Université de Montreal

EFFECT OF INTERFERON TAU ON THE SECRETION OF E-CADHERIN AND MACROPHAGE MIGRATION INHIBITORY FACTOR FROM BOVINE ENDOMETRIAL EPITHELIAL CELLS.

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EFFECT OF INTERFERON TAU ON THE SECRETION OF E-CADHERIN AND MACROPHAGE MIGRATION INHIBITORY FACTOR FROM BOVINE ENDOMETRIAL EPITHELIAL CELLS.

Présenté par

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RÉSUMÉ

Interferon-tau (IFN-τ) sécrété du concept allongé du bovin, exerce un effet paracrine sur la sécrétion d'une variété de protéines à partir de l`endométrium qui sont nécessaires pour l'adhésion de l'embryon, durant la période de préimplantation. L'objectif de ce travail était de déterminer si l'IFN-τ altérait les fonctions relatives à l'endomètre, en modifiant la sécrétion de protéines spécifiques importantes pour les fonctions utérines et/ou pour le développement de l'embryon. L'immunolocalisation du facteur d'inhibition de la migration du macrophage (MIF) était comparé sur des tissus endométrials provenant de vaches en cycles et enceintes. Les résultats démontrent une augmentation de la coloration de l'épithélium luminal et glandulaire de vaches enceintes. Le traitement des cellules épithéliales de l'endomètre bovin (BEEC) *in-vitro*, avec 100 ng/ml IFN-τ pour 24 h. démontre une plus forte accumulation du cytoplasme du MIF que les cellules contrôles. Donc les résultats *in vivo* observés sont probablement dû à cause de la stimulation du MIF par l' IFN-τ. Le milieu du BEEC traité *in vitro* avec 100 ng/ml IFN-τ pour 24 h. fut analysé par deux dimensions PAGE et Western blotting. Le résultat démontre l'apparence d'un point qui fut identifiée pour une forme soluble de E-cadherin.

Dans le présent ouvrage, en utilisant l'*in vitro* BEEC, il a été démontre que IFN-τ stimule le clivage protéolytique de l'E-cadherin et une subséquente accumulation dans le cytoplasme et accumulation du β-catenin à la membrane plasmatique. Un effet autocrine de MIF fut également observé sur E-cadherin et β-catenin dans BEEC *in vitro.* Ces changements du MIF et E-cadherin causés par IFN-τ, secrété par l'embryon jouent un rôle important dans l'attachement du trophoblaste sur le mur endométrial.

Adhésion, concept, embryon, utérus, enceinte, endométrium, préimplantation.

ABSTRACT.

Interferon-tau (IFN-τ) secreted from the elongated bovine conceptus exerts a paracrine effect on the secretion of variety of proteins from the endometrium that are necessary for adhesion of the embryo in the preimplantation period. The objective of this work was to determine if IFN-τ alters endometrial function by modifying the secretion of specific proteins important for endometrial function and/or embryo development. Immunolocalization of macrophage migration inhibitory factor (MIF) was compared in endometrial tissues from cyclic and pregnant cows compared to non pregnant cows. The results showed increased staining in luminal and glandular epithelium of pregnant cows. Treatment of bovine endometrial epithelial cells (BEEC) *in vitro* with 100 ng/ml IFN-τ for 24 h. showed stronger cytoplasmic accumulation of MIF than control cells. Therefore, the observed *in vivo* results are probably due to stimulation of MIF by IFN-τ. Medium from BEEC *in vitro* treated with IFN-τ for 24 h was analyzed by two-dimensional PAGE and Western blotting. The result showed the appearance of a spot that was identified as the soluble form of E-cadherin. In the present work using *in vitro* BEEC, it has been shown that IFN-τ stimulates the proteolytic cleavage of E-cadherin and subsequent accumulation in cytoplasm, and of β-catenin at the plasma membrane. An autocrine effect of MIF was also observed on E-cadherin and β-catenin in bovine endometrial epithelial cell *in vitro*. These data suggest that changes in MIF and E-cadherin induced by IFN-τ secreted by the embryo plays an important role in attachment of the trophoblast to the endometrial wall.

Adhesion, conceptus, embryo, uterus, pregnancy, endometrium, preimplantation

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LIST OF ABREVIATIONS

BEEC: bovine endometrial epithelial cells.

bHLH: basic helix-loop helix.

CL: corpus luteum.

COX: cyclooxygenase

CSFs: colony-stimulated factors.

E-17β: estradiol -17β.

EC: endothelial cells.

EC1-EC5: extracellular cadherin domains.

ECM: extracellular matrix.

EGFR: epidermal growth factor receptor.

ER: estrogen receptors.

ERK: extracellular signal-regulated kinase.

ERK1/2: extracellular-signal regulated kinase ½.

ET-1: endothelin-1.

Fzd: Frizzled proteins

GAS: interferon-g-activated sequence.

GCP-2: granulocyte chemotactic protein-2.

GE: glandular epithelium.

GM-CSF: Granulocyte-macrophage colony-stimulating factor.

hCG: Human corionic gonadotrophin.

HGF/SF: hepatocyte growth factor/scatter factor.

HGFRTKS: hepatocyte growth factor-regulated tyrosine kinase substrate.

HAV: His-Ala-Val

ICM: inner cell mass.

IFN-τ: interferon-tau.

IFN-R: interferon receptor.

IFNs: interferons.

IL-8: interleukin-8.

ILs: interleukins.

IP-10: interferon-gamma-inducible protein 10 kDa

IRF-1: interferon regulatory factor 1.

IRF-2: interferon regulatory factor 2.

ISG17: interferon stimulating gene 17.

ISGF: interferon-stimulated gene factor.

ISGF3: stimulated gene factor three

ISRE: interferon-stimulated regulatory element.

JAKs: tyrosine kinases, Janus kinases.

LE: luminal epithelium.

Lgs: Legless/BCL9

LH: luteinizing hormone.

LHr: luteinizing hormone receptors.

LLC: large luteal cells.

LPS: lipopolysacarides lipoproteins.

L-selectin: cell-surface selectin.

LEF/TCF: lymphoid enhancer factor/T-cell factor

MAP: mitogen-activated protein.

MAPK: MAP kinase.

MAPKs: mitogen-activated protein kinase

MHC: major histocompatibility complex.

MIF: Macrophage migration inhibitory factor.

MLC: myosin light chain

MLCK: myosin light chain kinase

MMPs: Matrix metalloproteinases.

MT1-MMP: Membrane type-1 matrix metalloproteinase.

MUC-1: Mucin glycoprotein 1.

NBCS: newborn calf serum.

Trp2: N-terminal b-strands

oIFN-τ: ovine interferon-tau.

OPN: osteopontin.

OT: oxitocin.

OTR: oxitocine receptors.

P: progesterone .

PBS: phosphate-buffered saline

PGF2α prostaglandin F2α.

PGE2 : prostaglandin E2.

PGFr: prostaglandin F receptors.

PKC: protein kinase C

PLs: placental lactogens.

PR: progesterone receptors.

PRPs: prolactin-related proteins.

Pygo: Pygopus

rMIF: recombinant MIF.

RTK: receptor tyrosine kinase.

SIBLING: small integrin-binding ligand, N-linked glycoprotein.

SLC: small luteal cells.

STATs: signal transducers and activators of transcription.

TBS: tris-buffered saline

TCF: transcription factors

TCF/LEF: T cell factor/lymphoid enhancer factor

TGFs: transforming growth factors.

TNF-α: tumor necrosis factor α.

TNFs: tumor necrosis factor.

Tr: Trophoblast.

UCRP: Ubiquitin cross-reactive protein.

VEGF: vascular endothelial growth factor.

AKNOWLEDGEMENTS.

Thanks to my *"Alma Mater"* University of Zacatecas, for this gift.

Acknowledge to the *University of Montreal*, for permitting me to do this work.

Sincerely thanks to my professor Dr *Alan K Goff,* for his kind help.

DEDICATION

For: Diana, Anabel y Luis Dario

"with all my affection".

Effect of interferon-tau on the secretion of E-cadherin and macrophage migration inhibitory factor from bovine endometrial epithelial cells.

INTRODUCTION

Early embryonic mortality causes a loss of 600 million dollars per year in reduced weaning weights and milk production (Austin, 2001) The embryo losses occur during the first 4 to 6 weeks of pregnancy in high producing dairy cattle, and during periods of nutritional or environmental stress, these losses can approach 80% (Ott, 2003a). The harmonic interactions between the hypothalamus, pituitary, ovary, uterus and conceptus are essential for normal embryo development and implantation. Disruptions in these interactions result in failure of the embryo to attach and embryo mortality. The basic events that occur in the uterus in response to pregnancy must be studied and understood so that future technologies may be designed to detect early pregnancy and decrease early abortion.

The successful implantation of the embryo in the cow requires a succession of coordinated events; these include progesterone-induced development of the uterus, and conceptus and placental formation. The communication between the conceptus and the maternal endometrium in the preimplantation period happens via the secretion of growth factors, hormones and cytokines and by several molecules including adhesion signaling, transcription, cell cycle and DNA replication proteins, that creates an environment propitious for attachment (Carson et al., 2000) When the conceptus and uterus are developing simultaneously, the attachment of trophectoderm to endometrium can occur. This event happens in ruminants, rodents and primates(Imakawa et al., 2004).

Steroid hormones secreted during the estrous cycle, estradiol -17β (E-17β) and progesterone (P), prepare the uterus to receive the embryo. The appropriate secretions of

the endometrium in the preattachment period permit the elongation and development of the conceptus in ruminants (Bazer and Roberts, 1983).

In the mammalian uterus the establishment of pregnancy is dependent on the corpus luteum (CL), which secretes P, the essential hormone of pregnancy. If the female does not become pregnant, the CL regresses due to a pulsatile secretion of prostaglandin F2α (PG F2α) from the endometrium, a process known as luteolysis and another new estrous cycle commences (Bazer and First, 1983). If the female becomes pregnant, the synchronous development of the endometrium and conceptus results in the trophectoderm secreting interferon tau (IFN-τ), which is the signal for the maternal recognition of pregnancy that prevents luteolysis, and allows the attachment and subsequent implantation (Bazer et al., 1997).

Ruminant blastocysts develop for up to three weeks in the uterine lumen, before implantation, during this period maternal-conceptus communication is established (Yamada et al., 2002). The trophoblast (Tr) secretes specific molecules such as placental lactogens, prolactin-related proteins, IFN-τ, and adhesion molecules such as integrins (Gumbiner, 1996; Hynes, 1987), glycoproteins and cadherins that serve as receptors for extracellular matrix ligand and act as modulator of cellular function (Lessey, 1994; Lessey et al., 1994a; Lessey et al., 1994b). After blastocyst elongation during the preimplantation period, IFN-τ acts on the bovine endometrium and increases secretion of several proteins that support conceptus-endometrium development necessary for adhesion.

Endometrial proteins such as interferon-gamma-inducible protein 10 kDa (IP-10) regulate the establishment of apical interactions between trophoblast and epithelial cells during early gestation (Nagaoka et al., 2003). Galectin-15 has an extracellular function to regulate Tr migration and adhesion to the endometrial epithelium, and intracellular function to regulate Tr cell survival, growth and differentiation (Gray et al., 2004). Granulocyte-

macrophage colony-stimulating factor (GM-CSF) (Emond et al., 2004) beta 2 microglobulin, interferon stimulate gene 17 (ISG17) (Johnson et al., 2002) osteopontin (OPN) I and OPN II may induce adhesion between luminal epithelium and trophectoderm to facilitate superficial implantation (Johnson et al., 1999a; Johnson et al., 1999b). In the early pregnancy, IFN-τ from bovine conceptus, stimulate secretion of uterine endometrial cytokines; ubiquitin cross-reactive protein (UCRP), bovine granulocyte chemotactic protein-2 (GCP-2) (Staggs and Dooley, 1998; Teixeira et al., 1997) and macrophage migration inhibitory factor (MIF) (Wang and Goff, 2003). Also IFN-τ may affect the cleavage of the external domain of E-cadherin, that is transmembrane protein of the adherents junctions and is responsible for homophilic cell to cell adhesion (Wheelock and Johnson, 2003a; Wheelock and Johnson, 2003b).

The role of proteins secreted by the uterus in trophoblast-epithelium adhesion has not been determined in domestic ruminants and remains a point of intense investigation. The effect of IFN-τ on cell adhesion in bovine endometrial epithelial cells is not understood. My goal was to determine if IFN-τ has an effect on the secretion of proteins such as MIF and E-cadherin and how these proteins are involved in the modification of epithelial cells for cell adhesion.

LITERATURE REVIEW

Conceptus development

In mammals after mating and fertilization the zygote develops into a blastocyst as it migrates through the oviduct into the uterus. Close to the uterus, the solid ball of cells, the morula, becomes fluid-filled and a cavity blastocoele appears, which enlarges rapidly and transforms the morula to a blastocyst. The blastocyst has peripheral layer of large flattened cells, the trophectoderm or trophoblast, and a knob of smaller cells to one side of the

central cavity, the so called inner cell mass (ICM) (Russell et al., 2006). The ICM will give rise mainly to the adult organism, while the cells of trophectoderm form the placenta and embryonic membranes (Koo et al., 2002; Maddox-Hyttel et al., 2003). After differentiation, the blastocyst hatches from the zone pellucida and acquires the ability to attach to the uterus (Brandao et al., 2004; Dalton et al., 1995; Hynes, 1987). This preimplantation stage varies in duration between species. In mice, implantation occur 4 days post coitum (McLaren, 1985), humans average 9 days (Lee and DeMayo, 2004), and in cow implantation does not occur until 30 days after fertilization (Xiang and MacLaren, 2002).

Tr cells express a number of extracellular matrix receptors and matrix-degrading activities that support interaction and invasion through the endometrium (Carson et al., 2000; Carson et al., 2002). During the development of the embryo, genes encoding for putative transcription factors, these transcription factors are expressed in ICM or trophoblast lineages, such as Rex-I (Rogers et al., 1991), GATA-3 (Ng et al., 1994), T-Box gene Eomesodermic (Hancock et al., 1999), the caudel related gene Cdx-2 (Beck et al., 1995), activating protein-2 gamma (Shi and Kellems, 1998), basic helix-loop helix (bHLH) (Sapin et al., 2000), Mash 2 (Rossant et al., 1998), Ets-2 that orchestrates modifications of cellular adhesion (de Launoit et al., 1998; Meyer et al., 1997), and a transcription factor protocadherins encodes a transmembrane cell adhesion molecule (Imakawa et al., 2004; Yamamoto et al., 1998). Comparison of day 7 and day 14 embryos revealed that blastocyst expresion of most genes increased during this period, and a small number of genes exhibited decreased expression. Clustering analysis demonstrated that trophoblast cells secrete specific molecules such as placental lactogens (PLs), prolactin-related proteins (PRPs), IFN-τ, and adhesion molecules that apparently all play pivotal roles in the

preparation needed for implantation, since their expression was remarkably enhanced during the pre-implantation period (Ushizawa et al., 2005a). Expression of ovine nterferon-tau (oIFN-τ) gene is restricted to the trophoblast and is not detected in any other cell types in ruminants. Substantial secretion of oIFN-τ, starts on day 12-13 of pregnancy, reaches the highest on day 16 –17, and then declines rapidly. Changes in the degree of DNA methylation could be one of the major mechanisms leading to downregulation of the oIFN-τ gene during early gestation (Nojima et al., 2004; Ushizawa et al., 2006). Expresion of gene eomesodermic, the caudel related gene Cdx-2, activating protein 2 gamma, bHLH, Mash 2, Hand I and Ets-2 increased up to implantation (Roberts et al., 2003). The change in expression of these genes propose novel molecules in trophoblast differentiation (Ushizawa et al., 2006; Ushizawa et al., 2005b).

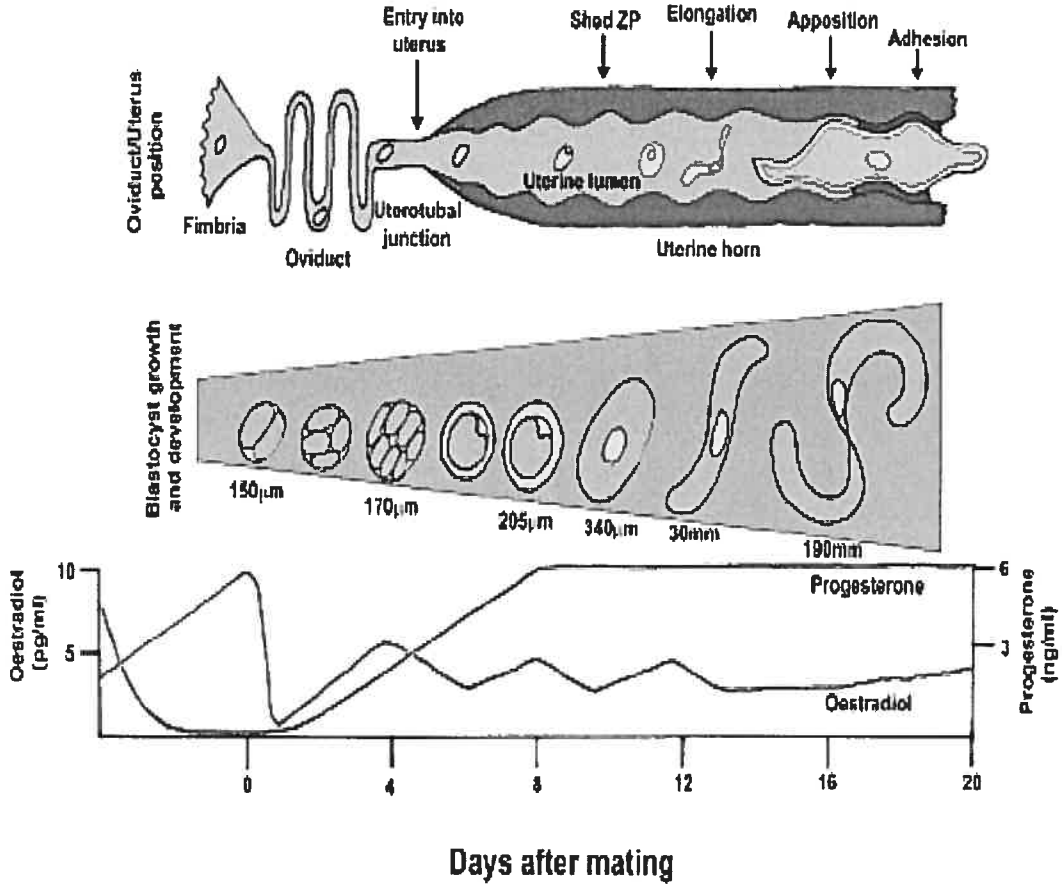

Figure 1. Early pregnancy events in sheep. This schematic summarizes the relative changes in embryo/blastocyst development after fertilization in relation to position in the female reproductive tract and circulating levels of ovarian steroid hormones. Fertilization occurs in the oviduct, and the morula stage embryo enters the uterus on day 4. The blastocyst is formed by day 6 and hatches from the zona pellucida on days 8–9. The blastocyst develops from a spherical to a tubular form by day 11 and then elongates to a filamentous conceptus between days 12 and 16. The elongation of the blastocyst marks the beginning of implantation, which involves apposition and transient attachment (days 12–15) and firm adhesion by day 16. Taken from Spencer et al., 2004.

Maternal preparation for implantation

The preparation of maternal tissue for implantation of conceptus implicates that the uterus undergoes dynamic changes, including differential and ordered activation or repression of gene expression and programmed changes in posttranscriptional and posttranslational modifications of mRNA and proteins (Carson et al., 2002). The steroid hormones secreted during estrous cycle E-17β and P, prepare the endometrium for the secretion of proteins and prostaglandins. The proteins nourish the embryo and the prostaglandins act on the corpus luteum and induce luteolysis if the animal does not have a viable embryo. The conceptus itself secretes growth factors, steroids, prostaglandins and cytokines depending on the species, which presumably act on the endometrium to prevent prostaglandin secretion, or directly on the ovary to stimulate protein secretion (Goff, 2002). Prostaglandins are also involved in implantation. IFN-τ elevated cycloxygenase (COX)-2 expression and selectively increased prostaglandin E2 (PGE2) secretion in epithelial cells at the time of pregnancy recognition, and may have a luteotropic effect (Asselin et al., 1997; Guzeloglu et al., 2004; Xiao et al., 1999).

In the estrous cycle, the coordinated secretion of hormones by the hypothalamus, ovary and uterus prepares the endometrium for the implantation of embryo. Gonadotropin-releasing hormone produced by hypothalamus regulates the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland. FSH and LH are synthesized and secreted by gonadotroph cells of the anterior pituitary gland (Wilson et al., 2004). These hormones regulate gametogenesis, steroidogenesis and ovulation in mammalian ovaries.

Corpus Luteum formation

The preovulatory LH surge initiates the differentiation of follicular cells into luteal cells (luteinization). The CL is a heterogeneous tissue containing endothelial cells, steroidogenic cells as large luteal cells (LLC) and small luteal cells (SLC), fibroblasts, smooth muscle cells and immune cells (O'Shea et al., 1989). The endothelial cells contribute approximately 50% of the total cell population of the CL (Meidan and Girsh, 1997). Studies by Mamluk et al., 1998 (Mamluk et al., 1998) showed that PGF2α is a major regulator of prostaglandin F receptor (PGFr) and luteinizing hormone receptor (LHr) expression in the two steroidogenic cell types, and all three major cell types of the CL (steroidogenic and endothelial) express PGFr and LHr mRNA (Mamluk et al., 1998). PGF2α induces an elevation in luteal expression of endothelin-1 (ET-1) (Girsh and Dekel, 2002) from endothelial cells, which may mediate the luteolytic action of PGF2α (Girsh et al., 1996; Levy et al., 2001).

The CL lasts for 17-18 days in the cyclic cow or for up to 200 days in the pregnant cow. Regression of the corpus luteum is essential for normal cyclicity as it allows the development of a new ovulatory follicle (Meidan and Girsh, 1997; Meidan et al., 1999). The CL produces P, required for the establishment and maintenance of pregnancy (Schams and Berisha, 2004). P also seems to play a luteotropic role by stimulating the synthesis of LHr in bovine CL (Jones et al., 1992). There is also evidence that P represses the onset of apoptosis in the CL by a progesterone receptor (PR) dependent mechanism (Rueda et al., 2000). During early diestrus, P from the newly formed CL stimulates accumulation of phospholipids in endometrial luminal epithelium (LE) and glandular epithelium (GE) that can liberate arachidonic acid for synthesis and secretion of PGF2α. During diestrus, progesterone levels increase and act via PR to block expression of estrogen receptors (ER) and oxytocin receptors (OTR) in the endometrial LE and GE (Spencer et al., 2004b).

Continuous exposure of the endometrium to P eventually down-regulates PR gene expression in the endometrial LE. The loss of PR terminates the P block to ERa and OTR formation. The increase in OTR expression is facilitated by increasing secretion of estrogen by ovarian follicles (Spencer and Bazer, 2002).

Luteolysis.

In ruminants and other large domestic animals PGF2α is the luteolysin secreted by the uterus that controls the length of the estrous cycle. Episodic release of PGF2α from the uterus reaches the CL through a counter current system between the uterine vein and the ovarian artery and induces luteolysis (Schams and Berisha, 2004). Luteolysis is initiated by increased expression of ERs and subsequently OTRs by the uterine endometrial epithelium. Oxytocin (OT) stimulates PGF2α secretion by cow endometrial cells (Ohtani et al., 2004; Tysseling et al., 1998; Tysseling et al., 1996) through activating the OTR (a 7-transmembrane, G-protein-associated receptor), increasing inositol triphosphate turnover, cytosolic calcium concentration, and activation of protein kinase C (Duras et al., 2005). In the uterus, these events result in activation of COX-2 (Asselin and Fortier, 1996). In cattle and sheep, luteolysis appears to be initiated by an increase in endometrial sensitivity to OT due to the increase of the number of OTR (McCracken et al., 1996; McCracken et al., 1999), and the existence of positive feedback loop between endometrial PGF2α and luteal OT secretion (Parkinson et al., 1992).

In contrast to PGF2α, PGE2 may be a luteotropic agent and could be a luteo-protective signal to antagonize potential luteolytic effects of PGF2α. Before implantation, PGE2 may also be responsible for the increase in vascular permeability and secretion of growth factors and nutrients, and it may be involved in the local regulation of immune responses (Emond et al., 1998).

Embryo endometrial interactions

The synchronous development of blastocyst and endometrial luminal epithelium receptivity initiates an adhesion cascade that results in implantation. In ruminants the blastocyst sheds the zona pellucida (day 8) and elongates to a filamentous form. The elongation of the blastocyst marks the beginning of implantation, which involves apposition and transient attachment (days 12–15) and firm adhesion by day 16 in sheep (Spencer et al., 2004a). Apposition of the conceptus involves the trophectoderm becoming closely associated with the endometrial LE followed by unstable adhesion. After day 14, the filamentous conceptus appears to be immobilized in the uterine lumen. The elongating blastocyst maintains close contact with the endometrial LE, which appears to imprint its rounded shape on the trophectoderm in fixed specimens (Gharib-Hamrouche et al., 1993). On day 15, apposition occurred: most microvilli on the surface of the trophoblast disappeared. Between days 16 and 18, adhesion began as a result of the interpenetration of the uterine microvilli and cytoplasmic projections of the trophoblast cells (Guillomot et al., 1981). Adhesion of the trophectoderm to the endometrial LE progresses along the uterine horn and appears to be completed around day 22 and 28, the establishment of an overall intimate epithelial contact of fetal binucleate cell with microvillar junction take place and continued binucleate cell migration at the time of mature bovine placenta formation (King et al., 1982; Wathes and Wooding, 1980).

The endometrium in ruminants consists of LE, GE, several types of stroma (stratum compactum and stratum spongiosum), blood vessels and immune cells. In sheep, the endometrium has two distinct areas: aglandular caruncular and glandular intercaruncular. The caruncular consist of LE and compact stroma and are the sites of superficial implantation and placentation (Amoroso and Perry, 1975; Lawn et al., 1969). Placentation

in ruminants such as cows and goats are noninvasive, or the extent of invasion is very limited (Hashizume et al., 2003; Hirata et al., 2003; Steven, 1975). Tr remain essentially in the uterine lumen and placentation involves only superficial physical contact with the maternal tissue (Carter and Enders, 2004). In contrast placentation in humans and mice is highly invasive as the Tr penetrates into endometrial stromal tissue (Bischof and Campana, 2000; Bischof et al., 2000a; Bischof et al., 2000b).

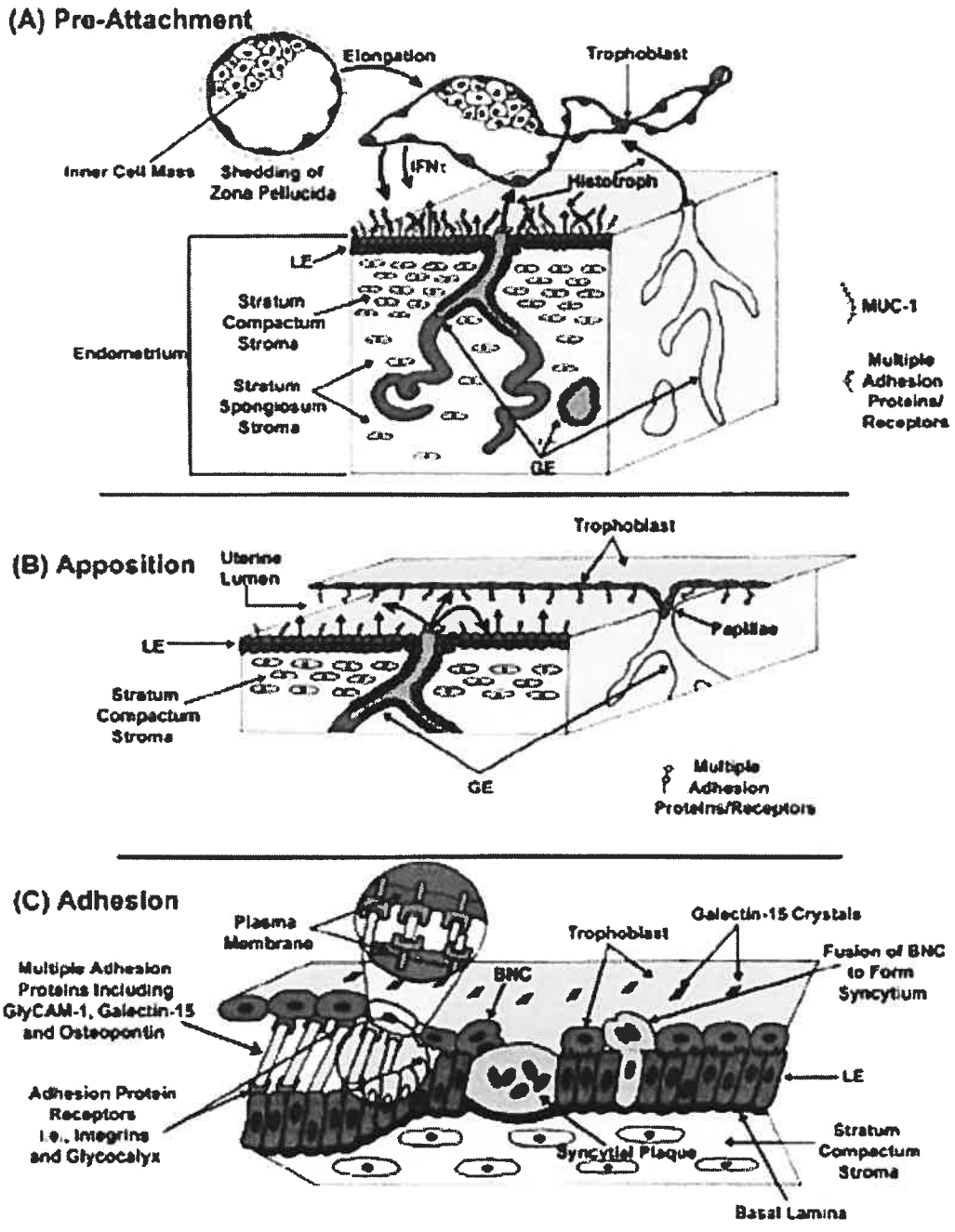
(A) Pre-Attachment
Elongation
Trophoblast
Inner Cell Mass
Shedding of Zona Pellucida
IFNt
Histotroph
LE
Stratum Compactum Stroma
Endometrium
Stratum Spongiosum Stroma
GE
MUC-1
Multiple Adhesion Proteins/ Receptors
(B) Apposition
Uterine Lumen
Trophoblast
Papillae
LE
Stratum Compactum Stroma
GE
Multiple Adhesion Proteins/Receptors
(C) Adhesion
Plasma Membrane
Trophoblast
Galectin-15 Crystals
Fusion of BNC to Form Syncytium
Multiple Adhesion Proteins including GlyCAM-1, Galectin-15 and Osteopontin
BNC
LE
Adhesion Protein Receptors i.e., Integrins and Glycocalyx
Syncytial Plaque
Stratum Compactum Stroma
Basal Lamina

Figure 2 Apposition and adhesion phases of blastocyst implantation in sheep. (A) Preattachment involving shedding of the zona pellucida (phase 1) and precontact and blastocyst orientation (phase 2). The antiadhesive mucin MUC-1 is present on the endometrial LE, thereby preventing contact of the Tr with adhesive receptors such as integrins. Histotroph, secreted from the endometrial LE and GE, nourishes the developing blastocyst. (B) Apposition and transient attachment (phase 3). After day 11, the tubular blastocyst elongates to form a filamentous conceptus. During this period, expression of MUC-1 declines on the LE, which exposes constitutively expressed integrins on the LE as well as trophoblast. Apposition occurs between the trophoblast and endometrial LE and between the Tr papillae and GE ducts. Elongation of the blastocyst probably requires apposition and transient attachment to the endometrial LE. (C) Adhesion (phase 4). Firm adhesion of the mononuclear cells of the Tr to the LE occurs between days 15 and 16. Available evidence indicates that several molecules (GlyCAM-1, galectin-15 and osteopontin) interact with receptors (integrins and glycoconjugates) on the apical surfaces of the Tr and LE to facilitate adhesion. Taken from (Spencer and Bazer, 2004).

Recognition of pregnancy

During the preimplantation period, pregnancy recognition signals from the conceptus to the maternal system are antiluteolytic and/or luteotrophic. The functional life span of the CL is controlled by release of PGF2α from the uterus and IFN-τ is the signal from the trophoblast acts in a paracrine or endocrine manner to interrupt endometrial production of luteolytic PGF2α (Kim et al., 2003).

Maternal recognition of pregnancy in ruminants (sheep, cattle, goats) requires that the conceptus elongate from a spherical to a tubular and then filamentous form to produce IFN-τ, which is the signal that prevents development of the endometrial luteolytic mechanism (Kim et al., 2003; Spencer and Bazer, 2002). IFN-τ is considered to prevent luteolysis by blocking the upregulation in OTR during diestrus (Spencer et al., 1996), through the activation and repression of genes responsive to Type I IFN (Green et al., 2005).

The IFN-τ secreted by conceptus inhibits OTRs and protects the CL and maintains secretion of P (Bazer and First, 1983; Bazer and Roberts, 1983). IFN-τ is released by the conceptus in the pregnant cow by day 12-28, reaches highest levels on days 15-19, after which time levels decrease from days 21 to 26 of pregnancy (Roberts, 1991; Roberts et al., 1991a; Roberts et al., 1991b). In the sheep, the quantities of oIFN-τ secreted are elevated (20–200 mg/day) by days 14-16 of pregnancy and 1-2 mg/day by the day 12 of pregnancy (Rooke et al., 2005). IFN-τ is reduced as definitive attachment of trophectoderm to the uterine epithelium is established (Day 21 or 25 of pregnancy in the sheep and cow, respectively) (Roberts et al., 1992; Spencer and Bazer, 2004).

The only maternal tissue immediately exposed to IFN-τ is the epithelium that borders the endometrium (Rosenfeld et al., 2002). IFN-τ binds to a dimeric interferon receptor (IFN-R)

in the cell membrane. The intracellular domain of the receptor binds tyrosine kinases, Janus kinases, (JAKs) which are activated after interferon binding, and subsequently phosphorylate other proteins named signal transducers and activators of transcription (STATs). The STATs dimerize and bind two other proteins to form a trimeric interferon-stimulated gene factor (ISGF) complex, which is translocated to the nucleus, where it binds an interferon-stimulated regulatory element (ISRE), resulting in the expression of the interferon regulatory factor 1 (IRF-1) gene. The product of this gene, in turn, activates expression of IRF-2, which interacts with other regulatory elements to control the expression of interferon-responsive genes, including the OTR and ER receptors (Demmers et al., 2001).

IFN-τ possess similar antiproliferative and antiviral activities to other Type I IFN (Mathialagan and Roberts, 1994). IFN-τ affects the synthesis of cytokines that contribute to the immunomodulation required to prevent rejection of the conceptus and stimulate blastocyst growth (Demmers et al., 2001; Gierek et al., 2006).

Adhesion mechanisms

Cell adhesion mechanisms are responsible for assembling cells together and, along with their connections to the internal cytoskeleton determine the overall architecture of the tissue (Gumbiner, 1992). Three general classes of proteins take part in cell adhesion: the cell adhesion molecules/adhesion receptors, the cell-extracellular matrix (ECM) proteins, and the cytoplasmic plaque/peripheral membrane proteins (Gumbiner, 1996). The cell adhesion receptors are glycoproteins that mediate binding interactions at the extracellular surface and determine the specificity of cell-cell and cell-ECM recognition. They comprise integrins, cadherins, immunoglobulins, selectins and proteoglycans (Bella and Berman, 2000). The cell adhesion receptors recognize and interact with either other cell adhesion

receptors on neighboring cells or with proteins of the ECM. The ECM is typically composed of large glycoproteins including collagen, fibronectins, laminins and proteoglycans. Cytoplasmic plaque proteins serve to link the adhesion systems to the cytoskeleton, to regulate the functions of the adhesion molecules, and to initiate transducer signals at the cell surface by the adhesion receptors (Gumbiner, 2005). Signals generated locally by the adhesion receptors themselves are involved in the regulation of cell adhesion. These regulatory pathways are also influenced by extrinsic signals arising from the classic growth factor receptors (Gumbiner, 1996).Adhesion mechanisms are highly regulated during tissue morphogenesis and are intimately related to the processes of cell motility and cell migration. In particular, the cadherins and the integrins have been implicated in the control of cell movement. Cadherin-mediated cell compaction; integrins-mediated cell spreading and motility on the extracellular matrix (Wheelock and Johnson, 2003a; Wheelock and Johnson, 2003b).

Adhesion molecules and implantation

Endometrial epithelium synthesize and secrete or transport a complex array of proteins and related substances termed "histotroph", that is a mixture of enzymes, growth factors, cytokines, lymphokines, hormones and other substances that act as primary regulators of conceptus survival, development, production of pregnancy recognition signals, implantation and placentation (Bazer et al., 1979; Burton et al., 2002) These uterine secretions establishing synchrony between development of the conceptus and uterine receptivity that remodeling the endometrial LE for conceptus adhesion (Burghardt et al., 2002). The LE that is a simple, polarized cell layer mediates cell-cell and cell-extracellular matrix interactions is normally nonadhesive; however, this characters is lost during

development of receptivity and begin apical adhesion between LE and Tr defines the onset of implantation (Lue et al., 2006).

In the early pregnancy, continuous exposure of the endometrium to progesterone down regulates the progesterone receptors in the epithelia, a process that is associated with loss of the cell-surface mucin glycoprotein 1 (MUC-1) and induction of several secreted adhesion proteins (Carson et al., 1998). The removal of mucin, that is an antiadhesive barrier is hypothesized to be necessary to expose other glycoproteins involved in the adhesion between Tr and LE (Aplin and Hey, 1995; Hey et al., 1995).Mucin is locally reduced at implantation sites, via the activity of cell-surface proteases that are triggered by the blastocyst or mediated by paracrine signals from blastocysts (Brayman et al., 2004; Thathiah et al., 2004). In sheep the implantation adhesion cascade is initiated after down regulation of MUC-1 (Johnson et al., 2001).

A number of endometrial proteins have been identified as potential regulators of blastocyst development and implantation in sheep, including glycosylated cell adhesion molecule 1, galectin-15, osteopontin, that binding to adhesion receptors such as integrin, cadherin and immunoglobulin and selectin, to proteins of ECM. These adhesion proteins are secreted for luminal epithelium and regulate for progesterone or IFN-τ produced by the Tr during blastocyst elongation (Spencer et al., 2004a).

Galectins are proteins with a conserved carbohydrate recognition domain that bind b-galactosides, thereby cross-linking glycoproteins as well as glycolipid receptors on the surface of cells and initiating biologic responses (Cooper, 2002). Functional studies of other galectins have implicated these proteins in cell growth, differentiation and apoptosis as well as in cell adhesion, chemoattraction and migration (Yang and Liu, 2003).

Integrins comprise a family of heterodimeric intrinsic transmembrane glycoprotein receptors that mediate cellular differentiation, motility and adhesion (Giancotti and Ruoslahti, 1999; Munger et al., 1998; Oktay et al., 1999). The central role of integrins in the implantation adhesion cascade is to bind ECM ligand(s) to cause cytoskeletal reorganization, stabilize adhesion, and mediate cell migration, proliferation and differentiation through numerous signaling intermediates(Burghardt et al., 2002; Pfarrer, 2006). Altered expression of integrins is correlated with several causes of infertility (Lessey, 1994; Lessey et al., 1994a), null mutations of several integrins leads to peri-implantation lethality (Hynes, 1996) and functional blockade of selected integrins reduces the number of implantation sites. In the sheep, receptivity to implantation does not appear to involve changes in either temporal or spatial patterns of integrin expression, but may depend on expression of other glycoproteins and ECM proteins, such as galectin-15, OPN and fibronectin, which are ligands for heterodimers of these integrins.

OPN is a member of the small integrin-binding ligand, N-linked glycoprotein (SIBLING) family of genetically related ECM proteins recognized as key players in a number of diverse processes such as bone mineralization, cancer metastasis, cell-mediated immune responses, inflammation, angiogenesis and cell survival (Johnson et al., 2003a; Sodek et al., 2000).OPN has also been linked to pregnancy (Johnson et al., 2003b). Microarray profiling identified OPN as the most highly upregulated ECM adhesion molecule in human endometrium that is receptive to implantation (Carson et al., 2002). Multiple integrin receptors for OPN are present on trophoblasts and LE of humans and domestic animals, some of which increase during the peri-implantation period (Damario et al., 2001; Lessey, 1994).

IFN induced protein secretion from endometrium

When the bovine conceptus makes contact with the uterine wall, IFN-τ is secreted and has an immunomodulatory activity that offers protection for the embryo from the immune system of the mother. IFN-τ is an apoptotic protein that induces the secretion of protein and cytokines and prepares the endometrium for embryo attachment. IFN-τ possesses potent antiviral, antiproliferative, and immunomodulatory activities (Demmers et al., 2001). It can also alter the synthesis of endometrial proteins; retard the growth of the endometrium during the preimplantation period (Roberts et al., 1992).

IFN-τ was shown to increase expression of beta 2 microglobulin, ISG17 (also known as Ubiquitin Cross Reactive Protein), which is expressed in the endometrium of Day 17 pregnant cows, prepares the uterine wall for the adhesion and implantation of the embryo (Austin, 2001; Hicks et al., 2003; Ott, 2003b; Ott et al., 1998). ISG17 controls cytosolic protein processing through the proteosome; osteopontin (Johnson, 1999), which promotes cell–cell attachment and may be involved in attachment of the blastocyst to the endometrial epithelial surface; and the antiviral Mx protein (Ott et al., 1998).

IFN-τ stimulate the expression of endometrial IP-10 that regulates the establishment of apical interactions between trophoblast and epithelial cells during early gestation (Nagaoka et al., 2003). Also Galectin-15 was discovered in the uterus of sheep, and secretion in to the uterine lumen increased between days 14 and 16 of pregnancy and galectin-15 mRNA was detected only in the endometrial LE and superficial ductal GE (Kuwabara et al., 2003; Purkrabkova et al., 2003). Galectin-15 is hypothesized to function extracellularly to regulate trophoblast migration and adhesion to the endometrial epithelium, and intracellularly to regulate trophoblast cell survival, growth, and differentiation (Gray et al., 2004).

Expression and secretion of OPN I and OPN II are induced by IFN-τ in uterine glands during the periimplantation period. Also OPN is a potential mediator of implantation in sheep, as a bridge between integrin heterodimers expressed by Tr and uterine LE responsible for adhesion for initial conceptus attachment (Johnson et al., 2001; Johnson et al., 1999a; Johnson et al., 1999b) Also IFN-τ induces adhesion molecules UCRP and GCP-2 during early pregnancy (Staggs and Dooley, 1998).

In the periattachment period IFN-τ increases the expression of GM-CSF in immune and nonimmune cells of the bovine endometrium (Emond et al., 2004). Furthermore, IFN-τ stimulates the production of PGE2 in bovine endometrial cells via the induction of COX-2, PGE2 increases the expression of GM-CSF, a cytokine that promotes conceptus growth and survival, from leukocytes and endometrial stromal cells (Emond et al., 2000; Emond et al., 2004).

Pleiotropic cytokine MIF was stimulate for IFN-τ from uterine epithelial cells (Arcuri et al., 2001; Wang and Goff, 2003). The pleiotropic activities of MIF are based upon transcriptional regulation of inflammatory gene products, modulation of cell proliferation, differentiation, and cell cycle control, inhibition of apoptosis, and several metabolic effects (Calandra et al., 2000; Lue et al., 2006; Walter et al., 2000; Wang and Goff, 2003; Wang et al., 2003) MIF is released by bovine endometrial epithelial, but not stromal, cells and is stimulated in response at IFN-τ (Wang and Goff, 2003). MIF has an autocrine effect in adhesion; it stimulates the formation of integrin clusters and accumulation of cytoplasmic β-catenin.

Endometrial cytokines

The cytokines are implicated in processes characteristic of inflammation, immunity, remodeling and the migration of various cellular components. In mammalian reproduction

processes like ovulation, blastocyst implantation and parturition resemble those of the inflammatory and reparative processes in which cytokines and chemokines act as autocrine and paracrine mediators. Actually, many reports have widely documented the involvement of cytokines in the intercellular signaling that affect reproductive events (Orsi et al., 2006). A recent study demonstrates the transcription of 16 different cytokines common in normal and tumor bearing ovaries (Burke et al., 1996).

The endometrium is source of cytokines; interleukins (ILs), tumor necrosis factors (TNFs), transforming growth factors (TGFs), colony-stimulating factors (CSFs), and interferons (IFNs) have been reported in cycling and pregnant endometrium (Arcuri et al., 1999; Arcuri et al., 2001). The cytokine interleukin-1 beta has a major effect on gene expression in stromal cells from human endometrium and plays a role in disorders of the endometrium, especially in implantation-related infertility and endometriosis (Rossi et al., 2005).

The recently recognized multifunctional cytokine MIF that modulates the immune response and acts as a growth and angiogenic factor (Baugh and Bucala, 2002), has possible functions in reproduction. MIF mRNA and protein have been identified in murine and human ovaries as well as in human follicular fluid. In rodents, MIF has been detected in the amniotic fluid and in the early embryo (Nishihira et al., 1998).

In human pregnancy, a sarcolectin-binding protein whose properties corresponded to those of MIF has been described in term placenta (Zeng et al., 1993) also MIF is expressed by first-trimester human trophoblasts (Arcuri et al., 1999). Similarly, in human endometrium hCG induces MIF synthesis and secretion by endometrial stromal cells, and results of nuclear transcription assays (run-on) revealed that hCG acts predominantly by up-regulating MIF gene transcription (Akoum et al., 2005; Kats et al., 2005). Human

endometrial epithelial cells can also secrete MIF (Chaisavaneeyakorn et al., 2005). Thus, as demonstrated by immunohistochemistry, in the secretory-phase glandular epithelium, the protein is mainly located on the luminal side, usually at the apical surface of the cells (Paulesu et al., 2005). Moreover, abundant immunoreactive material is present in the glandular secretion (Arcuri et al., 1999; Paulesu et al., 2005), and it has been suggested that endometrial epithelial cells secrete MIF during the luteal phase of the menstrual cycle (Arcuri et al., 2001).

MIF and the immune response

MIF has immunosuppressive activity and it has bee shown that MIF is capable of inhibiting Natural Killer cells in cell-mediated cytolysis of both neoplastic and normal cells of human endometrium (Apte et al., 1998). Also MIF represents an important effector of hCG-induced endometrial changes during embryo implantation, growth, and development (Akoum et al., 2005).

MIF is present in human serum at concentrations ranging from 2-6 ng/ml. Macrophages contain large quantities of stored, pre-formed MIF (2-4 fg/cell) that is released in response in lipoproteins (LPS) stimulation. Physiological concentrations of glucocorticoids stimulate macrophage secretion of MIF (Isidori et al., 2002; Nishihira et al., 1995). On release from macrophages, MIF can exert potent autocrine and paracrine effects, promoting cell activation, proinflammatory cytokine release and overriding glucocorticoid action at the site of inflammation (Chesney et al., 1999; Isidori et al., 2002; Liao et al., 2003). MIF is present in several tissues including T lymphocytes, anterior pituitary cells, monocyte /macrophages, eosinophils, endothelium, various epithelial cell types, fibroblasts, and muscle cells (Baugh and Bucala, 2002).

MIF is involved in the regulation of innate and adaptative immunity. It is a counter-regulator of glucocorticoid action within the immune system (Baugh and Donnelly, 2003), and inhibits the random migration of macrophages and promotes tumor cell growth. The pro-inflamatory effect of MIF may be explained by its ability to induce release of the pro-inflammatory cytokine tumor necrosis factor α (TNF-α) by macrophages, and form a positive feedback loop, as TNF-α is itself able to induce MIF secretion via tyrosine-kinase-dependent pathway (Mitchell et al., 2002).

MIF in proliferation and differentiation

MIF has the potential to suppress the action of the tumor suppressor gene p53, leading to cell growth (Fingerle-Rowson et al., 2003). MIF inhibits p53 activity in macrophages via an autocrine regulatory pathway, resulting in a decrease in cellular p53 accumulation and subsequent function. This mechanism to explain its critical proinflammatory action of MIF in conditions such as sepsis (Mitchell, 2002). MIF induces tumor cell growth in concert with other growth factors. It stimulates the proliferation of fibroblasts and also in wound repair (Takahashi et al., 1998). MIF could promote both tumor cell growth and angiogenesis induced by lysophosphatidic acid via mitogen-activated protein kinase (MAPK) signaling pathways (Sun et al., 2003). MIF is directly associated with the growth of lymphoma, melanoma, and colon cancer (Nishihira et al., 2003). Studies where treatments with either anti-MIF immunoglobulin therapy and/or MIF antisense oligonucleotide confer antitumor activity (Chesney et al., 1999) and the activity of MIF is associated with cancer angiogenesis, progression, and metastasis (Leng et al., 2003; Stephan et al., 2006; Wymann et al., 1999).

MIF in adhesion

MIF is implicated in adhesion, and MIF secretion is induced by cell adhesion to fibronectin in quiescent mouse fibroblasts (Mitchell et al., 1999). Therefore adhesion-mediated release of MIF subsequently promotes integrin-dependent activation of mitogen-activated protein (MAP) kinase, cyclin D1 expression, and DNA synthesis. MIF is secreted in a protein kinase C (PKC) dependent fashion as a consequence of cell adhesion to the ECM and plays a significant role in integrin-mediated signalling to sustained MAP kinase activation cyclin D1 expression, and cell cycle progression (Liao et al., 2003). MIF stimulates the proliferation of mouse fibroblasts (Mitchell et al., 2002) throught the activation of the p44/p42 extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK)s. Additional growth factors stimulate the rapid release of preformed MIF from adherent, quiescent fibroblasts including the sustained activation of MAPK in serum-stimulated fibroblasts is dependent upon MIF autocrine action (Ren et al., 2003). Hence growth factors and adhesion are required for efficient signaling to sustained ERK activation and subsequent cell cycle progression (Roovers et al., 1999). This is through cyclin D1 transcription and the subsequent activation of specific cyclin-dependent kinases (Welsh et al., 2001).

MIF secretion is induced by a variety of stimuli including growth factors and integrin engagement (Liao et al., 2003). Extra-cellular MIF then binds to its putative membrane bound receptor, CD74 (Leng et al., 2003), which can then initiate the activation of Rho GTPase activity via an unknown mechanism. Increased Rho activity is then thought to promote the activation of Rho kinase and myosin light chain (MLC) phosphorylation. Hyperphosphorylated MLC, in turn, induces stress fiber formation/integrin clustering and

subsequent Focal adhesion kinase-dependent sustained MAPK activation, cyclin D1 transcription, and retinoblastoma tumor suppressor inactivation (Swant et al., 2005).

The activation of Rho by integrins and growth factors is essential for modulating the sustained activation of ERK and subsequent cyclin D1 transcription (Welsh, 2004). Rho and Rho kinase-dependent stress fiber formation have been tightly linked to endothelial cell reorganization and mature blood vessel formation (Hoang et al., 2004), recruitment and clustering of integrins leading to focal adhesion formation (Roovers and Assoian, 2003). MIF production following growth factor or extracellular matrix stimulation would also perform in cell replication. In the case of neoplastic cells, internal (oncogenic) or external (growth factors, extracellular matrix) signals could serve to increase MIF production that, in turn, may facilitate anchorage independence and loss of contact inhibition (Meyer-Siegler et al., 2005; Meyer-Siegler et al., 2004; Rumpler et al., 2003).

MIF may be implicated in downregulation of E-cadherin because inflammatory cytokines can reduce E-cadherin mRNA levels, down-regulate E-cadherin surface expression, and induce a loss of E-cadherin-mediated adhesion (Jakob and Udey, 1998). IL-1, TNF-α and LPS induced increased expression of major histocompatibility complex (MHC) class II Ag, CD40 and CD86 complex and decreased E-cadherin expression that was temporally related to dissociation of aggregates (Jacob et al., 1999; Jakob et al., 1997; Jakob and Udey, 1998)

E-Cadherin in Epithelial cells

The epithelium is composed of a single layer of polarized epithelial cells, the membrane has one apical and one basolateral domain. In mammals, multiprotein junctional complexes mediate the adhesion between epithelial cells, which are comprised of the apical tight junctions, the subapical adherens junctions and basolateral desmosomes, linked to cytoskeletal filaments (Wang et al., 2006). The morphogenesis of the epithelium is

sustained by cadherins that are a superfamily of calcium (Ca++) dependent cell-cell adhesion molecules. E-cadherin that belongs to type 1 transmembrane protein of the adherens junctions, and are responsible for homophilic cell to cell adhesion (Wheelock and Johnson, 2003b). E-cadherin is a 120-kDa transmembrane gycoprotein (Takeichi, 1991) composed of five tandem extracellular cadherin domains (EC1–EC5), a single transmembrane domain and a distinct and highly conserved cytoplasmic tail that specifically binds catenins. Extracellular domains EC1 to EC4 are homologous cadherin repeats and include the well-known His-Ala-Val (HAV)-sequence that is conserved within the binding surface of the first domain (Renaud-Young and Gallin, 2002), and EC5 is a less-related membrane-proximal domain (Gooding et al., 2004).

Classical or type I cadherins (E-, N-, P-, R-, H-, EP cadherin) mediate adhesion at the adherens, cell–cell or cell–matrix adhesive junctions that are linked to microfilaments. A predomain (usually less than 80 amino acids) between the signal sequence and the start of the EC1 domain exists at the N terminus (Takeichi et al., 1990) and must be cleaved prior to adhesive function activation (Ozawa et al., 1990a; Ozawa et al., 1990b; Ozawa and Kemler, 1990). E-cadherins mediate both homotypic one type of cadherin on one cell surface interacting with the same type of cadherin on the surface of the opposing cell, and heterotypic cell–cell interactions. Homotypic adhesion involves N-terminal β-strands (Trp2) (Gooding et al., 2004) and the conserved HAV sequence as essential components of the EC1 adhesion recognition site (Blaschuk et al., 1990a; Blaschuk et al., 1990b). T-cells expressing integrins aEb7/aM290b7 specifically interact with E-cadherin in the lymphocyte adhesion system (Cepek et al., 1994).

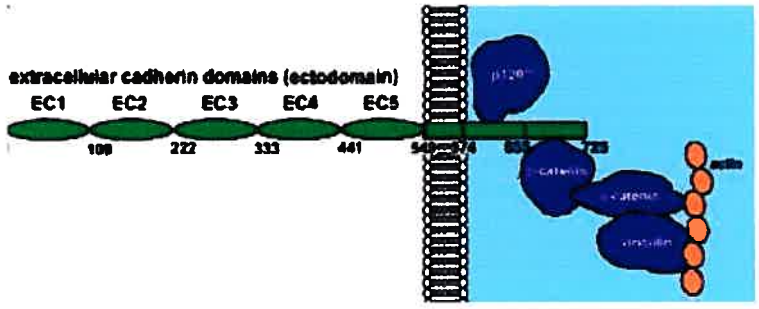

Figure. 3. Cadherin domain layout. A cadherin molecule (green) consists of five extracellular domains, a transmembrane domain and an intracellular domain, itself divided into a membrane proximal (residues 574–655) and catenin-binding (655–725) domain. In purple, interacting proteins p120ctn is bound to the membrane proximal region; b-catenin binds the catenin-binding domain, then binds a-catenin at its N terminus. a-catenin and vinculin form the direct link between the b-catenin–cadherin complex and the actin cytoskeleton (orange). Residue numbers shown are based on the sequence and structure of the C-cadherin ectodomain. Taken from (Gooding et al., 2004).

The extracellular domain provides Ca++ dependent adhesion, and interactions between the cadherin cytoplasmic tail and the cytoskeleton significantly increase the strength of cadherin-mediated adhesion (Yap et al., 1997a; Yap et al., 1997b). The cytoplasmic carvoxil tail is associated with a group of closely related but distinct inner-membrane proteins, termed the catenins (α, β and p120)(Gumbiner and McCrea, 1993). Cytoplasmic domain of E-cadherin interaction with a complex of β-catenin (92 kDa) or plakoglobin (γ-catenin), which form the link to the actin cytoskeleton via α-catenin (102 kDa) (Gooding et al., 2004; Nieset et al., 1997). The anchorage of catenines (α-, β-, γ- and p120) to the actin cytoskeleton forms stable cell–cell contacts (D'Souza-Schorey, 2005). p120ctn was originally identified as a substrate for receptor tyrosine kinases (Harrington and Syrigos, 2000) binds directly to the cytoplasmic domain of cadherin and regulate actin cytoskeleton modulators such as RhoA, Rac and Cdc42 (Goodwin et al., 2003). The maturation of initial weak adhesive events between adjacent cells is consolidated by clustering of p120 and RhoA (Noren et al., 2000). The p120ctn–E-cadherin interaction may itself be sufficient to stabilize the E-cadherin complex (Wang et al., 2005) and Cdc42 (actin cytoskeleton modulators). The adhesion of E-cadherin-β-catenin at the cytoplasmatic membrane provides stability at adherens junctions. The disassembly and loss of E-cadherin permits phosphorylation of β-catenin that results in a decrease in the association of β-catenin with E-cadherin and α-catenin, thereby weakening the adherens junctions (Huber et al., 2001).

β-catenin has also been found to serve as a key component in signalling processes during embryonic development and adult tissue homeostasis (Eger et al., 2000; Li et al., 2002a; Ponassi et al., 1999; Stockinger et al., 2001). Therefore β-catenin has emerged as a key effector of the Wnt pathway, and β-catenin, or Armadillo in Drosophila, is a switch

associated with epithelial–mesenchymal transitions and cancer (Bienz, 2005; Eger et al., 2000). Inappropriate activation of β-catenin in the intestinal epithelium, and in other tissues, often leads to cancer (Basta-Jovanovic et al., 2003; Polakis, 2000; Polakis, 2002). In the absence of Wnt signaling, β-catenin is phosphorylated by glycogen synthase kinase-3β, targeting it for ubiquitination and 26S proteosome-mediated degradation. Suppression of glycogen synthase kinase-3β following Wnt/Fzd (frizzled) binding allows β-catenin to accumulate and function in the nucleus as a transcriptional co-activator with T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors (Nelson and Nusse, 2004; Winn et al., 2005). Ubiquitination of E-cadherin is mediated by Hakai, an E3 ubiquitin ligase, and has been proposed to signal the internalization of cadherin molecules (Fujita et al., 2002). It is also possible that Hakai-mediated ubiquitination might control the transport of E-cadherin to late endosomes and lysosomes for degradation, although this has not been demonstrated (Cong et al., 2003; Serban et al., 2005; Yook et al., 2005).

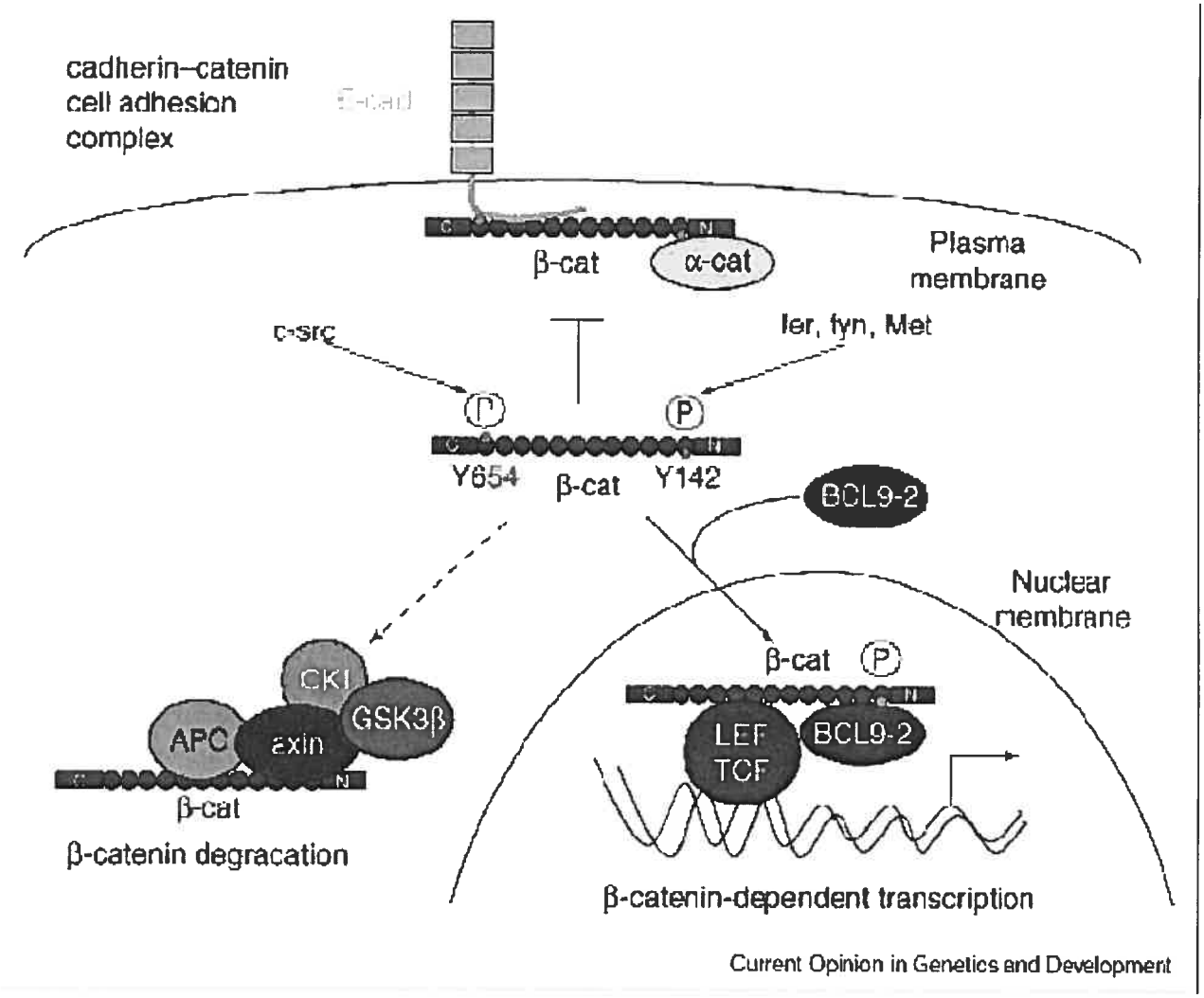
cadherin–catenin
cell adhesion
complex
E-cad
β-cat
α-cat
Plasma
membrane
c-src
Ier, fyn, Met
P
P
Y654
β-cat
Y142
BCL9-2
Nuclear
membrane
CKI
GSK3β
APC
axin
β-cat
β-catenin degracation
β-cat
P
LEF
TCF
BCL9-2
β-catenin-dependent transcription
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Figure 4. The dual function of b-catenin in cell adhesion and transcription. b-catenin (b-cat) functions in cell adhesion at the plasma membrane, by linking cadherins (E-cad) to a-catenin (a-cat). Cytoplasmic levels of b-catenin are tightly controlled by a destruction complex including the adenoma polyposis coli (APC) gene product, the scaffold molecules axin (conductin homolog; also known as axin2), glycogen synthase kinase (GSK3b) and casein kinase (CKI). b-catenin also functions in transcription, and this switch can be regulated by tyrosine phosphorylation. b- catenin contains two crucial tyrosine residues at positions 142 and 654. Phosphorylation of tyrosine residue 654, for instance by c-src, leads to loss of E-cadherin-binding. The tyrosine kinases Fer, Fyn or Met can induce phosphorylation of tyrosine residue 142 of b-catenin. This induces loss of binding to a-catenin and promotes the interaction with the nuclear co-factor BCL9-2. The b-catenin–BCL9-2 complex locates to the nucleus and regulates, in conjunction with the LEF/TCF DNA binding proteins, the transcription of crucial target genes. Taken from (Brembeck et al., 2006)

Cadherin recruitment

E-cadherin is recruited exclusively to the lateral membrane domain, the site of cell-cell contact. The process begins with the engagement of opposing E-cadherin molecules at the tips of filopodial or lamellopodial projections. Following the formation of this initial cluster of E-cadherin molecules, assemble with adjacent extremity , generating a zipper-like structure, which then develops into a mature, linear cell-cell contact (Adams et al., 1996), throughout this process, E-cadherin is transported from a cytoplasmic pool to the initial cluster (Hogan et al., 2004). Several proteins are known to interact with E-cadherin, including β-catenin, p120ctn, but appears that they not are involved in the recruitment of E-cadherin to nascent cell-cell contact sites (Fujita et al., 2002; Peinado et al., 2005).

The printing of small clusters of E-cadherin at the nascent cell-cell contact sites, the homophilic ligation of E-cadherin induces the binding of C3G (a guanine nucleotide exchange factor for Rap1) to the cytoplasmic tail of E-cadherin, which may in turn induce the activation of Rap1. Activation of Rap1 mediates the further recruitment of E-cadherin from the cytoplasmic or plasma membrane pool, facilitating the development of mature E-cadherin-based cell-cell contacts. Rap activity is required for the recruitment of E-cadherin into nascent cell-cell contact sites but not for the maintenance of mature E-cadherin (Cong et al., 2003; Wheelock and Johnson, 2003a). The interaction between C3G and E-cadherin is increased during formation of new cell-cell contacts and decreased as cell-cell contacts matur this interaction may be modulated by the competition between C3G and β-catenin for E-cadherin (Hogan et al., 2004). The interaction between β-catenin and E-cadherin is regulated by phosphorylation of E-cadherin (Huber et al., 2001). E-cadherin interacts with

the N terminus of C3G, which acts as an inhibitory domain suppressing the catalytic activity of the C terminus (Ichiba et al., 1999)..

Epithelial to mesenchymal transitions

Epithelial to mesenchymal transition, describes the morphological changes that occur in embryonic epithelia to individual migratory cells. The presence of E-cadherin at the cell surface is a key determinant in distinguishing epithelial cells from mesenchymal cells and in establishing epithelial cell polarity within tissue (Palacios et al., 2005). Several developmental processes in animals are controlled by the Wnt signaling pathway. These comprise early embryonic patterning, epithelial–mesenchymal interactions and maintenance of stem cell compartments (Bienz and Clevers, 2003). The sharing of a critical component between two fundamental processes, cell adhesion and cell signaling, may reflect a need for coordinate control between them. The adhesion to maintain the tissue integrity and cell signaling is coupled to a loosening of adhesion between epithelial cells during epithelial–mesenchymal transitions and other developmental processes (Bienz, 2005). The linke between these two processes seemed to reside in β-catenin, which potentially couples loss of cell adhesion to increased Wnt signaling if diverted from the plasma membrane to the nucleus (Wang et al., 2006). Studies on cases of pulmonary adenocarcinoma, have shown that the loss of expression of E-cadherin and β-catenin occurs prior to the structural destruction of the alveolar wall by invasion of carcinoma cells (Genda et al., 2000).

There is a continuous process of reassembly and disassembly of epithelial cell adherens junctions to maintain the dynamics of the epithelial monolayer. The intercellular adherens junctions are specialized subapical structures that function as principle mediators of cell–cell adhesion. Their disassembly correlates with a loss of cell–cell contact and an

acquisition of migratory potential. Oncogenes and growth factors might trigger individual cellular responses, and the concerted action of several such responses will be required to bring about the decisive changes that lead to the permanent dissolution of an adherens junctions (Palacios et al., 2005).

The development and regeneration of tissues is critically dependent on the balance of cell migration, cell–cell adhesion, and cell–matrix interactions (Vespa et al., 2005). Integrin-mediated cell-matrix adhesion and motility occur in the presence of cadherin-based intercellular adhesion (Grosheva et al., 2001; Larue and Bellacosa, 2005).A standard characteristic of epithelial tumor progression is the loss of the epithelial phenotype and acquisition of a motile or mesenchymal phenotype (Zhou et al., 2004). These transitions are accompanied by the loss of E-cadherin function by either transcriptional or posttranscriptional mechanisms (Liu et al., 2006b; Vogelmann et al., 2005). E-cadherin mediated adhesion inhibits receptor tyrosine kinase (RTK) activity. E-cadherin was found to interact through its extracellular domain with epidermal growth factor receptor (EGFR) and other receptor tyrosine kinases, thereby decreasing receptor mobility and ligand-affinity with the consequential loss of cell adhesion, and increased cell migration and invasion (Andl and Rustgi, 2005).

Internalization and recycling of cadherin

Posttranscriptional and posttranslational modifications regulate the cadherin adhesive activity. The trafficking of E-cadherin together with the endocytic pathway represents a cellular process that can regulate stability of adherens junctions. E-cadherin is internalized and then is recycled back to the lateral cell surface for adherens junctions reassembly or can be transported to the lysosome for degradation. Actin remodeling and posttranslational modifications of junctional components can also impact on the stability of the adherens

junctions (Wang et al., 2005) This traffiking of E-cadherin to the lysosome serves as a means to ensure that cells do not reform their cell-cell contacts and remain motile (Balzac et al., 2005; Palacios et al., 2005).

The spreading of epithelial cells is mediated by the proteosome (Tsukamoto and Nigam, 1999). Metalloproteases and γ-secretase cleave the extracellular and transmembrane domains of E-cadherin, dissolving adhesive contacts (Ii et al., 2006; Wildeboer et al., 2006), suggesting that fragments generated by metalloproteases promote cell invasion through paracrine mechanisms. Calpain and caspase-3 have been shown to target the cytosolic domains of E-cadherin (Carragher et al., 2006; Raynaud et al., 2006; Wells et al., 2005). The proteolytic cleavage of E-cadherin has been linked to destabilization of the adherens junctions during the metastatic progression of various cancers. Proteolysis of E-cadherin was reported almost two decades ago after the discovery that an 80-kDa E-cadherin extracellular domain fragment was released into the culture media of tumor cells (Billion et al., 2006; Chu et al., 2006; Kuefer et al., 2003). E-cadherin levels are increased in the serum of individuals affected with adenocarcinomas including prostate cancer (Kuefer et al., 2005).

Regulation of cadherins

Loss of the function or the expression of any of the elements of the E-cadherin/catenin complex makes the cell incapable of adhering, resulting in a loss of the normal architecture of tissues and acquisition of a motile or mesenchymal phenotype. The presence of E-cadherin at the cell surface is a key determinant that distinguishes epithelial cells from mesenchymal cells and establishes epithelial cell polarity within tissues. Silencing mutations in E-cadherin or its transcriptional repression have principally been attributed to the decrease in cellular levels of E-cadherin during epithelial to mesenchymal transitions

(Eger et al., 2004). In a significant percentage of invasive tumors, the genes encoding E-cadherin as well as the associated catenins are normal, suggesting that posttranscriptional processes regulating adherens junctions stability may account for cell-cell dissociation and acquisition of migratory potential (Larue and Bellacosa, 2005; Lu et al., 2003). To maintain the dynamics of epithelial monolayers, E-cadherin is rapidly removed from the plasma membrane and then subsequently recycled back to the cell surface to reform new cell-cell contacts. Thus, the recycling of E-cadherin through the endosomal recycling pathway represents an effective mechanism for the remodeling of adhesive contacts in dynamic situations where cell-cell contacts must be dissolved and reformed (Ivanov et al., 2004a; Ivanov et al., 2004b; Lock and Stow, 2005). When E-cadherin is internalized and then shuttled to the lysosome instead of being recycled back to the lateral membrane the cells do not reform their cell-cell contacts and remain motile (Kamei et al., 1999; Togashi et al., 2002). E-cadherin at cell-cell contact may stimulate or inhibit epithelial cell proliferation in different settings (Liu et al., 2006b).

There is some evidence that cadherin adhesion may be regulated by tyrosine phosphorylation. In cells transfected with the v-src oncogene, the observed increased tyrosine phosphorylation of β-catenin and E-cadherin resulted in functional changes such as decreased adhesion and increased migration, without affecting the overall expression of either the catenins or the cadherins (Liu et al., 2006a; Liu and Li, 1998). EGFR as well as hepatocyte growth factor have been found to induce β-catenin and γ-catenin tyrosine phosphorylation (Nishioka et al., 2003; Slater et al., 2005). Stability of cadherins are proportionate for proteins Rho. The Rho family are small GTPases regulate actin cytoskeletal dynamics in different cell types.(Betson et al., 2002; Nakagawa et al., 2001).Rap 1 is actived upon adherens junctions disassembly that is stimulate by E-cadherin

internalization and trafficking along the endocytic pathway and Rap1 is associated and required for the formation of integrin-based focal adhesions (Balzac et al., 2005).

Soluble E-cadherin.

Soluble E-cadherin is a 80 kDa fragment released during breakdown of extracellular domain of E-cadherin of the cell-cell adhesion as it could be a tumor marker is found in serum from patients with lung cancer, Reduced expression of E-cadherin is present in tumorigenic and metastasis (Charalabopoulos et al., 2004; Jonsson et al., 2005).

In prostate cancer cells hepatocyte growth factor/scatter factor mediate release of matrilysin furthermore matrilysin activate the extracellular cleavage of E-cadherin increased the concentration of soluble E-cadherin, resulting in the shedding of a soluble *Mr* 80,000 fragment in tissue culture medium. and the dissociation from the cadherin/catenin complex. (Davies et al., 2001).

E-cadherin is involved in inducing cell cycle arrest, at least partially through up-regulation of the cyclin-dependent kinase inhibitor, p27 (Schrier et al., 2006). Loss of E-cadherin has been found in premalignant conditions in a number of organs including colorectal adenoma, oesophagus and gastric dysplasia. (Bailey et al., 1998). Soluble E-cadherin levels could predict disease recurrence in patients with gastric carcinoma that underwent curative surgery. Serum soluble E-cadherin was a good marker for predicting disease recurrence in the first 3–6 months after surgery, with a median of 13 months before clinical recurrence (Chan et al., 2005). Reduced E-cadherin–catenin expression is associated with tumor dedifferentiation, infiltrative growth, and lymph node involvement (Park et al., 2006). It has been discovered that β-catenin can bind not only to E-cadherin but also to other molecules such as the EGFR (Viswanathan et al., 2006).

Mastrix metalloproteinases (MMPs) are implicated in the proteolytic cleavage of E-cadherin in endothelial cells, resulting in the release of a soluble *Mr* 90,000 fragment after growth factor deprivation-induced apoptosis (Herren et al., 1998). MMPs belong to a group of zinc-dependent transmembrane enzymes that function extracellularly at neutral pH, and are thought to play key roles in tissue remodeling, tumor invasion, and metastasis.

Cathepsins (CTS), peptidases that have biological roles in degrading ECM, are present in early pregnant ewes. CTS of endometrial and conceptus origin may regulate endometrial remodeling and conceptus implantation. Endometrial CTS genes are regulated by ovarian and placental hormones, and cathepsin L (CTSL) is a novel IFN-τ stimulated gene expressed only in luminal epithelium and superficial glandular epithelium of the endometrium of pregnant ewes by days 10 and 18 (Song et al., 2005), these catepsins may are implicates in cleavage of E-cadherin. Cleavage of the extracellular domain of E-cadherin promotes destabilization of adherens junctions and loss of adhesive contacts in dynamic situations where cell-cell contacts must be dissolved and reformed, as an example, in embryo implantation.

In epithelial cells E-cadherin-mediated cell adhesion helps establish the cell polarity, but may be dynamicaly regulated by IFN-τ in the preimplantation period and stimulate the remodeling of BEEC to non-polarized phenotype, essential for adhesiveness of Tr.

IFN-τ stimulate the secretion of MIF in BEEC (Wang and Goff, 2003) and MIF may be implicated in downregulation of E-cadherin because inflammatory cytokines can reduce E-cadherin mRNA levels, down-regulate E-cadherin surface expression, and induce a loss of E-cadherin-mediated adhesion (Jakob and Udey, 1998).IFN-τ is secreted by the elongated bovine embryo and stimulates the secretion of proteins from the endometrium that are probably important for embryo development and attachment. My purpose was to

understood the regulation of the secretion of two specific proteins, MIF and E-cadherin, stimulated by IFN-τ and determine if they can modify endometrial function.

HYPOTHESIS AND OBJECTIVES.

The hypothesis of present work is that IFN-τ from the embryo stimulates secretion of proteins from the endometrium that are important for embryo development and attachment. The main objective was to determine if IFN-τ altered endometrial function by modifying the secretion of specific proteins.

The specific objectives were:

1. Determine if MIF expression is different between pregnant and non pregnant cows.
2. Study the regulation of E-cadherin in endometrial epithelial cells.
3. Determine if MIF has autocrine effects in the endometrial epithelial cells.

MATERIALS AND METHODS

Chemicals and Reagents.

Cell culture medium (RPMI 1640), Hanks buffered saline solution (HBSS, calcium and magnesium free), newborn calf serum (NBCS), gentamicin and trypsin were purchased from Invitrogen. Collagenase (type II), trypsin (type III, from bovine pancreas), DNase I (type I, from bovine pancreas) were purchased from Sigma Chemical Co. (St. Louis, MO). Matrigel was obtained from VWR Canlab (Montreal, QC, Canada). Protein assay dye reagent concentrate and electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA). Mouse anti-human MIF antibody mouse IgG and rhMIF were purchased from Cedarlane Laboratories Limited (Hornby, ON, Canada). Bio trans nylon membranes (0.2 mm) were obtained from Bio-Rad. Tissue culture plates were obtained from Corning-Costar (Fisher Scientific, Montreal, PQ, Canada). BioMax film was obtained from Eastman Kodak Company (Rochester, NY). The recombinant bovine IFNτ was a generous gift from

Dr. R. Michael Roberts (University of Missouri). Vectastain ABC Kit for mouse IgG was purchased from Vector Laboratories Inc. (Burlingame, CA). All othr antibodies and normal serum were obtained from Santa Cruz Biotechnology (California).

Preparation and culture of cells

The epithelial cells were prepared as previously described (Xiao and Goff, 1998). Uteri from cows at days 1 to 3 of the estrous cycle (ovaries with a corpus hemorrhagicum) were collected at the slaughterhouse and transported on ice to the laboratory. Cells prepared from endometrium at this stage respond to IFN-τ in a physiological manner, Briefly, the two horns of the uteri were placed in sterile HBSS containing 50 μg / ml of gentamicin. The myometrial layers were dissected from the two horns, and the horns were then inverted to expose the epithelium. The everted horns were digested for 2 hr in HBSS with 0.3% (w/v) trypsin at 37°C to obtain epithelial cells. At the end of incubation, the digested horns were scraped lightly with forceps, washed twice in HBSS and then further digested to obtain stromal cells by incubating in HBSS with 0.016% (w/v) trypsin III, 0.016% (w/v) collagenase II and 0.008% (w/v) DNase I for 45 mins at 37°C. Immediately after each cell suspension was collected, 10% NBCS was added to inhibit the trypsin. For epithelial cells, the cell suspension was centrifuged at 60 g for 5 min and then the pellet was washed 3 more times with HBSS. For further purification, the epithelial cell pellet was suspended in 20 ml RPMI-1640 medium supplemented with 5% NBCS and 50 μg/ml of gentamicin and plated onto 100 x 20 mm Nunclon petri dishes (Grand Island, NY, USA) and 100 incubated at 37°C with 5% CO2, 95% air for 3 h. At the end of incubation, contaminating stromal cells adhered to the dish and the floating epithelial cells were collected. Cells were then plated onto Matrigel-coated culture dishes. 100 μg of 11% Matrigel was added to each well of 24-well plates and the plates were dried overnight. For stromal cells, the cell

suspension was centrifuged at 60 g for 5 min to remove clumps of cells and then the supernatant was centrifuged at 1000 g for 10 min. The pelleted cells were washed twice with HBSS. The stromal cell suspension was plated onto dishes and after a 3 h incubation, the floating cells were washed away by gentle pipeting. The cells were cultured at 37°C with 5% CO2, 95% air until they were confluent (about 7 days) in RPMI-medium supplemented with 10% NBCS. The culture medium was changed every two days. The homogeneity of the cell populations was examined by immunocytochemistry with pancytokeratin antibody. Epithelial cell contamination of stromal cells was about 3% and stromal cell contamination of epithelial cells was less than 1%.

Radioactive labeling of Secreted Proteins and 2D-PAGE

The confluent epithelial cells were incubated in the presence or absence of 100 ng/ml IFN-τ for 24 h at 37°C. Cells were washed and incubated with methionine-free RPMI-1640 medium for 30 min. The medium was then replaced with 500 ml of methionine-free RPMI-1640 medium containing 5 ml (50 mCi) of 35S-labeled methionine (specific activity .1200 Ci/mmol) and the cells were incubated in the presence or absence of 100 ng/ml IFN-τ for a further 24 h. The medium was removed and stored at –80 °C until protein extraction. Before separation and analysis of the proteins by means of 2D SDS PAGE, the culture medium was centrifuged (500 3 *g* for 10 min) to remove cell debris prior to protein extraction. The proteins were concentrated to 50 ml using Ultrafree-15 concentrators (5000 MW cutoff; Millipore, Bedford, MA) and added to IPG buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer [pH 3–10], bromophenol blue, 65 mM dithiothreitol [DTT]). Prior to loading, a 5-ml aliquot of each sample was removed for radioactive counts, and 5 μl internal protein standards were added to each sample so that the molecular mass and isoelectric points of found proteins could be estimated. The separation in first dimension

was carried out using Immobiline DryStrips (pH 3–10), which had been rehydrated for at least 10 h in an Immobilin Drystrip reswelling tray (Amersham Pharmacia Biotech AB, Baie d'Urfe′, QC, Canada). The samples were then separated on a MultiPhore II flatbed system (Amersham) for 16 h at 158C. The voltage was 300 V for the first 3 h, from 300 to 2000 V during the following 5 h, and finally 8 h at 2000 V. Before the second dimension was performed, the dry strips were first equilibrated for 10 min in equilibration solution 1 (0.5 M Tris/HCl pH 6.8, 3.6 g urea, 3 ml glycerol, 0.1 g SDS, 25 mg DDT, and distilled water up to 10 ml) and another 10 min in equilibration solution 2 (0.5 M Tris/HCl pH 6.8, 3.6 g urea, 3 ml glycerol, 0.1 g SDS, 0.45 g iodoacetamide, and distilled water up to 10 ml). The second dimension was performed after placing the strips on Pharmacia ExcelGel XL SDS 8–18 using the MultiPhore II flatbed system at 158C. After running, were immediately immersed in fixing solution (50% methanol, 10% acetic acid in water), and stained with Coomassie blue, destained, and incubated in a radiographic enhancer and then in a preserving solution. The gels were wrapped in cellophane, air dried, and exposed with Kodak radiographic film for various times. Protein spots on control and IFN-t gels were compared, and molecular weight and pI estimated using the computer program Phoretix 2D (version 4.00, Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK).

Protein Sequencing

To obtain proteins for sequencing, the procedure for culture and incubation of the cells was the same as described above except that the proteins were not labeled with 35S-methionine. The 2D gels were run, each loaded with 50 μg of unlabeled proteins. After 2D PAGE the proteins were stained with silver nitrate (silver staining kit, Amersham), and the spots of interest were excised. The sequence analysis was performed at the Harvard Microchemistry

Facility by Microcapillary reverse-phase HPLC nanoelectrospray tandem mass spectrometry on a Finnigan LCQDECA quadruple ion-trap mass spectrometer.

Western Blotting

The confluent epithelial cells and stromal cells, were incubated in the presence or absence of 100 ng/ml of IFN-τ. for 24 h at 37°C. The culture medium were recollected and centrifuged (600 × g for 10 min at 4°C) to remove insoluble material. The proteins were concentrated to 250 µl using Ultrafree-15 concentrators (5000 MW cutoff; Millipore). The supernatant was recovered and stored at -20°C pending analysis. The protein concentration was determined by the method of Bradford, with the Bio-Rad DC Protein Assay. Protein extracts (40 µg) were heated at 99°C for 5 min and resolved by 7.5% SDS-PAGE. The gels were equilibrated in transfer solution for 1h and then electro-transferred into a 0.45 µm nitrocellulose membrane, using a Miniprotean II transfer (Bio-Rad, Mississauga, ON). The membranes were then blocked 1 h at room temperature in tris-buffered saline (TBS) with Tween 20 containing 5 % milk powder, then incubated with polyclonal rabbit anti E-cadherin (1:500), and subsequently with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5000) in 1% skim milk at room temperature by 2 h. After that the membranes were washed with TBS with Tween 20, 3 X 20', then incubated with chemiluminescent detection reagents (ECL Plus, Amersham Biosciences) for 5 minutes. The membranes were wrapped, and exposed to Kodak radiographic film for various times.

Immunohistochemistry

Uteri (day 1-3 of the estrous cycle) were obtained from a slaughterhouse. Pieces of tissue were carefully excised and transferred in Zamboni's solution by 24 hs and embedded in paraffin. Tissue sections 5 µm thick were mounted on polylysine-coated slides, deparaffinized, rehydrated, and then heated with 10 mM citrate buffer (pH 6). After two

wash with PBS, slides were then incubated with 0.3 % hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase activity. After washing with TBS-Tween 20 0.05%, tissues were incubated with 20% of blocking normal serum (Vectastain Elit ABC Kit; Vector Laboratories) at room temperature for 1 h. Primary antibodies, polyclonal goat anti MIF; polyclonal rabbit anti E-cadherin and monoclonal mouse anti β catenin; all at (1/100) were added to the slides and incubated at 4° C overnight. After washing 3 X 5 min in TBS-Tween 20, tissue sections were incubated for 1 h room temperature, with 2 μg/ml biotinylated antibody (anti-rabbit, anti goat or anti-mouse). Subsequently, slides were washed with TBS-T and incubated with Vectastain ABC kit (Vector Laboratories) reagent containing horseradish peroxidase for 30 min. After further washing with TBS-T, color development was achieved using AEC (Vector Laboratories) peroxidase substrate. After washing the tissue sections were counterstained with haematoxylin and mounted with aquamount. Negative controls were performed using the same protocol without primary antibody.

Immunocytochemistry

Confluent uterine epithelial cells grown on cover slips (Fisher-Brand) were washed twice in phosphate-buffered saline (PBS) and incubated in 100% methanol and stored at –20 °C until used. After washing twice in PBS, cells were then incubated with 0.3 % hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase activity for 30min. After washing with TBS-T 0.05%, slides were blocked in 20% normal donkey serum or normal horse serum for 1 h at RT. They were further incubated in primary antibody (polyclonal goat anti MIF; polyclonal rabbit anti E-cadherin, and monoclonal mouse anti β catenin, each of (1/100) at 4° C overnight. After washing 3 x 5 min in TBS-T, slides were incubated for 1 h with 2 μg/ml biotinylated antibody (anti-rabbit, anti goat or anti-mouse).

Subsequently, slides were washed with TBS-T and incubated with Vectastain Elit ABC (avidin biotin complex) kit (Vector Laboratories) reagent containing horseradish peroxidase for 30 min washed again with TBS-T for 3 x 5 min and color development was achieved using AEC (Vector Laboratories) peroxidase substrate. After washing, the tissue sections were counterstained with haematoxylin, and mounted with aquamount. Negative controls were performed using the same protocol without primary antibody.

Statistical Analysis

Each experiment was carried out using the cells from one uterus and was repeated with three different uteri. Western blots were run in triplicate for each uterus. For E-cadherin, the data were analyzed by 2-way ANOVA, which included the main effects of time and treatment (control, IFN-τ). For E-cadherin by effect of MIF were at diferents dosage. m Differences between individual means were determined by Tukey HSD test. A probability of $P < 0.05$ was considered to be statistically significant. The data were analyzed using the computer program JMP (SAS Institute Inc., Cary, NC). All tests of significance were performed using the appropriate error terms according to the expectations of the mean squares.

RESULTS

Immunolocalization of MIF in bovine endometrium.

To determine if MIF production changed during the estrous cycle and if it was altered during pregnancy, immunolocalization of MIF was compared in endometrial tissue taken at the beginning at the cycle and at day 18 of the estrous cycle and pregnancy. The results Figure 5 a, c and e were negatives (no first antibody) do not showed immunostain for MIF, whilst in figure 5 b MIF was present in luminal and glandular epithelium of cyclic cow and figure 5 d, MIF was weakly present in the tissue of non pregnant cows at day 18. This is in contrast to the increase in the amount of MIF staining found in the luminal and glandular epithelium from pregnant cows, figure 5 f. Stromal tissue did not show stain for MIF.

Immunolocalization of MIF in bovine endometrium.

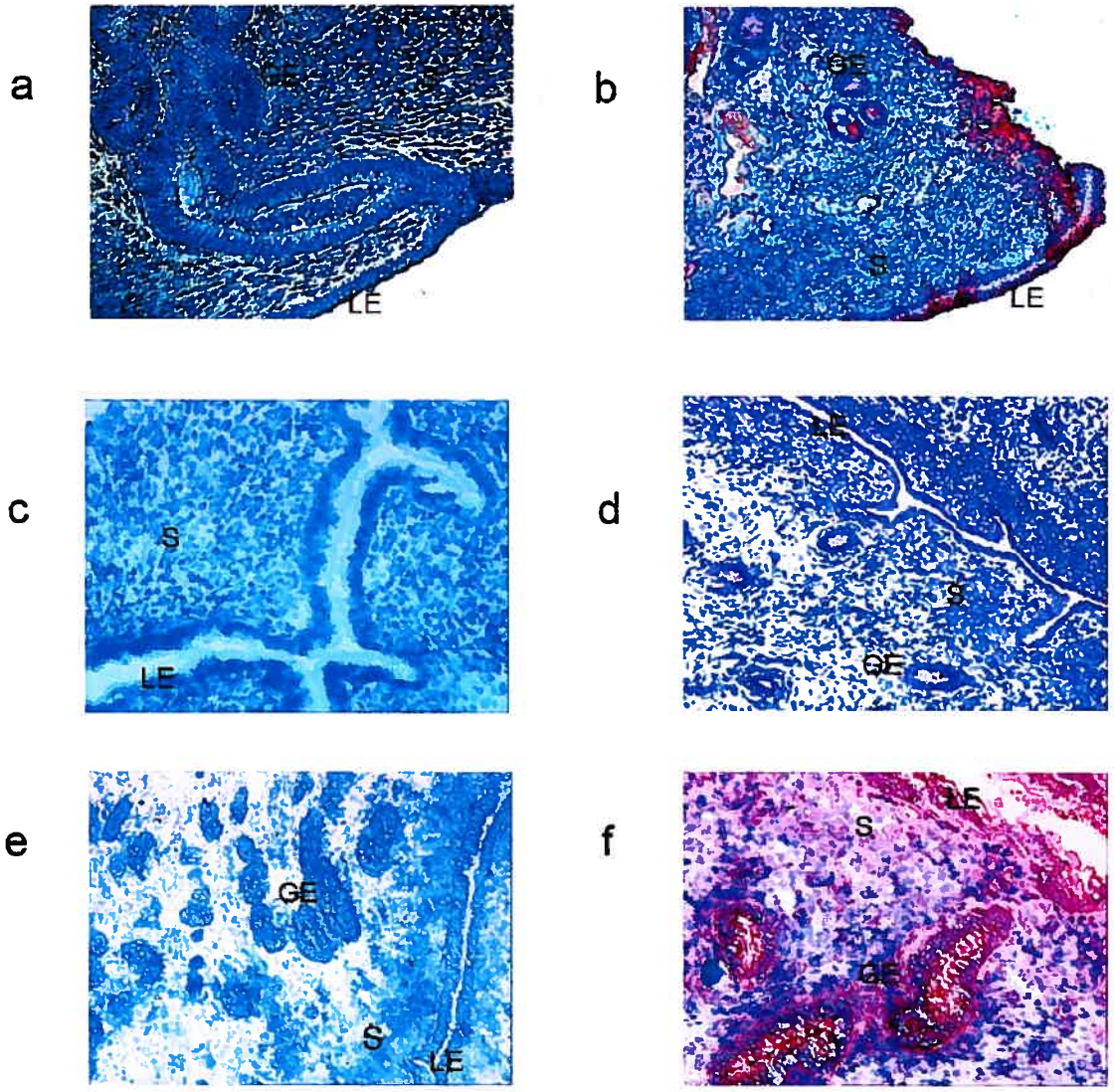

Figure 5. Immunolocalization of MIF in bovine endometrium. Bovine uteri were taken on days 1-3 of the cycle (a & b); on day 18 of the cycle (c & d); and day 18 of pregnancy (e & f). Figures a, c and e are negatives (no first antibody). Tissue samples from pregnant and non pregnant cows were kindly provided by Dr Leslie MacLaren. Magnification 20 x. LE Lumenal epithelium; S, stroma; GE, glandular epithelium. The analysis was performed on three different tissue samples.

MIF in bovine epithelial endometrial cells

To establish if IFN-τ stimulated the production of MIF, bovine epithelial endometrial cells, taken from uteri early in the cycle, were cultured on cover slips until confluent (9 days) and then treated with and without IFN-τ for 24 h. MIF was detected using immunocytochemistry with goat anti-MIF antibody. The results (Fig. 6) showed that epithelial cells treated with IFN-τ had a stronger staining in the cytoplasm than non-treated cells. Endometrial stromal cells that were cultured and treated similarly to the epithelial cells showed no positive staining.

MIF in bovine epithelial endometrial cells

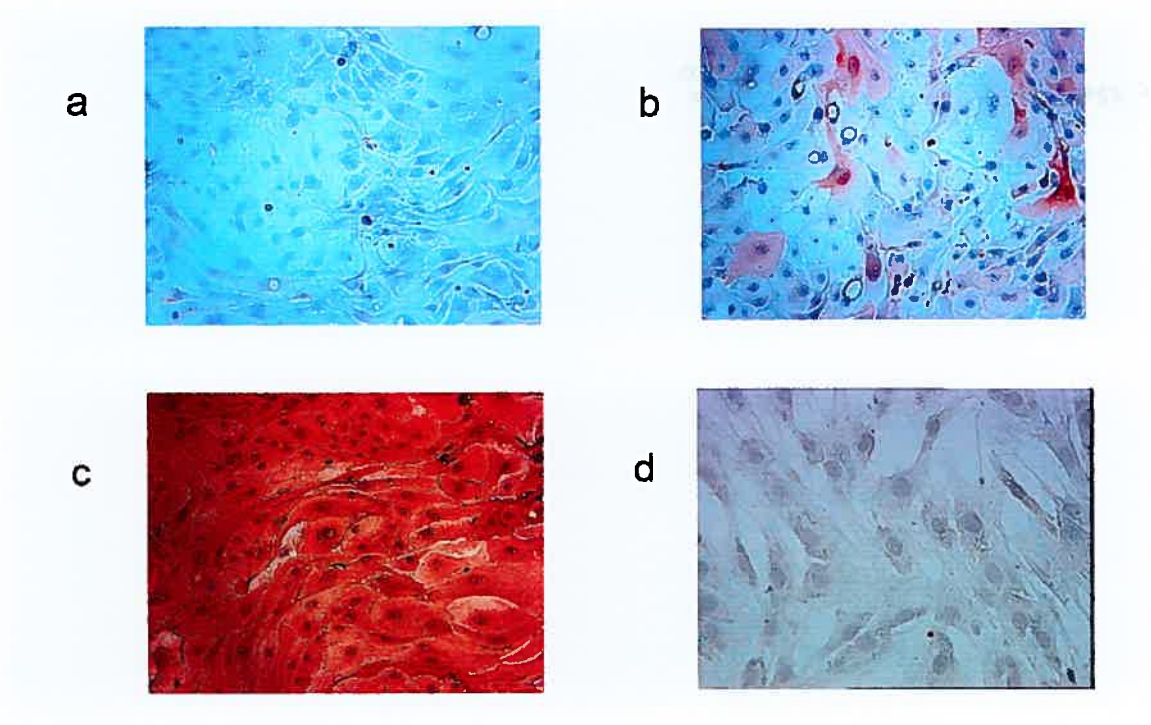

Figure. 6. Immunolocalization of MIF in bovine uterine endometrial cells cultured until confluence (9 d) on cover slips and treated with 100ng of IFN-τ for 24 h. (a) negative control (no first antibody), (b) control, epithelial cells no treatment. (c) epithelial cells treated with IFN-τ for 24 h, (d) stromal cells treated with IFN-τ for 24 h. Magnification 20 x. The analysis was performed on cells from three different uteri.

Identification of E-cadherin by 2D SDS PAGE.

In order to try to identify other proteins secreted by the endometrial epithelial cells, that are stimulated by IFN-τ and therefore could play a role in embryo development and implantation, confluent cultures of bovine endometrial epithelial cells were treated with or without IFN-τ and the 35S-methionine labeled proteins were analyzed by 2D PAGE. Figure 7 shows representative 2D gel autoradiographs of labeled proteins from control and IFN-τ treated cells. A comparison of control and IFN-τ-treated cells showed that a protein spot with an estimated pI of 4.8 and molecular mass of 76 kDa was present in IFN-τ-treated but not in control cells. This spot corresponded to the 80 kDa soluble fragment of extracellular domain of E-cadherin.

Identification of E-cadherin by 2D SDS PAGE.

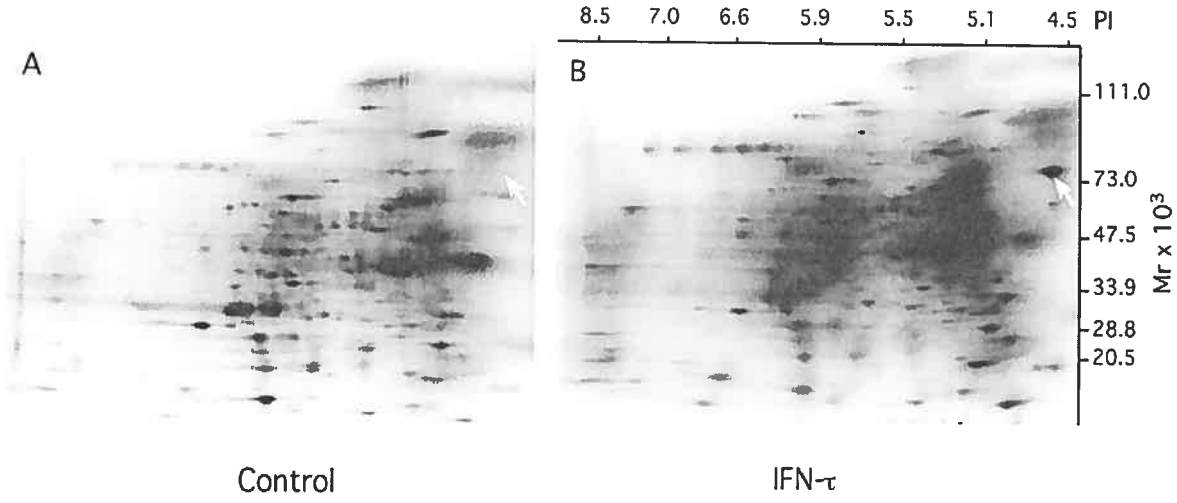

Figure 7. Identification of proteins secreted from endometrial epithelial cells and stimulated by IFN-τ. Epithelial cells were cultured with 35S-methionine incubated with and without 100 ng/ml of IFN-τ for 24 h. Radiolabeled proteins in the medium were separated by 2D-PAGE. The resulting representative autoradiographs were generated by exposure of x-ray film to the dried gels. Arrows show the position of protein spots upregulated by IFN-τ treatment.

To identify this protein, in-gel digestion and sequence analysis was performed at the Harvard Microchemistry Facility by microcapillary reverse-phase HPLC nanoelectrospray tandem mass spectrometry on a Finnigan LCQDECA quadruple ion trap mass spectrometer. The peptide sequences **(dwvippiscpenekgpfpk, gldfeak and epdtfmeqk)** that resulted in positive identification of the protein were located in the amino acid sequence of Bos taurus E-cadherin from Genbank Database [accession number AAR91598]):

MGPWSRSLSALCCCCRCNPWLCREPEPCIPGFGAESYTFTVPRRNLERGRVLGRVS
FEGCAGLPRTVYVSDDTRFKVHTDGVLTVRRPVHLHRPELSFLVHAWDSTHRKLS
TKVTLEVSAHHHHHHSHHDSPSGTQTEVLTFPGPHHGLRRQKR**DWVIPPISCPEN**
EKGPFPKSLVQIKSNKEKETQVFYSITGQRADTPPVGVFIIERETGWLKVTQPLDR
EQIAKYILFSHAVSSNGQAIEEPMEIVITVTDQNDNKPQFTQEVFKASALEGALPGT
SVMQVTATDIDDEVNTYTAAIGYTIPAQDPMLPHNKMFTINKETGVISVLTTGLDR
ESFPTYTLMVQAADLNGEGLSTTATAVITVLDTNDNAPRFNPTTYVGSVPENEAN
VAITTLTVTDADDPNTPAWEAVYTVLNDNEKQFIVVTDPVTNEGTLKTAK**GLDFE**
AKQQYILYVAVTNVAPFEVTLPTSTATVTVDVIDVNEAPIFVPPQKRVEVPEDFGV
GLEITSYTAR**EPDTFMEQK**ITYRIWRDTANWLEINPETGAISTRAELDREDVDHVK
NSTYTALIIATDNGSPPATGTGTLLLFLDDVNDNGPVPEPRTMDFCQRNPEPHIININ
DPDLPPNTSPFTAELTHGASVNWTIEYNDQERESLILKPKKTLELGDHKINLKLIDN
QNKDQVTTLDVHVCDCDGIVSNCRKARPAEAGLQVPAILGILGGILAFLILILLLLL
LVRRRRVVKEPLLPPEDDTRDNVYYYDEEGGGEEDQDFDLSQLHRGLDARPEVTR
NDVAPTLMSVPQYRPRPANPDEIGNFIDENLKAADSDPTAPPYDSLLVFDYEGSGS
EAATLSSLNSSESDQDQDYDYLNEWGNRFKKLADMYGGGEDD

E-cadherin and β-catenin in bovine endometrium.

Endometrial tissue taken at the beginning of the cycle (~day 3) was used to detect E-cadherin and β-catenin by immunohistochemistry (Figure 8): panel (b) shows immunoexpression of E-cadherin in LE and GE, but not in S. Panel (d) from the same uterus shows immunolocalization of β-catenin by mouse monoclonal anti IgG,to the LE. β-catenin was not present in GE and S. Panels (a) and (c) were negative controls, no first antibody was added.

E-cadherin and β-catenin in bovine endometrium.

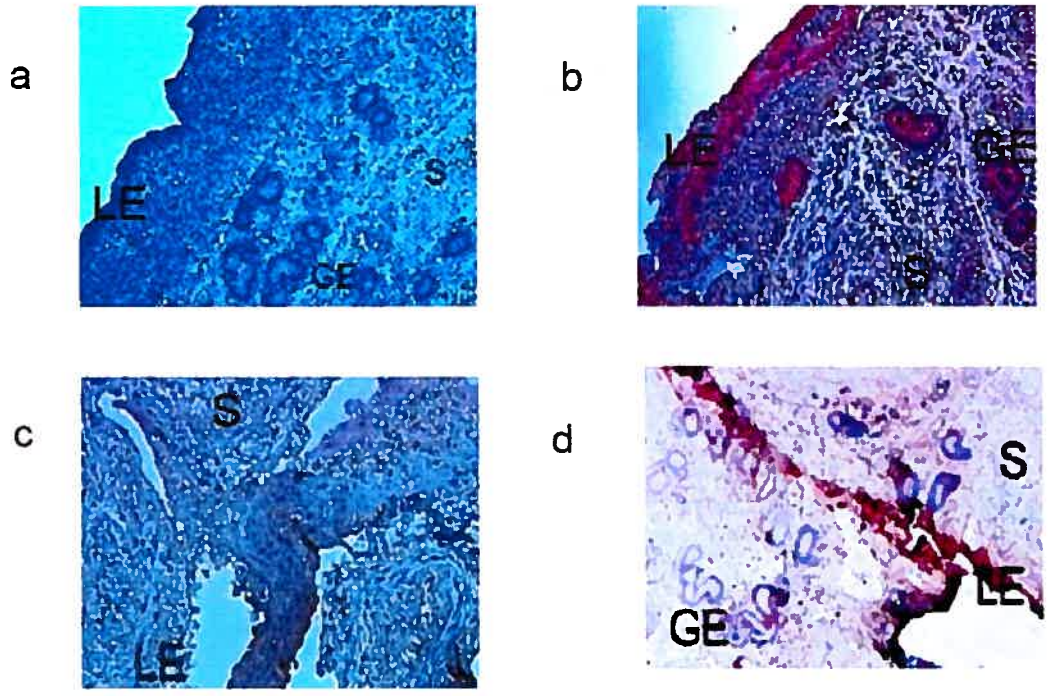

Figure 8. Immunolocalization of E-cadherin and β-catenin in bovine endometrium. Bovine uteri were taken early in the estrous cycle (~day 3). (a) Negative for E-cadherin (no first antibody was added). (b) Positive for rabbit polyclonal anti E-cadherin. (c) Negative for β-catenin (no first antibody). (d) Positive for mouse monoclonal anti β-catenin. Photomicrographs are at a magnification of 20 x. LE; Luminal epithelium; S, stroma; GE, glandular epithelium. The analysis was performed on tissue from three different uteri.

Effect of IFN-τ on soluble E-cadherin secretion.

To determine whether IFN-τ altered the secretion of soluble (80kDa fragment) E-cadherin protein from the endometrial epithelial cells *in vitro*, proteins present in the culture medium were analyzed by Western blotting using a polyclonal rabbit anti E-cadherin IgG. The results (Fig. 9) showed a significant ($P < 0.05$) increase in E-cadherin in the medium from bovine endometrial epithelial cells cultured in the presence of IFN-τ compared with control cells. Samples of culture medium of stromal cell from the same uterus did not demonstrate differences in the secretion of E-cadherin between the control and IFN-τ treatment groups.

Effect of IFN-τ on soluble E-cadherin secretion

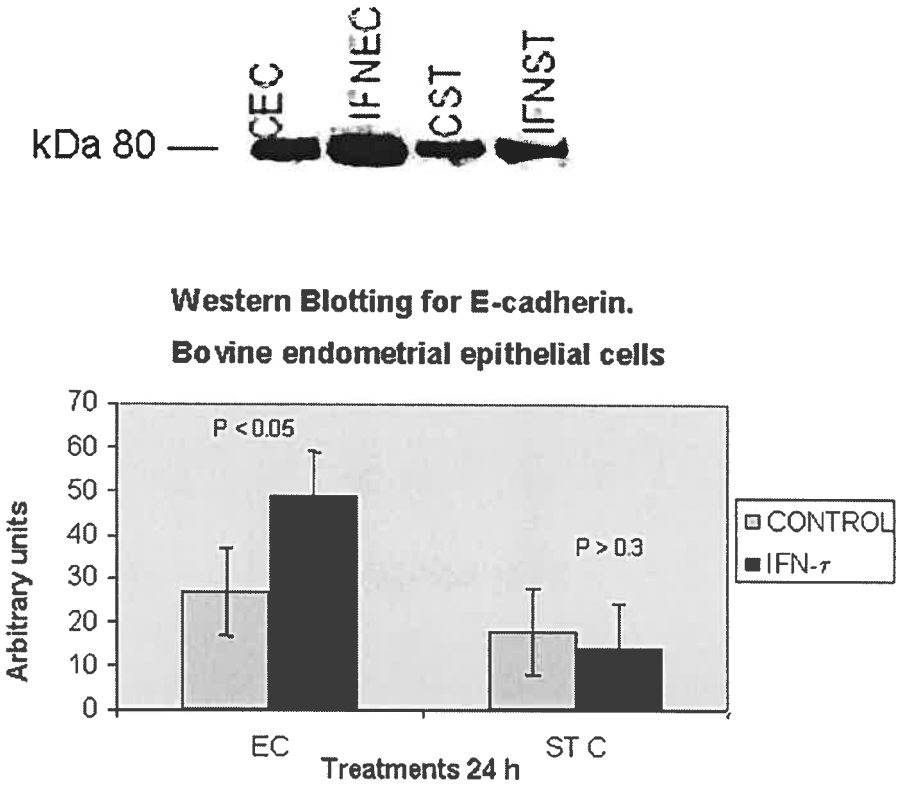

Figure 9. Effect of IFN-τ on soluble E-Cadherin secretion. Confluent bovine endometrial epithelial cells (EC) and stromal cells (STC) were cultured with and without 100 ng/ml IFN-τ for 24 h. After culture the medium was concentrated and E-cadherin measured by Western blotting. Upper panel shows a representative Western blot (CEC = control epithelial cells, IFNEC = IFN-τ epithelial cells, CST = control stromal cells, IFNST = IFN-τ stromal cells) and the lower panel shows the mean of four blots after quantification of spot density. Bars represent the SEM.

Effect of IFN-τ on E-cadherin in bovine uterine epithelial cells.

In order to establish the effect of IFN-τ on E-cadherin in bovine epithelial cells cultured and treated with 100 ng of IFN-τ by 24 h, the cover slips of cells in the absence of treatment and treated cells were immunostained with polyclonal rabbit anti E-cadherin. The results of figure 10 shown that panel (a) did not stain, was negative conrol. The panel (b) correspond at cells control without IFN-τ, shows low percent of cells stain, the panel (c) of cells treated with IFN-τ shows more intense staining for E-cadherin and found mainly in the cytoplasm of the cells. The panel (d) was for stromal cells of the same cultures and did not shows immunostain for E-cadherin. These immunocytochemistry results are concordant with the result of Western blotting that shown a positive effect of IFN-τ treatment on E-cadherin secretion.

Effect of IFN-τ on E-cadherin in bovine uterine epithelial cells.

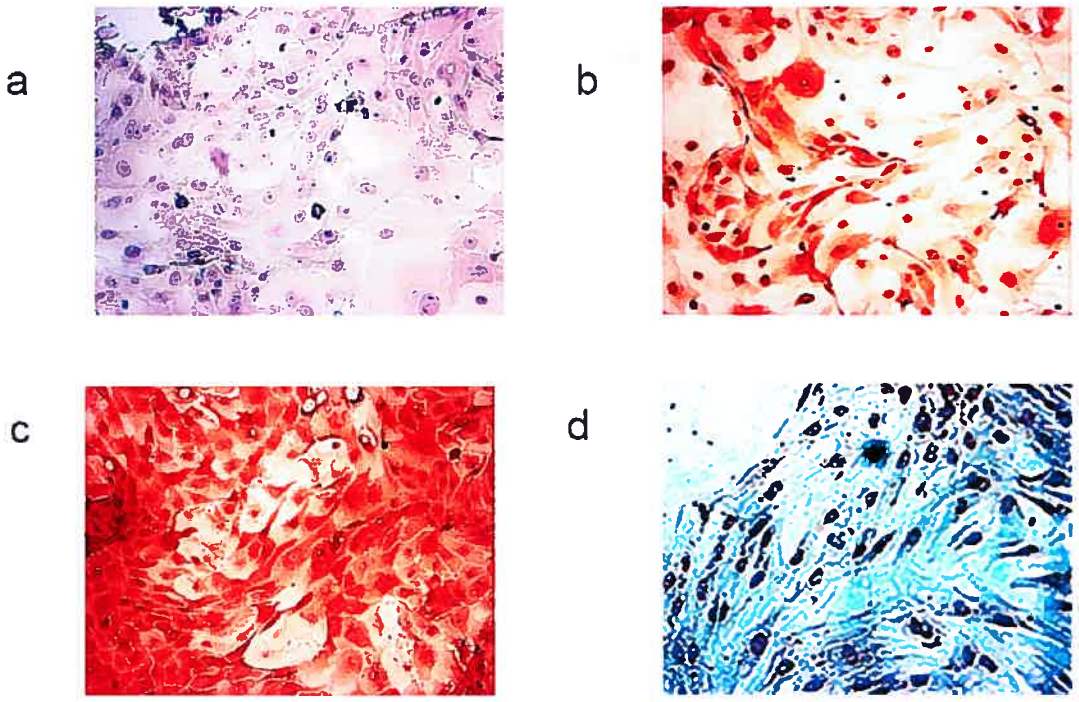

Figure. 10. Immunodetection of E-cadherin in bovine uterine epithelial cells cultured on coverslips until confluence (9 d) and treated with 100 ng of IFNt by 24 h. Panels a) negative control no first antibody was added, (b) cells in absence of treatment, (c) cells treated with IFNτ for 24 h, (d) stromal cells treated with IFNτ for 24 h. Magnification 20 x. The analysis was performed on cells from three different uteri.

Expression of β-catenin in bovine endometrial epithelial cells for effect of IFN-τ.

As β-catenin is directly associated with the highly conserved cytoplasmic domain of the cell adhesion protein E-cadherin. We examined β-catenin expression in bovine endometrial cells.The results of figure 11 shown that in panels: (a) negative control did not stain, no first antibody was added, (b) cell in absence of treatment shows low percent of immunoreactivity of β-catenin located at cell boundaries, and (c) cell treated with IFN-τ for 24 h., immunreactivity for β-catenin was increased at cell boundaries and cytoplasm.

Expression of β-catenin in bovine endometrial epithelial cells for effect of IFN-τ.

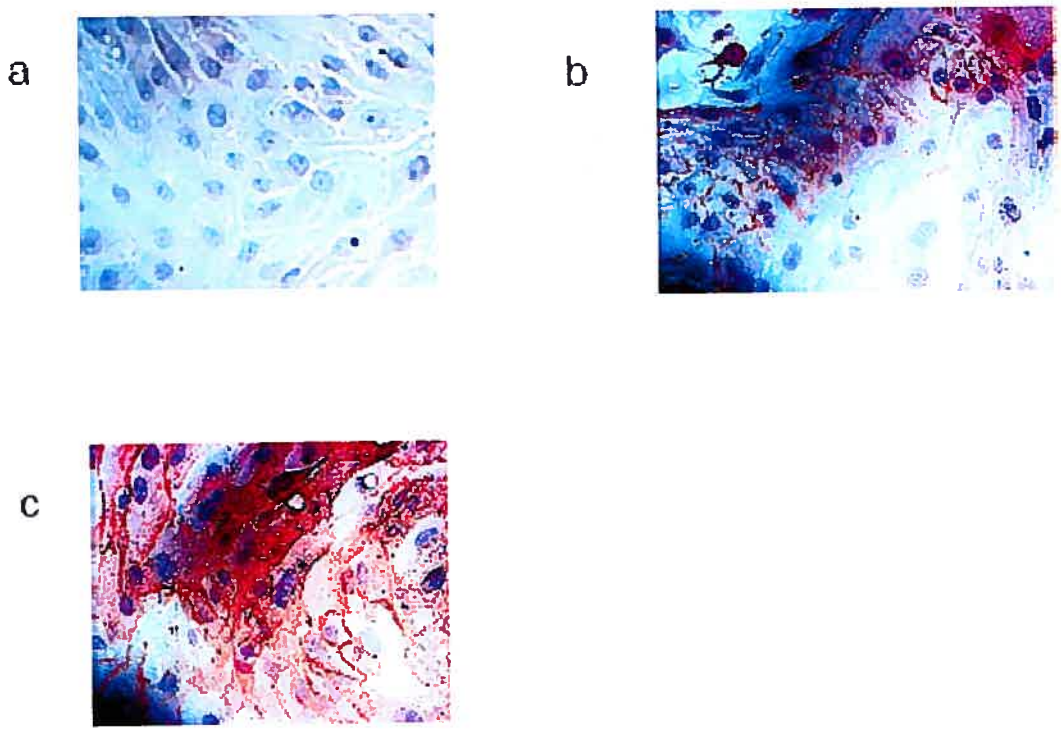

Figure 11. Immunolocalization of β-catenin in bovine endometrial epithelial cells. Epithelial cells were cultured on coverslips until confluence (9d) and then treated with 100 ng of IFNt for 24 h. Uteri were taken early in the estrous cycle. Panels (a) negative control no first antibody was added, (b) cell in absence of treatment, (c) cells treated with IFNτ for 24 h. Magnification 20 x. The analysis was performed on cells from three different uteri.

Response of MIF on E-cadherin in bovine endometrial epithelial cell.

In order to determine if MIF has autocrine effects in the cleavage of E-cadherin from endometrial epithelial cells in vitro, uterine epithelial cells were cultured until confluence (9d) and treated with several doses of MIF protein for 24 h. Proteins present in culture medium were concentrated and analyzed by Western blotting with a polyclonal rabbit anti E-cadherin IgG to determine if the soluble fraction (80 kDa) of E-cadherin was secreted into the culture medium. The results of Figure 12 shows that E-cadherin concentrations in culture medium were not significantly affected by treatment with MIF ($P > 0.7$). Data were subjected to least-squares ANOVA.

Response of MIF on E-cadherin in bovine endometrial epithelial cell.

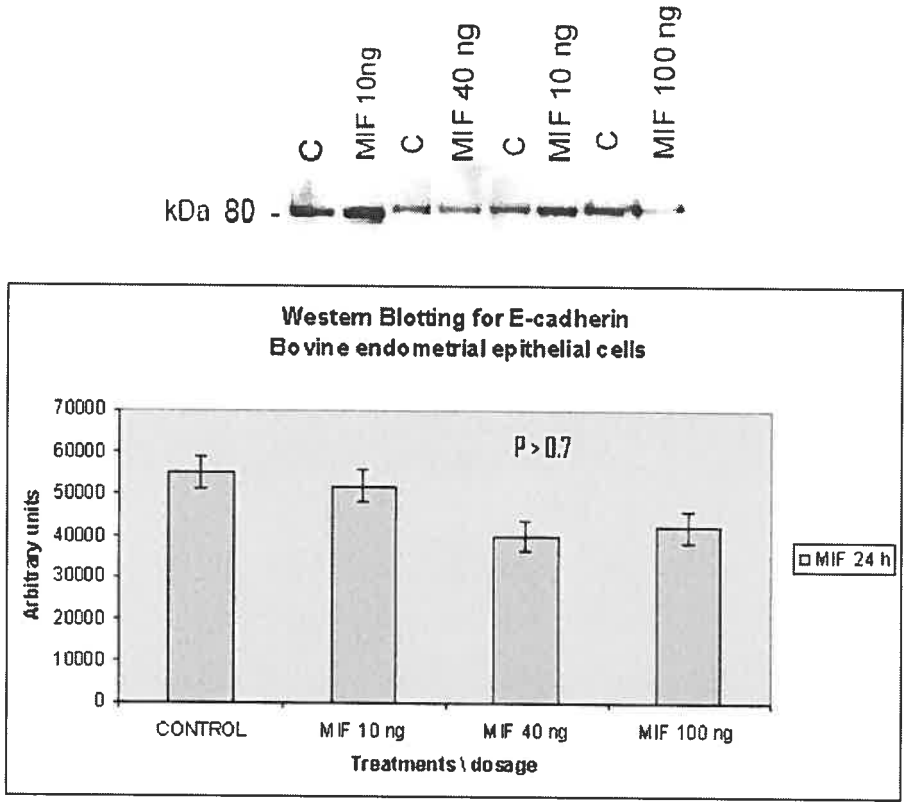

Figure 12. Effect of MIF on the secretion of the soluble fragment of E-cadherin. Epithelial cells were cultured until confluence and treated with several doses of MIF protein for 24 h. Western blotting was performed on the medium to quantify E-cadherin. The upper panel shows a representative Western blot. The lower panel shows the mean of four blots after quantification of spot density. The bars represent the SEM.

Effect of MIF on E-cadherin in bovine epithelial cells.

The presence of E-cadherin at the cell surface is a key determinant in distinguishing epithelial cells from mesenchymal cells and in establishing epithelial cell polarity within tissues. Internalization of E-cadherin is essential for adherens junction disassembly and increased cell migration. To determine if MIF had an autocrine effect on the internalization of E-cadherin, endometrial epithelial cells were cultured on coverslips and treated with 10 or 100 ng / ml of MIF for 24 h. The results of figure 13 shown: (a) negative control, no first antibody was added, (b) cells in absence of treatment presented a tenuous immunoreaction by the antibody (red color) at cell-cell contact region, (c) cells treated with 10 ng / ml of MIF, demonstrated immunostain located at cell boundaries of the plasma membrane and cytoplasm, (d) cells treated with 100 ng / ml of MIF immunoreactivity for E-cadherin was increased and concentrate at cytoplasm found an internalization of E-cadherin.

Effect of MIF on E-cadherin in bovine epithelial cells.

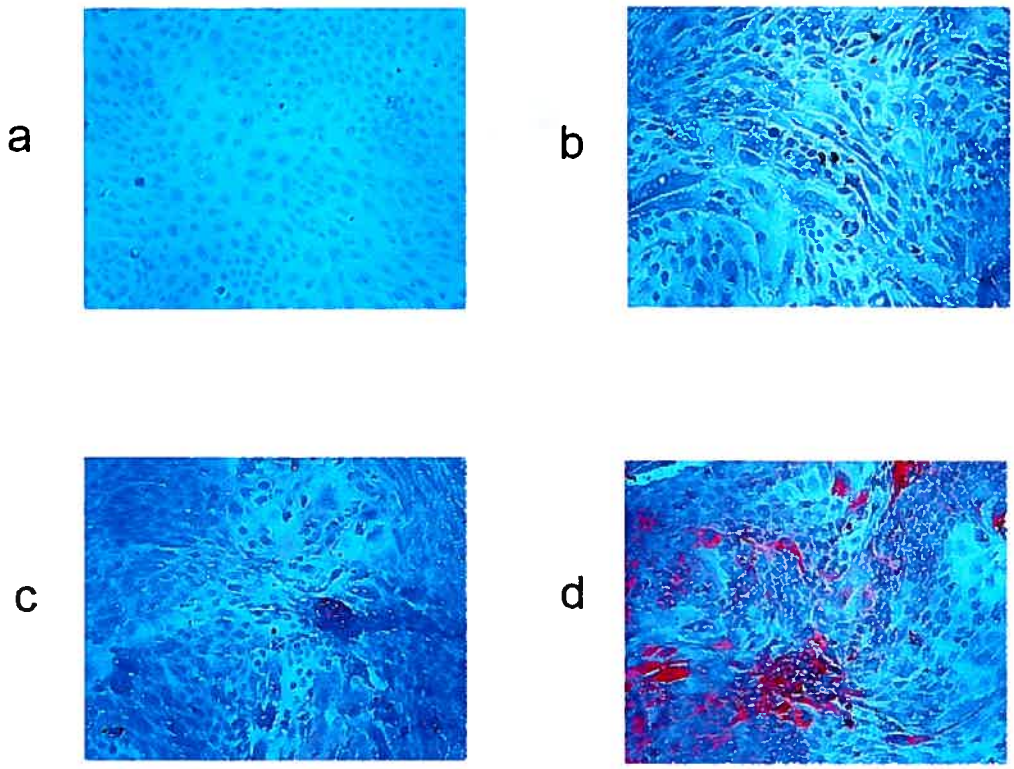

Figure 13. Effect of MIF on immunodetection of E-cadherin in bovine endometrial epithelial cells in culture. Epithelial cell were cultured to confluence on cover slips and then treated with different doses of MIF for 24 h. Immunocytochemistry was performed to detect E-cadherin using a polyclonal rabbit antibody. (a) cells control no first antibody was added, (b) cells control in the absence of treatment, (c) cells treated with 10 ng of MIF, (d) cells treated with 100 ng of MIF. Magnification 20 x. The analysis was performed on cells from three different uteri.

Effect of MIF on β-Catenin expression in bovine endometrial epithelial cells

In order to demonstrate if MIF had autocrine effect in destabilization of Adherens junctions by concentrating β-catenin in cytoplasm or nucleus, endometrial epithelial cells were cultured on coverslips until confluence and then treated with 10 or 100 ng of MIF by 24 h. The results of figure 14 showed that in panel (a) cell control no first antibody was added, (b) cells in absence of treatment were a slight immunostainning (red coloration) present to the cell membrane, (c) cells in presence of 10 ng / ml of MIF the positive immunoreactivity was increased, at the plasma membrane and cytoplasm, (d) cells after treatment with 100 ng/ml of MIF shows an increased of concentration of β-catenin in cytoplasm but not in the nucleus.

Effect of MIF on β-catenin expression in bovine endometrial epithelial cells

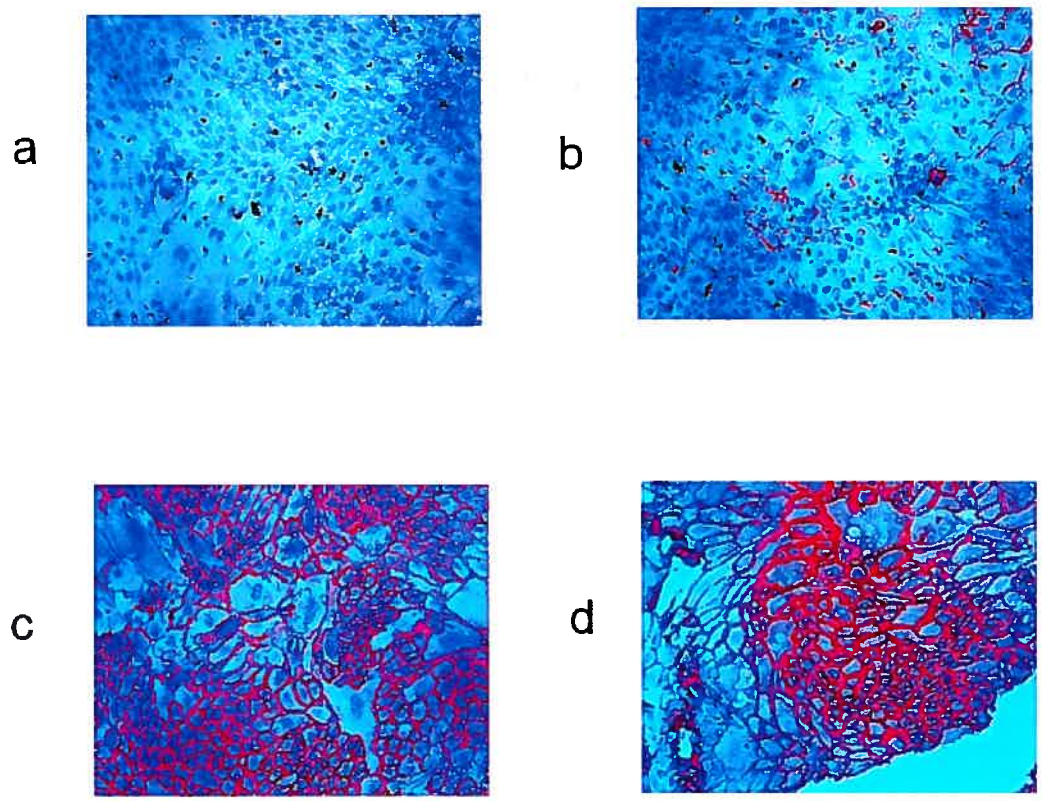

Figure 14. Effect of MIF on β-catenin expression in bovine endometrial epithelial cells in culture. Confluent cells grown on coverslips were treated with 10 or 100 ng/ml MIF for 24 h. Immunocytochemistry was performed to detect β-catenin using a monoclonal mouse antibody. a) negative (no first antibody was added). (b) control cell in absence of treatment, (c) cells treated with 10 ng of MIF, (d) cells treated with 100 ng of MIF. Magnification = 20 x. The analysis was performed on cells from three different uteri.

DISCUSSION.

In the cow, embryo implantation is characterized by migration, elongation into a filamentous conceptus, apposition and adhesion to caruncular areas that contain a considerable amount of glandular epithelium (Gray et al., 2001a; Gray et al., 2001b). The filamentous conceptus secretes IFN-τ, an embryonic cytokine that is known as a key factor for the maternal recognition of pregnancy. IFN-τ prevents PGF2α release and exerts a paracrine effect on the secretion of variety of proteins from the endometrium necessary for adhesion of the embryo in the preimplantation period (Hansen, 1998; Martal et al., 1998). The objective of this study was to investigate proteins whose secretion is modified by IFN-τ. In a previous study it was shown that MIF secretion from the luminal epithelium of bovine endometrial tissue is stimulated by IFN-τ (Wang and Goff, 2003). Results of the present study confirm that MIF is expressed in bovine endometrial epithelial cell *in vitro*; they also extend these findings to demonstrate that MIF is expressed in the ruminant endometrium *in vivo* during the estrous cycle and early pregnancy at the implantation period. One important finding of this study is that IFN-τ stimulated the cytoplasmic accumulation of MIF (fig 6-c) from endometrial epithelial cells. A possible explanation for this is that IFN-τ stimulates the synthesis of MIF protein and their accumulation in cytoplasm. The results of the *in vivo* immunohistochemical data showed that MIF was predominantly expressed in the luminal and glandular epithelium. Stromal cells of non-pregnant and pregnant cows did not show immunostaining for MIF. These result different with the study of Suzuki (Suzuki et al., 1996) in the mouse uterus, which showed that MIF is localized in the tunica muscularis in the preimplantation period. In humans, MIF is expressed in endometrial stromal cells (Akoum et al., 2005). Thus, these findings indicate the existence of differences in uterine MIF localization and response to regulatory factors

among species. The staining for MIF was stronger in the pregnant cow when compared to non-pregnant cows. This suggests that MIF is stimulated in the pregnant cow, presumably via the action of IFN-τ, confirming the *in vitro* data.

MIF was discovered as an activated T-lymphocyte-derived protein that inhibits the random migration of macrophages in vitro and is secreted by macrophages in response to cytokine stimulation, also MIF is recognized as a multifunctional cytokine that modulates the immune response, and stimulate pathophysiologic neovascularization and cell replication (Calandra et al., 2003; Mitchell, 2004). MIF is expressed in the human endometrium across the menstrual cycle and in early pregnancy in glandular epithelium (Arcuri et al., 2001). Studies showed that hCG, a glycoprotein hormone that plays a critical role in the initiation and maintenance of pregnancy, markedly stimulates MIF expression in endometrial stromal cells in a dose-dependent manner (Akoum et al., 2005). In addition MIF is released in endometriosis and acts as a potent mitogenic factor for human endothelial cells *in vitro* (Yang et al., 2000). This suggests an involvement of MIF in endometrial function.

When bovine endometrial epithelial cells were treated with IFN-τ for 24 h, 2 SDS PAGE showed an increase in the secretion of the 80 kDa soluble fragment of E-cadherin. This was confirmed by Western blotting of culture medium of uterine epithelial cells where IFN-τ increased the cleavage of E-cadherin. This study shows for the first time that IFN-τ stimulates the secretion of soluble fraction (80 kDa) E-cadherin from endometrial epithelial cells. This may be an effect of apoptosis induced by IFN-τ. Studies by Wang et al, found that IFN-τ can induce apoptosis in bovine uterine epithelial cells and that this effect is modulated by progesterone (Wang and Goff, 2003). Studies have shown that ischemia is associated with cadherin cleavage and causes a loss of cell-surface E-cadherin and degradation of E-cadherin to a fragment of 80 kDa (Bush et al., 2000; Covington et al.,

2005; Covington et al., 2006). Experiments showed that transient expression of exogenous E-cadherin in both epithelial and fibroblastoid cells arrested cell growth or caused apoptosis, depending on the cellular E-cadherin levels (Eger et al., 2000; Stockinger et al., 2001).

IFN-τ may induce apoptosis in bovine endometrial epithelial cells or stimulate protein synthesis. IFN-τ binds to type 1 IFN receptor on the endometrium and stimulates gene factor three induced IRF-1 and IRF-2 (Choi et al., 2001; Spencer et al., 1999). IRF-1 is a transcriptional activator and IRF-2 is its antagonistic repressor (Choi et al., 2001). IRF-1 may promote the synthesis of proteins in the endometrium. IFN-τ stimulates the endometrial remodeling by increasing cell proliferation because the effects of IFN-τ are mediated through type I IFN receptor located in the luminal epithelium of the endometrium (Godkin et al., 1997). IFN-τ can stimulate the transcription of uterine genes (Emond et al., 2000; Teixeira et al., 1997; Tuo et al., 1998) through the IFN-stimulated response element and interferon-g-activated sequence (GAS) (Stewart et al., 2001). Therefore IFN-τ may stimulate the secretion of endometrial proteins MIF and E-cadherin relate in implantationof embryo.

In ruminants, IFN-τ induced adhesion between the luminal epithelium and trophectoderm is essential for attachment and superficial implantation. The present experiments demonstrate that IFN-τ induced proteolytic cleavage of E-cadherin from endometrial epithelial cells *in vitro* and the results of immunohistochemistry showed that E-cadherin and β-catenin were present in endometrial tissue. E-cadherin was expressed on luminal and glandular epithelium, but not in stroma while β-catenin was present in luminal epithelium but not in glandular epithelium and stroma. When bovine epithelial cells were treated with IFN-τ for 24 h *in vitro* they showed an increase in E-cadherin staining in the cytoplasm and

β-catenin was concentrated at cytoplasmatic domain to cadenins-cadherin. This experiment showed that IFN-τ stimulates cleavage of 80 kDa fragment of E-cadherin and internalization of cytoplasmatic fraction of E-cadherin or increased protein production, and this process may facilitate the dissolution of adherens junctions (Palacios et al., 2005).

The down-regulation of the adherens junctions is a hallmark characteristic of an epithelial to mesenchymal transition, a process by which cells lose their polarized epithelial phenotype and concomitantly acquire a migratory or mesenchymal phenotype (Thiery, 2003).

Epithelial to mesenchymal transition have been shown to occur during normal embryonic development. Thus, the presence of E-cadherin at the cell surface is a key determinant in distinguishing epithelial cells from mesenchymal cells and in establishing epithelial cell polarity within tissues (Huber et al., 2005; Larue and Bellacosa, 2005). Kanako et al., (2006) studied the WNT signaling pathway in the ovine uterus during the estrous cycle and early pregnancy, and found that β-catenin GSK3B (glycogen synthase kinase-3 beta) E-cadherin mRNAs were abundant in the endometrial epithelia and also in conceptus trophectoderm. JUN, p-JUN and JNK proteins which mediates non-canonical WNT signaling pathway were also abundant in endometrial epithelia and conceptus trophectoderm (Kanako, 2006). These results implicate the canonical and non-canonical WNT signaling pathways in regulating conceptus differentiation and implantation in sheep

E-cadherin is type-I transmembrane protein is cleaved by a metalloproteinase *in vivo*, generating a soluble extracellular fragment and a carboxyl-terminal fragment associated with the cellular membrane (Haas et al., 2005). In the bovine endometrium, MMPs may be associated with ECM remodeling during implantation and placentation *in vivo* (Hirata et al., 2003). During peri-implantation period in the mouse, E-cadherin expression protein

was inhibited and the expression of MMP-2 and MMP-9 was increased at the transcriptional level (Liu et al., 2006a). Catepsins that are stimulates for IFN-τ and are implicates in endometrial remodelin and conceptus implantsation may promote the cleavage of E-cadherin.

MIF on secretion of E-cadherin.

These studies also provide preliminary evidence that MIF might have an autocrine effect on uterine epithelial cells *in vitro*. Although MIF did not significantly affect the cleavage of E-cadherin in bovine endometrial epithelial cell *in vitro* it did effect the cellular redistribution of E-cadherin from the membrane to the cytoplasm. Therefore the effect of MIF in immunoreactivity of β-catenin was a marked cytoplasmic accumulation.

An autocrine effect of MIF on E-cadherin and β-catenin in bovine endometrial epithelial cells may be explained by the autocrine effect of MIF on proliferation (Mitchell et al., 1999) and adhesion to fibronectin in quiescent mouse fibroblasts (Liao et al., 2003). Both exogenously added rMIF and endogenously released MIF induced the proliferation of quiescent mouse fibroblasts (Beswick et al., 2006; Mitchell et al., 1999; Ren et al., 2003). This proliferative response was associated with the phosphorylation and activation of the p44/p42 ERK kinases. ERK activation was sustained for a period of at least 24 h, and was dependent upon protein kinase A activity (Mitchell et al., 1999). Because cytokine mediators frequently function as mitogenic growth factors (Lang and Burgess, 1990), it was found that MIF is a growth factor-induced autocrine signaling factor (Mitchell et al., 1999) and that MIF is regulated by both growth factor and integrin activation and serves as an autocrine activator of MAP kinase, cyclin D1 expression, and DNA synthesis.

External (growth factors, extracellular matrix) signals could serve to increase MIF production that, in turn, may facilitate anchorage independence and loss of contact

inhibition. (Swant et al., 2005). Both growth factors and adhesion are required for efficient signaling to sustained ERK activation and subsequent cell cycle progression (Roovers et al., 1999). More recently, the autocrine effect of MIF on sustained ERK activation was proposed to occur through a receptor-mediated signalling pathway that involves the intermediate activation of MLCK (Wadgaonkar et al., 2005a). However, since intracellular MIF also binds to and activates MLCK (Wadgaonkar et al., 2005b) and since extracellular MIF is efficiently endocytosed and translocated into the cytosol it is possible that the autocrine action of extracellular MIF in sustained ERK signalling involves MIF endocytosis (Lue et al., 2006). MIF was identify as a natural ligand for CD74, a Type II transmembrane protein, is a high-affinity binding protein for MIF. MIF binds to the extracellular domain of CD74, and CD74 is required for MIF induced activation of the extracellular signal–regulated kinase–1/2 MAP kinase cascade, cell proliferation, and PGE 2 production (Leng et al., 2003). .

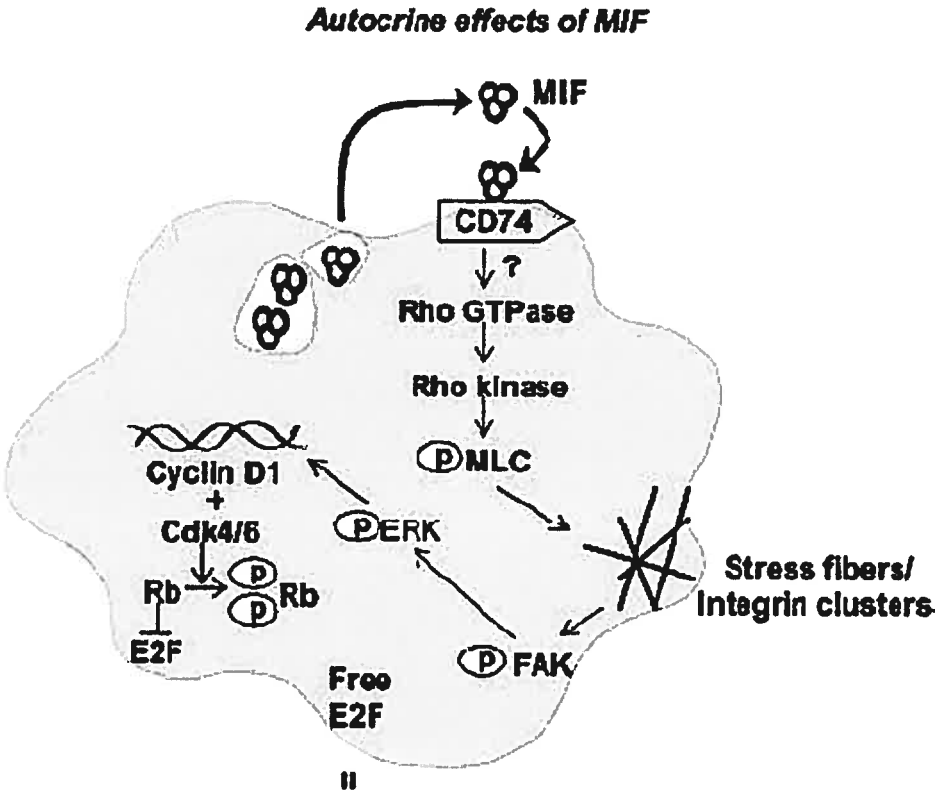

Figure 15 . Proposed scheme of MIF-dependent signaling to MAPK and cyclin D1 transcription. MIF secretion is induced by a variety of stimuli including growth factors and Integrin engagement (Liao et al., 2003; Mitchell et al., 1999). Extra-cellular MIF then binds to its putative membrane bound receptor, CD74 (Leng et al., 2003) that can then initiate the activation of Rho GTPase activity via an unknown mechanism. Increased Rho activity is then thought to promote the activation of Rho kinase and MLC phosphorylation. Hyperphosphorylated MLC, in turn, induces stress fiber formation/integrin clustering and subsequent FAK-dependent sustained MAPK activation, cyclin D1 transcription, and Rb inactivation. Taken from (Swant et al., 2005).

IFN-τ from the embryo during the preimplantation stage is involved in the reorganization of the luminal epithelium (Wathes and Wooding, 1980) and synthesis and secretion of histotroph by the glandular epithelium (Spencer and Bazer, 2004). Remodeling of the glycoprotein adhesion molecules of the apical surfaces of these cells precedes implantation (Aplin, 1997). Reduction of Muc-I (Johnson et al., 2001) precedes integrin activation (Maheshwari et al., 2000). In pregnant sheep at day 17, when intimate contact between LE and trophectoderm begins, a decrease in Muc-I was observed on uterine LE exposed apically at integrins to interaction with OPN during peri-implantation period (Johnson et al., 2001).

OPN protein is localized on the apical aspect of the endometrial LE, GE, and conceptus Tr (Johnson et al., 1999b; Johnson and and Bazer, 1999), and is a potential mediator of implantation in sheep, as a bridge between integrin heterodimers expressed by Tr and uterine LE responsible for adhesion for initial conceptus attachment (Johnson et al., 2003a). Integrin activation initiates multiple intracellular signaling pathways and results in regulation of cell functions such as motility, proliferation and differentiation (Maheshwari et al., 2000).

Members of the integrin family of cell surface adhesion molecules are now known to relay signals between extracellular matrix proteins in the microenvironment and intracellular signaling pathways in cells (Morozevich et al., 2002). Through interaction with the cytoskeleton, signaling molecules, and other cellular proteins, integrin cytoplasmic domains transduce signals from both the outside and inside of the cell and regulate integrin-mediated biological functions (Liu and Burridge, 2000; Liu et al., 2000). Integrin β chains, for example, interact with actin-binding proteins (e.g. talin and filamin), which form mechanical links to the cytoskeleton, might also link integrins to signaling

mechanisms and, in some cases (e.g. JAB1) mediate integrin-dependent gene regulation (Epler et al., 2000). E-cadherin and β-catenin also are implicated in the remodeling of endometrium during implantation; in pregnant mice, the β-catenin signaling pathway is inhibited in both blastocyst and uterus during the window of implantation (Li et al., 2005; Li et al., 2002b).

CONCLUSION.

In conclusion, embryo implantation involves endometrial remodeling for adhesion of the conceptus into the uterine wall. In ruminants, IFN-τ secreted from the embryo induces adhesion between luminal epithelium and trophectoderm essential for attachment and superficial implantation (Klein et al., 2006). In the present work using *in vitro* endometrial epithelial cells, it has been shown that IFN-τ stimulates the proteolytic cleavage of E-cadherin and subsequent accumulation in cytoplasm, and accumulation of β-catenin at the plasma membrane. Similarly MIF was increased in luminal and glandular epithelium from pregnant cows. MIF was stimulated supposedly via the action of IFN-τ. An autocrine effect of MIF was observed on E-cadherin and β-catenin cytoplasmic accumulation in bovine endometrial epithelial cell *in vitro*. These data suggest that changes in MIF and E-cadherin induced by IFN-τ secreted by the embryo play an important role in attachment of the trophoblast to the endometrial wall.

There is much information about IFN-τ in pregnancy recognition but the effect of IFN-τ in remodeling of bovine endometrium at the preimplantation period is not clearly understood. To further advances our knowledge on how MIF, E-cadherin and β-catenin affect attachment of the bovine embryo, these proteins should be loacated by immunohistochemistry in uterus bovine at different times of the estrous cycle and the implantation period in pregnant animals. Since proteolytic cleavage of E-cadherin or β-

catenin might induce apoptosis or cell growth. Therefore it is important verify their *in vivo* effect.

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