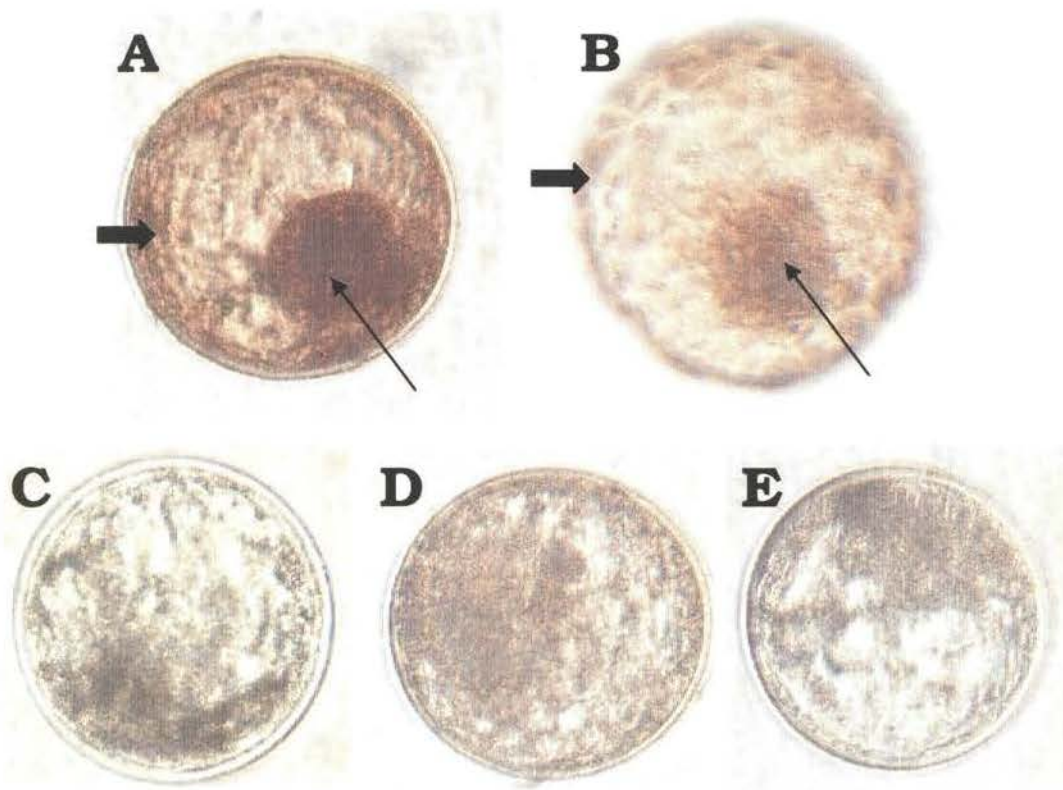

Figure 5. Whole mount immunolocalization of RARα protein in intact blastocysts and hatched blastocysts produced in vitro. In both blastocysts **(A)** and hatched blastocysts **(B)**, immunoreactive RARα was localized in both inner cell mass cells (thin arrow) and the trophectoderm (thick arrow). Control embryos were prepared with the primary antibody omitted **(C)**, secondary antibody omitted **(D)** or both primary and secondary antibody omitted **(E)**, and in all cases were devoid of immunostaining.

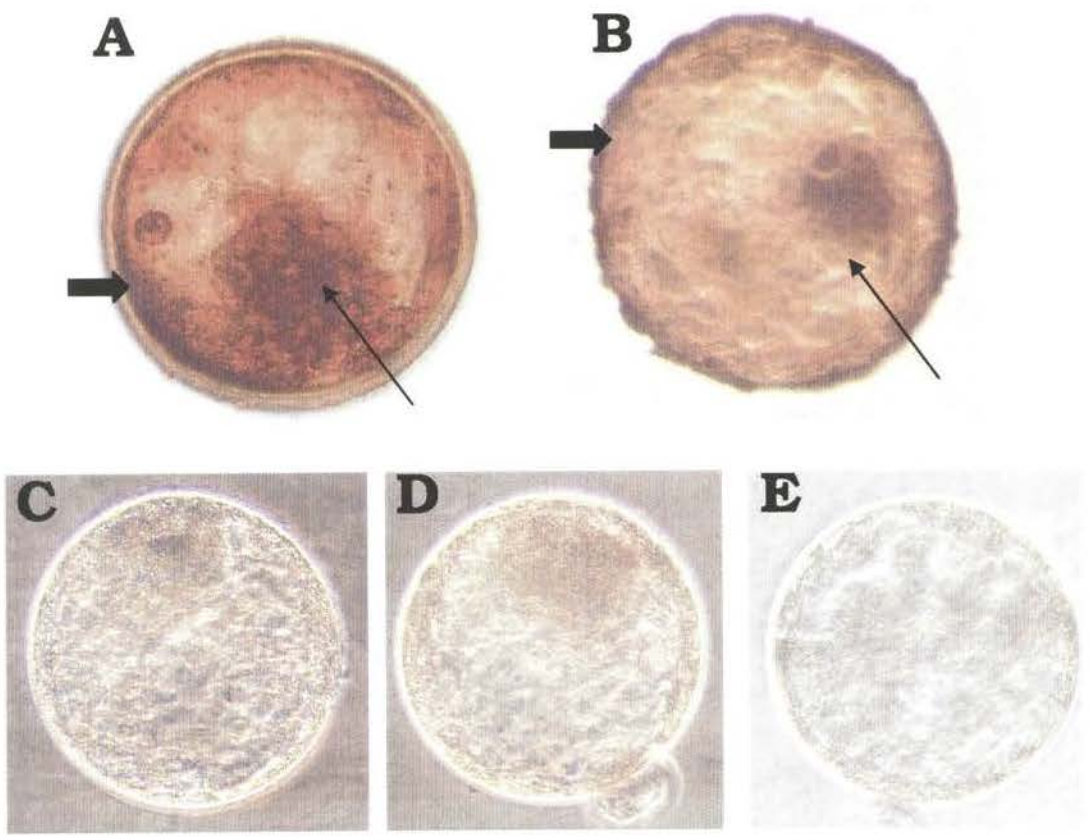

Figure 6. Whole mount immunolocalization of RARγ2 protein in intact blastocysts and hatched blastocysts produced in vitro. In both blastocysts **(A)** and hatched blastocysts **(B)**, immunoreactive RARγ2 was localized in both inner cell mass cells (thin arrow) and the trophectoderm (thick arrow). Control embryos were prepared with the primary antibody omitted **(C)**, secondary antibody omitted **(D)** or both primary and secondary antibody omitted **(E)**, and in all cases were devoid of immunostaining.

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Chapter IV

EXPRESSION PATTERNS OF RETINOID X RECEPTORS, RETINALDEHYDE DEHYDROGENASE, AND PEROXISOME PROLIFERATOR ACTIVATED RECEPTORS IN BOVINE PREATTACHMENT EMBRYOS

Abstract

In cattle, administration of retinol at the time of superovulation has been indirectly associated with enhanced developmental potential of the embryo. Vitamin A and its metabolites influence several developmental processes by interacting with two different types of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Given the limited information available concerning the RXR-mediated retinoid signaling system, particularly in species other than rodents, this study was performed to gain insight into the potential role of retinoid signaling during preattachment embryo development in the cow. Bovine embryos were produced in vitro from oocytes harvested from abattoir ovaries and frozen in liquid nitrogen at the oocyte, 2-, 4-, 8-, 16- to 20-cell, morula, blastocyst and hatched blastocyst stages. Reverse transcription polymerase chain reaction (PCR) and whole mount in situ hybridization were utilized to investigate mRNA expression for RXRα, RXRβ, RXRγ, alcohol dehydrogenase I (ADH-I), retinaldehyde dehydrogenase-2 (RALDH-2),

peroxisome proliferator activated receptor gamma (PPARγ), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Transcripts for RXRα, RXRβ, RALDH-2, PPARγ were detected in all stages beginning from the oocyte through to the hatched blastocyst. Whole mount in situ hybridization performed using digoxigenin labeled antisense probes detected all four transcripts in both inner cell mass and trophectoderm of hatched blastocysts. PCR products obtained for ADH1 exhibited very low homology to known human and mouse sequences. Immunohistochemistry was performed using polyclonal anti-rabbit antibodies against RXRβ and PPARγ to investigate if these embryonic mRNAs were translated to the mature protein. Strong immunostaining was observed for both RXRβ and PPARγ in the trophectoderm and inner cell mass cells of intact and hatched blastocysts. GAPDH mRNA was detected in all stages. Messenger RNA was not detected at any stage for RXRγ. Expression of mRNA for RXRα, RXRβ, RALDH-2, PPARγ suggests that the early embryo may be competent to synthesize retinoic acid and regulate gene expression during preattachment development in vitro.

Introduction

Apart from its crucial roles in cell growth and differentiation, vision and maintenance of epithelia, vitamin A and its physiological metabolites, collectively known as retinoids have profound effects on mammalian reproduction and embryonic morphogenesis [1]. The inclusion of adequate levels of vitamin A in

the maternal diet has been stressed because of its requirement for normal embryo development [2].

Retinol (vitamin A) metabolism in the cell can give rise to many physiologically active compounds [3]. Retinol is secreted into the circulation bound to a specific protein called retinol-binding protein (RBP), which is mainly responsible for its intercellular transport. Because retinol lacks appreciable water solubility, it is bound intracellularly to a second binding protein called cellular retinol binding protein (CRBP), which helps solubilize it in the aqueous cellular environment. Before exerting its biological effects, retinol is oxidized to retinaldehyde by a group of enzymes called retinol dehydrogenases; retinaldehyde is then oxidized to retinoic acid (RA) by a second group of enzymes called aldehyde dehydrogenases. RA and its isoforms are believed to interact with 2 separate subgroups of nuclear receptors, retinoic acid receptors (RARα, RARβ, RARγ) and retinoid X receptors (RXRα, RXRβ, RXRγ). The formation of ligand-receptor complexes will either activate or repress specific target genes by binding to specific response elements present in the vicinity of the promoter region.

Approximately a decade ago, the RXRs, the second class of nuclear retinoid receptors responsible for transduction of the differentiating properties of 9 *cis*-retinoic acid was discovered [4]. Similar to the RARs, three separate RXR genes (α, β, and γ) are known to exist. Retinoid receptors can give rise to isoforms through differential splicing or promotor usage; however, unlike the RARs, receptor isoforms have been isolated only for RXRγ. RXRs play a crucial

role in several nuclear receptor signaling pathways by homodimerizing or heterodimerizing with RARs, thyroid hormone receptors, vitamin D_3 receptors, peroxisomal proliferator activated receptors (PPAR) and a number of orphan receptors [5]. Thus, RXRs may be key players in several hormonal pathways.

The extreme sensitivity of embryonic development to vitamin A is clear; both hypovitaminosis and hypervitaminosis A can lead to abortion and embryonic malformation. Important vitamin A-mediated events occur during very early stages in the quail and around the first 2-3 wk of gestation in the human [6]. Retinol supplementation enhanced embryo survival in polytoccus species such as mice [7], rabbits [8] and swine [9]. Retinol administered at the time of superovulation increased the number of transferrable embryos in cattle [10] and increased blastocyst formation and hatching rates in sheep [11]. Recently, in the pig, administration of vitamin A to sows before ovulation enhanced embryonal survival by advancing meiotic resumption and altering follicular hormonal environment during follicle maturation [12]. Promotion of embryonic development in these studies suggested a possible direct or indirect interaction with the developing embryo. Hypothesizing a direct interaction, as a first approach, we detected the mRNAs for RBP, RARα and RARγ and the RARα and RARγ immunoreactive proteins in bovine preattachment embryos fertilized in vitro [13]. To better understand the retinoid signaling pathway, we used reverse transcription polymerase chain reaction (RT-PCR), whole mount in situ hybridization, and immunohistochemistry to characterize the expression and spatial distribution of RXRs, alcohol dehydrogenase I (ADHI), retinaldehyde

dehydrogenase 2 (RALDH-2) and PPARγ in the preattachment bovine embryo produced in vitro.

Materials And Methods

In Vitro Maturation, Fertilization, and Culture

Ovaries were collected from cows at a local abattoir and transported to the laboratory in 0.9% normal saline supplemented with penicillin-G (100 IU/ ml) and streptomycin sulfate (0.2 μg/ml) (Sigma Chemical Co., St. Louis, MO) at 26-30°C within 5 h. Oocytes were aspirated from follicles ranging in diameter from 2 to 5 mm using an 18-gauge needle into modified-PBS solution (Life Technologies Inc., Rockville Maryland). In vitro maturation, fertilization, and culture were performed according to protocols described by Mohan et al. [13].

RNA Extraction

Immature bovine oocytes and embryos at the 2-, 4-, 8-, 16 to 20-cell, morula and blastocyst stages were frozen in 250 μl of denaturing solution (4 M guanidium isothiocyanate (Promega, Madison, WI), 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-β mercaptoethanol; Sigma). Total RNA was extracted from a pool of 25 embryos at each stage according to protocols described by Mohan et al. [13].

Reverse Transcription-Polymerase Chain Reaction

In order to maximize the sensitivity of detection, RT-PCR was the method of choice for investigating gene expression from the small quantities of RNA obtained from bovine embryos. Ten microliters of the eluted total RNA was denatured by heating to 70°C and reverse transcribed in the presence of random hexamers (pdN_6; 100 pmole; Pharmacia, Piscataway, NJ, USA), dATP, dTTP, dCTP and dGTP (dNTPs, Pharmacia), $MgCl_2$, RNase inhibitor (20 U/reaction; Promega) and reverse transcriptase (Superscript™, 200 U/reaction; Gibco-BRL, Gaithersburg, MD) at 42°C for 1 hr. The RT reaction was terminated by heating to 70°C.

Reverse transcribed cDNA (1-2 μl) was denatured by heating to 95°C and subjected to PCR in the presence of picomole quantities of specific primers, $MgCl_2$, dNTPs, and Amplitaq™ DNA polymerase (0.5 U/reaction; Perkin-Elmer, Foster City, CA). Specific primers and the PCR conditions used to generate target cDNA fragments using a 2-step PCR procedure are shown in Tables 1 and 2. Because of the variation in published sequences for ADH-I, we used degenerate primers to amplify this target (Table 1). Products of RT-PCR were resolved on 1.5% agarose-TAE (40 mM Tris-acetate, 1 mM EDTA) gels and visualized on an ultraviolet transilluminator following ethidium bromide staining. Representative RT-PCR products from each primer were excised from agarose gels, subcloned, and subjected to dideoxy chain termination sequencing (Model 373A Automated Sequencer; Applied Biosystems, Foster City, CA). The identity of each product was confirmed in a sequence homology analysis using the Basic

Local Alignment Search Tool (BLAST) [14]. The analysis was repeated on 4 separate groups of embryos.

Whole Mount In Situ Hybridization

All RT-PCR products were cloned into pCR II vector (Invitrogen, Carlsbad, CA). The cDNA-containing plasmids were linearized with *Bam*HI and *Eco*RV depending on the orientation, to generate either the antisense or sense probe. The resulting fragments were phenol-choloroform extracted, ethanol precipitated and used as plasmid templates for riboprobe synthesis (Roche Diagnostics Corporation, Indianapolis, IN). In vitro transcription was performed with 1 μg of plasmid template in a final volume of 21 μl containing the digoxigenin RNA labeling mix, transcription buffer, 10 mM dithiothreitol, RNase inhibitor (1 unit), SP6 RNA polymerase or T7 RNA polymerase incubated for 2 h at 37°C. The template cDNAs were digested with RNase-free DNase (5 units) for 15 min at 37°C, and the reaction was stopped by adding 0.2 M EDTA, pH 8.0 (2μl). The riboprobes were ethanol precipitated in the presence of 4M LiCl and quantified with a series of digoxigenin labeled control RNAs according to the manufacturers instructions (Roche Diagnostics).

Day 9-10 hatched blastocysts produced in vitro were fixed overnight in 4% paraformaldehyde, washed in PBS containing 0.1% Tween- 20 (PBST), and dehydrated by an ascending methanol concentration series immediately followed by rehydration in the reverse order on ice. Rehydrated embryos were washed 3 times with PBST at room temperature. Embryos were permeabilized by 3

incubations in a cocktail of ionic and non-ionic detergents (RIPA: 150 mM NaCl, 1% Nonidet P-40, 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM Tris pH 8.0), with each incubation lasting 10 minutes. Embryos were then post-fixed with 4% paraformaldehyde/0.2% electronmicroscopy grade glutaraldehyde in PBS for 20 minutes. Prehybridization, hybridization and posthybridization were performed at 60°C for PPARγ, 65°C for RXRβ and 70°C for RXRα and RALDH-2. Embryos were washed five times in PBST and incubated for 15 minutes with a 1:1 mix of hybridization mixture (HB- 50% deionized formamide, 5X saline-sodium citrate [SSC], pH 7.0, 50μg/mL heparin, 0.1% Tween 20) and PBST followed by a brief wash with HB at room temperature. Embryos were incubated for 1-3 h in prehybridization mixture (HB containing 100 μg/ml tRNA and 100 μg/mL sheared denatured herring sperm DNA). The probes were denatured at 95 °C for 10 minutes and added to the HB mix at the following concentrations (1μg/100μl for PPARγ, RXRβ, RALDH-2 and 0.5μg/100μl for RXRα). Hybridization was carried out overnight in a box saturated with 50% formamide/5X SSC to prevent evaporation at the temperatures used for each probe. Posthybridization washes included 50% formamide in 2X SSCT (SSCT: SSC + 0.1% Tween 20) (30 min), 2X SSCT containing 0.5% SDS (2 x 15 min), 0.2X SSCT containing 1% SDS (2 x 15 min) and 0.1X SSCT containing 2% SDS (2 x 20 min).

Specific in situ hybridization signals were detected by incubation in a peroxidase conjugated anti-DIG antibody solution at a dilution of 1:100 (Roche Diagnostics) and 3,3' diaminobenzidine (DAB; Sigma) for color development. The

color reaction was stopped by moving embryos to PBS. Hatched blastocysts subjected to hybridization with the sense probe served as controls. This procedure was repeated on 4 separate groups of embryos.

Whole Mount Immunohistochemistry

In vitro produced bovine blastocysts and hatched blastocysts were washed in PBS and fixed in 4% paraformaldehyde overnight at 4°C. Fixed embryos were dehydrated in an ascending methanol series (5 min) and permeabilized in PBS containing 0.1% Triton X-100 (PBST) at room temperature for 40 min. Embryos were then incubated in blocking solution (PBST containing 1% casein) for 1 h. Embryos were incubated with the polyclonal primary antibody (either RXRβ or PPARγ: Affinity Bioreagents, Golden, CO) at a 1:500 dilution in blocking solution overnight at 4°C. Embryos were washed 5 times in PBST, with the final wash lasting 4 h. Detection of RXRβ and PPARγ primary antibody was performed using a goat anti-rabbit IgG conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersdurg, MD), diluted at 1:500 in blocking solution, and incubated for 2.5 h at room temperature. Embryos were again washed 5 times in PBST with the final wash lasting 4-5 h. Embryos were then transferred to a dish containing DAB tablets dissolved in 5 ml of water. Embryos were frequently observed for the appearance of a reddish brown color. The color reaction was stopped by moving the embryos to PBS. Control embryos were prepared by omitting primary antibody, secondary antibody or both primary and

secondary antibodies. This procedure was repeated for two separate groups of embryos.

Results

Presence of Transcripts for RXRs, RALDH-2 and PPARγ in the Early Bovine Embryo

Primer sequences and PCR conditions used to amplify RXRα, RXRβ, RXRγ, ADH-I, RALDH-2, PPARγ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are given in Tables 1 and 2. Products of the predicted size (Table 1) were detected for each target cDNA (Figures 1-3). Because mRNA was extracted from a small sample of 25 embryos, a second round of amplification was needed to visualize PCR amplicons. The identity of the PCR products was verified by sequence analysis. The isolated bovine cDNA sequences exhibited a very high (>90%) homology to published mouse and human RXRα, RXRβ, RALDH-2, and PPARγ cDNA sequences.

Transcript for GAPDH was detected at all stages of embryo development examined except the 16- to 20-cell stage (Fig. 1), suggesting that RNA populations suitable for RT-PCR amplification of specific cDNAs were produced. Although methods employed in this study are not quantitative, under similar conditions, the level of the message was apparently decreased from 2 cells to below the limit of detection in the 16- to 20-cell stage, increased to the morula stage, and remained elevated through the blastocyst stage. Disappearance of

the message between the 8- to 16-cell and the 16 to 20-cell stages suggests that utilization and/or degradation of all maternally-derived transcripts had occurred followed by reappearance at the initiation of transcription from the embryonic genome. This is in agreement with the model for transition from maternal to embryonic genome control in the bovine occurring around the 8- to 16-cell stage [19].

Transcripts for RXRα (Fig. 2A) and RXRβ (Fig. 2B) were also detected in all stages from the 2-cell embryo through to the hatched blastocyst. However, expression of RXRγ was not detected at any stage examined (data not shown). As in the case of GAPDH expression, the level of the message for both RXRα and RXRβ was apparently lower at the 16- to 20-cell stage and elevated again at the morula stage. RXRα message was very low from the 2-cell through the morula stages until the blasotcyst stage, when the transcript levels apparently increased. On the contrary, RXRβ expression was clearly evident from the 2-cell through to the 8- to 16-cell stage.

Transcripts for PPARγ (Fig. 3A) and RALDH-2 (Fig. 3B) decreased from the 2-cell to the morula stage. The level of the message was lower at the 8- to 16-cell and 16- to 20-cell stage and was undetectable at the morula stage. The message levels were elevated again at the blastocyst stage. PCR products obtained for ADH1 agreed in terms of the predicted size. Their sequence exihibited very low homology to mouse and human sequences. Transcripts for RXRα, RXRβ, RALDH-2 and PPARγ were also identified in immature oocytes (Data not shown).

In Situ Localization of Transcripts for RXRs, RALDH-2 and PPARγ

Identifying the cell type that expressed the RXR and PPAR genes was essential in determining the possible combinations of RXR homo/heterodimers that formed in the embryo during preattachment embryogenesis. RXRα transcripts were present in the trophectoderm cells and inner cell mass cells. The hybridization signal obtained with the antisense probe was much stronger than that seen with the sense probe (Fig. 4A). The sense probe, however, produced a mild background signal in the inner cell mass (Fig. 4B). RXRβ, RALDH-2 and PPARγ had a distribution similar to that of RXRα. All three transcripts were detected in both trophectoderm cells and inner cell mass cells (Fig. 4, C, E, and G). Detection was more specific compared with that of RXRα; no signal was obtained with the control sense probes (Fig. 4, D, F, and H). The hybridization signal for all 4 transcripts appeared stronger in the inner cell mass cells than in the trophectoderm cells.

Presence of RXR Proteins in the Early Bovine Embryo

Whole mount Immunohistochemistry revealed immunoreactive RXRβ and PPARγ proteins in the trophectoderm and inner cell mass of blastocysts and hatched blastocysts (Figs. 5 and 6). Both RXRβ and PPARγ immunoreactive proteins were expressed in the same region as their corresponding transcripts.

Discussion

Retinoic acid and its metabolites are believed to function as potent morphogens during early embryonic development [20]. Apart from the RARs, RXRs along with their ligand *9-cis* retinoic acid constitute a second retinoid signaling pathway, the role of which has been well dissected in the mouse, especially for later embryonic stages [21, 22]. Information on other vertebrate lineages, especially domestic animals, is very limited. Gene expression studies for RXRs and RA metabolizing enzymes have not been focussed towards preattachment embryonic stages. Preattachment embryo development in mammals involves the participation of a variety of growth factors, their receptors, cell adhesion molecules, etc. During the last 2 decades, it has become clear that these molecules fulfill a mandatory requirement in supporting the progression of embryos during the period of early preattachment development. Given that retinoids may induce cell differentiation in vitro by regulating the expression of homeobox genes, growth factors and their receptors [23], the existence of a master regulatory system involving retinoic acid seem plausible. In an earlier study, applied to in vitro fertilized preattachment bovine embryos, we detected mRNA for RBP, RARα and RARγ and the immunoreactive protein for RARα and RARγ [13]. In the present study, we investigated the importance of vitamin A during preattachment development more specifically by characterizing the expression patterns of RXRs, ADH-I, RALDH-2 and PPARγ in preattachment bovine embryos.

Although RXRβ and RXRα are ubiquitously expressed in every embryonic tissue during mouse development, RXRγ shows a restricted pattern during embryogenesis [21, 22]. In earlier studies on *Xenopus laevis*, RXRγ and RXRα were detected in unfertilized eggs and embryos until gastrulation [24]. Later, in the same species, a third RXR more closely resembling mammalian RXRβ was identified and shown to be expressed throughout early development [25]. Extending these studies to preattachment embryos of farm animals, in the present study we detected mRNA for RXRα and RXRβ in the unfertilized oocyte, indicating that these transcripts are synthesized and accumulated during oogenesis. These transcripts showed a steadily declining pattern through early cleavage indicating usage or degradation until the 16- to 20- cell stage, at which time the embryonic genome gets activated. Messenger RNA for the both RXR subtypes reappeared at the morula and persisted at higher levels until the hatched blastocyst stage. In the blastocyst, both the trophectoderm and inner cell mass cells expressed both RXR subtypes (Fig. 4, A and C). The results of immunohistochemistry suggest that, at least in the blastocyst stages, expression of transcripts for RXRβ translates into protein expression (Fig. 5A). Expression of the protein may also enable *all trans* RA/9-*cis* RA to exert effects at the level of the nuclear RXRs in both trophectoderm and inner cell mass cells. Messenger RNA for RXRγ was not detected, suggesting that RXRγ may not be important during very early stages of mammalian embryo development and that its lack can be compensated by the presence of the other two RXRs. Expression of these RXR subtypes suggests some essential function for these receptors during

maturation, fertilization, early cleavage, blastocyst development and/or hatching, most likely regulation of gene expression through nuclear receptor-mediated pathways.

The functional significance of RXR expression during embryogenesis has been investigated by creating entire subtype, double and compound mutant mice. Although RXRβ knockout mice appeared morphologically normal with the exception that males were sterile [26], homozygous RXRα mutant mice died around 13.5–16.5 days post coitum [27]. Double ($RXR\beta^{-/-}/RXR\gamma^{-/-}$) and triple ($RXR\alpha^{+/-}/RXR\beta^{-/-}/RXR\gamma^{-/-}$) RXR mutant mice, other than displaying marked growth deficiency and sterility in males, were postnatally normal [28]. These studies meant that early developmental processes could proceed normally provided a single copy of RXRα was available to heterodimerize with RARs and other nuclear receptors such as PPARs. Further, $RXR\alpha^{-/-}/RXR\beta^{-/-}$ mutant mice died between 9.5 and 10.5 days of gestation [29], again implicating RXRα as the main RXR during early development. Previously, we showed that the preattachment bovine embryo fertilized and cultured in vitro expressed both mRNA and the mature protein (immunoreactive) for RAR subtypes α, and γ [13]. This expression suggests possibilities for a functional interaction between RARs and RXRs in transduction of the retinoid signal around this critical period of development. Detailed analysis of RAR/RXRα double-null mutant phenotypes revealed that RXRα/RAR heterodimers are the most common functional units responsible in transduction of the retinoid signal during embryogenesis [30]. Messenger RNA for RXRα is strongly expressed in the placenta in the mouse [31] and human

[32]. Abnormalities of the chorioallantoic placenta have been the hallmark of RXRα$^{-/-}$ homozygous [33] and RXRα$^{-/-}$/RXRβ$^{-/-}$ compound mutant mice [29] suggesting a critical role for RXRα during placentation. During the formation of the blastocyst, retinoid signaling utilizing RXRα may contribute to the process of differentiation as loss of RXRα alters morphological endodermal differentiation of F9 cells [34].

Presence of the mRNA and protein for the nuclear receptors of the retinoid superfamily prompted us to investigate if the preattachment bovine embryo was capable of synthesizing retinoic acid, the biologically active ligand for retinoid receptors. ADH-I and ADH-IV along with several other enzymes, i.e., 3 forms of microsomal retinol dehydrogenase and short chain dehydrogenase/reductases, can oxidize all *trans*-retinol to all *trans*-retinal [35-38]. We investigated gene expression for ADH-I using degenerate primers in a nested PCR. Despite obtaining a PCR product for ADH-1 very close to the required size in all preattachment stages examined, homology searches revealed extremely low similarity to known alcohol dehydrogenases. Therefore, the conversion of retinol to retinaldehyde in bovine embryos needs further scrutiny. Retinaldehyde generated from retinol is oxidized to RA by another group of enzymes called aldehyde dehydrogenases (ALDHs) [39], among which ALDH-I is known to efficiently perform this function [40]. Recently, RALDH-2, a NAD-dependant dehydrogenase known to exhibit the greatest specificity for retinaldehyde, was cloned and its expression patterns characterized throughout embryogenesis [41]. RALDH-2 is essential for embryo survival and morphogenesis in the mouse, and

its complete absence or knockout results in embryonic mortality around midgestation [42]. Transcripts for RALDH-2 also occur in immature bovine oocytes. Although not quantitative, the message level started to diminish as early cleavage progressed, suggesting that these transcripts were either translated or degraded, and then disappeared but reemerged at the morula stage and remained constant all the way through to the hatched blastocyst stages. Using in situ hybridization, we localized mRNA for RALDH-2 to both the inner cell mass and trophectoderm in the present study (Fig. 4E). The bovine unfertilized oocyte does encounter retinol due its presence in higher concentrations in healthy follicles [43-44]. The bovine oocyte also contains transcripts for RBP, which may aid in the uptake of retinol in the follicle [13]. These observations and the detection of RALDH-2 in the present study suggest possible retinoic acid synthesis and, together with the presence of nuclear receptors, the presence of a retinoid signaling pathway from the oocyte through the hatched blastocyst stage.

Because PPARγ is a well established heterodimeric partner for RXRs, we also examined the embryonic expression and localization of this isoform of PPAR using PCR, in situ hybridization and immunohistochemistry. The PPAR family of nuclear receptors, which consist of α, β, and γ isoforms, has received much attention mainly because of its role in the regulation of lipid and glucose metabolism [45]. The 3 isoforms, which are encoded by different genes, differ in their metabolic effects, tissue specific expression, and response to pharmacological agents. PPARγ is specifically expressed in adipose tissue and acts to supresss adipocyte differentiation. It has two isoforms, γ1 and γ2, which

are derived from the same gene through differential splicing and promotor usage. PPARγ was given attention in this study because it is essential for placental development and differentiation in the mouse [46] and is expressed in syntiotrophoblasts and cytotrophoblasts in human placental villi [47], suggesting a similar function. We detected mRNA encoding for PPARγ in all stages of preattachment development in vitro, from the 2-cell to the hatched blastocyst. Transcripts were also detected in the oocytes, suggesting a likely role during in vitro maturation and fertilization. Expression of PPARγ would indicate the presence of a heterodimeric partner for RXRs during preattachment development. With the exception of PPARγ, mRNA encoding PPARα, β have been detected in *Xenopus* oocytes and embryos [48].

All three isoforms of PPAR can be activated by many of the same ligands and bind to the same peroxisome proliferator response element in the promoter regions of their target genes. They exert similar influence on transcriptional regulation of several enzymes involved in fatty acid oxidation in vitro [49]. Under in vitro conditions, all 3 PPAR subtypes can interact with either RXRα, RXRβ, or RXRγ [50, 51]. Thus, under specific conditions regulation of PPAR target gene transcription is contingent on its heterodimerization with RXRs. The occurrence of PPARγ-RXRα/RXRβ heterodimer can be expected because in the present study PPARγ mRNA was found to be coexpressed with RXRα and RXRβ in both trophectoderm and inner cell mass cells (Fig. 4 A, C, and G). The immunolocalization of PPARγ and RXRβ protein in both trophectoderm and inner

cell mass cells (Figs. 5 and 6) indicates that at least in the blastocyst these proteins are available for interaction.

The simultaneous expression of RARs [13], RXRs and RALDH-2 provides a compelling argument for the existence of a functional retinoid signaling pathway and support for the hypothesis that the transduced signal functions in the regulation of a subset of genes important during preattachment development in the cow. Providing more support to this argument is the presence of significant concentrations of retinoic acid in day 10 spherical blastocysts and trophectoderm cell lines in the pig, as revealed by HPLC and reporter assays [52]. A specific enzyme namely, 9-*cis* retinol dehydrogenase [53] can oxidize 9-*cis* retinol to 9-*cis* retinaldehyde, which may then be oxidized to 9-*cis* RA, a specific ligand that can activate RXRs. However, nothing is known about the activity of this enzyme in bovine preattachment embryos. In *Xenopus* embryos, RXRs respond to various natural vitamin A metabolites, including RA, albeit at higher concentrations than those of RARs [24]. In the *Xenopus* study, presence of both RARs and RXRs in a single cell was advantageous because it gave the embryo an option to respond to a shallow gradient of RA through differential activation of either RARs or RXRs. Whether the expression of RXRs and RARs along with PPARγ would encourage homodimerization or heterodimerization, thereby activating some putative retinoid responsive target genes during preattachment development in the cow, is largely unknown and needs future attention.

Acknowledgements

We thank David Goad and Joe Donnenhoffer (Roche Diagnostics) for excellent technical assistance and the staff of Wellington Quality Meats (Wellington, KS) for their assistance and generous donation of materials used in the study. We acknowledge the Oklahoma State University Recombinant DNA/Protein Resource Facility for the synthesis of synthetic oligonucleotides and the sequencing of cloned cDNA.

Table 1. Sequences of primers used in RT-PCR assay

mRNA Species	Primer Nucleotide Sequence	Product Size (bp) and Reference
RXRα	5'-GGACACCAAACATTTCCTGCC- 3' 5'-GATGTGCTTGGTGAAGGAAGCC-3'	415 (15)
RXRβ	5'-GGCTGGCAAACGGCTA- 3' 5'-TGGCCAGGCACTTCTG-3'	207 (15)
RXRγ	5'-GCAGGAATGCACAGAGAGCTAT- 3' 5'-GAAGGCCTGTTTCTGTGTCAT-3'	373 (16)
PPARγ	5'-TCCGTGATGGAAGACCACTC- 3' 5'-CCCTTGCATCCTTCACAAGC-3'	332 (17)
RALDH-2	5'-TCCCTGTCTGTAATCCAGCCAC- 3' 5'-GAAAGCCAGCCTCCTTGATGAG- 3'	531
ADH1	5'- GAGGATCCGAGGATATAGAAGTTGCACC-3' 5'- GATCTAGACCNACNCCNCCNARNCCRAA-3' 5'- GAGGATCCGGNRTNGTNGARAGYRTNGG-3' 5'- GATCTAGACCRWANCCNGTNGANAHNCC-3'	350 (18)
GAPDH	5'-CCTTCATTGACCTTCACTACATGGTCTA-3' 5'-GCTGTAGCCAAATTCATTGTCGTTACCA-3'	800

Table 2. PCR conditions for amplification of GAPDH, RXRα, RXRβ, RXRγ, PPARγ

mRNA Species	$MgCl_2$ (mM)	dNTPs (μM)	Primer (μM)	Annealing Temperature (°C)	PCR Cycles	
					PCR I	PCR II
GAPDH	1.5	0.1	1	60	10	20
RXRα	2.5	0.5	1.5	64	25	25
RXRβ	1	0.5	0.5	50	20	20
RXRγ	1.5	1.5	1.5	60	25	25
PPARγ	1	0.5	1.5	55	25	25
RALDH-2	1	0.5	0.5	60	25	25
ADH1	1	0.25	0.5	48 & 50	20	25

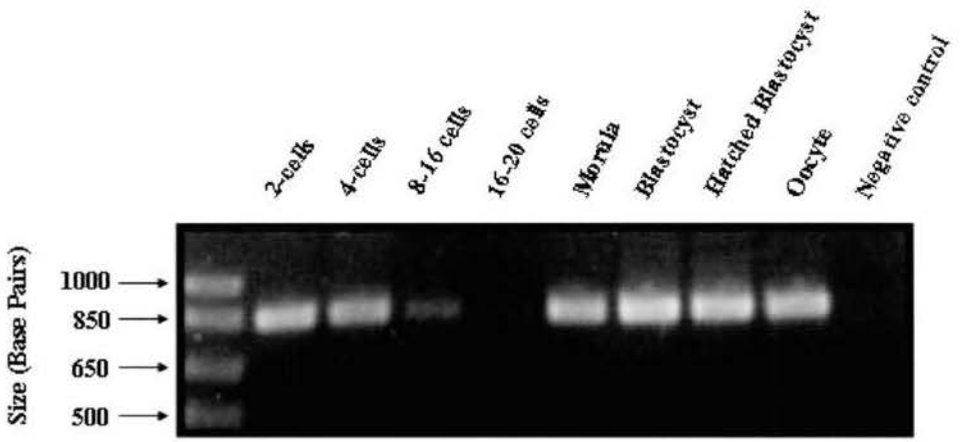

FIG 1. Expression of transcript for GAPDH (800 base pairs) using RT-PCR in 25 pooled embryos at the oocyte, 2-, 4-, 8-, 16 to 20-cell, morula, blastocyst, and hatched blastocyst stages. Negative control lane contains the product of RT-PCR under identical conditions in the absence of RNA template. Products of RT-PCR were resolved in 1.5% TAE-agarose gels and visualized by ethidium bromide staining.

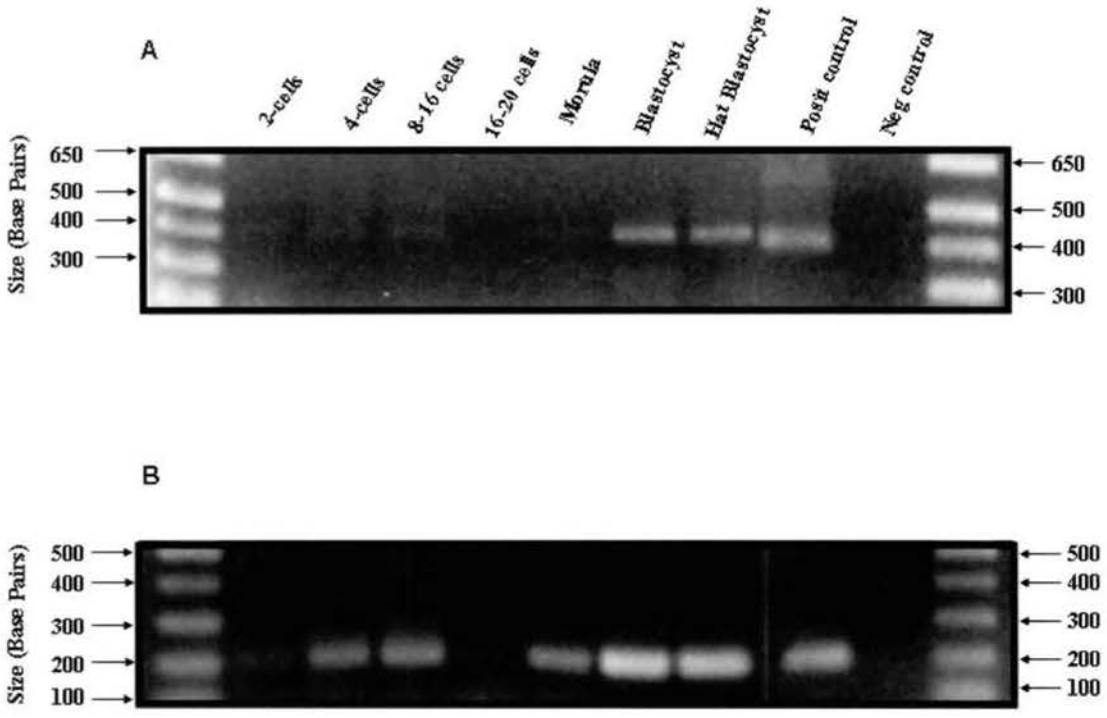

FIG 2. Expression of transcript for RXRα (415 base pairs) (**A**) and RXRβ (207 base pairs) (**B**) using RT-PCR in 25 pooled embryos at the 2-, 4-, 8-, 16 to 20-cell, morula, blastocyst, and hatched blastocyst stages. Negative control lane contains the product of RT-PCR under identical conditions in the absence of RNA template. Positive control lane contains the product of RT-PCR using RNA from hatched blastocysts. Products of RT-PCR were resolved in 1.5% TAE-agarose gels and visualized by ethidium bromide staining.

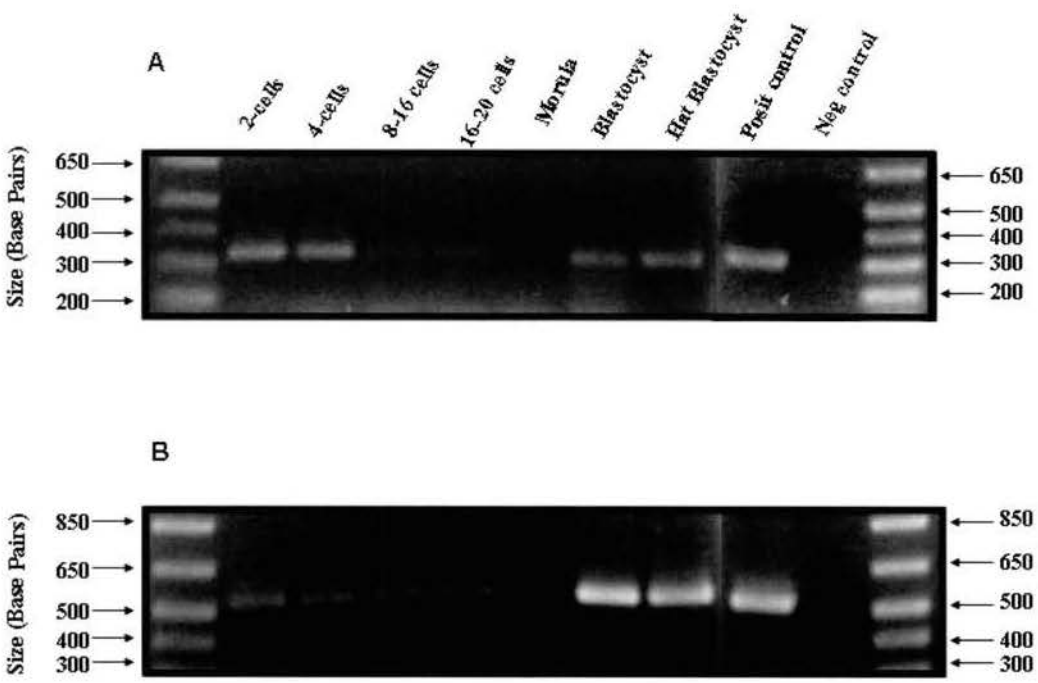

FIG 3. Expression of transcript for PPARγ (332 base pairs) (**A**) and RALDH-2 (531 base pairs) (**B**) using RT-PCR in 25 pooled embryos at the 2-, 4-, 8-, 16 to 20-cell, morula, blastocyst, and hatched blastocyst stages. Negative control lane contains the product of RT-PCR under identical conditions in the absence of RNA template. Positive control lane contains the product of RT-PCR using RNA from hatched blastocysts. Products of RT-PCR were resolved in 1.5% TAE-agarose gels and visualized by ethidium bromide staining.

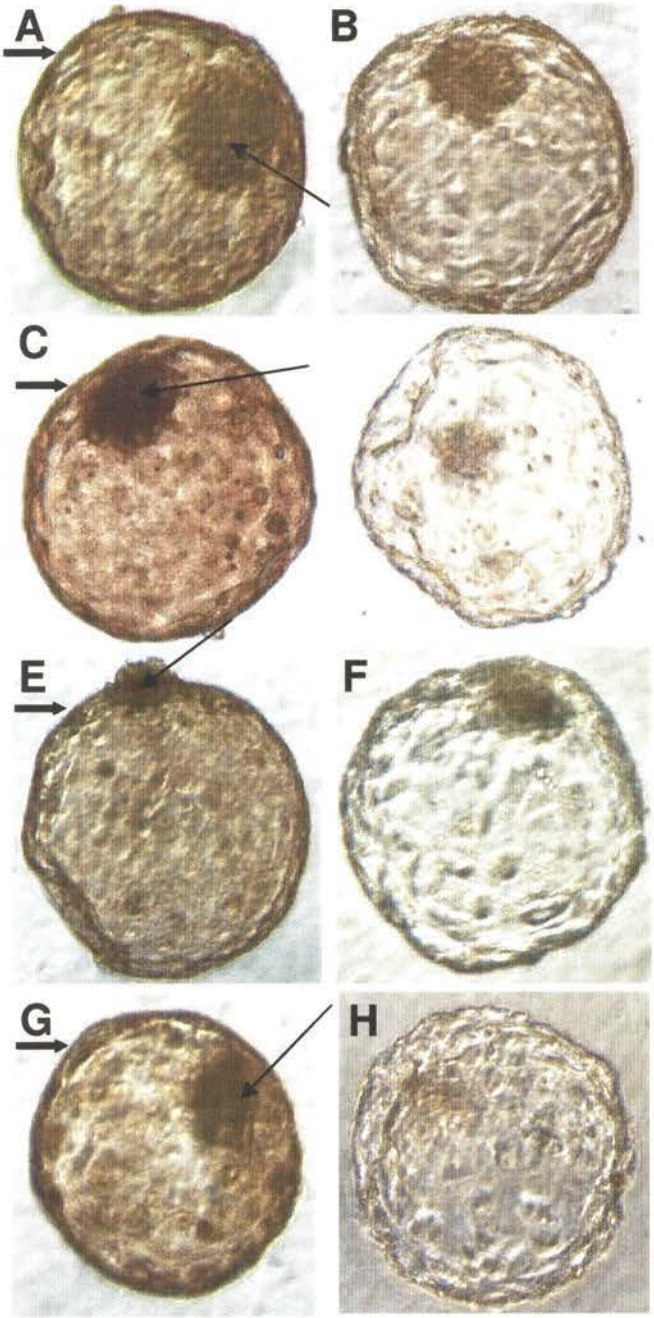

FIG 4. Whole mount in situ hybridization with the following digoxigenin-labeled bovine cRNA probes: RXRα antisense (**A**), RXRα sense (**B**), RXRβ antisense (**C**), RXRβ sense (**D**), RALDH-2 antisense (**E**), RALDH-2 sense (**F**), and PPARγ antisense (**G**), PPARγ sense (**H**) was performed on hatched blastocysts fertilized and cultured in vitro. RXRα antisense probe (**A**) demonstrated a much stronger signal in the trophectoderm (thick arrow) and inner cell mass cells (thin arrow), than in controls hybridized with a sense RXRα probe (**B**). RXRβ (**C**), RALDH-2 (**E**) and PPARγ (**G**) mRNA was expressed in the trophectoderm (thick arrow) and inner cell mass cells (thin arrow). Hybridization with the sense probe for RXRβ (**D**), RALDH-2 (**F**) and PPARγ (**H**) was devoid of staining.

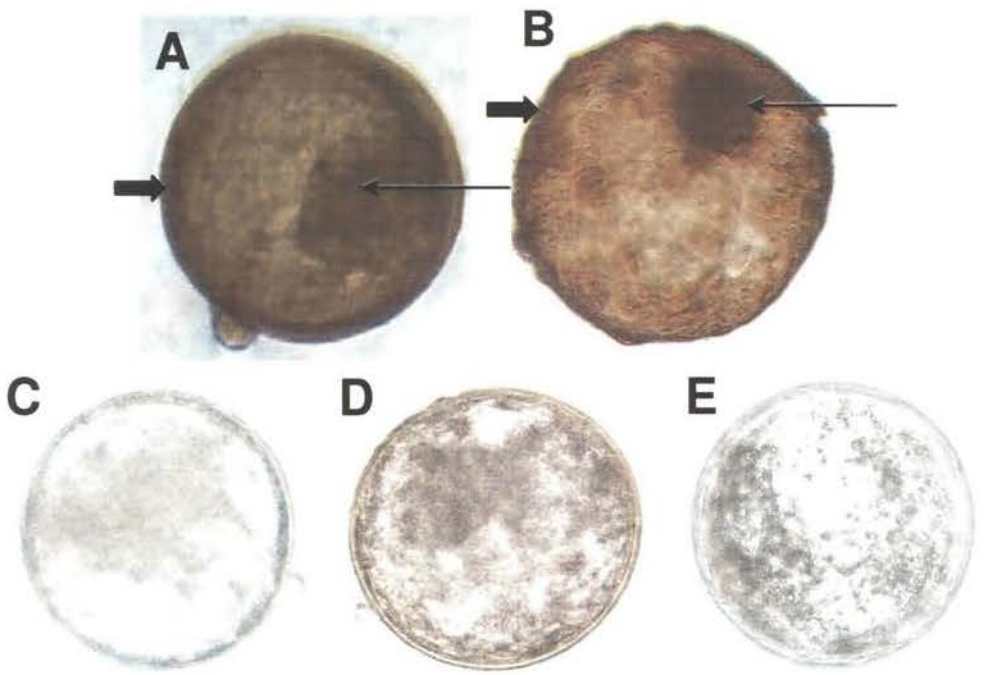

FIG 5. Whole mount immunolocalization of RXRβ protein in intact blastocysts and hatched blastocysts produced in vitro. In both blastocysts **(A)** and hatched blastocysts **(B)**, immunoreactive RXRβ was localized in both inner cell mass cells (thin arrow) and the trophectoderm (thick arrow). Control embryos were prepared with the primary antibody omitted **(C)**, secondary antibody omitted **(D)** or both primary and secondary antibody omitted **(E)**, and in all cases were devoid of immunostaining.

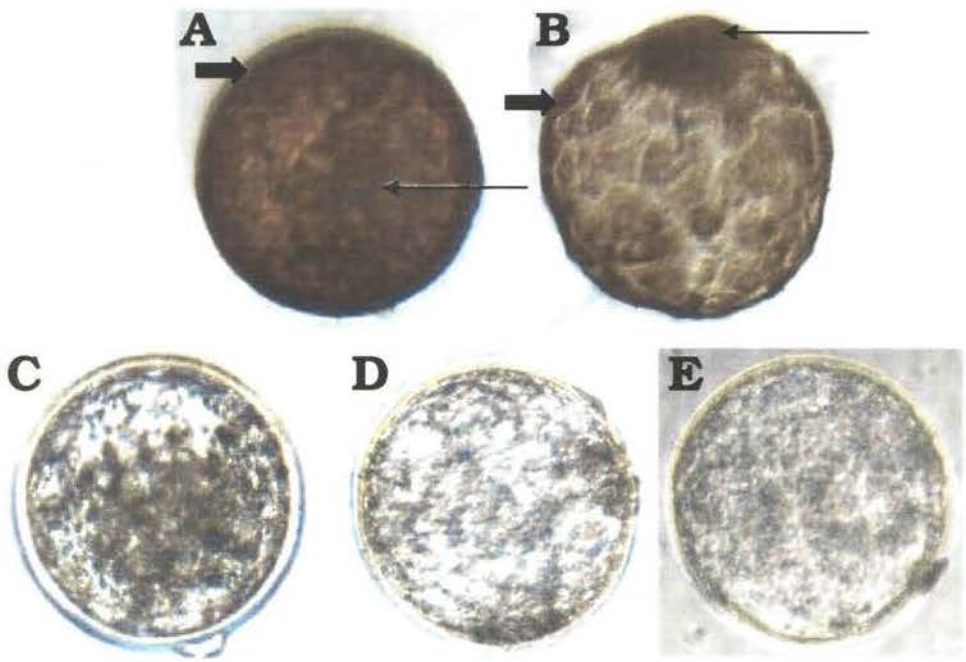

FIG 6. Whole mount immunolocalization of PPARγ protein in intact blastocysts and hatched blastocysts produced in vitro. In both blastocysts (**A**) and hatched blastocysts (**B**), immunoreactive PPARγ was localized in both inner cell mass cells (thin arrow) and the trophectoderm (thick arrow). Control embryos were prepared with the primary antibody omitted (**C**), secondary antibody omitted (**D**) or both primary and secondary antibody omitted (**E**), and in all cases were devoid of immunostaining.

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Chapter V

BOVINE CUMULUS-GRANULOSA CELLS CONTAIN BIOLOGICALLY ACTIVE RETINOID RECEPTORS THAT CAN RESPOND TO RETINOIC ACID

Abstract

Retinoids, a class of compounds which include retinol and its metabolite, retinoic acid, are absolutely essential for ovarian steroid production, oocyte maturation, and early embryogenesis. Previous studies have detected high concentrations of retinol in bovine large follicles. Further, administration of retinol in vivo and supplementation of retinoic acid during in vitro maturation results in enhanced embryonic development. In the present study, we hypothesized that retinoids administered either in vivo or in vitro interacts with both the cumulus-granulosa cells and the oocyte. Total RNA extracted from in vitro cultured cumulus-granulosa cells was subjected to reverse transcription polymerase chain reaction (RT-PCR) and mRNA expression for RBP, RARα, RARβ, RARγ, RXRα, RXRβ, RALDH-2, and PPARγ. Transcripts were detected for RBP, RARα, RARγ, RXRα, RXRβ, RALDH-2, and PPARγ. Expression of RARβ was not detected in cumulus cells. The biological acitivity of these endogenous retinoid receptors was tested using a transient reporter assay using the pAAV-MCS-βRARE-Luc vector. Addition of 0.5 and 1μM all-*trans* retinoic acid significantly ($P < 0.05$) increased

the activity of the pAAV-MCS-βRARE-Luc reporter compared to cells transfected with the control reporter lacking a retinoic acid response element. Addition of 5 or 10 μM all-*trans* retinol stimulated a mild increase in reporter activity, however, the increase was not statistically significant. Based on these results we conclude that cumulus cells contain endogenously active retinoid receptors and may also be competent to synthesize retinoic acid using the precursor, retinol. These results also indirectly provide evidence that retinoids administered either in vivo or in vitro may have exerted a receptor mediated effect on cumulus-granulosa cells.

Introduction

Retinoids, which include vitamin A and its active metabolites, are unstable hydrophobic compounds essential for cell growth and differentiation (Blomhoff, 1994) and more importantly, for embryonic and placental development (Bavik et al., 1996). Various retinoid binding proteins such as the 21 kDa plasma retinol binding protein (RBP), cellular retinol binding protein (CRBP-I & II) and cellular retinoic acid binding proteins (CRABP-I & II) both of ~16 kDa molecular weight, exist in the cell. RBP is extracellular and functions in the intercellular transport of retinol. On the other hand, CRBP-I & II functions in the intracellular transport of retinol and its metabolism to retinoic acid. CRABP-I & II not only regulates retinoic acid availability to retinoic acid receptors but also modulates its metabolism (Napoli, 1996). Biologically active retinoids mediate their effects on target cells through binding to two sets of nuclear receptors, namely, retinoic acid

receptors (RARs) and retinoid X receptors (RXRs), that are members of steroid/thyroid hormone nuclear receptor superfamily. Both RARs and RXRs have three subtypes, α, β, γ. Ligand-bound RARs and RXRs function as transcription factors by binding to *cis*-acting DNA sequences called retinoic acid response elements (RAREs). RAREs comprise directly repeated hexameric half-sites with consensus sequences (5'-PuG(G/T)TCA-3') and are located within the transcriptional regulatory regions of target genes and facilitate transcriptional regulation of these genes (Xiao et al., 1995). The first step in the synthesis of retinoic acid is the oxidation of retinol to retinaldehyde by alcohol dehydrogenases (Clagett-Dame and DeLuca, 2003). Both medium and short chain retinol dehydrogenases can perform this function. The next step involves the oxidation of retinaldehyde to retinoic acid by aldehyde dehydrogenases (Clagett-Dame and DeLuca, 2003). Several aldehyde dehydrogenases (ALDH) including three NAD-dependant enzymes specific for retinaldehyde called RALDH-1, -2 and -3, have been isolated and characterized (Clagett-Dame and DeLuca, 2003).

We had earlier shown that both immature oocytes and the early preattachment bovine embryo, from the 2-cell to the hatched blastocysts, express mRNA for RBP, RARα & γ, RXRα & β, and RALDH-2 (Mohan et al., 2001, 2002). In addition, we also detected the immunoreactive protein for RARα, γ2 and RXRβ in both inner cell mass and trophectoderm cells of intact and hatched blastocysts. Recently, Duque et al. (2002) showed that addition of 5nM 9-*cis* retinoic acid (9-*cis* RA) during prematuration of cumulus-oocyte complexes

(COCs) in the presence of roscovitine improved cytoplasmic maturation and had a positive effect on blastocyst development and freeze-thaw survival rates. COCs treated with 9-*cis* RA had higher total cell numbers than untreated controls. In addition, the same authors also provided evidence to show that 9-*cis* RA induced trophectoderm differentiation, altered inner cell mass to trophectoderm cell ratio and also increased pregnancy rates following transfer of 9-*cis* RA treated day 7 blastocysts (Hidalgo et al., 2003). Based on these and our earlier studies we hypothesize that the cumulus-granulosa cells may be the predominant targets for retinoic acid added during in vitro prematuration. The objective of the present study is to investigate the presence of the retinoid signaling pathway in cumulus-granulosa cells, and retinoic acid responsiveness in cumulus-granulosa cells.

Materials and Methods

Cell Culture

Experiments were carried out using cumulus cells harvested from follicles utilized for our routine in vitro fertilization studies. Cumulus cells that were removed following in vitro maturation and at the time of fertilization were maintained in Hyclone's high glucose Dulbecco's modified Eagle's Medium (Hyclone Laboratories Inc, Logan, UT) containing 1x antibiotic-antimycotic (Sigma Chemical Company, MO), and supplemented with 10% fetal calf serum

(FCS) (Hyclone Laboratories Inc, Logan, UT). Cultures were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2 and fed every 48 h.

RNA Extraction and Reverse Transcription Polymerase Reaction

RNA extraction and reverse transcription were performed according to the methods described in Mohan et al. (2001) with the following modifications. In the present study total RNA was extracted from cumulus-granulosa cells and only one round of PCR was performed owing to the large amount of RNA available from cumulus-granulosa cells.

Reporter Plasmid Constructs

The plasmid pGL3 promotor vector (Promega Corporation, Madison, WI) contains the SV40 promoter driving expression of the firefly luciferase reporter gene. To confer retinoic acid responsiveness, a 37 bp consensus retinoic acid response element (RARE) from the human retinoic acid receptor β2 (de The et al., 1990) carrying *Kpn*1 linkers on the 5' prime end of both strands was cloned into the multiple cloning region located upstream of the SV40 promoter to produce the pGL3 promoter βRARE plasmid. The expression cassette from the pGL3 promoter RARE plasmid was isolated by cutting with the restriction enzyme *Cla*1. A second vector, pAAVMCS (4.7 kb) (Stratagene Corporation, La Jolla, CA) containing inverted terminal repeats (ITRs) was digested with *Not*1 to remove the expression cassette (1.8 kb). The remaining backbone of the pAAVMCS vector containing the ITRs (2.9 kb) was ligated to the expression

cassette of the pGL3 promoter vector to generate the final reporter construct, hereafter called pAAVMCS-pGL3-βRARE-Luc vector (Fig. 1). pAAVMCS-pGL3-βRARE-Luc vector was digested with *Kpn*1 to remove the RARE for control studies.

Transient Transfection Assays

Cumulus cells were cultured in 48-well plates (Costar, Corning Inc., Corning, NY) at a density of 35,000 cells per well in 500 μl of DMEM (Hyclone Laboratories Inc., Logan, UT) containing 5% charcoal dextran-extracted FCS, 1x antibiotic-antimycotic and 2mM glutamine (Sigma Chemical Company). At 85-90% confluency, the cells were transfected with pAAVMCS-pGL3-βRARE-Luc or control reporter vector. For each well, approximately 1 μl of the transfection reagent (Lipofectamine 2000 Reagent, Invitrogen Corp, CA) was diluted in 37 μl of serum-and antibiotic-free DMEM. DNA was then diluted at a concentration of 500 ng in 37 μl of serum-and antibiotic-free DMEM in a separate tube. The DNA was then mixed with the liposomes and the transfection mixture was incubated at room temperature for about 20 minutes for the DNA-liposome complexes to form. Before transfection, the culture media in each well was replaced with 200 μl of serum-and antibiotic-free DMEM and 74 μl of the DNA-Liposome complex was added to achieve a final volume of 274 μl. The transfection media was removed 6-8 h later and replenished with 500 μl of antibiotic-antimycotic free fresh media and the cells were allowed to recover for 24 h. Twenty four hours post-transfection the cells received one of the five treatments: all-*trans* retinoic acid

(Sigma Chemical Co) at two different concentrations (0.5 and 1 μM), 5 or 10 μM all-*trans* retinol, or alcohol vehicle. One micromolar and five micromolar concentrations were selected for all-*trans* RA and all-*trans* retinol, respectively, because these concentrations have been previously shown to enhance in vitro bovine embryo development (Duque et al., 2002; Livingston et al., 2002). The specified concentration of each ligand was added to triplicate wells and the entire assay was repeated twice. Twelve hours post-treatment, the culture medium was removed and the cells were washed with Ca^{2+}-, Mg^{2+}- containing Dulbecco's phosphate buffered saline (DPBS) (Invitrogen Corporation, CA). Luciferase assays were performed using the LucLite Luciferase Reporter gene assay kit according to the manufacturer's instructions (Perkin Elmer Life Sciences, Boston, MA). The cell lysates were transferred to a 96-well Microtiter luminescence microplates (Microlite 1+,Thermo Biosystems, Vantaa, Finland). Luminescence in each well was recorded by counting the plates on a TopCount NXT Microplate scintillation and luminescence counter (Packard Instrument Company, IL) for 30 seconds. For each treatment, luminescence recordings were obtained from the mean of triplicate wells. Luminescence measurements were normalized to the protein content and are expressed as luminescence units per microgram protein. The protein content in the cell lysate was measured using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Statistical Analysis

Luminescence measurements for all five ligand concentrations including alcohol from cells transfected with either pAAVMCS-pGL3-βRARE-Luc or the control vector were analyzed as a 2 x 5 factorial experiment with subsamples in a completely randomized design using Proc Mixed. Mean luminescence mesurements for each ligand concentration for both pAAVMCS-pGL3-βRARE-Luc and the control vector transfected cells were compared using the tukey's procedure. A probability value of $P < 0.05$ was considered significant.

Results

Reverse Transcription Polymerase Chain Reaction

Primer sequences and PCR conditions used to amplify RBP, RARα, RARγ RXRα, RXRβ, RALDH-2 and PPAR were previously described (Mohan et al., 2001; 2002). Products of the predicted size were detected for each target cDNA (Fig. 2). The identity of the PCR products was verified by sequence analysis. The isolated bovine cDNA sequences exhibited a very high (>90%) homology to published mouse and human RBP, RARα and RARγ cDNA sequences.

Out of the three known isoforms for both RARs and RXRs, the expression of two were observed in cumulus granulosa cells. A 392 bp and 795 bp product was detected for RARα and RARγ (Fig. 2), respectively. In addition to the 795 bp product corresponding to RARγ, a second product migrating between 400 and

500 bp was also detected. We have not sequenced the lower molecular weight product and therefore, it is not clear if this product represents a different isoform of RARγ or if it is the result of non-specific amplification. A 311 bp product was detected for retinol binding protein. Similarly, a 415 bp and 207 bp product was detected for RXRα, RXRβ, respectively. On the other hand, expression of RARβ and RXRγ was not detected (data not shown). A 432 bp product representing the heterodimerization partner for RXRs, namely, PPARγ was also detected in cumulus-granulosa cells. Cumulus-granulosa cells also expressed RALDH-2, the enzyme responsible for the conversion of retinaldehyde to retinoic acid which was detected as a 531 bp fragment.

Transient Reporter Assays

To understand the transcriptional properties of the endogenous retinoid receptors, a retinoid responsive reporter construct, pAAVMCS-pGL3-βRARE-Luc vector was introduced using liposomes into cumulus granulosa cells. A similar vector lacking the βRARE was also transfected into cells to serve as a control. The assay was performed twice and a similar trend in luminescence activity as presented here was observed on both occasions. The data on fold increase provided here is from a single assay and is presented in Figure 3. There was a vector by ligand concentration interaction and therefore, for each vector, mean luminescence measurements at each of the ligand concentrations were compared using the tukey's procedure. In Figure 3, both 0.5 and 1 μM all-*trans* RA significantly increased ($P < 0.05$) pAAVMCS-pGL3-βRARE-Luc activity by

3.87- and 2.46- fold, respectively, compared to the cells transfected with the control vector. Supplementation of 5 μM retinol caused a mild increase in pAAVMCS-pGL3-βRARE-Luc activity (1.63- fold) ($P > 0.05$). Increasing the concentration of retinol from 5 μM to 10 μM accordingly increased pAAVMCS-pGL3-βRARE-Luc activity (2.14- fold) ($P > 0.05$) compared to the cells transfected with the control vector. However, the increase in reporter activity observed following retinol treatment at both concentrations was not statistically significant. Addition of alcohol vehicle did not stimulate an increase in pAAVMCS-pGL3-βRARE-Luc activity.

Discussion

Cumulus-granulosa cells play a very important role during maturation so that the oocyte acquires competence for further development (Fatehi et al., 2002). Since mammalian oocytes develop in the follicular environment tightly surrounded by cumulus cells, these cells directly exert their effects on the developing oocyte. Cumulus cells support oocyte maturation before ovulation, facilitate oocyte transport into the oviduct during ovulation and thereafter orchestrates the complex mechanisms that control the interaction of spermatozoa with the oocyte (Tanghe et al, 2002). At least under in vitro conditions these observations are exemplified by the fact that cumulus removal before IVF reduced cleavage rate in the cow (Fatehi et al., 2002). Administration

of retinol to cows in conjunction with superovulation protocols increased the number of transferable blastocysts on day 7 (Shaw et al., 1995) and more recently improved the yield of cumulus oocyte complexes from heifers following transvaginal ultrasound aspiration (Hidalgo et al., 2003).

Earlier, Scheigwert et al. (1988) and more recently, Brown et al. (2003) reported detecting retinol in the follicular fluid of bovine dominant follicles. Both these authors observed vitamin A concentrations to be highest in large nonatretic follicles and lowest in small atretic follicles and correlated their findings with follicular fluid estradiol concentrations. In the later study, both the mRNA and immunoreactive RBP was detected in granulosa, theca cells, and the blood vessels lining the follicle (Brown et al., 2003). In addition, RBP concentrations were also found to be elevated in follicles containing high retinol concentrations (Brown et al., 2003). These findings indicate that retinol is available in high concentrations in the follicular fluid of large ovulatory follicles and the follicular cells were equipped to take up retinol from the follicular fluid. In the present study, we also detected mRNA expression for RBP in cumulus cells which is in agreement with the findings of Brown et al. (2003), which suggests the capacity for intercellular binding and transport of retinol. The presence of RBP does not signify the possibility for retinoic acid synthesis or provide information about the fate of retinol following uptake by cumulus-granulosa cells from the follicular fluid. No effort was made in the present study to investigate the expression of alcohol dehydrogenases responsible for the oxidation of retinol to retinaldehyde. We earlier reported detecting a product very close to the expected size for alcohol

dehydrogenase IV using nested PCR in bovine preattachment embryos (Mohan et al., 2002). However, BLAST searches revealed less than 45% homology to alcohol dehydrogenase IV. Using RT-PCR, we detected RALDH-2 in cumulus granulosa cells indicating the possibility of retinoic acid synthesis at least from retinaldehyde. Data from our transient reporter assays show that addition of retinol at 5 and 10 μM concentrations to cells transfected with the pAAVMCS-pGL3-βRARE-Luc vector did not cause a significant increase in reporter activity compared to controls (Fig. 3). Ethanol was used as a solvent for retinoids used in this study and it is possible that ethanol decreased retinoic acid synthesis in competition with all-*trans* retinol to inhibit alcohol dehydrogenase (Molotkov and Duester, 2002). This reduction in reporter activity was not observed with all-*trans* retinoic acid since it acts at a step downstream of the inhibitory point. Based on these results it is not clear if cumulus cells can oxidize retinol to retinaldehyde or in other words if alcohol dehydrogenase activity is existent in cumulus cells. Therefore, in the future it may be a good idea to test the effect of retinol dissolved in another solvent such as dimethyl sulfoxide on activation of the reporter. The detection of RALDH-2, nevertheless, indirectly points towards the likely oxidization of retinol to retinoic acid by cumulus-granulosa cells since retinaldehyde, the substrate for RALDH-2 is generated from retinol. Retinoic acid thus generated may then activate the reporter.

Transcripts for RARα, RARγ, RXRα and RXRβ were also expressed in cumulus cells (Fig. 2). The presence of both RAR and RXRs would mean that both all-*trans* and 9-*cis* RA have the potential to exert receptor mediated effects

on transcriptional regulation in cumulus-granulosa cells. Interestingly, presence of the mRNA does not specify the presence of the mature protein. Furthermore, presence of the mature protein does not guarantee biological activity. Therefore, we tested the biological activity of both RARs and RXRs using a transient reporter assay. Addition of both 0.5 and 1 μM RA significantly increased reporter activity in cells transfected with the pAAVMCS vector carrying the RARE sequence compared to the cells transfected with the control vector. The main reason for performing a retinoid sensitive reporter assay is to only show that the retinoid receptors expressed by the cumulus-oocyte complexes are biologically active or in other words capable of binding retinoic acid. Therefore, caution should be exercised while interpreting the data from the reporter assay since it neither represents a dose-response nor a time course study. These results basically show that cumulus-granulosa cells contain endogenous retinoid receptors capable of binding all-*trans* retinoic acid and these receptors may transduce the retinoid signal further downstream. Based on our transient reporter assay results it appears that addition of 5 nM 9-*cis* and 1μM all-*trans* retinoic acid to the in vitro maturation medium (Duque et al. 2002) had a direct positive effect on the cumulus oocyte complex, thereby enhancing embryo development. We also detected transcripts for PPARγ in cumulus cells. The expression of PPARγ further suggests the possibility for heterodimerization between RXRs and PPARs.

The mechanisms by which addition of retinoic acid brought about positive effects on embryonic development in earlier studies is unclear and needs further

investigation. Exposure of immature porcine granulosa cells to 1 μM retinoic acid for 15 hrs inhibited mRNA expression of LH receptor via downregulation of c-fos mRNA (Hattori et al., 2000). Consequently, RA-treated immature granulosa cells failed to differentiate into mature cells. Similarly, RA also inhibited transcription of FSH receptor mRNA in a dose-dependant manner (Minegishi et al., 2000). Follicle stimulating hormone is partly responsible for the differentiation process initially through the induction of FSH receptors and later of LH receptors (Hseuh et al., 1989). It is likely that retinoic acid could have exerted a very similar negative effect on bovine cumulus-granulosa cells during in vitro maturation (Duque et al., 2002). However, the significance of retinoic acid induced downregulation of both LH and FSH receptor mRNA and its consequent effects on in vitro oocyte maturation needs future investigation. Further, retinoic acid receptors being ligand activated transcription factors are involved in regulating the transcription of several genes. One such target gene is midkine, initially identified in a teratocarcinoma cell line as a retinoic acid inducible gene (Kadomatsu et al., 1988). Midkine belongs to the family of heparin-binding growth/differentiation factors. Midkine has been detected and reported to be present at a concentration of 125 ng/mL in the bovine follicular fluid (Ohyama et al., 1994). Further, in situ hybridization studies have shown that midkine mRNA is restricted to the granulosa cells of healthy rat follicles (Karino et al., 1995) and RA has been shown to induce a 2-fold increase in midkine mRNA at a concentration of 0.3 μM (Minegishi et al., 1996). Similarly, treatment of bovine cumulus-granulosa cells with 5 nM 9-*cis* retinoic acid increased the expression of

midkine mRNA (Royo et al., 2003). More recently, addition of midkine during in vitro oocyte maturation influenced cytoplasmic maturation of oocytes and increased blastocyst yields compared to untreated controls (Ikeda et al., 2000a, 2000b).

In summary, we detected mRNA expression for RBP, nuclear retinoic acid and retinoid X receptors, PPARγ and RALDH-2 in bovine cumulus granulose cells. The activation of the reporter construct following the addition of all-*trans* retinoic acid would indicate that these endogenous retinoid receptors are competent to bind the ligand and may be capable of transducing the biologically active retinoid signal further downstream. Presence of biologically active retinoic acid and retinoid X receptors also suggests that retinol and its metabolite retinoic acid may exert transcriptional regulation during in vitro/in vivo oocyte maturation in the bovine. Retinoic acid is believed to be a potent regulator of cell differentiation, cell proliferation and apoptosis by regulating the expression of specific genes. Identification of functional retinoic acid responsive genes and their downstream products will throw more light into the mechanisms by which retinoic acid addition during prematuration enhanced blastocyst development rates and increased blastocyst cell numbers (Duque et al., 2002). Therefore, elucidation of the molecular pathways involved in retinoid-mediated regulation of gene expression in COCs remains a high priority in the future.

Acknowledgements

We thank David Goad, Leanne Wier and Abigail Farfan (Promega Corporation) for excellent technical assistance and the staff of Mikkelson Beef Inc., (Oklahoma City, OK) for their assistance and generous donation of materials used in this study. We also thank Dr. Larry Claypool of the Dept. of Statistics, Oklahoma State University for assisting us with the statistical analysis of the data. We acknowledge the Oklahoma State University Recombinant DNA/Protein Resource Facility for the synthesis of synthetic oligonucleotides and the sequencing of cloned cDNA and reporter vectors.

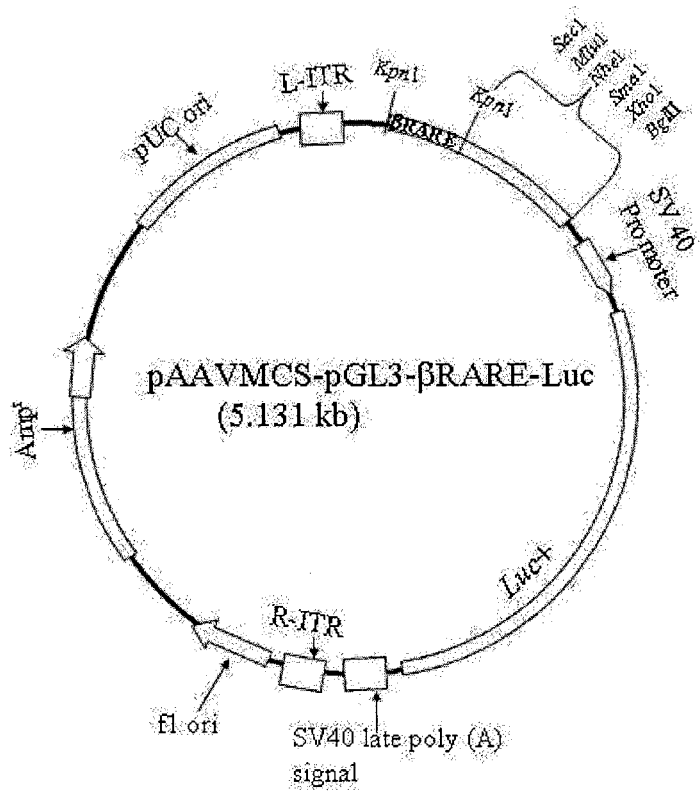

Figure 1. The plasmid pAAV-MCS-βRARE-Luc was generated by inserting a 37 bp consensus retinoic acid response element (RARE) from the human retinoic acid receptor β2 carrying *Kpn*1 linkers on the 5' prime end of both strands into the pGL3 promotor vector (Promega Corporation, Madison, WI). The expression cassette with the RARE sequence was then ligated to the back bone of the pAAVMCS vector carrying the ITR sequences. This construct was characterized by restriction mapping and by dideoxy chain-termination sequencing to verify that the β2 RARE was intact. The SV40 promoter drives expression of the firefly luciferase reporter.

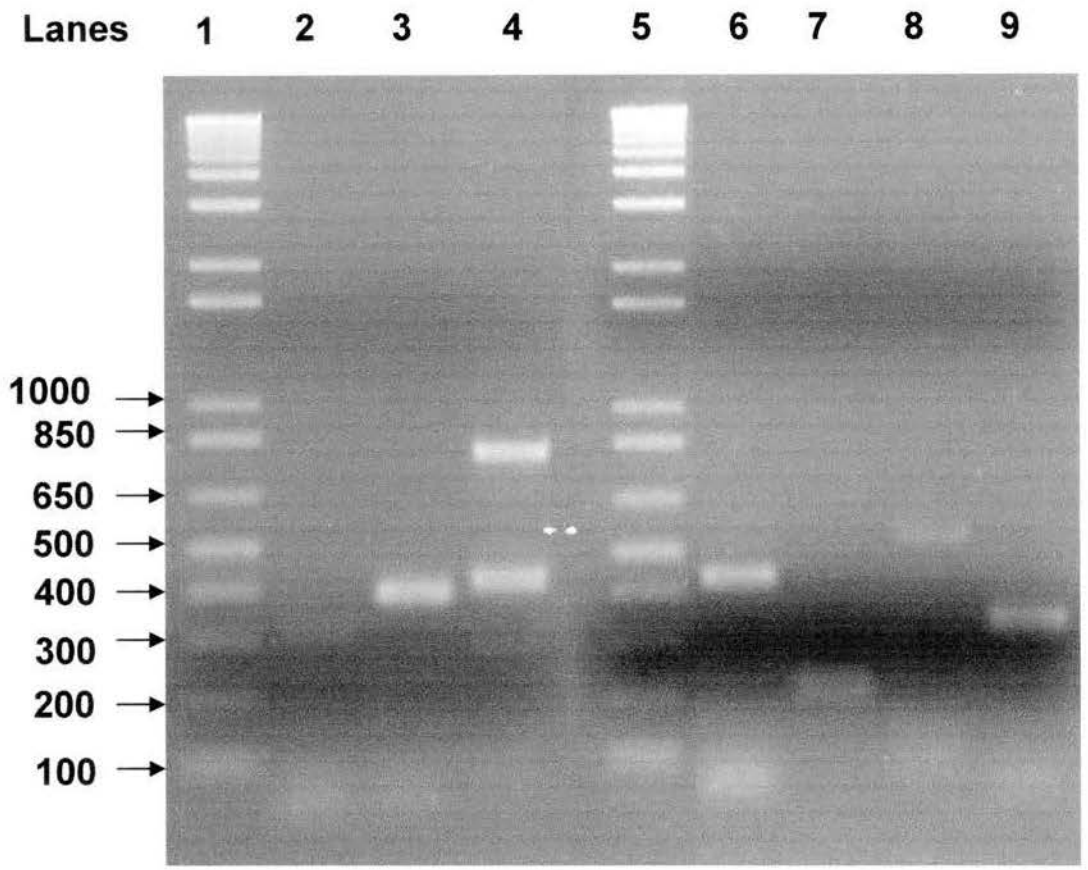

Figure 2. Expression of transcripts for RBP (311 bp, Lane 2), RARα (392 bp, Lane 3), RARγ (795 bp, Lane 4), RXRα (415 bp, Lane 6), RXRβ (Lane 7, 207 bp), RALDH-2 (531 bp, Lane 8) and PPARγ (332 bp, Lane 9) using RT-PCR in cumulus cells. Lanes 1 and 5 contain DNA marker. Negative control lane (not shown here) contained the product of RT-PCR under indentical conditions in the absence of RNA template. Products of RT-PCR were resolved in 2% TAE-agarose gel and visualized by ethidium bromide staining.

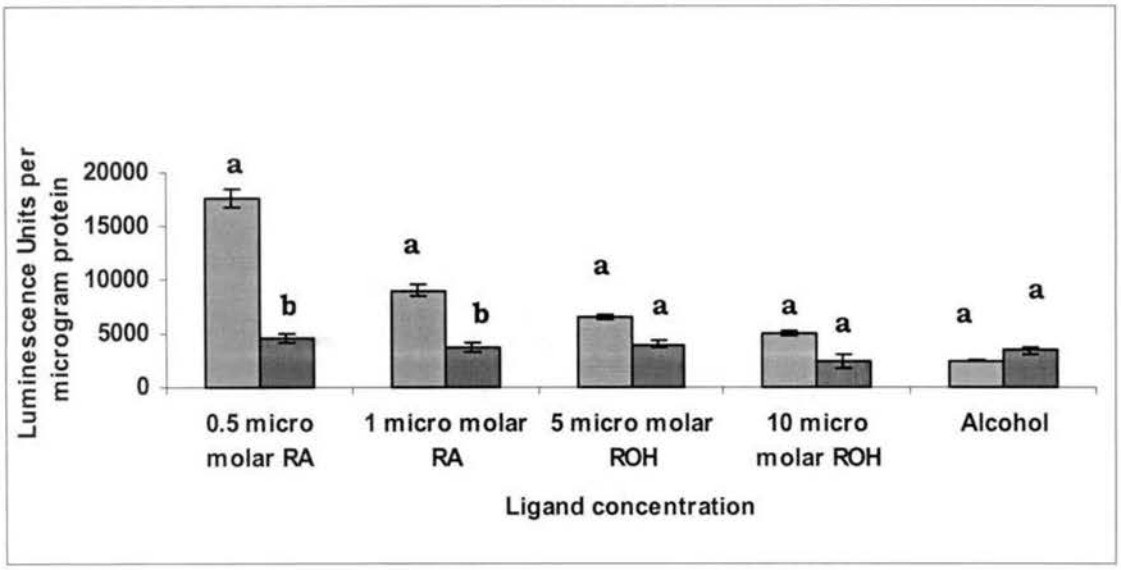

Figure 3. Transactivation of retinoid-responsive reporter gene (pAAVMCS-pGL3-βRARE-Luc) by endogenous retinoid receptors in bovine cumulus-granulosa cells. The Y axis represents luciferase activity in luminescence units (LU) per microgram total protein following treatment with all-*trans* retinoic acid (RA) (0.5 or 1 μM), all-*trans* retinol (ROH) (5 or 10 μM) and alcohol vehicle control. Each sample was accordingly normalized and means±SEs were evaluated for LU/μg of total protein. Within each treatment (ligand concentration) means with different superscripts differ significantly ($P < 0.05$). The assay was carried out in triplicate wells for each treatment and performed twice. The data shown here is from one such experiment.

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Chapter VI

ANALYSIS OF GENE EXPRESSION IN THE BOVINE BLASTOCYST USING SUPPRESSION SUBTRACTIVE HYBRIDIZATION

Abstract

Successful embryonic development is dependant on the stage-specific expression of the appropriate genes in the correct tissue at the right time. Creating a database on stage-specific gene expression patterns will not only throw light on the molecular control of preattachment development but also help us better understand those factors responsible for early embryonic mortality. Unfortunately, information on stage-specific gene expression during early embryonic development in the bovine is lacking. In the present study, we compared gene expression between in vitro-produced day 7-8 intact blastocysts (driver) and day 9-10 hatched blastocysts (tester) using suppression-subtractive hybridization. Pools of 30 embryos for both driver and tester were used in the RNA extraction process. From limited amounts of starting material (~400 ng of total RNA), a reverse transcription-polymerase chain reaction (RT-PCR) procedure was used to amplify the mRNA and generate sufficient cDNA to conduct suppression-subtractive hybridization. The subtracted cDNA products were cloned and, 126 cDNAs representing expressed mRNAs were isolated, sized, single-pass sequenced, and compared to known sequences in GenBank.

Ninety-two clones provided sequence information for further analysis. Among these, 31 clones exhibited high homology to known genes. Three clones, namely, 26S proteasomal ATPase (PSMC3), casein kinase 2 α subunit (CK2) and phosphoglycerate kinase (PGK) were selected and further characterized using real-time quantitative PCR to assess their differential expression in hatched blastocysts. Overall, a 1.3-, 1.6-, and 1.5-fold increase in expression level was observed in hatched blastocysts compared with intact blastocyst for PSMC3, CK2 and PGK, respectively. The results show that construction of subtracted cDNA libraries from small numbers of embryos is feasible and can provide information on gene expression patterns during preattachment embryogenesis.

Introduction

The development of a fertilized ovum into a highly complex individual requires the orchestrated expression of specific subsets of genes. In most mammals, including cattle, after fertilization the zygote undergoes several cleavage divisions, it compacts and cavitates to form a blastocyst, and hatches. These early events, collectively called preattachment embryogenesis, are initially supported by the utilization of transcripts and proteins synthesized during oogenesis until a stage when the embryonic genome becomes activated. In the bovine this occurs around the 8- to 16-cell stage [1]. Further development is dependant on the successful control of both temporal and spatial gene expression following activation of the embryonic genome.

Recent studies reveal that most embryo losses in heifers occur before day 14 after insemination [2]. Contemporary developments in the in vitro production (IVP) of bovine embryos have fostered basic studies aimed toward understanding the intricate pathways controlling early embryo development. Despite these advances, success rates in terms of blastocyst yields range between 30% and 40% following in vitro culture with 50% being able to initiate a successful pregnancy following transfer [3]. Furthermore, in vitro produced embryos continue to exhibit conspicuous morphological, biochemical and metabolic differences compared to their in vivo counterparts [4]. Other detrimental effects of in vitro embryo culture include fetal oversize and significant fetal loss after Day 35 due to failure of normal allantoic development within the conceptus [5]. These negative consequences have substantially hampered the field application of in vitro embryo production in the bovine.

It is presumed that successful preimplantation and early fetal development is reliant on the timely expression of approximately 10,000 genes [4]. Unfortunately, sequence information for only a few of these genes is currently known meaning that our basic understanding of gene expression patterns driving blastocyst development is very restricted. In many cases, our present knowledge of genes expressed during early embryogenesis in the bovine has been gained from studies using data extrapolated from mouse as the starting point. Thus, the identification of novel genes and analysis of their function during preimplantation embryogenesis in the bovine is necessary. Several modifications of the reverse transcription polymerase chain reaction (RT-PCR) have been used to quantify

the relative abundances of individual gene transcripts [4]. Differential display RT-PCR developed a decade ago [6] was recently applied to compare patterns of RNA expression from preattachment bovine embryos [7]. Large and representative subtractive cDNA libraries have been successfully constructed from preattachment murine embryos to identify novel genes critical for development [8].

The present communication describes the use of a modification of suppression-subtractive hybridization (SSH) originally developed in 1996 [9], to study differential gene expression in the bovine preattachment embryo. Use of SSH is advantageous for it enriches low abundance transcripts that are differentially expressed in the tester population. For a discussion of the basis of SSH and how it leads to the enrichment and isolation of differentially expressed transcripts, see [9]. In the present study, we attempted to identify changes in gene expression between in vitro produced, intact Day 7-8 blastocysts (driver) and day 9-10 hatched blastocysts (tester). A better knowledge of gene expression patterns during early preattachment development would yield insights into the molecular pathways controlling early development and as a preamble to understanding events that may be compromised in early embryonic mortality.

Materials and Methods

In vitro Maturation, Fertilization and Culture

Ovaries were collected from cows at a local abattoir and transported to the laboratory in 0.9% normal saline supplemented with penicillin-G (100 IU/ ml) and streptomycin sulfate (0.2 μg/ml) at 26-30°C within 5 h. Oocytes were aspirated from follicles ranging in diameter from 2 to 8 mm using an 18-gauge needle and transferred into a modified-PBS solution containing 0.3% BSA. In vitro maturation, fertilization and culture were performed according to protocols described in Mohan et al. [10].

RNA extraction

In vitro produced intact blastocyst (day 7-8) and hatched blastocyst (day 9-10) stages (n=30) were frozen in 250 μl of denaturing solution (4 M guanidium isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-β mercaptoethanol). Total RNA was extracted according to the method described in Mohan et al. [10] from a pool of 30 embryos at both stages.

Driver and Tester cDNA Synthesis

Due to the small amounts of mRNA extractable from bovine embryos, mRNA was reverse transcribed and the cDNA was subjected to the PCR using the SMART cDNA synthesis kit (Clontech Laboratories Inc., Palo Alto, CA).

Protocols were followed according to the manufacturers instructions. Briefly, about 400 ng of total RNA from both blastocysts (driver) and hatched blastocysts (tester) were denatured at 70°C for 2 min, then reverse transcribed in the presence of 1μM of cDNA synthesis primer (CDS), 1μM SMART II oligonucleotide, 1mM of 50X dNTPs and 200 units of reverse transcriptase (Superscript, 200 U per reaction; Invitrogen, Carlsbad, CA) at 42°C for 1 hr. The reaction was primed using an anchored oligo-dT primer called the CDS primer, carrying an internal PCR primer sequence at the 5' end. The SMART II oligonucleotide anneals with the CCC tailing left by the reverse transcriptase on the newly formed cDNA and contains the same internal primer as the CDS primer. The samples were diluted with 40 μl of Tris-EDTA (TE) buffer and the reaction was stopped by heating to 70° C for 7 minutes. Approximately, 4 μl of diluted driver and tester cDNA were denatured for 1 minute at 95°C and subjected to 34 PCR cycles in the presence of 0.2 mM of 50X dNTP; 0.2 mM of PCR primer, which anneals on both the SMART II oligonucleotide; and the CDS primer along with 2 μl of 50X Advantage 2 polymerase mix (Clontech). The cycling conditions were as follows: denauration at 95°C for 5 sec, annealing at 65°C for 5 sec and extension at 68°C for 6 min. Aliquots (15μl) were analyzed on a 2% agarose gel. PCR products were extracted once with 150 μl of phenol:chloroform:isoamyl alcohol (25:24:1). Approximately 120 μl of the aqueous phase was removed and concentrated to about 40-70 μl using 700 μl of n-butanol. The cDNA was then purified using CHROMA SPIN-350 gel exclusion columns (Clontech) to remove unincorporated dNTPs, primers and DNA

fragments less than 300 base pairs in length. Both driver and tester cDNAs were then digested with 15 units of *Rsa*I in a 500 μl reaction mixture at 37°C for 3 h, and the reaction was stopped by adding 8 μl of EDTA. *Rsa*I digested driver and tester cDNA were extracted once with phenol:chloroform:isoamyl alcohol. The aqueous phase was removed, precipitated with ethanol, and the pellet was redissolved in 7μl of TNE buffer (10mM Tris-HCl pH 8, 10mM NaCl, and 0.1 mM EDTA). The final concentration of both driver and tester was ~300 ng/μl. One microliter of *Rsa*I digested tester cDNA was diluted in 5 μl of sterile water, and 2 μl of diluted tester was then ligated with 2 μl of adapter 1 and adapter 2R (2 μM) according to the guidelines provided in PCR-Select cDNA subtraction kit (Clontech Laboratories) in separate ligation reactions in a total volume of 10 μl at 16°C overnight using 400 units of T4 DNA ligase in 2 μl of 5X ligation buffer. The ligation was stopped by adding EDTA/glycogen mixture and heated at 72°C for 5 min to inactivate the ligase. Samples were stored at –20°C. A PCR based ligation efficiency analysis to verify that at least 25% of the cDNAs had adaptors on both ends was performed according to the instructions detailed in the Clontech PCR-Select cDNA subtraction kit user manual.

Suppression Subtractive Hybridization

SSH (9) was performed using the Clontech PCR-Select cDNA subtraction kit. Briefly, 1.5 μl of driver ds cDNA (~450 ng) was added to each of two tubes containing 1.5 μl of adapter 1 and adapter 2R-ligated tester cDNA (~20 ng) in 1 μl of 4X hybridization buffer. The samples were denatured at 98°C for 1.5 min,

and then allowed to anneal at 68°C for 8 h. Following first hybridization, the two samples were combined in the presence of fresh excess denatured driver cDNA (~300 ng) in 1 μl of 4X hybridization buffer. The samples were allowed to hybridize overnight at 68°C. The hybridized samples were diluted in 200 μl of dilution buffer (20 mM Hepes, pH 8.3, 50 mM NaCl, and 0.2 mM EDTA), heated at 68°C for 7 min, and stored at -20°C.

PCR Amplification of Subtracted Products

Two PCR amplifications of the subtracted cDNAs were performed. The primary PCR contained 1μl of diluted, subtracted cDNA, and 24 μl of the PCR master mixture prepared using the reagents provided in the kit. PCR was performed at 75°C for 5 min to extend the adaptors; 94°C for 25 sec; and 27 cycles at 94°C for 10 sec, 66°C for 30 sec, and 72°C for 1.5 min. The amplified products were diluted 10-fold in sterile deionized water. The diluted primary PCR product was used as template in a secondary nested PCR for 10 cycles at 94°C for 10 sec, 68°C for 30 sec, 72°C for 1.5 min using two nested primers, 1 and 2R, provided in the kit. Primary and secondary PCR products were analyzed on a 2% agarose gel. A second PCR-based analysis was performed according to the instructions detailed in the Clontech PCR-Select cDNA subtraction kit user manual to test for the efficiency of subtraction.

Cloning and Analysis of Subtracted cDNA

Following PCR subtraction, the amplified products were cloned into the pCR II vector of the TA cloning kit (Invitrogen) and used to transform competent DH5α *Escherichia coli* cells. Colonies were grown for 16-18 h at 37°C on Luria broth (LB) agar plates containing ampicillin, X-gal (5-bromo 4-chloro 3-indoyl-β-D-galactopyranoside), and isopropyl-β-D-thiogalactopyranoside for blue/while colony selection. Plasmids were extracted and the inserts were subjected to dideoxy chain termination sequencing (Applied Biosystems, Model 373A Automated Sequencer, Oklahoma State University Recombinant DNA/Protein Resource Facility) and the identity of each product was confirmed in a sequence homology analysis using the Basic Local Alignment Search Tool [11].

Quantitative 1- step RT-PCR

Expression of three clones of interest: namely, 26S proteasomal ATPase (PSMC3), Casein kinase 2 α II subunit (CK2), and phosphoglycerate kinase (PGK) was evaluated by real-time quantitative RT-PCR utilizing a fluorescent reporter and 5' exonuclease assay system [12]. This technique from our previous experience, is capable of efficiently amplifying and detecting a product from as few as 10 copies of the target. Approximately 20 pooled embryos per group were grouped as early blastocysts, expanded blastocysts, early hatched blastocysts, and late hatched blastocysts (in culture until day 10). One sample from each of the 4 stages was assayed in triplicate wells. All 4 samples representing each of the 4 stages came from different embryo batches (i.e., embryos from 4

independent runs of the in vitro fertilization protocol were present in each pool). Reverse transcription of total RNA and PCR amplification was performed using the Taqman One-Step RT-PCR Master Mix Reagents Kit, Taqman fluorescent probe, and sequence detection primers (PE Applied Biosystems. Foster City, CA). Taqman probe specific for target was designed to contain a fluorescent 5' reporter dye (FAM) and 3' quencher dye (TAMRA). Each RT-PCR reaction (25 μl) contained the following: 2X Master Mix without uracil-N-glycosylase (12.5 μl), 40X Multiscribe and RNAse Inhibitor Mix (0.63 μl), target forward primer (200 nM), target reverse primer (200 nM), fluorescent labeled target probe (200 nM) designed from the mRNA sequence isolated from hatched blastocysts using SSH, and total RNA (40 ng) quantified spectrophotometrically based on A_{260}:A_{280} ratios. Forward and reverse primer and probe sequence for all three targets are shown in Table 1. The PCR amplification was carried out in the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). Thermal cycling conditions were 48°C for 30 minutes, 95°C for 10 minutes followed by 40 repetitive cycles of 95°C for 15 sec and 60°C for 1 minute. As a normalization control for RNA loading, parallel reactions in the same multiwell plate were performed using 18S ribosomal RNA as target (18S Ribosomal Control Kit, PE Applied Biosystems).

Quantification of gene amplification was made following RT-PCR by determining the threshold cycle (C_T) number for FAM fluorescence within the geometric region of the semi-log plot generated during PCR. Within this region of the amplification curve, each difference of one cycle is equivalent to a doubling of

the amplified product of the PCR. The relative quantification of target gene expression across treatments was evaluated using the comparative C_T method [13]. The ΔC_T value was determined by subtracting the ribosomal C_T value for each sample from the target C_T value of that sample. Calculation of $\Delta\Delta C_T$ involved using the highest sample ΔC_T value (i.e., sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔC_T sample values. Fold changes in the relative gene expression of target was determined by evaluating the expression, $2^{-\Delta\Delta CT}$.

Statistical Analysis

Quantitative RT-PCR ΔC_T values were analyzed using Proc Mixed [14] as a completely randomized design with 4 treatments. Blastocysts grouped as early and late (expanded) and hatched blastocysts grouped as early and late (in culture until day 10) were considered as four independent treatments. All possible comparisons among the different embryonic stages were performed for all three mRNAs quantified. A probability value of $P < 0.05$ was considered significant. Results are presented as arithmetic means ± SEM.

Results

Suppression Subtractive Hybridization

After 34 PCR cycles using the SMART cDNA synthesis kit, agarose gel electrophoresis revealed cDNA bands ranging in size from 250 bp to 1.5 kilobases (kb) for both blastocyst and hatched blastocyst (data not shown). Following size exclusion chromatography and *Rsa*I digestion, approximate yields of cDNA for the tester and driver ranged around 5-8 μg. Two different adaptors were ligated to the tester cDNA, and ligation efficiency was confirmed using a PCR-based assay employing two different sets of primers in two independent PCR reactions. The first set of primers included a glyceraldehyde-3-phosphate dehydrogenase-specific (G3PDH 3' primer) primer and PCR primer 1, which bound specifically to the adaptor sequence (product size ~1.2 kb). The second set of primers bound internally to the G3PDH gene (G3PDH 3' and 5' primer) (product size ~500 bp). The PCR product using both sets of primers resulted in bands of the expected size (data not shown). Further, the PCR products amplified using both primer sets were of the same intensity, indicating that the adaptor ligations worked successfully. Finally, a PCR-based subtraction efficiency analysis was done using specific primers provided in the kit by comparing the abundance of G3PDH before and after subtraction. For the unsubtracted sample, a G3PDH PCR product (500 bp) was seen after 18 cycles (data not shown). However, the same product in the subtracted sample appeared after 23 cycles (data not shown), indicating that G3PDH levels were reduced several fold in the subtracted product.

A total of 126 clones were selected after SSH, and we obtained partial sequence information on 92 clones following dideoxy chain termination

sequencing. After restriction digestion with *Eco*R1 and agarose gel electrophoresis it was found that approximately 18% of the clones were in the 100-300 bp size range, 40% in the 301-500 bp size range, 27% in the 501-800 bp size range and 15% ranged in size from 801-1500 bp. The partial sequence obtained from all 92 clones were compared with known sequences in the GenBank (National Center for Biotechnology Information, Bethesda, MD) database. Their putative identity, nucleotide homologies with other known sequences and insert size are shown in Table 2. Sequence data were submitted to the dbEST database (National Center for Biotechnology Information).

RT-PCR Quantitation Using Taqman PCR

The mRNA expression of PSMC3, CK2, and PGK was quantified using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). Specific primers designed for all three genes amplified mRNA in all embryo samples with alteration of probe fluorescence detected within 30 cycles (Table 3). The relative abundance of mRNAs encoding PSMC3, CK2 and PGK was calculated using the comparative C_T method (Table 3).

Ribosomal 18S RNA was used to normalize each sample for variation in RNA loading. As shown in Table 3, 18S rRNA was variable across developmental stages as a result of the dynamic nature of the RNA populations in the developing embryonic cells. We are not aware of any product suitable as a stable normalizer for this type of analysis in the preattachment embryo. Results

with G3PDH (not shown) reveal that it is also dynamic in these embryos, as are structural elements as actin.

Based on normalization with 18S rRNA levels, expression of 26S proteasomal ATPase (PSMC3) mRNA in early intact blastocysts was significantly different from late hatched blastocysts ($P < 0.05$). Similarly, expression of CK2 mRNA in late intact blastocysts was significantly different from late hatched blastocysts ($P < 0.05$). However, in the case of PGK, early hatched blastocysts exhibited significant differences compared to late hatched blastocysts ($P < 0.05$). Overall, hatched blastocysts had higher expression levels than intact blastocysts for all three genes examined as well as for 18S rRNA. Differences in expression for all three genes based solely on RNA loading in the PCR reactions were several fold greater between intact and hatched blastocysts (4- to 8-fold; Table 3) than when analysis was based on normalization. This can be evaluated based simply on the differences in average C_T values. We report the more conservative approach above, and recognize the technical limitations of using a normalization control.

Discussion

Early embryonic mortality is a well-recognized cause for reproductive failure in cattle leading to the loss of a large number of potential calves, retarded genetic progress, and significant loss of money and time in rebreeding cows. Owing to the even greater losses when employing in vitro fertilized embryos,

suggestions have been made to transfer at least two embryos to achieve a successful pregnancy [15]. It is very likely that several extrinsic and intrinsic embryonic factors could be at fault. Focusing our efforts to circumvent various factors intrinsic to the embryo would require us to have a better insight into the changes in gene expression occurring during early embryo development. Embryonic development is to a great extent controlled and regulated by differential gene expression and successful development to term is strictly contingent upon these changes in gene expression taking place at the right time in the appropriate tissue.

Since each stage of embryonic development is characterized by the activation of a diverse set of genes [16], it is becoming increasingly necessary that we have a better perception of stage-specific gene expression patterns. Much progress has been made in understanding normal embryonic development in mice through transgenic and knockout experiments [17], however, these developments are only beginning in domestic animals. Suppression subtractive hybridization is a very sensitive technique and has the advantage of greatly enhancing levels of mRNA sequences that are unique to the tissue of interest being investigated, while reducing sequences that are common to both tissues being compared. The entire task can be accomplished without prior knowledge of genes being expressed, and yields subtracted cDNA that is either upregulated or differentially expressed. Making use of SSH, stage specific gene expression patterns were recently described for the hatched blastocyst in human [18] and day 15 conceptuses in the equine [19]. Working along the same lines, in the

present study, we report the construction of a subtracted cDNA library from bovine hatched blastocysts. Approximately 92 from a total of 126 clones isolated provided sequence information. Homology searches revealed the identities of 31 clones with known genes (Table 2), among which three of them, namely, 26S proteasomal ATPase (PSMC3), CK2, PGK with known established roles during early embryogenesis were further characterized using real time quantitative PCR. In the rest of the discussion, a possible relationship between the known physiological functions of these three cDNAs and early embryogenesis in the bovine is described.

Protein synthesis in the early preattachment embryo has been subjected to a great deal of scrutiny in the recent past [20-22]. The protein content of in vivo derived preattachment cattle embryos from the two-cell through to the elongated blastocyst at day 16 has been described [20]. The protein content increased two fold from the morula to the expanded blastocyst stage followed by a 160-fold increase to the hatched blastocyst stage on day 13. While protein synthesis is critical, proteolysis on the other hand is equally vital to the upkeep of appropriate levels of short-lived and regulatory proteins mostly involved in basic cellular processes such as regulation of cell cycle and division, development and differentiation, cellular metabolism, heat shock and stress response, modulation of the immune and inflammatory responses, modulation of cell surface receptors and ion channels, transcription, and signaling factors, [23, 24].

In eukaryotic cells, degradation of intracellular proteins is mediated by a non- lysosomal ATP-dependant protease complex, the 26S proteasome. This

complex, present in both the nucleus and cytoplasm, consists of a proteolytic cylinder-shaped particle (20S proteasome) and an ATPase-containing complex (19S cap complex). The ubiquitin conjugated proteins are unfolded by the 19S regulatory subunit thereby facilitating their entry into the 20S proteasome cylinder particle (25, 26). Several ATPases with a highly conserved ATPase domain [27] such as PSMC1 (S4), PSMC2 (MSS1), PSMC3 (TBP1), PSMC4 (TBP7) and PSMC5 (TRIP1) comprise the 19S complex. While protein synthesis has been extensively researched in the early preattachment embryo, protein degradation or its control during preattachment bovine embryo development has never been looked into. One of the subtracted products isolated and further characterized in the present study revealed 90% similarity to the conserved ATPase domain of PSMC3 as well as tat binding protein 1 (TBP1). PSMC3 which is synonymous to TBP1 was earlier identified as a component of human immunodeficiency virus tat-binding protein and negatively regulated tat-mediated transcriptional activity. In Table 3, the C_T values for 26S proteasomal ATPase (PSMC3) decreased in the hatched blastocysts indicating that the target copy numbers were higher in the hatched blastocysts which agrees with the fact that SSH enables isolation of differentially expressed/upregulated sequences. After normalization with an internal control such as 18S rRNA we see that the fold differences when expressed with respect to late blastocysts, on an average scale increased by approximately 1.3 fold in the hatched blastocysts (Table 3). The differential expression of this ATPase may be indicative of an energy-dependant active protein degradative process in the hatched blastocyst to eliminate abnormal

proteins along with various cell cycle regulatory proteins so that development can continue uninterrupted. From recent studies in mice it appears that PSMC3 may be highly indispensable for early preattachment development because PSMC3 knockout mice fail to implant owing to defective blastocyst development [28]. E3.5 PSMC3$^{-/-}$ embryos when cultured in vitro for 5 days exhibited shrinking of embryonic cells and failed to differentiate into trophectoderm and inner cell mass cells. Taken together, these and our findings lead to the suggestion of a specific role for PSMC3 in blastocyst formation in the bovine.

Casein kinase-2, a pleiotropic serine-threonine specific growth related protein kinase is known to regulate a myriad of intracellular processes fundamental to maintaining cell viability, cell proliferation and differentiation, signal transduction, transcriptional control, apoptosis, cell cycle, etc. [29]. Not only it is ubiquitously expressed in every eukaryotic tissue but also in every cellular compartment [30]. Described to exist as a spontaneous heterotetramer (M_r of ~130,000) it is composed of two catalytic subunits namely, α, (M_r 42-44 kDa) and/ or α' (M_r 38 kDa) and two non-catalytic β-subunits whose M_r in animals is approximately 26 kDa. In contrast to other protein kinases, CK2 is unique in its ability to utilize GTP, in addition to ATP as a phosphate donor. Approximately 160 proteins are known to be phosphorylated by CK2 including several proteins involved in cell cycle control, transcriptional and translational processes (29).

To our knowledge, CK2 expression or activity has never been investigated during early embryogenesis in the bovine. A second cDNA clone isolated had very high homology to the regulatory alpha subunit of CK2 of several species

(Table 3) and its expression was quantified using the taqman PCR assay. Following quantification, an approximately 1.6 fold increase in CK2 mRNA levels was detected in hatched blastocyst compared to intact blastocysts (Table 3). Considered by some as a "proliferation marker", the high expression pattern of CK2 in hatched blastocyst is no surprise as the hatched blastocyst is a stage during embryonic development where naturally high proliferation rates prevail. An increase in CK2 has been reported during late embryogenesis in mice [31] and early embryogenesis in nematodes [32] and insects [33]. In sea urchins, almost all of the increase in phosphorylation during early development has been attributed to CK2 like activity [34]. Embryonic stage-specific changes in protein phosphorylation has been described for mice [35] and more recently in elongating blastocysts in cattle [36]. In the mouse, protein phosphoryation is associated in critical events such as zygotic genome activation [37], blastocyst expansion [38] and is required for preimplantation embryo development [39]. Interestingly, growth factors, whose participation in promoting early embryogenesis is quite transparent, have also been shown to satisfy this essential role at least in part by regulating the expression and activity of CK2 [40].

The current school of thought on blastocyst formation involves the establishment of a trans-trophectoderm ion gradient(s), contributed equally by Na/K-ATPase, which drives water through water channels called aquaporins [41]. Aquaporins belong to a critical group of genes whose members are integral membrane proteins and function to channel the movement of water through the

cell membrane [42]. A role for CK2 in blastocyst formation is further strengthened by the presence of supposed CK2 phosphorylation sites in the primary amino acid sequence of aquaporins [42]. Recently, it was reported that CK2 may be involved in the phosphorylation of the proteasome and is critical for its association with the 19S regulatory complex and activity [43]. Very interestingly, in sea urchins, inhibition of CK2 activity delayed hatching of the blastula from the fertilization envelope and the transition from blastula to gastrula [34]. It is an interesting question to ask whether a similar cause-and-effect relationship would be applicable to the bovine blastocyst. Taken together, the hitherto known functions of CK2 fit very well with the events leading to the formation and possibly hatching of the blastocyst implying that CK2 activity has important physiological roles during early embryogenesis in the bovine.

Two metabolic pathways, namely, the pentose phosphate pathway and the Embden-Meyerhof pathway play essential roles during embryo development [44]. Cattle embryos utilize very little glucose until the 16-cell stage [3, 44, 45]. However, utilization of glucose significantly increases at the morula stage [44] through to blastocyst expansion [45]. PGK catalyzes the first ATP-generating reaction in glycolysis by transferring a phosphoryl group from the acyl phosphate of 1,3-bisphosphoglycerate to ADP forming 3-phosphoglycerate and ATP. A third subtracted cDNA clone representing PGK was isolated and selected for further characterization using real-time PCR. Similar to the previous two products, the expression of PGK was 1.5 fold higher in hatched blastocysts compared with intact blastocysts (Table 3). The expression of PGK would suggest active

utilization of glycolytic substrates like glucose to meet the energy requirements of the early embryo. An increase in the utilization of glucose has been reported as a means to meet the growing energy demands of Na^+-K^+ ATPase required for the formation and maintenance of the blastocele [45] and one other report suggested it to be essential for hatching of the bovine blastocyst [46]. Higher glucose utilization has also been directly correlated with greater blastocyst viability [47] and better developmental potential in vitro in day 10 cattle embryos [46]. The trend towards higher expression of PGK as embryo development advances seem to agree with the aforementioned reports on glucose utilization/metabolism by the bovine embryo in vitro.

In summary, data presented here are the first description of the generation of an embryonic preattachment stage-specific cDNA library using SSH in the bovine. We successfully sequenced and identified several differentially expressed mRNAs from in vitro produced bovine hatched blastocysts which may play important roles during early embryogenesis. Expression levels of three of these mRNAs, namely, 26S proteasomal ATPase (PSMC3), CK2 and PSGK known to be associated with early embryogenesis were shown to increase in the hatched blastocyst using the Taqman real-time quantitative PCR assay. The information available on these three markers together with our findings suggest that they may have an imperative role to play during preattachment embryogenesis. The characterization studies done in the present study pertain to in vitro derived embryos and need to be extended to their in vivo counterparts and also to embryos produced in defined culture systems. The subtracted clones

analyzed in the present study were randomly picked and they certainly do not represent true positives meaning they are not solely specific to the hatched blastocysts. Hence, a different approach to follow in future studies would be to identify the true positive clones using a differential screening protocol. These subtracted stage specific cDNA clones can be spotted on a microarray and we anticipate that, in the future, mRNA phenotyping on embryos subjected to differing culture conditions will be profiled with greater ease and thereby, immensely augment our ongoing efforts to optimize culture environments in vitro. The use of these advanced molecular techniques to study preattachment embryogenesis in the bovine is expected to create new prospects for research and substantially contribute to the enduring efforts to understand embryo development so that assisted reproduction technologies can be successfully applied in the bovine.

Acknowledgments

The authors thank David Goad and Khurshid Gandhi (CLONTECH Labs, CA) for excellent technical assistance. We also thank the staff of Wellington Quality Meats, Wellington, KS for their assistance and generous donation of materials used in the study. The authors acknowledge the Oklahoma State University Recombinant DNA/Protein Resource Facility for the synthesis of synthetic oligonucleotides and the sequencing of cloned cDNA. This study was supported by the Oklahoma Agricultural Experimental Station.

Table 1. Primers and probe sequences used for Real time RT-PCR.

Gene	Primer sequence	Probe sequence
PSMC3	5'-AAGTCTCCCGGCTTCAGCTT-3' 5'-ACGCGGCAGACCTACTTCCT-3'	5'-FAM-TCGGCATCCACCAAGCCAATCACT-TAMRA-3'
CK2	5'-CCAAGGCAGGGAGTTCGT-3' 5'-GAGGCGGTCCCAACATCA-3'	5'- FAM-CACACTGGCAGACATTGTAAAAGACCCTGTGT-TAMRA-3'
PGK	5'-GATGTTTATGTCAATGATGCTTTTGG-3' 5'-TCTTTGGCAGATTTACTCCTACCA-3'	5'-FAM-TGCTCACCGAGCCCACAGCTCC-TAMRA-3'

Table 2. Identity, size and percentage homology of putative bovine hatched blastocyst subtracted cDNA clones compared to known sequences in GenBank.

Identity	Clone number	Accession number	Base pairs sequenced	Homologya
60S Ribosomal protein L7 (8 clones)	OKST HatBlast- 01-05, 14-16	BM076109	320	Human 92% (296/320)
Proteasome 26S subunit ATPase (PSMC3)	OKST HatBlast- 21	BM076114	672	Human 90% (366/403) Mouse 88% (350/397)
Phosphoglycerate kinase 1	OKST HatBlast- 23	BM076116	610	Human 93% (567/606) Mouse 89% (525/589)
40S Ribsomal protein S25	OKST HatBlast- 29	BM076118	616	Human 92% (421/457) Mouse 88% (391/443) Rat 90% (352/388)
Ribosomal protein L27	OKST HatBlast- 30	BM076119	320	Human 91% (245/267) Mouse 91% (239/262) Dog 95% (247/260)
Chaperonin 10	OKST HatBlast- 31	BM076120	588	Cow 96% (443/457) Human 90% (391/430) Rat 87% (342/392) Mouse 87% (322/369) Chicken 80% (180/224)
SH3- domain protein 5 (Ponsin)	OKST HatBlast- 36	BM076123	730	Human 91% (421/462) Mouse 87% (381/435)
Glycine receptor beta subunit	OKST HatBlast- 37	BM076124	350	Cow 89% (204/229)
Ribosomal protein L18a	OKST HatBlast- 42	BM076128	147	Human 90% (108/120)
Activated RNA polymerase II transcription cofactor 4	OKST HatBlast- 46	BM076131	490	Human 91% (368/401) Mouse 87% (359/411)
Casein kinase II alpha subunit (2 clones)	OKST HatBlast- 48, 88	BM076133	341	Cow 100% (308/308) Human 97% (299/307) Mouse 94% (290/308)
Cytochrome C oxidase subunit VII (COX7c1) (5 clones)	OKST HatBlast- 49, 103, V, Z, BB	BM076134	593	Cow 99% (317/320) Human 87% (185/212) Mouse 88% (156/176)
Tropomyosin pseudogene (2 clones)	OKST HatBlast- 51-52	BM076135	321	Human 83% (198/236)
Mannosidase alpha Class 2A member 1	OKST HatBlast- 57	BM076138	216	Human 85% (181/211) Mouse 83% (168/201)
Transcription elongation factor B	OKST HatBlast- 58	BM076139	621	Human 90% (313/346) Mouse 90% (284/315)
Mitochondrial ribosomal protein S21	OKST HatBlast- 59		336	Human 93% (213/228) Mouse 85% (188/219)
Chloride intracellular channel	OKST HatBlast- 70	BM076142	283	Human 91% (261/283) Mouse 88% (252/286)
KIAA0809 protein	OKST HatBlast- 73	BM076143	491	Human 92% (455/491)
Eukaryotic translation initiation factor (EIF5) (2 clones)	OKST HatBlast- 75, 92	BM076145	295	Human 92% (274/295) Rat 91% (269/295)

Zinc finger protein 277 (2NF 277)	OKST HatBlast- 85	BM076147	283	Human 98% (225/228)
Opa-interacting protein OIP2	OKST HatBlast- 93	BM076151	277	Human 93% (249/265)
RNA polymerase I (DNA directedl	OKST HatBlast- 97	BM076154	628	Human 91% (272/296) Mouse 86% (249/288) Rat 88% (255/287)
Nucleosome assembly protein 1 like (NAP ILI) (2 clones)	OKST HatBlast- 99, 102	BM076155	271	Human 86% (86/99)
Mitochondrial ribosomal protein (MRP L24) (2 clones)	OKST HatBlast- 100-101		239	Human 90% (137/151)
Ribosomal protein S26 (RPS 26) 40S (2 clones)	OKST HatBlast- 104	BM076158	216	Human 92% (179/194) Mouse 89% (170/190) Rat 90% (157/193)
DC 13 protein	OKST HatBlast- C	BM076162	650	Human 95% (159/166)
Deoxyguanosine kinase (2 clones)	OKST HatBlast- H, I	BM076163	318	Human 96% (308/318)
Mitochondrial ribosomal protein L20	OKST HatBlast- Q		388	Human 86% (124/143) Mouse 83% (113/135)
Mariners transposase gene	OKST HatBlast- R	BM076166	568	Human 93% (496/533)
Mitochondrial ATP synthesis d-subunit	OKST HatBlast- T		341	Human 99% (108/109)
Other Mitochondrial genes (8 clones)	OKST HatBlast- 28, 33, 35, 62, 63, 64, 65, W			
Unknown (36 Clones)	OKST HatBlast- 13, 17, 18, 19, 20, 22, 27, 32, 34, 38, 39, 41, 44, 45, 47, 53, 56, 66, 67, 68, 69, 74, 83, 84, 86, 87, 89, 90, 95, 96, 108, 109, 112, L, O, X	(Within the series BM076109-BM076168)		

[a]The percentages are based on BLAST searches of the GenBank database. The numbers in parethesis show the number of bases (query/subject) that were compared.

Table 3. Quantitative PCR analysis of specific mRNA expression in bovine blastocysts.

Embryonic stage	Subtracted product Average C_T †	18S rRNA Average C_T†	ΔC_T ‡ ¶	$\Delta\Delta C_T$§	Fold difference
PSMC3					
Blastocyst					
Early	25.75 ± 0.37	23.05 ± 0.08	2.7 ± 0.38[a]	-1.25 ± 0.38	2.4 (1.83-3.09)
Late	27.27 ± 0.07	23.32 ± 0.18	3.94 ± 0.19[ab]	0.00 ± 0.19	0.00
H. Blastocyst					
Early	24.88 ± 0.05	21.45 ± 0.16	3.43 ± 0.17[ab]	- 0.51 ± 0.17	1.4 (1.27-1.6)
Late	23.90 ± 0.15	20.25 ± 0.01	3.65 ± 0.15[b]	-0.3 ± 0.15	1.2 (1.23-1.37)
CK2					
Blastocyst					
Early	24.68 ± 0.31	23.05 ± 0.08	1.63 ± 0.19[ab]	-0.49 ± 0.19	1.4 (1.23-1.6)
Late	25.43 ± 0.03	23.32 ± 0.12	2.11 ± 0.12[a]	0.00 ± 0.12	0.00
H. Blastocyst					
Early	22.89 ± 0.59	21.45 ± 0.08	1.45 ± 0.59[ab]	-0.66 ± 0.59	1.6 (1.05-2.37)
Late	21.67 ± 0.08	20.25 ± 0.005	1.41 ± 0.08[b]	-0.7 ± 0.08	1.6 (1.54-1.72)
PGK					
Blastocyst					
Early	24.97 ± 0.04	23.05 ± 0.08	1.92 ± 0.09[ab]	0.00 ± 0.09	0.00
Late	24.87 ± 0.08	23.32 ± 0.12	1.55 ± 0.15[ab]	-0.38 ± 0.15	1.3 (1.17-1.44)
H. Blastocyst					
Early	22.93 ± 0.21	21.45 ± 0.16	1.48 ± 0.26[a]	-0.44 ± 0.26	1.4 (1.13-1.62)
Late	21.53 ± 0.26	20.25 ± 0.005	1.27 ± 0.26[b]	-0.65 ± 0.26	1.6 (1.31-1.88)

†C_T = Cycle threshold: cycle number in which amplification crosses the threshold set in the geometric portion of amplification curve.

‡ΔC_T = Subtracted gene product of interest C_T – 18s rRNA C_T : normalization of PCR cycles for subtracted gene target with 18s rRNA and the values are presented as a mean C_T ± SD

§$\Delta\Delta C_T$ = Mean ΔC_T – highest mean ΔC_T value: the mean value of either early or late blastocyst (highest ΔC_T; lowest expression of target in study) was used as a calibrator to set the baseline for comparing mean differences in values across all other stages.

¶[ab] values with a common superscript within same column for each of the three mRNAs do not differ significantly (P > 0.05)

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Chapter VII

COMPARATIVE GLOBAL GENE EXPRESSION ANALYSIS BETWEEN IN VIVO AND IN VITRO DAY 7 BOVINE BLASTOCYSTS

Abstract

In vitro produced bovine embryos (IVP) have darker cytoplasm, reduced buoyant density, fragile zonae pellucidae, chromosomal abnormalities, higher pregnancy failure rates and altered gene expression compared to embryos produced in vivo. Characterization of early deviations in gene expression would enable us to better understand the biology of early embryo development and improve in vitro culture systems. Here we compared gene expression between Day 7 blastocysts generated in TCM199 with 5% FBS and Day 7 in vivo derived blastocysts and using suppression-subtractive hybridization (SSH). Pools of 25 embryos for both driver and tester were used in the RNA extraction process. The subtracted products were cloned and subjected to differential hybridization screening analysis. cDNAs were isolated, single-pass sequenced and subjected to BLAST search. Of 32 in vivo ESTs (expressed sequence tags) that provided sequence information, 30 matched homologous sequences in GenBank. Of 32 in vitro ESTs, 22 provided specific matches while the remaining 10 represented novel transcripts. Two in vivo ESTs, galectin-1 and fibronectin, and one in vitro EST, filamin A, were further characterized using real-time quantitative PCR.

Significant increases in the expression level of galectin-1 and fibronectin were observed in the in vivo derived blastocysts compared to blastocysts produced in TCM199 with 5% FBS and CR1aa cultures. No significant difference in filamin A expression was found between blastocysts derived in vivo and those from either of the in vitro production systems. We conclude that these techniques are useful to characterize the transcriptome of the early prettachment embryo and observed deviations in mRNA expression may partially explain the differences in quality between in vivo and in vitro produced embryos.

Introduction

Following the birth of the first in vitro matured, fertilized and cultured calf in 1982 (Brackett et al., 1982), in vitro embryo production (IVP) technology showed great promise as a tool for both basic research and commercial applications. Improvement in IVP over the last decade has resulted in at least 30-40% of immature oocytes developing to the blastocyst stage following in vitro maturation, fertilization and culture (Niemann and Wrenzycki, 2000; Gutierrez-Adan et al., 2001). Unfortunately, the quality and developmental competence of in vitro produced bovine embryos has failed to keep up with those of their in vivo counterparts (Viuff et al., 1999, 2000).

To date, several differences have been recorded between in vivo and in vitro produced bovine embryos (Holm et al., 1998). In vitro produced blastocysts have gross morphological abnormalities such as having darker cytoplasm (Leibo

et al., 1995) and swollen blastomeres (Rizos et al., 2001), low buoyant density due to high lipid content (Leibo et al., 1995), high proportion of polyploid cells (Viuff et al., 1999, 2000; Slimane et al., 2000), a very delicate zona pellucida (Duby et al., 1997), reduced perivitelline space, accelerated development rates (Thompson et al., 1998; Lonergan et al., 1999), and aberrant expression of gap junctional proteins (Bony et al., 1999). In addition, differences have been observed in embryonic metabolism (Niemann and Wrenzycki, 2000; Khurana and Niemann, 2000) and gene expression patterns (Lazzari et al., 2002; Rizos et al., 2002, 2003). These differences might explain the high embryonic and fetal losses (Niemann and Wrenzycki; Thompson et al., 1998) that have been observed following the transfer of in vitro fertilized embryos.

While the innate quality of the oocyte to a great extent determines the fraction of oocytes developing to the blastocyst stage (Rizos et al., 2002), recent evidence suggests that the in vitro culture environment to which the embryos are exposed following fertilization is the key determinant of blastocyst quality (Rizos et al., 2002). Therefore, any inadequacies in the culture environment can seriously compromise the developmental potential of in vitro produced embryos. Suboptimal in vitro culture conditions have been previously shown to modify the expression patterns of several developmentally important genes (Lazzari et al., 2002; Niemann et al., 2003). It is presumed that successful preimplantation and early fetal development is reliant on the timely expression of approximately 10,000 genes. Therefore, we hypothesize that, in addition to the existing

information, differences in relative abundance exist for several hitherto unidentified developmentally important genes.

Several modifications of the reverse transcription polymerase chain reaction (RT-PCR) have been used to quantify the relative abundances of individual gene transcripts (Niemann and Wrenzycki, 2000) and these include DD-RT-PCR (Natale et al., 2000), semiquantitative RT-PCR (Wrenzycki et al., 2002), RT-AFLP (Rizos et al., 2002). In this study, we describe the use of SSH originally developed in 1996, (Diatchenko et al., 1996) and its applicability to studying differential gene expression in early preattachment bovine blastocysts. Use of SSH is advantageous for it enriches low abundance transcripts that are differentially expressed in the tester population. In the present study, we attempted to identify changes in gene expression between in vitro produced day 7 blastocysts (driver) and in vivo derived day 7 blastocysts (tester) and *vice versa*. A comprehensive knowledge of altered gene expression patterns induced by the post fertilization culture environment would help us better understand the factors responsible for poor embryo quality, high embryonic loss and abnormal embryonic development. These studies will further provide us an opportunity sometime in the future to utilize the expression patterns of these marker genes to modify and improve the composition of in vitro culture media.

Materials and Methods

In Vitro Maturation, Fertilization and Culture

Ovaries were collected from cows at a local abattoir and transported to the laboratory in 0.9% normal saline supplemented with penicillin-G (100 IU/ ml) and streptomycin sulfate (0.2 μg/ml) at 26-30 °C within 5 h. Oocytes were aspirated from follicles ranging in diameter from 2 to 8 mm using an 18-gauge needle and subsequently placed into a modified-PBS solution. In vitro maturation, fertilization and culture were performed according to protocols previously described in Mohan et al. (2001). Zygotes were cultured in CR1aa medium with 5% FBS introduced on day 5 of in vitro culture. For Real-time quantitative PCR analysis, three independent groups of embryos from each of the three production systems ie., in vivo-derived, in vitro produced embryos cultured either in TCM199 or CR1aa with 5% FBS with each group containing ten embryos were utilized. Total RNA was extracted from ~10 pooled day 7 in vivo blastocysts, day 7 in vitro blastocysts cultured in TCM199 with 5% FBS as well as CR1aa. In vitro produced embryos originating from either TCM199 or CR1aa cultures were visually monitored during in vitro culture and only unexpanded, morphologically clear and uniform looking intact blastocysts were selected RNA extraction.

In Vivo Embryo Production

For in vivo blastocyst production, Angus cows were superovulated by i.m. 300 mg of FSH (Folltropin; London, ON, Canada) given in a series of decreasing

doses over a 4-day period. On the morning and evening of the third day of FSH treatment an i.m. injection of 25 mg of prostaglandin $F_{2\alpha}$ was administered to induce luteolysis. Donor cows were artificially inseminated 12 and 24 h after first standing estrus with semen from a proven Hereford sire. Early stage blastocysts were recovered by nonsurgical uterine flushing on Day 7 after the first standing estrus (Day 0 = standing estrus).

RNA Extraction

In vivo and in vitro produced unexpanded blastocysts with intact zona pellucida (day 7) (n=25) were frozen in 250 μl of denaturing solution (4 M guanidium isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-β mercaptoethanol). In vitro produced embryos were visually monitored during in vitro culture and only unexpanded, morphologically clear and uniform looking intact blastocysts were selected for RNA extraction. Total RNA was extracted from a pool of 25 embryos at both stages according to the method described in Mohan et al. (2001).

Suppression Subtractive Hybridization and PCR Amplification of Subtracted Products

Subtractive hybridization was performed using the CLONTECH PCR-Select cDNA subtraction kit as described in Mohan et al. (2002) using cDNA synthesized with the SMART™ cDNA synthesis kit (CLONTECH Laboratories, Inc, Palo Alto, CA). After completion of PCR and before subjecting the amplified

products to SSH, 5 μl aliquots were run on a 2% agarose gel to verify that the amplified products ranged in size from 500 bp to about 6 kb and were not inadvertently overamplified. Subtraction was performed in two directions: cDNA derived from in vivo blastocysts served as tester and in vitro blastocyst cDNA served as driver and *vice versa*. In brief, both the tester and driver cDNAs were digested with *Rsa*I following which two different adaptors, namely, adaptor 1 or adaptor 2R were ligated to two separate pools of tester cDNA in independent ligation reactions. Adaptor ligated tester cDNAs were then allowed to hybridize in the presence of excess driver cDNA in two separate hybridization reactions. The two tester populations ligated with the two different adaptors were then mixed in the presence of excess driver and allowed to hybridize a second time. After the second hybridization, tester cDNA was subjected to a primary and a secondary PCR amplification. Following subtractive hybridization and PCR amplification, the amplified products were cloned into the pCR II vector of the TA cloning kit (Invitrogen, Carlsbad, CA) and used to transform competent DH5α *Escherichia coli* cells.

Differential Screening and Clone Analysis

Ninety six clones each from the in vivo and in vitro blastocyst-enriched libraries were randomly picked and grown overnight in 96-well plates. Plasmids from each clone were extracted and four identical dot blots were prepared for each subtraction. The forward subtracted, unsubtracted and reverse subtracted, unsubtracted PCR products were purified, digested with *Rsa*I to remove the

adaptor sequences, and used as templates for synthesizing probes for differential screening. The *Rsa*I digested products were purified and non-radioactively labeled with digoxigenin using the random-primed DNA labeling kit (Roche Diagnostics, Mannheim, Germany). Membranes were prehybridized for 30 min in DIG Easy Hyb (Roche) and hybridized overnight in the same solution at 42° C with the forward subtracted, unsubtracted forward driver, reverse subtracted and unsubtracted reverse driver probes. Post hybridization washes included two washes with 0.5% SSC+0.1%SDS (10 min) followed by two 30 minute washes in 0.1X SSC, 0.1% SDS. Membranes were rinsed briefly in washing buffer and detected with alkaline phosphatase-conjugated anti-digoxigenin antibodies (Roche) and CSPD as a chromogenic substrate according to the manufacturer's instructions. Based on the visual assessment of signal intensity by two different investigators, three different types of differentially expressed clones were picked: 1. Clones that hybridized to the forward subtracted and unsubtracted probes and not to the reverse subtracted and unsubtracted probes. 2. Clones that hybridized only to the forward subtracted probes. 3. Clones that hybridized to both forward and reverse subtracted probes, but with different intensities. Differentially expressed clones were grown overnight, plasmids extracted and the inserts were subjected to dideoxy chain termination sequencing (Applied Biosystems, Model 373A Automated Sequencer, Oklahoma State University Recombinant DNA/Protein Facility) and the identity of each product was confirmed in a sequence homology analysis using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).

Quantitative 1-Step RT-PCR

Expression of 3 ESTs of interest; namely, galectin-1 and fibronectin isolated from in vivo blastocysts and filamin- A found to be upregulated in in vitro produced blastocysts was evaluated by real-time quantitative RT-PCR using a fluorescent reporter and 5' exonuclease assay system as previously described in our laboratory (Mohan et al., 2002). Three independent groups of embryos for each of the three production systems ie., in vivo-derived, in vitro produced embryos cultured either in TCM199 or CR1aa with 5% FBS with each group containing ten embryos were utilized for real-time RT-PCR analysis. Total RNA was extracted from ~10 pooled day 7 in vivo blastocysts, day 7 in vitro blastocysts cultured in TCM199 with 5% FBS as well as CR1aa. In vitro produced embryos originating from either TCM199 or CR1aa cultures were visually monitored during in vitro culture and only unexpanded, morphologically clear and uniform looking intact blastocysts were selected for RNA extraction. Total RNA from all embryo samples was quantified at least twice using ribogreen assays and spectrophotometrically based on A_{260} values and 20 ng of total RNA was employed for real-time PCR analysis. In this way, we ensured that equal concentrations of RNA was loaded for all samples and that changes in gene expression did not arise as a result of differences in cell number. Total RNA from each group of embryos was assayed in triplicate wells (in total 27 wells).

Quantification of gene amplification was made following RT-PCR by determining the threshold cycle (C_T) number for FAM fluorescence within the

geometric region of the semilog plot generated during PCR. Within this region of the amplification curve, each difference of one cycle is equivalent to a doubling of the amplified product of the PCR. The quantification of target gene expression was done using the relative standard curve method. The quantification of target gene expression was normalized to the expression of 18S ribosomal RNA, and standard curves were generated for each of the three targets and the 18S ribosomal RNA control in order to determine the total input RNA. A best-fit line was generated using Microsoft Excel Software and the concentration was determined from the threshold cycle value of the known standards. For each culture system, the concentration of mRNA of each target and 18S ribosomal RNA were determined from their respective standard curves in order to assess the amplification efficiency. The concentration of target mRNA was then divided by the 18S ribosomal RNA to obtain a normalized value for each of the three target transcripts. The sample with the lowest value was considered the calibrator, and assigned a value of one, or 1x sample. The relative abundance was determined by dividing each of the normalized target values by the calibrator normalized target value.

Statistical Analysis

The normalized values for each target transcript were analyzed using SAS Proc Mixed (SAS. SAS/STAT User's Guide, 1989) as a completely randomized design with three treatments. Blastocysts obtained in vivo (a), and in vitro from TCM199 with 5% FBS (b) or CR1aa (c) cultures were considered as three

independent treatments. A probability value of $P < 0.05$ was considered significant. Results are presented as bar graphs representing relative abundance. Comparisons among the three different culture systems for all three genes assayed were made using least significant difference (LSD) and tukey's procedure.

Results

Suppression Subtractive Hybridization

After 27 PCR cycles using the SMART™ cDNA synthesis kit, agarose gel electrophoresis revealed cDNA bands ranging in size from 200 bp to 1.6 kb for both in vivo and in vitro produced blastocyst pools (data not shown). Following size exclusion chromatography and *Rsa* I digestion, approximate yields of cDNA for the tester and driver ranged approximately 3-4 μg. Two different adaptors were ligated to the tester cDNA and ligation efficiency was confirmed using a PCR based assay employing two different sets of primers in two independent PCR reactions according to the manufacturer's instructions. Finally, a PCR based subtraction efficiency analysis was done using specific G3PDH primers provided in the kit according to the manufacturer's instructions. After differential hybridization screening 45 in vivo and 51 in vitro clones were identified as differentially expressed. Differentially expressed clones were picked based on the criteria outlined in the materials and methods section. Approximately 32 from a total of 45 in vivo clones isolated provided sequence information. The partial

sequences obtained from all clones were compared with known sequences in the GenBank (National Center for Biotechnology Information, Bethesda, MD) database. Homology searches revealed the identities of 31 clones with known genes. Two clones represented novel uncharacterized transcripts. Similarly, 32 out of the 51 in vitro clones provided sequence information. Out of these, 22 clones matched homologous sequences in the GenBank and 10 did not provide any matches. Their putative identity, nucleotide homologies with other known sequences and number of base pairs sequenced are presented in table 2. Sequence data were submitted to the dbEST database (National Center for Biotechnology Information).

RT-PCR Quantitation Using Taqman PCR

The quantitation of mRNA expression for galectin-1, fibronectin and filamin A was done using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). The quantification of mRNA expression was done using the relative standard curve method.

Ribosomal 18S RNA was used to normalize each sample for variation in RNA loading. Galectin-1 expression levels in embryos cultured in TCM199 and CR1aa appeared very similar (Fig. 1), and the differences were not statistically significant. Comparison of relative abundance of galectin-1 transcripts between in vivo-derived and in vitro produced embryos was made using least significant difference and tukey's procedure. According to tukey's procedure, the *P* value for comparisons between in vivo and TCM199 cultured embryos and between in vivo

and CR1aa cultures was 0.053 and 0.08, respectively. Based on LSD, however, galectin-1 expression in in vivo derived embryos was significantly different ($P < 0.05$) from embryos derived from both in vitro culture systems. It is assumed that a P value between 0.05 and 0.10 may result in significant differences provided the sample size is increased. Based on the above assumption, we accepted the comparisons made using LSD. Expression of galectin-1 mRNA in vivo-derived embryos was significantly different from embryos cultured in either TCM199 and CR1aa media ($P < 0.05$) (Fig. 1). Based on normalization with 18S rRNA levels, expression of galectin-1 mRNA was 2.93 times higher in in vivo produced blastocysts compared to blastocysts obtained from both in vitro culture systems (Fig. 1). Similarly, expression of fibronectin in in vivo produced blastocysts was significantly different from in vitro produced embryos irrespective of the culture system used. Blastocysts produced in vivo exhibited approximately ~2.64 fold greater expression than those produced and cultured in vitro in TCM199 with 5% FBS ($P < 0.05$) (Fig. 2). The difference in relative abundance was almost 4.93-fold between in vivo blastocysts and those that were obtained from CR1aa cultures ($P < 0.05$) (Fig. 2). These observations further confirmed the results of the forward subtraction. In order to confirm the results of the reverse subtraction, filamin A, isolated from in vitro produced blastocysts was further characterized. Interestingly, in vitro produced blastocysts cultured in TCM199 showed an ~1.45-fold higher expression than those derived in vivo, however, the differences did not show statistical significance (Fig. 3). Unlike, galectin-1 and fibronectin, the

relative abundance of filamin A in blastocysts cultured in CR1aa was similar to those produced in vivo.

Discussion

The first 7 days of in vitro embryo culture is a very sensitive phase during early development because several important biological events occur during this window. These include timing of first cleavage division, embryonic genome activation, compaction of the morula, differentiation of the blastocyst into trophectoderm and inner cell mass (Rizos et al., 2003). Further development is dependant on the successful control of both temporal and spatial gene expression following the activation of the embryonic genome. Therefore, any deviation from normal gene expression pattern during this period may have developmental consequences later during embryogenesis.

Serum supplementation during in vitro culture, irrespective of duration of exposure has had a profound impact on the rate of blastocyst development, sex ratio (Gutierrez-Adan et al., 2001), blastocyst morphology (Abe et al., 1999; Fair et al., 2001) and metabolism (Khurana and Niemann, 2000). Serum, still a major component of in vitro culture media (Thompson et al., 1998; Lonergan et al., 1999) does exert a biphasic effect on embryo development. While serum hindered early cleavage divisions, its presence was found to be beneficial at the morula to blastocyst transition stage (Pinyopummintr and Bavister, 1994). The embryotrophic effects of serum, however, remain inexplicable due to its

undefined composition and batch to batch variation. Because a lot of attention is being paid to understanding the cell biology of blastocyst development, there is more evidence to indicate that prolonged serum treatment can adversely affect blastocyst morphology, biochemistry (Fair et al., 2001; Farin et al., 2001) and fetal development leading to increased birth weights in the long term (Thompson et al., 1998; Lonergan et al., 1999). Further, supplementation of the bovine in vitro culture medium with serum has been recently shown to alter the expression patterns of several developmentally important genes (Lazzari et al., 2002; Rizos et al., 2002, 2003). Therefore, the present study was done to help elucidate altered gene expression patterns in blastocysts induced by the inclusion of serum in the in vitro embryo culture media. The information generated from studies of this kind, will hopefully contribute to improving the efficiency of current in vitro embryo production systems. A few laboratories have addressed this critical issue (Niemann and Wrenzycki, 2000; Lazzari et al., 2002; Rizos et al., 2002, 2003; Niemann et al., 2002), and, more needs to be done in order to get a comprehensive understanding of this deviation.

Suppression subtractive hybridization is a very sensitive technique and has the advantage of greatly enhancing levels of mRNA sequences that are unique to the tissue of interest, while reducing sequences common to both tissues. The entire task can be accomplished without prior knowledge of genes being expressed, and yields subtracted cDNA pools that are either upregulated or differentially expressed. The SSH protocol, however, involves digesting the cDNA with *Rsa*I, a four base cutter, to generate smaller cDNA fragments with an

average size of about 600 bp (Diatchenko et al., 1996). These smaller sized fragments hybridize more efficiently since larger fragments tend to form complex secondary structures and are likely to hinder the hybridization process (Diatchenko et al., 1996). This step makes it impossible to obtain full length cDNAs representing differentially expressed genes. SSH also requires at least 2-4 μg of cDNA to begin with and this amount is difficult to obtain, especially, from preattachment embryos. Using the SMART PCR synthesis kit the required amount of cDNA for SSH was successfully generated. The final products of SSH are in the form of small cDNA fragments ranging in size from 250 bp to 1.5 kb called expressed sequence tags (ESTs). These ESTs can be used to discover novel genes (Adams et al., 1995), characterize gene function (Papadopoulos et al., 1994), and quantify the expression patterns of a particular gene of interest (Okubo et al., 1992).

Making use of SSH, we had earlier described stage specific gene expression patterns for the bovine hatched blastocyst (Mohan et al., 2002). In the present study, we report the construction of a subtracted cDNA library from day 7 in vivo derived and in vitro produced blastocysts cultured in TCM199 in the presence of 5% FBS. Out of the thirty-two ESTs representing in vivo blastocysts, roughly 19% were homologous to coding regions for products involved in protein synthesis, and included several ribosomal proteins and 16% represented products related to metabolism. The remaining identified ESTs were related to mitochondrial function (22%), transcription (6%), lectins (3%) and others (19%). Among the ESTs isolated from in vitro blastocysts, roughly 38% matched

products involved in transcription, and the remaining were related to signaling (10%), metabolism (23%), protein synthesis (10%) and others (19%). Of significance is the observation that 38% of the in vitro subtracted ESTs represented products related to transcription compared to 6% of the in vivo subtracted ESTs. The majority of these are nuclear proteins, possibly expressed as a defense mechanism to overcome the adverse environment arising from suboptimal in vitro culture conditions. Two in vivo subtracted ESTs, galectin-1, and fibronectin and one in vitro subtracted EST, filamin A with recognized roles during early embryogenesis were further characterized using real-time quantitative PCR. Expression of galectin-1 has been well characterized during embryogenesis in mice (Poirier and Robertson, 1993), human (Van den Brule et al., 1997) and birds (Levi and Teichberg, 1989). Recently, using SSH, Ponsuksili et al. (2002) isolated galectin-3 and fibronectin from in vitro produced bovine blastocysts. Using real-time PCR, they also showed that the mRNA expression for galectin-3 was three fold higher in blastocyst stage than in the morula stage. Galectin-1 can transform non-myogenic cells such as fibroblasts to muscle cells thereby implicating its role in cellular differentiation processes (Goldring et al., 2002). Filamin A was originally isolated from murine blastocysts and since two ESTs isolated from in vitro produced blastocysts showed greater than 95% homology to filamin A, we envisaged that filamin A probably had a regulatory role to play during early embryo development and hence characterizing its expression level was important.

Galectin-1 is a member of the galectin family of carbohydrate binding proteins and to date is known to include at least 14 members (Liu et al., 2002). Galectin-1 exists as a homodimer with two conserved carbohydrate recognition domains (CRDs) and has a binding affinity for β-galactosides. With the aid of two CRDs, galectin-1 can play important roles in mediating cell-cell and cell matrix interactions. Further, galectin-1 has been shown to cross link T-cell glycoproteins, by specifically recognizing galactose residues on CD45 and CD26. In this way, galectin-1 can induce apoptosis of activated T cells via ERK (extracellular regulatory kinase) phosphorylation and activation of specific transcription factors (Rabinovich et al., 2000). Based on this function a role for galectin-1 in providing immune privilege to the developing haploid sperm cells in the testis was recently proposed (Dettin et al., 2003). Other functions of galectin-1 include regulation of cell transformation, anchoring Ras to the cell membrane and nuclear splicing of pre-mRNA (Liu et al., 2002). In the present study, galectin-1 expression was found to be 2.93-fold higher in blastocysts produced in vivo compared to in vitro produced blastocysts (Fig. 1). In addition to the regulatory roles, expression of galectin-1 by the bovine preattachment embryo may function to protect the implanting embryo from the harmful effects of the maternal immune system. Reduced expression of galectin-1 by in vitro produced embryos as observed in the present study may be partially responsible for the high embryonic loss and poor pregnancy rates following transfer of IVP embryos.

A second in vivo subtracted clone that we identified as differentially expressed was fibronectin. Fibronectin is an extracellular matrix protein and

performs vital roles during cell proliferation, cell adhesion and cell mobility. The size of the fibronectin gene is about 50 kb and several isoforms exist for fibronectin due to alternative splicing of ED-A, ED-B and III-CS regions and post-translational modifications (Przybysz and Katnik-Prastowska, 2001). Fibronectin exists as a homodimer and has several domains such as, heparin binding domain, fibrin binding domain, collagen binding domain, cell recognition domain. The presence of many such domains provides fibronectin the opportunity to interact and bind several ligands such as, cells, heparin, fibrin, collagen, immunolgobins, DNA., etc (Yamada, 1991). Because events such as cell proliferation, cell adhesion and cell mobility are active during early embryogenesis, fibronectin does automatically qualify as an important participant during this phase of embryo development. Our results show that early preattachment embryos not only express fibronectin but also differ in their expression patterns depending on the source of production and type of in vitro culture system employed. In the present study, in vivo derived blastocysts had an approximately 2.64- fold increase in fibronectin expression compared to blastocysts cultured in TCM199 with 5% FBS (Fig. 2). However, fibronectin expression in in vivo derived blastocysts was at least 4.65- fold higher compared to blastocysts derived from CR1aa cultures (Fig. 2). This significant difference in expression between in vivo and in vitro produced embryos further indicates that the mRNA expression for fibronectin, a very important extracellular matrix protein is significantly reduced in bovine embryos produced in vitro. Considering the known functions of fibronectin during early embryogenesis, lower expression

patterns exhibited by in vitro produced embryos may be responsible for the poor quality of these embryos compared to their in vivo counterparts.

The expression patterns of galectin-1 and fibronectin in in vivo-derived embryos as revealed by real-time PCR confirmed the results of the forward subtraction. In order to confirm the validity of the reverse subtraction, we characterized a third subtracted clone, namely filamin A, isolated from in vitro produced embryos. Filamin A is a high molecular mass cytoplasmic actin binding protein. There are three known isoforms, filamin A, B and C, transcribed from 3 independent genes (Van der Flier and Sonnenberg, 2001). The primary function of filamin A is to organize actin filaments into networks and stress fibers. They also anchor various transmembrane proteins to the actin cytoskeleton to provide a scaffold for cytoplasmic signaling proteins (Van der Flier and Sonnenberg, 2001). Furthermore, filamin A also functions to transduce stress signals to the actin cytoskeleton by interacting with the cytoplasmic domain of integrins (Stossel et al., 2001). This association signals the actin cytoskeleton to stiffen so that the cell can now withstand any additional strain or stress. It is clear from recent studies that in vitro produced embryos are constantly subjected to cellular stress arising from suboptimal culture conditions. The in vitro culture system induced stress is indicated by the enhanced expression of HSP 70.1 by in vitro produced embryos (Lazzari et al., 2002). In the present study, in vitro produced blastocysts cultured in TCM199 with 5% FBS showed a 1.45-fold increase in filamin A expression compared to in vivo produced embryos and in vitro produced embryos cultured in CR1aa media (Fig. 3). Unlike galectin-1 and

fibronectin, only a very small increase in filamin A expression was observed in in vitro produced embryos cultured in TCM199 and the increase was not statistically significant. These observations mean that filamin A mostly likely falls under the so called " very low abundance transcript" category. Further, based on the real-time quantification data it was observed that while target C_t values were lower for both galectin-1 and fibronectin, these values representing filamin A were greater than thirty for both in vivo and in vitro produced embryos. The above finding further strengthens our claim that filamin A could possibly be a very low abundance transcript.

A few in vivo and in vitro ESTs did not match any homologous sequences in the GenBank initially, however, they did provide perfect matches when subjected to the blast search at a later date. Interestingly, six in vivo ESTs matched mitochondrial DNA and mitochondrial products coding for various metabolic enzymes (Table 2). Mitochondrial dysfunction/ degeneration has been identified as a conspicuous molecular pathology associated with in vitro embryo culture involving serum (Farin et al., 2001; Crosier et al., 2001; Abe et al., 2002). This finding therefore, may be significant because of the high redundancy (6 clones) and its uniqueness to the in vivo derived blastocyst. These results further corroborate the observations made earlier by others (Farin et al., 2001; Abe et al., 2002), using a different approach. Similarly, two unique in vitro ESTs gave >98% homology with pyruvate kinase, a glycolytic enzyme that catalyzes the conversion of phosphoenolpyruvate to pyruvate during which process an ATP molecule is generated (Table 2). The isolation and identification of pyruvate

kinase as a differentially expressed gene from in vitro produced blastocysts indirectly supports the findings of Khurana and Niemann (Khurana and Niemann, 2000) on early embryonic metabolism. In their study, in vitro blastocysts cultured in the presence of serum were found have high rates of glycolysis and produced high amounts of lactic acid. In addition, approximately seven in vitro specific ESTs were found to be homologous to a nuclear protein namely, aortic preferentially expression gene (APEG) found to be expressed in differentiated aortic vascular smooth muscle cells (Chen et al., 2001) (Table 2). The function of APEG during early preattachment embryogenesis under in vitro conditions is not clear and certainly requires scrutiny in the future as it may be of interest to researchers working in the field of early mammalian development.

In summary, we have performed a SSH analysis between bovine in vivo and in vitro produced embryos cultured in the presence of serum. Two in vivo subtracted clones representing galectin-1 and fibronectin were further characterized using real-time PCR and the expression levels were shown to be elevated in in vivo produced embryos compared to blastocysts derived from two in vitro culture systems. Filamin-A, an EST isolated from in vitro produced embryos using SSH was also characterized using real-time PCR. Interestingly, even though a slight increase (1.45- fold) was observed in embryos produced in TCM199 with 5% FBS this increase was not statistically significant. Therefore the expression pattern of filamin A based on real-time RT-PCR did not agree with SSH. The findings in the present study further supports the earlier generated hypothesis by others that there are several candidate genes whose expression

patterns differ as a result of employing suboptimal culture systems. In the future it is necessary to extend these studies to embryos cultured in other in vitro systems such as SOF, Gardner's G1 & G2 both in the presence and absence of serum. We suggest that deviations in gene expression patterns do exist between in vivo and in vitro produced bovine embryos.

Acknowledgments

The authors thank David Goad and Khurshid Gandhi (CLONTECH Labs, CA) for excellent technical assistance. We also thank the staff of Mikkelson's Beef, Oklahoma City, OK for their assistance and generous donation of materials used in the study. The authors also thank Dr. Larry Claypool from the Department of Statistics, Oklahoma State University for assisting with the statistical calculations. The authors acknowledge the Oklahoma State University Recombinant DNA/Protein Resource Facility for the synthesis of synthetic oligonucleotides and the sequencing of cloned cDNA. This study was supported by the Oklahoma Agricultural Experimental Station.

Table 1. Primers and probe sequences used for real time RT-PCR.

Gene	Primer sequence	Probe sequence	Product (bp)
Galectin-1	5'- CCTGGAAGTGTCGTGGAGGTAT- 3' 5'- TCGTATCCATCAGGCAGCTT- 3'	5'-CATCTCCTTCAACCAGACGGACCTAACCA- 3'	75
Fibronectin	5'- TGACAGAGAAGATTCCCGAGAGTAATAT- 3' 5'- CGAACACCACTCCAGTTTGGAT- 3'	5'-CAACCCAGAGAAACAAGCGTGGACCTC-3'	91
Filamin A	5'- CCTGAGGGCTACCGTGTCA- 3' 5'- GCCACCATACTTGATGGAGATG- 3'	5'- CCCATGGCACCTGGCAGCTACC- 3'	72

Table 2. Identity, size, and percentage homology of putative bovine in vivo and in vitro blastocyst specific subtracted cDNA clones compared to known sequences in GenBank.

Identity	Clone number	GenBank accession number	Base Pairs Sequenced	Homology[a]
In Vivo Blastocyst subtracted clones				
Galectin- 1	OKST InvivoBlast-18	CD28525	456	Bovine 98% (398/405) Human 86% (346/401) Mouse 83% (337/406)
Transcription elongation factor B (SIII) (2 clones)	OKST InvivoBlast-24, 41	CD28526, 30	591	Human 92% (425/460) Rat 91% (406/446) Mouse 90% (417/461)
Peroxiredoxin 2	OKST InvivoBlast-28	CD28527	610	Bovine 100% (173/173) Human 85% (135/154) Mouse 89% (116/129)
Ribosomal protein S4	OKST InvivoBlast-29		722	Cat 85% (248/291) Hamster 84% (237/282) Rat 83% (234/279) Human 84% (222/264)
Fibronectin (3 clones)	OKST InvivoBlast-31, 62, 71	CD28528, 34, 38	486	Bovine 98% (455/461) Equine 89% (212/238) Rabbit 81% (371/453) Human 87% (182/209)
Cytochrome C Oxidase (3 clones)	OKST InvivoBlast-32, 54, 75	CD28529	227	Bovine 99% (210/212) Human 86% (177/204) Mouse 84% (144/170)
Ribosomal protein L24	OKST InvivoBlast-47		514	Bovine 97% (427/439) Human 90% (430/473) Mouse 89% (392/439) Rat 89% (392/439)
Vacuolar protein sorting 29	OKST InvivoBlast-56	CD28532	694	Human 93% (562/602) Rat 88% (490/556) Mouse 87% (453/515)
Glucose transporter type 3	OKST InvivoBlast-60	CD28533	698	Ovine 95% (345/361) Bovine 97% (306/313)
Eukaryotic translation elongation factor 1 alpha	OKST InvivoBlast-68	CD28536	493	Bovine 89% (441/493) Dog 83% (414/493) Human 83% (414/493)
Ubiquinol-cytochrome c reductase binding protein	OKST InvivoBlast-69	CD28537	703	Human 90% (289/320) Mouse 88% (303/341)
Cytoskeletal tropomyosin TM30(nm)	OKST InvivoBlast-76	CD28539	433	Human 88% (257/289) Mouse 86% (123/143) Rat 85% (125/146)
Proteasome (prosome, macropain) subunit, alpha type, 2	OKST InvivoBlast-83	CD28540	282	Human 93% (237/253) Rat 89% (229/257) Mouse 87% (226/257)

Actin	OKST InvivoBlast-84	CD28541	694	Bovine 97% (394/404) Rat 92% (328/354) Human 92% (337/366) Mouse 92% (327/355)
Ribosomal protein S25 (RPS25)	OKST InvivoBlast-85		519	Human 91% (421/458) Rat 90% (353/388) Mouse 88% (396/447)
Ribosomal protein S27 (metallopanstimulin 1) (RPS27)	OKST InvivoBlast-86		317	Human 93% (275/293) Mouse 93% (270/290) Rat 92% (255/276)
Ring-box protein 1 (RBX1)	OKST InvivoBlast-92	CD28543	487	Human 89% (408/454) Mouse 92% (255/276) Rat 92% (255/276)
Chaperonin containing TCP1, subunit 3 (gamma)	OKST InvivoBlast-94	CD28544	571	Human 94% (534/565) Rat 90% (512/568) Mouse 89% (510/568)
Thymosin beta-10	OKST InvivoBlast-96	CD28545	301	Bovine 98% (280/284) Equine 88% (207/235) Human 89% (178/200) Mouse 93% (112/120) Rat 93% (112/120)
Mitochondrial genes and mitochondrial RNA products	OKST InvivoBlast-48, 59, 72, 90, 95, 82			
Unknown ESTs (2 clones)	OKST InvivoBlast-44, 63	CD28531, 35		
In Vitro Blastocyst Subtracted Clones				
Nuclear protein 220 (2 clones)	OKST InvitroBlast-10, 53	CD28547 CD038846	696	Mouse 95% (661/694) Human 91% (237/258)
28S ribosomal RNA (3 clones)	OKST InvitroBlast-17, 64, 71		303	Mouse 100% (282/282) Rat 99% (278/280)
Splicing factor, arginine/serine- rich 2 (SC-35) (3 clones)	OKST InvitroBlast-18, 22, 90	CD28549, 51, 69	256	Rat 99% (255/256) Mouse 94% (197/208) Human 95% (105/110)
Pyruvate kinase (2 clones)	OKST InvitroBlast-23, 74	CD28552, 66	487	Mouse 99% (465/469) Rat 99% (300/302) Human 85% (88/103)
Aortic preferentially expressed gene 1 (Apeg1) (7 clones)	OKST InvitroBlast-29, 75, 80, 83, 89, 93, 94	CD28554, 67, 68, 69, 71, 72	485	Rat 100% (250/250) Mouse 91% (230/252) Human 87% (74/85)
Filamin A like protein (3 clones)	OKST InvitroBlast-46, 60, 72	CD28557, 61, 65	695	Mouse 96% (673/695) Bovine 85% (595/692) Human 91% (357/389)
Clathrin	OKST InvitroBlast-65	CD28563	589	Rat 97% (325/334) Mouse 97% (313/322) Human 89% (283/316)
Heat shock protein 105 kDa alpha	OKST InvitroBlast-92	CD28570	702	Rat 99% (435/449) Mouse 90% (545/604) Human 83% (381/459)

Unknown ESTs (10 clones)	OKST InvitroBlast-5, 19, 20, 25, 32, 33, 48, 56, 59, 95	CD28546, 50, 53, 55, 56, 58, 59, 60, 73		

Percentages are based on BLAST searches of the GenBank database. The numbers in parentheses show the number of bases (query/subject) that were compared. Mitochondrial and ribosomal RNA sequences were not reported to dbEST.

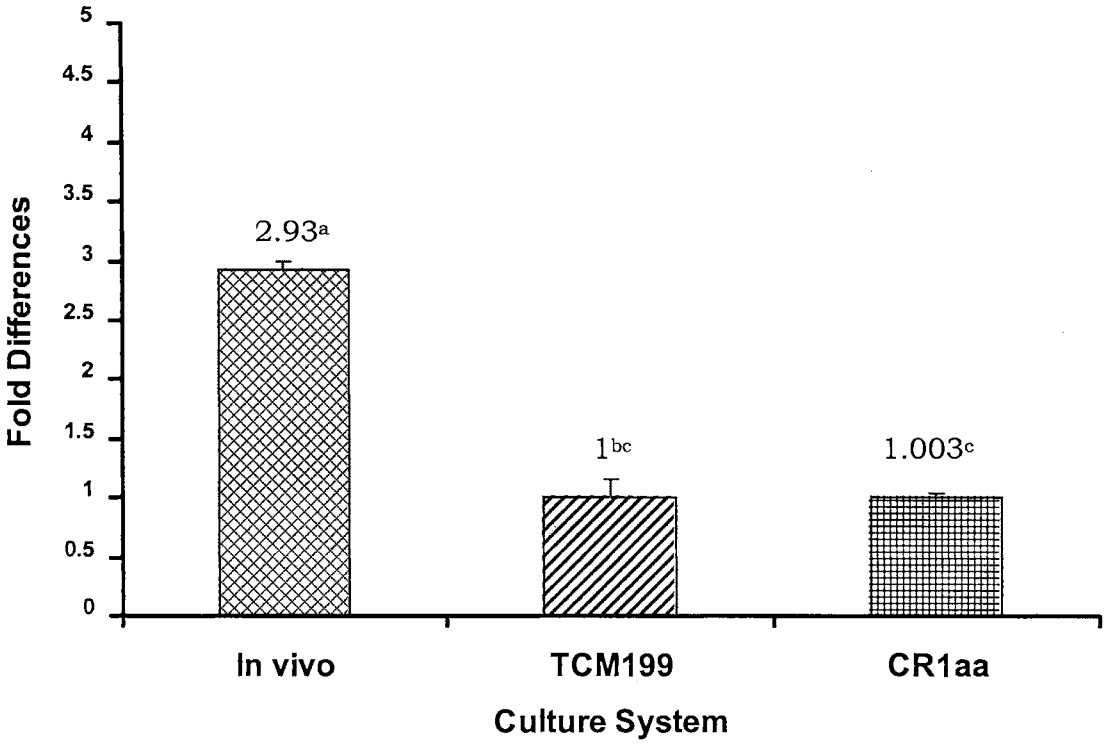

Figure 1. Relative abundance in gene expression for galectin-1 detected using one-step real-time RT-PCR (n= 3, representing 3 pools of ~10 blastocysts per treatment). The fold differences in gene expression were calculated as described in *Materials and Methods*. Bar graphs with different superscripts differ significantly ($P < 0.05$). Bars with a common superscript do not differ significantly from each other.

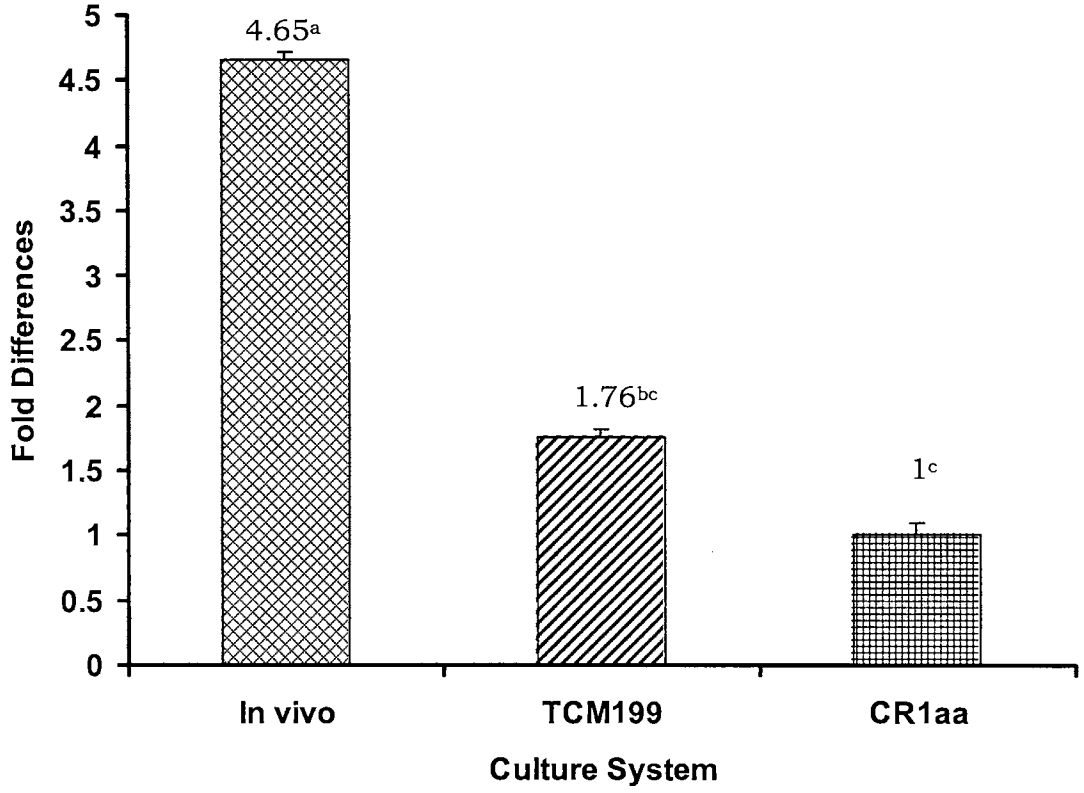

Figure 2. Relative abundance in gene expression for fibronectin detected using one-step real-time RT-PCR (n= 3, representing 3 pools of ~10 blastocysts per treatment). The fold differences in gene expression were calculated as described in *Materials and Methods*. Bar graphs with different superscripts differ significantly ($P < 0.05$). Bars with a common superscript do not differ significantly from each other.

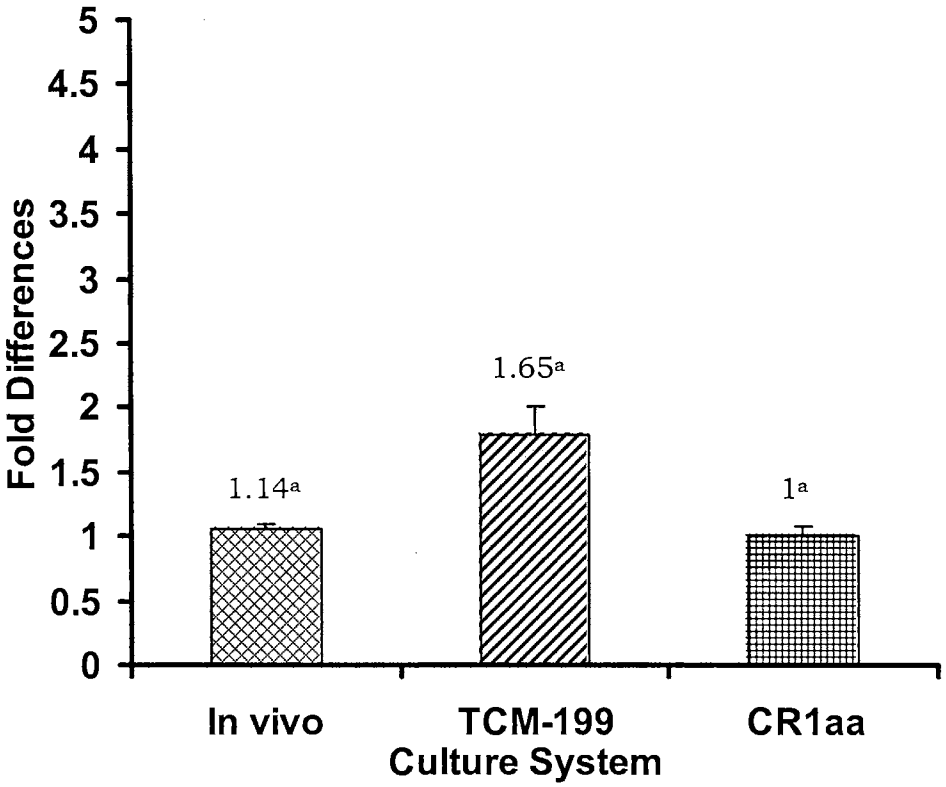

Figure 3. Relative abundance in gene expression for filamin A detected using one-step real-time RT-PCR (n= 3, representing 3 pools of ~10 blastocysts per treatment). The fold differences in gene expression were calculated as described in *Materials and Methods*. Bar graphs with similar superscript do not differ significantly ($P > 0.05$).

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General Discussion and Conclusions

Given the limited information available concerning the retinoid signaling pathway in the bovine, and the fact that previous work on the role played by this pathway has focused primarily on the rodent species, the present research work was carried out to generate information for developing an understanding of the role of the retinoid signaling system in early developmental processes in non-rodent species such as cattle. Irrespective of the species of interest, the current information available on the role played by the retinoid signaling pathway pertains predominantly to postimplantation stages and very little information is known during preattachment embryogenesis. Therefore, the successful execution of a project of this nature would require an unrestricted supply of preattachment stage bovine embryos. These early embryonic stages are normally found in the oviduct and are therefore, difficult to retrieve through non-surgical means. The ready availability of an in vitro bovine embryo production system was very encouraging as it alleviated this difficulty. Before we employed embryos produced from our in vitro system for research studies it was necessary to ascertain that the resultant embryos from our IVF system were competent enough to establish viable pregnancies and translate these pregnancies into viable calves. In order to fulfill the above objective, we non-surgically transferred three day 7 blastocysts into a single holstein friesian receipient cow in November 1998. Two embryos were transferred into the ipsilateral horn and one into the contralateral horn. A viable male calf was delivered on the 1st of August 1999.

Before we studied the functions of the retinoid signaling system in bovine preattachment embryos it was vital that we investigated the expression patterns of the different components of the signaling system. The different components comprise retinol binding protein, enzymes involved in the oxidation of retinol to retinaldehyde and then to retinoic acid, retinoic acid receptors, retinoid X receptors, other retinoid binding proteins such as cellular retinol binding protein, cellular retinoic acid binding protein., etc. In chapter III, as a first approach we investigated the expression of retinol binding protein, retinoic acid receptor α, β, and γ at the mRNA and protein level. Employing the sensitive RT-PCR method, transcripts for RBP, RARα, and RARγ were detected in all stages from the oocyte through to the hatched blastocyst. Expression of RARβ was not detected at any stage. Strong immunostaining was observed for both RARα and RARγ2 in the inner cell mass and trophectoderm of intact and hatched blastocysts. In chapter IV, we determined the expression patterns of the remaining components of the retinoid signaling pathway. These included the retinoid X receptors, alcohol dehydrogenases, retinaldehyde dehydrogenases and PPAR which is an important heterodimerization partner for RXRs. RT-PCR was again employed to investigate mRNA expression for RXRα, RXRβ, RXRγ, alcohol dehydrogenase I (ADH-I), retinaldehyde dehydrogenase 2 (RALDH-2), and peroxisome proliferator activated receptor gamma (PPARγ). In addition, whole mount in situ hybridization using digoxigenin labeled probes was also utilized to specifically determine which of the two cell types in the blastocysts actually expressed the transcripts. Transcripts for RXRα, RXRβ, RALDH-2, and PPARγ were detected in all stages

beginning from the oocyte through to the hatched blastocyst. Whole mount in situ hybridization performed using digoxigenin-labeled antisense probes detected all 4 transcripts in both the inner cell mass and the trophectoderm of hatched blastocysts. PCR products obtained for ADH-I exhibited less than 45% homology to known human and mouse sequences. Employing polyclonal antibodies strong immunostaining was observed for both RXRβ and PPARγ in the trophectoderm and inner cell mass cells of intact and hatched blastocysts. Based on in situ hybridization and immunohistochemistry it is clear that both cell types in the early blastocyst express these receptor proteins. Messenger RNA was not detected at any stage for RXRγ. Expression of mRNA for RBP, RARα, RARγ, RXRα, RXRβ, and PPARγ by the bovine embryo may suggest a role for retinoic acid in regulation of gene expression during preattachment development. Expression of mRNA for RALDH-2, suggests that the early bovine embryo may be competent to synthesize retinoic acid during preattachment development in vitro.

Similar expression patterns for retinoic acid receptors have been previously reported in murine preattachment embryos (Wu et al., 1992). In their study too, RARβ was also not detected at any stage of development. Therefore expression patterns, at least in the mouse and the bovine, agree and it is likely that these expression patterns might be conserved across the different mammalian species. Presence of the immunoreactive receptor protein for a few of the RARs and RXRs does not necessarily guarantee biological activity. Techniques such as transient reporter and electromobility shift assays are available to study biological activity of the receptor protein or in other words

protein-DNA interactions. Introducing transgenic reporter constructs into blastocysts is not an easy task due to the presence of the zona pellucida which can effectively block successful transfection. A second problem is that these early embryonic stages are very sensitive to mild insults and introduction of any foreign DNA can easily impede further development. Third, following hatching the embryo may be amenable to transfection but with reduced viability since hatched embryos do not survive longer under in vitro conditions. Fourth, given the limited number of blastocysts that hatch under in vitro conditions these numbers may not be sufficient to detect a significantly quantitative signal following transfection. To overcome some of these problems we decided to employ a cell line derived from the bovine trophectoderm generated by Talbot et al. (2000). This cell line is immortal and has gone through several passages. Our initial attempts to transfect reporter constructs into this cell line using different transfection reagents did not succeed. The trophectodermal cell line not only was difficult to transfect but also resisted lysis. We then packaged the reporter construct into replication incompetent adeno-associated viruses, purified and concentrated the virus using centrifugal concentrator filter devices with a molecular weight cut off of 100 kDa. Before we packaged the gene of interest into viral particles we generated viral particles carrying the coding region of the beta-galactosidase gene in order to ascertain that the virus was able to successfully transfect cells. Interestingly, while the virus successfully transfected bovine cumulus and 1080 mouse fibrosarcoma cell lines as revealed by the blue color following X-gal staining, it failed to infect the trophectodermal cell line. Adeno-associated viruses utilize

heparan sulfate proteoglycans as receptors for attaching and gaining entry into the cell. Our negative results may be explained by the fact that this cell line has gone through several passages and it is more likely that the cells no longer express heparan sulphate proteoglycans on the surface thus preventing successful entry of the virus. Therefore, at this point we do not have direct evidence to show that the receptor mediated retinoid effects exist in the bovine blastocyst.

Recently, improved cytoplasmic maturation, increased embryonic cell numbers, increased cryotolerance, enhanced embryo development and pregnancies were reported following addition of 5 nM 9-*cis* retinoic acid during prematuration in the presence of roscovitine (Duque et al., 2002; Hidalgo et al., 2003). Later, the same authors also showed that 9-*cis* retinoic acid treatment enhanced the expression of midkine, a growth factor belonging to the family of heparin binding growth/differentiation factors in cumulus granulosa cells (Royo et al., 2003). In addition, positive effects of supplemental retinol at 5 μM concentration during in vitro maturation on blastocyst cell number was also recently reported (Livingston et al., 2003). These studies show that the retinoid signaling pathway can be successfully exploited practically to improve in vitro embryo development and pregnancy rates in cattle. However, this study also raised a few questions as to how 9-*cis* retinoic acid brought about these positive effects. It will be advantageous to have a clear understanding of the mechanism of action and the cell types that were targets for the retinoids. It is possible that 9-*cis* retinoic acid had a direct effect on either the cumulus cells or the oocyte or

both. Similar positive effects on embryo development in the bovine have also been observed using 1 μM retinoic acid (Duque et al., 2002). In chapter V, we hypothesized that addition of retinoic acid during prematuration of bovine cumulus oocyte complex induced a receptor mediated effect particularly, on cumulus-granulosa cells. In chapter III and IV, we showed that the immature bovine oocyte expressed RARα, γ, RXRα, β, PPARγ and RALDH-2 meaning that the different components of the retinoid signaling system were in place at least at the mRNA level. In chapter V we detected RARα, γ, RXRα, β, PPARγ and RALDH-2 at the mRNA level in bovine cumulus-granulosa cells. Therefore, it appears that both cumulus-granulosa cells and the immature oocyte can possibly respond to retinoids. However, mRNA expression does not guarantee presence of the biologically active protein. mRNAs can either be translated to the functional protein or be destroyed and not be translated at all. Therefore, a transient reporter assay was performed to determine the existence of a biologically active retinoid signaling system in cumulus-granulosa cells. This assay not only confirms the existence of the receptor proteins but also provides evidence for their biological activity. The retinoic acid response element (RARE) from the human RAR β2 promoter (de The et al., 1990) was cloned into the *Kpn*1 site of the pGL3 promoter vector containing the firefly luciferase reporter driven by the SV40 promoter. The idea is that if cumulus-granulosa cells expressed active retinoic acid receptor proteins these proteins in the presence of the ligand should be able to form a complex with the ligand, bind to the RARE and activate the luciferase reporter. Using this reporter construct we have shown that

endogenous retinoid receptors expressed by cumulus cells are biologically active. Retinoic acid at 0.5 and 1 μM concentration significantly induced reporter activity in bovine cumulus- granulosa cells. In our reporter assays, the data was expressed as luminescence unit per microgram total protein. This method was followed to make sure that cells in all wells were completely lysed and that we were not unintentionally transferring incompletely lysed cellular contents for luminescence measurements. Still an important shortcoming with our luciferase assay protocol is that it does not minimize experimental variability caused by differences in cell viability or transfection efficiency. Therefore, the assay was performed several times in its present format to check for repeatability and ascertain that the increase in activity observed was due to retinoid receptor activation of the reporter and not due to the variations mentioned above. These shortcomings can be overcome in the future by switching to a dual reporter assay (Promega Corporation, Madison, WI). The dual reporter assay involves simultaneous expression and measurement of two individual reporter enzymes within a single cell. In this assay, the "experimental" reporter which is a firefly luciferase is correlated with the effect of specific experimental conditions, while the activity of the co-transfected "control" reporter which is a *Renilla* luciferase functions as an internal control. The activity of the experimental reporter can then be normalized to the control reporter. Performing a dual reporter assays calls for additional equipment such as a luminometer and due to the non-availability of a luminometer we were unsuccessful in performing a dual reporter assay.

Due to the hindrance imposed by the presence of the zona pellucida around the oocyte we were not successful in introducing reporter gene constructs into the oocyte and therefore, it was not possible to test the same hypothesis on immature oocytes. Finally, since RARs and RXRs are ligand activated transcription factors, the presence of any one or all the components of the retinoid signaling pathway together with our reporter assay results may necessarily mean that there is retinoid-mediated gene activation in the cumulus oocyte complex or at least in the cumulus-granulosa cells. Therefore, the objective of identifying target genes that are specifically regulated by retinoic acid in the cumulus oocyte complex remains. Identifying these genes will provide us a molecular insight into the role played by vitamin A and their metabolites during in vitro/in vivo oocyte maturation in cattle. Due to time constraints this objective was not pursued.

Therefore, combining the data from our studies, those of Shaw et al., 1995, Duque et al., 2002, Hidalgo et al., 2002, Brown et al., 2003, and Hidalgo et al., 2003 it is possible that retinol injections given to cattle during superovulation programs or retinoic acid treatments provided during in vitro maturation has had a direct positive effect on the follicular cells, predominantly the cumulus-granulosa cells and possibly the oocyte too. This positive interaction was likely facilitated by the expression of the binding proteins, retinoid receptors and retinoic acid synthesizing enzymes by the cumulus cells. The expression of RARs, RXRs along with PPARγ in both cumulus cells would also mean that these receptors can possibly homo or heterodimerize to transduce the retinoid signal.

Expression of RALDH2 also shows that cumulus cells may be competent to synthesize retinoic acid from retinol that is present in the follicular fluid (Brown et al., 2003).

It is also necessary to know whether cells in the early preattachment bovine blastocyst that have transcripts for the various retinoid receptors and the immunoreactive retinoid receptor proteins can interact with physiologically significant concentrations of retinoic acid from an endogenous or exogenous source. Endogenously produced retinoids have been measured using HPLC in various species including the porcine day 10 blastocyst (Cosaridis et al., 1996; Horton and Maden, 1995; Parrow et al., 1998). These studies show that the early embryo has the enzymatic capabilities to fully metabolize vitamin A. Studies in both young and adult animals show that a stringent homeostatic mechanism operates in controlling the systemic levels of vitamin A. It is therefore, assumed that a similar homeostatic system is existent in the embryo as excess and deficiency can lead to anomalies in the embryo. Recently, it was shown that the treatment of murine blastocysts with 10 μM retinoic acid induced apoptosis preferably in the inner cell mass cells and significantly reduced the average number of total cells in the blastocysts (Huang et al., 2003). As a result fewer retinoic acid treated blastocysts implanted compared to control untreated blastocysts. This study indirectly shows that retinoic acid most likely exerted a receptor mediated effect on the inner cell mass cells. As of now this is the only evidence available to show that a retinoid receptor mediated effect of retinoic acid is operational in the blastocyst. Therefore, biological activity of the retinoid

receptor proteins in the early preattachment blastocyst also need to be demonstrated in the future. The availability of the CT-1 trophectodermal cell line and techniques like non-radioactive electromobility shift assays should help resolve the issue.

Retinoids are expensive, unstable hydrophobic ligands, light sensitive and are therefore, easily oxidized. An alternative would be to use synthetic reduced-toxicity retinoids called heteroarotinoids that have been developed and used for the prevention and treatment of cancer (Benbrook et al., 1997). At the molecular level, these compounds act very similarly to the endogenous retinoids. The availability of a completely serum free in-vitro bovine embryo production/culture system effectively enables the possibility of examining the effects of heteroarotinoids on in vitro maturation, cleavage rates, blastocyst production, hatching rates and cell number. Studies can later be extended to understand which receptors and genes are regulated by the most active compounds. The information thus generated can then be used to design highly specialized retinoids to exclusively promote embryo development.

Early embryonic development in the bovine may be comparable to the human and many analogies could be drawn. It is assumed that identifying key genetic markers that are regulated by retinoic acid may improve our understanding of the events occurring during maturation, around preattachment / implantation period and will throw more light towards understanding the biology of preattachment development and accordingly develop suitable remedies to prevent early embryonic mortality.

In order to identify differentially expressed genes, techniques with high sensitivity and efficiency need to be optimized for the cell type of interest. Several techniques are currently available for this purpose. These include several modifications of RT-PCR that comprise DDRT-PCR, RT-AFLP., etc. Even though DDRT-PCR has been used extensively it has several disadvantages. These include the use of radioisotopes which can be a major health hazard. DDRT-PCR also has an inherent tendency to pick high abundance transcripts and has a high false positive rate. In the absence of a microarray, a second technique available to study differential gene expression is subtractive hybridization. Recently, a suppression PCR step was included and it is now called suppression subtractive hybridization (SSH) (Diatchenko et al., 1996). SSH has several advantages over DDRT-PCR as it does not require the use of isotopes, can pick both high and low abundance transcripts and does not produce false positive data. However, successful use of SSH requires at least 2-4 μg of total RNA to begin with. Our primary interest was to study differential gene expression in bovine preattachment embryos, however, obtaining 2-4 μg of total RNA became a major constraint. Employing SMART-PCR we were able to successfully generate the required amount of cDNA from intact expanded (driver) and hatched bovine blastocysts (tester) generated in vitro. This procedure enabled us to perform SSH uninterruptedly. In this way, a subtracted cDNA library from bovine hatched blastocysts was successfully constructed. Approximately, 92 from a total of 126 ESTs isolated provided sequence information. Homology searches revealed the identities of 31 of these expressed sequences (expressed sequence tag; EST)

with known genes, among which three, PSMC3, CK2, and PGK with predicted roles during early embryogenesis were further characterized using real-time quantitative PCR. Overall, the largest group of identified ESTs, roughly 40%, was homologous to coding regions for products involved in protein synthesis, and included several ribosomal proteins. The remaining identified ESTs were related to transcription (about 20%), metabolism (20%), and signaling (20%). The use of SMART-PCR, although useful can also prove disastrous if adequate precautions are not taken. Overamplification of the cDNA is a serious inadvertent error that the researcher is likely to commit. Therefore, in spite of taking adequate precautions it is always advisable and safe to validate quantitative differences of a few ESTs using another independent technique such as northern blot, competitive PCR, or real-time RT-PCR. In this study, employing real-time RT-PCR we observed an overall 1.3-, 1.6-, and 1.5-fold increase in expression level in hatched blastocysts compared with intact blastocyst for PSMC3, CK2, and PGK, respectively. More importantly, we only performed SSH in one direction. As a result we had to sequence almost all the clones that we got following transformation of bacterial cells with the subtracted products. This approach is certainly not very cost effective. Therefore, in the future it would be ideal to perform SSH in both the forward and reverse direction, and identify the true positive clones (i.e., transcripts unique to one or the other stage) using a differential hybridization screening protocol. In this way, we can narrow down the number of differentially expressed products to be sequenced which would not

only help avoid additional expenditure but also provide us some assurance that only the differentially expressed ESTs are picked.

The entire project involved the use of in vitro produced bovine embryos and it is now well-known that the quality of in vitro produced embryos continue to lag behind those of their in vivo counterparts. Several differences including deviations in early gene expression between the two production systems have been documented and those differences have been provided in detail in chapter VII. Since we had optimized methods to study differential gene expression we included an additional objective to identify and characterize some of the hitherto, unidentified genes between in vivo and in vitro produced embryos. Comparisons were made between Day 7 blastocysts generated in TCM199 with 5% FBS (driver) and Day 7 in vivo derived blastocysts (tester) and *vice-versa* using suppression-subtractive hybridization (SSH). In this study, SSH was performed in both forward and reverse directions and in order to identify the true differentially expressed genes we performed a differential hybridization screening analysis. Forty-five forward subtracted cDNAs and 51 reverse subtracted cDNAs were isolated, single-pass sequenced and subjected to BLAST search. Out of the 33 in vivo clones that provided sequence information, 31 clones matched homologous sequences in the GenBank and the remaining two did not provide any matches. On the other hand, out of the 31 in vitro specific clones, 21 clones provided specific matches while the remaining 10 clones represented novel transcripts. Two in vivo clones, galectin-1 and fibronectin and one in vitro clone namely, filamin A were selected and further characterized using real-time quantitative

PCR. The real-time PCR results obtained for galectin-1 and fibronectin were in agreement with SSH. Overall, a 2.93-, 2.64-fold increase in expression level was observed in in vivo derived blastocysts compared to blastocysts cultured in TCM199 with 5% FBS for galectin-1 and fibronectin, respectively. However, fibronectin expression in in vivo derived blastocysts was at least 4.65-fold higher compared to blastocysts derived from CR1aa cultures. On the other hand, filamin A expression in blastocysts derived from TCM199 with 5% FBS cultures was not significantly different from in vivo derived blastocysts. Transcriptome analysis performed between in vivo and in vitro blastocysts revealed deviations in mRNA expression and the differences may partially explain why the quality of in vitro embryos are inferior to that of their in vivo counterparts. Several mitochondrial genes were also found to be differentially expressed in blastocysts derived in vivo. One in vitro subtracted product had a high redundancy and revealed high homology to a nuclear protein namely, aortic preferentially expressed gene (APEG). The expression and the function of this gene during in vitro embryo development is not clear and needs further investigation. More importantly, the gene expression data we obtained from this study agreed well with the information already available on the morphological and functional differences between in vivo and in vitro generated embryos.

In majority of the cases, the SSH data should agree with the real-time RT-PCR data. There are instances where real-time data may not agree statistically with the SSH data. There are several possible reasons and one of them could be reduced sample size. Therefore, while performing real-time RT-PCR it is always

advisable to have multiple samples. Another important point to keep in mind is that subtracted products that show high redundancy (repeating clones) are better enriched and are more likely to be differentially expressed. Since SSH is a very sensitive technique and from my experience it will help isolate target genes that show differences is expression ranging from 1.3 to several fold higher. Therefore, it may be advisable to be very stringent while performing the differential screening analysis and this part of the technique is totally subjective. The investigator could make the choice to pick products that only hybridize to the forward subtracted probe or forward subtracted and unsubtracted probe. Again, differences in gene expression will depend on the type of tissue samples being compared. When comparisons are made, say for example, between preattachment embryos of the same stage originating from different culture environments it may be unwise to expect too many genes to be differentially expressed or genes that show very high differences in expression patterns as a result of differing culture conditions. In chapter VII, initially we did not get many products with good matches after homology searches, however, at a later date we did see several mitochondrial genes and APEG representing in vivo and in vitro embryos, respectively. Both these products showed very high redundancy (~6-7 clones) and would have been good targets for further characterization using real-time RT-PCR. Further, fibronectin was isolated thrice compared to galectin-1 which was represented by only one clone. Based on real-time PCR analysis, expression of fibronectin was not only high but also statistically significant from in vitro produced embryos.

The successful implementation of differential gene expression analysis using preattachment embryos opens up new opportunities for comparisons to be made in the immediate future involving embryos produced from other currently available culture systems. Further, some of these genes may serve as markers and aid in the modification/development of new in vitro embryo culture systems. However, additional studies involving embryos originating from other available in vitro culture systems and more characterization studies will be required before we identify and establish good marker genes. Differentially expressed genes from both subtractions may be immobilized on a microarray platform. Availability of a embryo specific microarray would permit large scale screening of embryos originating from different culture systems and will also give us the opportunity to modify/change or device new culture media based on the expression patterns of these genes.

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VITA 2

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