CHARACTERIZATION OF CADHERINS, ADAMTS, STEROID RECEPTORS, DURING BLASTOCYST FORMATION IN VITRO AND OF ADAMTS IN THE BOVINE OVARY

by

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ABSTRACT

In bovine, early embryonic mortality (EEM) results in low reproductive efficiency, the major cause for which is believed to be impaired development of the embryo, but mechanisms involved therein remain poorly understood. A lack of progesterone support from the corpus luteum (CL) also contributes to EEM. Recent studies demonstrate that regulated expression of distinct cadherins that mediate Ca^{2+}-dependent cell-cell adhesion and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) subtypes involved in cell-matrix interactions, and steroid receptors mediate important cellular events underlying the formation and organization of tissues, however, their role in preimplantation embryogenesis is poorly characterized. Understanding genes involved in the blastocyst and CL formation provides interesting possibilities in improving embryo viability. To address these issues, investigations into the expression of cadherins, ADAMTS, estrogen and progesterone receptor isoforms during the preimplantation embryogenesis in bovine embryos at timed stages of development has been undertaken, using reverse transcription-polymerase chain reaction (RT-PCR) or immunohistochemistry strategies. In addition, expression of ADAMTS subtypes in ovarian follicles and CL has also been characterized. These studies indicate that multiple cadherins, both type 1 (E-, N- and P-Cad) and type 2 (cad-8, -10 and -11), with their specific localizations, at least in part, provide the molecular basis for the formation and organization of the inner cell mass and the trophectoderm. The differential mRNA and protein expression of steroid receptor isoforms (ERα, ERβ, PRA and PRB) suggests distinct role(s) during early embryogenesis and provides evidence for direct

effect of steroids on the embryo. The regulated but complex expression of ADAMTS subtypes both in the embryo (ADAMTS-1, -4, -5, -6, -8, -9, -10, but not ADAMTS-2, -3, -7 or -12) and the ovary (ADAMTS-1, -2, -3, -4, -5, -7, -8, -9, but not ADAMTS-6, -10 or -12) suggests that these proteinases could be involved in cell-matrix interactions and contributing towards important cellular events like cell migration, differentiation and remodeling of the extracellular matrix. Though, the biological significance of these molecules remains unclear, these studies demonstrate that novel cellular molecules involved in cell-cell, cell-matrix and steroid interactions could be playing a key role during developmental processes like embryogenesis and/or CL formation.

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LIST OF ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AF	Activation function
ANOVA	Analysis of variance
ATD	1,4,6-androstatriene-3, 17-dione
BEHAB	Brain enriched hyaluronan binding
BLAST	Basic local alignment search tool
BMP	Bone morphogenetic protein
BO	Brackett and Oliphant
BSA	Bovine serum albumin
Cad	Cadherin
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine 3',5'-monophosphate
CAR	Cell adhesion recognition
cDNA	Complementary DNA
CL	Corpus luteum
CNS	Central nervous system
COX	Cycloxygenase
CP	Cytoplasmic
EGF	Epidermal growth factor
DBD	DNA binding domain
DD	Differential display
DDRT-PCR	Differential display reverse transcription-polymerase chain reaction
DERKO	Double estrogen receptor knockout
DES	Diethylstilbestrol
DF	Dominant follicle
DNA	Deoxyribonucleic acid
E2	17β-estradiol
EC	Extracellular
eCG	Equine chorionic gonadotropin
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetate
EEM	Early embryonic mortality
EGF	Epidermal growth factor
ER	Estrogen receptor
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
ERE	Estrogenic response elements
ERKO	Estrogen receptor knockout
ES	Embryonic stem
EST	Expressed sequence tag
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone

g	Grams
g	Gravity
G	Gauge
GAGs	Glycosaminoglycans
GnRH	Gonadotrophin releasing hormone
GR	Glucocorticoid receptor
HA	Hyaluronic acid
HAS	Hyaluronic acid synthetase
hCG	Human chorionic gonadotropin
HSD	Hydroxysteroid dehydrogenase
ICM	Inner cell mass
Ig	Immunoglobin
IGF	Insulin-like growth factor
IL	Interleukin
INF	Interferon
IVF	In vitro fertilization
kb	Kilobases
LBD	Ligand binding domain
LH	Leutinizing hormone
MDCK	Madine Darby canine kidney
MET	Maternal to embryonic transition
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MT-MMP	Membrane-type MMP
μl	Microliter
μm	Micrometer
μM	Micromolar
Na^+,K^+-ATPase	Sodium, potassium-adenosine triphosphatase
N-CAM	Neural-cell adhesion molecule
NLS	Nuclear localization signals
P	Probability
P4	Progesterone
PA	Plasminogen activator
PAI	PA inhibitor
PBS	Phosphate-buffered saline
PCNA	Proliferative cell nuclear antigen
PCR	Polymerase chain reaction
PGF	Prostaglandin
PKC	Protein kinase C
PLSD	Protected least significance difference
PR	Progesterone receptor
PRA	Progesterone receptor A
PRB	Progesterone receptor B
PRAKO	Progesterone receptor A knockout
PRBKO	Progesterone receptor B knockout
PRE	Progesterone response elements

PTH	Parathyroid hormone
QABD	Quantitative amplification and dot blot
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
S	Svedberg unit of flotation
SCS	Superovulated cow serum
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SERM	Selective estrogen receptor modulators
SSPE	Standard saline phosphate EDTA
SNuPE	Single nucleotide primer extension
SSH	Supression/substractive hybridization
TACE	TNF-α converting enzyme
TCM	Tissue culture media
TE	Trophectoderm
TGF-β	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinases
TM	Transmembrane
TNF	Tissue necrosis factor
tPA	tissue-type PA
Tris-HCL	Tris (hydroxymethyl)-aminomethane-hydrochloric acid
TSG	Tumor-necrosis factor stimulated gene
uPA	urokinase-type PA
UTP	Uracil triphosphate
VEGF	Vascular endothelial growth factor
WT	Wild type
ZO	Zonula occludens

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CHAPTER I: OVERVIEW

1.1: Introduction

Early embryogenesis, as a critical period during development, is a great determinant of embryonic health and reproductive efficiency. Early embryonic mortality (EEM) in cows (Ayalon, 1978; Diskin and Sreenan, 1980; Dunne et al., 2000; Roche et al., 1981) and humans (Edmonds et al., 1982) has been estimated to be anywhere between 30 to 62% and can account for up to 75 to 80% of all embryonic and fetal deaths. The major cause for this embryonic loss is attributed to the retarded development of the embryo (Goff, 2002).

Though embryonic development is attributed to be a result of intricate and precise spatial arrangement and interactive exchange between many different cell types, through activation of specific subset of genes, the ways by which developing embryonic cells change to form the blastocyst and subsequently the functional organs of the body are still not completely understood. Suggestions have been made that critical events of embryo development are brought about through a relatively limited repertoire of cellular processes involving primarily interactions between the cell surfaces of adjacent cells as well as by bringing about changes in the composition of the extracellular matrix (Fig. 1.1) (Damsky et al., 1993; Damsky et al., 1997; Werb and Chin, 1998; Zagris, 2001). Collectively, the molecular events mediating the selective recognition of cells and their formation into tissues and organs occurs on the cell surface (Buck and Horwitz, 1987; Gullberg and Ekblom, 1995). In addition, hormones, growth factors and morphogens have also been identified influencing the developmental processes (Loutradis et al., 2000; Schultz and Heyner, 1993; Stewart and Cullinan, 1997; Teruel et al., 2000). However, the role of cell-cell, cell-matrix adhesive molecules and steroid hormones during preimplantation embryogenesis is poorly understood. It is therefore intriguing to analyze the presence of these novel cellular molecules during very early embryonic development. Preimplantation embryonic

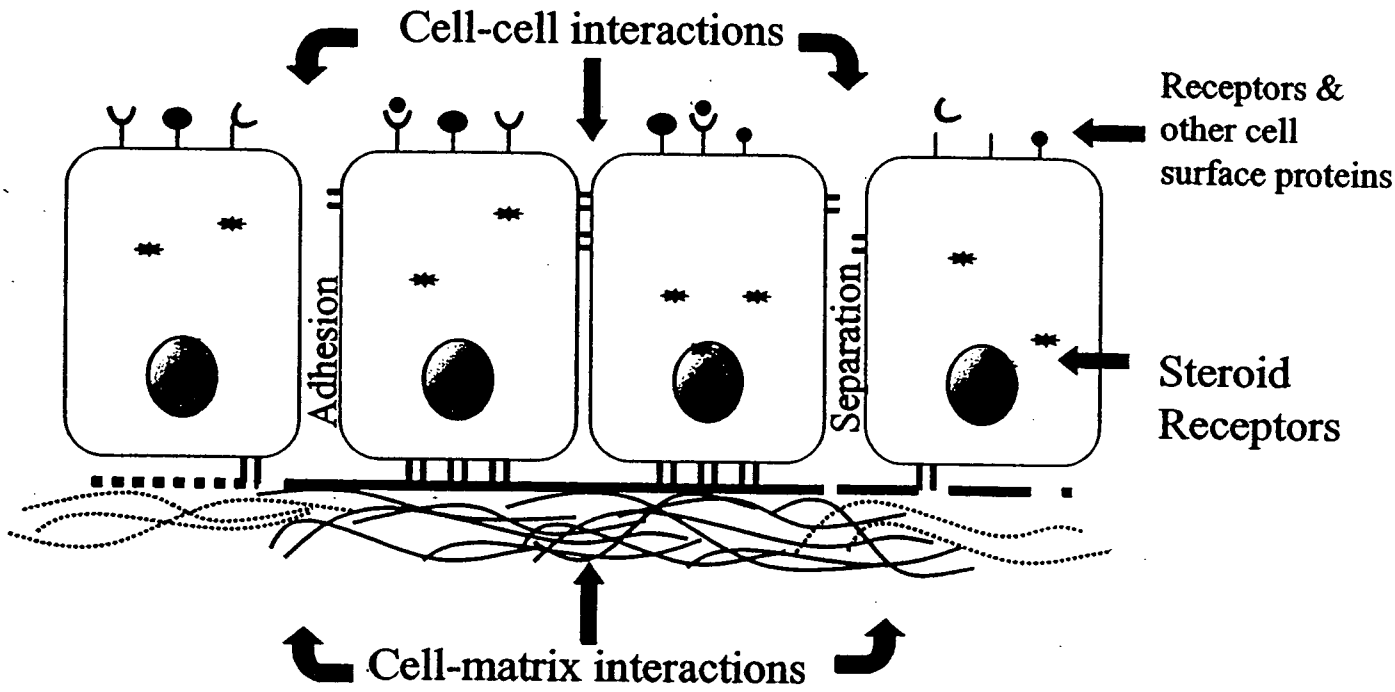

Fig. 1.1: Schematic representation of the potential mechanisms of cell-cell, cell-matrix and steroid receptor interactions.

survival being greatly dependent upon plasma progesterone (P4) profiles (Chagas e Silva et al., 2002; Thatcher et al., 2001a) and the corpus luteum (CL) being the critical entity in this interaction needs examination with reference to the cellular mechanisms of its formation and maintenance.

In this section, preimplantation embryogenesis is reviewed with specific emphasis on EEM. Subsequently, the morphogenetic events, which occur during preimplantation embryogenesis, are described with particular emphasis on gene expression. The role of cell-cell adhesion mediated by cell adhesion molecules (CAMs), known as cadherins, and of cell-matrix interactions during embryogenesis is also reviewed with specific reference to ADAMTS gene subfamily. Structure and function estrogen and progesterone receptor isoforms and their roles during embryogenesis are subsequently reviewed. Finally, the role of cell-matrix interactions during preovulatory follicle development, ovulation, CL formation, maintenance and development are also reviewed.

1.2: Preimplantation embryogenesis

The preimplantation period of development in mammals has been defined as the time interval from conception to nidation (implantation) or attachment of the embryo to the uterus. The preimplantation period lasts for about 30 d, 7 d, 4 d and 5 d in cattle, humans, mice and rats, respectively. Following fertilization, the zygote undergoes several cleavage divisions that divide the ooplasm into several compartments called blastomeres that are collectively surrounded by the zona pellucida.

This preimplantation or pre-attachment period is characterized by the development of the fertilized zygote through cleavage divisions, the activation of embryonic transcription, and the morphogenetic events of compaction and cavitation, which result in the formation of the blastocyst. The blastocyst is composed of the outer epithelial trophectoderm (TE), a fluid filed

cavity (blastocoel), and a small group of cells, the inner cell mass (ICM) (Handyside, 1978; Johnson, 1979b; Watson et al., 1990a). While TE is believed to be the progenitor of placenta, the ICM contributes to the formation of the embryo proper (Cross, 2001; Handyside, 1978; Johnson, 1979b; Watson et al., 1990a; Watson et al., 1992). The blastocyst stage must be reached in order for the embryo to hatch from the zona pellucida, thereby enabling the establishment of the maternal-embryonic interface associated with embryo implantation/attachment. Any aberration in the cascade of events during this critical period of blastocyst formation and hatching has an affect on the developmental potential and/or the survival of the embryo (Niemann and Wrenzycki, 2000; Niemann et al., 2002; Watson and Barcroft, 2001).

1.2.1: Survival of the early embryo

The importance of this critical period of embryo development is highlighted by the high incidence of embryo loss during early stages of embryogenesis. EEM has been suggested as a key determinant of the reproductive efficiency in cattle as it leads to loss of a large number of potential calves, retarded genetic progress, and significant loss of money and time in rebreeding cows, thereby making embryo survival of economic as well as fundamental importance. This is evident from the fact that, fertilization rates for cows are generally close to 90% (Sreenan and Diskin, 1983), but the average calving rate to a single insemination is only about 40-50% (Goff, 2002), suggesting that embryonic and fetal death account for most of the reproductive wastage following single breeding. EEM happening within three weeks of insemination, has been estimated to be around 30 to 40% in cattle (Ayalon, 1978; Diskin and Sreenan, 1980; Roche et al., 1981) and can account for up to 75% to 80% of all embryonic and fetal deaths, which results in substantial loss of production. In a recent study, embryo survival rates on day 14, 30 and at term have been found to be almost similar at 68%, 76% and 71.8% respectively indicating that most embryo losses occur during the first two weeks of bovine development (Dunne et al.,

2000). Furthermore, in cattle, embryos produced in vitro exhibit morphological, biochemical and metabolic differences compared with their in vivo counterparts (Abe et al., 1999; Crosier et al., 2002; Crosier et al., 2000; Crosier et al., 2001; Dieleman et al., 2002; Khurana and Niemann, 2000; Knijn et al., 2002; Niemann and Wrenzycki, 2000). High EEM has substantially hampered the field application of in vitro embryo production in bovine leading to the practice of transfering 2 embryos or more to achieve successful pregnancy (Farin et al., 2001; Farin and Farin, 1995; Kruip and den Daas, 1997).

Embryonic loss is even higher in humans where 62% of the pregnancies diagnosed by increases in human chorionic gonadotropin (hCG) do not come to term (Edmonds et al., 1982) and around 20% of these fail even before the pregnancy is detected clinically (Wilcox et al., 1999; Wilcox et al., 1988). There is evidence to support a similarity in some aspects of embryonic loss in humans and ruminants (Goff, 2002). For example, folliculogenesis, ovulation rate, rate of embryo development and the frequency of cleavage and blastocyst formation in vitro, timing of genome activation and metabolism are similar in bovine and human embryos (Adams and Pierson, 1995; Anderiesz et al., 2000; Kopecny, 1989; Menezo and Herubel, 2002; Niemann and Wrenzycki, 2000; Tesarik, 1988; Wrenzycki et al., 2001a). Even through the mechanisms involved in CL maintenance are different, formation and organization of CL and length of pregnancy in these two species are comparable (Adams and Pierson, 1995; Pierson and Adams, 1999) suggesting that bovine could be a useful model for understanding human embryo and CL development.

1.2.2: Factors involved in embryo loss

The causes of EEM are likely to be diverse, and can be either due to the problems with the embryo (Goff, 2002; Gustafsson and Larsson, 1983) or the events mediated through the CL (Chagas e Silva et al., 2002; Gustafsson and Larsson, 1985; Lafrance et al., 1989) or both. The

role of chromosomal abnormalities in EEM among ruminants is low (Hare et al., 1980; King, 1990; King, 1991). Chromosomal abnormalities also appear to be related to morphology of the embryo as only 13-15% of the poor quality embryos are cytologically abnormal whereas absolutely no good quality embryos demonstrate chromosomal abnormalities (King, 1987).

Environmental stress, especially hot climate, impairs reproductive performance in cattle leading to embryo mortality, as mammalian preimplantation embryos are sensitive to maternal as well as to direct heat stress (de la Sota et al., 1998; Ozawa et al., 2002; Wolfenson et al., 2000). The embryos appear to be affected during the second week of pregnancy suggesting that they are affected earlier in development and the consequences are only observed later (Ryan et al., 1992). Studies have demonstrated that maternal heat stress at the zygote stage has a direct effect on the developmental ability of mouse and bovine embryos (Arechiga and Hansen, 1998; Edwards and Hansen, 1997; Ozawa et al., 2002). Though, the mechanisms by which heat stress affects early embryonic development in vivo or in vitro are unknown, recent study has demonstrated that maternal exposure to heat stress at the zygote stage promotes disruption in gene activity (Ozawa et al., 2002). Recent studies have also demonstrated that heat shock compromises development of preimplantation bovine embryos (Paula-Lopes and Hansen, 2002a) and apoptosis is initiated when embryos are exposed to elevated temperature (Paula-Lopes and Hansen, 2002b). However, as the development proceeds, later embryo and fetal stages acquire a better ability to tolerate heat stress (Edwards et al., 2001). Moreover, plasma P4 concentrations are lowered in cows subjected to chronic heat stress (Howell et al., 1994) thereby, contributing to the EEM.

Similarly, infection, nutrition, oocyte quality have also been shown to affect embryo survival (Ain et al., 1998; Apelo and Kanagawa, 1989; Gardner et al., 2000; Knijn et al., 2002; Kwong et al., 2000; Martin, 2000). Irrespective of the factors leading to EEM, delayed development of the embryo, on account of failed cues from spatiotemporal expression of specific genes, has been opined as the major cause of embryonic losses (Goff, 2002; Niemann and

Wrenzycki, 2000; Niemann et al., 2002; Wrenzycki et al., 2001a). In addition, the developmentally challenged embryo is unable to produce sufficient quantities of interferon-τ (INF-τ) to initiate maternal recognition of pregnancy. In humans, measurement of hCG concentrations after in vitro fertilization (IVF) has revealed two possible causes of embryonic loss (Lenton et al., 1988). In one group, hCG rises at the normal time but then declines, indicating normal development but failure at implantation; in the other group, hCG increases later than normal before declining, indicating delayed embryo development.

Progesterone plays a vital role in early pregnancy as shown by higher P4 levels in pregnant than non-pregnant cows (Barnes, 2000; Diskin and Sreenan, 1986; Lukaszewska and Hansel, 1980; Sreenan and Diskin, 1983). Since, synchrony between the embryo and endometrium is regulated by the time of the postovulatory rise in P4 from the CL, it is quite likely that both the time of the increase in plasma P4 and its concentration during the luteal phase are important factors in maintaining pregnancy (Barnes, 2000).

Recent studies have demonstrated that cows with poorly developed embryos undergo a delayed P4 increase after ovulation, compared with cows having well-developed embryos (Mann, 2001; Mann and Lamming, 2001). Whereas, the time of P4 increase is important for embryo development, the concentration of P4 is important for preventing luteolysis, since cows with lower concentrations of plasma P4 are more susceptible to embryonic loss (Binelli et al., 2001; Chagas e Silva et al., 2002; Mann, 2001; Sreenan and Diskin, 1983). Collectively, these findings suggest that a healthy, developmentally competent embryo and a functional CL are two key components of embryo survival.

1.2.3: Possible ways to reduce early embryonic mortality

Blastocyst formation is an important milestone in the development of an embryo. Failure to reach this developmental stage is a principal cause of early pregnancy loss (Edwards and

Hansen, 1997; Watson, 1992) and is essential for implantation and subsequent development. Since each stage of embryonic development is characterized by temporal and spatial activation of specific subsets of genes (Kidder and McLachlin, 1985; Niemann and Wrenzycki, 2000; Watson and Barcroft, 2001), it is necessary that we have a better understanding of genes involved in embryogenesis. Thus, understanding the developmental process of the blastocyst has both economic and clinical importance.

During the last decade, production of bovine embryos in vitro has become a routine research tool in many laboratories, and is also being adopted in applied breeding programs. Moreover, towards the end of the last decade, the production of transgenic offspring in large domestic species by cloning based on nuclear transfer from cultured embryonic, fetal and adult somatic cells became a reality (Illmensee, 2002; Wilmut et al., 2000). The rapid expansion of these biotechnologies has not been without problems, leading to an increased focus on embryo quality and viability. Thus, although, thousands of calves have been born from embryos produced in vitro, problems with abortions, increased birth weights, dystocia and high rates of neonatal mortality have been widely reported (Farin and Farin, 1995; Kruip and den Daas, 1997; Walker et al., 1996). In the case of nuclear transfer, the situation is even more pronounced with only 1-2% of the reconstructed embryos resulting in offspring (Wilmut et al., 2000). With these substantial problems further investigations devoted to understanding the cell biological processes that regulate initial embryonic development and on which technological manipulations of the embryo may have detrimental impacts is warranted.

The other potential method of preventing EEM would be to prevent luteolysis from occurring. This can be achieved by administrated of P4 to support the embryo for secreting sufficient IFN-τ to prevent luteolysis (Martal et al., 1997). However, application of this protocol has met with only mixed success suggesting an asynchronous role of exogenous progestins (Diskin and Sreenan, 1986; Van Cleef et al., 1991). In contrast, promising results have been

achieved through the strategy of producing accessory CL using hCG and/or GnRH analogue treatments in cows (Rajamahendran et al., 1998; Rajamahendran and Sianangama, 1992; Thatcher et al., 2001b). In spite of species differences for pregnancy maintenance between humans and bovine, luteinization has been shown to be essential for the survival of the embryo in both the species (Chew et al., 1979; Hoffmann and Schuler, 2002; Johnson et al., 1981; Murphy, 2000). However, the process of luteinization by which the postovulatory follicle differentiates to become a CL, its maintainance and regression are poorly understood. Thus, it is important to study the cell biology of this important organ so significantly involved in maintaining the embryo during pregnancy.

1.2.4: Gene expression during embryo development

Preimplantation stage mammalian embryos are very simple morphologically, being composed of mostly round cells that exhibit only a limited range of changes in cell shape. This morphological simplicity, however, belies a much greater complexity of events that occur at a molecular level. Throughout preimplantation development, some essential gene regulatory events occur that are fundamental for the continued development of the embryo. These include, for example, the translational recruitment and/or degradation of maternal mRNAs, transcriptional activation of the embryonic genome resulting in a second stage of gene expression. Inhibiting gene expression during embryogenesis can completely arrest preimplantation development, indicating that expression of specific genes at certain times is crucial for continued embryonic development (King and Wall, 1988; Kopecny et al., 1989). It is presumed that successful preimplantation and early fetal development is reliant on the timely expression of approximately 10,000 genes (Niemann and Wrenzycki, 2000). Unfortunately, sequence information for only a few of these genes is currently known, thereby restricting our understanding of gene expression patterns during blastocyst development. In many cases, our present knowledge of genes

expressed during early embryogenesis in bovine has been extrapolated from the data available from studies in the mouse, which might be indicative, but independent studies in the bovine embryo are needed to ascertain the exact process of early embryogenesis.

Because preimplantation embryos are small and the amount of material available for analysis is limited, the analysis of gene expression in preimplantation embryos requires the most sensitive methods that can be applied to a small number of embryos. The paucity of biological material has hindered identification and analysis of zygotically activated genes and essentially restricted this analysis to housekeeping genes, i.e., mRNAs that are often abundant. The advent of reverse transcription-polymerase chain reaction (RT-PCR) has overcome the problem of limited amounts of embryo RNA available for analysis (Rappolee et al., 1988). In addition, several modifications of the RT-PCR technique like semi-quantitative RT-PCR (Latham et al., 1994; Manejwala et al., 1991), quantitative amplification and dot blot (QABD) (Rambhatla et al., 1995) have been used to quantify the relative abundance of individual genes. In situ hybridization, analysis of expressed sequence tags (ESTs) and the single nucleotide primer extension (SNuPE) method have been developed and used extensively in embryological studies (Singer-Sam et al., 1992; Singer-Sam and Riggs, 1993).

By employing qualitative or quantitative RT-PCR analysis, the pattern of expression of several classes of genes has been studied in preimplantation murine and bovine embryos. About 32 physiological functions involving the expression of ~250 different genes have been studied by RT-PCR in murine preimplantation development (Niemann and Wrenzycki, 2000). This is in contrast to the bovine embryo where not more than 15 physiological functions and expression of only ~60-70 different genes are known (Niemann and Wrenzycki, 2000). Differential display (DD) of mRNA is another RT-PCR based method that allows comparison of two or more mRNA samples prepared from small amount of tissues (Liang and Pardee, 1992). DDRT-PCR has now been used to compare patterns of RNA expression from preattachment bovine embryos (Natale

et al., 2001; Natale et al., 2000). It is particularly suitable for developmental studies involving temporal changes in gene expression in embryos. However, a majority of the DD bands are common among the different embryonic groups (Lee et al., 2001b). This is not surprising as they may represent genes with housekeeping functions. The DD amplicons that are differentially expressed in these embryos are selected and confirmed by semi-quantitative RT-PCR analysis. Recently, modification of the suppression/subtractive hybridization (SSH) technique (Diatchenko et al., 1996), to study differential gene expression has been used to study gene expression in the bovine (Mohan et al., 2002b; Ponsuksili et al., 2002), murine (Zeng and Schultz, 2003) and rabbit (Pacheco-Trigon et al., 2002) embryos. Since cDNA bandings between different stages of development using DD/SSH-RTPCR are largely conserved, only a small number of uniquely expressed genes can be studied (Natale et al., 2001; Natale et al., 2000). For studies reported in this thesis, the gene specific, sensitive semiquantitative RT-PCR and immunohistochemistry techniques have been used to determine, quantify and characterize the expression of novel genes and proteins being expressed during bovine preimplantation embryogenesis.

1.2.5: Maternal endowment to the early embryo and embryo gene activation

Beginning after the first few cleavage divisions, a critical transition occurs in the genetic control of development. Before this time, the embryo contains a host of maternally derived mRNAs and macromolecules that are sufficient to drive transcription and translation through the first few cleavage divisions. Further development, however is dependent on the activation of embryonic control of transcription and subsequent degradation of maternal mRNAs and proteins which occurs at a specific point of time, depending on the species (Schultz, 2002). This change from maternal to embryonic control of transcription also referred as maternal to embryonic transition (MET) occurs at one cell stage in mice (Aoki et al., 1997), four cell stage in pigs

(Kopecny, 1989) and approximately eight cell stage in cattle (Barnes and First, 1991; Camous et al., 1986; Frei et al., 1989; King et al., 1988; Telford et al., 1990), sheep (Crosby et al., 1988), rabbits (Manes, 1971) and humans (Braude et al., 1988).

In bovine embryos, the first few cell divisions are regulated by maternal mRNA and proteins accumulated in the oocyte prior to the resumption of meiosis. As MET occurs, the majority of maternal mRNA molecules accumulated during oogenesis are degraded and gradually replaced by the new mRNA molecules (De Sousa et al., 1998; Telford et al., 1990; Watson et al., 1999). During MET, there is a major turnover of the mRNA populations during this phase of early development and these changes are both qualitative and quantitative (Schultz, 2002; Watson and Barcroft, 2001; Watson et al., 1999). The first evidence of RNA polymerase I transcription in bovine embryos occurs with the formation of the functional nucleolar organizing region at the eight cell stage (King et al., 1988; Kopecny et al., 1989). However, recent studies using α-aminitin, which is a RNA polymerase II inhibitor, ^{3}H-uridine and ^{35}S-UTP labeling have shown that the first product of embryonic genome transcription can be detected at earlier stages of development, as early as the two-four cell stage (Bilodeau-Goeseels and Panich, 2002; Memili et al., 1998; Memili and First, 1998; Natale et al., 2000; Plante et al., 1994; Viuff et al., 1996), however, the early transcription is not required for the progression of development (Hyttel et al., 1996) as the development can still proceed to 8-cell stage in the presence of a transcriptional inhibitor (Liu and Foote, 1997). The effect of α-aminitin during MET is characterized by a major quantitative drop in the presence of cell stage specific proteins (Frei et al., 1989) and arrest in embryonic development (Crosby et al., 1988) which can be detected at the 8-cell stage in bovine embryos (Liu and Foote, 1997). While the detailed mechanism of genome activation is lacking, the acquisition of a transcriptionally repressive environment and changes in chromatin structure by alteration of histone deacetylase activity can block or stimulate repression of markers of genome activation (Stein et al., 1997; Tsukiyama and Wu, 1997; Worrad et al., 1995). Apart

from several genes that are involved in embryonic genome activation, transcription factors playing a role in MET have also been identified (De Sousa et al., 1998; Rosenfeld, 1991; Scholer, 1991).

1.2.6: Compaction

Once the embryonic genome as been activated, two important morphogenetic events occur in the embryo during the preimplantation period. The first, known as compaction, occurs late in the 8-cell stage when individual blastomeres condense and their boundaries become less prominent, thus forming a uniform cellular mass known as a morula (Ducibella et al., 1975; Reeve, 1981; Ziomek and Johnson, 1980). Compaction is a common feature of preimplantation embryos within all mammalian embryos, although the timing of this event varies greatly (McLaren, 1982). This process results in several changes in the embryo. Compaction symbolizes the onset of cellular differentiation during mammalian development (Pratt, 1982). During this time, several gene products are expressed that contribute to many of the morphogenetic manifestations of compaction. The cellular events associated with compaction include the development of cell-cell adhesion among blastomeres and other molecular processes occurring as a result of cellular adhesive mechanisms (Watson, 1992). The events of compaction are crucial for the further development of the preattachment embryo as it provides the foundation for the initiation of a chain of events leading to cavitation, which is the second major morphogenetic event during embryogenesis (Fleming et al., 1989; Lee et al., 1987; Watson et al., 1992; Watson et al., 1990b).

1.2.7: Cavitation

The second morphogenetic event to occur in the preimplantation development is known as cavitation. This process begins several days after conception (3 d in the mouse, 3-4 d in rat

and 6 d in the bovine and human) and culminates in the formation of the blastocyst. For cavitation to arise, the embryo appears to require the development of a polarized epithelium and leads to the formation of the first epithelium also called as the trophectoderm (TE) (Wiley, 1988). At this stage, the two cell types are easily distinguishable: the ICM cells are located internally at the embryonic pole of the embryo whereas the TE cells, which are extremely large, owing to the cytokinetic cell division, surrounds both the ICM and the blastocoel. The differentiation of the TE and ICM has direct ties to attachment/implantation, since the TE is also responsible for the maternal recognition of pregnancy, initiates attachment/implantation via direct contact with the uterus and eventually contributes to the formation of the placenta and the extraembryonic membranes (Chavez et al., 1984; Duc-Goiran et al., 1999; Stojkovic et al., 1995). The ICM differentiates into all tissues of the developing fetus. During the formation of blastocyst, cell adhesion has been shown to be of fundamental importance and also shown to be able to govern the viability of the conceptus (Fleming et al., 2001).

1.3: Cell adhesion during early embryogenesis

During embryogenesis blastomeres are organized in a specific manner to undergo compaction, cavitation and blastocyst formation. This spatiotemporal organization of the embryo has been suggested to be associated with adhesive interactions between cell-cell and cell-ECM. Such cell-cell and cell-ECM interactions employing a variety of CAMs have been suggested as the translators of basic genetic information into complex three-dimensional patterns of cells and tissues (Gumbiner, 1996).

1.3.1: *Cell-cell interactions*

The development of techniques to ascertain the ability of a cell to interact with its neighbors paved the way for modern analysis of morphogenesis. It was demonstrated that

dissociated animal tissues can assemble autonomously and reform into the original tissue-like structures (Townes and Holtfreter, 1955; Weiss and Taylor, 1960). In these studies, the embryonic cells of several amphibian species were dissociated by alkaline treatment and then allowed to reorganize under neutral pH conditions. It was established that the cells adhered to each other, forming aggregates in a tissue specific manner on agar-coated petri dishes. These studies however did not identify the molecules involved in these differential cell adhesions. Subsequently it was confirmed that in some animal species, dispersed embryonic cells could even reconstruct the complete embryonic body (Dan-Sohkawa et al., 1986; Guidice, 1962; Spiegel and Spiegel, 1975). It was subsequently demonstrated that differential cell types contained different cell adhesion molecules (Roth et al., 1971; Roth and Weston, 1967) but it was more recently that the molecular basis of cell adhesion was shown to be due to the cell surface expression of a family of glycoproteins that bind with high specificity to each other on adjacent cells (Edelman, 1984; Edelman, 1985; Edelman, 1986; Edelman and Crossin, 1991; Kemler, 1992; Takeichi, 1988; Takeichi, 1990). These authors concluded that the construction of tissues is thus, at least dependent on the intrinsic morphogenetic capacity of the individual cells and each cell has the ability to selectively recognize identical or different cell types thereby adhering preferentially to their own cell type when mixed with other cells (Roth and Weston, 1967). The molecular basis for this selective adhesion was subsequently identified in a series of studies examining the ability of antibodies directed against cell surface proteins to interfere with compaction in mouse preimplantation embryos, a developmental process that is dependent on the presence of Ca^{2+} and changes in cell-cell interactions (Ducibella et al., 1975; Ducibella and Anderson, 1975; Ducibella and Anderson, 1979). Inhibitory antibodies were used to purify a membrane glycoprotein, initially termed uvomorulin (Hyafil et al., 1981; Hyafil et al., 1980; Kemler et al., 1977). Similar approaches were used to identify chicken liver CAM (L-CAM) (Bertolotti et al., 1980), canine Arc-1 (Behrens et al., 1985; Imhof et al., 1983), and human

epithelial cell-CAM 120/80 (Damsky et al., 1983). These molecules appear to be species-specific homologues of the murine 124 kDa proteins species first referred to as E-cadherin (E-cad) in the studies of Yoshida-Noro et al. (Yoshida-Noro et al., 1984).

Significantly, these molecules are expressed in distinct patterns during tissue and organ morphogenesis, suggesting that they play important and direct roles in the temporal and spatial regulation of cell interactions and cell sorting during tissue formation (Takeichi, 1988). Furthermore, loss of expression of these proteins correlates with loss of intercellular adhesion (Behrens et al., 1989; Hashimoto et al., 1989; Takeichi, 1993; Vleminckx et al., 1991). During preimplantation embryogenesis, out of the four major cell adhesion molecule families namely, cadherins, integrins, selectins and the immunoglobulin (Ig) superfamily, the presence of the first three have been demonstrated in the early embryo (Bloor et al., 2002; Campbell et al., 1995; Damsky et al., 1993; Fleming, 1991; Kimber, 1990; Sutherland et al., 1993; Weitlauf and Knisley, 1992). While the cadherins, integrins and selectins demonstrate Ca^{2+}-dependent mechanisms, the members of the immunoglobulin (Ig) superfamily demonstrate Ca^{2+}-independent mechanisms of cell-cell adhesion (Edelman, 1985; Edelman, 1986; Edelman, 1992; Edelman and Crossin, 1991; Takeichi, 1988; Takeichi, 1990). To this date, only a few studies have focused on the role of the CAMs of Ig superfamily during preimplantation embryogenesis. For example, in mice, neural (N)-CAM is present during preimplantation embryogenesis at the expanded blastocyst stage, where they are present on the trophectoderm but absent on trophoblasts of day 6 blastocysts in utero suggesting a downregulation during implantation process (Kimber et al., 1994). In contrast, N-CAM, intercellular (I)-CAM-1 and vascular (V)-CAM-1 are detected on oocytes as well all on embryos in humans (Campbell et al., 1995). However, the biological significance of their expression patterns remains largely undefined.

Selectins are transmembrane carbohydrate binding proteins expressed by endothelial cells and leukocytes, with primary function in the recruitment of leukocytes to sites of inflammation

(Vestweber and Blanks, 1999). The presence of L-selectin has been demonstrated in the human oocytes, and also at the 4-cell stage during early embryonic development (Campbell et al., 1995). However, in a recent study, both L- and P-selectin could not be detected during human preimplantation embryonic development suggesting that these selectins could not be playing a role during early embryo development, at least in humans (Bloor et al., 2002).

Integrins are heterodimers composed of an α and β membrane glycoproteins subunits. At least 25 functional integrin dimers can be formed and expressed on the cell surface, from 19 α subunits and 8 β subunits identified and these have a diverse range of ligands (Humphries, 2000; Humphries, 2002). Integrins mediate cell-cell and cell-ECM interactions, anchoring the cell to the surrounding substratum and are also believed to provide the molecular framework for cellular migration and activate signal transduction pathways (Lafrenie and Yamada, 1996).

In mouse embryos, α5, α6B, αv, β1 and β3 integrin subunit expression is detected throughout preimplantation development, α3 expression is detected at the 8-cell stage, and the α2, α3, α7 and α6A subunits are first detected only in the late blastocyst stage embryos (Sutherland et al., 1993) which have been shown to be involved in formation of distinct dimers in the mouse embryos. Expression of the α6Bβ1, α5β1 and αvβ3 dimers during preimplantation murine development has been reported (Klambt et al., 1989; Sutherland et al., 1993). In humans, expression of the α3, α6, αv, β1, β3, β4 and β5 has been reported (Bloor et al., 2002; Campbell et al., 1995; Dubey et al., 2001; Turpeenniemi-Hujanen et al., 1992). Though, protein expression of β3 has been reported earlier (Campbell et al., 1995), no expression of α2, α3, α7 or β3 subunit mRNA was detected in human embryos (Bloor et al., 2002). Embryos from null mutants for β1 integrin exhibit ICM retardation and preimplantation lethality (Fassler and Meyer, 1995; Stephens et al., 1995). Expression of the αvβ3 and α5β1 subunits has been detected on the trophectoderm of the blastocyst making them likely candidates for embryo attachment and

implantation along with β1 subunit (Kimber and Spanswick, 2000; Schultz et al., 1997; Stephens et al., 1995; Wang and Armant, 2002; Wang et al., 2002). Integrin αvβ3 has been shown to bind to laminin while αvβ5 has been shown to bind to other ECM components namely, fibronectin, vitronectin and fibrinogen (Kimber and Spanswick, 2000) suggesting a role in ECM remodeling during embryogenesis and invasion/attachment. Collectively, a number of integrin molecules that may function in embryonic cell-cell adhesion or in interaction with ECM in the embryo or the uterine epithelium are known, however, functional studies are required to complement these observations in order to ascertain their biological role(s).

1.4: The cadherins

The cadherins are a gene superfamily of integral membrane glycoproteins mediating Ca^{2+}-dependent cell-cell adhesion in a homophilic manner and playing important roles in tissue organization of the multicellular organism (Gumbiner, 1996; Niessen and Gumbiner, 2002; Tepass et al., 2000). The specificity of their interactions is thought to underlie the sorting and segregation of cells into specific tissue layers and organ formation (Redies, 2000; Takeichi, 1995). Several subfamilies of cadherins have been defined and include classical type 1 cadherins or type 2 (atypical) cadherins, desmocollins, desmogliens, protocadherins and flamingo cadherins. In addition, several other cadherins occupy isolated positions in this superfamily and are referred to as unclassified cadherin related proteins. This classification is based on protein domain, composition, genomic structure and phylogenetic analysis of the protein sequences (Alattia et al., 2002; Kemler, 1992; Nollet et al., 2000; Suzuki, 1996a; Vleminckx and Kemler, 1999).

Type 1 cadherins include the best characterized members of this gene superfamily of CAMs, and include E-cadherin (E-cad), N-cadherin (N-cad), P-cadherin (P-cad) and the more recently cloned R-cadherin (R-cad) (Hatta et al., 1988; Hatta et al., 1985; Inuzuka et al., 1991a;

Nagafuchi et al., 1987; Nose et al., 1987; Nose and Takeichi, 1986; Suzuki et al., 1991; Yoshida-Noro et al., 1984). These cadherins subtypes were identified by immunological and cDNA cloning strategies and were subsequently named on the basis of their major tissue distributions in mouse embryonic development; E-cad for predominantly being present in the epithelial cells, N-cad for being expressed in neural calls, P-cad showing major expression in the placenta and R-cad for being expressed in the retina. In addition, novel cadherins were first identified using RT-PCR strategy, exploiting the structural homology between the conserved amino acid sequences (TAPPYD and FKKLAD; single letter amino acid code) of the cadherin structure (Suzuki et al., 1991). These new eight novel cadherins subtypes were named, cadherin-4 (cad-4) to cadherin-11 (cad-11). The amino acid sequence homologies of the cDNA sequences of these proteins (other than cad-4) provided sufficient evidence to categorize these new proteins into a distinct subfamily of cadherins and were named type 2 cadherins (Suzuki et al., 1991). Sequence analysis of cad-4 showed that it was a mammalian homologue of chicken type 1 cadherin subtype, R-cad (Inuzuka et al., 1991a; Tanihara et al., 1994a; Tanihara et al., 1994b). To date, several type-2 cadherins are known namely, cad-6, -7, -8, -9, -10, -11, -12, -14 and -20 (Kools et al., 2000; Selig et al., 1997; Shibata et al., 1997; Shimoyama et al., 1995; Shimoyama et al., 2000; Suzuki et al., 1991; Tanihara et al., 1994b).

1.4.1: Structure of type 1 and type 2 cadherins

In general, cadherins possess functional sites for adhesion recognition, calcium binding, membrane integration and cytoskeletal interactions (Grunwald, 1993; Takeichi, 1993; Takeichi, 1994) which is brought about by three domains, namely, an amino terminal extracellular domain, a single transmembrane domain and a carboxy terminal cytoplasmic domain (Fig 2).

In type 1 cadherins, the extracellular domain comprises of approximately 550 amino acid residues while each cadherin repeat motif in the extracellular domain (EC1-EC5) comprises of

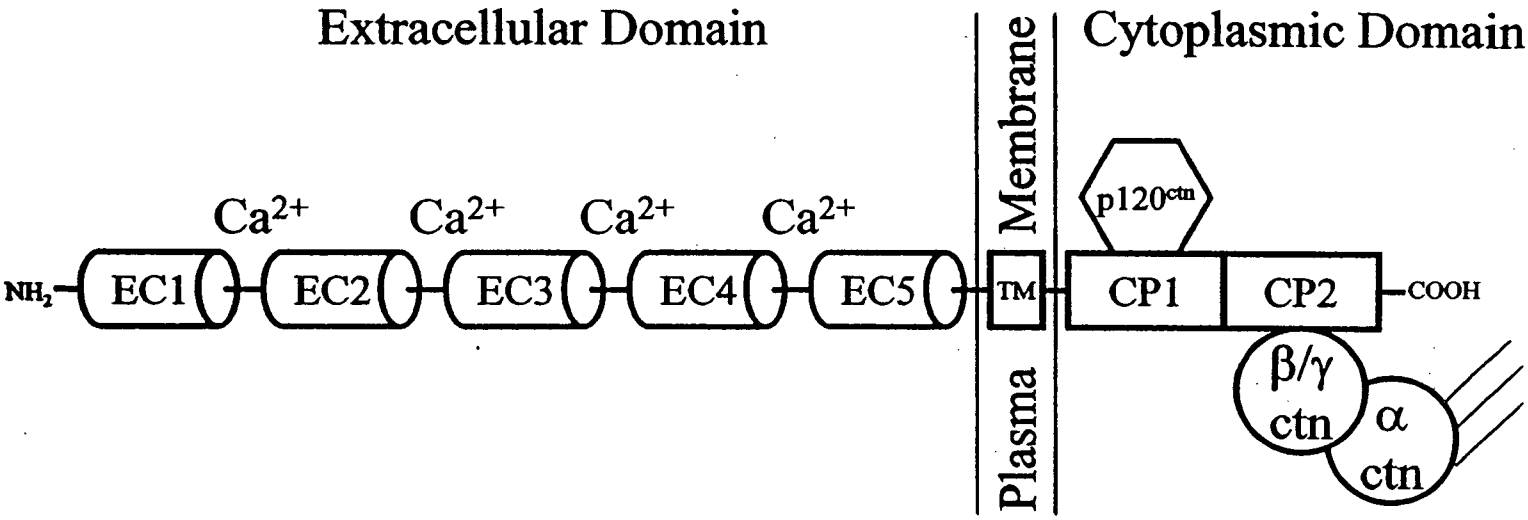

Fig. 1.2: Schematic representation of the basic structure of the classical cadherins in the plasma membrane. The cadherins are comprised of five extracellular domains (EC1-EC5), a single transmembrane domain (TM), and two cytoplasmic domains (CP1 and CP2). Ca^{2+}-binding sites are present at the interface between the extracellular domains. The cytoplasmic domains are the most highly conserved regions between members of the classical cadherins and interact with a family of cytoplasmic proteins known as the catenins (ctn). These interactions are believed to further link the cadherins to the actin-based microfilaments of the cytoskeleton.

approximately 110 amnio acids in length (Hatta et al., 1988; Mahoney et al., 1991; Sano et al., 1993; Tanihara et al., 1994b). Each motif has highly conserved amino acid sequences, LDRE, DXD, and DXNDN (single letter code for amino acids and X refers to any amino acid), which are responsible for dimerization and Ca^{2+} binding and thus play a key role in the structural conformation of the cadherin extracellular domain (Nagar et al., 1996; Pertz et al., 1999; Ringwald et al., 1987). EC1 mediates cell adhesion and this domain contains the evolutionary conserved HAV motif, which together with variable residues in its immediate vicinity, are implicated in selective cadherin binding (Blaschuk et al., 1990a; Blaschuk et al., 1990b; Gallin, 1998; Nose et al., 1990). Linear peptides harboring this sequence are capable of inhibiting a variety of cadherin-dependent processes (Blaschuk et al., 1990b; Doherty et al., 1991; Noe et al., 1999). EC1 has also been shown to be essential for the specification of lateral dimerization (Shan et al., 2000), a prerequisite for functional cadherin molecules (Brieher et al., 1996; Tamura et al., 1998) and this function may contribute to its role in determining cadherin specificity. However, a recent study examining lateral dimerization of E-cadherin and its adhesive activity by co-immunoprecipitation and aggregation assays has demonstrated that lateral dimerization is necessary, but not sufficient, for adhesive activity as the constructs retain the ability to associate laterally but are inactive in aggregation assays (Ozawa, 2002). However, recent studies have shown that binding and adhesion of cadherins involves other EC repeats besides EC1 (Chappuis-Flament et al., 2001; Sivasankar et al., 1999; Sivasankar et al., 2001). For example, a role of EC5 has also been suggested in dimerization, as four conserved cysteine residues in EC5 are likely to play a role in the formation of intramolecular disulfide bonds (Blaschuk et al., 1990a; Blaschuk et al., 1990b; Nose et al., 1990; Ozawa and Kemler, 1990; Ringwald et al., 1987; Takeichi, 1991; Williams et al., 2000).

In contrast to type 1 cadherins, type 2 cadherins share a similar extracellular domain structure but the over-all amino acid sequence homology between these two subfamilies is low (<40%) (Nollet et al., 2000; Shimoyama et al., 2000; Tanihara et al., 1994b). In particular, the EC1 domain, which demonstrates > 65% amino acid homology between members of the type 1 cadherins, shows < 50% amino acid homology when compared to the EC1 sub domain of the type 2 cadherins. In contrast, the EC1 sub domain of the type 2 classical cadherins exhibits a high degree of amino acid similarity (>75%) between members of this subfamily. Furthermore, the type 2 classical cadherins do not contain the CAR sequence, HAV. The functional significance of these amino acid sequence differences remains to be determined. However, the transfection of full-length cDNAs for several type 2 cadherin subtypes has been shown to promote the aggregation of murine fibroblastic L cells, demonstrating that these cadherin subtypes are capable of mediating Ca^{2+}-dependent cell adhesion in a homophilic manner (Kimura et al., 1995; Mbalaviele et al., 1998; Shimoyama et al., 1999; Shimoyama et al., 2000; Sugimoto et al., 1996).

The transmembrane domains of type 1 and type 2 cadherins also differ from each other, though only to a limited extent (Huber et al., 1999; Shimoyama et al., 2000). For example, the type 1 cadherin transmembrane domain is somewhat variable in length, consisting of 25-35 amino acid residues. In contrast, the transmembrane domain of the type 2 cadherins is comprised of exactly 33 amino acid residues and demonstrates high homology between members of this subfamily (Shimoyama et al., 2000). However, the type 1 and type 2 cadherins share the conserved leucine residues that are believed to regulate the lateral dimerization of these integral membrane glycoproteins at the cell surface (Gurezka et al., 1999; Huber et al., 1999).

The cytoplasmic domains of type 1 and type 2 cadherins are highly conserved between members of these two subfamilies (Hatta et al., 1988; Nollet et al., 2000; Shimoyama et al., 2000; Suzuki et al., 1991; Tanihara et al., 1994b). It has been proposed that the cytoplasmic

domains of both type 1 and type 2 cadherins interact with distinct cytoplasmic proteins a phenomenon found essential for the functioning of cadherins (Nollet et al., 2000). Catenins constitute one family of cytoplasmic proteins, which interact with the cytoplasmic domain of cadherins (Aberle et al., 1996; Gumbiner, 2000; Hirano et al., 1992; Oyama et al., 1994) and includes α-, β-, and γ-catenin (also known as plakoglobin) (Ozawa et al., 1989; Ozawa and Kemler, 1992). In addition, $p120^{ctn}$, a cytoplasmic protein that is distantly related to β- and γ-catenin (Peifer et al., 1994; Reynolds et al., 1992), has been identified as a component of these distinct cadherin-catenin complexes (Reynolds et al., 1994; Shibamoto et al., 1995). α-catenin is believed to link the cadherin-catenin complex to the actin-based cytoskeleton (Ozawa et al., 1990). This cytoplasmic protein in turn has been shown to bind directly to both β- and γ-catenin (Huber et al., 1997; Koslov et al., 1997; Nieset et al., 1997; Obama and Ozawa, 1997). $p120^{ctn}$ binds to a highly conserved region of the cadherin cytoplasmic domain that is more proximal to the plasma membrane than the carboxy terminal β- and γ-catenin binding region (Ohkubo and Ozawa, 1999; Ozawa and Kemler, 1998; Yap, 1998; Yap et al., 1997) and is not believed to link the cadherins to the cytoskeleton (Conacci-Sorrell et al., 2002; Daniel and Reynolds, 1995).

Cadherins are synthesized as precursor molecules containing a signal peptide and an amino terminal domain that is removed post-transcriptionally by the furin/subtilin family of proprotein convertases (Ozawa and Kemler, 1990; Posthaus et al., 1998). The extracellular domain of the cadherins is further glycosylated on asparagine residues prior to being transported to the cell surface (Geyer et al., 1999; Shore and Nelson, 1991).

1.4.2: Type 1 cadherins and embryogenesis

The spatiotemporal expression of type 1 cadherin subtypes is tightly regulated during several developmental processes such as cell-migration, cell rearrangements and sorting, tissue

remodeling, cell differentiation, and organogenesis (Boller et al., 1985; Gumbiner et al., 1988) and therefore, type 1 cadherins are believed to be key morphoregulators during embryonic development and maintaining tissue integrity in adults (Gumbiner, 1996; Takeichi, 1991; Takeichi, 1995). The role of cadherins in maintaining cell-cell interactions and mediating morphogenetic events during preimplantation embryogenesis was initially examined in the mouse embryo (Hyafil et al., 1981; Hyafil et al., 1980; Kemler et al., 1977; Takeichi, 1977).

E-cad has been shown to be present in both unfertilized and the one cell stage embryos in murine, porcine and bovine embryos (Barcroft et al., 1998; Reima, 1990; Reima et al., 1993; Shehu et al., 1996; Vestweber et al., 1987). The expression of this CAM at this stage of development has been attributed to the stored maternal mRNA transcripts. Compaction of preimplantation embryos, which is in the first sign of overt cell differentiation, is highly dependent on the changes in Ca^{2+}-dependent cell-cell adhesion mediated by E-cad (Hyafil et al., 1981; Johnson et al., 1988; Vestweber et al., 1987; Vestweber and Kemler, 1984; Vestweber and Kemler, 1985). E-cad is expressed even in the loosely adhering blastomeres before compaction (Bloom, 1989; Johnson et al., 1988) but then at compaction, E-cad mediated cell-cell adhesion is activated. Although, the mechanism(s) involved in the increase in adhesivity is poorly understood, there is evidence to suggest a role of protein kinase C (PKC) in activation of E-cad mediated compaction (Bloom, 1989; Winkel et al., 1990).

Although, cell-cell adhesion and commitment of some cells according to their mutual interactions was initially proposed as a key factor in blastocyst formation (Tarkowski and Wroblewski, 1967), experimentation lead to the notion that E-cad is the only type-1 cadherin expressed during preimplantation development in the mouse embryo (Ohsugi et al., 1997) as P-cad could not be detected in the murine embryo until the blastocyst stage, where it was localized in the trophectoderm and also in the deciduas following implantation (Kadokawa et al., 1989; Nose and Takeichi, 1986) and similarly, N-cad was first detected only at the gastrulation stage in

the murine and chicken embryo (Hatta et al., 1987; Hatta and Takeichi, 1986). However, a recent study has shown that in addition to the expression of E-cad, N-cad and P-cad are also expressed in the mouse embryo from 2-cell stage onwards (Harrouk et al., 2000). In rats, E- and N-cad are already present on the plasma membrane of immature unfertilized oocytes and their concentration increases after fertilization in early cleavage stage embryos where they continue to be localized at the plasma membrane (Ziv et al., 2002). The presence of mRNA transcripts encoding these cadherin subtypes during preimplantation embryogenesis suggests a prior role(s) of these subtypes. R-cad, which is one of the more recent inclusions in the subfamily, has so far been detected only at the post implantation stages of development and during formation of several tissues including the eye, brain, kidney, pancreas, gastro-intestinal tract, skeletal muscle and spleen (Goto et al., 1998; Inuzuka et al., 1991a; Inuzuka et al., 1991b; Matsunami and Takeichi, 1995; Rosenberg et al., 1997; Sjodin et al., 1995).

Interblastomeric adhesion is mediated by the redistribution of Ca^{2+} dependent cell adhesion molecule E-cad within the basolateral membrane surfaces of compacting 8-cell mouse embryos. This increase in interblastomeric contact provided by E-cad further plays an important role in blastomere polarization and the formation of the focal tight junctions (Watson et al., 1990a; Wiley et al., 1990). As interblastomeric contact increases, the formation of a free and apposed plasma membrane region occurs, and by the 16-cell stage, the embryo is composed of an outer layer of polar cells that completely encloses an inner group of four to seven apolar blastomeres. The degree of cell contact associated with cell position in thought to provide the necessary developmental cue for the maintenance of polarity with outer blastomeres (Ziomeck, 1987). This E-cad mediated polarization further leads to the development of a cytochalsin-D resistant microvillous cap (Fleming and Johnson, 1988), a Na^+-dependent amino acid transport system (Miller and Schultz, 1985), a Na^+-glucose cotransporter system (Wiley et al., 1991) and Na^+-channels (Manejwala and Schultz, 1989). The basolateral surfaces of the outer blastomeres

are displayed within the cell cortex by the appearance of an apical actin cap and also within the cytoplasm of the asymmetric distribution of lipid vesicles, mitochondria, and the nucleus towards the basal poles of the cells (Wiley, 1987; Wiley, 1988).

E-cad mediated cell adhesion also has a role to play in the process of cavitation. Firstly the E-cad mediated tight junctions that form between plasma membranes of the outer blastomeres not only provide an apical basolateral polarization of cells, but also prevent the paracellular leakage of fluid from the nascent blastocoel. Secondly, the cell adhesion properties of E-cad, which is located in the basolateral regions of the plasma membrane, are crucial in restricting the distribution of Na^+/K^+-adenosine triphosphatase (Na^+/K^+-ATPase) to the region (Barcroft et al., 2002). With these two factors in place, the polar distribution of Na^+/K^+-ATPase to the basolateral location causes a Na^+-gradient to be established within the interior of the embryo, and subsequently osmotic uptake of water occurs such that it accumulates in the extracellular space of the nascent blastocoel (Kidder, 2002). Because of the tight junctions prevent this fluid from leaking out; it accumulates until the blastocoelic cavity is fully expanded (Barcroft et al., 2002; Watson and Barcroft, 2001). In addition, E-cad has been shown to restrict the localization of the Na^+/K^+-ATPase to the basolateral membrane domain of a variety of polarized epithelial cells, including mural trophectodermal cells, MDCK cells, and thyroid epithelial cells (Piepenhagen and Nelson, 1998; Watson et al., 1990a; Yap et al., 1995). For example, the transfection of a full-length E-cad cDNA into fibroblastic cells and retinal epithelial cells was capable of localizing the Na^+/K^+-ATPase to the basolateral membrane in these respective cell cultures (Marrs et al., 1995; McNeill et al., 1990). The interaction between E-cad and the Na^+/K^+-ATPase may, at least in part, explain why E-cad deficient embryos fail to form a blastocoelic cavity in vivo (Larue et al., 1994). Expression of E-cad was markedly diminished in embryos injected with antisense RNA as compared to controls and underwent delayed compaction, suggesting that the observed delay is due to the inhibition of E-cadherin gene

expression by antisense RNA (Ao and Erickson, 1992). The dependence of compaction and cavitation on E-cad has been demonstrated experimentally in the mouse by treating precompaction embryos with blocking antiserum against E-cad. Disruption of E-cad mediated cell-cell adhesion delays polarization of blastomeres and the cell contact pattern is lost (Johnson et al., 1986; Pratt et al., 1982) suggesting that these events of compaction, particularly led by E-cad and responsible for the development of cell-cell contact and polarity within the outer blastomeres and are essential processes directly contributing to the formation of blastocyst. In addition, inhibition of E-cad mediated adhesion leads to a loss of tissue diversification with few of any cells differentiating into ICM (Johnson et al., 1986; Shirayoshi et al., 1983). Collectively, these observations suggest that E-cad is at the top of the molecular cascade of protein interactions that result in the establishment of the trophectoderm (De Sousa et al., 1998) and that E-cad mediates early adhesive events that serves as a prerequisite for the recruitment and organization of membrane components that are involved in maintaining the differentiated epithelial cell state.

1.4.3: Cell biology of type 1 and type 2 cadherins

The role of type 1 cadherins in selective adhesion between cells resulting in cell aggregation and sorting was evaluated using embryonic cells displaying different type 1 cadherins in vitro (Friedlander et al., 1989; Miyatani et al., 1989; Nose et al., 1988; Steinberg and Takeichi, 1994; Takeichi, 1988; Takeichi, 1991). Fibroblasts expressing either E- or P-cad were mixed and found to sort into separate group of cells, each of which expressed the same cadherin. Pair wise, combinations of cells expressing N-, P- and E-cad obtained similar results. A recent study has demonstrated that cell sorting is dependent on not only the type of cadherin being expressed by the transfected L cells, but also on the level of cadherin subtype expressed (Steinberg and Takeichi, 1994). Collectively, these studies lead to the conclusion, that

homophilic interactions of cadherins leading to specific interaction and thereby segregation and sorting of cell populations provide the molecular basis for the organization and formation of tissues during development.

In vivo studies with embryonic stem (ES) cells rendered genetically null for E-cad expression and then "rescued" with different cadherins have indicated that, to some extent, cadherins can directly dictate the formation of certain tissue subtypes (Larue et al., 1996; Rosenberg et al., 1997). Mouse ES cells in which both alleles of the E-cad gene was knocked out not only showed a disaggregated phenotype in culture but also had a specifically altered gene expression, for example, of the transcription factor T-Brachyury (Larue et al., 1996; Rosenberg et al., 1997). When injected into syngeneic animals, wild type ES cells form benign teratomas that are highly differentiated and show a remarkable degree of tissue organization, indicating derivates of all three germ layers, e.g., muscle, epithelium, neuroepithelium, bone, teeth and hair follicles. By contrast, the cadherin null mutant ES cells formed teratomas in which no organized structures could be observed. Several early and late differentiation markers were still expressed, in these tumors, indicating that, at least to some extent, the differentiation program of the ES cells were not impaired per se. Interestingly, when these genetically altered ES cells were "rescued" with an E-cad cDNA under the control of constitutive promoter; the teratomas generated were made up almost exclusively of epithelia (Larue et al., 1996). When N-cad was constitutively expressed in the E-cad negative ES cells, teratomas formed neuro epithelia and cartilage. Alternatively, expression of R-cad resulted in the formation of striated muscle and epithelia (Rosenberg et al., 1997). Since all these cadherins can similarly rescue the impaired adhesion of E-cad negative ES cells, their differential behavior in the teratomas suggests each cadherin has the ability to govern the development fate of these cells. The ability of P-cad and other type-2 cadherins to influence tissue formation in this model system has yet to be determined.

The biological significance of type 1 cadherins in early embryogenesis has been demonstrated in mice in which the specific gene has been ablated (Larue et al., 1994; Radice et al., 1997a; Radice et al., 1997b). The gene knockout for E-cad results in embryonic death prior to implantation. Although, compaction is initiated in early morula, the blastomeres subsequently lose intercellular aggregation and disintegrate. This formation of morula has been attributed to the maternal mRNA transcripts of E-cad present in the developing embryo. The embryo subsequently fails to hatch from the zona pellucida and fail to implant. Interestingly, embryonic stem cells derived from E-cad null mutant mice are viable, grow in culture and express the epithelial marker protein cytokeratin, indicating some differentiation occurs perhaps as a consequence of the presence of maternal E-cad (Larue et al., 1994). N-cad is believed to play a key role in neural tube formation, but the mutant mice continue to develop and form neural structures until 10 d of gestation (Radice et al., 1997b). However, cardiac defects and not neural abnormality, are the cause of mortality in these knock out mice. This indicates that other cadherins can substitute for the function during development, at least during developmental processes of neural tube formation and somatogenesis (Vleminckx and Kemler, 1999). The homozygous P-cad knockout mice are viable and fertile. However, the females display an aberrant differentiation and morphology of the mammary gland resembling the glands of pregnant animals (Radice et al., 1997a). The results were unique as P-cad is expressed at high levels in the mouse placenta and deciduas and plays an important role in the process of implantation (Kadokawa et al., 1989). Taken together, these observations suggest that other cadherin subtypes might be playing an important role in embryogenesis.

The mechanism(s) by which these cadherin subtypes modulate cellular differentiation remains poorly understood. However, recent studies have shown that the E-cad-mediated adhesion is capable of regulating the expression of different integrin subunits in isolated human epidermal keratinocytes (Hodivala and Watt, 1994; Sastry et al., 1996; Zhu and Watt, 1996).

Moreover, it has been demonstrated that E-cad can interact with integrin $\alpha 2\beta 1$ (Whittard et al., 2002) suggesting that the type 1 classical cadherins may modulate the repertoire of adhesion receptors expressed on the cell surface and thereby play an integral role in cellular differentiation. The confirmation of the role of E-cad being the primary modulator in the formation of tight, gap, and desmosomal junctions before themselves being localized to the adherens junction in well-differentiated epithelial cells has been convincingly demonstrated (Adams et al., 1996; Angres et al., 1996). Antibodies specific for E-cad disrupt the formation of gap junction, desmosomes, and tight junctions in epidermal keratinocytes (Wheelock and Jensen, 1992). Similarly the transfection of a full-length E-cad cDNA into retinal pigment cells mediates the formation of desmosomes in these cells (Marrs et al., 1995). However, in the embryo, E-cad may not be required for the absolute maintenance of desmosomes as the blastomeres of E-cad-deficient embryos are capable of forming desmosomes (Riethmacher et al., 1995). The importance of E-cad in maintaining epithelial cell differentiation, however, is highlighted by the failure of these cells to form a polarized epithelium despite the presence of these junctions in E-cad null-mutant embryos.

The cell biology of the type 2 cadherins has not been as well characterized, both functionally and structurally (Shimoyama et al., 2000). It was initially suggested that type 2 cadherins mediate weaker cell-cell adhesion than type 1 cadherins because of their expression in loosely associated cells (Takeichi, 1995). This was substantiated by expression of cad-5 and cad-8 in cell-aggregation experiments wherein they did not demonstrate significant cell-adhesion activity (Kido et al., 1998; Tanihara et al., 1994a). Other researchers have however contradicted these findings and shown that both cad-5 and cad-8 mediate cell-cell binding comparable to that of type 1 cadherins (Shimoyama et al., 2000). This discrepancy can be explained by the fact that the sequences for cad-5 and cad-8, used in former studies were incomplete. For example, the new cad-5 sequence isolated and used for the latter study had additional 12 nucleotides compared to

the one previously reported by Tanihara et al. (Tanihara et al., 1994b). Similarly, the cad-8 sequence used in the former study was shorter by seven amino acids. This could have resulted in derangement in the cleavage of the signal peptide of the precursor region or of the transport of cadherin to the cell surface leading to malfunction of the cadherins in the former study.

As the type 2 classical cadherins were first identified in the murine brain (Suzuki et al., 1991), the majority of the studies to date have focused on the role(s) of these cadherin subtypes, in addition to the role of N-cad, in the morphogenesis of the mammalian central nervous system (CNS). Several type 2 cadherin subtypes, including cad-6, -8, -10, -11 and -12, have been shown to be differentially expressed in the rodent brain and these expression patterns correlate with the formation of interconnected neuronal cell populations and synaptogenesis (Gil et al., 2002; Inoue et al., 1997; Kimura et al., 1996; Korematsu and Redies, 1997a; Marthiens et al., 2002b; Redies and Takeichi, 1996; Selig et al., 1997; Suzuki et al., 1997). These observations have led to the proposal that the complexity of neuronal circuitry in the mammalian CNS may be mediated, at least in part, by the spatiotemporal expression of the type 2 classical cadherin subtypes in this tissue.

The regulated expression of cad-6 in the rodent and human kidney suggests that this CAM also plays a key role in the formation and organization of this tissue during development (Cho et al., 1998; Paul et al., 1997; Shimazui et al., 2000; Xiang et al., 1994). In particular, the onset of cad-6 expression correlates with the formation of a polarized epithelium in the kidney (Cho et al., 1998; Paul et al., 1997). Function-perturbing antibodies specific for cad-6 are capable of inhibiting the formation of renal epithelial cell structures in vitro (Cho et al., 1998). Although embryos from cad-6 null mutant mice survive but exhibit minor defects in organ formation (Mah et al., 2000). Cad-6 has also been shown to mediate the heterotypic interactions that occur between osteoclasts and the surrounding stromal cells in vitro, a cellular event that is believed to promote osteoclast differentiation and bone morphogenesis (Mbalaviele et al., 1998). Expression

of cad-8 has been detected in the post implantation embryonic and postnatal mouse brain that is restricted specifically to the brain nuclei of the developing gray matter structures (Korematsu and Redies, 1997a; Korematsu and Redies, 1997b), striatal matrix (Korematsu et al., 1998a) and neural circuits (Korematsu et al., 1998b) during brain development. These results suggest that cad-8 is involved in formation of the striatal-compartmentalized structures during brain development. Recently, expression of cad-8 has been detected in some types of renal cell carcinomas and thus may be involved in both kidney morphogenesis as well as tumorigenesis (Blaschke et al., 2002). In the central nervous system (CNS), cad-10 expression is spatially restricted at all stages of development of the mouse embryogenesis (Bekirov et al., 2002; Fushimi et al., 1997). In addition to the expression N-cad, three type 2 cadherins, namely, cad-6, -8, and -10 are expressed contemporaneously in barrel cortex and are involved in the formation of afferent and intrinsic circuitries and synaptogenesis during embryogenesis in mice (Gil et al., 2002). Cad-11 is expressed in several mesenchymal cell subpopulations and in the epithelial cells of the renal tubules during embryonic development in the rodent (Hoffmann and Balling, 1995; Kimura et al., 1995; Simonneau et al., 1995). Recently, cad-11 expression has also been correlated with the terminal differentiation of rabbit corneal fibroblastic cells and human endometrial stromal cells in vitro (Chen et al., 1999a; Masur et al., 1999). The overexpression of cad-11 in Xenopus embryos was capable of disrupting anterior-posterior axis formation in a manner similar to that observed following the ectopic expression of other type 1 classical cadherins in these embryos (Dufour et al., 1994; Hadeball et al., 1998; Kuhl and Wedlich, 1996). Finally, the transfection of a full-length cad-11 cDNA into murine embryonic fibroblast cells resulted in an increase in vascular endothelial growth factor (VEGF) mRNA levels in these cell cultures (Orlandini and Oliviero, 2001). Recent studies have examined the role(s) of cad-11 during the formation of murine and human bone. For example, mRNA transcripts encoding this CAM have been detected in bone marrow-derived osteogenic precursor cells as well as in

differentiated osteoblasts in vitro (Cheng et al., 1998; Kashima et al., 1999; Lecanda et al., 2000; Okazaki et al., 1994; Shin et al., 2000). The addition of inhibitory peptides containing the HAV sequence only partially blocked the differentiation of isolated bone marrow-derived osteoblastic precursor cells, which also express the type 1 classical cadherins N-cad and R-cad in vitro (Cheng et al., 1998; Lecanda et al., 2000). Moreover, cad-11 expression is associated with the terminal differentiation and fusion of villous cytotrophoblasts isolated from the human term placenta and human myoblasts in vitro (Getsios et al., 1998b). Collectively, these data indicate that type 2 cadherins are sufficiently diverse and differentially distributed to support a role in cell surface recognition and adhesion during development.

Sequence analysis of human cad-10 shows very close homology with human, mouse and rat cad-6 (Kools et al., 1999). Similarly, close homology between cad-8 and cad-12 has also been reported (Kools et al., 1999). This suggests that there might be a close evolutionary relationship between cad-6 and cad-10, as well as, between cad-8 and cad-12, which could also mean that they might have some common, shared functions. Though, cadherins are considered to undertake homophilic interactions, it has been demonstrated that cad-6 interacts with cad-14, though only to a limited extent (Shimoyama et al., 1999). Heterophilic interactions, ranging from complete to partial have also been reported for other cadherin subtypes. For example, complete heterophilic interaction has been observed between cad-6 and cad-9; cad-7 and cad-14; cad-8 and cad-11 with latter two combinations showing almost indistinguishable binding specificity to that of E-cad. These data generated under in vitro conditions are indicative of the fact that one cadherin subtypes can undergo heterophilic interaction and might possibly explain the unexpected results of cadherin knockouts.

Recently, studies with human cancer specimen and functional experiments with cultured tumour cells and transgenic mouse models have indicated that the loss of the cell adhesion molecule E-cad is causally involved in the formation of epithelial cancers (Cavallaro et al.,

2002). Since, progression to tumour malignancy involves changes in a tumour cell's capabilities to adhere and communicate with neighboring cells, an aberrant cadherin expression resulting in altered phenotype and neoplastic state has been suggested (Cavallaro et al., 2002). While E-cad has been strongly implicated as an invasion suppressor in a variety of cancers, an inverse correlation between E-cad expression and invasion has been reported for a variety of carcinoma cell lines including bladder, breast, lung, pancreas, oesophagus, colon and endometrium (Okegawa et al., 2002). Similarly, down regulation of E-cad expression has been reported for a wide variety of solid carcinomas including skin, meninges, oesophagus, thyroid, mammary gland, stomach, liver, kidney, pancreas, colon, bladder, cervix, ovary and prostrate (Bracke et al., 1996; Hajra and Fearon, 2002).

However, impaired E-cad mediated interactions or loss of E-cad mediated adhesion should not be taken as the sole explanation for the observed correlation between cancer and poor clinical outcome (Cavallaro et al., 2002). In human prostate cancer cells, besides loss of E-cad, a number of other cads are detected, namely, N-, P-cad, cad-4, -6, and -11 (Bussemakers et al., 2000). Out of these, upregulation of N-cad and cad-11 has been reported and suggests that these two CAMs are involved in the acquisition of an invasive and/or metastatic phenotype (Bussemakers et al., 2000; Tomita et al., 2000). Similarly, during melanoma development, loss of functional E-cad accompanies gain of expression of N-cad (Li et al., 2001a; Li et al., 2002). In addition, while breast cancer cell lines express higher levels of N-cad (Hazan et al., 1997), squamous cell carcinomas of head and neck express N-cad but not E- or P-cad (Islam et al., 1996). A similar switch in cadherin expression has been reported recently during cancer progression in the human ovary with P-cad being the predominant subtype in the ovarian tumor mass with an concomitant decrease in E-cad levels (Patel et al., 2003). In addition, N-cad, cad-4 and cad-6 transcripts were detected from the ovarian tumor mass, but there were no significant changes in their levels with cancer progression. Moreover, Cad-11 expression has also been

detected in bone, breast, gastric, prostate, and renal cancer cell lines (Bussemakers et al., 2000; Kashima et al., 1999; Pishvaian et al., 1999; Shibata et al., 1996; Shimazui et al., 1996; Tomita et al., 2000). The coordinated expression of cad-11 in these carcinoma cells and the surrounding stroma suggests that this CAM may mediate carcinoma-stromal cell interaction during tumour cell invasion (Pishvaian et al., 1999; Shibata et al., 1996; Tomita et al., 2000). Though, the biological significance of these subtypes during cancer progression has not been determined, it is tempting to speculate that the neoplastic transformation of epithelial derived tumour cells involves a highly regulated switch in the expression of different classical cadherin subtypes in a manner similar to that observed during tissue morphogenetic processes.

In view of these observations, it is evident that cadherins mediate important cell adhesive mechanisms during development. Since the expression patterns of different cadherins often superimpose during morphogenesis, there is a distinct possibility that in addition to E-cad, other cadherins are also being expressed simultaneously during preimplantation embryogenesis and might be playing a key role in preimplantation embryogenesis.

1.5: Extracellular matrix deposition

Cells synthesize macromolecules that are secreted into the extracellular spaces and mediate the relationships among cells. These compounds are called substrate adhesion molecules and they form the extracellular matrix (ECM). The interactions between cells and the ECM initiate a flow of information that acts to regulate many fundamental processes which include cell migration in the early embryo, maintaining tissue integrity and modulating cellular differentiation during development (Adams and Watt, 1993; Lin and Bissell, 1993). For instance, the ECM serves as the substrate upon which cells migrate, or even induce and modulate growth and differentiation of cells (Zagris, 2001). The classical view of the ECM as a physical

support/barrier has therefore been extended by its additional role as an instructional entity (Streuli, 1999).

The concept of cells being held together in tissues by intercellular adhesive substance can be traced to the work of Schiefferdecker (schiefferdecker, 1886), who segregated the epidermis from the dermis by incubation in a pancreatic extract and therefore demonstrated that living cells could be separated. It was subsequently demonstrated that chick embryonic tissues could be dissociated with trypsin into single cells (Moscona, 1952; Moscona and Moscona, 1952). The success of these procedures clearly implied that a proteinaceous material connected these tissues and suggested that not only cells of the connective tissue but tissue cells in general, including epithelial cells, are held together by an "intercellular cement" or "ground substance" (Gray, 1926; Grobstein, 1954). Affinities and disaffiliations in cellular aggregation and disaggregation, mass cellular movements such as stretching and foldings, regulation and mutant developmental abnormalities, all could find rationale, at least in part, in this complex bio-physical-chemical matrix. The extracellular material in preparations of trypsin-dissociated cells was dubbed as ECM by Moscona (Moscona, 1960). The role of the ECM in development was indicated in experiments where mesenchymal cells derived from primitive epithelium, required remodeling events within the matrix, in order to traverse the stroma and reform specific types of epithelia. This indicated that during development, the generation and maintenance of cellular diversity is dependent on ECM remodeling (Hay, 1989; Hay, 1990). Remodeling of the ECM is also important for numerous differentiative processes in the adult, including neovascularisation, trophoblast invasion, uterine changes during the menstrual cycle, pregnancy, tumorigenesis and wound repair processes (Woessner, 2002).

1.5.1 Extracellular matrix components

The ECM is a complex cellular product comprising of glycoproteins, collagens, glycoaminoglycans (GAGs) and proteoglycans as major structural and functional components. The ECM is comprised of a meshwork of fibers embedded in a gel like ground substance made up of GAGs and proteoglycans. The fibrous component of glycoproteins reinforce the ground substance. This collective matrix provides attachments sites that could guide cells into defined pathways that can influence the extent and direction of their movements, and also affect the cell-matrix interactions thereby assisting in either the formation or maintenance of the proper architecture of the developing embryo (Adams and Watt, 1993; Chung, 1995; DeSimone, 1994; England, 1984; Harrisson, 1989; Timpl and Brown, 1994). A variety of glycoproteins such as fibronectin, tenasin, entactin/nidogen and laminin, have been suggested to be involved in cell and tissue adhesive processes during development. These glycoproteins are multidomain structures that interact with either one another, or with other ECM molecules or the cell surfaces and have been suggested to be responsible for organizing the collagen, proteoglycans and cells in an ordered manner. While fibronectin has been shown to bind to collagen, entactin, GAGs and cells, it has also been shown to be essential for mesodermal cell migration during gastrulation (Ruoslahti and Obrink, 1996). Tenascin expression is highly regulated during development and possesses both adhesive and anti-adhesive properties (Chiquet-Ehrismann, 1995; Chiquet-Ehrismann et al., 1986). Both fibronectin and tenascin have been shown to regulate collagenase gene expression in fibroblast cells in vitro suggesting a role in cell adhesion, migration and morphogenesis (Tremble et al., 1994). Entactin/nidogen binds to collagen IV, fibronectin and laminin (Chung, 1995; Martin and Timpl, 1987; Tsiper and Yurchenco, 2002; Yurchenco and O'Rear, 1994) and have been shown to be playing a key role in the mesodermal cell migration during gastrulation. Laminin is the first glycoproteins to appear in the ECM of the developing embryo (Cooper and MacQueen, 1983; Dziadek and Timpl, 1985; Leivo et al., 1980; Martin and Timpl, 1987). Though, chick embryos are able to synthesise laminin, they do not deposit it on

their surfaces during early stages (Zagris and Chung, 1990). Deposition of laminin to the ECM was first detected on the cell surfaces on the ventral surface of the epiblast and in the hypoblast in embryo of chick at the blastula stage (Zagris, 2001). In contrast, laminin is detected on the cell surface of blastomeres at the 2- cell stage itself (Dziadek and Timpl, 1985; Wu et al., 1983). The ability of laminin to self-associate and to interact with collagen IV, nidogen/entactin, perlecan and other proteins is essential to the assembly of the ECM (Martin and Timpl, 1987; Yurchenco and O'Rear, 1994). Cell binding to laminin has been reported to occur via a large diverse group of integrins (Giancotti, 2000; Haas and Plow, 1994; Hynes, 1992; Mecham, 1991) and this cellular interaction with laminin matrices plays an important role in cell attachment, directional migration, mitogenic modulation, neurite outgrowth, axon guidance, survival of cells, maintenance of differentiated cell phenotypes and the induction of new expression patterns (Adams and Watt, 1993; Chung, 1995; Engel, 1992; Luckenbill-Edds, 1997; Streuli, 1999; Timpl and Brown, 1994; Yurchenco and O'Rear, 1994).

GAGs are key components of the ECM in the embryo and have been suggested to play a key role in cellular proliferation, migration, differentiation, and maintenance of morphogenetic structures. Hyaluronate, chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate are some of the GAGs being expressed during early embryogenesis. While hyaluronate is the predominant GAG in the early chicken and mouse embryos, it has been shown to interact with sulfated proteoglycans and collagen to form gel fiber networks. Though it has been identified that the cells bind and respond to hyaluronate through the cell surface protein CD44 and receptor for hyaluronan-mediated motility or RHAMM (Akiyama et al., 2001; Sherman et al., 1994), the presence of these surface proteins has been detected only on the chick embryo (Corbel et al., 2000).

The proteoglycan superfamily comprises of three families, namely, the basement membrance proteoglycans comprising of perlecan, agrin and bamacan, the hyalectans comprising

of versican, aggrecan, neurocan and brevican and the small leucine-rich proteoglycans comprising of decorin, and biglycan. Though, these molecules are involved in diverse functions, collectively, proteoglycans are involved in tissue arrangement and organization, as biological filters and modulators of growth factor activities and regulate collagen fibrillogenesis (Iozzo, 1998). For example, perlecan is detected in preimplantation mouse embryos and has been suggested to be fundamental in cell differentiation and tissue morphogenesis (Carson et al., 1993; Dziadek and Timpl, 1985). While perlecan has been reported to be a key player in regulating fibroblast growth factor (FGF)-2 signalling and as a blocker of several growth factors to their target cells (Iozzo, 1998), its splice variant, has been shown to be present in murine embryos and controlling the amounts of FGFs expression during early neural development (Joseph et al., 1996).

The hyalectants have been shown to bind complex carbohydrates such as hyaluronate at their N-terminus, less complex sugars at their C-terminus and a varied number (2-100) GAG chains at their central non-homologous regions. Moreover, the different splice variants of hyalectants are often expressed spatio-temporally during development (Dours-Zimmermann and Zimmermann, 1994). Versican is selectively expressed in the embryonic tissues that act as barriers to neural crest cell migration and motor and sensory axonal outgrowths (Landolt et al., 1995) and neurocan has been shown to bind to N-CAM and Ng-CAM, inhibiting their homophilic interactions and blocking neurite outgrowth (Grumet et al., 1993). Though the mechanism of this anti-adhesive activity of proteoglycans remains uncertain, it has been proposed that these molecules bearing chondrotin sulfate epitope might prevent access to substrate molecules by literally covering them up by providing an anti-adhesive function through steric hindrance at the cell surface (Oakley et al., 1994). Decorin, binds TGF-β and it's isoforms with high affinity and functions as a reservoir for these growth factors in the ECM (Hildebrand et al., 1994).

The highly diverse group of fibrillar proteins called collagens constitutes the most abundant structural component of the ECM. The first collagen to appear in the early embryo is the collagen IV, which is one of the constituents of the ECM and is considered the major constituent of basement membranes and forms by end to end and lateral interactions, open or planer or polygonal networks to which other glycoproteins and proteoglycans are bound, though only at specific sites (Zagris, 2001).

Several of these ECM constituents are expressed spatiotemporally during preimplantation embryogenesis in mouse. Laminin and heparan sulfate proteoglycans are detected on the surface of 2-cell stage embryos (Dziadek and Timpl, 1985; Wu et al., 1983). In contrast, nidogen/entactin is first detected only at the morula stage in embryos undergoing compaction, while collagen IV and fibronectin are first detected at the blastocyst stage and localized only in the ICM (Adamson and Ayers, 1979; Leivo et al., 1980; Wartiovaara et al., 1979). The appearance of collagen IV at the blastocyst stage coincides with the appearance of the first basement membrane in the developing preimplantation embryo. It has been suggested that all these ECM components interact with each other to form a thin, tightly packed network of fine cords in a highly cross linked network of collagen IV (Martin and Timpl, 1987).

1.5.2: Extracellular matrix degradation-proteinases and their inhibitors

Efficient degradation and turnover of the ECM is fundamental to the ECM remodeling required during biological procedures such as tumor invasion, wound healing, inflammation, ovulation and development (Zagris, 2001). ECM is degraded by several families of proteinases, including those from the plasminogen activator (PA) and the metzincins that includes the matrix metalloproteinases (MMP) family and the adamlysins (Davis et al., 2002; Sternlicht and Werb, 2001; Werb and Chin, 1998).

1.5.3: Plasminogen activators and their inhibitors

The plasminogen activators are substrate-specific serine proteinases that mediate cleavage of plasminogen to plasmin exhibiting a broad range of serine protease activity (Liu, 1999; Preissner et al., 1999). The proteinase activator system includes the urokinase-type plasminogen activator (uPA), the tissue-type PA (tPA), the PA inhibitor-1 and -2 (PAI-1 and PAI-2, respectively) and the uPA receptor.

tPA and uPA are expressed during early development in several mammalian species. tPA is expressed by mouse oocytes during maturation (Huarte et al., 1995) and has been suggested to play a key role in preventing polyspermy during fertilization (Huarte et al., 1993; Zhang et al., 1992). Mouse embryos do not express tPA beyond the two-cell stage (Zhang et al., 1994). In contrast, uPA is first expressed by mouse blastocysts coinciding with the initiation of implantation and subsequent trophoblast invasion (Harvey et al., 1995; Sappino et al., 1989; Strickland et al., 1976). uPA expression has also been detected in blastocyst stage embryos of several other species including the cow (Dyk and Menino, 1991; Menino and Williams, 1987), sheep (Bartlett and Menino, 1993) and pig (Fazleabas et al., 1983). The onset of uPA expression and secretion during the peri-implantation period has implicated that it is an ECM degrading proteinase (Sappino et al., 1989). Recent study has revealed that uPA is expressed and secreted by early in vitro produced bovine embryos (Whiteside et al., 2001b). This expression pattern is quite different from that in other species where uPA is expressed at the blastocyst stage, i.e., only at the time of implantation, suggesting a prior role of uPA in bovines. Moreover, total PA production by cow blastocysts has been linked to hatching efficiency. It has been demonstrated that hatched blastocysts secreted more total PA than those blastocysts that did not hatch from their zona pellucidae (Kaaekuahiwi and Menino, 1990). In addition, the membrane-bound uPA receptor is expressed by the invasive trophoblasts in mouse, macaque and humans during

implantation (Feng et al., 2001; Multhaupt et al., 1994; Pierleoni et al., 1998; Teesalu et al., 1996).

The expression of PAI-1 and PAI-2 appears to be differentially regulated during embryogenesis as ovine embryos produce a PAI-2 like protein that associates with PA and forms a PA-PAI-2 complex, while treatment with PAI-1 antibodies does not lead to elevated levels of PA activity suggesting absence or low expression of PAI-1 (Bartlett and Menino, 1993). Similarly embryonic PA activity is suppressed by PAI-2 in bovine preimplantation embryos (Coates and Menino, 1994) and has been suggested to be playing an important role in zona pellucida hatching. In human term placenta, the expression of PAI-1 and PAI-2 appears to be differentially regulated during the terminal differentiation and fusion of villous cytotrophoblasts (Feinberg et al., 1989). In particular, PAI-1 expression is high in freshly isolated mononucleate cytotrophoblasts whereas maximal PAI-2 expression is detected in the multinucleated syncytial structures that form in these cultures at later time points. However, the functional significance of PAI subtypes during embryogenesis or the switch in their expression during terminal differentiation remains to be elucidated.

1.5.4: The Metzincin family of the metalloproteinases

The metzincin superfamily is characterized by a highly conserved motif, HEXGHXXGXXHZ (single letter code for amino acids, X refers to any amino acid and Z is a family-specific amino acid), containing three histidines that bind zinc at the catalytic site and a conserved methionine turn that is in close proximity to the active zinc site (Stocker and Bode, 1995). The metzincins are further divided into four multigene families namely, the serralysins, astacins, MMPs, and ADAMs, based primarily on the Z residue, which is serine in all but few MMPs (Chang and Werb, 2001; Stocker and Bode, 1995).

The serralysins, containing proline in their Z position, are bacterial enzymes playing an important role in bacterial virulence and pathogenicity (Bode et al., 1993). Astacins contain glutamic acid in the Z position and include bone morphogenetic protein-1 (BMP-1), which is the procollagen C-proteinase that removes the C-terminal propeptides of fibrillar procollagens (Bode et al., 1993). They also include *Drosophila* and mammalian tolloid and tolloid like proteins, which activate certain growth factors, and transmembrane meprins A and B that can process peptide hormones (Sternlicht and Werb, 1999).

1.6: Matrix metalloproteinases and their inhibitors

The matrix metalloproteinases (MMPs) are a large gene subfamily of zinc-dependent proteinases that mediate a variety of tissue remodelling processes (Fata et al., 2000; Nagase and Woessner, 1999; Woessner, 1991). The MMPs are synthesized as latent precursors that must be cleaved following secretion in order to become activated. The activity of MMPs can be further regulated by the secretion of specific tissue inhibitors of MMPs (TIMPs).

To date, 25 vertebrate and 9 non vertebrate MMPs have been identified (Llano et al., 2000; Lohi et al., 2001; Nagase and Woessner, 1999; Sternlicht and Bergers, 2000; Sternlicht and Werb, 2001). Individual MMPs are referred to by their common names or according to a sequential numeric nomenclature reserved for the vertebrate MMPs. Each of the vertebrate MMPs have distinct but often overlapping substrates, including virtually all ECM proteins (Sternlicht and Werb, 2001). MMPs have been found to be expressed in pathological conditions such as arthritis and tumor invasion (Birkedal-Hansen, 1993) and have also been implicated as facilitators of implantation in the mouse (Alexander et al., 1996; Harvey et al., 1995; Whiteside et al., 2001a) and in humans (Bischof and Campana, 2000; Rawdanowicz et al., 1994; Schatz et al., 1999). MMP-9 is first expressed at the blastocyst stage in mouse (Behrendtsen et al., 1992; Brenner et al., 1989; Harvey et al., 1995). Expression of MMP-9 has also been demonstrated in

peri-implantation ovine (Salamonsen et al., 1995) and porcine embryos (Menino et al., 1997). In bovine, MMP-9 is expressed throughout the first 7 d of development i.e. from fertilization to the blastocyst stage (Whiteside et al., 2001a). This expression phenotype is novel in comparison to murine embryos, which only express embryonic transcripts of MMP-9 at the blastocyst-stage (Harvey et al., 1995; Zhang et al., 1994). Early murine embryos during in vitro culture produce several MMPs, including MMP-1, -2, -3, -9 and -11 (Das et al., 1997; Kim et al., 2002; Lefebvre et al., 1995) and a similar repertoire excluding MMP-11, has been reported in sheep (Riley et al., 2000; Salamonsen et al., 1995). It has been demonstrated that disruption of MMP-9 in murine blastocysts outgrowths inhibits ECM degradation (Behrendtsen et al., 1992) demonstrating the importance of MMP-9 in ECM degradation during trophoblast invasion. In situ hybridization studies have substantiated these findings by showing the presence of MMP-9 being expressed by the extraembryonic trophoblast giant cells, which are highly invasive during day 5 through day 8 during murine embryogenesis however, mice with null mutation for MMP-9 gene are viable and fertile (Das et al., 1997; Leco et al., 1996). Similar results have been obtained with MMP-11 null mutants as they are perfectly viable and fertile as well (Masson et al., 1998). Since the mouse blastocyst secretes a repertoire of MMPs (Das et al., 1997; Kim et al., 2002; Lefebvre et al., 1995), it is tempting to speculate that other enzymes might compensate for the lack of MMP-9 or -11. In addition, membrane-type (MT)1-MMP also called as MMP-14, is also expressed by blastocysts and trophoblast giant cells on day 6.5 and 7.5 murine embryos respectively, substantiating the multiple role(s) of MMPs during trophoblast invasion (Tanaka et al., 1998). However, the functional role of many of these MMPs during developmental processes remain to be elucidated.

Apart from a MMP-11 and -14, which are activated by furin, most of the other MMPs are secreted as inactive proteinases (Nagase and Woessner, 1999). This is facilitated in vivo by other proteinases such as uPA (Kleiner and Stetler-Stevenson, 1993). Using transgenic mice deficient

in uPA, Carmeliet et al. (1997), demonstrated that uPA is a significant activator of pro-MMPs. In addition, MT1-MMP has been demonstrated to be an activator of pro-MMP-2 (Sato et al., 1994). Researchers have however proposed that this activation process requires both active MT1-MMP and the TIMP-2 bound MT1-MMP (Butler et al., 1998; Kinoshita et al., 1998; Strongin et al., 1995).

Many of the MMP genes being expressed have been shown to be regulated by a number of factors including growth factors, cytokines, chemical agents, physical stressors, ultraviolet B irradiation and oncogenic cellular transformation, while the MMP gene expression is downregulated by growth factors, retinoic acids and glucocorticoids (Nagase and Woessner, 1999). In particular, MMP-1, -2, and -3 are regulated by extracellular matrix metalloproteinase inducer (EMMPRIN), a member of the Ig family expressed on tumor cell surface in fibroblasts (Guo et al., 1997; Li et al., 2001b). In addition, TNF α and IL-1 are able to regulate the expression of MMP-1 (Fini et al., 1998; Spiegel et al., 1996). Induction of MMP-2 and -9 has been reported by intercellular VCAM-1 and ICAM-1 mediated cell adhesion respectively (Aoudjit et al., 1998; Romanic and Madri, 1994). More evidence of a role for steroids in ECM remodelling comes from the fact that the administration of estrogen to double estrogen receptor knockout (DERKO) mice shows upregulation of MMP-9 and α1 chain of collagen VIII (Lindberg et al., 2002). Although, this effect of estrogen in the DERKO mouse could also be due to some low affinity binding to other known or unknown nuclear receptors (Kousteni et al., 2001) or incomplete inactivation of ERα in mice (Pendaries et al., 2002) it still demonstrates that steroids have a direct effect on ECM.

With unabated invasiveness, the trophoblast cells might progress unimpeded (Kirby et al., 1966) as when murine blastocysts are implanted under the kidney capsule, trophoblast cells invade the surrounding tissue and cause considerable damage to the kidney. One of the potential

mechanisms in countering the effect of proteases is the expression of TIMPs which are major endogenous regulators of MMP activities in the tissues.

The TIMPs represent a family of at least four secreted proteins to date (TIMPs 1-4) (Brew et al., 2000; Gomez et al., 1997) that reversibly inhibit the MMPs in a 1:1 stoichiometric fashion (Edwards, 2001; Edwards et al., 1996; Gomez et al., 1997; Sternlicht and Werb, 1999). They share about 37-51% overall sequence identity, a conserved gene structure, and 12 similarly separated cysteine residues (Huang et al., 1997; Murphy et al., 1991; O'Shea et al., 1992; Williamson et al., 1996; Williamson et al., 1993). TIMP-2 shows structural similarity to TIMP-1 (Boone et al., 1990), but individual TIMPs differ in their ability to inhibit various MMPs (Woessner, 1999). TIMP-1 is a relatively poor inhibitor of MT3-MMP, and TIMP-3 appears to be a more potent inhibitor of MMP-9 than other TIMPs. Similarly, TIMP-2 and -3 inhibit MT1-MMP, whereas TIMP-1 does not. TIMP-3, which differs from TIMP-1 and -2 being a protease inhibitor that remains bound to the ECM after secretion (Leco et al., 1994; Uria et al., 1994) has been shown to induce apoptosis of human colon carcinoma and melanoma cells (Ahonen et al., 1998; Smith et al., 1997). This has been attributed to the stablization of tumor necrosis factor (TNF)-α receptors as TIMP-3 has been reported to inhibit the TNF-α converting enzyme and receptor (Smith et al., 1997). TIMP-3 has also been shown to be involved in the shedding of cell surface-anchored molecules such as L-selectin (Borland et al., 1999), interleukin 6 receptor (Hargreaves et al., 1998) and syndecans-1 and -4 (Fitzgerald et al., 2000). TIMP-4 expression has been identified in murine and humans, in a tissue specific manner suggesting unique roles (Greene et al., 1996; Leco et al., 1997). Though, TIMPs have been postulated to be specific inhibitors of MMPs, many biological activities of TIMPs are independent of MMP-inhibitory activities (Chesler et al., 1995; Hayakawa, 2002; Hayakawa et al., 1994; Sternlicht and Werb, 2001). Fore example, TIMP-2 and not TIMP-1, inhibits basic fibroblast growth factor-induced human endothelial cell growth (Murphy et al., 1993). The TIMP-1-procathepsin L complex has

been reported to stimulate steroidogenesis in the rat testis and ovary in vitro (Boujrad et al., 1995). However, TIMP-1 deficient mice provide no evidence for regulation of steroidogenesis in vivo (Nothnick et al., 1997). The reasons for this difference are yet to be ascertained. Collectively, TIMPs have been shown to inhibit cell invasion in vitro, metastasis in vivo, tumorigenesis and angiogenesis (Gomez et al., 1997). TIMP-1 and TIMP-2 have mitogenic activities and overexpression of these two inhibitors causes reduced tumor cell growth (Gomez et al., 1997). Although all these TIMPs have been shown to be abundantly expressed in female reproductive tissues including, ovary, uterus and mammary tissue (Fata et al., 2000; Inderdeo et al., 1996; Khokha and Waterhouse, 1994; Leco et al., 1997; Waterhouse et al., 1993) studies showing their role in early embryonic development are limited (Das et al., 1997).

1.7: The adamlysins

The adamlysins are soluble snake venom enzymes with strong ECM degrading abilities. The ADAM (a disintegrin and metalloproteinase) are transmembrane cell surface proteins that have a disintegrin and metalloproteinase domain (Primakoff and Myles, 2000; Wolfsberg et al., 1995). Each of the ADAMs has an N-terminal signal sequence followed by a propeptide domain, a functional or non-functional metalloproteinase domain, a disintegrin-like domain, a cysteine-rich domain, EGF-like repeats, a transmembrane domain, and a C-terminal cytoplasmic tail. Individual ADAMs may participate in proteolysis via their metalloproteinase domain, adhesion via their disintegrin domain, cell-cell fusion via a putative hydrophobic fusion peptide in their cysteine-rich domain, and cell signalling via SH3-recognition sequences that are sometimes present in their intracellular domain. Seventeen of the 30 known ADAMs have functional zinc-binding motifs, including ADAM-17 (TNF-α converting enzyme, TACE), which cleaves membrane bound TNF-α to generate active soluble TNF-α. TACE also probably contributes to the shedding of several other cell surface molecules and appears to be an essential activator of

TGF-α in vivo (Peschon et al., 1998). Considering their localization, other ADAMs are also likely to regulate the shedding of several other important cell surface molecules (Werb and Yan, 1998).

The ADAMs have also been implicated in diverse processes in the cellular adhesion and proteolytic processing of important cell surface molecules. For example, ADAM-1 (fertilin α) may be involved in spermatogenesis in a number of mammals, and is found as a heterodimer with ADAM-2 (fertilin β) on the mature guinea pig and bovine sperm (Myles and Primakoff, 1997; Waters and White, 1997). Although, the function of protease domain in ADAM-1 has not been clearly defined, but the presence of ADAM-1 with the protease domain on even the immature sperm suggests a role in the development of sperm (Myles and Primakoff, 1997). On mature sperms however, the pro- and protease domains of both ADAM-1 and -2 are cleaved (Myles and Primakoff, 1997; Waters and White, 1997) . These heterodimeric sperm's proteins ADAMs 1 and 2 are essential for sperm egg fusion during fertilization via integrin α6β1 and this Cys-rich domain of ADAM-1 has been suggested to mediate the sperm-egg interaction (Blobel et al., 1992; Evans, 1999; Huovila et al., 1996; Waters and White, 1997). On the basis of their expression patterns, ADAMs 20 and 21 have been suggested to be involved in spermatogenesis and displays close similarity to ADAM 1 (Hooft van Huijsduijnen, 1998). ADAM 9 (Meltrin γ), ADAM 12 (Meltrin α) and 19 (Meltrin β) have a role in myosin fusion during muscle development and/or osteogenesis (Gilpin et al., 1998; Inoue et al., 1998a; Kurisaki et al., 1998; Yagami-Hiromasa et al., 1995). ADAM 10 (Kuzbanian), which was first identified as a protease in bovine brain showed its tendency to cleave myelin basic protein (Howard et al., 1996), and also has the capability to cleave type IV collagen (Millichip et al., 1998). Subsequent work however established that ADAM 10 is important for neural development through its processing of Notch and the Notch ligand Delta (Pan and Rubin, 1997; Qi et al., 1999; Rooke et al., 1996).

Recently ADAM 10-deficient mice have been produced which die at about day 9.5 of embryogenesis with multiple defects of the developing central nervous system, somites and the cardiovascular system suggesting a role in developmental processes (Hartmann et al., 2002). ADAM 9 has also been shown to promote the migration of fibroblasts in vitro (Nath et al., 2000) whereas ADAM-13 expression has suggested to be essential for cranial neural crest cell migration, in the Xenopus embryo (Alfandari et al., 2001; Alfandari et al., 1997). ADAM 15 may be involved in regulating blood vessel function (Herren et al., 1997; Zhang et al., 1998). ADAM 23 has been shown to play important roles in the development and progression of inflammatory and tumor processes (Cal et al., 2000; Yavari et al., 1998). ADAM 17 (TACE) (Black, 2002; Black et al., 1997), ADAM 9 (Mahimkar et al., 2000) and ADAM 10 (Pan and Rubin, 1997) mediate the shedding of cell surface proteins and are called sheddases. Interestingly, TIMP-3 has been reported to be an endogenous ADAMs inhibitor as it blocks certain shedding phenomena of ADAM 17 and also inhibits the activity of recombinant ADAM 17 (Amour et al., 1998; Hargreaves et al., 1998; Smith et al., 1997). The role of other TIMPs or of other endogenous ADAMs inhibitors remains to be elucidated. Collectively, these observations demonstrate that ADAMs are playing a key role in cell adhesion and proteolytic processing of important cell surface molecules.

The structural and functional complexity of the ADAM family of cellular disintegrins has grown considerably after the recent cloning studies have identified new members of the ADAM family known as ADAMTS (a disintegrin and metalloproteinases with thrombospondin motifs), in *C. elegans*, *Drosophila* and mammals (Kaushal and Shah, 2000; Kuno et al., 1997b; Tang, 2001). These new members are characterized by the presence of thrombospondin repeats in their amino acid sequence but do not contain the EGF like, transmembrane and cytoplasmic domains characteristic of other members of the ADAM gene family (Kaushal and Shah, 2000; Tang, 2001). To date, twenty ADAMTS genes have been reported in the human genome along with

four homologues being found in *C. elegans*, and three in *D. melanogaster* (Cal et al., 2001; Cal et al., 2002; Clark et al., 2000; Kuno et al., 1997b; Tang and Hong, 1999; Vazquez et al., 1999).

1.7.1: Structure of ADAMTS

The primary sequence of an ADAMTS is organized into modular structures. Similar to the ADAMs, there is a signal sequence at the N-terminus, followed by a putative prodomain, a catalytic domain, a disintegrin-like domain and an ECM binding domain, composed of thrombospondin (TSP) type 1 motifs, a spacer region and a variable number of TSP-like motifs at the carboxyl terminal of the protein (Abbaszade et al., 1999; Cal et al., 2001; Cal et al., 2002; Hurskainen et al., 1999; Kuno et al., 1997b; Tang, 2001; Tang and Hong, 1999; Vazquez et al., 1999) (Fig 3).

All ADAMTS subtypes show a clear hydrophobic signal sequence, which is followed by a prodomain presumably involved in maintaining the latency of these enzymes. The length of this prodomain is variable but demonstrates short stretches of sequence similarities containing three cysteine residues within each prodomain. Two of these conserved consensus motifs are CXYXGXV and CXGLXG and can be found generally in most of the ADAMTS. However, these cysteines are absent in ADAMTS-13, which can be explained due to the short length of the prodomain, making it the shortest ADAMTS subtype among ADAMTS subtypes described to date. The third Cys residue charachterisitic of ADAMTS prodomain is located close to the end of this region. Interestingly in ADAMTS-12 and -15, it lies within a sequence that resembles the Cys-switch (PRCGVPD) present in MMPs and is also responsible for maintaining their enzymatic latency. However, this Cys residue is absent in ADAMTS-2, -3 and -14 making them structurally closer to each other and suggesting a special feature of subset of ADAMTS. The prodomain ends in a basic region (RXR/RR) that mediated the intracellular activation of these enzymes by proprotein convertases such as furin and furin like proteases. The furin cleavage site

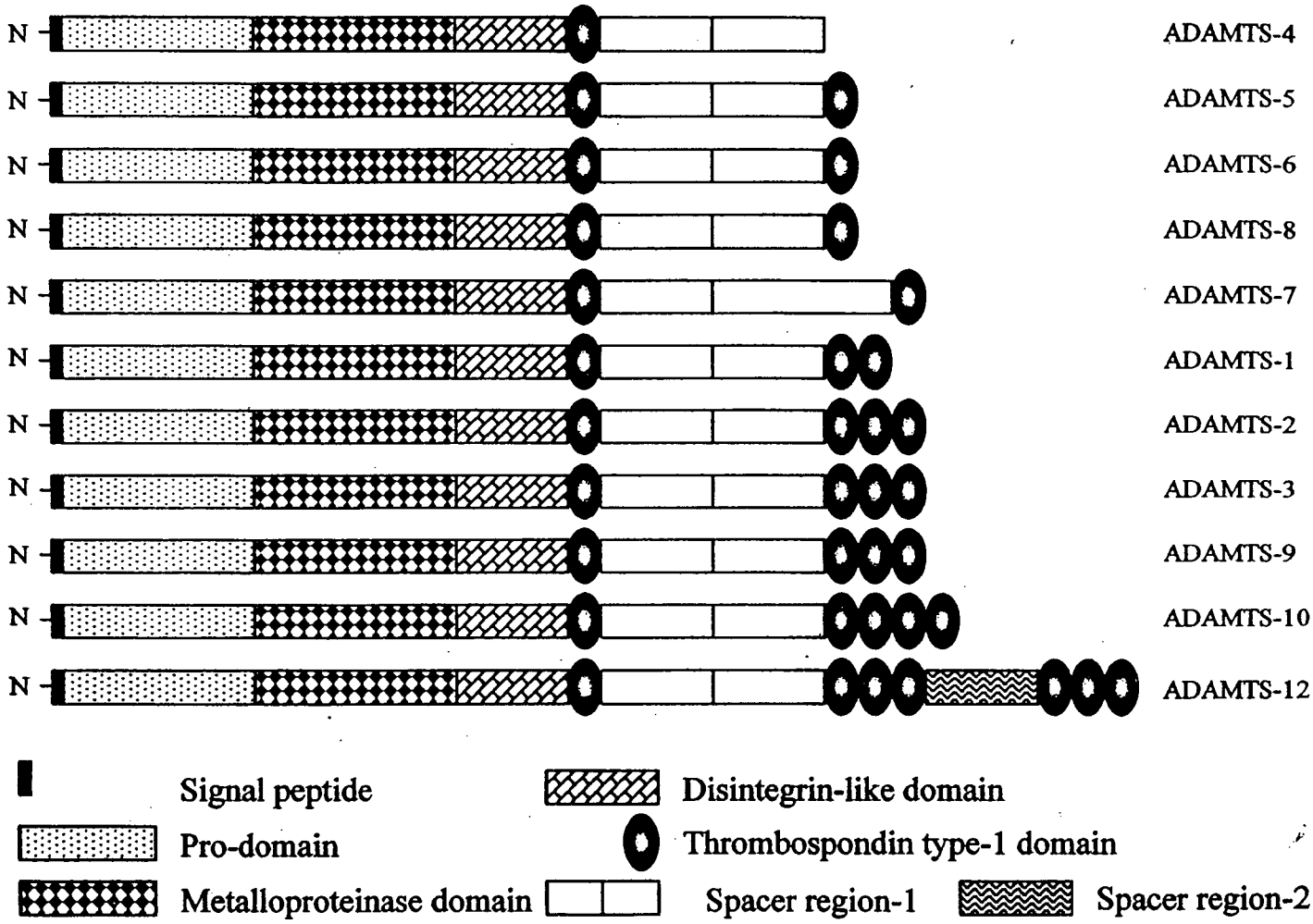

Fig. 1.3: Schematic representation of the basic structure of ADAMTS subtypes

predicts the processing site for generation of the mature enzymes (Cal et al., 2001; Kuno et al., 1997b).

The catalytic domains are conserved amongst the ADAMTS subtypes and contain eight cysteine residues and a typical reprolysin-type zinc binding structure. Five cysteine residues are upstream of the zinc binding sequence, while three residues are downstream, an arrangement that is shared with other ADAMTS members. Like all MMPs and reprolysins, the zinc binding signature (HEXGHXXGXXHD) is followed in all ADAMTS proteins by a methionine residue within a conserved sequence context. This has been designated as being the methionine of the "Met-turn" which is a structural landmark present in all the MMPs and ADAMs. The "Met-turn" is a tight turn arranged as a right handed screw in the adamlysin and MMPs COOH-terminal to the third zinc-binding histidine (Black and White, 1998; DeClerck, 2000; Stocker and Bode, 1995). The presence of a complete zinc protease catalytic site consensus sequence suggests an intact catalytic activity of this domain (Blobel, 1997; Blobel, 2002). Zinc binding motifs generally end up with an Asp residue that distinguishes the ADAMs and ADAMTS from MMPs. However, the second Gly residue of this motif, strikingly conserved in ADAMs, ADAMTS and MMPs is replaced by Asn in ADAMTS-1, -4 and -15. A Thr residue at position 3 of the zinc binding site has been reported to be present in the catalytic domain of ADAMTS-2, -3 and -14 making it a characteristic feature of all the procollagen N-propeptidases (Cal et al., 2002). Moreover, recent molecular modeling experiments based on the crystal structure have confirmed that the overall fold of the catalytic domains of ADAMTS-14, ADAMTS-2 and -3 are very similar topologically (Cal et al., 2002) suggesting that these three ADAMTS could be closely related and that in addition to ADAMTS-2 and -3, ADAMTS-14 could also be an procollagen N-propeptidase.

The disintegrin domain is comprised of about 60-90 residues with 35-45% similarity to snake venom disintegrins, but without canonical cysteine arrangement seen in the latter (Cal et

al., 2001; Cal et al., 2002; Clark et al., 2000; Hurskainen et al., 1999; Kuno et al., 1997b; Tang and Hong, 1999; Vazquez et al., 1999). The disintegrin domain contains eight cysteine residues which is characteristic of this region (except for ADAMTS-6 which has six) (Hurskainen et al., 1999). This domain has been so named in the SVMPs where they have been shown to disrupt integrin functions (Huang, 1998; Paine et al., 1992). Moreover, in ADAMs, this domain has been shown to mediate cell-cell and cell-matrix interactions via integrin molecules (Almeida et al., 1995; Chen et al., 1999b; Chen et al., 1999c; Yuan et al., 1997; Zhang et al., 1998) and also cell fusion events (Myles and Primakoff, 1997; Waters and White, 1997). Expression of this domain of ADAMTS-1 as Glutathione S-transferase (GST) fusion protein had no effect on the platelet aggregation, as compared to hemorrhagic toxin, Ht-e, which share about 21% homology in their disintegrin domain (Kuno et al., 1997b). To date, the biological functions of this domain in distinct ADAMTS subtypes remain to be elucidated.

TSP type 1 motif has been identified as a conserved repeat element between TSP 1 and 2, which are known mutifunctionary secertory glycoproteins to support blood coagulation, to regulate proliferation, adhesion and migration of a number of normal and tumor cells (Agah et al., 2002; Bornstein, 1992; Frazier, 1991) and to some extent also a physiological inhibitor of angiogenesis (Armstrong et al., 2002; Rastinejad et al., 1989). The biological activities of TSPs are attributed to their binding to cell surfaces and many matrix macromolecules including heparan sulfate, proteoglycans, fibronectin, laminin and collagen (Bornstein, 1992; Bornstein, 2001; Frazier, 1991). The TSP-1 motif of thrombospondin 1 and 2 has been thought to be involved in binding matrix molecules. In particular, CS-(A/V)-TCG element within the TSP type 1 motif is an important determinant in binding of cells (Prater et al., 1991; Tuszynski et al., 1992). In addition, the W-(S/G)-X-W motif found in the NH_2-termincal of TSP-1 motif, has shown to promote the adhesion of melanoma cells through heparan sulfate (Guo et al., 1992). This central thrombospondin domain comprises of three Trp residues within a conserved

sequence context of 20 amino acids (Cal et al., 2001). The third Trp residue is replaced by Phe in ADAMTS-2, -3 and -14 providing another reason for a structural link between them (Cal et al., 2001; Fernandes et al., 2001). The third Trp is replaced by Arg in ADAMTS-13 and Tyr in ADAMTS-15. ADAMTS-19 has a unique property of having three residue deletions including the second conserved Trp in the central part of the thrombospondin like repeat (Cal et al., 2001). Since ADAMTS are secreted proteins, they may be incorporated into the ECM through interactions of TSP-1 domains with several components of the ECM.

The TSP-1 domain is followed by a conserved cysteine rich sequence termed the cysteine-rich domain (to distinguish it from the cysteine free spacer domain). It contains ten conserved cysteine residues and each demonstrated a high sequence homology with the cysteine rich domain of other ADAMTS proteins. This domain has been suggested to play a key role in intrachain disulfide bond formation and ADAMTS protein folding (Hurskainen et al., 1999).

The spacer domain is of variable length and with no specific structural features. It shows the least homology of all the domains. The length of this region varies for different ADAMTS subtypes ranging from 127 aa for ADAMTS-6 to 221 aa for ADAMTS-7. However, there is evidence of a second spacer region in ADAMTS-12 (Cal et al., 2001). Besides providing the most variability in sequence and length to the ADAMTS protein, the spacer domain also may also provide the desired flexibility to the ADAMTS protein, in order to achieve desired folded structure in a modular fashion (Hurskainen et al., 1999).

The sequence of the second TS module is more variable between the members of the ADAMTS family than the first TS module, despite the conservation of the number and spacing of cysteine residues. ADAMTS-15 exhibits two TS-like repeats, ADAMTS-14, -16, -17 and -18 contain three repeats, ADAMTS-19 contains four repeats and ADAMTS-13, seven TS-like repeats. The biological significance of these structural variations remains to be elucidated but they could be playing a key role in interaction with the ECM components. Some of the

ADAMTS show in their C-terminal region a copy of the recently described PLAC domain (Nardi et al., 1999). This domain, characterized by the presence of six conserved cysteine residues, can be clearly recognized in ADAMTS-2, -3, -12, -14, -17 and -19 (Cal et al., 2001; Fernandes et al., 2001). The biological significance of this domain is yet to be ascertained.

Collectively, the data suggests that the conserved catalytic domains of this subfamily could mean similar catalytic mechanisms, while the differences in their ancillary domains (i.e. the TSP, disintegrin-like, cysteine-rich and spacer domains) may affect substrate preferences, intermolecular interactions or remodelling of the ECM.

1.7.2: Cell biology of ADAMTS

In general, most of the ADAMTS being detected seem to be highly regulated in their expression (Cal et al., 2001; Cal et al., 2002; Colige et al., 1997; Hurskainen et al., 1999; Kuno et al., 1997b). It has been demonstrated that ADAMTS-1 is dramatically upregulated in carchexigenic carcinoma cells and in mice stimulated with liposaccharide (Kuno et al., 1997a). Liposacchrides increase ADAMTS-1 mRNA levels in renal and cardiac tissues of adult mice (Kuno et al., 1999). Similarly, expression of ADAMTS-5 appears to be highly regulated around the peri-implantation period, as this ADAMTS is totally undetectable before and after the peri-implantation period. TIMPs have been shown to regulate the activity of ADAMTS-4 and -5 (aggrecanases). TIMP-3, but not TIMPs-1, -2 or -4 is a potent inhibitor of ADAMTS-4 and -5, that plays a key role in degradation of articular cartilage aggrecan (Kashiwagi et al., 2001). A recent study has validated the role of TIMP-3, but not of TIMP-1 and -2, in inhibition of ADAMTS-4 (Westling et al., 2002). Whether TIMP-3 or other TIMPs are able to regulate the activities of other ADAMTS has not been ascertained to date, however, due to their diverse roles in inhibiting a repertoire of matrix metalloproteinases, it is quite likely that they might be playing a role in regulation of other ADAMTS as well.

ADAMTS-1 has been shown to be regulated by hormones. Parathyroid hormone (PTH) has shown to upregulate the levels of ADAMTS-1 expression in both metaphyseal and diaphyseal bone within one hour of PTH injection in vivo and in vitro (Miles et al., 2000). Moreover, studies by Robker et al. (2000a), and Espey et al. (2000), indicate that ADAMTS-1 is regulated by P4 in ovarian tissues (Richards, 2002; Robker et al., 2000b). $1\alpha,25\text{-}(OH)_2D_3$ has been shown to upregulate ADAMTS-1 in the osteoblasts (Miles et al., 2000). However, data regarding regulation of other ADAMTS by hormones are lacking at the present time. Upregulation of the expression of ADAMTS-12 by transforming growth factor (TGF)-β (Cal et al., 2001) and aggrecanases (ADAMTS-4 and -5) by IL-1, IL-6, and TNF-α has been recently reported. Moreover, TGF-β1 but not TGF-α, IL-α, IL-1β, αFGF or EGF increased ADAMTS-12 mRNA levels in human fibroblasts (Cal et al., 2001). The finding that TGF-β, widely assumed to be inhibitory for matrix metalloproteinases expression, is able to induce ADAMTS-12 expression is not surprising because recent studies have demonstrated that this growth factor up-regulates expression of potent metalloproteases associated with tumor progression such as MMP-2 and MMP-13 (Overall et al., 1991; Uria et al., 1998).

Members of the ADAM and the ADAMTS family of proteins are proteolytically processed to remove the prodomain. Kuno et al., have shown that this is most likely achieved by endogenous furin endoproteases (Kuno et al., 1999). Further processed forms of ADAMTS-1, -8 -12 and -13 have been identified (Cal et al., 2001; Georgiadis et al., 1999; Kuno et al., 1999). The size of these smaller fragments is consistent with further processing of these proteins, possibly to remove the metalloproteinase domain. During spermatogenesis, the metalloproteinase domain of ADAM1/fertilin α is known to be cleaved, leaving only the carboxy terminal disintegrin and transmembrane domains on the mature sperm (Myles and Primakoff, 1997; Waters and White, 1997). A similar deletion of the metalloprotease domain is seen with

ADAM12/ meltrin α (Yagami-Hiromasa et al., 1995). Hence it is possible that the ADAMTS family of proteins is also processed to forms that do not contain metalloprotease activity. This processing may be developmentally regulated, as seen with ADAM1/fertilin α, leading to different activities for these proteins during development and in the adult. Since most of the members of this family are likely to be processed by furin and are therefore likely to be activated prior to secretion, it is likely that regulation at the transcriptional level may be required to control their activity.

Recent studies have demonstrated that ADAMTS-1, -4 and -12 are able to undergo a second proteolytic cleavage, which results in the formation of a truncated form of these proteins (Cal et al., 2001; Rodriguez-Manzaneque et al., 2000; Tortorella et al., 2000b). These truncated forms possess an amino terminal fragment containing only the metalloproteinase, disintegrin-like and the central TSP-1 domains of the mature proteins and therefore are likely to have different biological functions. The truncated form of ADAMTS-1 binds to the ECM with a lower affinity and is less effective at regulating the proliferation of endothelial cells from the mature form (Rodriguez-Manzaneque et al., 2000). Thus the removal of TSP motifs may serve as a "self check mechanism" or an endogenous mechanism of regulating the catalytic activity of this ADAMTS subtype. Conversely, the carboxy terminal fragments of ADAMTS-4 and -12 have been shown to possess the capability to bind to the ECM, suggesting a role as an competitive inhibitor of the enzymatic action of the mature proteins (Cal et al., 2001; Tortorella et al., 2000b).

A recent study has shown that agents leading to increased aggrecanase activity in cartilage explants, recombinant human interleukin (rhIL)-1α and all-*trans*-retinoate (Ret), do not upregulate the expression of bovine synovial ADAMTS-5 transcirptionally, nor do they lead to a major increase in the amount of secreted ADAMTS-5 or an increase in synovium derived aggrecanase activity (Vankemmelbeke et al., 2001). These results suggest that control of

synovial aggrecanase activity is not exerted at the transcriptional level and points to the potential importance of post transcriptional controlling mechanisms (Vankemmelbeke et al., 2001).

Proteolytic activity of ADAMTS-1 under in vitro conditions is inhibited by papilin (Kramerova et al., 2000). Papilin is an ECM glycoprotein, identified in *Drosophila*, *C elegans*, and mammals and shares a set of domains, called the papilin cassette, with ADAMTS (Kramerova et al., 2000). Papilin regulates the proteolytic activity of ADAMTS subtypes by binding to it's spacer region and/or TSP-1 region, thereby preventing the interactions with ECM. Though, alterations in the expression levels of papilin have a profound effect on the development of the *Drosophila* embryo (Kramerova et al., 2000), the mechanisms of its action or its biological role in the mammalian embryo is yet to be elucidated.

To date, the specific substrate(s) of many of ADAMTS subtypes has not been identified. However, ADAMTS-1 has been shown to interact with heparin (Kuno et al., 1997b) and can also cleave aggrecan, a large chondrotin sulphate and veriscan (Kuno et al., 2000; Sandy et al., 2001) in vitro. Moreover, ADAMTS-4, -5 and in addition, based on the structural similarity of ADAMTS-15 to ADAMTS-1, it has been speculated that these ADAMTS subtypes could also be involved in aggrecan, brevican or versican degradation (Cal et al., 2002; Tortorella et al., 2001). ADAMTS-2 cleaves native triple-helical, but not denatured procollagen-I, and has an affinity for collagen XIV (Colige et al., 1995). Whereas, the bovine procollagen I N-proteinase functions to process procollagen into collagen while ADAMTS-4 and -8 have been shown to degrade aggrecan (Tortorella et al., 2000a; Tortorella et al., 2001; Tortorella et al., 2000b). Procollagen-I and -II have been identified as substrates for ADAMTS-1, -2 and -3 (Li et al., 2001c). However, it has been suggested that a distinct NH_2-terminal processing event, mediated by an enzyme other than ADAMTS-2 occurs in procollagen III (Nusgens et al., 1980). ADAMTS-4 has also shown abilities to degrade versican and the brain specifc ECM protein, brain-enriched hyaluronan binding (BEHAB) (Matthews et al., 2000). Importantly, recombinant aggrecanase-1

and -2 cleaved the aggrecan IGD at Glu373-Ala374 without cleaving the MMP-susceptible Asn341-Phe342 bond (Abbaszade et al., 1999; Tortorella et al., 1999). In addition recent studies have shown that recombinant aggrecanase-1 is also able to cleave the relevant aggrecanase sites (Glu-Xaa) located in the C-terminal of the aggrecan CS-2 domain (Tortorella et al., 2000b). Although the substrates of ADAMTS-10 and -12 have not been identified, the metalloproteinase domains of these ADAMTS subtypes have been shown to be proteolytically active using the α2-macroglobulin complex formation (Cal et al., 2001). TSP type-1 motifs of ADAMTS-1 bind to sulphated GAGs and thus, may serve to influence substrate availability and/or specificity for ADAMTS proteins (Kuno and Matsushima, 1998). Moreover, it has been demonstrated that inhibition of aggrecanase activity by exogenous chondrotin sulphate or heparin, suggesting that these GAGs may compete for the binding of aggrecanases to their aggrecan substrate. Collectively, these findings demonstrate that multiple types of substrates can be targeted by ADAMTS.

ADAMTS subtypes show a large diversity in their expression across various tissues in mouse, adult human and fetal tissues. ADAMTS-1 and -8 have angio-inhibitory activities (Vazquez et al., 1999) and based on the structural similarity of ADAMTS-15 with ADAMTS-1 and -8 it could be speculated that even ADAMTS-15 could be involved in exhibiting angio-inhibitory activities (Cal et al., 2002) ADAMTS-2 and -3 are procollagen N-propeptidases (Colige et al., 1995; Fernandes et al., 2001) and a deficiency of ADAMTS-2 causes Ehlers-Danlos syndrome VIIC in humans and dermatosparaxis in cattle (Colige et al., 1999; Fujimoto et al., 1997; Lapiere and Nusgens, 1993; Reardon et al., 1995). ADAMTS-4 has also been found to be responsible for brevican degradation in glioma cells, which is critical step in the invasive properties of the tumors (Nakamura et al., 2000). It has also been demonstrated that ADAMTS-1 and -4 can catalyze the hydrolysis of versican in the human aorta (Sandy et al., 2001). ADAMTS-4 has also been proposed to play a role in the degradation of the ECM in the brains of

patients diagnosed with Alzheimers disease (Satoh et al., 2000). A recent study has demonstrated the presence of ADAMTS-5/11 in the human and bovine synovium (Vankemmelbeke et al., 2001). ADAMTS-4 and ADAMTS-5 are aggrecanases involved in cartilage aggrecan degradation in arthiritic diseases (Abbaszade et al., 1999; Bayliss et al., 2001; Tortorella et al., 1999; Vankemmelbeke et al., 2001). ADAMTS-12 has been shown to be highly expressed in the colorectal, renal and pancreatic carcinomas (Cal et al., 2001). ADAMTS-6, -7 (Hurskainen et al., 1999), -9 (Clark et al., 2000) and -16 to -19 (Cal et al., 2002) have been characterized at the structural level and their putative role(s) remain unknown. Collectively, these findings suggest that these subtypes could be involved in pathogenesis of certain diseases besides being involved in normal physiological functions in the body. Moreover, since members of ADAMTS family possess the TSP domain containing TSP-1 motifs, it can be presumed that most if not all ADAMTS could be mediating cell adhesion, and also playing a role in cell proliferation and migration. Some additional clues to the putative roles of these ADAMTS can be concluded from the analysis of their expression patterns in tissues.

In general most of the ADAMTS genes show low levels of expression; however using northern blot analysis and sensitive RT-PCR procedures a diverse range of subtypes has been detected across a variety of tissues both in adults and fetuses. ADAMTS-1 is expressed during mouse development and the transcripts are detected in E-10 to E-18 yolk sac, placenta, brain, heart, lung, limb bud, liver, spleen and kidney (Thai and Iruela-Arispe, 2002). In humans, expression for ADAMTS-1 was highest in the arthritic tissues, bladder, aorta, cervix and uterus (Abbaszade et al., 1999). ADAMTS-2 and -3 expression can also be detected during mouse embryogenesis their expression was noted in mouse at day 7, 15, 17 but not on day 11 (Fernandes et al., 2001). However, in adult human tissues, ADAMTS-2 transcripts can be identified in the human placenta, lung and liver (Fernandes et al., 2001; Hurskainen et al., 1999), while the highest expression of ADAMTS-3 was noted in placenta with lower levels of

expression observed in the lung, brain, and heart (Fernandes et al., 2001). Using sensitive RT-PCR techniques, ADAMTS-4 expression has been detected in arthritic tissues, ovaries, spinal chord, uterus, bladder, brain, testis, lung and heart (Abbaszade et al., 1999). Expression for ADAMTS-5/11 was highest in placenta with weaker expression noted in heart and brain (Abbaszade et al., 1999). In addition, ADAMTS-5/11 were seen in a variety of normal tissues including cervix, uterus, bladder, esophagus and placenta (Abbaszade et al., 1999). ADAMTS-5 has been shown to be expressed around the peri-implantation period in the 7 d old mouse embryo and thus has been given the name "implantin", suggesting a role in the process of implantation (Hurskainen et al., 1999). However, this role has not been substantiated to date. ADAMTS-6 mRNA are expressed at low levels in placental tissue, but is barely detectable in other adult human tissues. In contrast, ADAMTS-7 mRNA is found in a number of adult tissues (Hurskainen et al., 1999). However, while ADAMTS-6 expression could not be detected during mouse embryo development, ADAMTS-7 is expressed at low levels throughout the embryonic stages (Hurskainen et al., 1999). Similarly, low levels of ADAMTS-8 expression are detected during mouse development. There is selective expression of ADAMTS-8 in adult lung and heart (Georgiadis et al., 1999). ADAMTS-9 shows as much homology to *C. elegans* GON-1 as to other ADAMTS proteins (20-40%), suggesting that ADAMTS-9 might be the human homologue of GON-1 (Clark et al., 2000). The expression of ADAMTS-9 has also been detected in the ovary, pancreas, heart, kidney, lung, placenta, and strikingly in all fetal tissues. ADAMTS-9 expression has also been detected in the spinal cord and brain but not in colon, leukocytes, prostrate, small intestine, testis, liver, skeletal muscle, spleen or thymus (Clark et al., 2000). Such diverse expression, of ADAMTS-9 suggests a global role of ADAMTS-9 in development. ADAMTS-12 has been predominantly expressed in fetal lungs suggesting that this enzyme could participate in some of the tissue remodeling processes taking place in this tissue during development. ADAMTS-13 to -19, interestingly show a high expression in fetal tissues,

suggesting a role in cell adhesive events or tissue remodelling process during organ development. In contrast, ADAMTS-15 and -19 are almost undetectable in adult tissues, suggesting a specific and significant role only during embryogenesis (Cal et al., 2002). Weak expression of ADAMTS-14 has been reported from adult brain, lung, retina, skin fibroblasts, liver, stomach, and placenta (Bolz et al., 2001; Colige et al., 2002). The highest levels of ADAMTS-14 have been found in collagen rich tissue, supporting its role as an aminoprocollagen peptidase. However, significant levels were also detected in various other tissues, indicating that ADAMTS-14 may have many other novel functions.

In *C. elegans*, GON-1, a member of the ADAMTS family, has been shown to be essential for gonadogenesis (Blelloch and Kimble, 1999). Although, the substrate for GON-1 has not been identified, it is speculated that GON-1 participates in gonad formation via remodelling of the basement membrane. Similarly, recent work has identified new *C. elegans* ADAMTS family gene, adt-1, playing an important regulatory role in ray morphogenesis which is their copulatory organ (Kuno et al., 2002). Mutations of the adt-1 gene results in the morphological changes in the rays as well as the appearance of abnormal protuberances around the rays. Collectively, these findings suggest that ADAMTS family members play an important role in the organogenesis of *C. elegans*.

More insight into the biological roles of ADAMTS came from the recent targeted disruption of the gene in mouse for ADAMTS-1 (Shindo et al., 2000) and ADAMTS-2 (Li et al., 2001c). Both phenotypes have demonstrated a key role of these ADAMTS in embryonic development and tissue morphogenesis. ADAMTS-1 knockout mice exhibit significant growth retardation and aberrant development of urogenital tract, kidneys and the adrenal glands. A particular distinctive feature of the ADAMTS-1 gene knockout mouse is the impairment of fertility. This is accompanied by obvious abnormalities of the uterus and ovaries. The endometrium shows large cysts that have been a factor contributing towards the reduced fertility

in these mice. Since the ADAMTS-1 gene has been shown to be regulated by P4 during ovulation (Robker et al., 2000b), it is not at all surprising that ADAMTS-1 has a function in shaping the female genital organs. In contrast, mice null mutants for ADAMTS-2 do not exhibit any phenotypic abnormality, being grossly normal at birth. However, as the mice mature, their skin becomes very thin and soft and begins to tear with slight trauma or handling. Structurally, these mice show many similarities to animals with dermatosparaxis and patients with Ehlers-Danlos syndrome type VIIC (Colige et al., 1997; Colige et al., 1999; Fujimoto et al., 1997; Giunta et al., 1999; Lapiere and Nusgens, 1993; Nusgens et al., 1992; Reardon et al., 1995). Moreover, sterility and decreased spermatogenesis was observed in the homozygous male mice suggesting a role of this ADAMTS subtype in male gametogenesis. Similarly, mutations for ADAMTS-2 causes a recessively inherited connective tissue disorder dermatosparaxis in animals and the Ehlers-Danlos syndrome (EDS)-VIIC in humans (Colige et al., 1999; Fujimoto et al., 1997; Lapiere and Nusgens, 1993; Reardon et al., 1995). Clinically, these disorders present cases with severe fragility of skin and are characterized by irregular, thin, branded collagen fibrils in the dermis, which appears "hierographic" in cross section and contain collagen I with an intact N-propeptide, termed pN-collagen I (Li et al., 2001c; Nusgens et al., 1992). Collectively, the data suggest an imperative role for ADAMTS-1 and -2 during development.

Based on their structural features, some speculation about the putative roles of ADAMTS has been attempted. For example, based on the high structural resemblance with ADAMTS-2 (procollagen I N-propeptidases) (Colige et al., 1995), and ADAMTS-3 (procollagen II N-propeptidase) (Fernandes et al., 2001), ADAMTS-14 is likely the third member of the procollagen N-propetidase subfamily of ADAMTS (Bolz et al., 2001). The finding that three procollagen N-propeptidases provide an interesting parallelism between ADAMTS subfamily and the MMPs, which is also comprised of three subtypes involved in cleaving fibrillar collagen, namely MMP-1, -8 and -13 (Freije et al., 1994). Interestingly, MMP-1 degrades preferentially

type III collagen, MMP-8 type I collagen and MMP-13 type II collagen (Knauper et al., 1996). This parallelism has lead to the speculation that ADAMTS-14 could be the procollagen III N-propeptidase (Cal et al., 2002). However this hypothesis needs to be verified, but gives an indication of two different protease systems being operative in remodelling of fibrillar collagen. ADAMTS-13 has been identified as a von Willebrand factor-cleaving protease and any mutation in ADAMTS-13 gene causes thrombotic thrombocytopenia purpura, a life threatening disease characterized by intravascular destruction of erythrocytes and blood platelets (Levy et al., 2001; Zheng et al., 2001; Zheng et al., 2002).

Taken together, multiple ADAMTS could be involved in a variety of processes involving cell migration, changes in cell adhesion and remodelling of connective tissues. Based on the imperative role they play during development, it is likely that they might be playing a role during preimplantation embryogenesis as well. However, to date, expression of ADAMTS in preimplantation embryogenesis has not been determined. Therefore, in view of our current understanding of the adhesive mechanisms involved in the ECM degradation and cell-ECM interactions, we have chosen to focus our studies on the family of ADAMTS.

1.8: Cell-matrix interactions during folliculogenesis, ovulation and luteinization

The endocrine units within the ovary-follicles and corpora lutea-continually develop and regress, thus giving rise to day to day variation in hormones secreted by the ovary (Rodgers and Irving Rodgers, 2002). Tissue remodeling, cellular replication and specialization contribute to the formation of follicles and corpora lutea. While folliculogenesis is characterized by a dramatic increase in the follicular surface area within the confines of the ovarian stroma (Smith et al., 1999), as each primordial follicle develops into preovulatory follicles, ovulation is characterized by local ECM degradation at the apex of the preovulatory follicular wall. Immediately, following ovulation, the process of luteinization is characterized by the reorganization of the remaining

follicular cells into a highly vascularized CL that undergoes twenty-fold increase in tissue mass within a few days. Moreover, in the absence of maternal recognition of pregnancy, the CL undergoes regression and the ovarian cycle resumes (Smith et al., 2002). Components of ovarian ECM can be altered through the action of MMPs and TIMPs (Smith et al., 2002). In addition, a role for serine proteinases (tPA and uPA) and plasmin in remodeling ovarian ECM has also been reported (Beers et al., 1975; Canipari et al., 1987; Reich et al., 1985; Reich et al., 1986).

1.8.1: Bovine ovarian follicular dynamics

The bovine estrus cycle is characterized by a series of follicular waves (Evans et al., 1997; Fortune et al., 2000; Fortune et al., 1991; Ginther et al., 1996; Mihm et al., 2002; Perry et al., 2002; Rajamahendran et al., 1998). Cohorts of gonadotropin-responsive antral follicles are stimulated to initiate increased growth during each follicular wave. A dominant follicle (DF), defined as a follicle, which achieves a size of about $\geq$10 mm in diameter after being recruited and selected, emerges from this group of follicles and as it continues to grow, secretes increased amounts of 17β-estradiol (E2), inhibin and several other factors (Adams et al., 1993; Bigelow and Fortune, 1998; Evans et al., 1997; Ginther et al., 2002; Ginther et al., 1997; Ginther et al., 1996; Mihm et al., 2000; Mihm et al., 2002; Mihm et al., 1997; Nicholas et al., 2002). These secretions from the DF cause the remaining subordinate follicles to become atretic and regress (Adams et al., 1993; Ginther et al., 2000; Ginther et al., 1996; Mihm and Austin, 2002). The dominant follicle remains functional for several days while the CL is present but then undergoes atresia. This loss of functionality of the DF results in a transient rise in plasma FSH, which stimulates the emergence of a new follicular wave (Adams et al., 1992; Gibbons et al., 1997; Sheldon et al., 2002). This pattern of follicular waves, dominance and regression is repeated until a DF is present, coincident with regression of CL, which marks the beginning of the follicular phase of the estrus cycle and continued growth of that DF to a preovulatory stage (Lucy et al.,

1992; Savio et al., 1988; Savio et al., 1993; Sirois and Fortune, 1988; Taylor and Rajamahendran, 1991b). It has been demonstrated that the period of corpus luteum determines the number of follicular waves that occur during each estrus cycle (Fortune et al., 2001; Roche et al., 1999; Taylor and Rajamahendran, 1991a; Taylor and Rajamahendran, 1991b). From two to four follicular waves can occur during an estrus cycle with about 75% of the cycles comprising three waves and it is generally the second or the third wave follicle that ovulates.

1.8.2: Extracellular matrix remodeling during folliculogenesis and ovulation

In the bovine ovary, the surface area of each preovulatory follicle increases by about 19 times during follicular development, implying that continual remodeling of the follicular components occurs (van Wezel and Rodgers, 1996). Composition of the follicular basal lamina has been shown to change during folliculogenesis in both murine (Frojdman et al., 1998) and bovine (Rodgers et al., 1998; van Wezel et al., 1998). While the laminin $\alpha 1$ and $\beta 1$ chains are present during all stages of follicle development, presence of laminin $\alpha 2$ and $\beta 2$ chains are detected only in the antral follicles. In addition presence of $\gamma 1$ chains has been detected in the antral follicle. However, it has not been ascertained if all three chains are able to link together to form the stable structure of laminin 3. In contrast, the levels of type IV collagens $\alpha 1$-$\alpha 6$ are all detected in the primordial follicle but the levels are decreased at the antral follicular stage (Rodgers et al., 1998). Perlecan has been detected both in the follicular fluid (Eriksen et al., 1999) as well as in the basal lamina (McArthur et al., 2000). However, the roles of these changes in the basal lamina compostion have yet to be ascertained.

A number of studies have shown that levels for different MMPs are upregulated by exogenous administration of equine chorionic gonadotropin (eCG). In particular, expression of MMP-2 and MMP-9 has been shown to be upregulated in rat preovulatory follicles after the adminstration of eCG (Bagavandoss, 1998). Similarly, expression of MMP-2, -9, -13 (Cooke et

al., 1999) and TIMP-1 (Kennedy et al., 1996) is upregulated in rat ovaries in response to eCG administration. The biological signifance of this increase in MMP and TIMP expression is not known as yet.

Ovulation is a unique process by which the mature DF responds to the surge of leutinizing hormome (LH) and ruptures to release the oocyte. During ovulation, functional changes occur in several cells of the follicle including granulosa, theca, stroma (fibroblasts and endothelial) cells, as well as on the ovarian surface epithelium (Espey, 1994). The remodeling process of ovulation also requires structural changes within the ECM where regulated changes in the proteinases occurs. At the time of ovulation, a precise area of the ovarian surface adjacent to the apical region of the ovulatory follicle disintegrates to allow release of the oocyte (Espey, 1994). The follicular wall at the apex is composed a single layer of surface epithelium, two collagenous layes (theca externa and tunica albugenia), the vascular theca interna containing differentiated fibrocytes active in steroidogenesis and granulosa cells separated from the theca interna by the basement membrane. Therefore, three layers of ECM must be compromised for the follicle to rupture and accordingly, decrease in the collagenous matrix has been seen ultrastructurally to occur at the apical region of the dominant follicle prior to ovulation (Bjersing and Cajander, 1974a; Bjersing and Cajander, 1974b).

The oocyte is surrounded by a specialized group of cells called the cumulus cells. These cells are directly in contact with the oocyte and comprise a specialized functional unit during follicular growth. In response to the preovulatory LH surge, the cumulus cells begin to synthesize special ECM proteins that are essential for the process of cumulus expansion a step important for ovulation (Eppig, 1979; Salustri et al., 1999). Hyaluronic acid (HA) and two HA binding proteins namely, tumor-necrosis factor stimulated gene (TSG)-6 and the serum derived inter-α-inhibitor (IαI), also known as inter-α-trypsin inhibitor (ITI) or serum-derived hyaluronic acid binding protein (SHAP) are synthesized (Eppig, 1979; Fulop et al., 1997; Hess et al., 1999;

Salustri et al., 1999; Sato et al., 2001; Yoshioka et al., 2000; Zhuo et al., 2001). The cumulus derived matrix may also contain other factors such as the proteoglycans, brevican and versican (McArthur et al., 2000). The LH surge also initiates cumulus expansion by inducing the expression of specific genes. Cyclooxygenase-2 (COX-2), the rate limiting enzyme in the synthesis of prostaglandins such as prostaglandin E_2 (PGE_2) (Joyce et al., 2001; Sirois and Richards, 1993); HA synthatase-2 (HAS-2) which catalyzes the production of HA (Weigel et al., 1997) and TSG-6 (Fulop et al., 1997; Lee et al., 1993; Lee et al., 1992; Yoshioka et al., 2000). Though, expression of these genes has been shown to be important for ovulation, the mechanisms by which LH induces the expression of the COX-2, HAS-2 and TSG-6 genes remain to be clearly defined. Moreover, both TSG-6 and HA are expressed several hours prior to any visible physical expansion of the matrix which can be assessed by the dispersion of the cumulus cells away from the oocyte. This suggests that the presence of these molecules is not sufficient for the matrix changes during follicular development and other molecules could be mediating the matrix remodeling.

The preovulatory LH surge is also known to stimulate the production of serine proteinases (tPA and uPA), plasmin (Beers et al., 1975; Canipari et al., 1987; Reich et al., 1985; Reich et al., 1986) and MMPs in preovulatory follicles. Ovarian targets for plasmin include fibrin, fibrinogen, type III and IV collagen, fibronectin, laminin and proteoglycans (Barnathan et al., 1990; Mignatti et al., 1986; Werb et al., 1992). Plasmin has been shown to decrease the tensile strength of the bovine follicular wall (Beers et al., 1975). Antibodies, specific for uPA (Colgin and Murdoch, 1997) or tPA (Reich et al., 1985; Tsafriri et al., 1989) reduce the ovulation rate in sheep and rats respectively. Before ovulation, tPA is the primary plasminogen activator induced in the pig preovulatory follicles (Smokovitis et al., 1988). In contrast, uPA is the predominant PA induced in mouse and sheep preovulatory follicles before ovulation (Colgin and Murdoch, 1997; Hagglund et al., 1996). Thus regulation of tPA and uPA is species specific. In a

recent study, tPA was localized in the granulosa cells of the follicle while uPA was detected in the bovine theca, granulosa and stromal cells (Dow et al., 2002). mRNA expression and enzyme activity of both tPA and uPA are increased in a spatio-temporal manner in bovine preovulatroy follicles after exposure to a gonadotropin surge (Dow et al., 2002) suggesting that increased PA and plasmin activity may be a contributing factor in the mechanisms of follicular rupture and formation of CL.

In addition to playing a direct role on ECM degradation, plasmin can also activate the proenzyme form of several MMPs implicated in follicular rupture including MMP-1, -2, -3 and -9 (DeClerck and Laug, 1996; Lijnen et al., 1998; Liu et al., 1998; Makowski and Ramsby, 1998; Mazzieri et al., 1997; Murphy et al., 1999). These metalloproteinases have been suggested to promote a cascade of proteolytic interactions and enzymatic digestion in the localized area near the follicular apex and presumed site of ovulation by degradation of the collagen matrix (Espey, 1994) that leads to the release of follicular fluid and subsequent ovulation. The importance of these proteases in the process of ovulation can be gauged from the fact that administration of synthetic or pharmacological MMP inhibitors blocks ovulation (Brannstrom et al., 1988; Butler et al., 1991; Ichikawa et al., 1983; Tsafriri et al., 1989). Furthermore, a loss or downregulation of localized matrix degradation in the preovulatory follicle leads to unruptured luteinized follicle (Katz, 1988). MMP-2 is expressed in the theca cells of preantral, preovulatory and ovulating follicles (Liu et al., 1998) and could play a role in degradation of the basement membrane that separates the granulosa cells from the theca cells. In addition, they may also be able to hydrolyze the denatured collagen fibrils in the follicle wall after the initial cleavage by the collagenase (Bakke et al., 2002). MMP-2 expression been reported in the follicular fluid of preovulatory follicles suggesting that even the constituent factors in the follicular fluid could be aiding in the degradation of the matrix leading to ovulation (Espey, 1967). In a recent study, effects of preovulatory gonadotropin surge on MMP-2, MMP-14 and TIMP-2 expression has been

determined in the bovine periovulatory follicular and early CL tissue (Bakke et al., 2002). Although expression of MMP-2 was localized in the thecal cells, MMP-14 expression was localized in the thecal as well as adjacent stromal tissues. Interestingly, the expression of MMP-14 was detected in the granulosa cells, after the preovulatory gonadotropin surge. In contrast, TIMP-2 expression was detected predominantly in the granulosa cells with maximum expression being detected in the antral portion of the granulosa layer in response to the gonadotropin surge. These data collectively suggest that while MMP-2 could be playing a role in breakdown of the basement membrane which separates the granulosa and thecal cells, while MMP-14 and TIMP-2 may be involved in the follicular rupture and formation of the CL. Northern analysis revealed statistically significant cyclic expression patterns for the mRNAs coding for type III, IV and VI collagens as well as for the small proteoglycan, biglycan, and for syndecan-1 and osteonectin. The cyclic changes observed in the levels of mRNAs for these structural components exceeded those for matrix metalloproteinases (MMP)-2, -9 and -13, and for tissue inhibitors of matrix metalloproteinases (TIMP)-1, -2 and -3, where the changes were not statistically significant, despite their apparent role in ECM remodelling in the ovary (Oksjoki et al., 1999). Inspite of the complex expression patterns of MMPs and their inhibitors, mice null for many of these genes remain fertile (Curry and Osteen, 2001).

Regulation of extensive proteolytic acitivity in the perifollucular matrix is thought to be brought about by LH upregulation of TIMPs (Curry et al., 1988; Ichikawa et al., 1983; Mann et al., 1991; Moor and Crosby, 1987; Zhu and Woessner, 1991), PAIs (Reinthaller et al., 1990) and α_2-macroglobulin (Curry et al., 1990). TIMP-1 expression has been detected in both the human granulosa cells as well as in the follicular fluid (Reinthaller et al., 1990). In contrast, the TIMP-2 expression is very species specific as it is constitutive in rat (Curry and Nothnick, 2000) and mouse (Hagglund et al., 1999; Inderdeo et al., 1996) ovaries and sheep preovulatry follicles (Smith et al., 1995) after the LH surge, but is increased in macaque granulosa cells (Chaffin and

Stouffer, 1999). TIMP-2, though is known to bind to MMPs in a 1:1 stoichometery and inhibit their activation, has been suggested to play a role in pro-MMP-2 activation and localization of MMP-2 activity at the cell surface (Bakke et al., 2002). TIMP-1 deficient mice do not demonstrate any changes in ovarian morphology, oocyte numbers or ovulation when compared to wild type mice (Nothnick et al., 1997). Therefore, the primary function of TIMP-1 with abundant expression in the ovary, remains undetermined. Though, TIMP-1 stimulates progesterone and estrogen (Boujrad et al., 1995) production by granulosa cells in vitro, TIMP-1 deficient mice exhibit no differences in the circulating levels of these hormones (Nothnick et al., 1997). This suggests, that TIMP-1 might not be a steroidogenic inducer in vivo, but it may function as a corregulator of steroidogenesis in the ovary. Further studies are needed to ascertain its role in steroidal regulation. A recent study has demonstrated the expression of TIMP-4 in the ovary and showed cyclic expression during murine estrus cycle (Rahkonen et al., 2002). Thought the diverse roles of these TIMPs have not been ascertained, it has been recently suggested that MMPs and TIMPs might be acting as protective shields to ensure that toxic levels of compounds do not reach the granulosa cells or the oocyte at inappropriate times during folliculogenesis (Richards et al., 2002).

1.8.3: Extracellular matrix remodeling during corpus luteum formation, maintenance and regression

Luteinization is the process by which the postovulatory follicle differentiates to become CL. It has been suggested to be a highly complex remodeling event as it involves differentiation and integration of cells derived from different cell types of the ovary (Murphy, 2000). Based on all these cellular, morphological and biochemical events, major ovarian functions including oocyte and hormone production have been integrally linked to extensive tissue and ECM remodeling wherein proteases are thought to play a key role (Espey, 1980; Fata et al., 2000;

Tsafriri, 1995). Although, the preovulatory gonadotropin surge leads to ovulation and precedes luteinization (Filicori, 1999; Tsafriri and Reich, 1999), ovulation is not a prerequisite for luteinization as numerous instances of luteinization in unruptured follicles from different species have been reported (D'Hooghe et al., 1996; Douglas et al., 1994; Hall et al., 1993; Mattheij and Swarts, 1995; Westfahl, 1993; Zaidi et al., 1995).

During luteinization in bovine, theca cells develop further as small luteal cells while the granulosa cells become large luteal cells (Meidan et al., 1990). However studies have demonstrated that in bovine some of the small luteal cells have the potential to differentiate into large luteal cells (Lei et al., 1991). Though, these large and small luteal cells make up the two steroidogenic cell types, they differ morphologically, endocrinologically and biochemically (Fitz, et al., 1982). However, the factors governing the differentiation of these two cell types remain to be elucidated.

Bovine granulosa cells grown in anchorage-independent conditions do not spontaneously acquire luteal morphology, suggesting that attachment is a prerequisite for luteinization to occur (Lavranos et al., 1994). Though serum has been proposed as a provider of important ECM constituents under in vitro conditions, granulosa cells being cultured in vitro undergo attachment to the culture wells because of endogenous production of ECM components specially fibronectin (Morley et al., 1987; Skinner et al., 1985). Integrin expression has been demonstrated to be playing an important role in luteinization. For example, antibody to the integrin $\beta 1$ subunit inhibited differentiation of granulosa cells. Similarly, integrin $\alpha 2$ is expressed in early CL and its ligand, collagen type IV, is expressed in granulosa cells before ovulation and persists even in the newly formed CL (Yamada et al., 1999). Moreover, integrin $\alpha 5$ and $\alpha 6\beta 1$ are expressed in the early human CL (Honda et al., 1997; Yamada et al., 1999). Luteinizing human granulosal cells have been shown to surround themselves with a basement membrane (Richardson et al., 2000) and Matrigel promotes luteinization of granulosa cells (Hwang et al., 2000). Collectively, these

findings suggest that differentiation of granulosal cells into large luteal cells is likely to require a sequential exposure to different ECM components.

It has been suggested that CL regression also involves extensive remodeling (Paavola, 1979). MMP-2 activity has been found associated with CL regression (Curry et al., 1992; Endo et al., 1993). In a recent study, the role of MMPs in collectively degrading ECM and regulating luteal function was determined by studying the expression of MMP-2, -9 and -14 in the ovine CL from day 2 to day 15 of the estrus cycle (Ricke et al., 2002a). While expression of MMP-2 and-9 proteins were localized predominantly to large luteal cells, expression of MMP-14 was localized primarily to cells other than large luteal cells. These results support the hypothesis that ECM remodeling occurs throughout the luteal phase and may therefore help in tissue remodeling during CL formation, maintenance and regression.

TIMP-1, -2 and -3 expression has been assessed during CL formation, maintenance and regression in sheep (Endo et al., 1993). While TIMP-1 is the predominant inhibitor during CL formation, TIMP-3 is predominant during CL maintenance and regression (Endo et al., 1993). Although expression of TIMP-2 was detected during all the three stages of CL development, it's levels were lower than that of other two TIMPs, indicating a less critical role (Smith et al., 1994). A recent study using in situ hybridization has demonstrated the strongest expression of TIMP-4 mRNA in the corpus luteum during luteal phase (Rahkonen et al., 2002). The data suggest that TIMP-4 plays a role in maintenance of the CL in the ovary, most likely by maintenance of the delicate balance between MMPs and TIMPs.

Recently, role of $PGF_{2\alpha}$ in ECM remodeling during luteal regression was determined by studying its effect on the expression of TIMP-1 and TIMP-2 and MMP-2 and MMP-9 in sheep (Towle et al., 2002). There was a rapid decrease in TIMP-1 and -2 levels and an increase in MMP-2 activity, which correlated to the low levels of P4 after luteal regression, while the MMP-9 was found to be expressed in its latent form at this stage. In another study, the effect of $PGF_{2\alpha}$

on the expression of mRNA encoding MMP-1, -2, -3, -7, -9, -13 and -14 during $PGF_{2\alpha}$-induced luteolysis in sheep was investigated (Ricke et al., 2002b). Expression of mRNA for these MMPs increased significantly within 30 min to 6 h after $PGF_{2\alpha}$ administration suggesting that MMP mRNA expression and activity were significantly increased during prostaglandin induced luteolysis. Increased MMP activity may promote ECM degradation during luteolysis. However, contrasting results have been reported for the expression of TIMP-1, where ovarian TIMP-1 mRNA upregulation is detected only at mid-pregnancy suggesting that TIMP-1 could be playing a role in maintenance as opposed to CL formation (Woessner et al., 1989). However, TIMP-1 expression is unregulated during regression of the CL in mice and after $PGF_{2\alpha}$-induced luteolysis in the cow (Juengel et al., 1994). During the estrus cycle in cows and sheep, the highest levels of TIMP-1 are detected during the midluteal phase (Goldberg et al., 1996; McIntush et al., 1997). However, in primates a decrease in TIMP-1 expression is observed after $PGF_{2\alpha}$-induced luteolysis, while humans show no changes in TIMP-1 expression during the life span of the CL (Duncan et al., 1996). The reasons for such discrepancy between species have not been determined. Studies have also localized TIMP-1 expression in the large luteal cells of the CL (Smith et al., 1994) in both pregnant and non-pregnant CL (Freudenstein et al., 1990; Inderdeo et al., 1996; Tanaka et al., 1992). Since, TIMP-1 deficient mice do no exhibit any altered signs of CL functionality or overt morphological differences (Nothnick et al., 1997), the functional significance of TIMPs during CL formation and regression still remains unclear. Since, ECM remodeling has been considered important for CL formation, maintenance and regression, it is tempting to speculate that other proteinases could be playing a significant role in the remodeling process of this dynamic tissue.

1.8.4: Role of ADAMTS in ovulation and luteinization

In the ovary, LH has been shown to induce progesterone receptor (PR) in mural granulosa cells of the preovulatory follicles (Natraj and Richards, 1993; Park and Mayo, 1991; Robker et al., 2000a), however, the mechanisms by which LH is able to induce progesterone receptor (PR) have not yet ascertained. Moreover, mice null for PR fail to ovulate even when stimulated by exogenous hormones (Lydon et al., 1995; Robker et al., 2000a), unlike estrogen receptor knock out for α isoforms (ERαKO) mouse, where impaired ovulation is restored by controlling pituitary secretion of LH (Couse et al., 1999; Couse and Korach, 1999). These findings suggest that progesterone is a key player in the ovulatory process (Lydon et al., 1995; Robker et al., 2000a). Mice null for PRA but not PRB exhibit impaired ovulation, indicating a sub type specificity of PR action in the ovulation process (Conneely et al., 2002; Mulac-Jericevic et al., 2000). Despite failure of ovulation to occur in PRKO/PRAKO mice, the expression of COX-2, cumulus expansion and luteinization proceeds normally (Robker et al., 2000a) suggesting that the follicular/cumulus mechanisms are operational and processes mediating granulosa cell differentiation in response to LH are intact. Studies have identified two distinct type of proteases which are likely candidates of the PR action. These are ADAMTS-1 (Espey et al., 2000; Vazquez et al., 1999) and cathepsin L (Robker et al., 2000a) and expression of both ADAMTS-1 and cathepsin L mRNA is markedly reduced in granulosa cells of PR null mice. Moreover, the expression of ADAMTS-1 is markedly reduced in rats when the preovulatory synthesis of progesterone is inhibited by epostane (Espey et al., 2000) or in mice that are null mutants for PR (Robker et al., 2000a). Whether PR acts directly or indirectly to control the expression of these two distinct proteases remains to be determined. Expression and function of other members of ADAMTS gene family in the ECM remodeling that occurs during folliculogenesis and luteinization has not been characterized. It is quite likely that several of the known ADAMTS are being expressed and playing a role in ovarian follicular and luteinization dynamics.

1.9: Steroid Hormone Receptors

Steroid hormone receptors belong to the nuclear receptor gene family, a large group currently totaling approximately 150 different proteins, which function as transcription factors in many different species including both invertebrates and vertebrates (Escriva et al., 2000; Laudet et al., 1992). Nuclear receptors are ligand-activated transcription factors, which play critical roles in modulating expression of target genes involved in reproduction, development and metabolism (Evans, 1988; Mangelsdorf et al., 1995). The receptors have been classified into four distinct families namely type I, II, III and IV. Type I receptors, also referred to as steroid receptors, include the estrogen receptor (ER), the progesterone receptor (PR), the glucocorticoid receptor, the mineralocorticoid receptor, and the androgen receptor. Type II receptors; include the thyroid hormone receptor, the retinoic acid receptor, the retinoid X receptor, and the vitamin D receptor. Finally, the type III and type IV receptors encompass a group of nuclear proteins known as orphan receptors, the ligands for which have yet to be identified (Mangelsdorf et al., 1995; Parker, 1993). All the nuclear receptors are highly related in both primary amino acid sequence and the organization of functional domains suggests that many aspects of their mechanisms of action are conserved (Laudet et al., 1992).

1.9.1: Estrogen Receptors

Estrogen action within a cell is mediated via specific receptors, the first of which was cloned in the human breast cancer cell line in 1986 (Green et al., 1986a). In the classical concept, the ER is rapidly transferred to the nucleus (King and Greene, 1984), where it is stored in an inactive but primed state until a proper stimulus is received. The priming step is accomplished by association of the ER with a variety of heat shock proteins including hsp 90 and hsp 56 (Jensen and De Sombre, 1969).This complex of ER and heat shock proteins rapidly dissociates after

binding to hormone (Kumar et al., 1987). After the activation, protein-protein interactions between the ER have been reported to be required for high affinity DNA binding (Wang and Miksicek, 1991). The receptor interacts via specific DNA binding domains with ER binding sites, the estrogen receptor responsive elements (EREs) in the promoter region of sensitive genes. This binding leads to an initiation of transcription, thereby initiating the hormonal effect (Gronemeyer, 1992).

However, new findings have made it mandatory to modify the understanding of the mechanism of steroidal actions as a second ER was cloned, from murine (Kuiper et al., 1996) and humans tissues (Mosselman et al., 1996) and named estrogen receptor beta (ERβ) to distinguish it from the previously identified ER which has been re-named estrogen receptor alpha (ERα) (Kuiper et al., 1997). These receptors are products of two genes located on different chromosomes (Enmark et al., 1997), but both proteins are encoded by eight exons. Splice variants of each of these receptors have been observed, allowing them to express various isoforms of ERα and β in different tissues (Denger et al., 2001; Flouriot et al., 1998; Ogawa et al., 1998; Vladusic et al., 1998). In the cell, both receptors are localized in the nucleus. Interestingly, a recent report suggests a small (3%) fraction of the nuclear receptors (ERα and β) also target to the cell membrane (Razandi et al., 2000; Razandi et al., 2002), indicating a possible function of these receptors in rapid, non-genomic effects.

1.9.1-A: Structure of Estrogen Receptor Isoforms

ER isoforms resemble the typical nuclear receptor structure as shown by cloning and functional studies. ERα and β cDNAs from different species comprise of 6 functional regions (A-F) exhibiting different degrees of amino acid sequence conservation (Krust et al., 1986; Kumar et al., 1987) (Fig 1.4). For example, Human ERα, the best-studied ER, is composed of

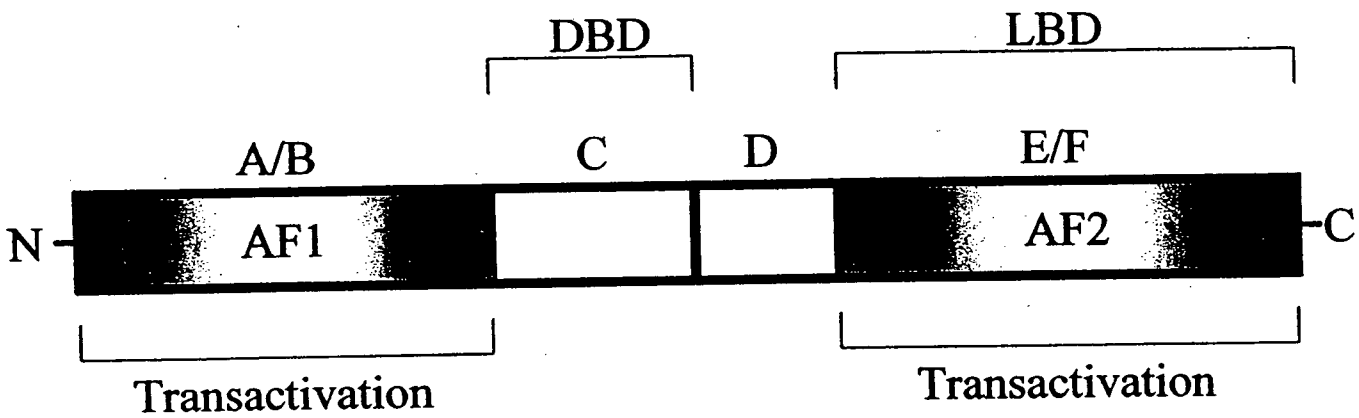

Fig 1.4: Schematic representation of the functional domains of the nuclear receptors. DBD and LBD refer to the positions of the DNA binding domain and the ligand binding domain respectively. The relative positions of the two transactivation domains AF-1 and AF-2 are also shown.

595 amino acids and displays an approximate molecular weight of 66-70 kDa. Functional analysis of ERα demonstrated conserved domain organization responsible for specific functions: DNA binding, ligand binding, dimerization, protein binding and transcriptional activation (Kumar et al., 1987; Mitchell and Tjian, 1989; Tjian and Maniatis, 1994). The hypervariable A/B domain in the amino terminal (N-terminal) region of ERα exhibits little or no conservation between species (Krust et al., 1986) and among the nuclear receptor superfamily (Segraves, 1991). The N-terminal A/B region contains a chromosome-independent transactivation function (AF-1) (Giguere et al., 1986; Kato et al., 1995; Kumar et al., 1987) and is thought to be important for transactivation and is also responsible for cell specificity (Bocquel et al., 1989; Lees et al., 1989a). The C region, also named as the DNA binding domain (DBD), is the most conserved between receptors and in both ERα and β, the position of cysteine residues that coordinate the two zinc fingers of the C domain are fully conserved between receptors and between species (Evans and Hollenberg, 1988; Freedman and Luisi, 1993; Green et al., 1986b; Gronemeyer, 1991; Kumar et al., 1987; Schwabe et al., 1990). The sequence EGCKAF at the base of zinc finger is essential for specific binding to estrogenic response elements (EREs) on target genes (Forman and Samuels, 1990; Schultz et al., 2002), and the region of the second zinc finger believed to contribute to specific receptor dimerization CPATNQC, are identical in both ER α and β (Gustafsson, 2000b; Schultz et al., 2002; Schwabe et al., 1993). That could be the reason why both ER α and β have been shown to be interacting with similar DNA binding sites (EREs). The DBD is followed by a short region D called the hinge region and separates the DBD from the ligand-binding domain (LBD) (Evans, 1988). The flexibility in the structure of LBD region is thought to allow conformational changes in the receptor molecule during activation and is important in receptor dimerization (Kumar and Chambon, 1988). Moreover, the LBD is involved in ERα dimerization, binding of co-activators and co-repressors, as well as, heat shock

proteins where ERα is in the inactivated state. Region D, along with carboxyl-terminal (C-terminal) portion of region C, is thought to contain nuclear localization signals (NLS) and is responsible for nuclear localization (Picard et al., 1990). Region E consists of 12α-helices, which form a hydrophobic pocket responsible for ligand binding (Berry et al., 1990; Bocquel et al., 1989; Bourguet et al., 1995; Danielian et al., 1992; Lees et al., 1989a). This region contains a second activation function domain (AF-2) (Danielian et al., 1992; Lees et al., 1989b; Tasset et al., 1990; Tora et al., 1989) and is involved in transactivation in conjunction with the A/B domain (Berry et al., 1990; Bocquel et al., 1989; Metzger et al., 1992). Consistent with the ability of both ERs to bind a similar range of steroid ligands (Kuiper et al., 1997), key amino acids within the LBD are conserved between the subtypes (Enmark et al., 1997; Kuiper et al., 1997). However, several studies have also demonstrated that ERα and β exhibit different affinities for some ligands notably genestein, rolaxifene, tamoxifen (Barkhem et al., 1998; Kuiper and Gustafsson, 1997) and novel ligands that act as selective estrogens or anti-estrogens for ERα or β have been identified (Sun et al., 1999). The exact functional role of region F is not clear at present. However, in some members of the steroid receptor family, this region is thought to play a role in distinguishing between agonist and antagonist binding to the receptor molecule (Montano et al., 1995).

In contrast, structural and functional studies with ERβ have shown that the DBD of ERα and ERβ are highly homologous, approaching 96%, whereas, the LBD showed only 59% homology. The N-terminal A/B domain, hinge region and the F domain are distinct in sequence between ERα and ERβ. ERβ is similar to ERα and contains a functional AF-2 region located in the LBD. Studies have described ERβ containing a similar AF-1 region located in the A/B domain (Tremblay et al., 1999a; Tremblay et al., 1999b). However, a recent report (Hall and McDonnell, 1999) demonstrates that ERβ doesn't contain a strong AF-1 activation region within

its amino-terminus but contains a repressor domain that upon deletion increases the overall transcriptional activity of the receptor.

The crystal structure of the ligand binding domains of ERα and β has been recently determined and their overall arrangement shown to be similar with both containing 12 helices (Hubbard et al., 2000; Pike et al., 2000a). Ligand binding to ERs induces a conformational change in the structure of the protein resulting in recruitment of interacting proteins (co-activators or co-repressors) that can have a profound effect on ER mediated gene transcription (Klinge, 2000). Detailed structural analysis has shown that following ligand binding the position of helix 12, identified as containing the AF-2 domain, can result in exposure of residues critical of co-activator recruitment (Brzozowski et al., 1997; Pike et al., 2000b). Interestingly, a differential recruitment of specific cofactors by ERα and β has been reported (Routledge et al., 2000; Warnmark et al., 2001).

Depending upon whether one or both of the receptors are present in the cell, homodimers (ERα-ERα or ERβ-ERβ) or heterodimers (ERα-ERβ), can be formed (Cowley et al., 1997; Pettersson et al., 1997). The binding affinity of ERα-ERα and ERα-ERβ dimers for a consensus DNA estrogen hormone response element is reported to be higher than that of a ERβ-ERβ homodimer (Cowley and Parker, 1999). Both receptor homodimers are reported to induce similar transactivation profiles in vitro when using a luciferase reporter gene linked to an ERE when they were activated with E2 or diethylstilbesterol but no signal in opposite ways at an AP-1 site (Pettersson et al., 2000). Interestingly, it has been established that one role of ERβ is to modulate ERα transcriptional activity. Specifically, they showed that the A/B domain of ERβ acts as a repressor and when it is removed there is an increase in the transcriptional activity of the receptor. Moreover, ERβ had the capacity to interact with target gene promoters in the absence of a ligand. At sub-saturating levels of ligand (10 pM E2) ERβ was able to act as a dominant

inhibitor of ERα. Taken together with the differential affinities of ERα and β for ligands, it appears that the structure of the ligand, its concentration and also the subtype(s) of ER expressed in each cell will influence gene expression.

In addition, several coactivators (Onate et al., 1995) and corepressors (Lavinsky et al., 1998) have been shown to be involved in the initiation and regulation of the gene transcription by ER. Such cofactors are believed to determine the agonistic and antagonistic properties of compounds and to be responsible for the tissue specific action of the so-called selective estrogen receptor modulators (SERMs)-like tamoxifen or raloxifene. The recent identification of several transcription coactivators and corepressors, which can associate with nuclear hormone receptors and enhance or repress their transcriptional activities, respectively, has further complexed the understanding of the mechanisms of ER action (Chen and Evans, 1995; Leo et al., 2000; McKenna and O'Malley, 2001; McKenna et al., 1999; Onate et al., 1995)

1.9.1-B: Differential expression of ERα and β

ERα and β are expressed in multiple cell types across various reproductive tissues in mice, rats, bovines and humans. In particular, ERα is predominantly expressed in tissues such as breast, uterus and vagina and is involved in regulation of reproductive processes (Enmark and Gustafsson, 1999; Gustafsson, 2000a; Korach, 1994; Kuiper and Gustafsson, 1997). ERβ, however, is expressed in diverse tissues and when both ERβ and ERα are present, ERβ is generally predominant (Enmark and Gustafsson, 1999; Gustafsson, 1999; Kuiper and Gustafsson, 1997). For example, human uterus co-express ERα and β in the stroma and epithelial cells lining the glands, but only ERβ is present in the endothelial cells lining the blood vessel walls (Critchley et al., 2001a; Critchley et al., 2001b). Expression of ERα declines in the glands and stroma of the functionalis during the secretory phase of the menstrual cycle. In

contrast, expression of ERβ declined in glandular cell nuclei, but not stroma, within the functionalis during the late secretory phase. Levels of expression of ERα and ERβ in all cellular compartments remained unchanged in the basalis. Collectively suggesting that estrogen action(s) within the vascular endothelium in the endometrium may be mediated via direct binding to the ERβ isoform and that these cells could therefore be a target for agonists or antagonists that selectively target ERβ. In addition, differential expression in stroma and epithelial cells lining the glands suggest a key and specific role during the menstrual cycle.

Similarly, in human ovary preantral follicles, ERα is not detectable but ERβ is present and forms ERβ homodimers (Pelletier and El-Alfy, 2000; Saunders et al., 2000). In mature follicles, both ERα and β are expressed and therefore homo and or heterodimers can be formed and this may result in altered gene expression compared with that in the granulosa cells of pre-antral follicles. In the human and primate testis, ERβ protein is expressed in multiple cell types including germ cells whereas ERα is undetectable using immunohistochemistry or western analysis (Saunders et al., 2001). ERβ is also the predominant form of ER expressed in the epithelial cells lining the vas deferens, seminal vesicles, prostrate, bladder (Saunders et al., 2001). However, in some epithelia such as that lining the efferent ductules, a major site of fluid resorption, high levels of ERα receptor are also present (Hess et al., 2001; Saunders et al., 2001). Differential expression of ERα and β has also been reported to occur in the prostrate (Royuela et al., 2001) and in normal and cancerous breast tissues (Pelletier and El-Alfy, 2000; Saunders et al., 2002). Besides a differential expression there appears to be a dramatic switch between ERα and β expression in the myometrium during pregnancy as ERβ expression is increased in human term myometrial tissue compared with non-pregnant tissue (Wang et al., 2001; Wu et al., 2000). In contrast, ERα is highly expressed in non-pregnant myometrium but not in term myometrium suggesting that ERβ plays a role in the timing of onset of labor (Benassayag et al., 1999; Wang

et al., 2001; Wu et al., 2000). It has been speculated that myometrial ERβ may inhibit AP-1 activity and thus block induction of labor-associated genes and that loss of myometrial ERβ expression is necessary for labor to occur (Benassayag et al., 1999; Wang et al., 2001; Wu et al., 2000).

The rat tissue distribution and/or relative level of ERα and the ERβ mRNA expression is quite different with ERα showing moderate to high expression in uterus, testis, pituitary, ovary, kidney, epididymus, adrenal and ERβ being the predominant isoforms in prostrate, ovary, lung, bladder, brain, testis (Kuiper et al., 1997). Examination of ERβ mRNA expression at the cellular level, by in situ hybridization, shows that rat prostrate ERβ is highly expressed in the epithelial cells at the secretory alveoli, whereas in the ovary the granulosa cells of the primary, secondary and preovulatory follicles show expression of ERβ (Byers et al., 1997; Kuiper et al., 1996). In contrast, examination of ERα in the ovary reveals expression at low levels throughout the ovary with no particular cellular localization (Byers et al., 1997). In bovine ovary, ERα was not observed in cells of primordial, primary and secondary follicles whereas, very weak expression was noticed in cells of healthy and atretic tertiary follicles (Van Den Broeck et al., 2002). In contrast, ERβ is localized within the granulosa cells of small, medium and large antral follicles suggesting that ERβ may play on important role in ovarian follicle growth, development, and subsequent ovulation (Manikkam et al., 2001; Rosenfeld et al., 1999). Immature rats undergoing exogenous hormonal administration demonstrate that the prevoulatory leutinizing hormone (LH) surge down regulates ERβ mRNA in the ovary (Mann, 93). These experiments clearly demonstrate that differential expression contributes to the physiological roles of ERβ in female reproductive functions.

ERβ binds to the natural hormone, E2 with affinity similar to that of ERα (Kuiper et al., 1997; Kuiper et al., 1998). However, it was postulated that these two receptor isoforms have

different biological activity inherent to their distribution and structural similarities. Further evidence that ERα and ERβ may elicit different biological responses comes from the fact that E2 binding to ERα activated transcription, whereas with ERβ, E2 binding inhibited transcription (Paech et al., 1997). Furthermore, the ER agonists, tamoxifen, raloxifene, and ICI 164384 were potent transcriptional activators when complexed with ERβ at an AP1 site but inhibit transcription through ERα (Paech et al., 1997). It was concluded that the two ER isoforms signal in different ways depending on ligand and response element suggesting that ERα and ERβ may play different roles in regulating gene expression (Paech et al., 1997).

1.9.1-C: Cell biology of ER isoforms

Gene targeting methods have been used to generate mice lacking either functional ERα or β, and more recently mice lacking both ERs, to provide a stable genetic model for evaluating ER actions. The resulting estrogen receptor knockout (ERKO) models provide an insight into the biological roles of each ER. The loss of ERα leads to severe gonadal and behavioral phenotypes that result in infertility in female and reduced fertility in male mice, respectively (Lubahn et al., 1993). In contrast, disruption of the ERβ gene results in subfertility in females whereas male fertility appears unaffected (Krege et al., 1998). This is inspite the fact that both ERα and β knockouts show no gross effect on ovarian differentiation, since females of each line possess normal appearing ovaries at birth and during neonatal development (Krege et al., 1998; Lubahn et al., 1993; Schomberg et al., 1999). However, at the commencement of sexual maturity, distinct ovarian phenotypes become apparent in the αERKO and βERKO females (Krege et al., 1998; Lubahn et al., 1993; Schomberg et al., 1999). βERKO females produce less frequent litters and a reduced number of pups per litter. In contrast, αERKO females exhibit a complete inability to spontaneously ovulate and become pregnant (Dupont et al., 2000; Krege et al., 1998; Lubahn et

al., 1993; Sar and Welsch, 1999). Although, the actions of both receptors are necessary for normal ovarian function, it can be concluded that the pathways affected by the respective ER gene disruption differ (Couse et al., 1997; Eddy et al., 1996; Korach, 1994; Rissman et al., 1997; Smith, 1994).

In a recent study, the role of ER isoforms in mediating the effects of estrogen in ovariectomized mice was analyzed (Lindberg et al., 2002). Moreover, the increased effect of estrogen on DERKO mice might be explained as a consequence of unopposed ERα activity (Lindberg et al., 2002). Surprisingly, both wild type (WT) and double knockout (αβERKO) mice showed increase in cortical bone dimensions and increased uterine and liver weights, suggesting that estrogen exerts some effects independent of ERα and β. It was however unclear whether the mutation generated by disruption of the gene for ERα in αERKO mice actually represents a null mutation, as a transcriptionally active form of ERα is present in low amounts in these mice (Couse et al., 1995) suggesting that the ERKO mouse used in these studies were not completely ERα inactivated and acted via functional ERα A/B domain, which may be responsible for minor ERα activity (Pendaries et al., 2002). Therefore, to further understand the roles of ER isoforms new ERα and ERβ null mutants fully lacking ERα (ERαKO), ERβ (ERβKO), or both (ERαβKO) have been generated (Dupont et al., 2000) and have been compared to the previous αERKO, βERKO and αβERKO mutants. The new look at the role of ER isoforms has substantiated the concept that ERβ plays an important role in mediating stimulatory effects of estrogen on granulosa cell proliferation while ERα is not required for follicle growth under wild conditions, while it is indispensable for ovulation and ERα is also necessary for interstitial glandular cell development (Dupont et al., 2000). Although, ERα is the predominant ER expressed in the uterus and vagina, ERβ could compensate for the loss of ERα at least in the genital tract as the ERαKO uterine and vaginal hypoplasia is exacerbated in ERαβKO mice,

whereas the ERβKO genital tract appears normal (Dupont et al., 2000). Interestingly, ERαβKO granulosa cells localized within degenerating follicles transform into cells displaying junctions that are unique to testicular Sertoli cells (Dupont et al., 2000) suggesting that these receptors could be playing a key role in regulating developmental processes. In addition, ERβ has an antiproliferative effect in the immature uterus and in the prostrate, at least partially, by balancing the proliferative activity of ERα (Weihua et al., 2002; Weihua et al., 2001; Weihua et al., 2000) however, direct repression of ERα expression by ERβ has also been demonstrated (Windahl et al., 2002; Windahl et al., 2001) suggesting that these isoforms may have distinct functions in regulating development.

1.9.2: Progesterone Receptors

PRs belong to the large family of related hormone dependent nuclear proteins (Evans, 1988; Mangelsdorf et al., 1995) and are responsible for mediating the actions of progestins (Pfaff, 1997). Upon binding progesterone, the receptor complex becomes activated and facilitates the interaction of the PR-ligand complex with progesterone response elements (PREs) within the regulatory regions of target genes. The DNA-bound receptor can either positively or negatively modulate transcription. In contrast to estrogen, receptors for progesterone are expressed as distinct isoforms, PRA and PRB that arise from a single gene (Conneely et al., 1987a; Conneely et al., 1989; Conneely et al., 1987b; Kastner et al., 1990). These isoforms may arise as a result of either initiation of translation from alternative sites in the same messenger RNA (Conneely et al., 1987b) or by transcription from alternative promoters (Kastner et al., 1990; Kraus et al., 1993). The selective physiological roles of the two isoforms of PR are predicted to differ based on differential structural and functional properties of the individual proteins observed using in vitro assay systems.

PRA and B isoforms are expressed in a number of vertebrate species, including rodents and humans (Bethea and Widmann, 1998; Giangrande and McDonnell, 1999; Schrader and O'Malley, 1972; Shyamala et al., 1990). The conservation of these two receptor isoforms and the complex genomic mechanisms for the generation in different species, as well as the differential hormonal regulation in reproductive tissues, suggest that their differential expression may be critical for the appropriate cellular responses to P4 (Bouchard, 1999). Structurally, the amino-terminal region is poorly conserved among species and contains transactivation functions (AF-1 and AF-3) that regulate the level of promoter specificity and target gene activation (Dobson et al., 1989; Meyer et al., 1992; Sartorius et al., 1994b; Tora et al., 1988) (Fig 1.4). The highly conserved DBD is located centrally and contains sequences that contribute to receptor dimerization as well as specificity of DNA binding (Freedman, 1992; Luisi et al., 1991). Downstream of DBD lies a small hypervariable region containing nuclear localization signals as well as an additional transactivation domain (AF-2) (Dobson et al., 1989). The LBD is located downstream of this region and is functionally complex. In addition to its progesterone binding function, it contains sequences important for interaction of inactive receptors with cellular heat shock proteins, sequences required for receptor dimerization, transactivation, and inter- and molecular silencing (Pratt et al., 1988; Vegeto et al., 1992).

The PRA and PRB receptor isoforms differ only to the extent that PRB contains an additional stretch of amino acids located at the amino terminus of the receptor. This region has been shown to encode a transactivation function that is specific to the PRB protein (Giangrande and McDonnell, 1999) and plays an essential role in specifying target genes that can be activated by the PRB but not PRA protein. Thus both the PRA and PRB proteins of PR are capable of binding progesterone, and dimerizing and interacting with progesterone responsive elements, as well as the transcriptional machinery to regulate gene expression. When expressed in equimolar ratios in cells, the A and B proteins can dimerize and bind DNA as three species: A:A or B:B

homodimers or A:B heterodimers. The specific contributions of each of these species in mediating the regulatory effects of P4 will depends on the differential transactivation properties contributed to these complexes by the B-specific domain. Clearly, these differences would be expected to expand the repertoire of physiological responses to P4. The absolute levels of PR as well as the stoichiometric ratio of PRA to PRB in reproductive tissues thus may vary as a consequence of developmental and hormonal status. Further, the ratios of A and B proteins within a specific target cell under specific physiological conditions would be expected to alter the relative complement of dimeric complexes and exert significant impact on the overall cellular responses to P4 (Conneely et al., 2002).

Although, the exact functions for each of these isoforms are still unclear, there is increasing evidence that the PRA and PRB proteins are functionally different (Kastner et al., 1990; Tora et al., 1988). First, when expressed individually in tissue cultured cells, PRA and PRB display different transactivation properties that are specific to the cell type and target gene promoter used (Giangrande and McDonnell, 1999; Meyer et al., 1992; Vegeto et al., 1993). Specifically, PRB has been shown to function as a strong activator of transactivation of several PR dependent promoters and in a variety of cell types in which PRA is inactive. These findings suggest that A and B may regulate different physiological target genes in response to P4, and each protein may display different transactivation capabilities in different target tissues. Second, when the A and B proteins are coexpressed in cultured cells, in cell and promoter contexts in which agonist-bound PRA is inactive, the protein can act as a dominant repressor of the PRB activity (Kraus et al., 1995; Tung et al., 1993; Vegeto et al., 1999; Vegeto et al., 1993). These findings suggest that PRA has the ability to diminish overall progesterone responsiveness of specific target genes in specific tissues. This repressor capability, which appears to be a selective property of the PRA protein, extends not only to PRB but also to other steroid receptors. Thus PRA has been shown to inhibit estrogen, glucocorticoid and mineralocorticoid receptor-

dependent gene activation presumably through competition or common limiting coactivators (McDonnell et al., 1994; Wen et al., 1994). Agonist bound PRB functions as a strong activator of transcription of several PR dependent promoters and in a variety of cell types in which PR-A is inactive (Musgrove and Sutherland, 1993; Sartorius et al., 1994a; Sartorius et al., 1993). Finally, the PRA and PRB proteins also respond differently to progesterone antagonists (Giangrande and McDonnell, 1999). While antagonist bound PRA is inactive, antagonist bound PRB can be converted to a strongly active transcription factor by modulating intracellular phosphorylation pathways (Beck et al., 1993; Musgrove and Sutherland, 1993; Sartorius et al., 1994a). Although, the sequence of the ligand binding domain of PRA and PRB is identical, the ability of different ligands to induce different conformational changes in PR, together with the synergistic activity of the amino and carboxy terminal activation domains (Tetel et al., 1999), predicts that PRA and PRB selective transcriptional regulation can be achieved by manipulating ligand interactions with the carboxy terminal.

1.9.2-A: Cell biology of PR isoforms

The expression of both isoforms overlaps spatiotemporally in female reproductive tissues, however, the ratios of the individual isoforms may vary as a consequence of development (Bigsby, 2002; Quadros et al., 2002), hormonal status (Duffy et al., 2000; Malayer and Woods, 1998) and during carcinogenesis (Akahira et al., 2000; Murphy and Watson, 2002). In rat ovarian (Kraus and Katzenellenbogen, 1993), and uterine (Natraj and Richards, 1993) tissues, PRA levels exceed those of PRB. However, in the human breast cancer cell line T47D, the two isoforms exist in approximately equimolar ratios (Lessey et al., 1983). Fiel et al. (Feil et al., 1988), indicated that the human uterus contains both PRA and PRB without clear evidence of changes in the ratio, however a study by Mangal et al. (1997), showed that both isoforms are highest at the periovulatory period, but levels of PRA were higher than those of PRB. Between

day 2 and 8, PRB is almost undetectable and the A:B ratio is >10:1. From day 9 to 13 the ratio is about 5:1 and it is between 2:1 between day 14 and 16. Thereafter, PRB levels decrease rapidly and are virtually undetectable at the end of the cycle, suggesting a cycle specific presence of PR isoforms. The dominancy of PRB mRNA expression is associated with advanced clinical stages of ovarian cancer (Fujimoto et al., 1995). Studies have also confirmed the dominance of PRB but not PRA mRNA during ovarian carcinoma (Akahira et al., 2000). Therefore, the dominant expression of PRB is considered to be characteristic feature of ovarian cancer, in contrast to breast and endometrium carcinoma, in which PRA is the dominant subtype of PR (Graham et al., 1996; Kumar et al., 1998). Collectively, these data suggest that progesterone action is a result of not only ligand concentration, but may also be a reflection of the proportion of progesterone receptor isoforms in specific tissue types. The differences in transcriptional activities and corregulator interactions between the PRA and PRB observed in vitro predict that these activator proteins also might mediate different physiological responses to P4 (McKenna et al., 1999; Rowan and O'Malley, 2000).

Null mutants of PR gene encoding both isoforms have provided evidence of an essential role of PRs in a variety of female reproductive and non reproductive tissues (Lydon et al., 1995). Female mice lacking both PRs exhibit impaired sexual behavior, non-endocrine gonadortropin regulation, anovulation, uterine dysfunction and ductal morphogenesis and lobuloalveolar differentiation of the mammary gland. Recent studies have further addressed the individual contributions of the PRA and PRB proteins to the physiological actions of P4 using mouse mutants in which expression of the PRA (PRAKO) or PRB (PRBKO) isoforms has been selectively ablated. In PRAKO mice, the PRB isoforms function in a tissue specific manner to mediate a subset of the reproductive functions of PRs (Mulac-Jericevic et al., 2000). Ablation of PRA in PRAKO uteri revealed an unexpected progesterone dependant proliferative activity of PRB in the epithelium and demonstrated that PRA is essential to diminish both progesterone

(acting via PRB) and estrogen mediated proliferative responses in this tissue. The observation that PRA is essential to inhibit estrogen induced proliferation in the uterus is consistent with previous observations that agonist bound PRA is capable of inhibiting estrogen dependant transcriptional activation in cell based transcriptional activation assays (McDonnell et al., 1994). Notably, this inhibitory activity of PRA was tissue specific and did not extend to the mammary gland where both PRA and PRB act as proliferative mediators of P4.

Consistent with the distinct tissue- and promoter-specific activities of PRA and PRB observed in in vitro studies, the tissue-selective activities of PRB observed in PRAKO mice were associated with an ability of this isoform to regulate a subset of progesterone responsive target genes rather than to differences in its spatiotemporal expression relative to the PRA isoform (Mulac-Jericevic et al., 2000). In contrast to the reproductive defects observed in the PRAKO mice, more recent studies using PRBKO mice have shown that ablation of PRB does not affect ovarian, uterine or thymic response to P4 but results in reduced mammary ductal morphogenesis (Conneely and Jericevic, 2002). Thus, PRA is both necessary and sufficient to elicit these progesterone dependent responses while the PRB isoform is required to elicit normal proliferative responses of the mammary gland to P4. Therefore, it is conceivable that the alterations in the ratio of PRA and PRB in a certain target tissue may modify the overall P4 action via differential regulation of specific progesterone response genes. Collectively, the data provide evidence of the central role of the PR proteins in the coordination of important reproductive functions in a tissue specific manner.

1.9.3: Expression of steroid receptors during preimplantation embryogenesis

After fertilization, the mammalian embryo undergoes a series of changes, which allow rapid cellular proliferation and differentiation leading to the formation of the blastocyst. Intrinsic and extrinsic factors influencing preimplantation development have been described (Johnson,

1979a), but little is known about the involvement of steroid hormones that are present in the immediate vicinity of the embryo. Roblero and Garavago (Roblero and Garavagno, 1979) have reported that in ovarectomized mice, there is a need for E2 and P4 in synchronizing embryo transport, and cleavage rates with the physiological state of the reproductive tract. Cleavage rates and transformation from morula to blastocyst were stimulated in mice by synergistic effects of estrogen and progesterone in these mice. Similarly, preimplantation embryo development was arrested at the premorula stage in hamsters ovarectomised on Day 1 of pregnancy. This effect was reversed when small amounts of P4 alone or a combination with E2 and P4 were administered together (Sengupta et al., 1983). In addition, hypophysectomy of pregnant rats was shown to result in the delayed entry of eggs into the uterus, expulsion of eggs from uterus and retarded development of the embryo, which is reversed on administration of P4 (Vinijsanun and Martin, 1991; Wu et al., 1972). Reactivation of dormant blastocysts by the administration of E2 has also been reported (Moore and Carter, 1984). Maintenance of proper concentrations of E2 and P4 at appropriate levels and also relative to each other has been suggested to be an important factor governing embryogenesis. Elevated ratios of E2 to P4 inhibit the blastocyst metabolism, development and subsequent implantation (Safro et al., 1990). Antagonism of P4 by antibody treatment was able to prevent implantation and normal embryo transport in mice (Vinijsanun et al., 1990). Since the majority of these experiments were conducted on ovariectomized animals receiving exogenous hormones, it could not be determined if the hormones were acting directly on the embryo or indirectly though the uterine environment (Hou and Gorski, 1993).

Histochemical evidence of Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) in mouse preimplantation embryos suggested that embryos are capable of synthesizing steroids with maximum amounts being present just before implantation (Dey and Dickmann, 1974). The conversion of ^{3}H-testosterone to ^{3}H-E2 is also maximal in rabbit blastocysts at this time (George and Wilson, 1978). This observation

suggested that steroidogenesis in morulae and blastocysts may play a role in embryonic development and in the preparation of the endometrium for implantation (Dickmann and Dey, 1974) whose target could be both the embryo itself as well as the uterus.

However, the mechanisms of steroidal action on embryo development are still not well understood. It has been suggested that the effects of E2 on embryos were indirect and preimplantation embryogenesis may be controlled by certain paracrine factors originating from the reproductive tract under the influence of E2 (Wu et al., 1992). However, the direct effect of estrogen on the embryo could be determined by observation that in the mouse, the development of morulae into blastocysts in vitro was inhibited by CI-628 citrate and nafoxidine, estrogenic antagonists (Gupta et al., 1977; Roy et al., 1981). Mouse embryos grown in culture with 1,4,6-androstatriene-3,17-dione (ATD), an inhibitor of estrogen synthetase (aromatase) stop developing (Sengupta et al., 1982). This inhibition of embryo development by ATD is overcome by E2 suggesting that mouse embryos contain machinery for synthesis of E2 and secondly, the availability of E2 in blastomeres is essential for morula to blastocyst transition (Sengupta et al., 1982). Interestingly, E2 was also shown to affect the uptake and incorporation of nucleic acid precursors (Harrer and Lee, 1973; Lau et al., 1973) and amino acids (Smith and Smith, 1971) by mouse blastocysts supporting the notion that E2 affects the embryo directly.

In porcine embryos, E2 has been shown to be essential for the transformation of the compacted morula to the cavitated blastocyst stage (Niemann and Elsaesser, 1986). Administration of antiestrogen in culture media impaired the transformation of pig morulae to blastocysts (Niemann and Elsaesser, 1987). The maternal recognition of pregnancy in pigs, which is established about 11-12 days after the start of estrus, is influenced by estrogen (Geisert et al., 1990). Implantation in the pig has also been found to be preceded by synthesis of estrogen by the conceptus to maintain functional corpora lutea throughout pregnancy (Gadsby et al., 1980; Geisert and Yelich, 1997). The main target of embryonic estrogen is likely to be the embryo

itself as well as the endometrium. While estrogen may have potential regulatory roles in embryonic growth, development or both, the effect of estrogen on the endometrium, which exhibits functional ERs (Geisert et al., 1993), along with P4 stimulation, has been to secrete a myriad of growth factors, cytokines, proteases and protease inhibitors, which are requisite for implantation and pregnancy success (Simmen et al., 1992). Studies that address the embryo as a direct target of estrogen action via ER mediated mechanisms are limited (Hou and Gorski, 1993; Hou et al., 1996; Ying et al., 2000a) and conflicting (Yelich et al., 1997).

ER and PR are spatiotemporally expressed during development of the murine or porcine embryos. In particular, ERα is present in the oocyte and two-cell stage embryo but not the eight-cell stage embryo or morula (Hou and Gorski, 1993). However, in another study, ERα mRNA was detected in whole ovaries, cumulus-oocyte complexes, denuded oocytes, 2-cell and 4-cell embryos, whereas it was undetected at 8-cell stage embryos, but reappeared in morulae and blastocysts (Hiroi et al., 1999). Similarly, expression of ERα has been described in human oocytes (Wu et al., 1993). In contrast, ERβ expression in mouse embryos is detected throughout the early stages of development except at the morula stage (Hiroi et al., 1999). A recent study has demonstrated that rising levels of E2 affects embryonic adhesion in mice at the embryo-endometrial interface, most likely through its effect on the embryo (Valbuena et al., 2001). Expression of ERα has been reported during preimplantation embryogenesis in pigs (Dekaney et al., 1998; Ying et al., 2000a). Moreover, in a recent study (Kowalski et al., 2002), expression of both ERα and β subtypes have been detected but expression of the ERβ gene is significantly higher than ERα. ERα expression has been found in 2-, 4- cell porcine embryos and blastocysts while its expression could not be detected at the 8-cell or at the morula stage (Ying et al., 2000a). However, in this study, ER protein could only be detected in the one-cell stage to the five-eight cell stages. Despite the appearance of ER mRNA at the blastocyst stage, ER protein was not

detected in the blastocyst stage. This has been suggested to be due to a delay between the transcription of the ER gene into mRNA and the translation of ER mRNA into proteins in the pig embryo (Ying et al., 2000a).

In contrast, PR mRNA transcripts have been detected during preimplantation development of the mouse embryos, but only at the blastocyst stage (Hou and Gorski, 1993). However, a recent study has shown that the PR is expressed only at the 2- and 4-cell stages of development in pig embryos and no expression is detected at the 8-cell, morula or the blastocyst stages of development (Ying et al., 2000b).

Expression patterns of mRNA for both ERs in organ formation during mouse late embryogenesis (day 9.5 to day 16.5 embryos) has revealed a characteristic differential expression during development (Lemmen et al., 1999). While ERα expression is detected in the heart atrium on day 9.5, ERβ expression can be detected in the mesonephric tubules, mesonephric duct, heart mysentry and brain on day 10.5. This differential expression continues to be seen during the remaining stages of development. However, on day 16.5 most of the tissues seem to coexpress ERα and β. Thus, taken together, distinct expression patterns observed for ER and PR isoforms during preimplantation and post implantation embryogenesis suggests specific functions for these receptors during development. However, to date, no information about the expression of steroid receptors in the bovine preimplantation embryos exists.

Previous studies have reported direct effects of ^{3}H-estrogen or DES binding in the mammary gland, brain, larynx, connective tissue and several other tissues during development (Holderegger and Keefer, 1986; Stumpf et al., 1980). Detection of the specific ER isoforms in these tissues suggests that the effects of steroids could be due to the presence of these receptor isoforms. Furthermore, E2 treatment of day 12 filamentous porcine embryos in vitro upregulated ERβ and embryo (type III)-specific (Choi et al., 1997) P450 aromatase mRNA levels, respectively, but decreased those of the proliferative cell nuclear antigen (PCNA), and had no

effect on cyclin D1 mRNA levels, a gene positively correlated to growth, suggesting a functional linkage between estrogen, ERβ and embryo development as ERβ appears to be mediating the autocrine functions of estrogens in the dynamic regulation of embryonic growth and development during early embryogenesis (Kowalski et al., 2002).

In addition, ER mediated estrogen action has long been implicated in the control of cell growth and development (Clark and McGuire, 1988) and PR mediated P4 action has been considered important for implantation and pregnancy (Sengupta and Ghosh, 2000; Spencer and Bazer, 2002). Besides the expression of ER and PR in the murine and porcine embryo, recent data have provided increasing evidence to the notion that positive or negative regulation of cell development by estrogen is a function, in part, of the ER subtype expressed in target cells (Kahlert et al., 2000; Weihua et al., 2000).

Thus, the identification of ER and PR isoforms in bovine preimplantation embryos, if present, is necessary for determining whether and how progestins and/or estrogens might be playing a role during early embryogenesis. Moreover, since the response of a cell/tissue to a hormonal stimulus will be dependent on the ratio(s) of the expression levels of these two distinct ER and PR isoforms, their identification in bovine embryos at distinct developmental stages will likely provide important insights into possible molecular mechanisms through which estrogen and progesterone may be correlated with embryo development.

1.10: Hypothesis and specific objectives

In view of the background information on the role of cadherins, ADAMTS and steroid receptors during tissue morphogenesis and embryogenesis, it is hypothesized that multiple cadherin subtypes, ADAMTS and steroid receptor isoforms are being expressed during bovine preimplantation embryogenesis. It is also hypothesized that a regulated expression of ADAMTS could be involved in other dynamic developmental processes, particularly, folliculogenesis and

corpus luteum formation, maintenance and regression in bovine. Therefore, the specific objectives of the study are, a) to identify, characterize and localize the expression of cadherin subtypes in the bovine embryo at timed stages of development, b) to identify, characterize and localize the expression of estrogen and progesterone receptors expressed in the bovine embryo during preimplantation embryogenesis, c) to identify and characterize the expression of ADAMTS subtypes in the bovine embryo during preimplantation embryogenesis d) to identify and characterize ADAMTS expression during folliculolgenesis, ovulation, CL formation, maintenance and regression in bovines.

The work done to achieve these objectives is presented in the following sections.

CHAPTER II: EXPRESSION AND LOCALIZATION OF TYPE 1 AND TYPE 2 CADHERINS DYRING PREIMPLANTATION EMBRYOGENESIS IN BOVINES

2.1: Preface

This chapter describes a series of studies undertaken to characterize the identity, expression patterns and localization of the cadherin subtypes during preimplantation embryogenesis in bovines.

Though previous studies have demonstrated a pivotal role of a type 1 cadherin, E-cad, in mediating blastocyst formation, recent studies have identified other type 1 cadherins (N- and P-cad) being expressed in the preimplantation murine embryo. These CAMs are believed to mediate selective association and arrangement of embryonic cells for specific spatial arrangement of the embryo. In contrast, the functions of type 2 cadherins remain poorly understood, though there is increasing evidence to suggest that these CAMs are involved in several developmental processes including morphogenesis. However their specific role(s) during preimplantation development has not been examined to date. Therefore, we have undertaken a comprehensive examination of the roles of cadherins during preimplantation embryogenesis.

In these studies we have demonstrated that in addition to type 1 cadherins, multiple type 2 cadherins, cad-8, cad-10 and cad-11, in particular, are expressed during bovine preimplantation embryogenesis. The expression of type 2 cadherins is almost similar to that of type 1 cadherins suggesting that the type 2 cadherins could be mediating equally important cellular events during embryogenesis. Moreover, the localization studies demonstrate the presence of both type 1 and type 2 cadherins at the plasma membrane of the blastomeres with maximum expression at the region of cell-cell contact during the early stages of development. However at the blastocyst stage, both type 1 and type 2 cadherins demonstrate specific sublocalizations with E-cad and cad-11 being expressed both in the TE and ICM region, while N-, P-cad and cad-8 being

expressed only in the ICM region of the blastocyst. This specific localization suggests a key role in the cell allocation during blastocyst formation. Collectively these observations suggest that both type 1 and type 2 cadherins could be regulating blastocyst formation.

These studies are the first to demonstrate that type 2 cadherins are expressed during preimplantation embryogenesis in any mammalian species. In addition this is the first demonstration of the localization of multiple type 1 and type 2 cadherin subtypes during preimplantation embryogenesis in any species. These studies are also the first report of the presence of N-cad and P-cad in the bovine embryo. The results of these studies are in preparation as a manuscript entitled "Characterization of type 2 cadherins in preimplantation embryos undergoing development in vitro" for submission in the *Biology of Reproduction.*

2.2: CHARACTERIZATION OF TYPE 1 AND TYPE 2 CADHERINS IN PREIMPLANTATION BOVINE EMBRYOS

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Introduction

The organization of the blastocyst requires selective association and arrangement of embryonic cells during preimplantation development. Cell recognition, sorting and arrangement are mediated by different classes of cell adhesion molecules (CAMs) that are present at the surface of embryonic cells. In particular, the regulated expression of CAMs, known as cadherins, play a key role during embryogenesis (Takeichi, 1995). The cadherins are a gene superfamily of integral membrane glycoproteins, which mediate calcium dependent cell-cell adhesion (Blaschuk

et al., 1994; Takeichi, 1995; Tepass et al., 2000). This gene superfamily is composed of two evolutionary distinct subfamilies: type 1 cadherins and type 2 cadherins (also known as atypical cadherins)(Suzuki et al., 1991; Suzuki, 1996b; Tanihara et al., 1994b).

The subfamily of type 1 cadherins, which includes three originally identified cadherins, E-cadherin (E-cad), N-cadherin (N-cad) and P-cadherin (P-cad), mediate cell adhesion through homophilic interactions (Takeichi, 1991; Takeichi, 1995). Embryonic cells expressing different classical cadherins segregate from one another and it is believed that these CAMs provide the molecular basis for the segregation of discrete populations of cells during development (Kadokawa et al., 1989; Ogou et al., 1982; Takeichi, 1991; Takeichi, 1995). It was suggested that E-cad is the only type 1 cadherin expressed during preimplantation development in the mouse embryo (Ohsugi et al., 1997), which was detectable from one cell stage (Hyafil et al., 1981). P-cad and N-cad were first identified only at the blastocyst and gastrula stages, respectively (Hatta and Takeichi, 1986; Kadokawa et al., 1989; Nose et al., 1987; Takeichi, 1988), demonstrating that the spatial and temporal patterns differ from each cadherin subtype during preimplantation embryogenesis . However, recently, P-cad and N-cad have also been detected from 2-cell stage onwards in the preimplantation murine embryo (Harrouk et al., 2000) suggesting a much prior role of these cadherins during embryogenesis.

In contrast, the function(s) of type 2 cadherins in general remain poorly characterized. Although, the type 2 cadherins are structurally similar to type 1 cadherins, there is low overall amino acid homology between the two cadherin subfamilies (Nollet et al., 2000; Shimoyama et al., 2000; Takeichi, 1995; Tanihara et al., 1994b). Type 2 cadherins have also been shown to mediate cell adhesion through homophilic interactions (Kimura et al., 1995; Shimoyama et al., 1999; Shimoyama et al., 2000). However, there is increasing evidence to suggest that members of type 2 subfamily are involved in tissue and organ formation during embryogenesis. Several type 2 cadherins including cadherin-6 (cad-6 or K-cadherin), cadherin-8 (cad-8), cadherin-10

(cad-10) and cadherin-11 (cad-11 or OB-cadherin) and cadherin-12 (cad-12) are differentially expressed in the rodent and chicken brain and these expression patterns correlate with the formation of various neural structures (Blaschke et al., 2002; Cho et al., 1998; Fushimi et al., 1997; Inoue et al., 1998b; Kimura et al., 1996; Korematsu and Redies, 1997b; Simonneau and Thiery, 1998; Tanihara et al., 1994b). Cad-6 is believed to play a role in the formation of the human and rodent kidney (Mah et al., 2000; Shimoyama et al., 1995). Expression of cad-11 has also been associated with bone formation (Okazaki et al., 1994) and the formation and organization of the placenta (MacCalman et al., 1996; MacCalman et al., 1998).

Cell adhesion has a profound influence on early implantation development for differentiation of the trophectoderm and the morphogenesis of the blastocyst (Fleming et al., 2001) as demonstrated using gene-targeting methods. E-cad null mutant embryos develop into abnormal blastocysts (Larue et al., 1994) undergoing compaction and possessing the ability to form tight junctions and dermatomes (Riethmacher et al., 1995), cellular processes that are believed to be partly mediated by E-cad. The ability of these mutant embryos to undergo compaction has been attributed to the maternally derived E-cad transcripts (Riethmacher et al., 1995). In contrast, N-cad gene knock out mice develop normally before undergoing mortality around day 10 of gestation due to cardiac defects (Radice et al., 1997b). Irrespective of the final outcome, these N-cad mutants expected not to form any neural tissues are still capable of forming neural structures. Furthermore, it has been shown recently that E-cad could functionally substitute for N-cad during cardiac development in mice (Luo et al., 2001). Similarly, P-cad gene knockout mice survive till term and beyond with only minor aberrations in mammary gland development (Radice et al., 1997a). This result is all the more surprising given the high level of P-cad expression in the early embryo, mouse placenta and deciduas and its putative role in mediating placental-uterine interactions (Kadokawa et al., 1989; Nose et al., 1987). Similarly,

null mutants for cad-6 and cad-11 develop normally until blastocyst stage, with minimal effects in the adult as well.

Taken together, these observations suggest compensation or an overlap function by other cadherins and a critical role of cadherin mediated cell adhesion during development. Since the expression patterns of different cadherins often superimpose during morphogenesis, there is a distinct possibility that other cadherins are expressed simultaneously during preimplantation embryogenesis as well. Therefore, in order to better define the role of cadherin subtypes in early embryonic development, we have performed a comprehensive analysis of cadherin subtypes present in the bovine embryo at timed stages of development.

Materials and methods

Production of Bovine Embryos In Vitro

Bovine ovaries were brought from a local abattoir in sterile saline at 32-37°C for oocyte collection and in vitro embryo production as previously described (Giritharan and Rajamahendran, 2001). Briefly, cumulus-oocyte complexes from small follicles (3-6 mm) were aspirated into the follicular aspiration medium containing Dulbecos's phosphate buffer saline (Gibco BRL; Invitrogen, Burlington, ON), 0.3% bovine serum albumin (Sigma-Aldrich Canada Ltd, Oakville, ON) and 50 μg/ml Gentamycin (Sigma-Aldrich Canada Ltd.) using an 18G needle attached to a 10 ml syringe. Oocytes with an evenly granulated cytoplasm and surrounded by more than three layers of cumulus cells were selected for maturation. The oocytes were cultured in maturation medium containing Tissue Culture Media-199 (Sigma-Aldrich Canada Ltd.) supplemented with 0.01 μg/ml follicle stimulating hormone (Folltropin; Vetrepharm Canada Inc., Belleville, ON), 5% superovulated cow serum (SCS) and antibiotics (50μg/ml Gentamycin) at 38.5°C in humidified air containing 5% CO_2 for 24 h. Frozen semen from a single bull was

thawed at 37°C, washed twice by centrifugation at 500 x g for 5 min, diluted to a final concentration of $5x10^6$ sperm/ml in Brackett and Oliphant medium supplemented with 2.5 mM caffeine sodium benzoate (Sigma-Aldrich Canada Ltd.) and 20 μg/ml heparin (Sigma-Aldrich Canada Ltd.). Twenty to 30 oocytes were incubated with 100 μl semen droplets, at 38.5°C and 5% CO_2 for 16-18 h. The presumptive zygotes were washed twice with TCM-199 to remove excess sperm, placed in a four well culture dish containing TCM-199 supplemented with 5% SCS, 5μg/ml insulin (Sigma-Aldrich Canada Ltd.) and 50μg/ml Gentamycin and cultured at 38.5° C and 5% CO_2. The culture media was changed every 72 h. Batches of embryos were harvested at the timed stages of development [2-, 4-, 8-, 16- cells, morulae, and blastocysts].

Isolation of Total RNA

Total RNA was extracted from bovine embryos (25-30 embryos at 2-, 4-, 8-, 16- cell, morula and blastocyst stages) using the phenol chloroform method of Chomczynski and Sacchi (1987). The total RNA extracts were then treated with deoxyribonuclease (DNAse)-1 to eliminate possible contamination from genomic DNA. The purification of total RNA present in each of the extracts was determined by optical densitometry (260/280 nm) using a DU-64 UV-spectrophotometer (Beckam Coulter, Mississauga, ON).

Degenerate Reverse-Transcription-Polymerase Chain Reaction

To identify the cadherins present in the bovine embryo, the reverse transcription-polymerase chain reaction (RT-PCR) strategy was used, according to the methods of Suzuki et al. (1991). RT-PCR was performed using degenerate oligonucleotides corresponding to two conserved regions in the cytoplasmic domain of cadherins as primers and total RNA extracted as template. RT-PCR was performed using Titan™ one tube RT-PCR kit (Mannheim Boehringer, Laval, QC) wherein RT was performed at 50°C for 30 min and PCR stages namely, denaturation,

annealing and elongation were performed at 94°C for 1 min, 50°C for 2 min and 72°C for 3 min for 35 cycles and a final extension of 15 min was performed at 72° C. The resultant RT-PCR products (155 bp) were sub-cloned into the PCR-II vector (Invitrogen, Burlington, ON) and subjected to DNA sequence analysis using an automated DNA sequencer employing Taq DiDeoxy sequencing reagents.

Primer Design and cDNA Preparation

Nucleotide sequences for non-conserved regions within the bovine cadherin cDNA sequences and/or human/murine homologue(s) deposited in the Genbank were identified using BLAST (Basic Local Alignment Search Tool) computer program (NCBI, Bethesda, MD). Primers were designed using the 'Primer3' computer program (Whitehead Institute, Cambridge, MA) and the corresponding oligonucleotides and primers specific for bovine 18 S ribosomal RNA subunit (Table 2.2.1), which served as an internal control for these studies were synthesized (Invitrogen, Burlington, ON). The use of 18 S ribosomal RNA subunit as an internal control for understanding bovine preattachment embryonic gene expression has previously been undertaken (Mohan et al., 2002b).

PCR was performed on three separate occasions using the specific cadherin primers or 18 S ribosomal RNA and first strand cDNA generated from mRNA extracted from bovine embryos at different stages of development using First-strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Baie d` Urfe`, QC). These PCR products were sub cloned into the PCR II vector (Invitrogen, Burlington, ON). DNA sequence analysis of these clones was performed to confirm the specificity of these primers. These plasmids were also used to generate cDNA probes for each of the cadherin subtypes identified in the embryo and the 18 S rRNA subunit using standard molecular biology techniques.

Semiquantitative Polymerase Chain Reaction

PCR was performed using primers specific for either the cadherins or 18 S ribosomal RNA and template cDNA (2 μl) synthesized from total RNA extracted from bovine embryos at timed stages of development. Non-reverse transcribed RNA or water were included with the primer sets and served as negative controls. The PCR cycles were repeated 20-40 times to determine a linear relationship between the yield of PCR products and the number of cycles.

Southern Blot Analysis

Following standardization with the amount of 18S ribosomal RNA present in each sample, equivalent amounts of the specific cadherin PCR products were used for Southern blot analysis. Aliquots (20 μl) of PCR products were generated from the bovine embryos and separated by electrophoresis in a 1.2% agarose gel and subsequently denatured with 0.5 M NaOH for 5 min, neutralized with 1 M TRIS-HCl for 5 min. and transferred onto a charged nylon membrane (Hybond+, Amersham Canada Ltd. Oakville, ON) using standard Southern blot procedures. The blots were probed with a radiolabeled ^{32}P cDNA specific for embryonic cadherin subtypes according to the methods of MacCalman et al. (MacCalman et al., 1992). The blots were then washed twice with 2x SSPE at room temperature, twice with 2x SSPE containing 0.1% SDS at 55°C and twice with 0.2x SSPE at room temperature. The resulting autoradiograms were then scanned and subjected to densitometry (Scion Corporation, Frederik, MD). The absorbance values obtained for the cadherin PCR products were normalized relative to corresponding ribosomal 18S RNA absorbance value.

Immunocytochemical Analysis

To analyze the distribution of E-cad, P-cad, N-cad, cad-8 and -11 in the bovine embryo during the preimplantation stages, a whole mount peroxidase-diaminobenzidine (DAB)

immunohistochemistry technique previously described by Gestios et al. (1998a; 2001) for immunolocalization of cadherins in human cytotrophoblast cells was used. The embryonic zona pellucida (except for the hatched blastocyst) was removed by incubating the embryos in acid tyrode's buffer (pH 2.1) for 1-3 min. Subsequently, the embryos were washed in TCM 199 medium at 39°C for 10 min and fixed in 4% w/v paraformaldehyde for 1 h at room temperature prior to being immunostained.

The fixed embryos were immunostained using mouse monoclonal antibodies [E-cad, N-cad (Transduction Laboratories, Mississauga, ON), cad-11 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)] and goat polyclonal antibody [P-Cad, cad-8 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)] directed against human cadherin subtypes. Sequential incubations were performed according to the methods of Cartun and Pedersen (1989) and included 10% normal horse/goat serum for 30 min, primary antibody at 37°C for 1h, secondary biotinylated antibody at 37°C for 45 min, streptavidin-biotinylated horseradish peroxidase complex reagent at 37°C for 30 min, and three washes (5 min each) in PBS. The embryos were then exposed to a chromagen reaction solution (0.035% diaminobenzidine and 0.03% H_2O_2) for 10 min, washed in water for 5 min and counterstained with haematoxylin and viewed under Nikon Optiphot-2 phase contrast microscope. The procedure was repeated as many as three times for each embryonic stage and in total approximately, 100 embryos for each stage were examined per cadherin subtype. For each trial, secondary antibody controls and IgG controls were conducted to ensure optimal experimental conditions for the immunoassay protocol. Primary antiserum was omitted for secondary antibody controls in order to determine the background DAB staining. For IgG controls, the first antibody was replaced by normal mouse IgG (Dako Corporation, Carpinteria, CA).

Statistical Analysis

The results are presented as the mean relative absorbance (± SEM) for three independent experiments. Statistical differences between time points were assessed by the analysis of variance (ANOVA). Differences were considered significant for $p \leq 0.05$. Significant differences between the means were determined using the least significant difference test.

Results

Characterization of the Cadherin Subtypes during Early Embryonic Development

Multiple cadherin subtypes were identified in the preimplantation bovine embryo from the 2-cell to the blastocyst stage using degenerate primers. Transcripts encoding for E-cad, P-cad and N-cad mRNA were detected in 2-cell bovine embryos through to the blastocyst stages. In addition, mRNA transcripts encoding the type 2 cadherins known as cad-8, cad-10 and cad-11 were also detected in total RNA extracted from embryos at different stages of development.

Regulated Expression of Cadherin Subtypes mRNA during Early Embryogenesis

Southern blot analysis demonstrated that levels of cadherin subtypes are regulated during embryonic expression (Figure 2.2.1). In general, mRNA levels for all cadherin subtypes were high during the early stages of development (2-cell stage) and decreased as development progressed to 4-cell (cad-10), 8-cell (N-cad, P-cad, cad-8 and cad-11) or 16-cell (E-cad) stage (Figure 2.2.2; A and B). There was a significant increase in all of the cadherins mRNA levels (Figure 2.2.2; A and B) at the morula stage. The mRNA levels of these cadherins continued to increase with the formation of the blastocyst.

Immunolocalization of cadherin proteins during early embryonic development

Whole mount immunohistochemistry revealed immunoreactive E-cad, N-cad, P-cad (Figures 2.2.3) and cad-8 and cad-11 (Figure 2.2.4) subtypes across early stages of bovine

embryonic development. Staining for all cadherin subtypes was detected at the cell periphery with maximum concentration being in the cleavage furrow between the blastomeres during 2- and 4-cell stages of development. However, at 8- and/or -16 stage, in addition to the presence of cadherin subtypes at the cell-cell contact, some blastomeres showed intense staining at the free apical surface. At the blastocyst stage, E-cad immunostaining was localized at the trophectodermal cell junctions, perinuclear region of the trophectodermal cells and also in the regions of the inner cell mass (ICM) (Figures 2.2.3 d). Cad-11 showed a similar pattern of immunostaining in the blastocyst (Figures 2.2.4 d). In contrast to the distinct trophectoderm associated localization of E-cad and Cad-11 in the blastocyst, no significant trophectodermal presence was seen for N-cad, P-cad and Cad-8 (Figures 2.2.3h, 2.2.3l and 2.2.4h respectively). The presence of N-cad, P-cad and Cad-8 protein was observed only in the ICM region of the blastocyst (Figures 2.2.3h, 2.2.3l and 2.2.4h respectively).

Discussion

Multiple cadherin subtypes are expressed during bovine preimplantation development. Using the degenerate RT-PCR technique, which was first used to identify cadherin subtypes in the embryonic brain (Suzuki et al., 1991) and subsequently to characterize the cadherin expression in the human trophoblast cells (MacCalman et al., 1996; MacCalman et al., 1997), we have been able to identify a gamut of cadherin subtypes, both type 1 and type 2, being expressed during early embryonic development in the bovine embryo. In particular, transcripts encoding E-cad, P-cad, N-cad, cad-8, cad-10 and cad-11 are present from early cleavage to the blastocyst stage in the bovine embryo. Although, the pattern of E-cad distribution agrees with the findings reported earlier (Barcroft et al., 1998; Natale et al., 2001; Niemann and Wrenzycki, 2000; Shehu et al., 1996; Wrenzycki et al., 1998), this is the first report of P-cad, N-cad, cad-8, cad-10 and cad-11 distribution in bovine embryos. While the expression of N- and P-cadherin has been

studied in mouse embryos (Collins and Fleming, 1995; Fleming et al., 1994; Vestweber et al., 1987); to our knowledge this is the first report demonstrating the presence of type 2 cadherins in preimplantation embryogenesis.

Each member of the classic cadherin family exhibits a specific spatiotemporal pattern of expression during embryogenesis (Radice et al., 1997a). A switch in cadherin expression coincides with major morphological events such as gastrulation, neuralation, cardiogenesis and somitogenesis (Duband, 1990; Hatta et al., 1987; Hatta and Takeichi, 1986). Expression of cadherins in developing embryos has been studied in the mouse and chicken (Hatta et al., 1987; Nose and Takeichi, 1986; Takeichi, 1988). In mice, E-cad is first expressed at the one cell stage (Ogou et al., 1982) and subsequently plays an important role in compaction at the 8-16 cell stage (Hyafil et al., 1981; Johnson et al., 1986; Vestweber and Kemler, 1984). At the blastocyst stage, E-cad is expressed in all cells of the mouse embryo (Takeichi, 1988). Our findings for E-cad correlate with the above results and are in accordance with those of Hyafil et al. (Hyafil et al., 1981) and Vestweber et al. (Vestweber et al., 1987), who observed that E-cad is present in mouse embryos from one cell stage to the blastocyst stage. Although, Shehu et al. (Shehu et al., 1996) reported the presence of E-cad from eight-cell stage through to the blastocyst stage in bovine embryos, Barcroft et al. (1998) conclusively demonstrated that in bovine, E-cad is present throughout the embryonic stages i.e. from the single cell stage to the blastocyst stage.

In bovine embryos, P-cad and N-cad are initially seen at the first cleavage which is in accordance with the findings of Harrouk et al. (Harrouk et al., 2000) in mice. However, these findings differ from the earlier reports of P-cad (Kadokawa et al., 1989) and N-cad expression in the mouse embryos. The reason for this discrepancy could be the use of the more sensitive RT-PCR protocol, than the northern blots as used in previous studies. Expression of cad-8, cad-10 and cad-11 mRNAs from 2-cell stage to the blastocyst stage in a manner similar to that of type 1 cadherins collectively suggests that both type 1 and type 2 cadherins are present throughout

blastocyst formation and there is no switching or differential expression of these cadherins in the bovine embryo during the period of investigation.

We also observed a distinct regulated spatio-temporal expression of cadherin subtypes from the 2-cell stage to the blastocyst stage. As the maternal to embryonic transition occurs around the 8-cell stage in bovine (Wrenzycki et al., 2001a), our results also show that the transcripts for all the cadherins decrease upto the 4- or 8- or 16-cell stage and then increase up to the blastocyst stage. This signifies that the transcripts for all the cadherin subtypes have both maternal and embryonic origin. Maternal to embryonic transition is a crucial phase in the early development which is characterized by onset of transcription and subsequent translation of mRNA transcripts and on the other hand degradation of maternal messages and proteins in a coordinated fashion (Natale et al., 2000). The transition from maternal to embryonic genome control in the early mammalian embryo is not fully understood (Lee et al., 2001a), however evidence suggests that acquisition of a transcriptionally repressive environment and changes in chromatin structure by alteration of histone deacetylase activity can block or stimulate repression of markers of genome activation (Davis et al., 1996; Worrad et al., 1995).

Cell adhesion in early embryos has been shown to play an important role in allocation of blastomeres to the two cell lineages of the blastocyst i.e. the TE and the ICM and also in ensuring that appropriate number of cells enter the trophectoderm and ICM cell populations (Fleming et al., 2001). Our findings clearly demonstrate that cadherin subtypes show a distinct pattern of aggregation and sublocalization during preimplantation embryogenesis and thereby could be playing an important role in cell differentiation and cell allocation. All cadherin subtypes show cell-cell contact between blastomeres during 2-, 4- cell stage embryos, but with varying intensity. It is around the 8- or 16-cell stage that the blastomeres take up irregular intensity of reaction with some blastomeres staining more at the cell margins than the others. This could be explained, as cell fates in bovine preimplantation development are less stringently

regulated than in murine (Betteridge and Flechon, 1988; Telford et al., 1990). For example, according to the inside/outside hypothesis, the two cell populations producing ICM and trophectoderm in the mouse are derived from the inner and outer cell layers of the morula (Johnson et al., 1986). In porcine and bovine embryos, compaction and ICM allocation seems to be an independent processes and allocation may even be random. It is therefore, tempting to speculate that this distinct localization of cadherin subtypes in the embryo during preimplantation development could be playing a key role in cell allocation. At the blastocyst stage, P-cad is localized in the ICM and not in the trophectoderm. This could be explained by the fact that bovine placenta does not express P-cad (Liaw et al., 1990) and hence its presence cannot be detected in the trophectoderm. Like P-cad, significant levels of N-cad and cad-8 are localized predominantly in the ICM of the bovine blastocyst. At the time of cavitation onset presence of E-cad and to some extent cad-11, get involved in the formation of both TE and the ICM while N-cad, P-cad and cad-8 get involved with the formation of the ICM cells. While E-cad has been established as an important marker for blastocyst development (Barcroft et al., 1998; Niemann and Wrenzycki, 2000; Watson and Barcroft, 2001), identification of N-, P-cad and cad-8 specifically in the ICM region of the blastocyst suggests that collectively, these cadherin subtypes could be playing an integral role in blastocyst formation and in laying down the foundation for subsequent embryogenesis.

Studies have proven that the normal pattern of cell lineage and allocation can be disturbed in different species by altering the environment by certain growth factors (Lea et al., 1996), steroids and antisteroidal drugs (Greenlee et al., 1999; Greenlee et al., 2000; Juneja and Dodson, 1990), culture conditions (Lane and Gardner, 1997; Van Soom et al., 1996) and levels of maternal diet. Since steroids are key regulators of cadherins (Chen et al., 1998; Chen et al., 1999a; Getsios et al., 1998b; MacCalman et al., 1995; Monks et al., 2001), it would be interesting to ascertain if the changes in cell lineages and allocation are due to the alterations in

the cadherin expression coordinated by steroids which are richly present in the uterine milieu. Further work needs to be done to understand this correlation.

In addition to the gene knockout studies for E-, N- and P-cad, recent knockouts for type 2 cadherins analyzed the direct role of these cadherins in early embryogenesis and provided useful insights into their biological roles. In the absence of cad-6 mediated cell adhesion in cad-6 knockout mice, the embryo develops normally. However, subtle defects are prevalent in developing kidneys resulting in significant loss of nephrons (Mah et al., 2000). However, this reduction in nephron number is not sufficient to compromise renal function and therefore results in normal development. Similarly, using double gene knockouts for N-cad and cad-11 it has been demonstrated that loss of both these cadherins did not affect the patterned expression of somites as they still underwent clustering, suggesting that other adhesion molecules are being expressed and functioning (Horikawa et al., 1999). In a recent study, overlapping roles of cadherins during nephrogenesis were examined using R-cadherin (R-cad)$^{-/-}$, P-cad$^{-/-}$ and N-cad$^{+/-}$ mice (Dahl et al., 2002). Surprisingly, kidney development was not affected to a greater extent than within the individual knockout strain suggesting a compensatory or an overlap role. Heterophilic interactions ranging from incomplete to complete have been observed in type 1 (Renaud-Young and Gallin, 2002) and type 2 cadherins (Shimoyama et al., 2000). The combinations of cad-8 and -11, cad-9 and -10, cad-6 and -9, and cad-7 and -14 have been shown to interact in a complete manner, and in particular cad-7 and -14 and cad-8 and -11 showed indistinguishable binding properties (Shimoyama et al., 2000). Even though the data for heterophilic interactions have been obtained from in vitro studies, it is useful in explaining, at least in part, the reason for the unexpected results from cadherin gene knockouts. Further studies with multiple cadherin knockouts or individual functional assays are needed to support the compensatory role(s) of these cadherins.

In conclusion, these studies are the first to demonstrate that multiple cadherins are expressed in the early bovine embryo. The regulated expression of these cadherins suggests that they may play distinct role(s) in the development of the preimplantation embryo. The expression of multiple cadherins subtypes in bovine preimplantation embryos at all stages from 2-cell stage to the blastocyst stage strongly suggests a regulatory role of these cadherins in embryonic growth, differentiation and development. Based on their specific sublocalizations, it is tempting to speculate that these CAMs could be playing an important role in the allocation of blastomeres to the two cell lineages of the blastocyst. Future studies will define the biological significance of this repertoire of cadherins in the cellular events underlying embryogenesis.

Acknowledgments

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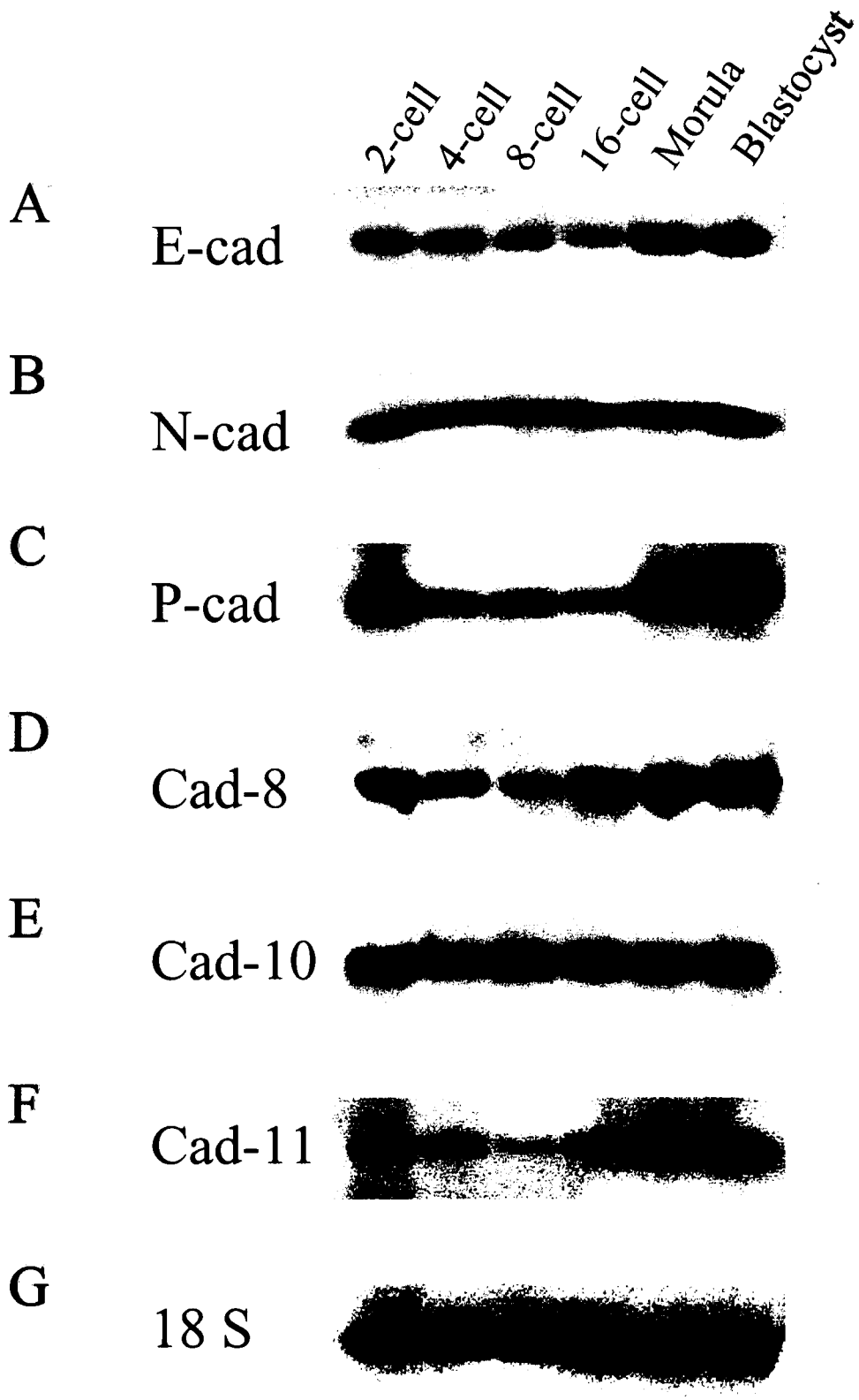

Fig. 2.2.1: Representative autoradiograms of Southern blots containing PCR products generated using template cDNA synthesized from total RNA extracted from timed stages of embryonic development (2-, 4-, 8-, 16-cell, morula or blastocyst) and primers specific for E-cad (Panel A), N-cad (Panel B), P-cad (Panel C), Cad-8 (Panel D), Cad-10 (Panel E), Cad-11 (Panel F) or 18 S ribosomal RNA (Panel G).

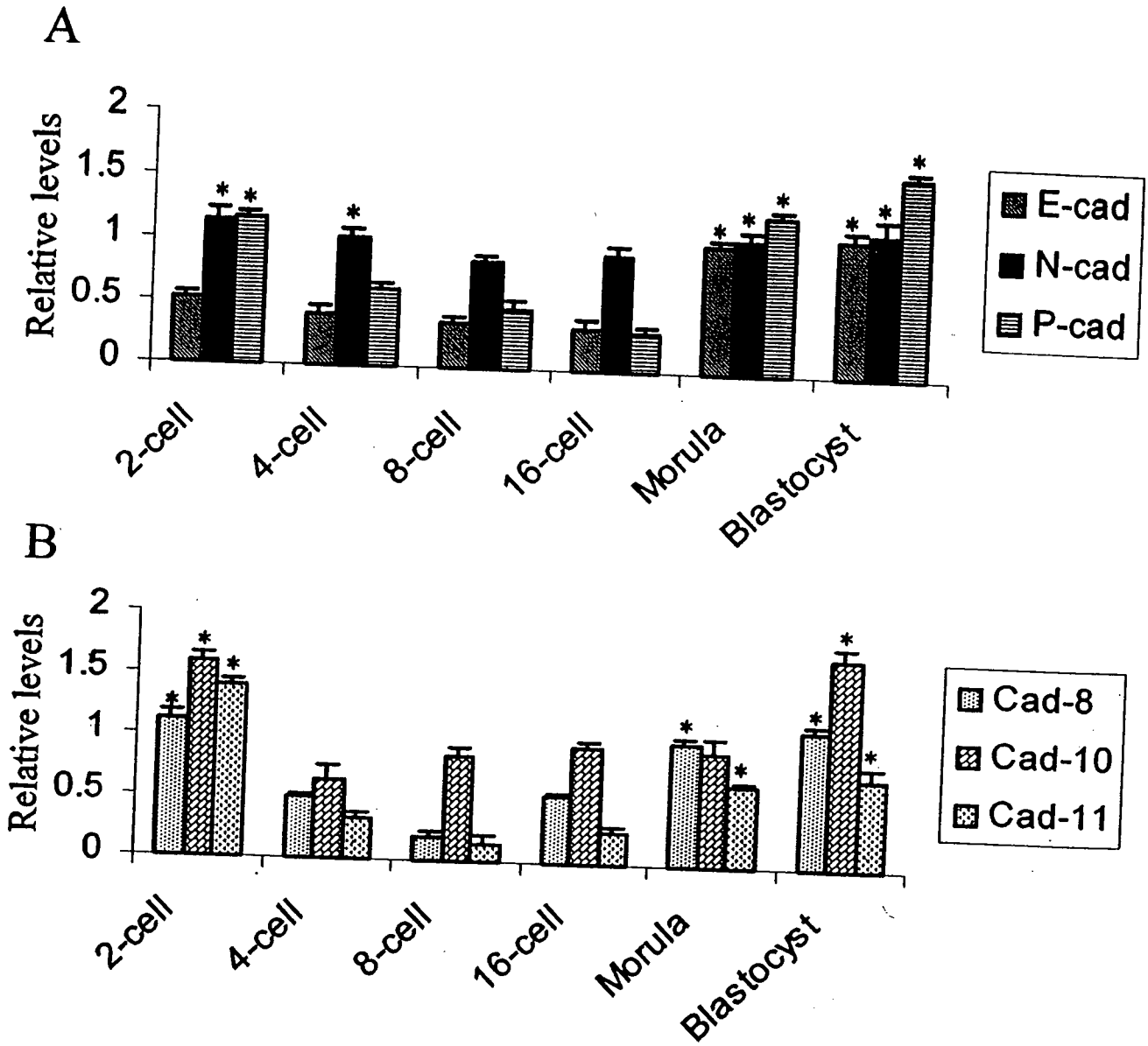

Fig. 2.2.2: Relative levels of the cadherin mRNA transcripts in the early preimplantation bovine embryos. Autoradiograms of Southern blots containing PCR products generated using template cDNA synthesized from total RNA extracts prepared from bovine embryos at timed stages of development (2-, 4-, 8-, 16-cell, morula or blastocyst) and primers specific for E-cad, N-cad, P-cad, Cad-8, Cad-10, Cad-11 or 18 S ribosomal mRNA were scanned using a laser densitometer. The absorbance values obtained for the distinct type 1 and type 2 cadherins (Panel A and B, respectively) were then normalized to the absorbance value obtained for the 18 S ribosomal RNA. The results are represented as mean± SEM in the bar graphs (n=3). Significant differences within the means of a cadherin subtype ($P \leq 0.05$) are represented as asterisks.

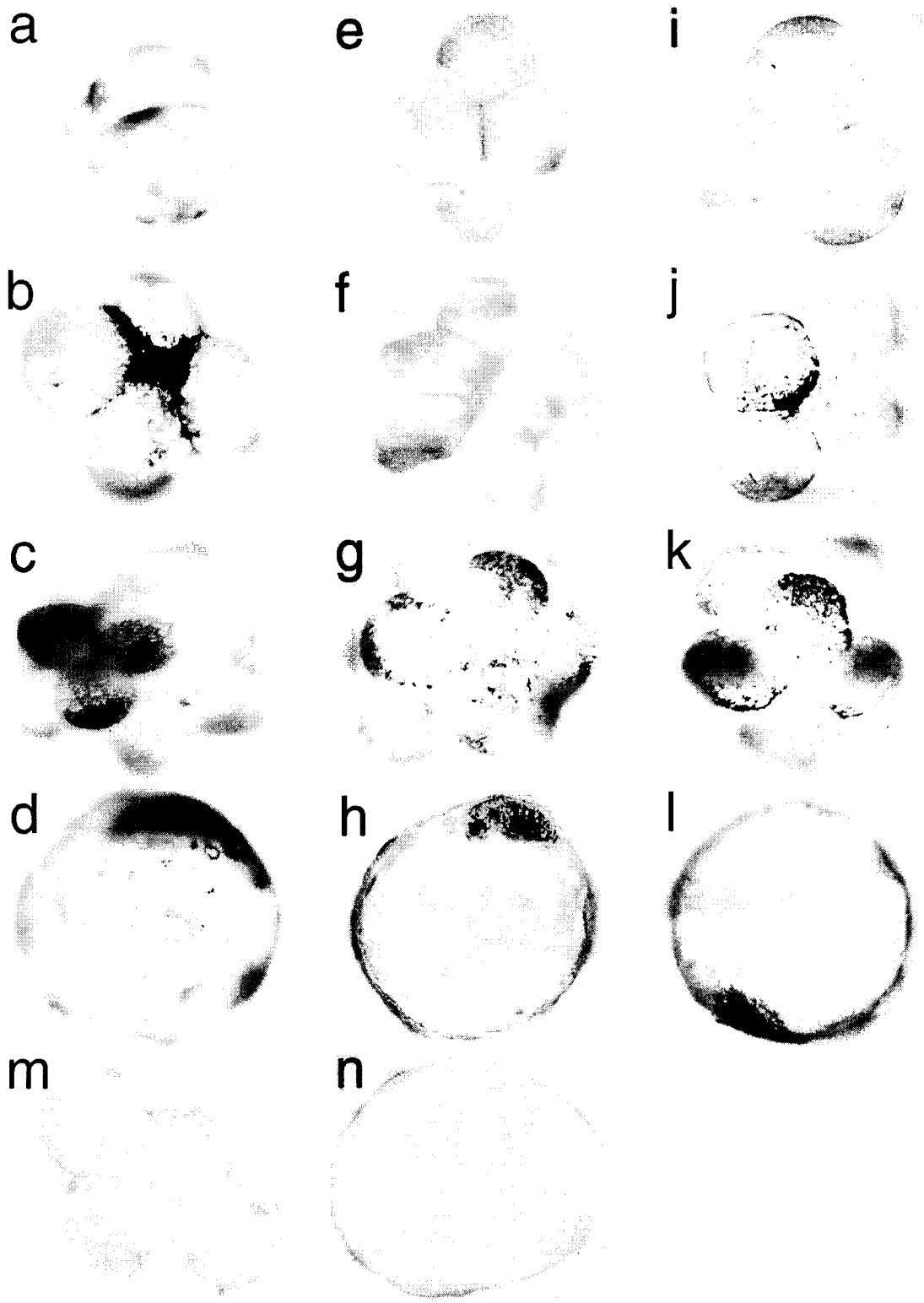

Fig. 2.2.3: Whole mount peroxidase diaminobenzidine (DAB) staining of bovine early preimplantation embryos for type 1 cadherins (E-cad, N-cad and P-cad) at timed stages of development. Representative 2-4 cell (a, e, i), 4-8 cell (b, f, j), 8-16 cell (c, g, k) and blastocyst (d, h, l) stage embryos immunostained with E-cad (a, b, c, d), N-cad (e, f, g, h) or P-cad (i, j, k, l) antibodies. Negative controls for cleavage and blastocyst stage embryos are represented in m and n.

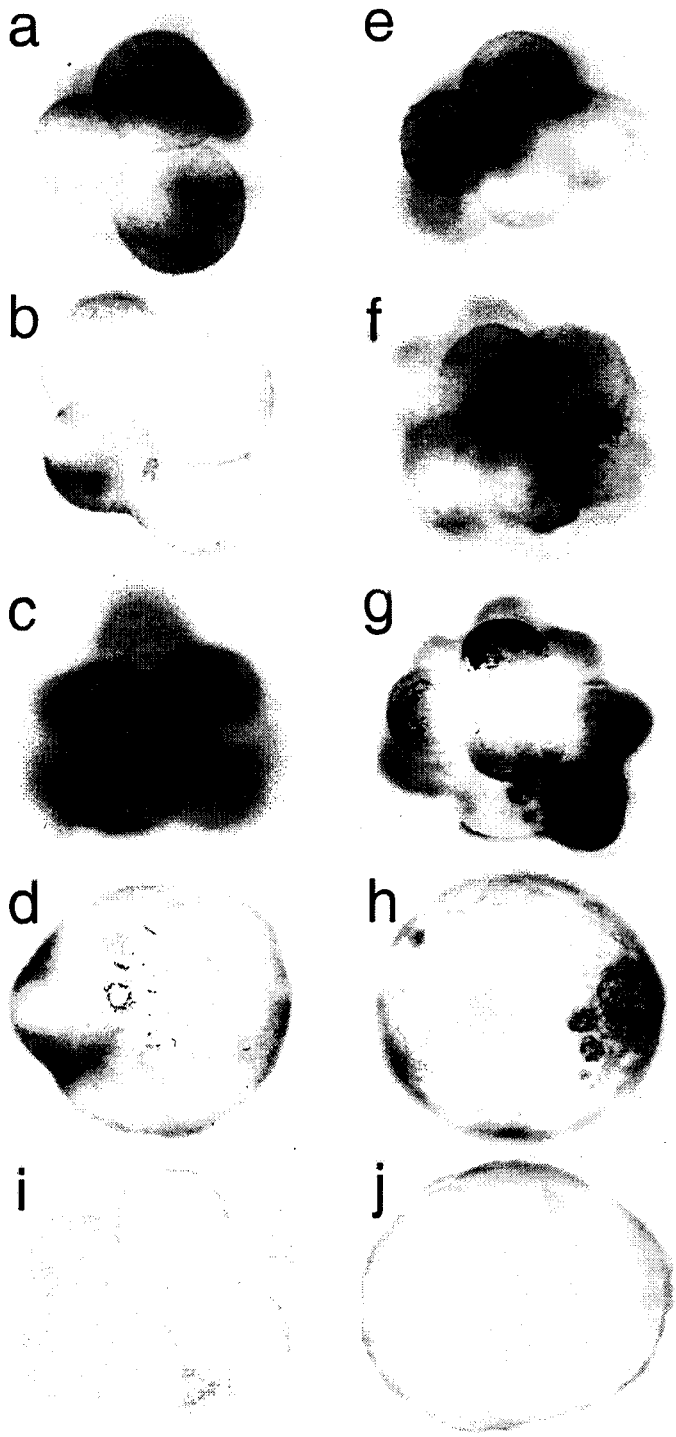

Fig. 2.2.4: Whole mount peroxidase diaminobenzidine (DAB) staining of bovine early preimplantation embryos for type 2 cadherins (cad-11 and cad-8) at timed stages of development. Representative 2-4 cell (a, e), 4-8 cell (b, f), 8-16 cell (c, g) and blastocyst (d, h) stage embryos immunostained with cad-11 (a, b, c, d) or cad-8 (e, f, g, h) antibodies. Negative controls for cleavage and blastocyst stage embryos are represented in i and j.

Cadherin Subtype	Primer Sequence	Estimated PCR Product Size	PCR Conditions
E-cad	Forward: 5'-TGAGGCCAAGCAGCAATACA -3' Reverse: 5'- TGCTGTTCTTCACATGCTCA-3'	350 bp	Denaturing: 94°C 30s Annealing: 60 °C 30s Extension: 72 °C 60s 35 cycles
N-cad	Forward: 5'- ACAATGGCTACCTGCAGAGG -3' Reverse: 5'-GGTTCTCCACTTGGTTTCCA-3'	300 bp	Denaturing: 94°C 30s Annealing: 60°C 30s Extension: 72 °C 60s 35 cycles
P-cad	Forward: 5'-CTCTCTGAGCTCGCTCACCT-3' Reverse: 5'- AAATCAAACTGCCCACGTTC-3'	303 bp	Denaturing: 94°C 30s Annealing: 55 °C 30s Extension: 72 °C 60s 35 cycles
cad-8	Forward: 5'-GACACGTGGCAGAGCTGTAA-3' Reverse: 5'TTTGGAGCGGTTCAAAATTC-3'	300 bp	Denaturing: 94°C 30s Annealing: 60 °C 30s Extension: 72 °C 60s 35 cycles
cad-10	Forward: 5'-ATGTTCAGAAGGACGCCTGT-3' Reverse: 5'-CCTTCAATGGTCCCGCCAATG-3'	298 bp	Denaturing: 94°C 30s Annealing: 55 °C 30s Extension: 72 °C 60s 35 cycles
cad-11	Forward: 5'-CTAGTAATTCTAACTGTATGT-3' Reverse: 5'-CCTTCAATGGTCCCGCCAATG-3'	750 bp	Denaturing: 94°C 30s Annealing: 60 °C 30s Extension: 72 °C 60s 35 cycles
18 S	Forward: 5'-GCTCGCTCCTCTCCTACTTG-3' Reverse: 5'-GATCGGCCCGAGGTTATCTA-3'	150 bp	Denaturing: 94°C 30s Annealing: 55 °C 30s Extension: 72 °C 60s 18 cycles

Table 2.2.1: Primer sequences and PCR conditions for the semiquantitative analysis of cadherin subtype mRNA levels in early preimplantation bovine embryos

CHAPTER III: IDENTIFICATION AND LOCALIZATION OF ESTROGEN AND PROGESTERONE RECEPTOR ISOFORMS DURING PREIMPLANTATION EMBRYOGENESIS IN BOVINES

3.1: Preface

In this section we have characterized and localized the expression of both estrogen and progesterone receptor isoforms during preimplantation bovine embryogenesis.

Blastocyst formation is a highly dynamic process wherein several intrinsic and extrinsic factors have been shown to influence preimplantation development. However, very little is known about the involvement of steroid hormones in regulating embryogenesis. Though it was demonstrated that both estrogen and progesterone are able to synchronize the development of the embryo, the effects were thought to be indirect. However, recent demonstrations of the presence of estrogen and progesterone receptors in preimplantation embryos of mice and pigs, has provided key evidence for the direct effect of these steroid hormones. However, such a demonstration is lacking for the bovine embryos. Moreover, with the identification of two isoforms for estrogen and progesterone, understanding the complex regulation of steroidal hormones during preimplantation embryogenesis has become more imperative. In order to address these outstanding issues, we have undertaken this study to characterize and localize the expression of estrogen receptors α and β, as well as of, progesterone receptors A and B.

Our studies demonstrate a differential expression of these steroidal receptors. While ERα mRNA levels are detected in the bovine embryo only at the blastocyst stage, where it is found localized more predominantly in the ICM region, ERβ mRNA levels can be detected throughout the early stages of development with ERβ protein being localized in the nucleus of the blastomeres as well as of the TE and ICM cells. Though, the expressions of PRAB and PRA

proteins are nuclear and perinuclear in the early cleavage stages, it becomes completely perinuclear at the blastocyst stage.

This study is the first demonstration of expression of ER and PR isoforms in the bovine embryo. In addition, this is the also the first attempt to understand the role of steroids on embryogenesis in an holistic manner since response of a cell to a hormonal stimulus would depend upon the ratio(s) of the expression of ER and PR isoforms. Moreover, this study also provides the first report of the localization of these steroid receptor isoforms on the bovine embryo. The results of these studies are in preparation as a manuscript entitled "Differential expression of estrogen and progesterone receptor isoforms during development of the preimplantation bovine embryos in vitro" for publication in the *Biology of Reproduction.*

3.2: DIFFERENTIAL EXPRESSION OF ESTROGEN AND PROGESTERONE RECEPTOR ISOFORMS DURING DEVELOPMENT OF THE PREIMPLANTATION BOVINE EMBROYS*

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Introduction

Estrogen and progesterone play key roles in the development and implantation of preimplantation embryos (Hou and Gorski, 1993). The effects of these steroids are mediated through interaction with specific intracellular receptors that are members of the nuclear receptor superfamily of transcription factors, which on attachment to their specific ligands play critical roles in modulating expression of target genes. Elimination of either estrogen or progesterone or both hormones from pregnant animals causes deleterious effects on the development and

implantation of embryos. Hypophysectomey or ovariectomy of pregnant rats results in retarded development of the embryos and delayed entry of the embryos into the uterus. However, this effect is reversed by the administration of estrogen and progesterone. Ovariectomized mice treated with progesterone only exhibited delayed implantation and dormant blastocysts could also be reactivated by administration of estrogen. Since the majority of these demonstrations were conducted on ovariectomized animals receiving exogenous hormones, it was unclear if the hormones were acting directly on the embryo or indirectly through the uterine environment.

Preimplantation embryos from different species including bovine have been shown to produce measurable amounts of steroid hormones and recently, the expression of key enzymes of steroid metabolic pathways has been detected in the bovine embryos (Chiappe et al., 2002). Under in vitro conditions and in the presence of estrogenic antagonists CI-628, nafoxidine and an aromatase inhibitor 1,4,6-androstatriene-3,17-dione reversibly inhibited the development of mouse embryos. In contrast, RU486 the potent progesterone antagonist, has been shown to have an inhibitory effect on the development of preimplantation embryos in mice (Juneja and Dodson, 1990). Interestingly, while estrogen has been shown to affect the uptake and incorporation of nucleic acid precursors and amino acids into mouse blastocysts (Harrer and Lee, 1973; Lau et al., 1973) and is routinely added to maturation media for bovine oocyte and embryo culture across many laboratories, addition of progesterone during in vitro maturation has shown to improve blastocyst production (Ryan et al., 1999).

Collectively these data suggest that steroids may be necessary for the development of the embryo and whose target could be both, the embryo itself and the uterus. However, for estrogen and progesterone to have any direct effect on the embryos, their receptors (ER and PR respectively) must be present on the embryo. This has been demonstrated in a number of studies in the mice and pig (Hiroi et al., 1999; Hou and Gorski, 1993; Hou et al., 1996; Kowalski et al., 2002; Ying et al., 2000a; Ying et al., 2000b). However, the presence of ER and PR has not been

reported in the bovine embryo, though both have been detected on the bovine ovary (Manikkam et al., 2001; Rosenfeld et al., 1999; Van Den Broeck et al., 2002; Van den Broeck et al., 2002) as well as in the uterus (Robinson et al., 2001). Based on the above evidence, it is highly unlikely that, the bovine embryo is merely a passive player during the early stages of development and its is quite possible that the intrinsic and extrinsic factors shown to influence preimplantation development could be mediated, at least in part, through the ER and PR.

Moreover, with the identification of two distinct estrogen (ER α and ERβ) (Green et al., 1986b; Kuiper et al., 1996; Mosselman et al., 1996) and progesterone (PRA and PRB) (Conneely et al., 1987b; Conneely et al., 1986; Kastner et al., 1990; Kraus et al., 1993) isoforms, understanding the biological response of estrogen and progesterone during embryogenesis has become more complex. The response of the embryo is likely to be dependent on the ratio(s) of the expression levels of these distinct ER and PR isoforms. In order to address these outstanding issues and also to get further insight into the estrogen and progesterone action during embryogenesis, we have examined the expression of ER and PR isoforms during development of the bovine embryo.

Materials and methods

In vitro production of bovine embryos

Bovine embryos were brought from local abattoir in sterile saline at 32-37°C for oocyte collection and in vitro embryo production as previously described (Giritharan and Rajamahendran, 2001). Briefly, cumulus-oocyte complexes were aspirated from small follicles and cultured in Tissue Culture Media 199 (TCM 199) containing 5% supervoulated cow serum (SCS), supplemented with FSH (0.01 μg) and antibiotics (50 μg/ml Gentamycin) at 38.5°C for 24 h. Approximately 30 of these matured oocytes were then incubated with 100 μl of frozen thawed semen, diluted to a final concentration of $5x10^6$ sperm/ml with Brackett and Oliphant

(BO) medium, for a further 16 -18 h (Brackett and Oliphant, 1975). The fertilized oocytes were washed twice with culture medium to remove excess sperm, placed in a four-well culture dish containing TCM 199 supplemented with 5% SCS, 5 μg/ml insulin and 50 μg/ml Gentamycin and allowed to undergo development before being harvested at timed stages of development (2-, 4-, 8-, 16-cells, morulae and blastocysts) for total RNA extraction.

Isolation of Total RNA

Total RNA was extracted from a pool of bovine embryos (25-30 embryos per stage) at timed stages of development using the phenol-chloroform method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). In order to eliminate possible contamination with genomic DNA, the total RNA extracts were treated with Deoxyribonuclease (DNAse)-I. The purity and concentration of total RNA present in each of the extracts were determined by optical densitometry (260/280 nm) using a DU-64 UV-spectrophotometer (Beckman Coulter, Mississauga, ON).

Primer Design

Nucleotide sequences specific for bovine/human estrogen and progesterone isoforms and 18 S rRNA subunit deposited in the GenBank were identified using the BLAST (Basic Local Alignment Seach Tool) computer program (NCBI, Bethesda, MD). Primers were designed using the "Primer 3" computer program (Whitehead Institute, Cambridge, MA). Forward and reverse oligonucleotide primers corresponding to these DNA sequences were synthesized (Invitrogen, Burlington, ON). The nucleotide sequences for each primer, the optimized conditions using a primer set for each PCR reaction and the expected size of PCR products are listed in Table 3.2.1.

Semiquantitative Polymerase Chain Reaction

PCR was performed on three separate occasions using primer sets specific for ERα, ERβ, PRAB or PRB and template cDNA synthesized from total RNA extracts of embryos and CL tissues, which served as technical controls for these studies. Non-reverse transcribed RNA or water was included with the primer sets and served as negative controls. Each of the steroid receptor isoforms were co-amplified with the 18 S rRNA. The PCR cycles were repeated 20-40 times to determine a linear relationship between the yield of PCR products and the number of cycles. The number of cycles used to amplify each steroid receptor isoforms from the embryo are listed in Table 3.2.1. To confirm the specificity of the primers, the resultant PCR products were subcloned into the PCR II vector (Invitrogen, Carlsbad, CA) and subjected to nucleotide sequence analysis using an automated DNA sequence analyzer (Applied Biosytems, Foster City, CA) employing DiDeoxy reagents. These clones were subsequently used to generate cDNA probes specific for each of the steroid receptor isoforms and the 18 S rRNA subunit using standard molecular biology techniques.

Southern Blot Analysis

Aliquots (20 μl) of the PCR products generated from the bovine embryos at different stages of development and CL tissues were separated by electrophoresis in a 1.2% agarose gel and visualized by ethidium bromide staining. The gels were then denatured with 0.5 M NaOH for 5 min, neutralized with 1M TRIS-HCl for 5 min and transferred onto a charged nylon membrane (Hybond$^+$, Amersham Canada Ltd., Oakville, ON). The Southern blots were probed with a radiolabeled-cDNA specific for each of the distinct steroid receptor isoforms or 18 S rRNA according to the methods of MacCalman et al. (MacCalman et al., 1992). The blots were then washed twice with 2 x SSPE at room temperature, twice with 2 x SSPE containing 0.1% SDS at 55°C and twice with 0.2 x SSPE at room temperature. The blots were subjected to

autoradiography to detect the hybridization of the radiolabled probes to the PCR products. The resultant autoradiograms were then scanned using a laser densitometer (Scion Corporation, Fredrick, MD) and the absorbance values obtained for each of the distinct steroid receptor isoform was normalized relative to the corresponding 18 S rRNA absorbance value.

Immunocytochemical analysis

To analyze the distribution of estrogen and progesterone receptor isoforms in the embryo, during the preimplantation stages, a whole mount peroxidase-diaminobenzidine (DAB) immunohistochemistry technique as described previously by Gestios et al. (1998a; 2001) was used. The embryonic zona pellucida was removed by incubating the embryos in acid tyrode's buffer (pH 2.1) for 1-3 min. Subsequently, the embryos were washed in TCM-199 medium at 39°C for 10 min and fixed in 4% w/v paraformaldehyde for 1 h at room temperature prior to being immunostained.

The fixed embryos were immunostained using the following, commercially avaiblabe antibodies: a polyclonal antibody directed against ERβ (Affinity Bioreagents, Golden, CO) and monoclonal antibodies directed against ERα, PRA or PRB (Dako Corporation, Carpinteria, CA). Sequential incubations were performed according to the methods of Cartun and Pedersen (1989) and included 10% normal horse/goat serum for 30 min, primary antiserum at 37°C for 1h, secondary biotinylated antibody at 37°C for 45 min, streptacidin-biotinylated horseradish peroxidase complex reagent at 37°C for 30 min, and three washes (5 min each) in PBS. The embryos were then exposed to chromagen reaction solution (0.035% diaminobenzidine and 0.03% H_2O_2) for 10 min, washed in water for 5 min and counterstained with haematoxylin and viewed under Nikon Optiphot-2 phase contrast microscope. The procedure was repeated as many as three times for each embryonic stage in total approximately, 100 embryos for each stage were examined per receptor isoforms. For each trial, secondary antibody controls and IgG controls

were conducted to ensure optimal experimental conditions for immunoassaying. Primary antiserum was omitted for secondary antibody controls in order to determine the background DAB staining. For IgG controls, the first antibody was replaced by normal mouse IgG (Dako Corporation, Carpinteria, CA).

Statistical Analysis

The results are presented as the mean relative absorbance ($\pm$ SEM) for three indepenent experiments. Statistical differences between time points were assessed by the analysis of variance (ANOVA). Differences were considered significant for $P \leq 0.05$.

Results

Regulated expression of steroid receptor isoforms during bovine preimplantation embryogenesis

Semiquantitiative PCR followed by Southern blot analysis using primers for the distinct ER and PR isoforms or 18 S ribosomal RNA demonstrated that ER and PR isoforms are highly regulated in their expression (Figure 3.2.1). ERα mRNA transcripts were detection only at the blastocyst stage of development. In contrast, ERβ, PRA and PRB mRNA levels were high at the 2-cell stage but declined at the 4-cell stage (Figure 3.2.2). The mRNA levels for ERβ increased at the 8-cell and maintained at the 16-cell and morula stages but declined at the blastocyst stage. Furthermore, ERβ mRNA levels decrease at the blastocyst stage with concomitant rise in ERα levels. PRA transcript levels increase significantly at the 8-cell stage, decrease at the 16-cell stage and then continue to decrease upto the blastocyst stage (Figure 3.2.2). We failed to detect PRB mRNA transcripts at the 16-cell and morula stages of preimplantation development but these transcripts were again detectable at the blastocyst stage.

Immunolocalization of steroid receptor proteins during bovine preimplantation embryogenesis

To determine if the mRNA for steroid receptor isoforms present in the bovine embryo is indeed translated into proteins during early embryogenesis, we used the immunohistochemistry to visualize the presence of ER and PR isoforms (Figures 3.2.3 and 3.2.4). ERβ proteins are present in the nucleus from the 2-cell stage to the blastocyst stage (Figure 3.3 e, f, g and h). Both the inner cell mass (ICM) and trophectoderm (TE) show the presence of this protein at the blastocyst stage (Figure 3.2.3 h). ERα proteins are seen predominantly in the ICM region of the blastocyst, though some ERα presence can be appreciated in the trophectodermal giant cell cytoplasm (Figure 3.2.3d). PRAB and PRB immunostaining showed that both these isoforms are present from 2-cell to the blastocyst levels (Figure 3.2.4). Expression for both PRAB and PRB is cytoplasmic at 2-cell stage but by 4-cell stage, nuclear/perinuclear appearance of both isoforms is detected. However, at the 8-cell stage, while the appearance of PRB is strongly nuclear, PRAB shows predominant perinuclear localization. At the blastocyst stage, both PRAB and PRB proteins can be detected in the TE giant cells in the perinuclear region, as well as in the ICM region of the blastocyst.

Discussion

Previous studies have demonstrated the presence of ER isoforms and PR in the murine and porcine embryos (Hiroi et al., 1999; Hou and Gorski, 1993; Hou et al., 1996; Kowalski et al., 2002; Ying et al., 2000a; Ying et al., 2000b), however, a direct comparison of the expression patterns of ER and PR during the preimplantation development in these species or, for that matter, in any mammalian species has not been reported. This is also the first study to show the expression of steroid receptors in the bovine embryo. In the present study, the developmental expression patterns of both ER and PR isoforms in bovine embryos, at timed stages of development have been determined by semiquantitative RT-PCR and their localization

ascertained using immunocytochemical technique. Our results indicate that all four isoforms are expressed during bovine preimplantation development, though this expression is differential.

The presence of ERα expression at the blastocyst stage partly correlates with the findings of previous studies in mouse and pig embryos (Hiroi et al., 1999; Hou and Gorski, 1993; Ying et al., 2000a). Though, in these studies ERα mRNA was found at 2-cell and 4-cell stages of development, we could not detect the expression of ERα mRNA in these early stages of bovine embryo development. The presence of ERα mRNA at the blastocyst stage correlates to the recent findings of Kowalski et al. (2002), who demonstrated that ERα gene expression was barely detectable in the porcine preimplantation embryos between day 11 and 14 of pregnancy, at the time of attachment. Our finding of ERβ expression partly correlates with the findings of Hirori et al. (1999) as they observed the expression of ERβ mRNA only at the 2-, 4-cell and blastocyst stages of development. However, our study shows that in addition to above stages, ERβ gene expression could also be detected at the 8-, 16-cell and morula stages of development. Interestingly, these levels of ERβ mRNA decline at the blastocyst stages, which correlates with the decline observed in ERβ levels in the procine embryos around day 12 of pregnancy (Kowalski et al., 2002). These differences in expression patterns between different studies could be either due to the use of different detection methods and/or sensitivity of the respective RT-PCR techniques used or because of species specific differences (Hiroi et al., 1999; Ying et al., 2000a).

Conflicting reports exist regarding the presence of PRA in the preimplantation embryo. While Hou et al. (1993) demonstrated that PRA is present only at the blastocyst stage of murine development, Ying et al. (2000b) reported the presence of PRA at the 2- and 4-cell stage of embryonic development in pigs. However, our results demonstrate that both PRA and PRB isoforms are present during all stages of bovine preimplantation embryo development, though

their levels vary from the 2-cell to the blastocyst stage. Collectively, ERβ, PRA and PRB mRNA are present at both early and late stages of the bovine preimplantation embryo suggesting that these isoforms of steroid receptors are both maternal and embryonic in expression. Transcripts for all these three subtypes decrease from the 2-cell stage to the 4-cell stage suggesting depletion of maternal transcripts in correlation with other studies reporting that maternal to embryonic transition occurs around the 8-cell stage in bovines. While transcripts of ERβ and PRA increase at the 8-cell stage, suggesting immediate contribution from the embryonic genome, mRNA transcripts for PRB and ERα rise only at the blastocyst stage. Emergence of ERα and reemergence of PRB mRNA transcripts at the blastocyst stage suggests important roles during this stage of development.

The results of the immunocytochemical analysis demonstrate that these steroid receptor mRNAs are being translated into specific proteins. Distinct ERβ immunoreactivity was observed specifically in the nucleus of each blastomere suggesting that these isoforms are functional at this stage. Moreover, at the blastocyst stage presence of ERβ protein in the nucleus of trophectoderm suggests an intergral role during trophectoderm formation and a putative role in attachment and or formation of the placenta. In contrast, immunoreactivity for ERα proteins is detected only at the blastocyst stage, where they seem to be localized predominantly in the inner cell mass region of the blastocyst. However, they are detected in the trophectoderm as well, but the immunostaining was weaker compared to the ICM suggesting a possible role in the formation of the embryo proper. As immunoreactivity for PRAB and PRB is cytoplasmic at the 2-cell stage but by the 4-cell stage it is predominantly nuclear/perinuclear, it suggests that both isoforms could be active in the embryo at this stage. The strong presence of PRAB in the nuclear region at the 8-cell stage suggests that PRA isoforms could be playing a role in the maternal to embryonic transition (MET) as expression for PRB at this stage is less nuclear and more perinuclear. At the

blastocyst stage, both isoforms are seen localized in the perinuclear region of the TE cells as well as in the ICM region. The cytoplasmic presence of PR nuclear receptor isoforms is not surprising as distinct intracellular distribution for PR isoforms has been reported previously (Lantinga-van Leeuwen et al., 2000; Lim et al., 1999). Moreover, an increase in cytoplasmic PR has been suggestive of an increase in the synthesis of the receptor (Turgeon et al., 1999) suggesting an active production of the receptor isoforms at this stage. Although mRNA levels for ERβ decrease at the blastocyst stage, the ERβ proteins expression can still be detected at this stage. Similarly, even though, no mRNA levels were detected for PRB during the 16-cell and morula stage, PRB protein could still be detected using immunohistochemistry. This discrepancy could be explained to the fact that messenger RNA levels in preimplantation embryos have relatively short lives (Kidder and Pedersen, 1982; Niemann and Wrenzycki, 2000) as compared to proteins (Brinster et al., 1979).

ERα and ERβ, are both expressed in multiple cell types across various reproductive tissues and are involved in regulation of various reproductive processes in mice, rats, humans and bovine (Enmark and Gustafsson, 1999; Gustafsson, 2000a; Korach, 1994; Kuiper and Gustafsson, 1997). Although, ERβ is the more predominant subtype in tissues where both ERα and β are present (Enmark and Gustafsson, 1999; Gustafsson, 1999; Kuiper and Gustafsson, 1997), a differential expression of ERα and β has also been reported to occur in several normal and cancerous tissues (Critchley et al., 2001b; Pelletier and El-Alfy, 2000; Royuela et al., 2001; Saunders et al., 2002; Saunders et al., 2000; Saunders et al., 2001). Moreover, a dramatic switch in the expression of both receptor isoforms has also been reported in several physiological and pathological events like pregnancy (Benassayag et al., 1999; Wang et al., 2001; Wu et al., 2000) and breast cancers (Saunders et al., 2002; Tong et al., 2002). In addition, our findings of declining trends of ERβ and appearance of ERα at the blastocyst stage suggests that the embryo

could be countering the repressive effects of ERβ at this stage as direct repression of ERα expression by ERβ has also been demonstrated during bone formation (Windahl et al., 2001).

Both ERs have been shown to play different roles in regulating gene expression based on their distribution and structural similarities, though ERβ binds to estrogen with affinity similar to that of ERα (Kuiper et al., 1997; Kuiper et al., 1998). The finding that estrogen binding to ERα activates transcription, whereas ERβ-estrogen binding inhibits transcription (Paech et al., 1997) provides evidence of their different biological roles. Though, the estrogen receptor knockouts (ERKO) provide an insight into the biological roles of each ER, they fail to address the role of ERs during preimplantation embryogenesis as both ER knockouts are viable but exhibit severe gonadal and behavioural phenotypes (Lubahn et al., 1993). While αERKO mice of both sexes are infertile (Eddy et al., 1996), βERKO mice exhibit subfertility in females whereas the male fertility appears unaffected (Dupont et al., 2000; Krege et al., 1998). Recent findings of the viability of a double estrogen receptor knockout mice (DERKO) further complexes their role as its suggests that estrogen might be exerting some of its effects independent of either ERα and β (Dupont et al., 2000; Lindberg et al., 2002). Though, it has been pointed out that the ERKO mice used in the latter study might not be completely ERα inactivated (Pendaries et al., 2002), it does not rule out the possibility of several other estrogen isoforms or other nuclear receptors interacting or compensating for the specific loss or losses as studies have shown that steroids are capable of inducing retinoid receptors (Celli et al., 1996; Sucov and Evans, 1995). Recently, retinoid receptors have been detected in the preattachment bovine embryos (Mohan et al., 2001; Mohan et al., 2002a), however, further studies are needed to ascertain their interrelationship with steroids or steroid receptors in the embryo.

Although, recent findings have shown that altered ratios of PR isoforms are closely associated with modulations of various progesterone actions, the precise functions of PRA and

PRB have yet not been characterized (Bouchard, 1999; Conneely and Jericevic, 2002). In the majority of progesterone responsive cells, PRB is the dominant activator of progesterone responsive target genes, whereas PRA may inhibit the activity of PRB (Tung et al., 1993; Vegeto et al., 1993). This repressive activity also extends to other nuclear receptors including ER (McDonnell et al., 1994; Wen et al., 1994). In addition, PRA has also shown to suppress the expression of ERα through the H19 promoter in both hormone sensitive and hormone insensitive breast cancer cell lines (Adriaenssens et al., 1999). Our findings of the presence of higher levels of PRB at the blastocyst stage than those of PRA suggest that this could be the time, when the embryo could be becoming receptive to the progesterone responsive genes and could be preparing for the next step i.e. attachment. This timely expression of PRB gene and protein in blastocyst implies that the effect of progesterone can be exerted directly on the embryo. Conversely, the higher levels of PRA during earlier stages of development could mean that this protein could be involved in some inhibition of gene transcription induced by other families of steroid receptors including, glucocorticoids, androgens and mineralocorticoid receptors. However, distinct biological roles or the significance of PRA and PRB have yet to be elucidated in the embryo and in other progesterone target tissues.

Interactions and cross talk between estrogen and progesterone receptors in the embryo could be playing an important role in embryogenesis. The interrelationship of ER and PR has been well documented in the uterus, where it has been shown that estrogens increase cell proliferation, progesterone receptor mRNA and protein levels (Kraus et al., 1995; Kraus et al., 1997). These effects are largely antagonized by progesterone. As no such information on the cross talk of ER and PR is available during embryogenesis, a better understanding of how steroids are able to co-function and exert their effects, especially during the process of embryogenesis is required.

In summary, the differential expression of ER and PR isoforms during preimplantation development suggests that both ER and PR may have different functions during this critical window of embryogenesis and therefore, may play important roles(s) in the development of the preimplantation embryo. However, their relative physiological significance and specific roles during embryogenesis remain to be further elucidated.

Acknowledgments

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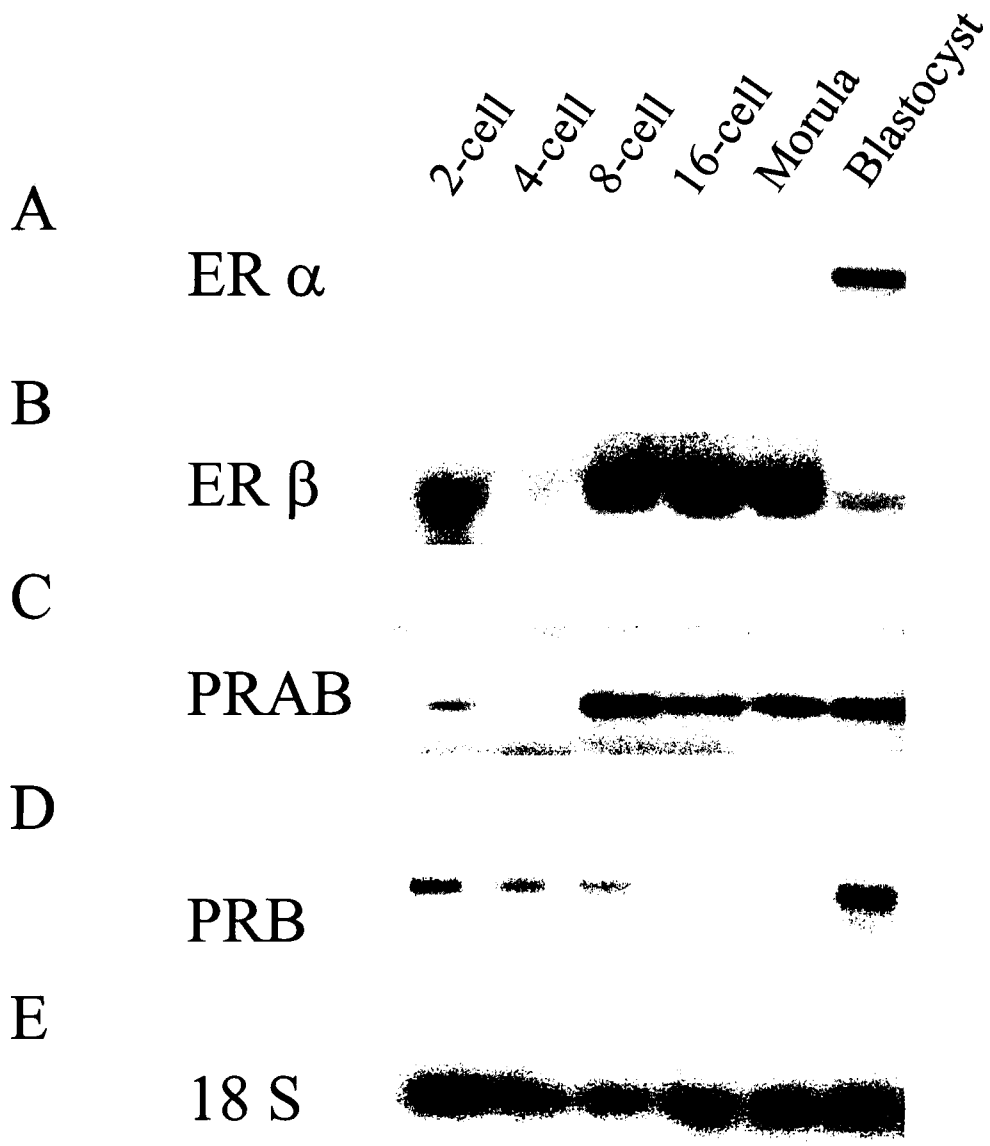

Fig. 3.2.1: Representative autoradiograms of Southern blots containing PCR products generated using template cDNA synthesized from total RNA extracted from timed stages of embryonic development (2-, 4-, 8-, 16-cell, morula or blastocyst) and primers specific for ERα (Panel A), ERβ (Panel B), PRAB (Panel C), PRB (Panel D) or 18 S ribosomal RNA (Panel E).

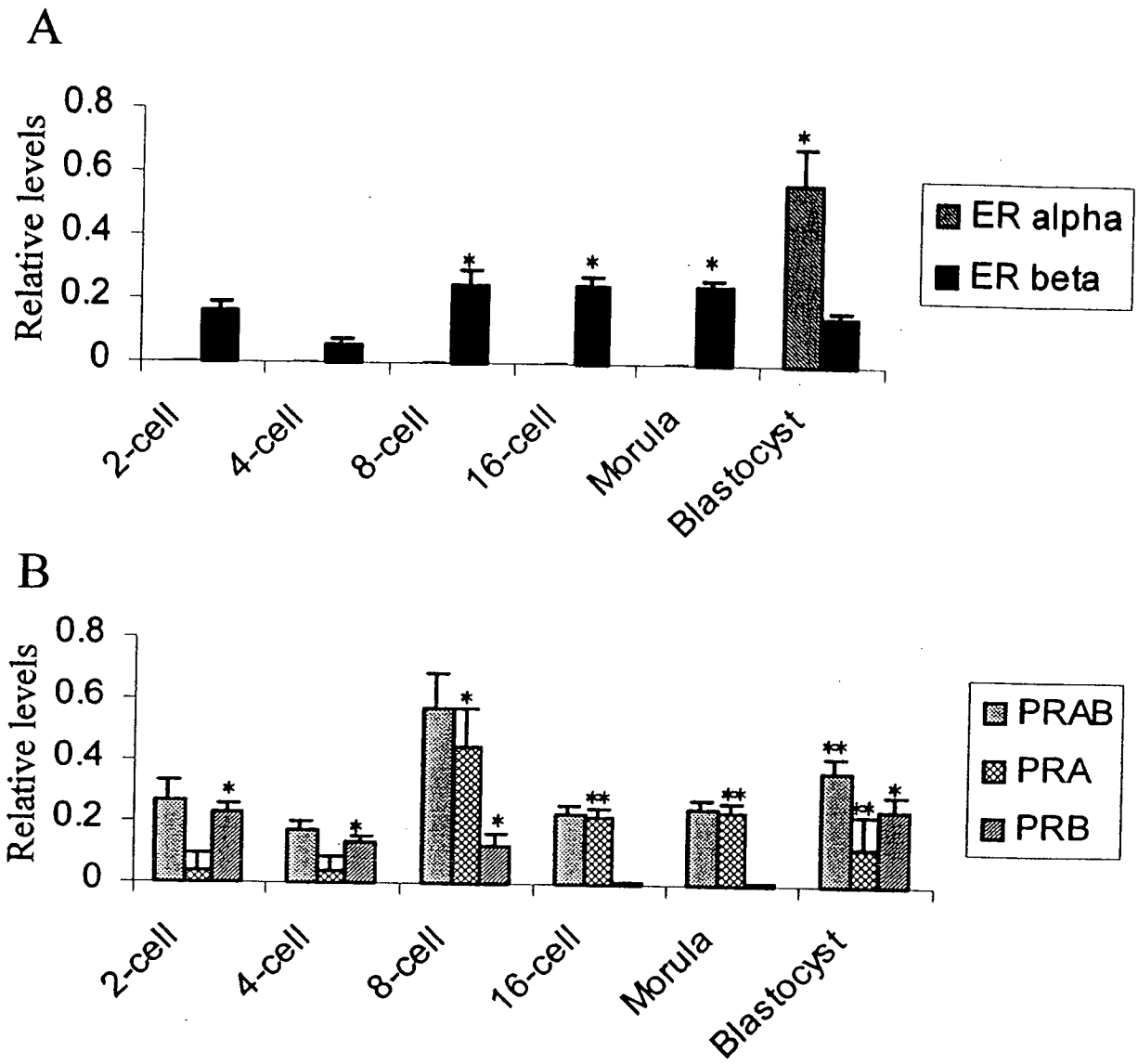

Fig. 3.2.2: Relative levels of the steroid receptor mRNA transcripts in the early preimplantation bovine embryo. Autoradiograms of Southern blots containing PCR products generated using template cDNA synthesized from total RNA extracts prepared from bovine embryos at timed stages of development (2-, 4-, 8-, 16-cell, morula or blastocyst) and primers specific for ERα, ERβ, PRAB, PRB or 18 S ribosomal mRNA were scanned using a laser densitometer. The absorbance values obtained for the distinct estrogen (ERα and ERβ) and progesterone receptor (PRAB and PRB) isoforms (Panel A and B, respectively) were then normalized to the absorbance value obtained for the 18 S ribosomal RNA. PRA expression levels were obtained by subtracting the intensity of common signal of PRAB amplification product minus the intensity of PRB amplification signal. The results are represented as mean± SEM in the bar graphs (n=3). Significant differences within the means of steroid receptor isoform ($P \leq 0.05$) are represented as asterisks.

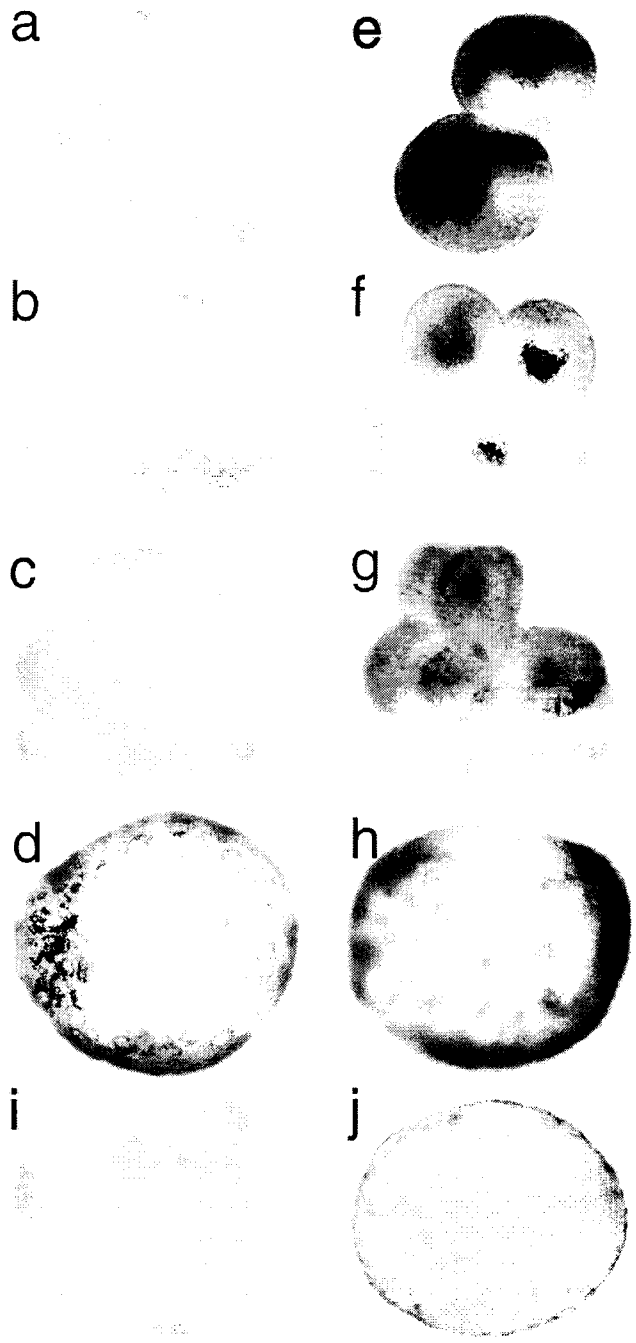

Fig. 3.2.3: Whole mount peroxidase diaminobenzidine (DAB) staining of bovine early preimplantation embryos for ERα and ERβ at timed stages of development. Representative 2-4 cell (a, e), 4-8 cell (b, f), 8-16 cell (c, g) and blastocyst (d, h) stage embryos immunostained with ERα (a, b, c, d) or ERβ (e, f, g, h) antibodies. Negative controls for cleavage and blastocyst stage embryos are represented in i and j.

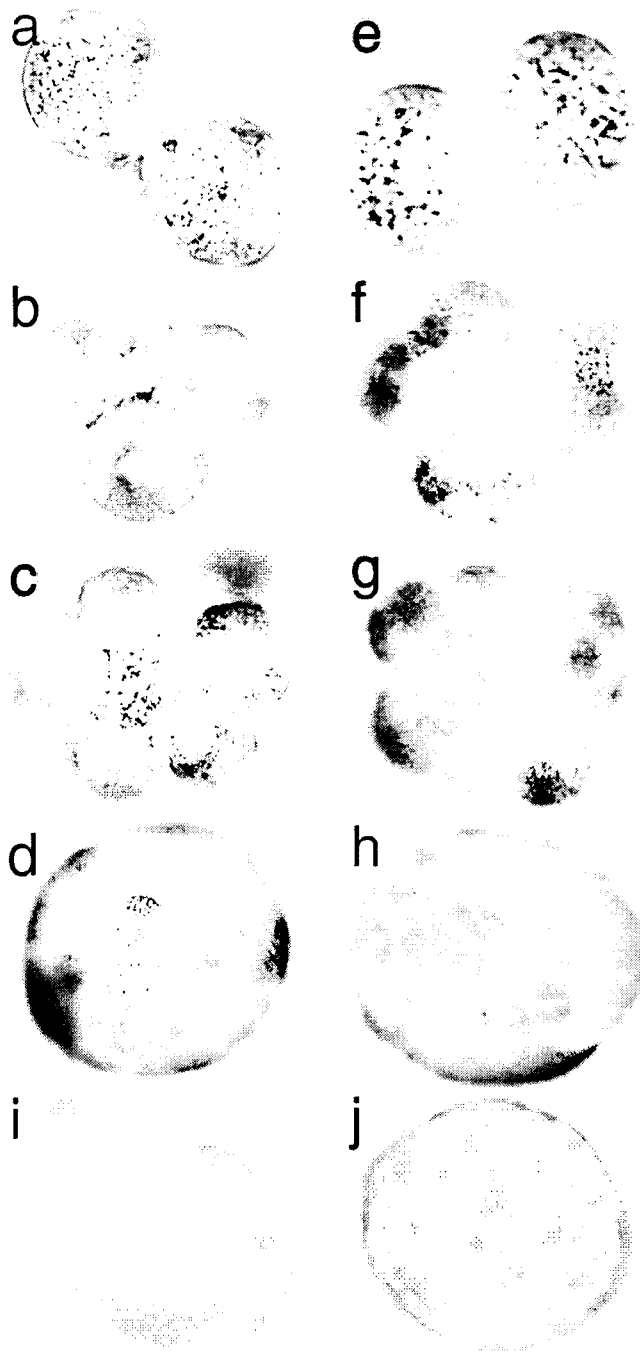

Fig. 3.2.4: Whole mount peroxidase diaminobenzidine (DAB) staining of bovine early preimplantation embryos for PRAB and PRB at timed stages of development. Representative 2-4 cell (a, e), 4-8 cell (b, f), 8-16 cell (c, g) and blastocyst (d, h) stage embryos immunostained with PRAB (a, b, c, d) or PRB (e, f, g, h) antibodies. Negative controls for cleavage and blastocyst stage embryos are represented in i and j.

Steroid receptor isoform	Primer Sequence	Estimated PCR Product Size	PCR Conditions
ERα	Forward: 5'-GAGATCCTGATGATTGGTCT -3' Reverse: 5'- CATCTCCAGCAGCAGGTCAT-3'	477 bp	Denaturing: 94°C 30s Annealing: 60 °C 30s Extension: 72 °C 60s 35 cycles
ERβ	Forward: 5'- GGACAGGGATGAAGGGAAAT -3' Reverse: 5'-AGACAGGAGCATCAGCAGGT-3'	304 bp	Denaturing: 94°C 30s Annealing: 65°C 30s Extension: 72 °C 60s 35 cycles
PRAB	Forward: 5'-ACAGAATTCATGAGCCGGTCCGGGTGCAAG-3' Reverse: 5'- ACAAGATCTCCACCCAGAGCCCGAGGTTT-3'	243 bp	Denaturing: 94°C 30s Annealing: 56 °C 30s Extension: 72 °C 60s 35 cycles
PRB	Forward: 5'-ACAGAATTCATGACTGAGCTGAAGGCAAAGGGT-3' Reverse: 5'-ACAAGATCTCAAACAGGCACCAAGAGCTGCTGA-3'	410 bp	Denaturing: 94°C 30s Annealing: 56 °C 30s Extension: 72 °C 60s 35 cycles
18 S	Forward: 5'-GCTCGCTCCTCTCCTACTTG-3' Reverse: 5'-GATCGGCCCGAGGTTATCTA-3'	150 bp	Denaturing: 94°C 30s Annealing: 55 °C 30s Extension: 72 °C 60s 18 cycles

Table 3.2.1: Primer sequences and PCR conditions for the semiquantitative analysis of steroid receptor isoform mRNA levels in early preimplantation bovine embryos

CHAPTER IV: EXPRESSION OF ADAMTS SUBTYPES DURING PREIMPLANTATION EMBRYOGENESIS IN BOVINES

4.1: Preface

In this study, we have characterized the expression of novel cellular molecules named ADAMTS, involved in cell-matrix interactions during preimplantation bovine embryogenesis.

The role of ECM and the importance of ECM remodeling during blastocyst formation is poorly understood. Recent studies have shown that ECM surrounding the cells is imperative in segregating cell types as well as in physically supporting cells and tissues. Though several proteolytic enzymes belonging to diverse protease families and playing a key role in embryonic ECM remodeling have been identified, functional roles of many of these remain poorly characterized. Moreover, evidence suggests that diverse members of the novel ADAMTS family are involved in key processes during tissue morphogenesis and post implantation embryogenesis. However, their role during early embryogenesis has not been ascertained. In view of these observations, we have studied the diverse subtypes of this family from ADAMTS-1 to-12 and characterized their expression during timed stages of development during bovine preimplantation embryogenesis.

Our results indicate that multiple ADAMTS subtype are expressed during bovine preimplantation embryogenesis, suggesting that major ECM remodeling could be playing a key role in cell migration, segregation and differentiation. Since, expression of these subtypes have been reported for numerous physiological and pathological processes, the highly regulated expression of these subtypes during the early cleavage and blastocyst stages suggests important biological roles, many of which remain to be elucidated. These studies are the first to report the presence of a diverse gamut of ADAMTS subtypes during preimplantation embryo development for any species. This is also the first demonstration of a regulated expression of these subtypes

from the 2-cell to the blastocyst stage during bovine preimplantation embryogenesis. The results of these studies are being submitted as a manuscript entitled "Regulated expression of ADAMTS subtypes during preimplantation bovine embryogenesis" in the *Biology of Reproduction.*

4.2: REGULATED EXPRESSION OF ADAMTS SUBTYPES DURING PREIMPLANTATION EMBRYOGENESIS IN BOVINES*

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Introduction

During embryogenesis, selective adhesive interactions, both cell-cell as well as between cell and the extracellular matrix (ECM) play an important role in determining the final organization of the early embryo (O'Shea et al., 1990). The ECM that surrounds each cell is imperative in segregating cell types and physically supporting tissues and organs, but in addition has recently been shown to play an active role as an instructional entity (Streuli, 1999). Moreover, dynamic biological processes like embryo development require efficient degradation and remodeling of the ECM and hence such proteolytic modification of the ECM are pivotal for the diverse array of biological processes including embryogenesis and implantation (Shindo et al., 2000). The proteolytic enzymes responsible for these morphogenetic events include members of diverse protease families and may work in concert or in cascades to degrade or process molecules (Hurskainen et al., 1999). Several key proteinases, including ones from the

* The work was conducted by PM under the supervision of RR and CDM. AGB provided the primer sequences for ADAMTS subtypes.

plasminogen activator (PA) family, and matrix metalloproteinases (MMPs) are known to be involved in ECM degradation (Sternlicht and Werb, 2001; Werb et al., 1996; Werb and Chin, 1998) and have been shown to be expressed during preimplantation development in several species including bovines.

Specifically, urokinase-type plasminogen activator (uPA), has been detected in blastocyst stage embryos from mouse, sheep, pigs and cows suggesting a role in implantation/attachment (Bartlett and Menino, 1993; Dyk and Menino, 1991; Fazleabas et al., 1983; Harvey et al., 1995; Menino and Williams, 1987; Sappino et al., 1989; Strickland et al., 1976; Zhang et al., 1994). However, a recent study has revealed that uPA is expressed and secreted from in vitro produced bovine embryos from the 2-cell stage onwards suggesting a much prior role of these proteinases (Whiteside et al., 2001b). Expression of MMP-1, -2, -3, -9 and -11 and has been observed in early murine embryos (Das et al., 1997; Kim et al., 2002; Lefebvre et al., 1995) and a similar repertoire excluding MMP-11 has been observed in ovine embryos (Riley et al., 2000; Salamonsen et al., 1995). In bovines, MMP-9 is expressed throughout the first 7 d of development (Whiteside et al., 2001b). In spite of the presence of these proteinases during early development, the functional roles of many of them during embryogenesis remain poorly characterized. Evidence suggests that MMP-9 and MMP-11 are involved in ECM degradation during implantation in mice but mice with null mutations for both MMP-9 and -11 are viable and fertile (Masson et al., 1998; Vu et al., 1998) suggesting that other enzymes might be involved in ECM degradation.

Recent cloning studies have identified a family of novel metalloproteinases, known as ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs), in C. *elegans*, *Drosophila* and mammals (Cal et al., 2002; Kaushal and Shah, 2000; Moerman, 1999; Tang, 2001; Tang and Hong, 1999). ADAMTS proteins have a characteristic amino terminal prodomain, followed by a putative prodomain, a catalytic domain, a disintegrin-like and a

cysteine-rich domain. In addition, ADAMTS proteins contain a thrombospondin type 1 (TSP-1) repeat found between the disintegrin-like and cysteine-rich domains. This is also followed by a varying number of TSP-1 like repeats at the C-terminus. ADAMTS proteins lack a transmembrane domain and are therefore secreted into the ECM (Kuno et al., 1997b; Vazquez et al., 1999). To date, 20 members of ADAMTS family have been identified in vertebrates (Cal et al., 2002). However, the majority of these ADAMTS subtypes have only been characterized at the structural level and their biological significance remains undetermined.

Recent knock out studies have highlighted the importance ADAMTS in tissue morphogenesis and development. ADAMTS-1 gene knock out studies shows that ADAMTS-1 is necessary for normal growth, development and function of kidneys, adrenal glands and the female reproductive organs (Shindo et al., 2000). ADAMTS-2 null mutants showed skin lesions having remarkable similarity to dermatosparaxis and Ehlers-Danlos syndrome type VIIC in animals and humans, respectively (Li et al., 2001c). In addition, homozygous male mice were sterile and showed decreased spermatogenesis. Collectively, these observations suggest that the ADAMTSs play a key role(s) in tissue morphogenesis during development and maintain the integrity of tissues in the adult. ADAMTS-2, -3, -5 and -8 mRNA have been shown to be tightly regulated during embryonic development and organ formation in the mouse (Fernandes et al., 2001; Georgiadis et al., 1999; Hurskainen et al., 1999). Despite the obvious importance of proteinases in adhesion and severance during morphogenetic processes of development and their presence during embryogenesis, the expression and function of the members of ADAMTS gene family involved in the early stages of embryogenesis have not been determined. In view of these observations, we have examined the expression of these novel sets of metalloproteinases, encoding ADAMTS-1 through -12, across different developmental stages of the preimplantation embryo in bovines.

Materials and methods

In Vitro Production of Bovine Embryos

Ovaries were collected from an abattoir and transported to the laboratory in sterile saline at 32-37°C for oocyte collection and in vitro embryo production. An in vitro fertilization protocol as previously described (Giritharan and Rajamahendran, 2001) was used for producing embryos in vitro. Briefly, oocyte cumulus complexes (COCs) from small non-atretic follicles (3-6 mm) were aspirated into the follicular aspiration media (Phosphate buffer saline [PBS] containing 0.3% bovine serum albumin [BSA] and 50 μg/ml Gentamycin) using an 18G needle attached to 10ml syringe. High quality, COCs were selected, based on stereomicrosopic examination, for maturation and were cultured in maturation media (Tissue Culture Media 199 [TCM 199]) supplemented with 0.01μg/ml follicle stimulating hormone (FSH), 5% supervoulated cow serum (SCS) and antibiotics (50 μg/ml Gentamycin) at 38.5°C and 5% CO_2 in a conventional incubator for 24 h. After 24 h of culture, 20 to 30 oocytes were incubated with 100 μl of semen droplets diluted with BO (Brackett and Oliphant) (Brackett and Oliphant, 1975) media to a final concentration of $5x10^6$ sperm/ml, at 38.5°C and 5% CO_2 for 16 to 18 h. Oocytes were washed twice with TCM 199 to remove excess sperm, placed in a four well culture dish containing TCM 199 supplemented with 5% SCS, 5 μg/ml insulin and 50 μg/ml Gentamycin and cultured at 38.5° C and 5% CO_2. The culture media were changed every 72 h. Batches of embryos were harvested at the timed stages of development [2-, 4-, 8-, 16-cells, morulae, and blastocysts].

Collection of bovine CL tissues

Ovaries containing CL were collected from Holstein cows at a local abattoir within 10-20 min of exsanguination. The CL tissues were classified as early- (Stage I), mid- (Stage II), or late- (Stage III) luteal phase by macroscopic examination of the ovaries and uterus as previously

described (Taniguchi et al., 2002). The CL were excised from the ovaries, cut in half through the papilla and sliced into pieces representative of this complex tissue (approximately 1g in weight). The CL samples were then snap-frozen, transported to the laboratory and only stage II samples were processed for RNA isolation for this study.

Isolation of total RNA and generation of first strand cDNA

Total RNA was extracted from bovine embryos (25-30 embryos at 2-, 4-, 8-, 16- cell, morula and blastocyst stages) and CL tissue using the phenol-chloroform method of Chomczynski and Sacchi (1987). To verify the integrity of the RNA from CL, aliquots of the total RNA extracts were electrophoresed in a 1% (w/v) denaturing agarose gel containing 3.7% formaldehyde and visualised by eithidium bromide staining. The purity and concentration of the total RNA present in each of the extracts from embryos were quantified by optical densitometry (260/280nm) using a DU-64 UV-spectrophotometer (Beckman Coulter). Aliquots (1μg) of the total RNA extracts prepared from the CL tissue and embryos, and were reverse-transcribed into cDNA using a First Strand cDNA Synthesis Kit according to a protocol recommended by the manufacturer (Amersham Pharmacia Biotech, Oakville, Canada).

Primer design and cDNA Preparation

Nucleotide sequences specific for human ADAMTS-1 through -12, which are conserved in the murine or rat homologues deposited in Genbank, were identified using the BLAST (Basic Local Alignment Search Tool) computer program (NCBI, Bethesda, MD). Forward and reverse oligonucleotide primers corresponding to these DNA sequences were synthesized at the NAPS Unit, The University of British Columbia. Primers specific for the bovine 18S ribosomal RNA subunit, which served as an internal control for these studies. The specific nucleotide sequences

of these primers, the optimized PCR conditions for each of these primer sets and the expected sizes of the PCR products are listed in Table 4.2.1.

PCR was performed using the specific ADAMTS primers sets and first strand cDNA generated from total RNA extracted from small pools of bovine preimplantation embryos or CL tissues. To confirm the specificity of the primers, the resultant PCR products were subcloned into the PCR II vector by blunt-end ligation (Invitrogen, Carlsbad, CA) and subjected to nucleotide sequence analysis using an automated DNA sequence analyzer (Applied Biosystems, Foster City, CA) employing Taq DiDeoxy reagents. These clones were subsequently used to generate cDNA probes specific for each of the bovine ADAMTS subtypes identified in the preimplantation embryos and/or CL tissues and the 18S rRNA subunit using standard molecular biology techniques.

Semiquantitative RT-PCR and Southern Blot Analysis

Semiquantitative PCR was performed using cDNA synthesized from total RNA extracted from bovine embryos at timed stages of development and primers specific for either the ADAMTS subtypes or 18S ribosomal RNA. Following standardization with the amount of 18S ribosomal RNA present in each sample, equivalent amounts of the specific ADAMTS PCR products were used for Southern blot analysis. The PCR products were separated by electrophoresis in a 1.2% agarose gel and subsequently denatured and transferred onto a charged nylon membrane (Hybond+, Amersham Canada Ltd. Oakville, ON) using standard Southern blot procedures. The blots were probed with a radiolabeled ^{32}P cDNA specific for ADAMTS subtypes as previously described (MacCalman et al., 1992). The blots were then washed twice with 2x SSPE at room temperature, twice with 2x SSPE containing 0.1% SDS at 55°C and twice with 0.2x SSPE at room temperature. The resulting autoradiograms were then scanned and subjected to densitometry (Scion Corporation, MD). The absorbance values obtained for the

ADAMTS PCR products were normalized relative to corresponding ribosomal 18S RNA absorbance value.

Statistical Analysis

The results are presented as the mean relative absorbance ($\pm$ SEM) for three independent experiments. Statistical differences between time points were assessed by the analysis of variance (ANOVA). Differences were considered significant for $p \leq 0.05$.

Results

Characherization of the ADAMTS subtypes present in the bovine embryos

Multiple ADAMTS subtypes were identified in the preimplantation bovine embryo from the 2-cell to the blastocyst stage of development (Figure 4.2.1). Transcripts encoding ADAMTS -1, -4, -5, -6, -8, -9, -10 subtypes are expressed during bovine preimplantation embryogenesis (Figure 4.2.1). In contrast, transcripts for ADAMTS -2, -3 and -7 could not be detected during the embryonic stages under study (Figure 4.2.2) however, they could be detected in the total RNA extracts prepared from the bovine CL tissue. ADAMTS-12 mRNA were not identified in the bovine embryo or the CL tissue by RT-PCR using a multiple set of primers, including those that have been used to detect this ADAMTS subtype in human placental tissues (Data not shown).

Regulated expression of ADAMTS subtypes during bovine preimplantation embryogenesis

Semiquantitative PCR and Southern blot analysis revealed that ADAMTS mRNA levels were high during the early stages of development (2- cell stage) and decreased as development progressed to the 4- cell (ADAMTS-5, -6), the 8- cell (ADAMTS-4, -8, -10) or morula (ADAMTS-1) stages, with the exception of ADAMTS-9 where the levels increased at the 4-cell

stage to decrease again at the 8-cell stage (Figure 3). There was a significant increase in ADAMTS -5, -8 and -9 mRNA levels at the morula stage and the mRNA levels of these ADAMTS continued to rise with the formation of blastocyst development in vitro. While the transcript levels for ADAMTS -1 and -6 rise sharply at the blastocyst stage, levels for ADAMTS-4 remain high throughout the embryonic stages under study. Like the other ADAMTS, mRNA transcripts for ADAMTS-10 increase to the blastocyst stage but this increase is not statistically significant.

Discussion

Multiple ADAMTS subtypes were detected in the bovine embryo during early embryonic development. Using the semiquantitative PCR and Southern blot analysis, we have been able to identify a gamut of ADAMTS subtypes being expressed during the preimplantation development of the bovine embryo. To the best of our knowledge, this is the first report of the expression of ADAMTS subtypes during the various stages of development of preimplantation embryogenesis.

In agreement with our observation, expression of ADAMTS-1, -5, and -8 mRNA has been reported during embryonic development in the mouse (Georgiadis et al., 1999; Hurskainen et al., 1999; Thai and Iruela-Arispe, 2002). ADAMTS-1 transcripts were present in the placenta, brain, heart, lung, limb bud, live, spleen and kidney from day 10 to day 18 in the mouse embryo. However transcripts were much reduced in adult organs (Thai and Iruela-Arispe, 2002). ADAMTS-5 is specifically expressed in the 7 d old embryo (the peri-implantation period) and hence has been given the name "Implantin" (Hurskainen et al., 1999) for its putative role in implantation and its levels are low or undetectable before and after this period. Low levels of ADAMTS-8 have been detected during mouse embryo development but selective expression is observed in adult lung and heart (Georgiadis et al., 1999). Though, we failed to detect the presence of ADAMTS -2, -3 and -7 in the bovine embryo, mRNA for ADAMTS-2 was detected

in mouse embryos at 7, 15 and 17 d but not on day 11 (Fernandes et al., 2001). Similarly, ADAMTS-3 mRNA was also detected in mouse embryos at days 7, 15 and 17 only (Fernandes et al., 2001). On the other hand, ADAMTS-7 showed only low levels of expression during the post implantation embryogenesis in the mouse (Hurskainen et al., 1999). Our findings also differ for the expression for ADAMTS-6, which could not be detected in the mouse embryo using northern blot analysis (Hurskainen et al., 1999), but its expression was readily detectable in the bovine embryo using RT-PCR. Expression of ADAMTS-4 and -9 has also been detected in diverse tissues in the adult mouse (Abbaszade et al., 1999; Clark et al., 2000). Collectively, these findings suggest that these ADAMTS are highly regulated during bovine embryogenesis and may have distinct but different roles during development as compared to their role in murine.

Since, transcripts encoding ADAMTS -1, -4, -5, -6, -8, -9, -10 subtypes were detected in the bovine embryo from the 2-cell stage to the blastocyst stage, it signifies that the transcripts of these ADAMTS are of both maternal and embryonic in origin (Kidder and McLachlin, 1985; Kidder and Pedersen, 1982). As this maternal to embryonic transition occurs around the 8-cell stage in bovine, transcripts for these ADAMTS in general, decrease up to the 4-, or 8-cell stage, until the maternal transcripts are depleted. Subsequently, their presence in the 16-cell stage signifies that these subtypes might be playing an important role during the preimplantation period. Findings in this study are in accordance with other gene expression studies done in the bovine embryo (Bilodeau-Goeseels and Panich, 2002; De Sousa et al., 1998; Memili et al., 1998; Memili and First, 1998). At the blastocyst stage, although the expression levels of all the ADAMTSs were the maximum with the exception of ADAMTS-10, suggesting a very important role during blastocyst formation and during subsequent development. It was interesting to note ADAMTS-4 had the maximum expression throughout embryonic stages and might be involved in important cell-matrix interactions during early embryogenesis.

In general, extracellular proteinases are required for numerous developmental and disease related processes. The ability to degrade extracellular proteins is essential for any individual cell to interact properly with its immediate surrounding and for multicellular organisms to develop and function normally. Cell-ECM interactions are important in mediating diverse physiological events such as lineage decisions during embryogenesis, differentiation, cell migration and programmed cell death (Tang, 2001). In view of these observations, it is tempting to speculate that these ADAMTS subtypes could be involved in a diverse array of cellular events in relation to development. However, the biological significance of the distinct expression patterns of the ADAMTS subtypes observed during preimplantation embryogenesis in bovines remains to be elucidated.

Total PA production by cow blastocysts has been linked to the hatching efficiency, based on the fact that hatched blastocysts produce more total PA than those blastocysts that fail to hatch from their zona pellucida (Kaaekuahiwi and Menino, 1990). MMPs have been implicated in the implantation process in many species including mice, sheep, pigs and cows (Bartlett and Menino, 1993; Dyk and Menino, 1991; Fazleabas et al., 1983; Harvey et al., 1995; Menino and Williams, 1987; Sappino et al., 1989; Strickland et al., 1976; Zhang et al., 1994). In ruminants such as cattle and sheep, the blastocyst stays in the lumen of the uterus and does not invade the stromal layers. This results in an epitheliochorial placenta that is fully established at around 5 wk post coitum. Although, within the uterus, the cow blastocyst is essentially non invasive, some of the trophoblast cells differentiate to form binucleate cells that fuse with the endometrium, enabling the trophoblast to be in contact with the maternal connective tissue. Hence, there is still a degree of ECM remodeling, though not to the same extent as in the haemochorial placentation exhibited by humans and rodents (Salamonsen et al., 1995). In light of the results presented in the study, it is easy to envisage a role of the ADAMTS subtypes in contributing towards ECM remodeling during early embryogenesis. Moreover, the presence of ADAMTS subtypes in the

early preimplantation embryo and their regulated expression until the blastocyst stage suggests that these proteases could be playing a crucial role in development even prior to implantation i.e, in embryonic biological processes including blastocyst formation and hatching.

Relatively few ECM or cell surface components have been localized during preimplantation embryogenesis. Laminin (Dziadek, 1995; Timpl et al., 1984) and heparin sulfate proteoglycan (Dziadek, 1995) appear as early as the 2-cell stage, with nidogen appearing in the 8-16 cell compact morula stage in the mouse. Both fibronectin (Timpl et al., 1984) and type IV collagen (O'Shea et al., 1990) were first observed between cells of the ICM in the late blastocyst. The extracellular matrix protein thrombospondin (TSP) was present within the cytoplasm of unfertilized eggs, in fertilized one-cell to four-cell mouse embryos; by the eight-cell stage, TSP was also densely deposited at the cell-cell borders (O'Shea et al., 1990). In the expanded blastocyst, the TSP was present in all three-cell layers, the trophectoderm, endoderm and inner cell mass. ADAMTS-1 has been shown to bind to heparin and ADAMTS-1, -4 and -5 also cleave large chondrotin sulphates, aggrecan, versican and brevican (Sandy et al., 2001; Tortorella et al., 2000a; Tortorella et al., 2001). Moreover, the bovine ADAMTS-2 has been shown to process procollagen to collagen. Procollagen-I and -II have been identified as substrates for ADAMTS-1, -2 and -3 (Fernandes et al., 2001; Prockop et al., 1998). Although, the substrates for many of the ADAMTS subtypes are currently not known it is tempting to speculate that the ECM remodeling during preimplantation embryogenesis could be mediated through the activity of distinct ADAMTS subtypes.

Inhibitors of PAs (PAI), along with tissue inhibitors of matrix metalloproteinases (TIMPs) provide potential mechanisms in countering the effects of proteinases. PA activity is suppressed by PAI-2 in bovine preimplantation embryos and affects the hatching of the embryos (Coates and Menino, 1994). Similarly, expression of TIMPs (TIMP-1, -2 and -3) has been reported in the embryos of mice and pigs suggesting these inhibitors function in cell-ECM

interactions during growth, development, and implantation of mammalian embryos (Brenner et al., 1989; Harvey et al., 1995; Menino et al., 1997). ADAMTS-4 and ADAMTS-5 are inhibited by TIMP-3 and not TIMP-1 and TIMP-2 (Kashiwagi et al., 2001; Westling et al., 2002). While Das et al.(1997), reported that TIMP-3 could be the most important inhibitor of the invasive process during embryo-uterine interaction, Menino et al. (1997) reported that abundant expression of TIMP-1, -2 and -3 in pig embryos might explain, at least in part, the absence of invasive implantation in pigs as compared to rodents and primates. Whether the TIMPs are able to mediate a similar action on several ADAMTS subtypes and thereby preventing an invasive process in bovine is yet to be determined.

In summary, we have determined a unique pattern of expression of ADAMTS mRNA during the development of bovine preimplantation embryo. Since the levels of these mRNA transcripts for distinct ADAMTS subtypes appear highly regulated, it suggests a multitude of possible roles for these novel matrix components, the majority of which remain to be determined.

Acknowledgments

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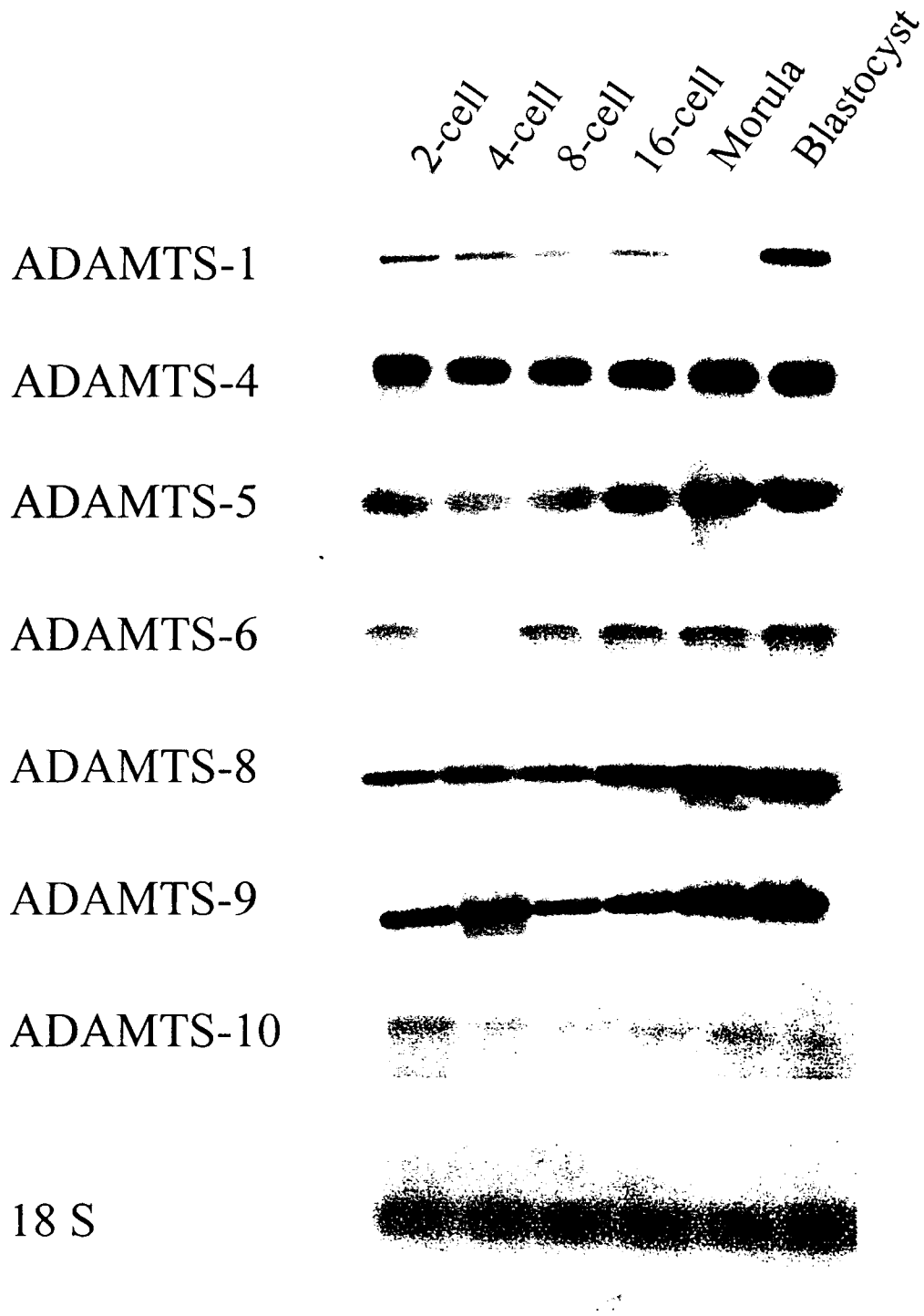

Fig. 4.2.1: Characterization of the ADAMTS mRNA transcripts present in bovine preimplantation embryos. Representative autoradiograms of Southern blots containing PCR products generated using template cDNA synthesized from total RNA extracted from preimplantation embryos at timed stages of development (2-, 4-, 8-, 16-cells, morula and blastocyst) and primers specific for ADAMTS-1, -4, -5, -6, -8, -9, -10 or the 18 S rRNA subunit.

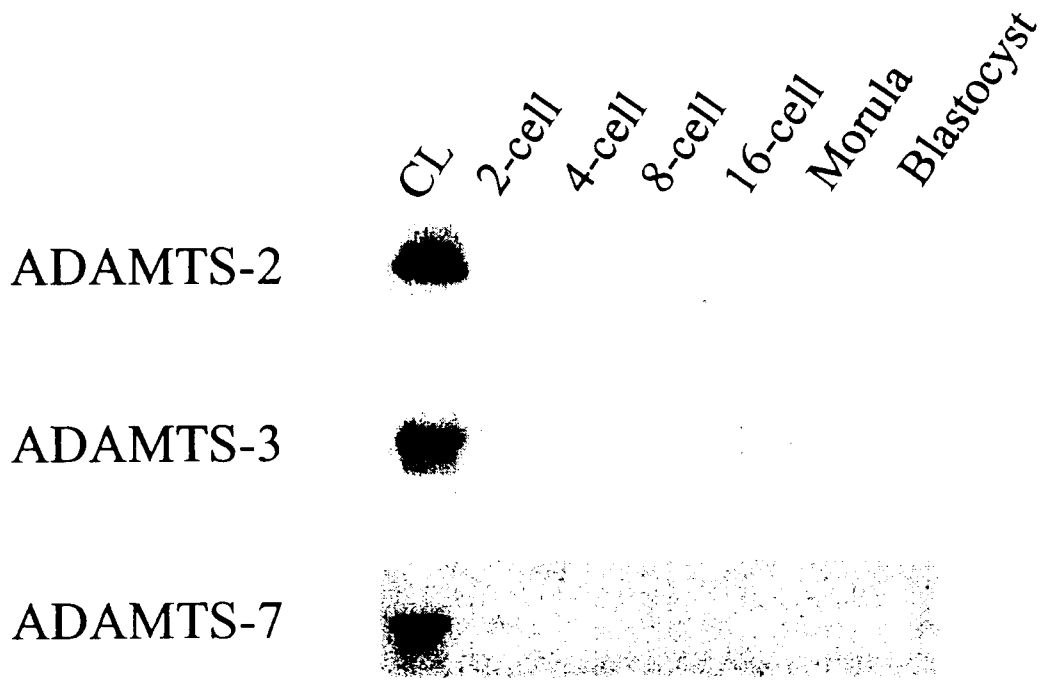

Fig. 4.2.2: Characterization of the ADAMTS-2, -3 and -7 mRNA transcripts present in bovine preimplantation embryos. Representative autoradiograms of Southern blots containing PCR products generated using template cDNA synthesized from total RNA extracted from bovine preimplantation embryos at timed stages of development (2-, 4-, 8-, 16-cells, morula and blastocyst stage) and primers specific for ADAMTS-2, -3 or -7. Template cDNA synthesized from bovine CL tissue served as positive controls for these studies.

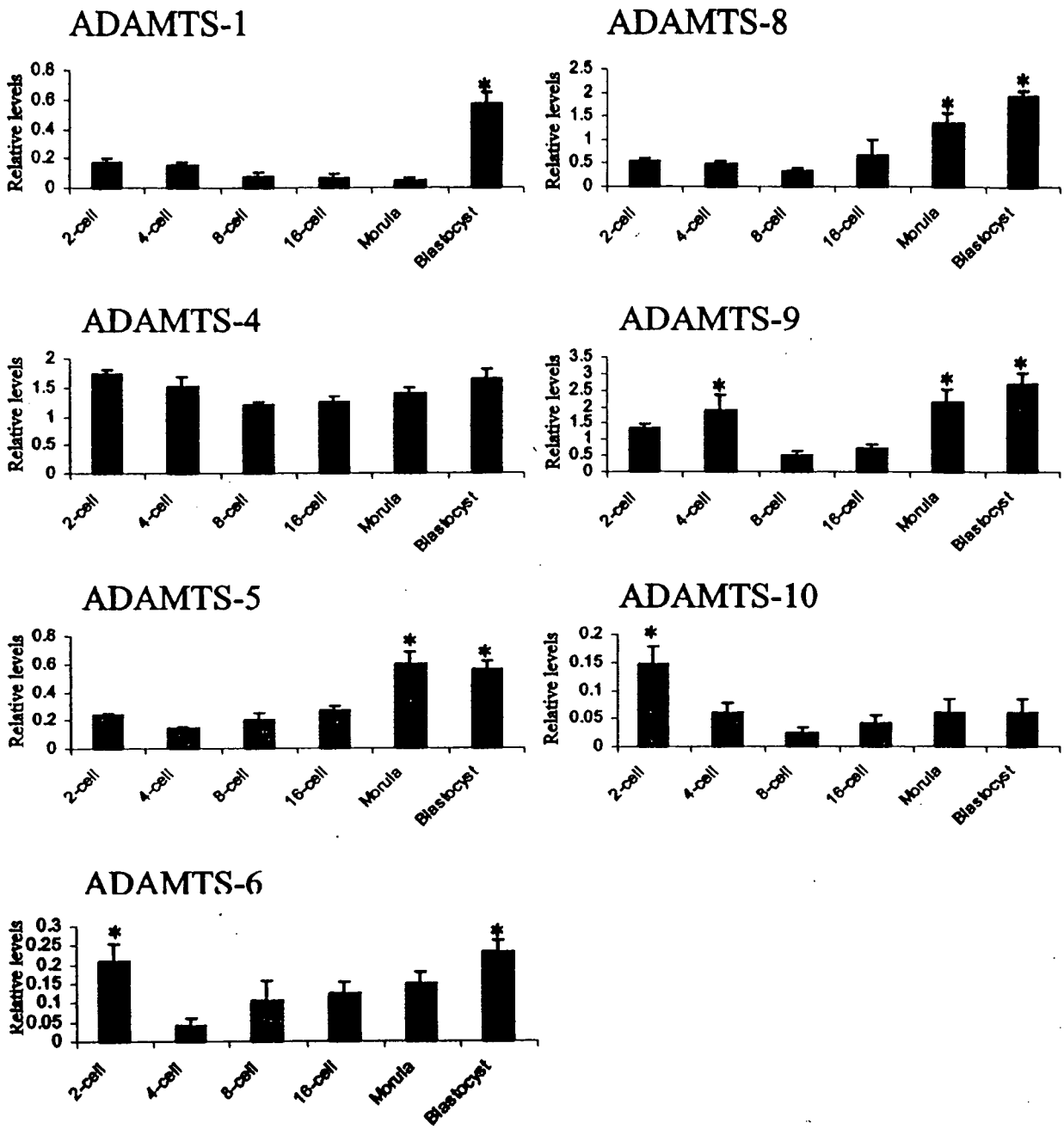

Fig. 4.2.3: Relative levels of the distinct ADAMTS mRNA transcripts in preimplantation bovine embryos. Autoradiograms of Southern blots containing PCR products generated using template cDNA synthesized from total RNA extracts from embryos at timed stages of development (2-, 4-, 8-, 16-cell, morula and blastocyst) and primers specific for the ADAMTS subtypes identified in these embryos or the 18 S rRNA subunit were scanned using a laser densitometer. The absorbance values obtained for the distinct ADAMTS subtypes were then normalized to the absorbance value obtained for the 18 S rRNA. The results are represented as mean $\pm$ SEM (n=3) in the bar graphs. Significant differences between the means ($P \leq 0.05$) are represented by asterisks.

ADAMTS Subtype	Primer Sequence	Estimated PCR Product Size	PCR Conditions
ADAMTS-1	Forward: 5'- CGAGTGTGCAAAGGAAGTGA-3' Reverse: 5'- CTACCCCCATAATCCCACCT-3'	339 bp	Denaturing: 94°C 30s Annealing: 65 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-2	Forward: 5'-CCTATGACTGCCTGCTGGAT-3' Reverse: 5'-TCCCAAAGTGCTGGGATAAC-3'	310 bp	Denaturing: 94°C 30s Annealing: 65 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-3	Forward: 5'-TCAAGGCCTTCCAGGTCCGACTCTC-3' Reverse: 5'- GGGAGCCTGTTCTACAGCTGATCTC-3'	299 bp	Denaturing: 94°C 30s Annealing: 60 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-4	Forward: 5'-ACCTTTCCCTGGGTAGCACT-3' Reverse: 5'-TCCTTGCATACCTCACTGCG-3'	320 bp	Denaturing: 94°C 30s Annealing: 60 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-5	Forward: 5'-GGCCATGGTAACTGTTTGCT-3' Reverse: 5'-CCTCTTCCCTGTGCAGTAGC-3'	444 bp	Denaturing: 94°C 30s Annealing: 65 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-6	Forward: 5'-TGACAGTCCAGCACCTTCAG-3' Reverse: 5'-CTACGTGCTTGCATTCTCCA-3'	340 bp	Denaturing: 94°C 30s Annealing: 60 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-7	Forward: 5'-CCATGTGGTGTACAAGCGTC-3' Reverse: 5'-GGTCCTCCTCCTCATCTTCC-3'	389 bp	Denaturing: 94°C 30s Annealing: 55 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-8	Forward: 5'-AAGAAGAGGAGGCAGAAGGC-3' Reverse: 5'-TCTGTCTGGTGAGCAGGATG-3'	380 bp	Denaturing: 94°C 30s Annealing: 65 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-9	Forward: 5'-CCCAGCCTGGACACATTACT-3' Reverse: 5'-CCCAGCCTGGACACATTACT-3'	428 bp	Denaturing: 94°C 30s Annealing: 65 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-10	Forward: 5'-TCTCTCAGTCACTTGGCCCT-3' Reverse: 5'-AGAGCGACCAGTTCCCTACA-3'	495 bp	Denaturing: 94°C 30s Annealing: 65 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-12	Forward: 5'-GTGCAGCGAGGAGTACATCA-3' Reverse: 5'-GCGTTTTCTTTCTCCAGTGC-3'	488 bp	Denaturing: 94°C 30s Annealing: 65 °C 30s Extension: 72 °C 60s 35 cycles
18 S	Forward: 5'-GCTCGCTCCTCTCCTACTTG-3' Reverse: 5'-GATCGGCCCGAGGTTATCTA-3'	150 bp	Denaturing: 94°C 30s Annealing: 55 °C 30s Extension: 72 °C 60s 18 cycles

Table 4.2.1: Primer sequences and PCR conditions for the semiquantitative analysis of ADAMTS subtype mRNA levels in bovine early preimplantation embryos or ovary.

CHAPTER V: EXPRESSION OF ADAMTS SUPTYPES IN THE PERIOVULATORY FOLLICLE AND CORPUS LUTEUM OF THE BOVINE OVARY

5.1: Preface

In this study, we have demonstrated the role of multiple ADAMTS subtypes during the important periovulatory period and CL formation in the bovine.

Corpus luteum formation being a complex process still remains poorly understood. While ovulation is marked by ECM degradation of the follicular wall, excessive tissue remodeling, cellular replication and specialization characterize luteinization. Although several proteases have been shown to play a role in this tissue remodeling, their biological roles remain poorly understood. Recent studies have identified ADAMTS (A Disintegrin And Metalloproteinase with ThromboSpondin motifs) as novel proteases playing a key role in diverse tissue remodeling processes during development. However their role in ovarian tissue remodeling remains unclear. In view of these observations, we have ascertained the role of these ADAMTS during ovulation and CL formation. In particular, ADAMTS-1, -2, -3, -4, -5 (also known as ADAMTS-11), -7, -8 and -9 but not ADAMTS-6, -10, or -12 mRNA transcripts were detected in non-atretic ovarian follicles and CL. The levels of mRNA for these ovarian ADAMTS subtypes were highly regulated in the granulosa and/or theca cells of the dominant follicle following the preovulatory surge of gonadotropins and in the CL during the luteal phase of the estrous cycle. The complex expression patterns observed for the distinct ADAMTS subtypes in the granulosa and theca cells of the periovulatory follicle and in luteal tissues of the bovine ovary suggest that these novel proteases mediate, at least in part, the remodeling events underlying folliculogenesis and ovulation and the formation, maintenance and regression of the CL.

This is the first demonstration of the role of multiple ADAMTS during the periovulatory stages on the bovine ovary. In addition, it is the first demonstration of regulation of these

ADAMTS by GnRH. The results of these studies have been submitted as a manuscript entitled "Regulation of ADAMTS messenger ribonucleic acid levels in the periovulatory follicle and corpus luteum of the bovine ovary" in the *Biology of Reproduction.*

5.2: REGULATION OF ADAMTS MESSENGER RIBONUCLEIC ACID LEVELS IN THE PERIOVULATORY FOLLICLE AND CORPUS LUTEUM OF THE BOVINE OVARY*

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Introduction

The preovulatory surge of gonadotropins triggers a myriad of morphological and biochemical changes within the dominant follicle that culminates in ovulation and the subsequent formation of the corpus luteum (CL). This highly regulated series of inter-related developmental processes is mediated, at least in part, by the extensive remodeling of the ovarian extracellular matrix (ECM). In particular, ovulation is associated with the degradation of the follicular basement membrane and the fragmentation of the extracellular matrix at the apex of the follicle wall, resulting in the release of the oocyte (Richards et al., 1998; Robker et al., 2000b). The ovarian ECM is subject to further remodeling, both deposition and degradation, during the formation, maintenance and regression of the CL which in turn, modulates proliferation,

* The work was conducted by PM under the supervision of RR and CDM. AGB provided the primer sequences for ADAMTS subtypes. PJB, CKM and JEF provided the RNA samples from theca and granulosa cells of the bovine ovary.

differentiation and/or apoptosis in the distinct cell populations that constitute this dynamic tissue (Richards et al., 1998; Robker et al., 2000b).

Matrix metalloproteinses (MMPs) and their endogenous inhibitors, tissue specific inhibitors of matrix metalloproteinases (TIMPs), are believed to play integral roles in the degradation of the follicular ECM during ovulation and to be operative in CL tissues throughout the luteal phase of the estrous cycle (Fata et al., 2000; Silvester and Luck, 1999; Smith et al., 1999). However, the specific roles of the MMPs and TIMPs in these developmental processes remain poorly defined. Furthermore, the spatiotemporal expression of distinct MMPs and TIMPs in the ovary often appears to vary among species (Fata et al., 2000; Silvester and Luck, 1999; Smith et al., 1999). In the bovine ovary, MMP-2, and -14 have been detected in the periovulatory follicle whereas MMP-2 and -9 are expressed primarily in the CL (Bakke et al., 2002; Curry and Osteen, 2001). The activity of these ovarian MMP subtypes is regulated by the constitutive expression of TIMP-1 and TIMP-2 in the follicle and CL (Bakke et al., 2002; Curry and Osteen, 2001; Goldberg et al., 1996; Smith et al., 1996). Recent cloning studies have identified a rapidly expanding family of novel metalloproteinases known as ADAMTS (A Disintegrin And Metalloproteinase with ThromboSpondin motifs) (Tang, 2001; Tang and Hong, 1999). ADAMTS are characterised by four structural and functional subunits: an amino terminal prodomain, a catalytic domain, a disintegrin-like domain, and an ECM binding domain composed of a central thrombospondin (TSP) type 1 motif, a spacer region and a variable number of TSP-like motifs) at the carboxyl terminal of the mature protein. To date, 20 members of the ADAMTS family have been identified in vertebrates (Cal et al., 2002). However, the majority of these ADAMTS subtypes have been characterized only at the structural level. Consequently, the biological function(s) of many of these novel proteases remain poorly understood.

Gene-knockout studies have provided useful insight into the important biological roles that members of the ADAMTS gene family play in embryonic development, tissue morphogenesis and reproduction. Mice null-mutant for ADAMTS-1 exhibit growth retardation and aberrant development of the kidneys, adrenals and urogenital tract (Shindo et al., 2000). In addition, fewer mature follicles were formed in the ovaries of these mice supporting previous observations that suggested a role for ADAMTS-1 in folliculogenesis (Espey et al., 2000; Robker et al., 2000a). Similarly, ADAMTS-2 gene-knockout mice develop structural defects in their skin (Li et al., 2001c). Although folliculogenesis is normal in these mice, testicular function is compromised suggesting that ADAMTS-2 plays a key role in spermatogenesis. To date, the expression and function of other members of the ADAMTS gene family in the ECM remodeling events that occur in the ovary during each estrous cycle have not been characterised. In view of these observations, we have characterised the ADAMTS subtypes present in small and large bovine follicles, in the theca and granulosa cells of the dominant follicle during the periovulatory period, and in CL tissues obtained at different stages of the luteal phase.

Materials and methods

Collection of Bovine Follicles and CL Tissues

Ovaries were collected from Holstein cows at a local abattoir within 10-20 min of exsanguination and transported to the laboratory in sterile saline at 32-37°C.

Small (3-6 mm) or large (12-16 mm) non-atretic follicles were aspirated through an 18 G gauge needle into a 10 ml syringe containing follicular aspiration medium (PBS supplemented with 0.3% BSA and 50 μg/ml Gentamycin). The aspirates were then centrifuged at 200 x g for 10 min and washed with follicular aspiration medium. This process was repeated twice before the ovarian cell pellets were finally collected for total RNA extraction.

CL tissues were classified as early- (Stage I), mid- (Stage II), or late- (Stage III) luteal phase by macroscopic examination of the ovaries and the corresponding uterine tissues (Ireland et al., 1980). The CL were excised from the ovaries, cut in half through the papilla and sliced into pieces representative of this complex tissue (approximately 1 g wet weight). The tissues were then snap-frozen for later extraction of total RNA.

Production of Bovine Embryos in vitro

Small pools of bovine embryos (n=25-30) were produced using an in vitro fertilization protocol previously described (Giritharan and Rajamahendran, 2001). Briefly, cumulus-oocyte complexes were aspirated from small follicles and cultured in Tissue Culture Media 199 (TCM 199) containing 5% supervoulated cow serum (SCS) and supplemented with FSH (0.01 μg) and antibiotics (50 μg/ml Gentamycin) at 38.5°C for 24 h. Approximately 30 of these matured oocytes were then incubated with 100 μl of bull semen, diluted to a final concentration of $5x10^6$ sperm/ml with Brackett and Oliphant (BO) medium, for a further 16 -18 h (Brackett and Oliphant, 1975). The fertilized oocytes were washed twice with culture medium to remove excess sperm, placed in a four-well culture dish containing TCM 199 supplemented with 5% SCS, 5 μg/ml insulin and 50 μg/ml Gentamycin and allowed to undergo development into morulae or blastocysts before being harvested for total RNA extraction.

Isolation of Bovine Granulosa and Theca cells from Periovulatory Follicles

Holstein heifers with regular estrous cycles were used in accordance with procedures approved by Cornell University's Animal Care and Use Committee. An experimental protocol that we have described and validated previously (Komar et al., 2001) was used to obtain preovulatory follicles before or after the preovulatory gonadotropin surge. Briefly, heifers (n = 3/group) were injected with prostaglandin $F_{2á}$ ($PGF_{2á}$; 25 mg Lutalyse; Pharmacia and Upjohn

Co, Kalamazoo, MI) on the evening of Day 6 of the estrous cycle (Day 0 = day of estrus) to regress the CL and thereby induce the follicular phase and further differentiation of the dominant follicle of the first follicular wave of the cycle. Injection of a GnRH analogue (100 ì g Cystorelin i.m.; Sanofi Animal Health, Inc., Overland Park, KS) 36 h later induces an LH/FSH surge and ovulation follows at about 29 h after GnRH (Komar et al., 2001). In the current study, the ovary bearing the preovulatory follicle was removed by colpotomy 36 h after injection of $PGF_{2\grave{a}}$ (i.e., time 0 after GnRH injection) or 24 h after injection of GnRH (before ovulation). The ovaries were examined daily by transrectal ultrasonography and blood samples were collected before and during the experimental protocol to verify the progression of luteal regression and follicular development in response to the treatments.

The ovary was transported to the lab (approximately 10 min) where the preovulatory follicle was dissected from the ovary and the follicular fluid removed by aspiration. In some experiments, the follicle wall (i.e. theca interna + attached granulosa cells) was isolated and cut into small pieces. In other experiments theca and granulosa cells were separated by dissection, as described previously (Fortune and Hansel, 1979). The theca interna was cut into small pieces and the granulosa cells were collected by centrifugation. Pieces of follicle wall and theca interna and the granulosa cell pellet were snap-frozen for later extraction of total RNA.

Isolation of Total RNA and Generation of First-Strand cDNA

Total RNA was extracted from the ovarian tissues and cells and from bovine embryos at timed stages of development using the phenol-chloroform method of Chomczynski and Sacchi (1987). The total RNA extracts were then treated with deoxyribonuclease (DNAse)-1 to eliminate possible contamination with genomic DNA. To verify the integrity of the RNA, aliquots of the total RNA extracts were electrophoresed in a 1% (w/v) denaturing agarose gel containing 3.7% formaldehyde and the 28 S and 18 S rRNA subunits visualized by ethidium

bromide staining. The purity and concentration of the total RNA present in each of the extracts were determined by optical densitometry (260/280nm) using a DU-64 UV-spectrophotometer (Beckman Coulter, Mississauga, ON).

Aliquots (approximately 1 μg) of the total RNA extracts prepared from the ovarian tissues and cells or the preimplantation embryos were reverse-transcribed into cDNA using a First Strand cDNA Synthesis Kit according to a protocol recommended by the manufacturer (Amersham Pharmacia Biotech, Oakville, Canada).

Primer Design and cDNA preparation

Nucleotide sequences specific for human ADAMTS-1 through -12, which were also conserved in the murine or rat homologues deposited in GenBank, were identified using the BLAST (Basic Local Alignment Search Tool) computer program (NCBI, Bethesda, MD). Forward and reverse oligonucleotide primers corresponding to these DNA sequences and primers specific for the bovine 18 S rRNA subunit, which served as an internal control for these studies, were synthesized at the NAPS Unit, University of Columbia. The specific nucleotide sequences of these primers, the optimized PCR conditions for each of these primer sets and the expected sizes of the PCR products are listed in Table 5.2.1.

PCR was performed on three separate occasions using the specific ADAMTS primers sets and first strand cDNA generated from total RNA extracts (n=6) prepared from small or large ovarian follicles. Total RNA extracts (n=6) prepared from the small pools of bovine embryos served as technical controls for these studies. To confirm the specificity of the primers, the resultant PCR products were subcloned into the PCR II vector by blunt-end ligation (Invitrogen, Carlsbad, CA) and subjected to nucleotide sequence analysis using an automated DNA sequence analyzer (Applied Biosystems, Foster City, CA) employing Taq DiDeoxy reagents. These clones were subsequently used to generate cDNA probes specific for each of the bovine ADAMTS

subtypes identified in the follicles and/or preimplantation embryos and the 18 S rRNA subunit using standard molecular biology techniques.

Semiquantitative Polymerase Chain Reaction

PCR was performed using the primer sets specific for ADAMTS-1 through -12, and template cDNA (2 μl) generated from the total RNA extracts prepared from the tissues and cells obtained from the dominant follicle before and after the preovulatory surge of gonadotropins or CL tissues obtained during the early-, mid- or late- stage of the luteal phase of the estrous cycle. Each of the distinct ADAMTS subtypes was co-amplified with the 18 S rRNA subunit. Non-reverse transcribed RNA or water were included with the primer sets and served as negative controls. The PCR cycles were repeated 20-40 times to determine a linear relationship between the yield of PCR products and the number of cycles. The numbers of cycles used to amplify the distinct ADAMTS subtypes from these ovarian cells and tissues are listed in Table 5.2.1.

Southern Blot Analysis

Aliquots (20 μl) of the PCR products generated from the ovarian tissue samples and cells or bovine embryos were separated by electrophoresis in a 1.2% agarose gel and visualized by ethidium bromide staining. The gels were then denatured with 0.5 M NaOH for 5 min, neutralized with 1M TRIS-HCl for 5 min and transferred onto a charged nylon membrane (Hybond^{+}, Amersham Canada Ltd., Oakville, ON).

The Southern blots were probed with a radiolabeled-cDNA specific for each of the distinct ADAMTS subtypes or 18 S rRNA according to the methods of MacCalman et al. (1992). The blots were then washed twice with 2 x SSPE (20 x SSPE consists of 0.2 M sodium phosphate, pH 7.4 containing 25 mM EDTA and 3M NaCl) at room temperature, twice with 2 x SSPE containing 0.1% SDS at 55°C and twice with 0.2 x SSPE at room temperature. The blots

were subjected to autoradiography to detect the hybridization of the radiolabled probes to the PCR products. The resultant autoradiograms were then scanned using a laser densitometer (Scion Corporation, Fredrick, MD) and the absorbance values obtained for each of the distinct ADAMTS PCR products normalized relative to the corresponding 18 S rRNA absorbance value.

Statistical Analysis

The results are presented as the mean relative absorbance value ($\pm$ SEM) obtained using tissues and cells harvested from different animals (n=2-3) on three separate occasions. Statistical differences between the time points were assessed by the ANOVA. Significant differences between the means were determined using the Fisher's Protected Least Significant Difference (PLSD) test. Differences were considered significant for $P \leq 0.05$.

Results

Characterization of the ADAMTS Subtypes present in Bovine Ovarian Follicles.

ADAMTS-1, -2, -3, -4, -5 (also known as ADAMTS-11), -7, -8 and -9 mRNA transcripts were detected in the total RNA extracts prepared from the small and large ovarian follicles (Fig 5.2.1). In contrast, ADAMTS-6 and -10 mRNA transcripts were not identified in the bovine follicles but were detected in the total RNA extracts prepared from the preimplantation embryos (Fig 5.2.1). ADAMTS-12 mRNA transcripts were not detected in the bovine ovarian tissues or embryos by RT-PCR using multiple sets of primers (data not shown), including those used to detect this ADAMTS subtype in human and murine tissues and cells (Cal et al., 2001). Nucleotide sequence analysis of the PCR products generated from the ovarian follicles and/or bovine embryos demonstrated that these cDNA fragments exhibited high sequence homology (86-100%) to the human and/or murine homologues deposited in GenBank.

Effects of the Gonadotropin Surge on ADAMTS mRNA Levels in the Preovulatory Follicle

The repertoire of ADAMTS subtypes identified in the small and large follicles was maintained in the dominant follicle before and after the preovulatory surge of gonadotropins induced by the adminstration of GnRH (Fig 5.2.2). There was a significant increase in ADAMTS-1, -2, -3 and -9 and a concomitant decrease in ADAMTS-5, -7 and -8 mRNA levels in the dominant follicle 24h after the administration of GnRH (Figs 5.2.2 and 5.2.3). In contrast to the other ADAMTS subtypes, ADAMTS-4 mRNA levels in the periovulatory follicle remained relatively constant, at least during the time points examined in these studies.

Regulation of ADAMTS mRNA in the Granulosa and Theca Cells of the Periovulatory Follicle

The preceding results for follicle wall samples indicated that the gonadotropin surge induces specific changes in the levels of mRNA for most of the ADAMTS subtypes examined in these studies. Therefore, the levels of the mRNA transcripts encoding these ovarian ADAMTS subtypes were next examined in granulosa and theca cells to localize these changes to specific cell types. The ADAMTS subtypes identified in the ovarian follicles were found to be present in granulosa and theca cells isolated from the dominant follicle during the periovulatory period (Fig 5.2.4). In agreement with our findings using samples of the follicle wall, the preovulatory surge of gonadotropins significantly increased ADAMTS-1 mRNA levels in granulosa and theca cells isolated from the dominant follicle but decreased the levels of the mRNA transcripts encoding ADAMTS-7 and -8 in both of these ovarian cell types (Figs 5.2.4 & 5.2.5). Similarly, GnRH had no significant effect on ADAMTS-4 mRNA levels in granulosa or theca cells, at least at the time points examined in these studies. However, levels of ADAMTS-2 and -5 mRNA levels were higher in granulosa cells 24 h after GnRH, whereas ADAMTS-2 was unchanged and ADAMTS-5 had decreased in theca cells at 24 h post-GnRH. In contrast, the preovulatory surge of gonadotropins had no significant effect on ADAMTS-3 mRNA levels in granulosa cells but

significantly increased the levels of this mRNA transcript in theca cells. Finally, there was a decrease in ADAMTS-9 mRNA levels in granulosa cells and a concomitant increase in the levels of the mRNA transcript encoding this ADAMTS subtype in theca cells 24 h after the administration of GnRH.

ADAMTS mRNA Levels are Regulated in CL Tissues during the Luteal Phase

ADAMTS-1, -2, -3, -4, -5, -7, -8, and -9 but not ADAMTS-6, -10, or -12 mRNA transcripts were detected in CL tissues at all stages of the luteal phase (Fig 5.2.6). ADAMTS-1, -7 and -8 mRNA levels were highest in CL tissues obtained during the early stage of the luteal phase (Figs 5.2.6 & 5.2.7). There was a significant and progressive decline in the levels of these mRNA transcripts in CL tissues as the estrous cycle entered the mid- and late-stages of the luteal phase. In contrast, ADAMTS-3 and -4 mRNA levels in CL tissues increased as the luteal phase progressed with maximum levels being observed in the late-stage CL. ADAMTS-5 and -9 mRNA levels were observed to increase between the early- and mid-stages of the luteal phase. The levels of the mRNA transcripts encoding these two ADAMTS subtypes in CL tissues subsequently declined as the estrous cycle entered the late luteal phase. In contrast to the other ADAMTS subtypes, only small fluctuations in the levels of ADAMTS-2 mRNA transcripts were observed in CL tissues obtained at different stages of the luteal phase.

Discussion

Multiple ADAMTS subtypes were detected in bovine ovarian follicles. This repertoire of ADAMTS subtypes was further localized to both the granulosa and theca cells of the large periovulatory follicle and was found to be maintained in CL tissues obtained at different stages of the luteal phase. In agreement with these observations, ADAMTS-1, and -2 but not ADAMTS-12 mRNA transcripts have been previously detected in total RNA extracts prepared

from adult human, mouse and/or rat ovaries (Cal et al., 2001; Espey et al., 2000; Li et al., 2001c). In addition to these ADAMTS subtypes, mRNA transcripts encoding ADAMTS-7, -8, and -9 but not ADAMTS-6 or -10 are present in the bovine ovary. To date, the presence or absence of these ADAMTS subtypes in the ovarian tissues of other species has not been determined.

The biological significance of the distinct expression patterns of the ADAMTS subtypes observed in the bovine ovary remains to be elucidated. However, the preovulatory surge of gonadotropins is believed to decrease the expression of ovarian genes involved in folliculogenesis and simultaneously increase in the levels of those involved in ovulation and luteinization (Richards, 2001; Richards et al., 1998; Robker et al., 2000b). In view of these observations, it is tempting to speculate that the ADAMTS subtypes identified in the ovarian follicles play distinct role(s) in folliculogenesis and ovulation. In particular, the presence of ADAMTS-5, -7, -8, or-9 mRNA transcripts in small and large follicles and the subsequent decline in the levels of these mRNA transcripts in granulosa and/or theca cells following the preovulatory surge of gonadotropins suggests that these ADAMTS subtypes may be involved in the ECM remodeling events underlying the structural and functional maturation of the dominant follicle. In contrast, the increase in ADAMTS-1, -2, -5 mRNA levels in the granulosa cells and ADAMTS-1, -3, and -9 mRNA levels in the theca cells of the dominant follicle following the preovulatory surge of gonadotropins suggests that these ADAMTS may mediate, at least in part, the degradation of the follicle wall during ovulation and/or the dissolution of the granulosa cell basement membrane underlying the formation of the CL. However, a similar increase in ovarian ADAMTS-1 mRNA levels was observed in GnRH-primed rats treated with indomethacin, an anti-inflammatory agent capable of inhibiting ovulation (Espey et al., 2000) and mice null-mutant for the ADAMTS-1 gene are capable of ovulating (Shindo et al., 2000). Similarly, female mice null-mutant for ADAMTS-2 have normal ovarian function (Li et al., 2001c). Taken together, these observations suggest that an increase in the expression of ADAMTS-1 or

ADAMTS-2 in the dominant follicle during the periovulatory period is neither necessary nor sufficient to mediate ovulation. The biological significance of the increase in ADAMTS-3, -5 and -9 mRNA levels in the granulosa or theca cell layers of the periovulatory bovine follicle remains unclear, but their up-regulation after the gonadotropin surge suggests potential roles for these ADAMTS subtypes, alone or in combination with ADAMTS-1 and -2, in the ovarian ECM remodeling events underlying ovulation.

ADAMTS mRNA levels were highly regulated in bovine CL during the luteal phase suggesting that these novel proteases may also play key integral roles in the formation and organization of this dynamic tissue. Remodeling of the ECM in CL tissues throughout the luteal phase not only modulates the biochemical differentiation of luteal cells but also promotes the formation and organization of a complex vasculature network (Curry and Osteen, 2001; Fata et al., 2000; Smith et al., 1999). After ovulation, there is an extensive growth of blood vessels in bovine CL tissues that peaks at the mid-stage of the luteal phase (Modlich et al., 1996; Zheng et al., 1993). These blood vessels subsequently undergo regression during luteolysis. Several of the ADAMTS subtypes identified in the bovine CL have been shown to have both angio-inhibitory and/or angiogenic activity in vivo and in vitro. In particular, ADAMTS-1 and ADAMTS-8 specifically inhibit endothelial cell proliferation and are capable of reducing growth factor-induced vascularization of tissues in vitro (Carpizo and Iruela-Arispe, 2000; Vazquez et al., 1999). Increased ADAMTS-5 expression has also been detected in the cells surrounding blood vessels in osteoarthritic synovium and is believed to be responsible for the extensive degradation of the surrounding ECM associated with the onset of this disease (Vankemmelbeke et al., 2001). However, ADAMTS-1 appears to be necessary for the development of the adrenomedullary capillary network in vivo (Shindo et al., 2000) suggesting that ADAMTS subtype(s) may have differential effects on angiogenesis that are tissue-specific. Similarly, ADAMTS-4 expression has been associated with the metabolism of vascular proteoglycans during endothelial tube

formation in vitro (Kahn et al., 2000). Although the increase in ADAMTS-4 mRNA levels in CL tissues during the late luteal stage suggest a role for this ADAMTS subtypes in luteolyis, the angiogenic activity of bovine luteal tissues in vitro has been shown to increase with the age of the CL (Redmer et al., 1996). To date, it is not clear whether the ADAMTS subtypes identified in the bovine CL exhibit angiogenic and/or anti-angiogenic activities in this highly vascularized tissue at different stages of the luteal phase.

The effects of the preovulatory surge of gonadotropins on follicular ADAMTS mRNA are mediated by either the coordinate or non-coordinate regulation of the levels of the distinct ADAMTS mRNA transcripts present in the granulosa and theca cells. These observations suggest that the regulation of ADAMTS subtypes in the ovary is complex and involves the activation/inhibition of local regulatory factors that may act in an autocrine and/or paracrine manner. To date, the factors capable of regulating ADAMTS expression in mammalian tissues and cells remain poorly characterized. TGF-β1 has been shown to regulate ADAMTS-12 mRNA levels in human fetal fibroblasts (Cal et al., 2001), whereas lipopolysaccharides increased ADAMTS-1 mRNA levels in renal and cardiac tissues of adult mice (Kuno et al., 1999). The ability of the progestin synthesis inhibitor, epostane, to inhibit the preovulatory increase in ADAMTS-1 mRNA levels rat follicles (Espey et al., 2000), which was also not observed in mice null mutant for the progesterone receptor (Robker et al., 2000a), has provided indirect evidence that progesterone is a key regulator of this ADAMTS subtype in the ovary. Although a similar increase in ADAMTS-1 mRNA levels was observed in the preovulatory bovine follicle, the levels of the mRNA transcript encoding this ADAMTS subtype were found to be high in early stage CL tissues when progesterone levels are low and subsequently decline during the mid-luteal phase of the estrous cycle when the circulating levels of this gonadal steroid are high (Tsang et al., 1995). Taken together, these observations suggest that factors, in addition to

progesterone, regulate the temporal expression of ADAMTS-1 mRNA levels in the bovine ovary.

The role(s) of the distinct ADAMTS subtypes in the remodeling of the ovarian ECM remain unclear as the specific substrate(s) of many ADAMTS subtypes have not, as yet been identified. To date, ADAMTS-1, -4 and -5 have been shown to degrade the large chondroitin-sulphate glycoproteins, aggrecan, brevican and versican (Sandy et al., 2001; Tortorella et al., 2000a; Tortorella et al., 2001). Although aggrecan and brevican have not been detected in bovine ovarian follicles, versican has been localized to the thecal layers, particularly in areas adjacent to the follicular basal lamina (McArthur et al., 2000). Procollagen-I and -II have also been identified as substrates for ADAMTS-1 and -2 and ADAMTS-2 and -3 respectively (Fernandes et al., 2001; Prockop et al., 1998). The accumulation of collagen I in the CL that occurs during the luteal phase (Fernandes et al., 2001) may thus be attributed, at least in part, to the progressive decline in ADAMTS-1 and ADAMTS-2 mRNA levels observed in this tissue during the luteal phase.

In summary, we have determined that mRNAs for multiple ADAMTS subtypes are present in the bovine ovary. As the levels of the mRNA transcripts encoding these distinct ADAMTS subtypes are highly regulated in the granulosa and theca cell layers of the dominant follicle following the administration of GnRH and in CL tissues during the luteal phase, it is tempting to speculate that members of this novel family of proteases are involved in the ECM remodeling events required for ovulation and/or the formation, maintenance and regression of the bovine CL.

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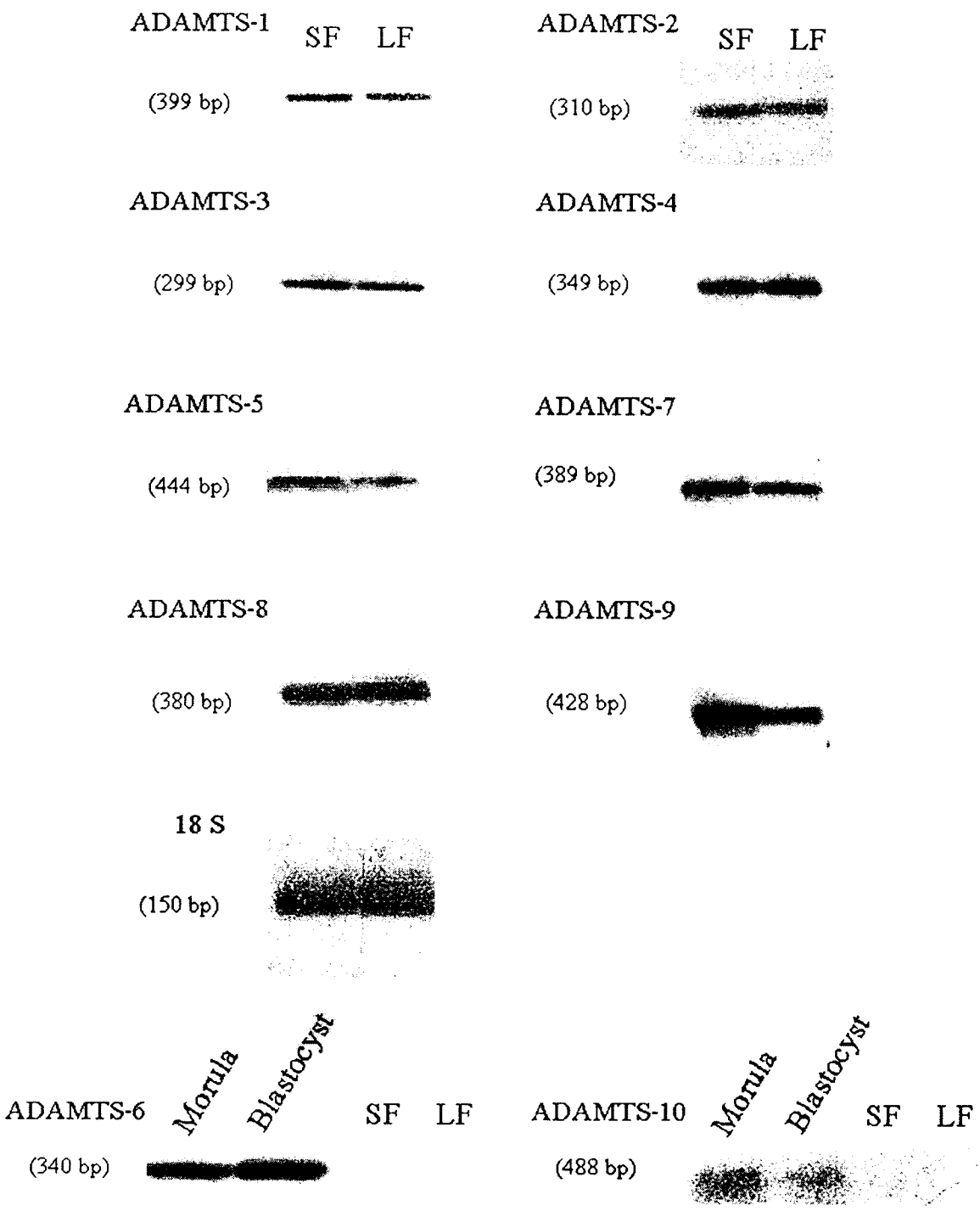

Fig. 5.2.1: Characterization of the ADAMTS mRNA transcripts present in bovine ovarian follicles. Representative autoradiograms of Southern blots containing PCR products generated using template cDNA synthesized from total RNA extracted from small or large ovarian follicles (lanes SF and LF, respectively) and primers specific for ADAMTS-1 through -11 or the 18 S rRNA subunit. Template cDNA synthesized from bovine morulae or blastocysts served as positive controls for these studies. The sizes of the distinct PCR products are shown to the left of the panels.

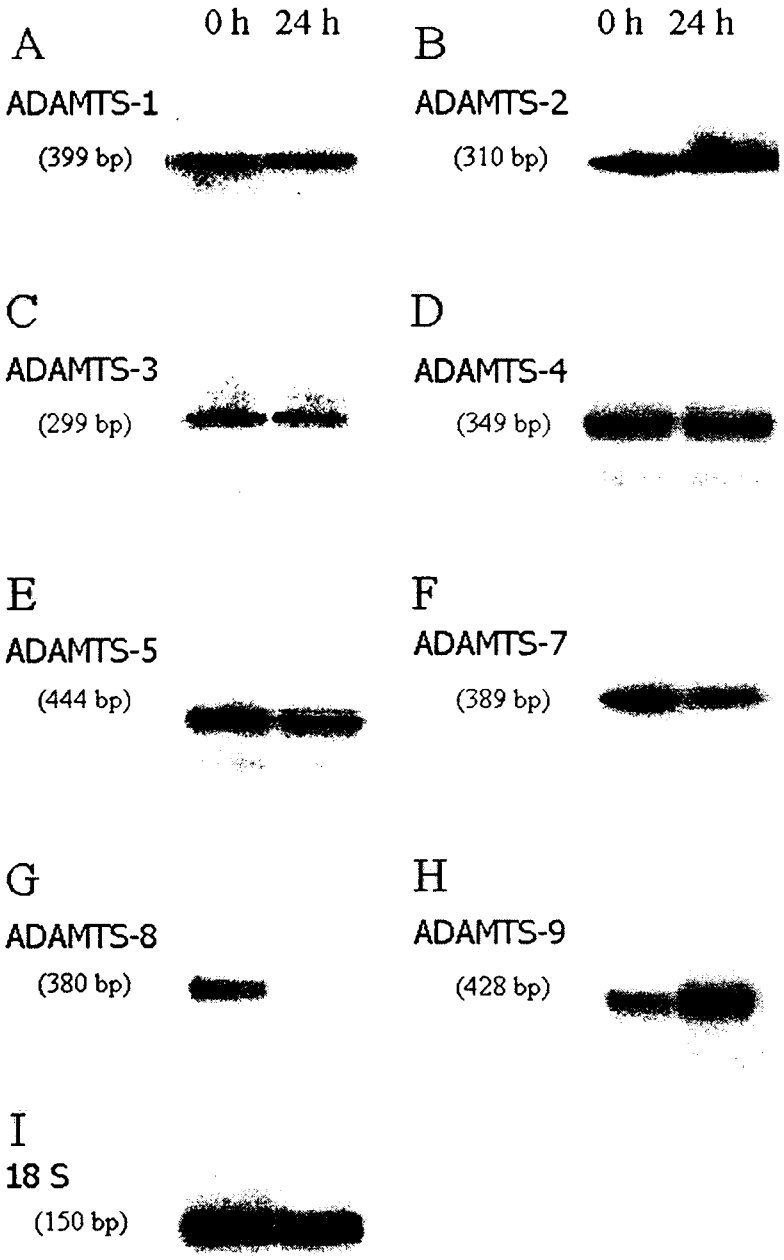

Fig. 5.2.2: Characterization of the ADAMTS subtypes present in the preovulatory follicle. Representative autoradiograms of Southern blots containing PCR products generated using template cDNA synthesized from total RNA extracted from the preovulatory follicle at 0 or 24 h after the administration of GnRH (lanes 0 h and 24 h respectively) and primers specific for ADAMTS-1, -2, -3, -4, -5, -7, - 8 or -9 (Panels A-H, respectively) or 18 S rRNA (Panel I). The sizes of the distinct PCR products are shown to the left of the panels.

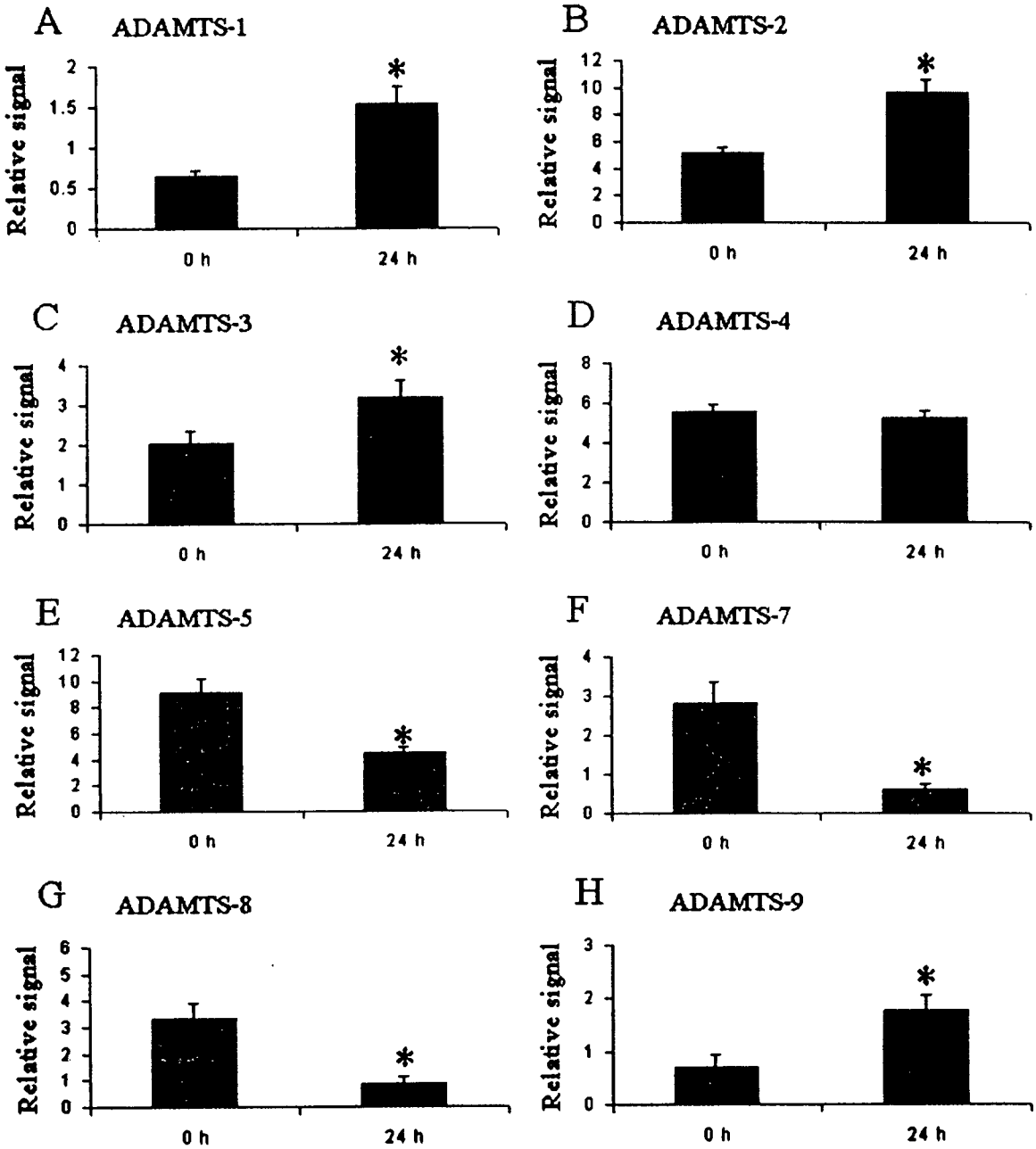

Fig. 5.2.3: Relative levels of the distinct ADAMTS mRNA transcripts in periovulatory follicles. Autoradiograms of Southern blots containing PCR products generated using template cDNA synthesized from total RNA extracts prepared from the preovulatory follicle at 0 or 24 h after GnRH was administered to induce the preovulatory gonadotropin surge and primers specific for the ADAMTS subtypes identified in these ovarian tissues or the 18 S rRNA subunit were scanned using a laser densitometer. The absorbance values obtained for the distinct ADAMTS subtypes were then normalized to the absorbance value obtained for the 18 S rRNA. The results are represented (mean $\pm$ SEM; n=3 follicles) in the bar graphs. Significant differences between the means ($P \leq 0.05$) are represented by asterisks.

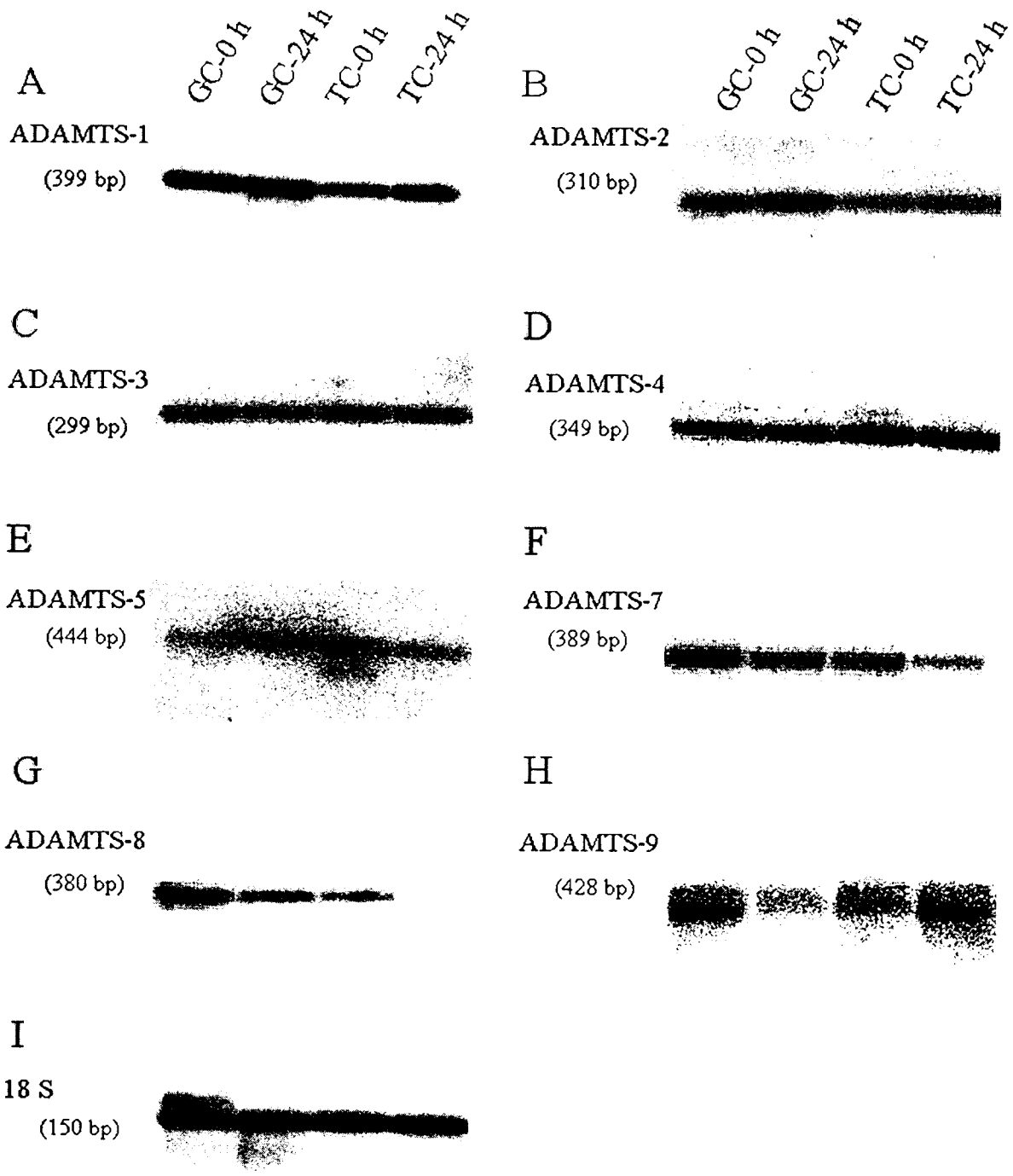

Fig. 5.2.4: ADAMTS mRNA levels in granulosa and theca cells isolated from periovulatory follicles. Representative autoradiograms of Southern blots containing PCR products generated using template cDNA synthesized from total RNA extracted from granulosa or thecal cells isolated from the preovulatory follicle at 0 and 24h after the administration of GnRH (lanes GC 0 h, GC 24 h, TC 0 h and TC 24 h respectively) and primers specific for ADAMTS-1, -2, -3, -4, -5, -7, -8 or -9 (Panels A-H respectively) or the 18 S rRNA subunit (Panel I). The sizes of the distinct PCR products are shown to the left of the panels.

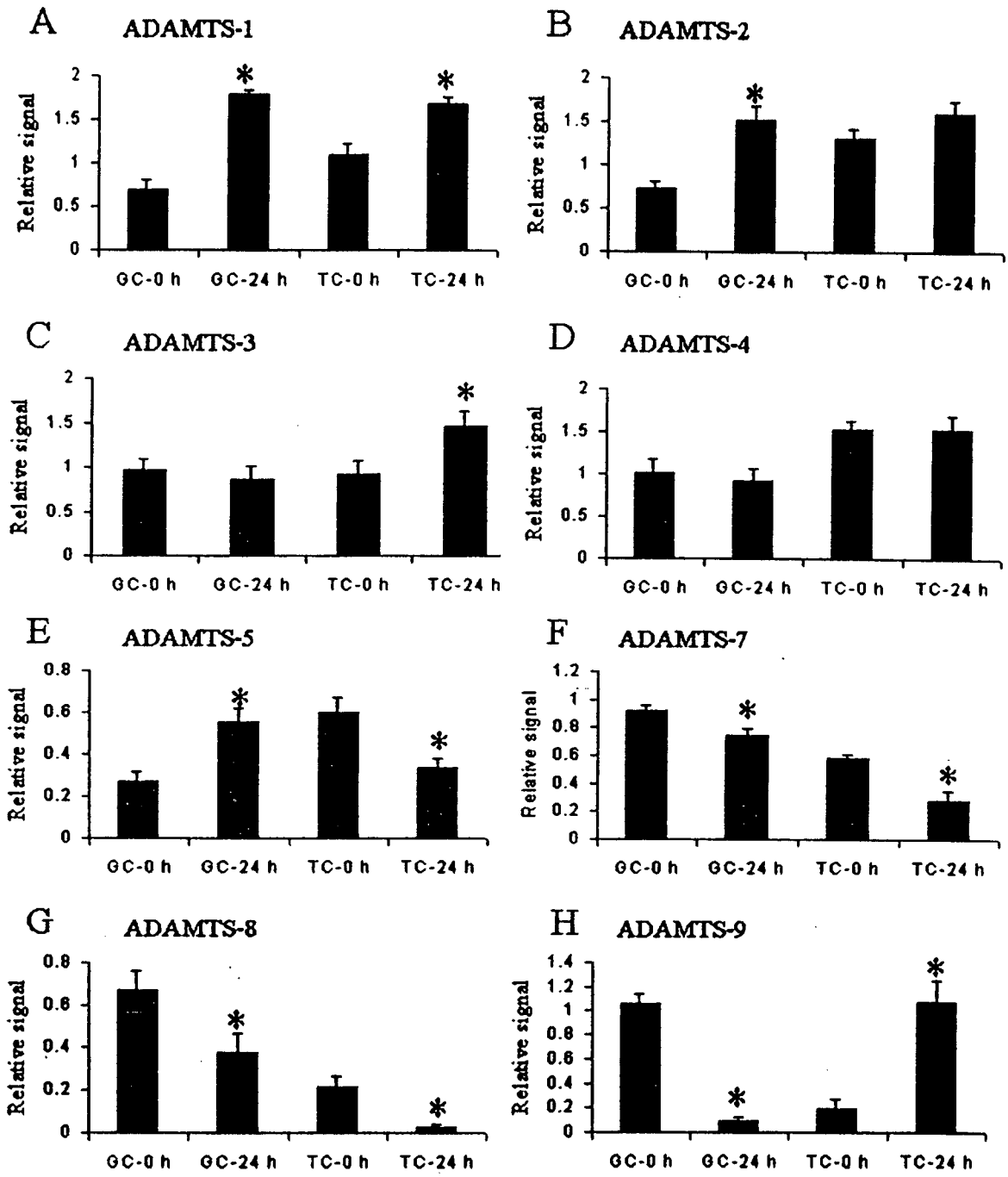

Fig. 5.2.5: Relative levels of the distinct ADAMTS mRNA transcripts present in granulosa and theca cells isolated from preovulatory follicles. Autoradiograms of Southern blots containing PCR products generated using template cDNA synthesized from total RNA extracts prepared from granulosa or thecal cells isolated from the preovulatory follicle at 0 or 24 h after the administration of GnRH (GC 0 h, GC 24 h, TC 0 h and TC 24 h respectively) and primers specific for the ADAMTS subtypes identified in these two cell types or the 18 S rRNA subunit were scanned using a laser densitometer. The absorbance values obtained for the distinct ADAMTS subtypes were then normalized to the absorbance value obtained for the 18 S rRNA. The results are represented (mean ± SEM; n=3 follicles) in the bar graphs. Superscript letters represent significant differences between the means ($P \leq 0.05$).

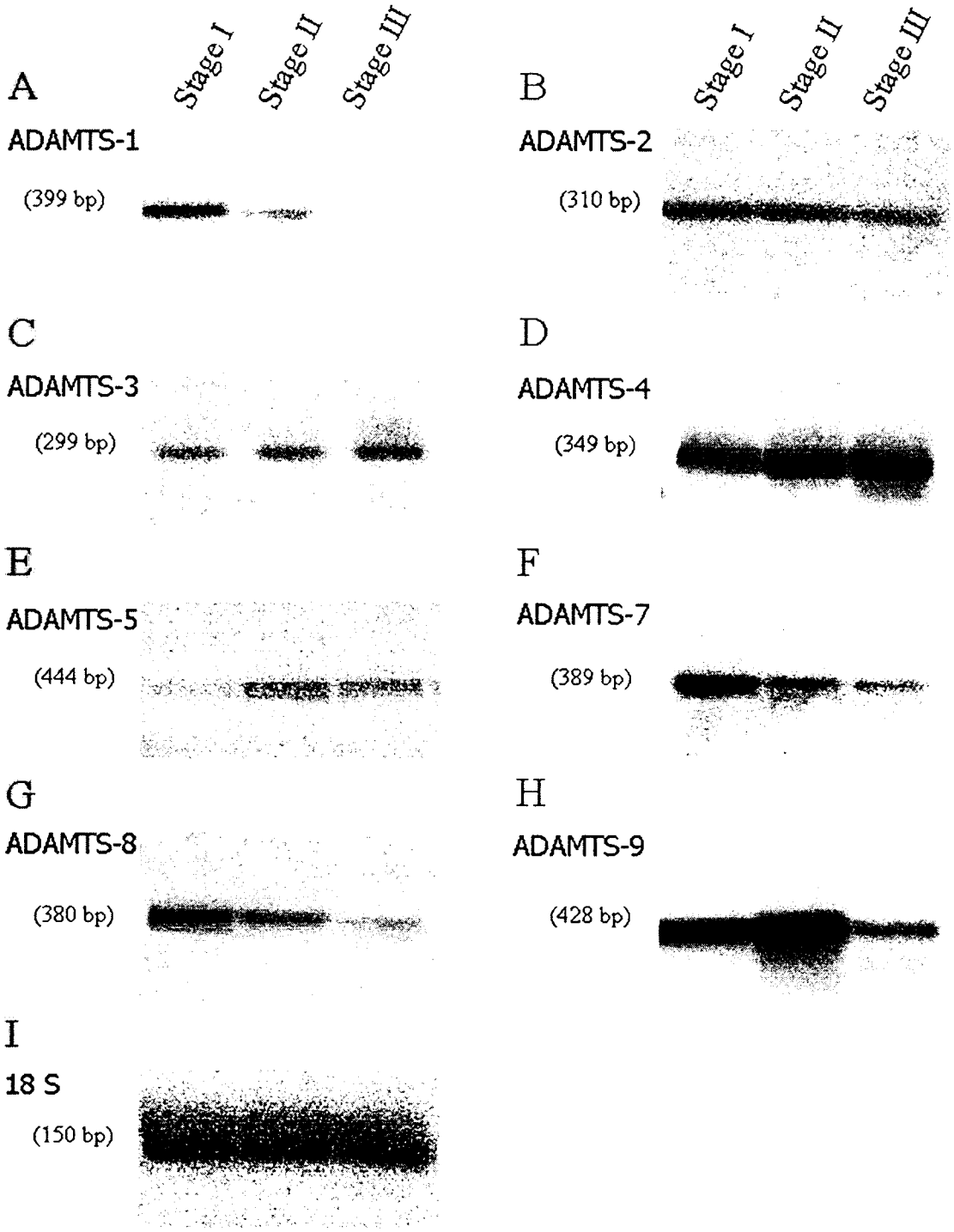

Fig. 5.2.6: Characterization of the ADAMTS subtypes present in CL tissues. Representative autoradiograms of Southern blots containing PCR products generated using template cDNA synthesized from total RNA extracted from CL tissues obtained during the early- (stage I), mid- (stage II), or late-(stage III) luteal phase and primers specific for ADAMTS-1, -2, -3, -4, -5, -7, or -9 (Panels A-H respectively) or the 18 S rRNA subunit (Panel I). The size of the distinct PCR products are shown to the left of the panels.

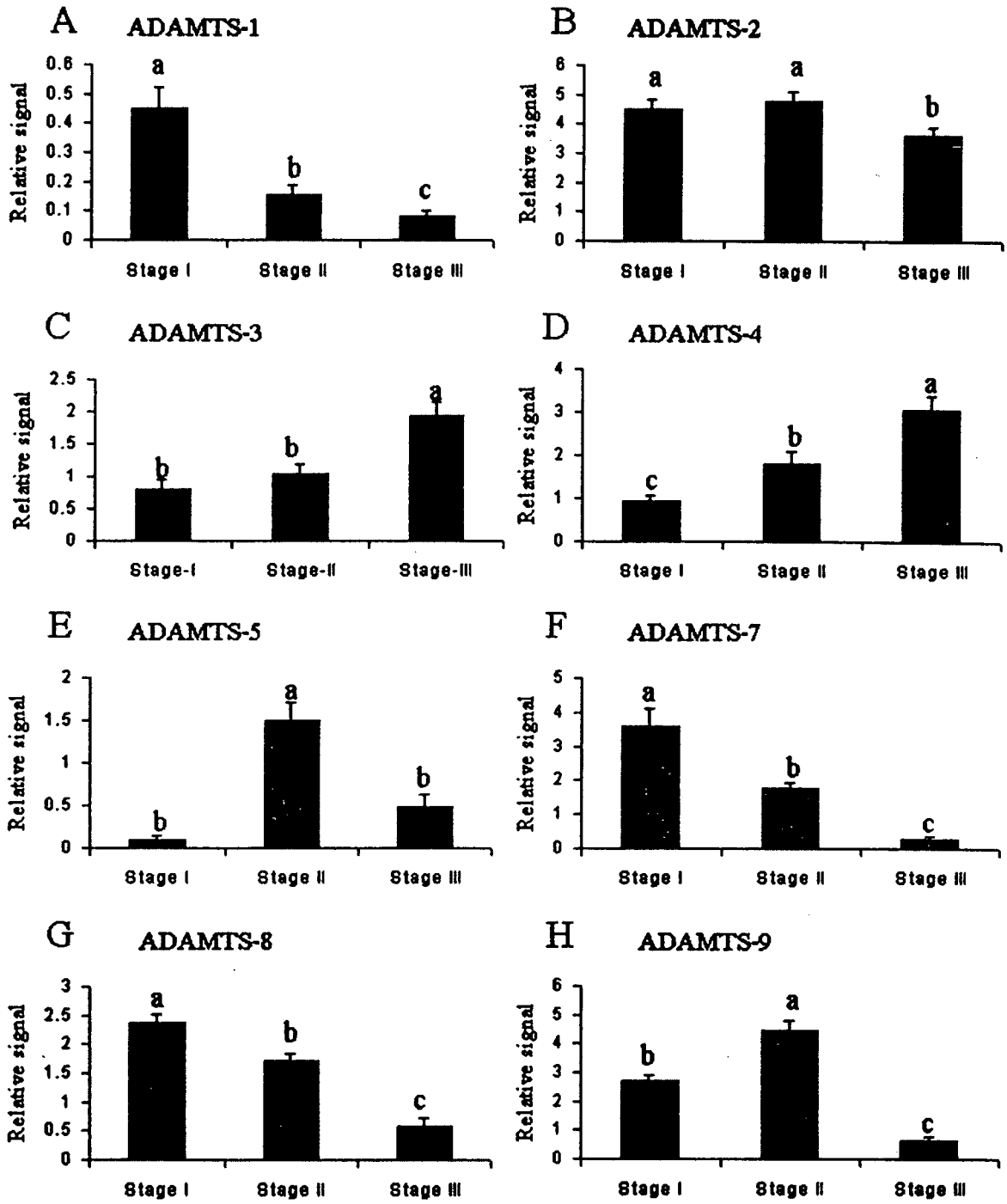

Fig. 5.2.7: Relative levels of the distinct ADAMTS mRNA transcripts present in CL tissues obtained at different stages of the luteal phase of the estrous cycle. Autoradiograms of Southern blots containing PCR products generated using template cDNA synthesized from total RNA extracted from CL tissues obtained during the early- (stage I), mid- (stage II), or late-(stage III) luteal phase and primers specific for the ADAMTS subtypes identified in these ovarian tissues or the 18 S rRNA subunit were scanned using a laser densitometer. The absorbance values obtained for the distinct ADAMTS subtypes were then normalized to the absorbance value obtained for the 18 S rRNA. The results are represented (mean $\pm$ SEM; n=3 CL/stage) in the bar graphs. Means with different superscripts are significantly different ($P \leq 0.05$).

ADAMTS Subtype	Primer Sequence	Estimated PCR Product Size	PCR Conditions
ADAMTS-1	Forward: 5'- CGAGTGTGCAAAGGAAGTGA-3' Reverse: 5'- CTACCCCCATAATCCCACCT-3'	339 bp	Denaturing: 94°C 30s Annealing: 65 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-2	Forward: 5'-CCTATGACTGCCTGCTGGAT-3' Reverse: 5'-TCCCAAAGTGCTGGGATAAC-3'	310 bp	Denaturing: 94°C 30s Annealing: 65 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-3	Forward: 5'-TCAAGGCCTTCCAGGTCCGACTCTC-3' Reverse: 5'- GGGAGCCTGTTCTACAGCTGATCTC-3'	299 bp	Denaturing: 94°C 30s Annealing: 60 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-4	Forward: 5'-ACCTTTCCCTGGGTAGCACT-3' Reverse: 5'-TCCTTGCATACCTCACTGCG-3'	320 bp	Denaturing: 94°C 30s Annealing: 60 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-5	Forward: 5'-GGCCATGGTAACTGTTTGCT-3' Reverse: 5'-CCTCTTCCCTGTGCAGTAGC-3'	444 bp	Denaturing: 94°C 30s Annealing: 65 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-6	Forward: 5'-TGACAGTCCAGCACCTTCAG-3' Reverse: 5'-CTACGTGCTTGCATTCTCCA-3'	340 bp	Denaturing: 94°C 30s Annealing: 60 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-7	Forward: 5'-CCATGTGGTGTACAAGCGTC-3' Reverse: 5'-GGTCCTCCTCCTCATCTTCC-3'	389 bp	Denaturing: 94°C 30s Annealing: 55 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-8	Forward: 5'-AAGAAGAGGAGGCAGAAGGC-3' Reverse: 5'-TCTGTCTGGTGAGCAGGATG-3'	380 bp	Denaturing: 94°C 30s Annealing: 65 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-9	Forward: 5'-CCCAGCCTGGACACATTACT-3' Reverse: 5'-CCCAGCCTGGACACATTACT-3'	428 bp	Denaturing: 94°C 30s Annealing: 65 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-10	Forward: 5'-TCTCTCAGTCACTTGGCCCT-3' Reverse: 5'-AGAGCGACCAGTTCCCTACA-3'	495 bp	Denaturing: 94°C 30s Annealing: 65 °C 30s Extension: 72 °C 60s 35 cycles
ADAMTS-12	Forward: 5'-GTGCAGCGAGGAGTACATCA-3' Reverse: 5'-GCGTTTTCTTTCTCCAGTGC-3'	488 bp	Denaturing: 94°C 30s Annealing: 65 °C 30s Extension: 72 °C 60s 35 cycles
18 S	Forward: 5'-GCTCGCTCCTCTCCTACTTG-3' Reverse: 5'-GATCGGCCCGAGGTTATCTA-3'	150 bp	Denaturing: 94°C 30s Annealing: 55 °C 30s Extension: 72 °C 60s 18 cycles

Table 5.2.1: Primer sequences and PCR conditions for the semiquantitative analysis of ADAMTS subtype mRNA levels in bovine ovary or preimplantation embryos

CHAPTER VI: GENERAL DISCUSSION, SUMMARY AND CONCLUSIONS

6.1 General discussion

Preimplantation embryogenesis and formation of CL being developmentally dynamic processes require spatiotemporal integration of cells brought about by a complex system of cell-cell and cell-matrix interactions. These interactions between cells and the ECM initiate a flow of information that acts to regulate many fundamental processes of development that includes cell migration, growth and differentiation. Several different classes of CAMs play a regulatory role in development. In particular, this study has focused on the role of cadherins, and a new family of proteinases, ADAMTS, during preimplantation embryogenesis. Since steroids serve to coordinate a complex series of interactive cellular events like leading to synchronized development of the embryo, the study has also determined whether receptors of estrogen and progesterone are expressed on the bovine embryo. In addition this study specifically examines the role of ADAMTS in folliculogenesis, CL formation and regression.

Previous studies have implicated the type 1 classical cadherin subtype, E-cad, in playing a key role during compaction, cavitation and blastocyst formation in several species including bovine (Barcroft et al., 1998; Reima, 1990; Reima et al., 1993; Shehu et al., 1996; Vestweber et al., 1987) and in addition, expression of N- and P-cad has also been reported in mice and it has been suggested to play a key role in blastomeres adhesion (Harrouk et al., 2000). In these studies, we have examined the expression pattern(s) of both type 1 and type 2 cadherins during preimplantation embryogenesis in the bovine embryo at timed stages of development from the 2-cell to the blastocyst stage. We have demonstrated that in addition to the expression of E-cad, two other type 1 cadherins, N-cad and P-cad are also expressed in the bovine embryo. In addition, to date, this is the first report of the presence of type 2 cadherins, specifically, cad-8, cad-10 and cad-11 in the preimplantation embryos of any species. Our findings also demonstrate that cadherin subtypes show a distinct pattern of aggregation and sublocalization during

preimplantation embryogenesis and thereby could be playing an important role in cell differentiation and cell allocation. The regulated expression of both type 1 and type 2 cadherins has previously been correlated with several other developmental process like formation of myotubules in skeletal muscles (Charlton et al., 1997; Knudsen et al., 1990; Marthiens et al., 2002a; Padilla et al., 1998; Pouliot et al., 1990; Sanes et al., 1986), osteoclasts during bone formation (Kawaguchi et al., 2001a; Kawaguchi et al., 2001b; Lecanda et al., 2000; Mbalaviele et al., 1995; Mbalaviele et al., 1998; Okazaki et al., 1994; Shin et al., 2000), in the formation and organization of CNS in mice (Fercakova, 2001; Goda, 2002; Hirano et al., 2003; Marthiens et al., 2002b; Togashi et al., 2002; Yagi and Takeichi, 2000) and in the process of invasion and placenta formation in humans (Floridon et al., 2000; Fujimoto et al., 1998; Getsios et al., 1998a; Getsios et al., 2000; Li et al., 2003; MacCalman et al., 1996; MacCalman et al., 1998; Merviel et al., 2001) and rat (Reuss et al., 1996). Based on the diverse roles of both type 1 and type 2 cadherins in regulating key developmental processes, it is tempting to speculate key roles of these diverse arrays of cadherins during preimplantation embryogenesis. The significance of this expression and localization in the embryo however is yet to be determined.

The expression of the repertoire of cadherins during preimplantation embryogenesis provides useful insights into the unexpected results obtained from cadherin knockout mice. Since heterophilic interactions ranging from incomplete to complete have been observed in type 1 (Renaud-Young and Gallin, 2002) and type 2 cadherins (Shimoyama et al., 2000), it is tempting to speculate that the unanticipated results obtained in cadherin knockout mice could be due to compensatory effect of other cadherins present in the embryo. The combinations of cad-8 and -11, cad-9 and -10, cad-6 and -9, and cad-7 and -14 have been shown to interact in a complete manner, and in particular cad-7 and -14 and cad-8 and -11 showed indistinguishable binding properties (Shimoyama et al., 2000). Since data for heterophilic interactions have been obtained

from in vitro studies, further studies with multiple cadherin knockouts or individual functional assays are needed to support the compensatory role(s) of these cadherins.

ECM that surrounds each cell is imperative in segregating cell types and physically supporting tissues and organs, has recently been shown to play an active role as an instructional entity as well (Streuli, 1999). Moreover, dynamic biological processes like embryo development require efficient degradation and remodeling of the ECM that are brought about by the proteolytic enzymes belonging to diverse protease families. In these studies, gamuts of ADAMTS subtypes being expressed during the preimplantation development of the bovine embryo have been identified. To the best of our knowledge, this is the first report of the expression of ADAMTS subtypes during preimplantation embryogenesis. Transcripts encoding ADAMTS -1, -4, -5, -6, -8, -9, -10 subtypes but not ADAMTS -2, -3 and -7 are expressed during bovine preimplantation embryogenesis up to the blastocyst stage of development. Since, the cell-ECM interactions are important in mediating diverse physiological events such as lineage decisions during embryogenesis, differentiation, cell migration and programmed cell death (Tang, 2001), it is tempting to speculate that these ADAMTS subtypes could be involved in a diverse array of embryonic biological processes including blastocyst formation and hatching. The regulated expression of diverse ADAMTS has been previously correlated with diverse morphological processes during development (Abbaszade et al., 1999; Cal et al., 2001; Cal et al., 2002; Clark et al., 2000; Colige et al., 1999; Fernandes et al., 2001; Hurskainen et al., 1999; Kuno et al., 1999; Li et al., 2001c; Shindo et al., 2000). The presence of these subtypes in the preimplantation embryo suggests that these ADAMTS subtypes could be involved in diverse processes during preimplantation embryogenesis and thus could be playing a key role in the formation of the blastocyst. However, the biological significance of the distinct expression patterns of the ADAMTS subtypes observed during preimplantation embryogenesis in bovines remains to be elucidated.

Elimination of both estrogen and progesterone leads to embryonic mortality. However, for estrogen and progesterone to have any physiological effect on embryos, their receptors (ER and PR respectively) must be present on the embryo. Previous studies have demonstrated the presence of ER and PR in mouse and pig embryos (Hiroi et al., 1999; Hou and Gorski, 1993; Hou et al., 1996; Kowalski et al., 2002; Ying et al., 2000a; Ying et al., 2000b). However, the presence of ER and PR has not been reported in the bovine embryo, though both have been detected on the bovine ovary (Manikkam et al., 2001; Rosenfeld et al., 1999; Van Den Broeck et al., 2002; Van den Broeck et al., 2002) as well as in the uterus (Robinson et al., 2001). Since it is highly unlikely that, the bovine embryo is merely a passive player during the early stages of development, it is quite possible that the intrinsic and extrinsic factors shown to influence preimplantation development could be mediated, at least in part, through the ER and PR. In view of these observations, we have examined the expression of ER and PR isoforms during development of the bovine embryo. Our results indicate that all four isoforms are expressed during bovine preimplantation development, though their expression is differential. ERβ mRNA levels decrease at the blastocyst stage with concomitant rise in ERα levels suggesting that in the early stages of development embryo could contain ERβ:β dimers. However, at the blastocyst stage since ERα is also expressed, the formation of homo- or heterodimers containing ERα may influence the pattern of gene activation within the developing embryo. Similar differential expression has previously been reported in several tissues including ovary (Jefferson et al., 2000; Sar and Welsch, 1999; Saunders et al., 2000; Slomczynska et al., 2001; Yang et al., 2002) and testis (Jefferson et al., 2000; Nie et al., 2002; Pelletier, 2000; Saunders et al., 2001). Moreover since ERα-estrogen binding has been shown to activate transcription and ERβ-estrogen binding inhibits transcription (Paech et al., 1997), it is tempting to speculate that blastocyst stage could be the period during development that some important estrogen dependent genes could be

activated after a period of repression under ERβ. However, further work needs to be done to ascertain this hypothesis. A predominant role for ERβ during preimplantation embryogenesis is further supported by immunodetection of ERβ during embryogenesis in our study. Our studies also demonstrate a differential role of PR isoforms during preimplantation embryogenesis, however the biological significance of this differential expression remains to be elucidated. In previous studies, an altered ratio of PR isoforms has been closely associated with modulations of various progesterone actions. For example, in the majority of progesterone responseive cells, PRB is the dominant activator of progesterone target genes, whereas PRA may inhibit activity of PRB (Tung et al., 1993; Vegeto et al., 1993). This repressor activity of PRA could also extend to other nuclear receptors including ER (McDonnell et al., 1994; McDonnell et al., 1992; Wen et al., 1994). Our findings of higher levels of PRB at the blastocyst stage could suggest that the embryo could be becoming more receptive to the progesterone responsive gene after a phase of inhibition by PRA. This correlates with the importance of progesterone for the development of the blastocyst, viability of the blastocyst as well as for the process of implantation.

Studies have demonstrated that the normal pattern of cell lineage and allocation can be disturbed in different species by altering the environment by certain growth factors (Lea et al., 1996), steroids (Greenlee et al., 1999; Greenlee et al., 2000; Juneja and Dodson, 1990), culture conditions (Lane and Gardner, 1997; Van Soom et al., 1996) and levels of maternal diet (Kwong et al., 2000). Since steroids are key regulators of cadherins (Chen et al., 1998; Chen et al., 1999a; Getsios et al., 1998b; MacCalman et al., 1995; Monks et al., 2001) and have also been shown to regulate the expression of ADAMTS (Miles et al., 2000; Richards, 2002; Robker et al., 2000a), it would be interesting to ascertain if the changes in growth, cell lineages and allocations during development are due to the alterations in the cadherin or ADAMTS expression coordinated by steroids through the receptors which we have identified to be present on the embryo. However, further work needs to be done to validate this correlation in the embryo.

There is increasing evidence to suggest that in vitro cultured preimplantation embryos demonstrate aberrant growth and specific phenotypic abnormalities during preimplantation, fetal and postnatal development (Bavister, 1995; Kruip and den Daas, 1997; Niemann and Wrenzycki, 2000; Walker et al., 1996). For example, in vitro cultured preimplantation embryos differ from their in vivo counterparts in many respects but specifically demonstrate darker cytoplasm (Pollard and Leibo, 1994; Rizos et al., 2002a) as a consequence of higher lipid content (Abd El Razek et al., 2000; Kikuchi et al., 2002), reduced intercellular communication (Boni et al., 1999), different metabolism (Khurana and Niemann, 2000) and more chromosomal abnormalities (Slimane et al., 2000; Viuff et al., 1999) . In addition, differences in relative abundances of developmentally important genes have also been reported (Khosla et al., 2001a; Khosla et al., 2001b; Lee et al., 2001b; Niemann and Wrenzycki, 2000; Rizos et al., 2002b). A recent study has determined that blastocysts cultured under non defined and defined culture conditions display different mRNA transcript patterns (Natale et al., 2001; Rizos et al., 2003) suggesting that the ingredients of the culture media regulate development. Although, mechanisms responsible for the deregulation of development as a consequence of in vitro culture remains elusive, the presence of cadherins, ADAMTS and steroid receptors working in their own capacities or in tandem provides an interesting possibility to understand the mechanisms involved in these aberrations. It is tempting to speculate that both cell-cell adhesive mechanisms and cell-matrix interactions being mediated by cadherins and ADAMTS respectively could be playing a role in cell-migration, segregation and allocation of embryo into a specific organized structure. It could also be possible that steroids could be regulating and governing their interactions. Any change in culture conditions, could mean that the embryos do not receive the appropriate cues leading to deregulation of embryonic development by inappropriate expression of cadherins, ADAMTS, steroid receptors and/or other yet unidentified factors. In addition, dissociation of blastomeres and fragmentation of cytoplasm, well documented in cultured embryos (Shamsuddin, 1994), and

has been suggested to be a possible reason for deregulation of development, it is tempting to speculate that cadherins and ADAMTS could be involved in this process. Further studies are however required to validate these hypotheses.

The present findings could also contribute to the understanding of the large offspring syndrome, which is characterized by increased fetal growth and newborns being heavier at birth than in vivo produced calves (Kruip and den Daas, 1997; Lazzari et al., 2002). The culture systems with which large offspring syndrome has been associated are varied suggesting a distinct role of the contents of the culture medium to contribute towards this syndrome (Khosla et al., 2001a; Natale et al., 2001; Rizos et al., 2003; Rizos et al., 2002b; Wrenzycki et al., 2001a). Similarly in humans, fetal development is affected by IVF procedures, i.e. the incidence of babies that are too small for their gestational age is significantly greater in singleton IVF pregnancies than in the normal obstetric populations (Tanbo et al., 1995; Wang et al., 1994; Wennerholm and Bergh, 2000). The steroid content could be a common factor in several culture media and our demonstration of steroid receptors on the embryo could possibly explain why different culture conditions are able to alter development. Therefore, the findings of these studies might also have strong implications for the application of assisted reproductive technologies in both bovines and humans. Moreover, it has been shown that the basic culture medium and supplementation with serum significantly affected the relative abundance of E-cad mRNA transcripts (Wrenzycki et al., 1999; Wrenzycki et al., 2001a). Similarly, it has been demonstrated that in vivo produced embryos express higher amounts of E-cad than in vitro produced embryos cultured in different culture medias (Wrenzycki et al., 2001a; Wrenzycki et al., 2001b) demonstratating that there is likely a difference in cadherin expression between in vitro and in vivo produced embryos.

Cloning of bovine embryos using nuclear transfer (NT) of fetal and adult somatic cells has also demonstrated a high rate of large offspring syndrome (Cibelli et al., 1998; Kato et al.,

1998; Kruip and den Daas, 1997; Niemann et al., 2002; Wells et al., 1999; Wells et al., 1998). Recent studies have demonstrated that modifications of NT protocols can alter the expression patterns of developmentally important genes in NT-derived embryos compared to their in vitro and in vivo produced counterparts (Wrenzycki et al., 2001b). It would be interesting to ascertain if the aberrant growth observed in NT embryos and calves could be explained due to the altered expression of cadherins, ADAMTS and steroid receptor isoforms working in isolation or in tandem. Such information will likely aid to reduce the incidence of the large offspring syndrome and should increase the proportion of viable NT calves.

Though, it can be argued that the present work was also carried out in an in vitro culture system, it needs to be mentioned that in vitro produced embryo provide a fast, economical and reliable method of obtaining the biological material required for this study. Since the purpose of our studies was to characterize the spatiotemporal expression of these novel set of molecules, and required a large number of embryos at timed stages of development to work with, in vitro produced embryos provided an efficient method of obtaining a regular harvest of embryos. Having now known the expression pattern of these novel cellular molecules, a comparison of their expression to that of in vivo produced embryos or embryos produced under a different set of culture conditions can be undertaken easily.

In the bovine ovary, ADAMTS-1, -2, -3, -4, -5 (also known as ADAMTS-11), -7, -8 and -9 but not ADAMTS-6, -10, or -12 mRNA transcripts were detected in non-atretic ovarian follicles and CL. The complex but regulated expression of these ovarian ADAMTS subtypes was highly regulated in the granulosa and/or theca cells of the dominant follicle following the preovulatory surge of gonadotropins and in the CL during the luteal phase of the estrous cycle suggests that these novel proteases mediate, at least in part, the remodeling events underlying folliculogenesis and ovulation and the formation, maintenance and regression of the CL. Since expression of ADAMTS has been shown to be associated with ECM remodeling in diverse

developmental processes, it is tempting to speculate that the ADAMTS subtypes identified in the ovarian follicles also play distinct role(s) in folliculogenesis and ovulation. Regulated expression of these distinct ADAMTS subtypes in granulosa and theca cells of the dominant follicle following the preovulatory surge of gonadotropins suggests that these ADAMTS may mediate, at least in part, the degradation of the follicle wall during ovulation and/or the dissolution of the granulosa cell basement membrane underlying the formation of the CL. A similar increase in ovarian ADAMTS-1 mRNA levels was observed in GnRH-primed rats treated with indomethacin, an anti-inflammatory agent capable of inhibiting ovulation (Espey et al., 2000) and mice null-mutant for the ADAMTS-1 gene are capable of ovulating (Shindo et al., 2000). Similarly, female mice null-mutant for ADAMTS-2 have normal ovarian function (Li et al., 2001c). Taken together, these observations suggest that an increase in the expression of ADAMTS-1 or ADAMTS-2 in the dominant follicle during the periovulatory period is neither necessary nor sufficient to mediate ovulation. The biological significance of the increase in specific ADAMTS subtypes in the granulosa or theca cell layers of the periovulatory bovine follicle remains unclear, but their up-regulation after the gonadotropin surge suggests potential roles for these ADAMTS subtypes, alone or in combination with ADAMTS-1 and -2, in the ovarian ECM remodeling events underlying ovulation.

6.2 Summary and conclusions

This study is an integrated and holistic effort to understand certain morphological and cellular aspects of cell-cell, cell-matrix and steroidal regulation during preimplantation embryogenesis and thereby understand the mechanisms regulating the formation of the blastocyst. In addition, this work provides an insight into the cell biology of ADAMTS during folliculogenesis, ovulation and CL formation and regression. The regulated expression of multiple type 1 and type 2 cadherins suggests that they may play distinct regulatory role(s) in the

development of the preimplantation embryo. Based on their specific sublocalizations, it is tempting to speculate that these CAMs could be playing an important role in the allocation of blastomeres to the two cell lineages of the blastocyst. Similarly, a unique but regulated pattern of expression of ADAMTSs mRNA in the bovine preimplantation embryo suggests a multitude of possible roles for these novel matrix components, including cell migration, growth and differentiation, but the majority of which remain to be determined. The characterization and cellular distribution of differentially expressed steroid receptor isoforms provides new insight into the possible mechanisms of steroidal regulation of embryo development. Furthermore, we have determined that mRNAs for multiple ADAMTS subtypes are present in the bovine ovary. As the levels of the mRNA transcripts encoding these distinct ADAMTS subtypes are highly regulated in the granulosa and theca cell layers of the dominant follicle following the administration of GnRH and in CL tissues during the luteal phase, it is tempting to speculate that members of this novel family of proteases are involved in the ECM remodeling events required for ovulation and/or the formation, maintenance and regression of the bovine CL. In conclusion, these observations provide the first demonstration of a possible biological role(s) of these novel cell molecules in the process of embryogenesis. Collectively, these studies not only further our understanding of the cellular mechanisms that mediate the formation and organization of the embryo, follicles, ovulation and CL formation but also give us useful insight into the cell biology of cadherins, ADAMTS and steroid receptors. A better understanding of the mechanisms by which cadherins, ADAMTS and steroids regulate formation of the blastocyst and the CL would have future implications in improving embryo quality and/or embryo viability.

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