UNIVERSITÀ
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DEPARTMENT OF MOLECULAR AND DEVELOPMENTAL MEDICINE

DOCTORATE SCHOOL OF MOLECULAR MEDICINE

CYCLE XXIX

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Glucocorticoids and proteases in the pathophysiology of pregnancy and parturition

Research field: Pathophysiology of reproduction

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Academic Year 2015-2016

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ABSTRACT

Understanding the physiology of pregnancy and parturition enables effective management of pregnancy complications that could otherwise be life threatening for both mother and fetus. A large body of evidence suggests that glucocorticoids (GC) and matrix metalloproteinases (MMPs) play a key role in the pathophysiology of pregnancy and parturition. Glucocorticoids influence several aspects of embryo implantation process including the maternal immune response, embryo attachment and fetal development. Prolonged exposure to high levels of glucocorticoids in pregnancy is dangerous for both placental and fetal development indicating that tight control of GC levels at the fetal-maternal interface is essential for establishment and maintenance of a healthy pregnancy. GC exert their actions via the glucocorticoid receptor, an ubiquitously expressed nuclear receptor comprised of 9 exons. Previous studies have demonstrated that differential promoter usage and alternative splicing-generated GR mRNA transcripts result in differential cellular GC responses.

Matrix metalloproteinases are involved in the extracellular matrix remodeling and they contribute to the success of a pregnancy from embryo implantation to labour and postpartum involution. A misregulation of their expression or their activity is observed in adverse outcomes of pregnancy such as preterm labour.

The aim of this thesis was to investigate 1) the expression of GR transcripts and the effect of inflammatory cytokines on the control of GR transcripts expression in cultured first trimester human decidua cells; 2) the expression of the two major groups of MMPs (stromelysins and gelatinases) and their inhibitors TIMP-1 and TIMP-2 in a) the mouse uterus throughout normal gestation, at labour and during inflammation-induced preterm birth and b) the human myometrium at term and preterm, both non-labouring and labouring.

This study demonstrated that first trimester human decidua cells are characterized by a complex pattern of GR mRNA transcripts expression. Among the several GR isoforms expressed, GRβ, GRγ, GRP and GRα-D might be correlated with the development of the glucocorticoid resistance. Moreover, the significant increase in GR isoforms mRNA expression and protein levels observed in IL-1β or TNF-α- treated FT-DCs suggest a role for inflammatory cytokines in regulating decidual GR isoforms abundance.

Furthermore, this study demonstrated that the majority of MMPs (Mmp-3, Mmp-2, Mmp-9, Mmp-10) studied in the mouse uterus displayed higher expression in early gestation compared to late gestation and labour; only Mmp-10 exhibited increased expression at labour suggesting a role in

parturition. In addition, Mmp-3 and Timp-1 increased in the uterus of mice destined to undergo premature labour due to LPS-induced intrauterine inflammation, in contrast to Timp-2, which decreases. These observations may suggest a role of MMP-3 and TIMP-1 in preterm labor. Finally, this work reported a decreased expression of MMP-10 in the human myometrium with the onset of term, but notably not preterm labour. On the other hand, human TIMP-1 is dramatically increased with the onset of term and preterm labour.

In conclusion, this study provide key novel findings in the pathophysiology of pregnancy and parturition highlighting 1) the importance of GCs and their receptors GRs in the establishing of pregnancy and that the identification, characterization and regulation of the several GR isoforms involved in the modulation of glucocorticoid action may lead to potential strategies for the treatment or prevention of a variety of pregnancy-related disorders; 2) the important involvement of MMPs and TIMPs in uterine remodeling during pregnancy and labour and that the balance between MMP and TIMP expression may be critical to controlling the timing of normal labour, alterations in MMP/TIMP ratio lead to multiple pregnancy complications such as preterm birth.

1 INTRODUCTION

Pregnancy is the period of gestation from the fertilization of an egg and implantation of the zygote, through development of a fetus, and ending at birth.

Human pregnancy lasts about 280 days (it averages between 266 and 294 days). Pregnancy is divided into three trimesters that culminate with parturition. The term birth is defined as delivery between 38 and 42 weeks of gestation. Preterm birth is defined as a delivery before 37 complete weeks of gestation (*Romero et al., 2006, Challis et al., 2009*).

The first trimester is from week one through 12 and includes fertilization and implantation of the zygote. The placental tissue that initially surrounds the fetus and the amniotic sac become concentrated in one circular area on the womb wall to form the placenta. During the first 12 weeks, the embryo begins to develop internal organs and body structures. The second trimester is from week 13 through 28. During this stage the fetus continues to grow and the fully developed placenta provides all the fetus' needs until birth; oxygen, nutrients and protective antibodies. The third trimester is from 29 weeks through 40 weeks in which the fetus grows quickly and reaches its full size until birth (*Hurt et al., 2010*).

The uterus is an important organ of pregnancy and parturition. The human uterus is a hollow organ surrounded by a thick uterine wall. The uterine wall consists of three layers; the endometrium (or decidua during pregnancy), which is the glandular inner lining of the uterus, the muscular layer consisting predominantly of smooth muscle cells (SMCs) known as the myometrium which is the principal site of uterine growth during pregnancy and parturition, and the outer epithelial layer that covers the uterus called perimetrium (*Smith,2007*).

During pregnancy the uterus undergoes dramatic changes that are required for successful pregnancy and labour. The endometrium (differentiated in decidua through the process of decidualization) adapts to accommodate the developing fetus and forms part of the placenta. The myometrial smooth muscle cells (MSMCs) undergo hypertrophy and hyperplasia to thicken and strengthen the uterine muscle in readiness for labour. During labour the myometrium develops powerful contractions to help expel both the fetus and the placenta (*Young and Hession, 1999*). These preparatory changes result in significant enlargement of the uterus, which at term will weigh approximately 20 times its non-pregnant weight (*Smith et al.,2007*). Following delivery, the uterine layers undergo rapid remodelling via phagocytosis and autolysis to help return the uterus to its prepregnant size (*Matsuzaki et al., 1971*). The degradation and remodeling of uterine extracellular matrix (ECM) during pregnancy and

parturition is regulated by matrix metalloproteinases (MMPs). An imbalance in the MMPs and their tissue inhibitors activity ratio in the uterine microenvironment may result in aberrant degradation of ECM and account for premature onset of labor (*Geng et al., 2016*).

The mechanisms involved in human pregnancy maintenance and parturition are highly complex and involve both maternal and fetal systems. Neuronal, hormonal, and immune pathways participate in both pregnancy and parturition (*Petraglia et al.,2010, Challis et al.,2000*).

This work is focused on the study of two stages of pregnancy, implantation and parturition, and important mediators, glucocorticoids and proteases, involved in their pathophysiology.

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1.1 Implantation

Implantation of the embryo takes place in a receptive uterus and occurs approximately six or seven days after fertilization (*Vigano et al.,2003*).

Human uterus is receptive during the midle secretory phase, between days 19 and 23 of the menstrual cycle. The endometrial receptivity period is also known as the window of implantation (WOI) (*Psychoyos et al., 1986*). Endometrial receptivity represents a physiological state in which the endometrium allows a blastocyst to attach, stably adhere, penetrate, and induce localized changes in the endometrial stroma under the influence of progesterone resulting in decidualization. Upon decidualization, the fibroblast-like endometrial stromal cells are characterized by a progressive cell enlargement, rounding of the nucleus, expansion of the rough endoplasmic reticulum and Golgi complex and cytoplasmatic glycogen accumulation (*Bergh et al., 1992; King, 2000*).

During implantation the trophoblast differentiates into two layers: the syncytiotrophoblast and the cytotrophoblast. The syncytiotrophoblast forms an external layer with multinucleated cells (syncytium) and invades the the uterine wall. The cytotrophoblast consists of an irregular layer of ovoid, mono-nucleated cells and lies directly below the syncytiotrophoblast (*Norwitz et al.,2001*).

The process of implantation consists of three different stages: apposition, adhesion and invasion. Apposition is an initial unstable adhesion of the blastocyst to the endometrial surface. This stage is characterized by the appearance of microprotrusions from the apical surface of the epithelium, termed pinopodes (*Strowitzki et al., 2006*). The pinopods express chemokines and cell adhesion molecules (CAMs), which attract the blastocyst floating within the endometrial cavity to appose. In addition, the smooth surface of the pinopodes facilitates the apposition of the blastocyst to the endometrium. The blastocyst is able to appose to the pinopods with the removal of adhesion inhibiting mucin, while the areas between pinopods have been shown to express mucin 1 (MUC-1), which prevents embryo adhesion (*Gipson et al., 2008*). Once the blastocyst is apposed, a stronger attachment is achieved though local paracrine signaling between the embryo and the endometrium. At this stage, the blastocyst is sufficiently adherent to the endometrium. The first sign of the attachment reaction coincides with a localized increase in stromal vascular permeability which is manifested as stromal edema at the site of blastocyst attachment (*Sharkey et al., 2003*). Following adhesion, the embryo invades through the luminal epithelium into the stroma to establish a relationship with the maternal vasculature (*figure 1*). Defective trophoblast invasion is implicated in several pregnancy disorders such as spontaneous preterm birth (PTB), preeclampsia (PE) and intrauterine growth restriction (IUGR) (*Staun-Ram et al., 2005*).

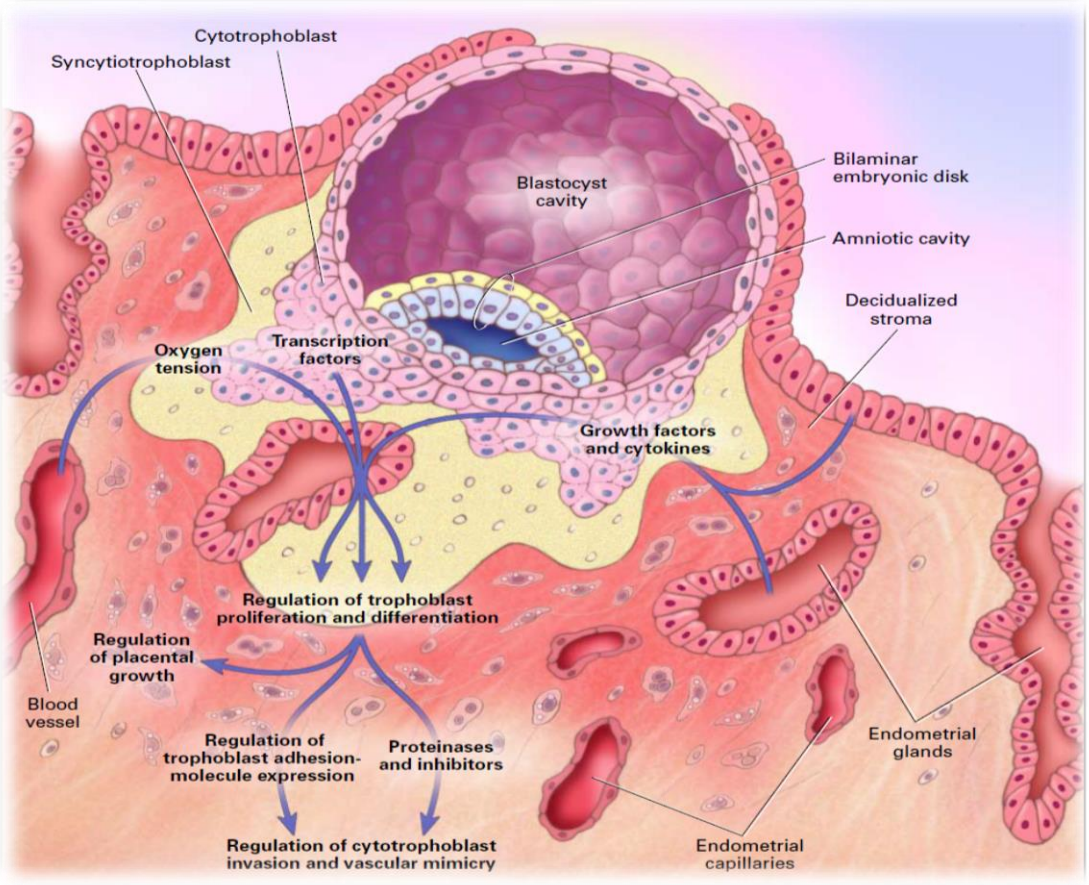

Figure 1. Blastocyst implantation (*Norwitz et al.,2001*)

1.2 Mediators of implantation

Implantation is associated with inflammatory changes and tissue remodeling by matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). Moreover, embryo implantation is strongly influenced by ovarian steroids and by an elaborate network of prostaglandins, proinflammatory citokines, corticosteroids, hormones, growth factors that act by regulating endometrial receptivity and in establishing the embryo-maternal dialogue (*Kodaman and Taylor, 2004; Makrigiannakis et al.,2011*).

1.2.1 Cytokines

A gradient chemokines and cytokines, produced by the endometrial cells, guide the blastocyst to the implantation site allowing its interaction with the uterine lining. During invasion, trophoblast cells break through the epithelial and stromal cells. The endometrial tissue is then repaired and remodeled by the growing placenta. This local 'wound healing-like' process is characterized by a strong Th1, pro-inflammatory response in which high levels of pro-inflammatory cytokines such as IL-6, IL-1, LIF, IL-8 and TNF-α are involved (*Dominguez et al., 2005, van Mourik et al., 2009*).

Leukemia inhibitory factor (LIF) is a proinflammatory cytokine which interacts with the receptor LIF-βR and initiates a variety of intracellular signaling pathways. LIF is expressed in the luminal epithelium during mid-late secretory phase of the mestrual cycle, supporting a role in implantation (*Cheng et al., 2001*; *Dimitriadis et al., 2005*). During the secretory phase, LIF-βRs is expressed in the luminal epithelium. Decidual stromal cells also produce LIF (*Kimber et al., 2005*). LIF may control the proportions and amount of immune cells in the endometrium at the time of implantation and may mediate interactions between decidual leukocytes and the invading trophoblast (*Dimitriadis et al., 2005*). In contrast, low levels of LIF in uterus have been shown to be dangerous for successful implantation (*Ledee-Bataille et al., 2002*). Interleukin 6 (IL-6) is a proinflammatory cytokine produced mainly by endometrial epithelium and stromal cells at the time of implantation (*Cork et al., 2002*). The levels are the highest during the window of implantation and menstruation and the expression is under the control of several factors, including IL-1 (*Perrier et al., 2004*). Furthermore, steroid hormones, especially estrogen, induce the expression of IL-6 (*Tabibzadeh et al., 1995*). Recent study has demonstrated that low IL-6 mRNA expression is associated with recurrent miscarriages, possibly as a result of a decrease of the functions of IL-6 in tissue remodeling, decidualization, and placenta/trophoblast development (*Jasper et al., 2007*).

Another cytokine system often implicated in embryonic implantation is the interleukin 1 (IL-1) system, which belongs to the IL-1/TLR superfamily. The IL-1 system contains two agonists, IL-1α and IL-1β, two cell-surface receptors, IL-1R1 and IL-1R2 (*Krussel et al., 2003*). IL-1R1 is found throughout the body in low levels whereas IL-1R2 is expressed in white blood cells only; IL-1R2 is a decoy target for IL-1, thus indicating that IL-1R2 may have an inhibitory role on IL-1 activity (*Boucher et al., 2001*). IL-1 signals through MAPK and NF-kB pathways (*Lessey et al.,2000*). Expression of IL-1 can be found in stromal cells, macrophages, leukocytes (including uNK cells), and endothelial cells. IL-1 has several functions in the window of implantation. It stimulates the production of LIF by the endometrium, increases the expression of the integrin 3 subunit, an adhesion

molecule that plays an important role in apposition and adhesion (*Gonzalez et al., 2004; Krussel et al., 2003*). IL-1 also appears to have an important function in decidualization (*Fazleabas et al., 2004*). The embryo may use the IL-1 system to communicate with maternal tissues and to influence endometrial conditions. IL-1β modulates trophoblast invasion (*Karmakar and Das, 2002*), promote angiogenesis (*Choi et al., 2002*) and mediate macrophage infiltration of the decidua (*Lockwood et al., 2006*).

Another cytokine that appears to have a role in implantation is tumor necrosis factor alpha (TNF-α). The pleiotropic cytokine TNF-α and its two receptors are present in the endometrium, as well as in placental trophoblasts (*Hunt,1993*). TNF-α was shown in vitro to increase urokinase-type plasminogen activator (uPA) secretion from cytotrophoblasts, thereby properly enhances the degradation of fibronectin during trophoblast penetration of the endometrial ECM. The up-regulation of uPA increases activation of MMP-9 through the plasminogen activation system, thereby enhancing trophoblast invasiveness (*Monzón-Bordonaba et al., 2002*). At the same time, TNF-α decreases MMP-2 concentration and activity as well as human human chorionic gonadotropin (hCG) secretion (*Meisser et al., 1999*). High TNF-α levels presented during inflammatory responses, could be found in pathologic processes such as pregnancy loss, preterm delivery and preeclampsia (*Monzón-Bordonaba et al., 2002*). In a recent study, despite increased MMP-9 expression, TNF-α was found to inhibit in vitro trophoblast migration and invasion. However, the plasminogen activator inhibitor-1 (PAI-1) that blocks the plasminogen activator system, was found to be increased (*Bauer et al., 2004*). Thus, TNF-α seems to exert different in vitro effects, depending on individual trophoblast preparation, type of cell-line and cytokine concentration. A link between elevated PAI-1 levels detected in plasma and syncytium of preeclamptic women, and elevated TNF-α found in preeclamptic sera, villi and deciduas has been suggested (*Bauer et al., 2004*). The elevated TNF-α level could result from local hypoxic conditions developing under reduced maternal and fetal vascular perfusion (*Benyo et al.,1997*).

Among their other activities, these cytokines recruit immune cells to the decidua. Infiltration of large populations of decidual leukocytes to the implantation site has been observed. Of these cells, 65–70% are uterine-specific natural killer (uNK) cells and 10–20% are antigen-presenting cells (APC) such as macrophages and dendritic cells (DCs) (*Kammerer,2005; Hanna et al., 2006*). Unlike NK cells, macrophages and DCs stay in the decidua throughout pregnancy and have a crucial role at the maternal–fetal interface (*Gardner and Moffett, 2003, Fest et al., 2007*) . Specifically, these cells form the initial contact with external antigens, mediating the antigen specific adaptive immune response. Several lines of evidence suggest that APCs play a key role in shaping the cytokine profile in the

maternal–fetal interface (*Mor, 2008*). Macrophages and DCs have the ability to secrete an array of antiinflammatory cytokines/chemokines (IL-4, IL-10, and IL-13) and enzymes that are involved in tissue remodeling and angiogenesis. In addition, macrophages have been suggested to regulate trophoblast invasion and may play a crucial role role in cleaning up the debris, resulting from apoptosis of the trophoblast in the various stages of pregnancy (*Abrahams et al., 2004, David Dong et al., 2009*). Accumulation of DCs in the maternal tissue surrounding the implanting embryo has been reported and their involvement in the establishment of tolerance toward the semi-allograft fetus has been extensively investigated. These cells are responsible for changing the Th1, pro-inflammatory, into the Th2, anti-inflammatory environment also during later stages of pregnancy (*Miyazaki et al., 2003; Nagamatsu and Schust, 2010*).

1.2.2 Ovarian hormones

The ovarian steroids, progesterone and estrogen, have a major regulatory role by mobilizing several molecular modulators in a spatiotemporal manner, which supports embryo implantation They play important roles in blastocyst activation, establishment of uterinal receptivity, and implantation of the blastocyst to receptive endometrium via binding to their specific nuclear receptors (*Carson et al., 2000; Lim et al., 2002*).

Following the increase of estrogen levels, the uterus enters into receptive phase and then embryo implantation occurs. Estrogen is capable of increase endometrial capillary permeability by stimulating expressions of several genes related to vascular alterations in the uterus where the embryo implantation occurs (*Kodaman and Taylor, 2004*).

Secreted estrogen from ovary uses two types of nuclear receptors (ER-α and ER-β), which belong to the steroid receptor super family. The phenotype of the ER-α knockout model in uterus indicates that ER-α has an essential role in uterine physiology. It is essential for blastocyst attachment but is dispensable for subsequent decidualization. ER-α is expressed by endometrial epithelial and stromal cells during the proliferative phase, but decreases during the secretory phase (*Lessey et al., 1988*). The cellular proliferation of the endometrial epithelium in response to estrogen is dependent upon stromal expression of ER-α. There is little endometrial expression of ER-β; it is limited to glandular epithelial cells and seems to modulate ER-α–mediated gene transcription in the uterus (*Wiehua et al., 2000*).

Progesterone has a crucial role in preparing the uterus for the developing embryo and for a successful pregnancy. Stromal cells differentiate into decidual cells in respond to progesterone during the

decidualization process, characterized by morphological changes and secretion of prolactin *(Dunn et al., 2008*). Progesterone is also required for maintenance of the pregnancy by stimulating and maintaining uterine functions, necessary for early embryonic development, implantation, placentation and fetal development. Hormones such as hCG, produced by the trophoblast, maintain this progesterone production in early pregnancy (*Spencer et al., 2004*). Progesterone is known to restrain endometrial breakdown by inhibiting MMPs. This inhibitory effect implies that progesterone impedes the invasion of trophoblast cells into the endometrial tissue. In a recent report progesterone was found to decrease invasion and gelatinase expression in early first trimester trophoblast cells through the down-regulation of MMP-9 and to increase cell invasion and MMP-2 expression in late trophoblast cells (*Goldman et al., 2005*). Progesterone plays a role in proliferation, differentiation, and maintenance of endometrial stromal and glandular cells and myometrial cells (*Huet-Hudson et al., 1990*). Progesterone acting through two isoforms of the progesterone receptor (PR), PR-A and PR-B. PR-A is expressed in the stroma and epithelium during the proliferative and secretory phases of the menstrual cycle; however, epithelial levels of PR-A gradually decrease during the secretory phase. PR-B is expressed in glandular and stromal nuclei only during the proliferative phase (*Wang et al., 1998*). PR levels are increased by estrogens and growth factors (*Krauss et al., 1993*) The down-regulation of PR during the window of implantation is a prerequisite for endometrial receptivity (*Lessey et al., 1996*).

1.2.3 Glucocorticoids

Glucocorticoids (GC) are hormones involved in the regulations of numerous physiologic processes, they are synthesized and released by the adrenal cortex in a circadian manner and in response to stress (*Oakley et al., 2013*). Glucocorticoids regulate many critical processes for successful embryo implantation such as modulation of materal immune response, trophoblast proliferation and invasion, subsequent growth and development of the fetus and placenta (*Michael and Papageorghiou, 2008*). The endometrium, placenta and embryo/fetus are exposed to the major physiological glucocorticoid, cortisol, rising from either the maternal or fetal adrenal glands. Glucocorticoids exert several anti-inflammatory actions which could impair the cytokine-prostaglandin signalling cascade required for implantation. GC can inhibit the activator protein AP-1 and nuclear factor NF-kB signalling pathways that mediate the cellular responses to pro-inflammatory cytokines (*Barnes, 1998; Adcock and Caramori, 2001; Hayashi et al., 2004*).

Glucocorticoids can act several positive effects that promote pregnancy such as stimulation of hCG secretion, suppression of uterine natural killer cells and promotion of trophoblast growth and invasion and a range of adverse effects that can compromise the pregnancy (*Gennari-Moser et al., 2011*).
The range of adverse effects exert by glucocorticoids that would be expected to impede pregnancy include inhibition of cytokine-prostaglandin signalling, restriction of trophoblast invasion following up-regulation of plasminogen activation inhibitor-1, induction of apoptosis and inhibition of embryonic and placental growth. Excess GCs has been shown to lead to abnormalities in placental development indicating that tight control of GC levels at the fetal-maternal interface is essential for establishment and maintenance of a healthy pregnancy. A growing body of evidence indicates that increased exposure of the fetus to glucocorticoids in early pregnancy may result in adverse outcomes, which include intra-uterine growth restriction (IUGR), increased risk of pre-term labour, post-natal hypertension, effects on fetal brain *(Michael and Papageorghiou, 2008; Singh et al.,2012).*

Local tissue GC levels are modulated by 11β-hydroxysteroid dehydrogenases (11β-HSD). The 11-hydroxysteroid dehydrogenase family (11HSDs) are enzymes that catalyze glucocorticoid metabolism, regulating the availability of active glucocorticoid (cortisol) and their inactive metabolites (cortisone). The type I isoform (11β-HSD1) is a bidirectional enzyme, which acts predominantly as an oxidoreductase to generate the biologically active glucocorticoid, cortisol, by using nicotinamide adenine dinucleotide phosphate (NADP+/NADPH) as a cofactor (*Courtney et al., 2008*). In contrast, the type II enzyme (11β-HSD2) uses nicotinamide adenine dinucleotide (NAD+) to unidirectionally inactivate cortisol (*Ferrari et al., 1996*). The main cellular targets for cortisol are glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). However, GR normally colocalizes with 11β-HSD1 in vivo, whereas 11β-HSD2 is commonly found in MR-expressing tissues (*Kuroda et al., 2013*).

1.2.3.1 Glucocorticoid receptor

Glucocorticoid receptor (GR) is a ubiquitously expressed member of the nuclear receptor transcription factor super family. The GR is a modular protein composed of 3 major domains: an N-terminal transactivation domain (NTD), a central DNA binding domain (DBD), and a C-terminal ligand-binding domain (LBD) (*Kumar et al., 2005*). The DBD is the most conserved region of the nuclear receptor family and contains two zinc finger motifs that recognize and bind target DNA sequences named glucocorticoid-responsive elements (GREs). The NTD contains a transcriptional

activation function (AF1) that interacts with coregulators and the basal transcription machinery and is the primary site for posttranslational modifications. The LBD has a hydrophobic pocket for binding glucocorticoids and an a second activation function (AF2) domain that interacts with coregulators in a ligand dependent manner (*Bledsoe et al., 2002*). In the absence of hormone, the GR resides mainly in the cytoplasm of cells as part of a large multiprotein complex that includes several chaperone proteins such as hsp90, hsp70 and p23 (*Grad et al.,2007*). These proteins maintain the receptor in a conformation that is transcriptionally inactive. On binding glucocorticoids, the GR undergoes a conformational change, resulting in the dissociation of the heterocomplex, the exposure of the two nuclear localization signals and subsequently the GR is rapidly translocated into the nucleus through nuclear pores. Once in the nucleus, the GR can binds directly to GREs and regulates the expression of target genes. It can regulate the transcription of target genes by directly interacting with other transcription factors or binding directly to DNA and interacting with neighboring DNA-bound transcription factors (*Rogatsky et al., 2006*). In contrast, the interaction of the GR with the proinflammatory transcription factors activator protein 1 (AP-1) and nuclear factor kB (NF-kB) inhibits their activity and is considered to be a primary mechanism through which glucocorticoids suppress inflammation. The GR directly binds the Jun subunit of AP-1 and the p65 subunit of NF-kB and interferes with the transcriptional activation function of these two proteins (*Nissen et al., 2000*). The repression is accomplished on some genes by GR tethering itself to these DNA-bound proteins. For other genes, however, the GR acts in a composite manner, binding directly both a GRE and the transcription factor on an adjacent site (*Chinenov et al., 2012*). This is the classic GR signaling pathway but GR can also act through nongenomic mechanisms to induce rapid cellular responses that occur within a few seconds to minutes and do not require changes in gene expression (*Samarasinghe et al., 2012*). Multiple mechanisms appear to be involved in these signaling events that affect the activity of various kinases, such as phosphoinositide 3-kinase and mitogen-activated protein kinases (MAPKs). The glucocorticoid-dependent release of accessory proteins associated with unliganded GR in the cytoplasm, such as the nonreceptor tyrosine kinase c-Src, can mediate nongenomic actions of glucocorticoids. When liberated from the GR complex, c-Src activates multiple kinase cascades that lead to the phosphorylation of annexin 1, inhibition of cytosolic phospholipase A2 activity, and impaired release of arachidonic acid (*Solito et al., 2003*).

The GR-encoding gene (NR3C1) is located on chromosome 5q31.3 and has nine exons. The untranslated exon 1 of the GR gene can be spliced into 9 different promoter variants that function in a tissue specific manner to regulate GR protein expression including GR-1A1, GR- 1A2, GR-1A3, GR-1B, and GR-1C. Promoters 1B and 1C are ubiquitously expressed and are probably responsible

for the basal GR expression. Promoters 1A, 1A1 and 1A2 are marginally expressed, 1A3 is mainly expressed in cells of the hematopoietic lineage. (*Russcher et al., 2007; Breslin et al., 2011*)

Alternative splicing or alternative initiation of translation in exons 2 and 9 can generate different isoforms of GR resulting in the expression of GRα, GRβ, GRγ, GR-A and GR P proteins (*Hollenberg et al., 1985*) (*figure 2*).

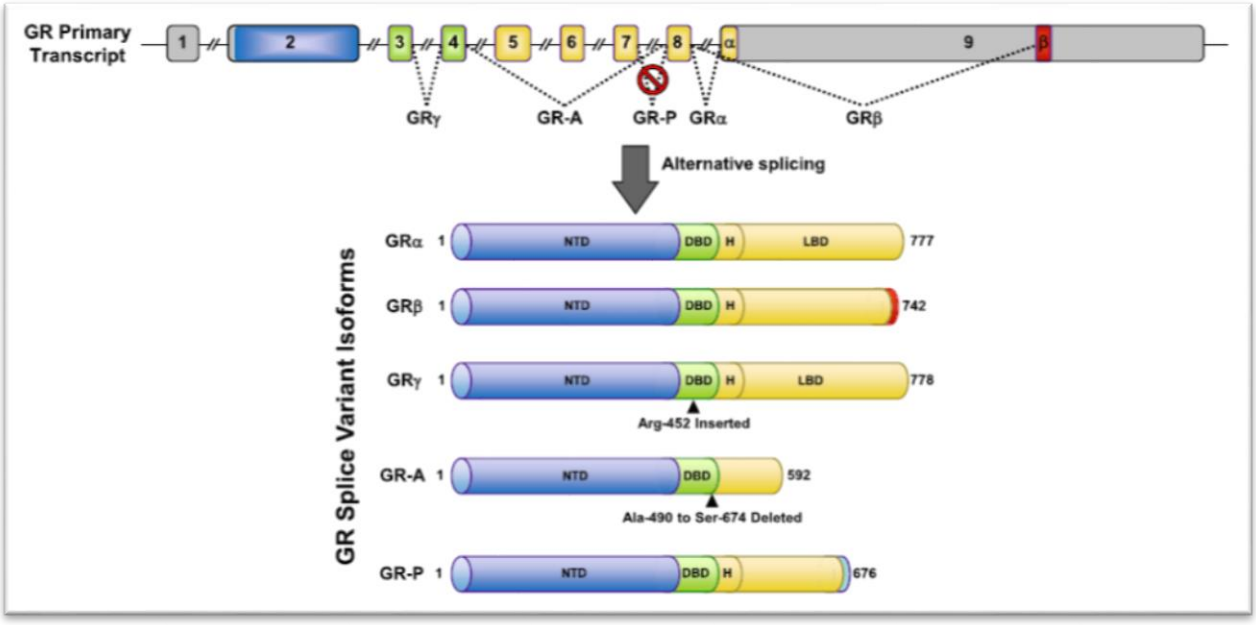

Figure 2. GR splice variants (*Oakley et al.,2013*)

GRα, the classic GR protein that mediates the actions of glucocorticoids, is produced from a primary mRNA transcript in which the terminal end of exon 8 is joined to the beginning of exon 9. In contrast, GRβ is translated from a transcript in which exon 8 is spliced to a downstream sequence in exon 9 (*Bamberger et al., 1995*). Although GRα and GRβ are identical through the N-terminal amino acids sequence, but they diverge at the C-terminus determining GRβ distinct functions and properties.

GRβ resides constitutively in the nucleus and does not bind glucocorticoid agonists and GRβ inhibits activation of GRα through a dominant negative mechanism including competition for GREs, interference with coregulators, and formation of inactive GRα/GRβ heterodimers (*Oakley et al., 1997*). In most tissues and cell lines, GRβ is expressed at lower levels than GRα although in some cell types, including human neutrophils and certain epithelial cells, GRβ is abundant (*Pujols et al., 2002*). Proinflammatory cytokines and microbial superantigens enhance GRβ expression in

mononuclear cells, an effect associated with glucocorticoid resistance. The remaining GR splice variants, GRγ, GR-A, and GR-P, were discovered in glucocorticoid-resistant cancer cells, and later found to be expressed in healthy tissues. GRγ, first identified in cancer cells then characterized in blood mononuclear cells. GRγ functions as transcription factor, but only exhibits approximately 50% of the activity of GRα for canonical glucocorticoid target genes (*Kasai et al., 1990;Ray et al., 1996*). GRγ expression in acute lymphoblastic leukemia has been shown to correlate with resistance to dexamethasone treatment (*Beger et al., 2003*). GR-A and GR-P were discovered in glucocorticoid-resistant multiple myeloma cells as GR isoforms that failed to bind glucocorticoid ligands Although little is known about GR-A, GR-P is detected in normal human tissues and is the predominant splice variant in several glucocorticoid-resistant cancers (*Moalli et al., 1993*).

In addition to alternative splicing, alternative translation initiation sites further diversify the repertoire of potential GR proteins (*de Lange et al., 2001*; *Krett et al., 1995*). A single GRα mRNA transcript can generate eight GRα isoforms: GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2, and GRα-D (*Lu et al., 2005*) (*figure 3).* All GRα translational isoforms bind glucocorticoid ligands and interact with GRE but they have distinct capacities to mediate glucocorticoid-induced cell death. The GRα-C3 isoform is the most transcriptionally active of GRα isoforms whereas the GRα-D isoform is the less active (*Lu et al., 2006*). Moreover, cells expressing GRα-C3 are most sensitive to dexamethasone-induced killing whereas those expressing the GRα-D3 isoform are relatively insensitive to glucocorticoid-induced death. GRα-D does not repress NF-κB activity like other GRα isoforms and fails to inhibit the transcription of certain antiapoptotic genes. Unlike GRα-A, -B, and -C isoforms that are predominantly found in the cytoplasm, GRα-D isoform resides in the nucleus (*Lu et al., 2007*). GRα translational isoforms are expressed throughout the body but the relative abundance of each isoform varies substantially across cell types, likely contributing to the tissue and cell-specific effects of glucocorticoids. GRα-C isoforms are highly expressed in the pancreas lung, and colon but are expressed at low levels in the liver (*Lu et al., 2005; Lu et. al., 2007*). GRα-D isoforms, in contrast, are abundant in spleen and bladder, but are relatively low in the heart and pancreas. The profile of GR isoforms can also change with maturation or activation within a cell lineage. GRα-D isoforms are abundant in immature dendritic cells but GRα-A are expressed in high levels in mature dendritic cells, resulting in differential susceptibility to glucocorticoid-induced cell death. (*Cao et al., 2013*).

GRs are highly expressed in endometrial stromal fibroblast and endothelial cells (*Bamberger et al., 2001*), in first trimester (*McDonald et al., 2006*) and term (*Sun et al., 1996*) decidual stromal cells and in isolated population of uterine natural killer (uNK) cells (*McDonald et al., 2006*). GR has also been detected in term and first trimester placenta (*Speeg and Harrison, 1979*).

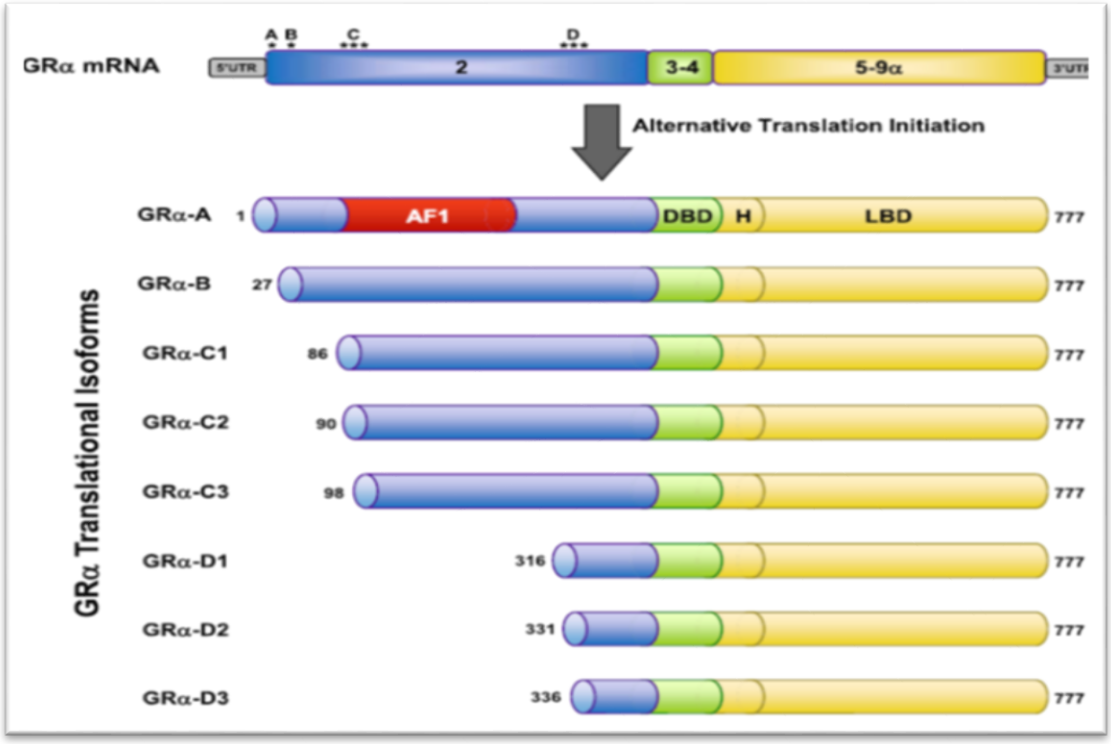

Figure 3. GR translational isoforms (*Oakley et al.,2013*)

1.2.4 Matrix metalloproteinases in pregnancy

Matrix metalloproteinases (MMPs) comprise a large family of zinc dependent proteinases, capable of degrading all extracellular matrix (ECM) components. The proteolytic activities of MMPs influence several cellular processes like cell proliferation, migration and adhesion, as well as many physiological events involving tissue remodeling, such as angiogenesis, bone development, wound healing and uterine involution (*Visse and Nagase, 2003*). MMPs have a multidomain structure consisting of a prodomain, a catalytic domain, a hinge region and a hemopexin domain. The propeptide is essential for maintaining the pro- MMP in a latent form, the catalytic domain contains the highly conserved Zn^{2+} -binding site, the proline-rich hinge region links the catalytic domain to the C–terminal hemopexin-like domain, which determines the substrate specificity of the MMP and mediates the interactions with endogenous inhibitors (*Overall, 2002*). On the basis of substrate specificity, sequence similarity, and domain organization, vertebrate MMPs can be distinguished into six groups: collagenases, gelatinases, stromelysines, matrilysins, membrane type- MMPs, and other MMPs (*Brummer et al., 2002; Marchenko et al., 2003*) (*figure 4*).

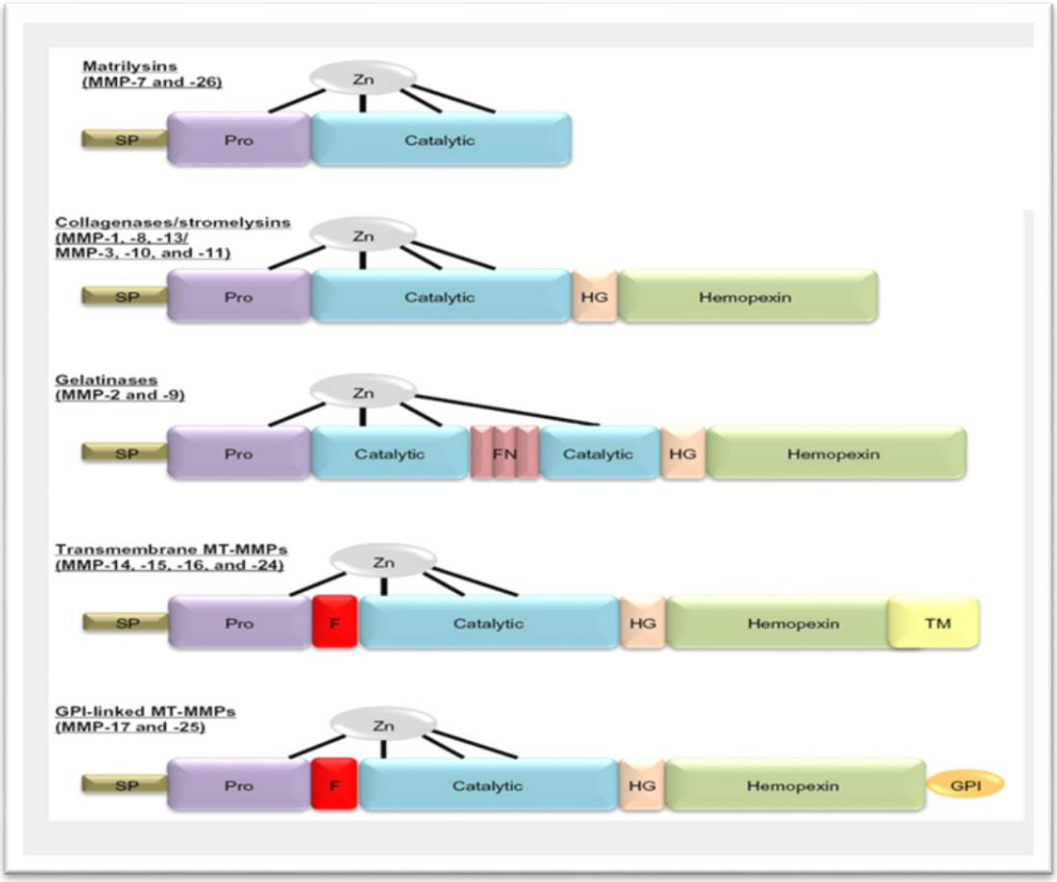

Figure 4. Classification of MMPs (*Nagase et al., 2006*)

Collagenase–1 (MMP–1), collagenase–2 (MMP–8), and collagenase–3 (MMP–13) are the main secreted proteinases involved in the degradation of native fibrillar collagens of types I, II, III, V and XI in the extracellular space (*Owen et al., 2004*). Gelatinases are MMP–2 and MMP–9, they degrade type IV, V, VII, X, XI, and XIV collagens, gelatin, elastin, proteoglycan core proteins, myelin basic protein, fibronectin, fibrillin–1. Gelatinases have an important role in ECM degradation and remodeling in various physiological states, such as implantation and wound healing. (*Folgueras et al.,2004;Hashizume et al., 2007*). Stromelysins are MMP–3 (stromelysin–1), MMP–10 (stromelysin–2), and MMP–11 (stromelysin–3) are capable of degrading types IV and IX collagens, laminin, fibronectin, elastin and proteoglycans. MMP–3 exhibits an higher proteolytic efficiency than that of MMP–10. MMP–3 and MMP-10 activate a number of pro-MMPs and they are expressed by fibroblastic cells and by normal epithelial cells (*Visse and Nagase, 2003*). The matrilysins are represented by matrilysin–1 (MMP–7) and matrilysin–2 (MMP–26 or endometase). MMP-7 is synthesized by epithelial cells and secreted apically. It degrades ECM components, but it also cleaves cell surface molecules such as Fas–ligand, protumor necrosis factor α, syndecan 1 and E-cadherin to generate soluble forms; MMP-26 is expressed in normal cells such as endometrial cells and in some

carcinomas and digests several ECM molecules (*Il et al., 2006*). Membrane-type MMPs (MT–MMPs) are transmembranary or anchored proteins, in a total number of six. MT1–MMP activates latent MMP–2 and MMP–13 at the cell membrane. MT1–MMP has collagenolytic activity on type I, II, and III collagens. MT1–MMP also cleaves gelatin, fibronectin, laminin–1, vitronectin, cartilage proteoglycans and fibrillin–1. MT1–MMP also plays an important role in angiogenesis and is expressed by dermal fibroblasts, and osteoclasts (*Zucker et al., 2003; Thomas et al., 2002*).

Most MMPs are not constitutively expressed by cells in vivo, but their expression is induced by exogenous signals, e.g., cytokines, growth factors, or altered cell-matrix and cell-cell contacts. Since MMPs are produced as zymogen forms, expression of the MMPs genes and proteins is not enough for their functioning but activation of MMPs is one of the key steps for their in vivo proteinase action (*Shiomi et al., 2003*). MMP expression is strictly controlled at the levels of transcription, secretion, activation, and inhibition of the activated enzyme. Proenzymes and active forms of MMP are controlled by stoichiometric binding of specific, locally produced tissue inhibitors of metalloproteinases (TIMPs) (*Visse and Nagase, 2003*).

At present, four members of the TIMP gene family are known: TIMP–1, TIMP–2, TIMP–3, and TIMP–4. TIMP–1 inhibits the activity of most MMPs, with the exception of MT1–MMP and MMP–2. TIMP–1 and TIMP–2 can bind to the hemopexin domain of latent MMP–9 and MMP–2, respectively. TIMP–1 binds to and inactivates MMP–1, stromelysin MMP–3 and MMP-10 with higher affinity for MMP-3 In addition, TIMP–1 has the capacity to form a complex with pro-MMP–9, thereby blocking the activation of the enzyme (*Collette et al., 2004*). TIMP–2 inhibits the activity of most MMPs, except MMP–9, but also to the latent form pro MMP–2. Expression of TIMP–2 is constitutive. TIMP–3 inhibits the activity of MMP–1, MMP–2, MMP–3, MMP–9, and MMP–13. Expression of TIMP–3 is induced in response to mitogenic stimulation and during normal cell cycle progression. TIMP–4 inhibits the activity of MMP–2, MMP–9, and MMP–7. Expression of TIMP–4 in vivo is especially abundant in the heart, but it is also expressed at the sites of tissue injury (*Collette et al., 2004; Folgueras et al., 2004*). In addition to metalloproteinase-inhibiting activities, TIMPs have other biological functions. TIMP–1 and TIMP–2 have erythroid-potentiating activity and cell growth–promoting activities. TIMP–2 inhibits endothelial cell proliferation in vitro and angiogenesis in vivo through a MMP–independent mechanism. TIMP–3 has proapoptotic activity, possibly through the stabilization of TNF-α cell receptor 1, Fas, and TNF related apoptosis, inducing ligand receptor–1. On the other hand, TIMP–1 and TIMP–2 have antiapoptotic activity (*Yu et al., 2000; Mannello et al., 2001*).

The establishment of the maternal-fetal interactions during implantation requires focal MMP expression and comunication pathway, involving both fetal tissue and the maternal endometrium.

Early pregnancy is associated with the expression of multiple MMPs, including MMP-2, MMP-3, MMP-7, MMP-9, MMP-11, MMP-13, and MT1-MMP (*Alexanader et al., 1996, Waterhouse et al., 1993*). MMP-2 and MMP-9 represent the most important MMPs expressed by invading fetal cells during establishment of pregnancy. Recent studies confirmed that primary cultures of first trimester human trophoblasts constitutively express both MMP-2 and MMP-9 on a gelatin matrix, with higher levels of MMP-9 expression and activity compared with cells from the third-trimester (*Shimonovitz et al., 1994*). Other MMPs have also been associated with the invasive characteristics of human trophoblasts including MMP-3, MMP-11, MT1-MMP, and MT2-MMP (*Riley et al., 1999*). The coexpression and subsequent interactions of multiple MMPs are likely required for the rapid turnover rate of ECM during early establishment of the maternal-fetal relationship. In mice, TIMP-1 and TIMP-2 are expressed in undifferentiated stroma toward the outside of the decidua, whereas TIMP-3 was reported in mature decidual cells specifically associated with trophoblast invasion (*Alexander et al., 1996*). Expression of an appropriate MMP/TIMP ratio at the maternal-fetal interface appears to be important in controlling the invasive behavior of cytotrophoblasts as well as maintaining a stabile uterine environment throughout pregnancy. For example, mRNAs for TIMP-1, TIMP-2, and TIMP-3 are coexpressed with MMP-2 and MMP-9 in the uterus of mice in a manner that suggests an important role during establishment and maintenance of pregnancy (*Das et al., 1997*). In humans, expression of TIMP-1, TIMP-2, and TIMP-3 has been observed in isolated stromal and epithelial cells obtained from nonpregnant endometrium, although the most intense expression occurs in decidualized stromal cells associated with pregnancy establishment (*Zhang et al., 1997*).

Among the cytokines that appear to impact the MMP family during pregnancy establishment, the IL-1 family and TNF-α are the most well-characterized. TNF-α and IL-1β, can increase gelatinolytic activity of first trimester cytotrophoblast cells via an increase in MMP-9 (*Meisser et al., 1999*). During the establishment and maintenance of pregnancy, the direct or indirect actions of progesterone appear to be key to the regulation of both MMPs and TIMPs. For example, even before establishment of pregnancy, the stimulation of MMP-3 expression in response to IL-1β can be blocked in human stromal cells by progesterone exposure either in vivo or in vitro (*Curry et al., 2003*).

1.3 Parturition

Parturition is the physiologic and coordinated process by which the fetus and placenta are expelled from the uterus. Parturition is the culmination of a number of events that include cervical ripening, myometrial contraction, fetal membrane rupture and placenta separation. Normal parturition occurs at term, which is anytime between 37-42 weeks of gestation, and is considered to be spontaneous in onset and low-risk for both mother and fetus (*Liggins et al., 1989; Smith et al. 2007*).

Although the timing of parturition relative to fetal maturation varies among viviparous species, important common pathways and their associated hormonal controls exist in the birth process. Parturition involves the transition of the myometrium from a relaxed to a highly excitable state in which the muscle rhythmically and forcefully contracts. Softening of the cervical extracellular matrix (ECM) allows the distensibility needed for dilation of the cervix and a weakening of the fetal membranes that facilitates their rupture. Another common parturition feature is inflammation within the gestational tissues, cervix, decidua/fetal membranes and myometrium (*Kota et al., 2013; Smith et al., 2007*) (*figure 5).*

The onset of labour comprises a fetal endocrine cascade involving the fetal hypothalamic–pituitary–adrenal axis (HPA) which, in most species, leads to an increase in estrogen and decrease in progesterone in maternal plasma. This endocrine cascade ultimately leads to both the ‘activation’ (contraction-associated protein (CAP) expression) and the ‘stimulation’ of the myometrium through the increased production of uterotonic agonists such as oxytocin and prostaglandins (*Kota et al., 2013*).

During labor, the uterine myometrium is converted from a tissue with relatively low connectivity between individual myocytes into a tissue with extensive physical connections. The physical connections occur through pores formed by multimers of connexin 43. Connections between myocytes during labor are also formed by paracrine release of prostaglandin F2α and local release of calcium. This physical and biochemical connectivity allows the depolarization in individual myocytes to be passed to neighboring cells and thus form extensive waves of depolarization and contraction over large areas of the uterus. This causes increased intrauterine pressure and progressive distention of the cervix, leading to expulsion of the fetus. Progesterone withdrawal increases the attachment of myocytes to the intercellular matrix, through integrins, and this process promotes activation of mitogen-associated protein kinase and increases contractility (*Young et al., 2005, Lye et al., 2001*).

As term approaches, there are increasing concentrations of placental orticotropin-releasing hormone (CRH) and increased steroidogenesis in the fetal adrenal glands. The deidroepiandrosterone produced in increasing amounts in the fetal zone is rapidly metabolized by the placenta into estrogens.

Concurrently, cortisol production is increased in the definitive zone of the fetal adrenal glands (*Yoon et at al., 1998*). The increased fetal concentrations of cortisol induce maturation of many fetal tissues, especially the lungs. The maturing fetal lungs increase production of the surfactant proteins and phospholipids that are critical for lung function. The surfactant proteins also enter the amniotic fluid, where they have macrophage-activating properties. In the mouse, surfactant protein A activates amniotic fluid macrophages, and these cells play a critical role in the onset of labor. In humans, the amniotic fluid surfactant proteins may stimulate the inflammation that is observed in the fetal membranes, cervix, and underlying myometrium at the time of labor. There is a clear evidence that this inflammatory process is one of the key elements leading to the onset of labor (*der JM et al., 2006, Keenlan et al., 1999*). The production of surfactant proteins, phospholipids, and inflammatory cytokines in the amniotic fluid increases as cyclooxygenase-2 (COX-2) activity and prostaglandin E2 production increase in the amnion. Levels of cortisol and CRH, both of which stimulate the production of COX-2, rise in the amniotic fluid (*Alvi et al., 1999, Challis et al., 2001, Whittle et al., 2001*). These redundant actions increase prostaglandin E2 and other mediators of inflammation in the amnion. The chorion underlies the amnion and it produces the enzyme prostaglandin dehydrogenase (PGDH), which is a potent inactivator of prostaglandins. Late in pregnancy, chorionic PGDH activity falls, exposing the underlying decidua, cervix, and myometrium to the proinflammatory actions of prostaglandin E2 (*Johnson et al., 2004*). Prostaglandins mediate the release of the metalloproteases that weaken the placental membranes, thereby facilitating membrane rupture (*Li et al., 2005*). A critical component of normal parturition is the softening of the cervix. Parturition is associated with movement of an inflammatory infiltrate into the cervix and the release of metalloproteases that degrade collagen, thus changing the structure of the cervix. During this process, the junction between fetal membranes and the decidua breaks down, and an adhesive protein, fetal fibronectin, enters vaginal fluids (*Ledingham et al.,1999*).

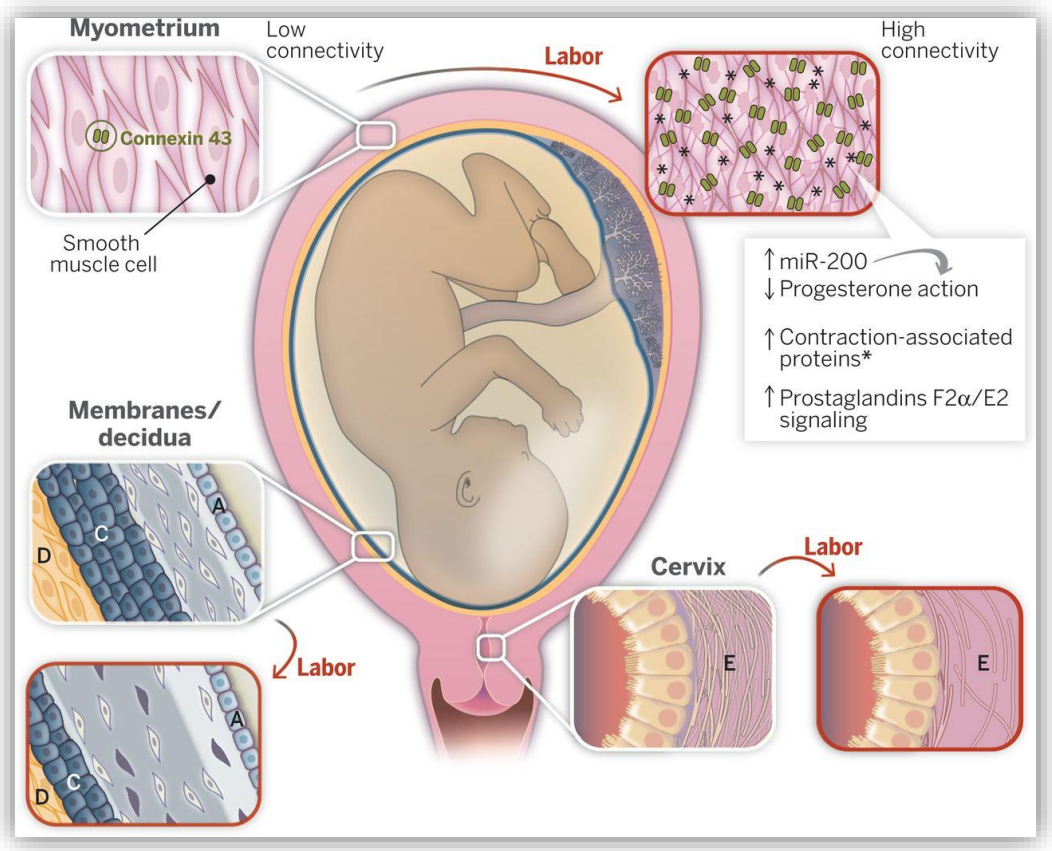

Figure 5. Mechanisms implicated in human parturition (*Romero et al.,2014*)

1.4 Mediators of parturition

Labour is a multifactorial process where inflammatory and endocrine pathways orchestrate the transition of the uterus from a quiescent to an active stage. These pathways favour the transition to an active uterus by promoting the expression of a cassette of contraction-associated proteins (CAPs) and the concomitant activation of contraction cascades in the term myometrium (*Smith et al., 2012*).

1.4.1 Cytokines

A growing body of literature suggests that inflammation is a key feature of human parturition (*Challis et al., 2009; Norman et al., 2007*). A number of studies have observed an influx of inflammatory cells into the myometrium, cervix and fetal membranes at the onset of labour (*Bollapragada et al., 2009;*

Thomson et al., 1999). The invading leucocytes have been shown to increase the production of pro-inflammatory cytokines, such as Interleukin 6/8 and 1β (IL-6; IL-8; IL-1β) in both cervix and myometrium (*Elliott et al., 2000; Young et al., 2002*). Notably, IL-1β has been shown to induce calcium transients and, hence, promote excitation of MSMCs (*Tribe et al., 2003*). In addition to its direct effect on calcium entry, IL-1β has been reported to promote arachidonic acid and cyclo-oxygenase-2 (COX-2) production in the myometrium, resulting in an increase in prostaglandins (PGs) synthesis and concomitant augmentation of myometrial contraction (*Belt et al., 1999; Molnar et al., 1993*). Similar effects have been reported for tumour necrosis factor alpha (TNF-α) *(Fitzgibbon et al., 2009; Molnar et al., 1993*). Besides the roles of pro-inflammatory cytokines in regulation of myometrial contraction, these cytokines have roles in cervical remodelling via stimulation of cervical ripening (*Roh et al., 2000; Sennstrom et al., 2000*). The NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) protein complex has also been shown to play an important role in the labour cascade (*Cookson and Chapman, 2010*). There is evidence that the activity of NF-kB in myometrium and fetal membranes increases at term and regulates labour via numerous mechanism, including induction of IL-8, upregulation of CAPs, upregulation of a cassette of genes involved in immunity and inflammation and antagonism of the relaxant effect of P4 on the myometrium (*Allport et al., 2001; Allport et al., 2000; Chapman et al., 2004; Khanjani et al., 2011; Khanjani et al., 2012; Lim et al., 2012*). The pro-inflammatory role of NF-kB in the myometrium suggests that MSMCs are not only contractile but also immune cells, supporting the notion that the activation of myometrial contractile apparatus is an inflammation associated response. Although numerous studies have suggested ways in which endocrine and inflammatory mediators may drive labour, the exact sequence of these events remains poorly understood. The disruption of one or more of these processes may be associated with impaired parturition, which encompasses spontaneous PTB.

1.4.2 Hormones

There is robust evidence to suggest that labour is driven by activation of fetal hypothalamic-pituitary-adrenal axis (fHPA), whereby an increase in fetal cortisol increases the placental availability of specific enzymes that favour the conversion of C21 steroids (i.e. progesterone) to C18 steroids (estrogens) (*Smith et al., 2002*). In women, concentrations of both sex hormones increase in circulation throughout gestation with no notable change occurring at onset of labour (*Tulchinsky D, 1980*). This is in contrast to the significant drop in circulating P4 concentration, the so-called P4 withdrawal, observed shortly before the onset of labour in rodents and many other animals (*Mesiano and Welsh, 2007*). Although human labour is not preceded by a fall in P4, is it hypothesised that a

'functional P4 withdrawal' at this time enables a decrease of P4 activity and promotes initiation of labour *(Mesiano et al., 2002*). It has been proposed that a decrease in P4 activity may be achieved through a range of mechanisms, including antagonism of progesterone receptor (PR) from other molecules, expression of different isoforms of PR in gestational tissues depending on the gestational time, altered PR cofactor expression and local metabolism of P4 to estrogens (*Condon et al., 2003; Mesiano et al., 2002*). The importance of P4 signalling in parturition has been highlighted in reports where administration of the PR antagonist RU486 induced softening of the cervix and increased the sensitivity of uterus to uterotonic agents (*Hapangama and Neilson, 2009*). Other studies have revealed that the two isoforms of the PR, the PR-A and PR-B, are differentially expressed in various gestational tissues (*Pieber et al., 2001a; Pieber et al., 2001b*). In particular, there is evidence that the binding of P4 to the myometrial PR-B promotes uterine quiescence whereas an epigenetically-regulated switch in predominance of PR-A at term is thought to trigger the expression of CAPs and activate pathways that lead to contraction (*Chai et al., 2012; Conneely et al., 2001; Mesiano, 2004; Mesiano et al., 2002*). Therefore, a high PR-A/PR-B ratio in the myometrium is associated with the onset of labour. Studies in the placenta and fetal membranes have also demonstrated an increase in PR-A/PR-B ratio with the onset of labour (*Merlino et al., 2009; Merlino et al., 2007; Ziyan et al., 2010*). In contrast to P4, which favours uterine quiescence via PR-B-dependent signalling, estrogens are thought to promote uterine activation by binding to estrogen receptor alpha (ER-α) (*Mesiano et al., 2002*). As mentioned above, the concentration of estrogen in maternal circulation does not change with the onset of labour. However, an increase in ER-α expression, detected in the human myometrium at term, is hypothesised to result in 'functional estrogen activation'. Numerous studies, which investigated the role of estrogens in the myometrium at term, have shown that estrogen-dependent signalling drives the expression of CAPs, including oxytocin receptor (OXTR) and connexin 43 (con-43) and, therefore, plays a crucial role in the mechanism of labour onset (*Welsh et al., 2012*). In addition to the sex hormones, oxytocin (OXT) and prostaglandins are thought to play key roles in the regulation of parturition. OXT is a neurohypophysial hormone secreted by the posterior pituitary gland and is believed to be necessary for stimulation and maintenance of myometrial contractions. The binding of OXT to the OXTR, has an excitatory effect on myometrial smooth muscle (*Arrowsmith and Wray, 2014*). In human myometrium, the concentration of OXTR is low during most of the pregnancy but increases 12-fold at term (*Fuchs et al., 1984*). This increase is thought to enhance the sensitivity of myometrium to OXT (*Soloff et al., 1979*). Prostaglandins (PGs) are hormone-like lipid compounds derived from arachidonic acid and are well characterised for their roles as central mediators in regulation of myometrial smooth muscle excitation (*Arulkumaran et al., 2012*). Studies have demonstrated that the primary prostaglandin 2 (PGE2) is significantly increased

in amniotic fluid at term and further increased with labour (*Dray and Frydman, 1976; Olson et al., 1985*). PGE2 is synthesised in the fetal membranes from where it diffuses to the decidua, and reaches the myometrium to stimulates local myometrial PGs production, which act in an autocrine manner to stimulate myometrial contraction (*Kandola et al., 2014; Slater et al., 1999; Smith, 2007*). Another PG, Prostaglandin F2α (PGF2α) has been reported to favour the expression of PR-A in myometrium, consistent with the role of PR-A in induction of labour (*Madsen et al., 2004*).

1.4.3 Matrix metalloproteinases in the pathophysiology of parturition

At the end of gestation and at parturition the matrix metalloproteinases (MMPs) play an important role in myometrial extracellular matrix remodeling, in fetal membrane breakdown and in cervical ripening. Elevated expression of MMP components in the amniotic fluid is related to declines in membrane tensile strength, which leads to fetal membrane rupture (*Geng et al., 2016*). During human parturition the rupture of fetal membranes is associated with a significant increase in the concentration of MMP-9 in the amniotic fluid and a significant decrease in the concentrations of active TIMP-1 and TIMP-2 (*Athayde et al., 1999; Maymon et al., 2000*).

Accumulation of MMPs has also been observed directly in fetal membranes. In human amnion and choriodecidua, MMP-9 levels are high during labor (*Tsatas et al., 1999; Chai et al., 2013*). Similarly, increases in MMP-9 abundance in the rat amnion approximately 12 hr prior to delivery is believed to partially contribute to structural changes of the fetal membranes before parturition (*Lei et al., 1995*). MMP-2 and -9 activity is also reported to dramatically increase in human fetal membrane during labor and to be higher at preterm birth (*Xu et al., 2002; Yonemoto et al., 2006*). MMP-2 expression increases gradually during cervical repening in order to aid in collagen denaturation. The involvement of other MMPs (MMP-3, MMP-1, MMP-9, MMP-7 and MMP-8) in cervical ripening has also been reported (*Sennstrom et al., 2003; Rahkonen et al., 2010*). Cervical ripening has been likened to an inflammatory-like process. The associated expression of numerous proinflammatory cytokines, including IL-1, IL-8, and TNF-α, has been found to increase the expression of cervical MMPs that facilitate dilatation preceding normal and preterm delivery. In particular, MMP-1, MMP-3, MMP-8, and MMP-9 and TIMP-1 all are increased during cervical ripening and are critically important to ECM remodeling at this time (*Osmers et al., 1995*).

The probability of preterm birth is increased when there is an imbalance between the activities of MMPs and their inhibitors. In amniotic fluid, increased expression and activity of multiple MMPs related with reduced levels of TIMP-1 or TIMP-2 expression has been associated with PROM (*Curry*

et al., 2003). Several studies have shown that preterm labour is associated with elevated MMP-9 and MMP-3 in amniotic fluid and fetal membranes (*Maymon et al.,2000*; *Fortunato et al.,1999b*). Moreover, placental MMP-2 and MMP-3 levels are reportedly higher in women delivering preterm compared with term (*Sundrani et al., 2013*). Comparison of MMP and TIMP concentrations during term and preterm birth in maternal serum revealed that the MMP-9 concentration is higher in women with preterm birth. Furthermore, increases in the MMP-9-to-TIMP-1 and -to-TIMP-2 ratios were observed in women who delivered preterm compared to women with term labor. (*Tency et al., 2012*). Several data indicate that cervicovaginal and intrauterine infection is associated with PPROM and spontaneous preterm birth, likely due to the triggered inflammatory response (*Romero and Mazor, 1988*). Women with PPROM followed by microbial infection in the amniotic cavity have elevated concentrations of MMP-1, -8, and -9 and a lower concentration of MMP-2 in the amniotic fluid (*Athayde et al., 1999; Biggio et al., 2005*). Furthermore, the abundance of MMP-3 increases and TIMP-2 reduces in the host response to intrauterine infection, which may also contribute to parturition (*Maymon et al., 2001; Park et al., 2003*).

Successful cervical ripening and parturition at the end of pregnancy require the inhibition of progesterone to allow normal delivery. Progesterone, a physiological suppressor of MMPs, can down-regulate the transcription of MMP-9 and MMP-10. Cyclic stretch was recently shown to increase IL-8 and proMMP-1 production. When human uterine smooth muscle cells are pretreated with progesterone for 24 hr, stretch effects are reduced (*Zhao et al., 2013*). Therefore, uterine contraction induced by progesterone withdrawal may induce MMP production. Corticotropin-releasing hormone and urocortin also regulate MMP-9 protein secretion from cultured cells of human chorionic trophoblast, amnion epithelium, and syncytiotrophoblast, thus contributing to the control of human pregnancy and parturition (*Li and Challis, 2005*).

Prostaglandin, prolactin, and relaxin can also stimulate the expression of MMPs. Prostaglandin F2a increases MMP-1 secretion in cultured human uterine cervical fibroblasts (*Yoshida et al., 2002*). Increased prolactin concentrations together with the overexpression of other pro-inflammatory cytokines, causes a premature switch from a favorable T helper 2-type immune response, which is associated with normal parturition and delivery, to a noxious T helper 1-type immune response in the intrauterine environment, which is associated with PPROM (*Raghupathy et al., 2001*). This prolactin-dependent change in immune response induces a premature imbalance between the synthesis and degradation of collagen in the chorioamniotic membrane, likely by upregulating MMP-2 and -9. Finally, decidual and placental production of relaxin, whose overproduction is associated with PPROM has been linked to MMP synthesis (*Casanueva et al., 2005*) .

Taken together, these evidences suggest that ECM remodeling plays a crucial role in multiple biological events during parturition and that numerous cytokines and small molecules expressed by maternal and fetal tissues act in concert with steroids to mediate the balance between MMPs and their inhibitors during parturition. Alterations in MMP/TIMP ratio may lead to multiple pregnancy complications such as preterm birth.

1.5 Preterm Birth

Preterm birth (PTB) is defined as a delivery before 37 weeks of gestation, affects 5 to 18% of pregnancies (*Romero et al., 2014*) and is cause of mortality and morbidity in newborn infant: neonates born preterm are at an increased risk of short-term complications attributed to immaturity of multiple organ systems as well as neurodevelopmental disorders such as cerebral palsy, intellectual disabilities and vision and hearing impairments (*Mwaniki et al., 2012*).

The complex etiopathogenesis of PTB is still unclear but it may be considered a clinical syndrome arising from different pathological processes that activate prematurely one or more components of the mechanisms leading to parturition (*Romero et al., 2006*). Proposed obstetric disorders implicated in spontaneous preterm labor are represented by placental vascular diseases, inadequate decidualization with consequent premature decidual senescence, uterine overdistension associated with multiple gestation or polyhydramnios, disorders in maternal-fetal tolerance, premature decline in P4 action, maternal stress and cervical diseases (*Romero et al., 2014*).

However, over 60% of PTB are unexplained and are called "idiopathic" PTB (*Green et al., 2005*). Between proposed mechanism leading preterm delivery the intrauterine infection represent a leading cause; one of every four preterm infants is born to mothers with an intra-amniotic infection that is largely subclinical (*Romero et al., 2001*).

Placental infections are primarily caused by microorganisms, usually bacteria, that reach the uterus ascending from the vagina and the cervix or by hematogenous dissemination with transplacental passage (*Madianos et al., 2013*) and produce an acute inflammatory reaction in the fetal membranes (chorioamnionitis), umbilical cord (funisitis) and ultimately within the fetus (*Goldenberg et al., 2000*). Microorganisms and their products are sensed by pattern recognition receptors, such as toll-like receptors (TLRs), which induce the production of chemokines (C-C motif ligand 2 (CCL2), cytokines (IL-1β and TNF-α), PGs, and proteases leading to activation of the common pathway of parturition (*Agrawal and Hirsch, 2012; Elovitz et al., 2003; Romero et al., 2001*).

At the fetal-maternal interface, the inflammatory response is manifested primarily by neutrophils and secondarily by macrophages accumulation and activation. Accordingly, the amniotic fluid from patients with intra-amniotic infections contains elevated levels of leukocyte chemoattractants and activators including IL-8, chemokine (C-C motif) ligand 2 (CCL2), colony stimulating factors (CSF1 and CSF2), as well as elevated levels of the classic pro-inflammatory cytokines, TNF-α and IL-1β (*Saji et al., 2000*). The microenvironment resulting from the maternal response to infection is rich in uterotonic compounds and extracellular matrix-degrading enzymes, that are thought to contribute to preterm labor, preterm premature rupture of membranes (PPROM) and to the onset of PTB (*Arechavaleta-Velasco et al., 2002; Goldenberg et al., 2000; Van Meir et al., 1996).* Indeed, previous studies demonstrated an association between preterm labor and systemic activation of maternal granulocytes and monocytes (*Gervasi et al., 2001*). Epidemiological data also demonstrate an association between infiltration of the genital tract by maternal granulocytes and monocytes and an increased risk of PPROM, suggesting that leukocyte accumulation and activation play a key role in its pathogenesis (*Parry and Strauss, 1998*). Other studies demonstrated a strong association between PPROM and increased protease activity in the fetal membranes *(Draper et al.,1995; Vadillo-Ortega et al., 1990*). Activated neutrophils and macrophages are a well documented source of pro-inflammatory cytokines, elastase, collagenase and MMP-9 (*Witko-Sarsat et al.,2000, Parry and Strauss, 1998*). Furthermore, the increased release of arachidonic acid by activated neutrophils would result in enhanced availability of prostaglandins (*Di Persio et al.,1988*). These bioactive lipids together with pro-inflammatory cytokines such as IL1-β, target the myometrium to increase contractility. Thus, enhanced proteolytic degradation of the extracellular matrix of the decidua and fetal membranes is likely to synergize with increased contractility to provide a potent signal that promotes PPROM and PTD. Chronic chorioamnionitis and villitis of unknown etiology are also common placental lesion in late spontaneous preterm birth, both in cases with PPROM or preterm labor with intact membranes (PTL) and are characterized by maternal T cell infiltration of the chorioamnionitis with trophoblast apoptosis. Recently a link between chronic chorioamnionitis and maternal anti-fetal rejection was shown (*Kim et al., 2010*); maternal sensitization to fetal human leukocyte antigens is frequently found in patients with chronic chorioamnionitis and is accompanied by C4d deposition in umbilical vein endothelium (*Lee et al., 2011, Lee et al., 2013*).

2 AIMS

Since important roles for glucocorticoids and matrix metalloproteinases in the pathophysiology of pregnancy and parturition were observed over the past few years, the aims of the present work were:

- To evalutate the expression of GR transcripts and the effect of inflammatory cytokines on the control of GR transcripts expression in cultured first trimester human decidua cells

- To evaluate the expression of the two major groups of MMPs (stromelysins and gelatinases) and their inhibitors TIMP-1 and TIMP-2 in a) the mouse uterus throughout normal gestation, at labour and during inflammation-induced preterm birth and b) the human myometrium at term and preterm, both non-labouring and labouring

2.1 AIM 1

Expression of glucocorticoid receptor isoforms: regulation by IL-1β and TNF-α in first trimester human decidua cells

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Abstract

Background: Cytokines and glucocorticoids (GC) influence several aspects of embryo implantation process including maternal immune response, angiogenesis and trophoblast invasion. GC exert their actions via the glucocorticoid receptor, an ubiquitously expressed nuclear receptor comprised of 9 exons. Previous studies have demonstrated that differential promoter usage and alternative splicing-generated GR mRNA transcripts, results in differential cellular GC responses.

Aim: The aim of the present study was to evaluate the expression of GR transcripts and the effect of inflammatory cytokines on the control of GR transcripts expression in cultured first trimester decidual cells.

Methods: First trimester decidua specimens were obtained from uncomplicated, elective terminations of pregnancy between 6 and 12 gestational weeks. Isolated, purified first trimester decidual cells (FT-DC) were pre-treated for 8 days with 10-8 mol/L E2 + 10-7 mol/L MPA in order to mimic the hormonal milieu of pregnancy and then exposed for 5 and 24 hours to TNF-α and IL-1β at different concentrations (n=6). RNA was purified and relative mRNA levels of GR mRNA transcript were assessed by quantitative real-time RT-PCR. Protein expression of GR isoforms was assessed by Western Blotting.

Results: GR mRNA transcripts produced by alternative splicing were detected in first trimester decidua cells and included GRα, GRγ, GR P and GRβ. Among the five untranslated exon 1 regions, mRNAs for exon 1C and 1B were observed. The highest expressions was observed for GRα and GRβ. After treatment with different doses of IL-1β a significant increase of GRα ($p<0,01$), GRβ ($p<0,01$) and GR P($p<0,01$) mRNA levels was observed in FT-DC. Treatment with different doses of TNF-α enhanced mRNA levels of GRα ($p<0,01$), GR P,($p<0,01$), GRγ ($p<0,05$) and GRβ ($p<0,05$). An increase in GRβ and GRα-D protein levels was detected following a 24h-treatment with TNF-α and IL-1β.

Conclusion: The present study demonstrated that first trimester human decidua cells are characterized by a complex pattern of GR mRNA transcripts expression. The significant changes in GR mRNA and protein levels observed in IL-1β or TNF-α- treated FT-DCs suggest a role for inflammatory cytokines in regulating decidual GR isoforms abundance. The increase in GRβ, GRγ, GR P and GRα D expression may be correlated with the development of glucocorticoid resistance.

Keywords: decidua, glucocorticoid receptor, TNF-α, IL-1β, inflammation

Introduction

Implantation of the blastocyst is a critical steps leading to establishment of pregnancy and involves extensive cross-talk between the developing embryo and the receptive endometrium (*Granot et al.,2012*). Human endometrium becomes receptive during the mid-secretory phase of menstrual cycle and it undergoes extensive morphological and biochemical changes under the control of ovarian hormones in a process termed decidualization (*van Mourik et al.,2009*).

During this early embryo–uterine interaction, implantation involves various classes of molecules. Among these are cytokines (IL-1β,INF-γ,TNF-α) and glucocorticoids (GC) that influence several aspects of the embryo implantation process including the maternal immune response, embryo attachment and fetal development (*Makrigiannakis et al., 2011*; *Michael et al., 2008*).

GC are hormones of the steroid family, products of the adrenal gland synthesized in the zona fasciculata that exert their actions via their intracellular receptor, the glucocorticoid receptor (GR). In its inactive state, GR resides in the cytoplasm in a chaperone complex; upon binding to the ligand, GR dissociates from its chaperones, dimerizes and translocates into the nucleus, where it binds specifically to DNA sequences known as glucocorticoid-responsive elements (GREs) (*Vandevyver et al., 2012*). GR subsequently recruits transcriptional coactivators and chromatin remodeling factors, to initiate transcription in a process termed transactivation. Conversely GR can also interfere with important tissue factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1) and repress transcription of several inflammatory genes (*Oakley et al., 2013*).

The GR-encoding gene (NR3C1) is located on chromosome 5q31.3 and has nine exons. The untranslated exon 1 of the GR gene can be spliced into 9 different promoter variants that function in a tissue specific manner to regulate GR protein expression including GR-1A1, GR-1A2, GR-1A3, GR-1B, and GR-1C. Promoters 1B and 1C are ubiquitously expressed and are probably responsible for the basal GR expression. Promoters 1A, 1A1 and 1A2 are marginally expressed, 1A3 is mainly expressed in cells of the hematopoietic lineage (*Russcher et al., 2007; Breslin et al., 2011*).

Alternative splicing or alternative initiation of translation in exons 2 and 9 can generate different isoforms of GR resulting in the expression of GRα, GRβ, GRγ, GR-A and GR P proteins (*Hollenberg et al., 1985*). GRα is the functional isoform that mediates the actions of glucocorticoids. GRβ does not bind glucocorticoid agonists, resides constitutively in the nucleus of cells, and by itself is inactive on glucocorticoid responsive reporter genes (*Kino et al.,2009*; *Lewis-Tuffin and Cidlowski, 2006*).

However, when coexpressed with GRα, GRβ functions as a dominant negative inhibitor and antagonizes the activity of GRα suggesting that high levels of GRβ might lead to glucocorticoid resistance. GRβ is abundant in certain cell types, such as neutrophils and epithelial cells, and its expression is selectively increased by proinflammatory cytokines and other immune activators that lead to reduced glucocorticoid sensitivity (*Tliba et al., 2006*; *Webster et al., 2011*).

GRγ binds glucocorticoids and DNA in a manner similar to GRα. However, GRγ expression is associated with glucocorticoid resistance in patients with small cell lung carcinoma. GR-P and GR-A are found in many tissues and appear to be the predominant receptor variant expressed in glucocorticoid-resistant cancer cells (*Cain et al., 2015*). The presence of multiple isoforms of GR may confer sensitivity or resistance to glucocorticoids and this response is not limited to the well characterized GRα but a far more complex process of several GR isoforms being co-expressed and potentially interacting with one another.

Previous study identified additional GRα isoforms generated from translational mechanisms : GRα A (94 kDa) and GRα B (91 kDa), GRα C1e C3 (82 e 84 kDa) and GRα D1e D3 (53 e 56 kDa). All GRα isoforms are functional receptors because they contain the identical intact ligand-binding domain that can bind glucocorticoids. Upon ligand activation, the GRα isoforms are located in the nucleus. However, in the absence of ligand all GRα isoforms, except GRα-D isoforms, localize in the cytoplasm. Interestingly, the GRα-D isoforms are localized primarily in the nucleus in the presence or absence of glucocorticoids (*Lu et al., 2005*).

These GRα isoforms have distinct tissue distribution patterns. The pancreas and colon have the highest amounts of the GRα-C isoforms, whereas spleen and lung have the highest amounts of the GRα-D isoforms. Immature dendritic cells have predominantly the GRα-D isoforms, whereas mature dendritic cells have predominantly the GRα-A isoform. Each GRα subtype possesses a distinct gene regulatory profile that lead to functional differences in glucocorticoid-induced apoptosis. Cells expressing GRα-C3 were the most sensitive to the cell-killing effects of glucocorticoids whereas cells expressing GRα-D3 were the most resistant (*Gross et al., 2011*). Preferential increases in the expression of the GRα-C or GRα-D isoforms might lead to glucocorticoid hypersensitivity or hyposensitivity, respectively.

In the context of pregnancy, GR is expressed in first trimester decidua (*McDonald et al., 2011*) and term (*Sun et al., 1996*) decidua and GR has also been detected in term and first trimester placenta (*Speeg and Harrison, 1979*). GR mRNA transcripts produced by alternative splicing including GRβ, GR P and GR γ are all detectable in the human placenta with GRα and promoter 1C showing the

highest expression (*Johnson et al., 2008*; *Saif et al., 2014; Saif et al., 2015*). There are currently no data on the expression of the multiple GR splice variants in first trimester decidua and the mechanisms implicated in their regulation. In order to better understand the mechanisms of glucocorticoid action in decidua, in this present study, the expression of GR transcripts and the potential role of inflammatory cytokines in regulating decidual GR isoforms abundance in early pregnancy were evaluated in cultured first trimester decidual cells.

Material and Methods

Isolation and culture of first trimester decidual cells

Decidual specimens were obtained from elective terminations between 6 and 12 weeks of gestation (n=6). All patients gave a written informed consent and the study was approved by the local Human Investigation Committee. In order to evaluate the effect of IL-1β and TNF-α on GRα, GRβ, GR P, GR γ mRNA expression, decidual cells were cultured (n = 6). Tissues were minced and digested with 0.1% collagenase type IV, as well as 0.01% DNase in phenol free DMEM-F12 containing 1% of penicillin/streptomycin, 1% of L-glutamine 200 mM and 10% of FBS (SIGMA Aldrich) in a 37°C shaking water bath for 30 min. After washing with sterile phosphate-buffered saline (PBS), the digestate was washed three times and subjected to consecutive filtration through 100, 70 and 40 millipore filters, respectively. Cells were then resuspended in phenol free DMEM-F12 containing 1% of penicillin/streptomycin, 1% of L-glutamine 200 mM and 10% of FBS (SIGMA Aldrich) and seeded on polystyrene tissue culture dishes. Cells were analyzed by IHC analysis with anti-CD45 mouse Abs (BD Pharmingen, San Diego, CA) to monitor the presence of leukocytes. After 3–4 passages, cell cultures were found to be leukocytefree (<1%). Consistent with published results of stromal cells isolated from cycling endometrium, decidual cells isolated from first trimester were vimentin-positive and cytokeratin-negative. These cells display characteristic decidualization related morphological changes and increased secretion of prolactin in response to treatment with 10^{-8}M Estradiol (E2) plus 10^{-7} M Medroxiprogesteroneacetate (MPA) (SIGMA Aldrich) in phenol free DMEM-F12 containing 1% of penicillin/streptomycin, 1% of L-glutamine 200 mM and 10% of Charcoal FBS (SIGMA Aldrich); MPA was used in place of progesterone because of its greater stability in culture. After isolation decidual cells were grown to confluence in a standard 95% air:5% CO_2 incubator at 37°C.

For evaluation of mRNA of GRα, GRβ, GRγ and GR P by inflammatory cytokines, confluent decidual cells were incubated for 7 days in phenol free DMEM-F12 containing 1% of penicillin/streptomycin, 1% of L-glutamine 200 mM and 10% of Charcol FBS with 10^{-8} M E2 plus10^{-7} M MPA, in order to simulate classical hormonal milieu of pregnancy;. each treatment was performed in triplicate. After 7 days cells were washed two time with Hanks' balanced salt solution (HBSS) (SIGMA Aldrich) to remove residual serum elements and then treated for 5 and 24 hours with steroids alone or with 5ng/ml of IL-1β and TNF-α, or with 10 ng/ml of IL-1β and TNF-α (R&D System) in phenol free DMEM-F12 containing 1% of penicillin/streptomycin, 1% of L-glutamine 200 mM, 7.5% NaHCO3 ITS+ , 5µmol/L Fe SO4, 0.5 µmol/L ZnSO4. 1 nmol/L CuSO4, 20 nmol/L Na2SO3, trace elements,

50µg/ml ascorbic acid and 50 ng/ml epidermal grow factor (SIGMA Aldrich).

RNA extraction and complementary DNA preparation

Total RNA from purified cells was extracted with the SV Total RNA Isolation System (Promega), according to manufacturer's instructions (Promega, USA). RNA was quantified by an ND-1000 Nanodrop Spectrometer (Thermo Fisher Scientific, Wilmington, Delaware) and RNA integrity was checked prior to downstream analysis with the FlashGel System (Lonza Group, Ltd, Switzerland). Complementary DNA (cDNA), from one µg of total RNA was generated using the ImProm-II ReverseTranscriptase kit (Promega, USA).

Quantitative Real Time-PCR

The expression of target genes was analyzed by qRT-PCR. Quantification of GRα, GRβ, GRγ, GR P, GR-1C and GR-1B mRNA expression was performed in triplicate of each cDNA in 20 µL reactions using the 2X Sybr Select Master Mix for CFX (Applied Biosystem) according to the manufacturer's protocol, on a CFX Connect 96 (Bio-Rad Laboratories, Waltham, MA) with appropriate 300 nmol/L forward and reverse primers. Gene-specific primers chosen for amplification of GRα, GRβ, GRγ, GR P, GR-1A1, GR-1A2, GR-1A3, GR-1B, and GR-1C are summerized in *Table 1*.

Amplifications were carried out at 95°C for 5 min, followed by 10 s at 95°C and 30 s at 60°C for 40 cycles, with fluorescence detection at the end of each extension step. Melt curve analysis was also performed to confirm specific products and standard curves were performed to confirm efficient amplification of each gene before final analysis of all samples. Target genes relative mRNA expression levels were determined by comparison to the HPRT1 and plotted as ratio to HPRT1 expression values. Final results were expressed as fold differences in gene expression relative to the normalized calibrator, calculated by the DDCt method as follows: n-fold = $2^{\wedge\text{-(DCtsample - DCtcalibrator)}}$ where DCt values of the sample and calibrator were determined by subtracting the average threshold cycle (Ct) value of the transcript under investigation from the average Ct value of the HPRT1 gene for each sample.

TABLE 1. Primer sequences for the GR

Gene	Forward 5'-3'	Reverse 5'-3'
GR-1A1*	CCAAATCACTGGACCTTAGAAGTTG	GGAGTTAATGATTCTTTGGAGTCCAT
GR-1A2*	TCCAACGGAAGCACTGGG	CATCAGTGAATATCAACCTCTTTCTGTT
GR-1A3*	CATTAAAGTGTCTGAGAAGGAAGTTGAT	TACCAGGAGTTAATGATTCTTTGGAGT
GR-1B*	CCCGGGCCCAAATTGA	GGAGTTAATGATTCTTTGGAGTCCAT
GR-1C*	TTGTTTATCTCGGCTGCGG	CCATCAGTGAATATCAACTCTGGC
GR-α*	CTTGGATTCTATGCATGAAGTGGTT	TTGGAAGCAATAGTTAAGGAGATTTTC
GR-γ*	TTCAAAAGAGCAGTGGAAGGTAGA	TTTATCGATGATGCAATCATTCCT
GR-P*	TTACTGCTTCTCTCTTCAGGTTGGT	CATGCTTTTGACATAAGGTGAAAAG
GR-β	AAGAGCAGTGGAAGGACAGC	CCTGTAGTGGCCTGCTGAAT
HPRT	CGTGATTAGTGATGATGAACCAG	CGAGCAAGACGTTGAGTCCT

* From reference *18*

Western Blot

Cell pellets were lysed in a 20 mM Tris-HCl pH 8, 135 mM NaCl, 10% glycerol, 1% NP-40 and 10 mM EDTA buffer, together with protease inhibitors (Thermo Fisher Scientific) for 30 minutes on ice. After centrifugation for 10 minutes at 1000 rpm, the supernatant was assessed for total protein content using the Bradford method (Quantum Protein Kit; Euroclone, Milan, Italy).

Western blot was used to detect GR protein in first trimester decidua cells which were treated with TNF-α or IL-1β for 24h as stated or vehicle. Proteins were separated by SDS-PAGE and then transferred to nitrocellulose. Membranes were blocked for 1 hour at room temperature with 2,5% dry skimmed milk and 2,5% BSA in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20, and then probed overnight at 4°C with anti-glucocorticoid receptor rabbit polyclonal Antibody (Thermo Fisher Scientific) or anti-glyceraldehyde 3-phosphate dehydrogenase rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA), as a housekeeping protein. Immunoreactive bands were detected by 1 hour incubation with anti-rabbit IgG HRP-conjugated antibody (Cell Signalling Technology, Danvers, USA) followed by detection with ECL Western Blotting Detection Reagent

(GE Healthcare, Wauwatosa, USA) or SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

Statistical analysis

All data were assessed for normality of distribution using a computer program (Prism 4; Graphpad Software, La Jolla, CA, USA). Where the data were normally distributed, differences among three or more groups were analyzed by ANOVA with Dunnet's Multiple Comparision Test. Two groups were analyzed using a t-test. The statistical significance was achieved when $P < 0.05$. Data are expressed as mean ± SEM

Results

GR mRNA transcripts produced by alternative splicing were detected in purified first trimester decidua cells (FT-DCs) and included GRα, GRγ, GR P and GRβ. Among the five untranslated exon 1 regions, mRNAs for exon 1C and 1B were observed. GRα (1,8-fold increase) and GRβ (0,9-fold increase) were the most highly expressed isoforms. Decidua GRα mRNA levels was higher than those of GRβ (*figure 1*).

The effects of inflammatory cytokines IL-1β and TNF-α on the GR mRNA transcripts expression levels in FT-DCs, assessed 5 hours after treatment, are shown in *figure 2*. There was a significant increase of mRNA levels for GRα ($p<0,05$), GRβ ($p<0,01$), GRγ ($p<0,05$) and GR P ($p<0,05$) after treatment with IL-1β (5ng/ml) (*figure 2 A*) and of mRNA levels for GRα ($p<0,01$), GRγ ($p<0,01$), GR-P ($p<0,01$) and GRβ ($p<0,01$) after treatment with IL-1β (10 ng/ml) (*figure 2 B*) in FT-DCs. Treatment of FT-DCs with TNF-α 5ng/ml (*figure 2C*) enhanced mRNA levels expression of GRα ($p<0,05$), GRγ ($p<0,05$) and GR P ($p<0,01$) whereas treatment with TNF-α 10 ng/ml (*Figure 2D*) has shown a stimulation in mRNA levels expression for GRα ($p<0,01$), GRβ ($p<0,05$), GRγ ($p<0,05$) and GR P, ($p<0,01$).

To determine whether cytokines mediated-enhancement of GR mRNA splice variants is accompanied by protein changes, GR protein levels were misured by Western blot analysis in FT-DCs treated with IL-1β and TNF-α or PBS (control). GR antibody identified 3 specific bands in protein extracts from FT-DCs. Some of these MWs are equivalent to known isoforms including GRα (97 kDa), GRβ (91 kDa), GRα D1-3 (50-55 kDa). As shown in *figure 3*, we observed an evident increase in GRβ and GRα-D protein levels following a 24h treatment with TNF-α and IL-1β (10 ng/ml).

FIGURE 1

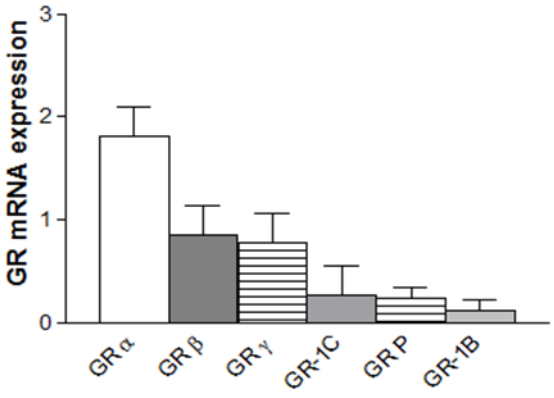

Figure 1. Expression of GR isoforms mRNA in FT-DCs. mRNA expression of GRα, GRβ, GRγ, GR P, GR-1C and GR-1B was quantified with RT-PCR. (n=6). Fold change (y axis) represents mRNA expression normalized to HPRT.

FIGURE 2

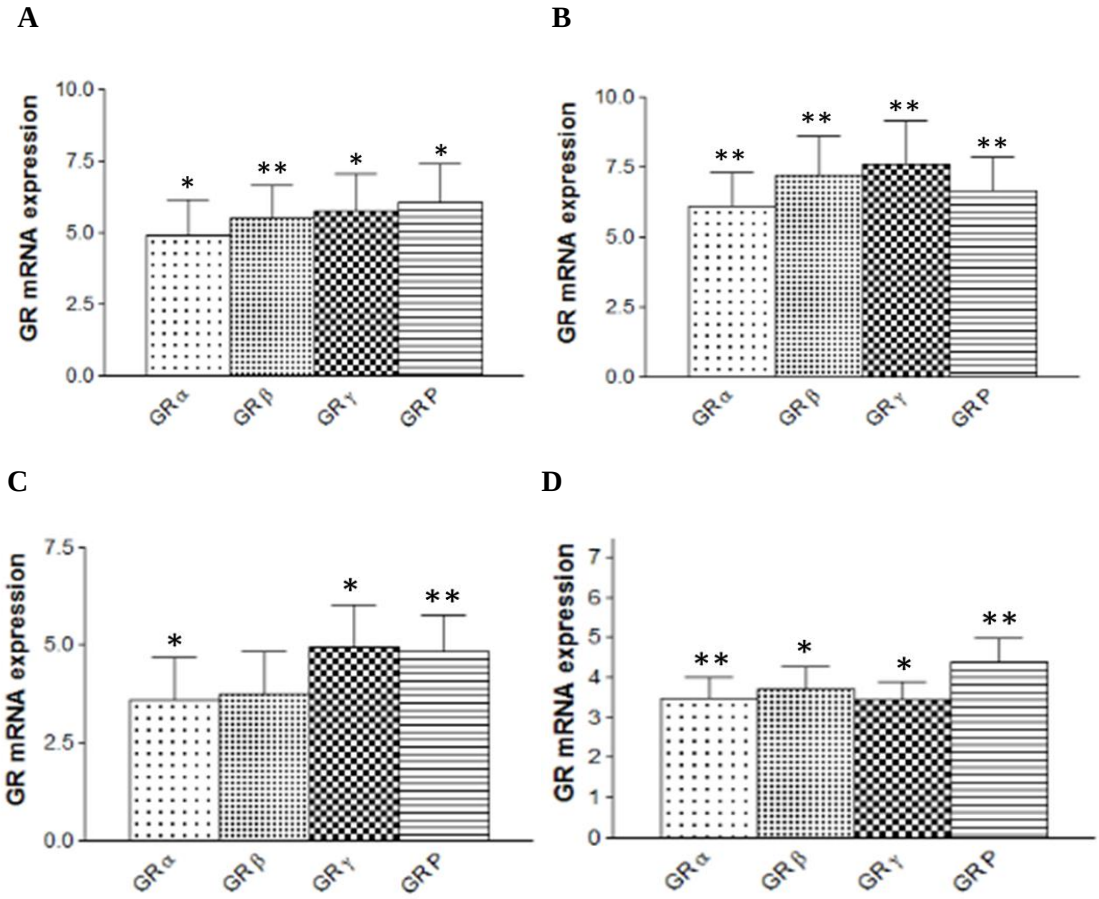

Figure 2. Expression of GR isoforms mRNA in FT-DCs after treatment with proinflammatory cytokines. The expression of GRα, GRβ, GRγ, GR P, GR-1C and GR-1B mRNAs were assessed 5 hours after treatment with 5 ng/ml and 10 ng/ml of IL-1β and TNF-α or vehicle alone. GRα, GRβ, GRγ, GR P, GR-1C and GR-1B mRNAs were significantly upregulated after treatment with IL-1β 5 ng/ml (A), IL-1β 10 ng/ml (B), TNF-α 5 ng/ml (C) and TNF-α 10 ng/ml compared to the control. Fold change (y axis) represents mRNA expression normalized to HPRT. Data were analysed using one-way ANOVA with Dunnet's Multiple Comparision Test . (n=6) (*p< 0,05; **p< 0,01).

FIGURE 3

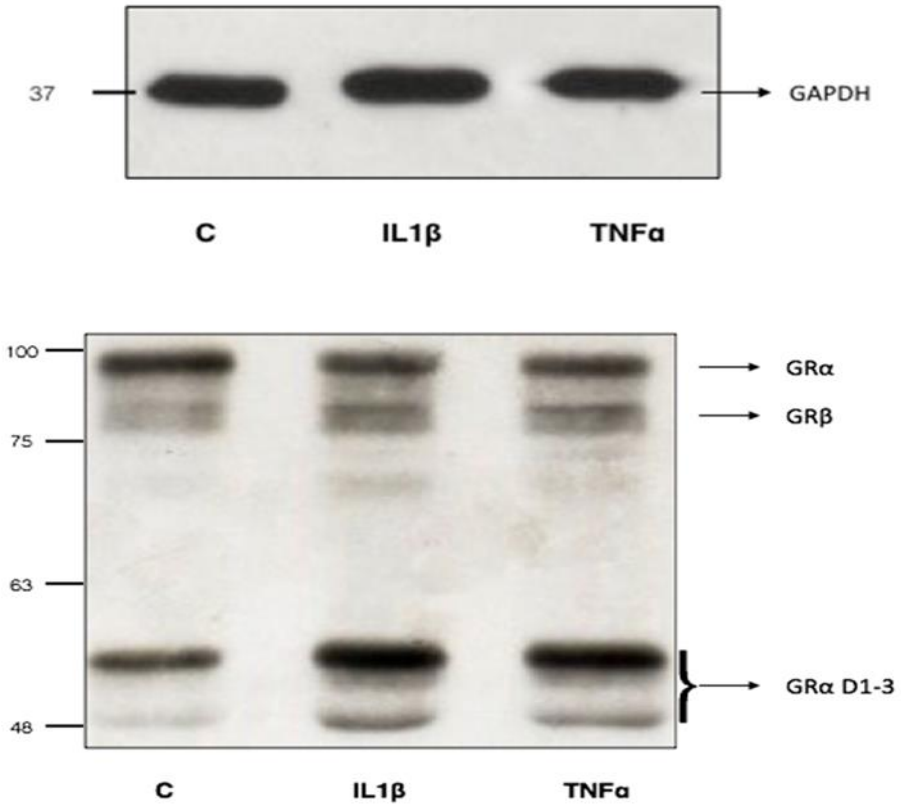

Figure 3. Representative Western blot of expression of GR isoforms in FT-DCs after treatment with proinflammatory cytokines. FTDCs were treated with vehicle alone (CTR), IL-1β and TNF-α for 24h and protein extracts were exposed to GR total antibodies. Protein bands were detected ranging from 49 to 97kDa. Blots were probed with GAPDH as a loading control.

Discussion

Implantation is an inflammatory process that depends on local release of proinflammatory cytokines and prostaglandins. Early pregnancy dysregulations are reflected in reduced blastocyst implantation and altered stromal cell decidualization, including aberrant apoptosis and altered gene expression (*Whirledge et al., 2015*). Glucocorticoids (GC) can modulate cytokine actions inhibiting the activator protein (AP)-1 and nuclear factor (NF)-kB signaling pathway that typically mediate the cellular responses to pro-inflammatory cytokines (*Barnes, 1998; Adcock and Caramori, 2001; Hayashi et al., 2004*). Previous studies have demonstrated that impaired uterine glucocorticoid receptor (GR) signaling leads to an exaggerated inflammatory response in decidualization and implantation, including altered immune cell recruitment (*Whirledge et al., 2015*).

The extensive distribution of GR across the feto-maternal interface suggests the engagement of glucocorticoid actions on both maternal and fetal sides. Glucocorticoids can exert a range of positive effects to promote early pregnancy, such as suppression of uterine NK cells and stimulation of hCG secretion, as well as promotion of trophoblast proliferation and invasion (*Michael and Papageorghiou, 2008*). Furthermore, glucocorticoids have also been shown to be involved in the activation of decidual matrix degrading proteases such as collagenase and plasminogen activator to facilitate the implantation of the embryo (*Tomlinson et al.,2004*). Despite the aforementioned beneficial effects of GC, other studies have shown that prolonged exposure to high levels is detrimental to both placental and fetal development (*Gennari-Moser et al., 2011*). Compelling evidence indicate that a tight control of GC levels at the fetal-maternal interface is essential for establishment and maintenance of a healthy pregnancy (*Michael and Papageorghiou, 2008; Singh et al.,2012*).

GRs are highly expressed in endometrial stromal fibroblast and endothelial cells (*Bamberger et al., 2001*), in first trimester (*McDonald et al., 2006*) and term (*Sun et al., 1996*) decidual stromal cells and in isolated population of uterine natural killer (uNK) cells (*McDonald et al., 2006*), as well as in term and first trimester placenta (*Speeg and Harrison, 1979*). GR is a nuclear receptor comprised of nine exons. Alternative splicing or alternative initiation of translation in specific exon can generate multiple GR isoforms (*Oakley et al., 2013*). Previous works (*Lu and Cidlowski, 2005*) demonstrated that the different GR isoforms expression varies between cell types and this may mediate the differential regulatory patterns of glucocorticoids.

Several isoforms of GR have previously identified in term human placenta and placental trophoblast (*Johnson et al., 2008*; *Zaif et al., 2014*). Our study represents the first report evaluating GR transcript

expression in first trimester decidual cells. We have demonstrated that there are six isoforms of the glucocorticoid receptor present in cultured first trimester decidual cells. Of the mature mRNA variants, GRα GRβ, GR P and GRγ, are all detectable in first trimester decidual cells with GRα mRNA represented at the highest level. Of the untranslated exon 1 GR mRNA splice variants, mRNA transcripts 1A1, A2, A3, 1B, and 1C were examined since previous studies suggest that these exon 1 mRNA transcripts may play a role in responsiveness of cells and tissues to GCs (*Breslin et al., 2001*). Our data indicate that the two alternative promoters 1B and 1C are expressed in first trimester decidual cells with promoter 1C being the most represented. These results further extend those obtained in the human placenta (*Johnson et al., 2008; Zaif et al., 2014*), and contribute to delineate a complete picture of GR transcript expression at the fetal-maternal interface.

A major goal of the present study was to examine changes in GR mRNA transcript expression after treatment of cultured first trimester decidual cells with inflammatory cytokines. The results highlighted a significant increase in GRα, GRβ, GR P and GRγ mRNA levels and an increased protein level expression of GRβ and GRα D in response to IL-1β and TNF-α.

Several studies in hematological malignancies, solid tumors and rheumatoid arthritis suggest that GRγ and GR P are associated with glucocorticoid resistance (*de Lange et al.,2001*; *Oakley et al., 2013*). Moreover, GRα-D translational isoforms are related to an hyposensitive response to glucocorticoids (*Oakley et al., 2013*). In other experimental systems, exposure of cells to proinflammatory cytokines and other immune activators selectively increased the expression of GRβ, leading to glucocorticoid resistance (*Lewis-Tuffin and Cidlowski, 2006*). Thus, the cytokine-dependent increase in resistance-related GR isoforms expression observed in FT-DCs suggests the existence of a previously unrevealed link between peri-implantation inflammatory reaction and decreased decidual response to GC.

Tissue levels of active endogenous glucocorticoids depend on the expression of 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes. Although the 11β-HSD1 isoform is a bidirectional enzyme, it predominantly catalyzes the conversion of inert cortisone to active cortisol, thus increasing tissue levels of active glucocorticoids (*Courtney et al., 2008*). The 11β-HSD2 acts as a dehydrogenase, converting cortisone to cortisol (*Ferrari et al., 1996*). Both 11β-HSD isozymes are present in gestational tissues in the early pregnancy and at term, and their expression is considered crucial for maintaining a proper GC balance at the fetal-maternal interface. Elevated levels of 11β-HSDs are present in early placental tissues homogenates (*Muneyyirci-Delale et al., 1996*) with 11β-HSD2 being the predominant isoform expressed in first trimester trophoblasts *(Arcuri et al., 1998*). Increased 11β-HSD expression during in vitro decidualization of human endometrial stromal cells (HESC) has also

been described (*Arcuri et al., 1996*). Moreover, our recent work indicated the predominance of 11β-HSD1 over 11β-HSD2, and an up-regulation of 11β-HSD1 expression by IL-1β, in cultured first trimester decidual cells (*Funghi et al., 2016*). These latter results are considered as a novel mechanism by which inflammatory mediators can affect implantation by increasing the availability of biologically active GCs (*Funghi et al.,2016*).

As previously mentioned, excess GCs has been shown to lead to complications of pregnancy, including IUGR, pre-term labour, chorioamnionitis and pre-eclampsia suggesting that the tight control of GC levels at the fetal-maternal interface is necessary for establishment and maintenance of a healthy pregnancy (*Michael and Papageorghiou, 2008*). More specifically, in decidua cells, GC repress the expression of major components of the embryo-endometrial interactome (*Kuroda et al., 2013*) and induce programmed cells death (*Tian et al., 2009*). Thus, it can be hypothesized that, in the inflammatory milieu of early pregnancy, increased expression of GC resistance-related GR isoforms could represent a novel mechanism to protect decidua cells against the GC overexposure driven by proinflammatory cytokine-dependent 11β-HSD1 induction.

In summary, the results of the present study indicate that 1) first trimester decidua cells are characterized by a complex pattern of GR mRNA transcripts expression; 2) the significant changes in GR mRNA and protein levels observed in IL-1β or TNF-α- treated FT-DCs suggest a role for inflammatory cytokines in regulating decidual GR isoforms abundance; 3) the increase in GRβ, GRγ, GR P and GRα D expression may be correlated with the development of glucocorticoid resistance. Future experiments will need to determine and verify the precise role GR isoforms in altering GC responsiveness and downstream signaling pathways activation in decidua cells.

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2.2 AIM 2

Expression of matrix metalloproteinases in the mouse uterus and human myometrium during pregnancy, labor and preterm labor

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Abstract

Background: Uterine extracellular matrix (ECM) remodeling occurs throughout pregnancy and at parturition. Inappropriate expression and activity of key mediators in ECM degradation, namely matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), is implicated in the pathogenesis of preterm labor.

Objectives: The purpose of this study was to characterize the expression of the two major groups of MMPs (stromelysins and gelatinases) and their inhibitors TIMP-1 and TIMP-2 in a) the mouse uterus throughout normal gestation, at labour and during inflammation-induced preterm birth and b) the human myometrium at term and preterm, both non-labouring and labouring.

Methods: The expression levels of Mmp-2, Mmp-9, Mmp-3, Mmp-10, Timp-1 and Timp-2 were determined in the uterus of C57BL/6 mice (n=6/group) during pregnancy (d5, d8, d12, d15, d17, d18), at normal labour and in the mouse uterus with lipopolysaccharide (LPS)-induced preterm labour or with phosphate-buffered saline (PBS) as control (n=6/group). The expression levels of MMP-10 and TIMP-1 were determined in human term myometrium prior to the onset of labour (TNL, n=7) or during active labour (TL, n=8) and the preterm myometrium prior to the onset of labour (PTNL, n=7) or during active labour (PTL, n=8). Gene expression and tissue localization were assessed by quantitative RT-PCR and immunohistochemistry.

Results: *Mmp-10* was significantly induced during murine labour (53-fold increase) in contrast to the majority of Mmps, which followed a decreasing pattern of expression from early gestation reaching a nadir at labour. *Mmp-3* ($p<0,001$) and *Timp-1* ($p<0,001$) mRNA significantly increased in the preterm mouse uterus. Mmp-3, Mmp-10 and Timp-1 were expressed in the uterine epithelium and stroma/myometrium. In the human myometrium, *TIMP-1* mRNA was higher in TL compared to TNL ($p<0,001$), PTL ($p<0,05$) and PTNL ($p<0,01$) but *MMP-10* was significantly lower at TL compared to TNL and PTL, PTNL. MMP-10 and TIMP-1 were localized to the myometrial smooth muscle cells, interstitial fibroblasts and inflammatory cells around the myometrial vasculature.

Conclusions: MMPs are plausible facilitators of physiological and inflammation-induced preterm labour. These data suggests key functions for MMP-3, TIMP-1 and MMP-10 in the uterine ECM remodeling during physiological and pathological parturition.

Keywords: metalloproteinase, TIMP, mouse, uterus, human, myometrium, labour, pregnancy

Introduction

Preterm birth (PTB), delivery before 37 completed weeks of gestation, is recognized as the leading cause of neonatal morbidity and mortality worldwide (*McCormick et al., 1985*). PTB is as a multifactorial syndrome and it is now well established that approximately 75% of PTBs result from premature onset of labor and only 25% of cases have an iatrogenic etiology (*Goldenberg et al., 2008*). During physiological pregnancy, the uterus expands to accommodate the fetus, amniotic fluid and placenta. At parturition, the myometrial extracellular matrix (ECM) re-organizes to enable contraction and undergoes postpartum involution to return to its pre-pregnancy state. Postpartum involution is characterized by rapid degradation of collagen bundles that were gradually assembled during pregnancy (*Monga and Sanborn, 1995*; *Manase et al., 2006*). The degradation and remodeling of ECM is regulated by matrix metalloproteinases (MMPs). MMPs belong to a large family of proteolytic zinc-dependent enzymes which are usually synthesized as proenzymes and distinguished by their structure and substrate specificities into collagenases (MMP-1, 8 and 13), gelatinases (MMP-2 and 9), stromelysins (MMP-3, 10 and 11), matrilysins (MMP-7), metalloelastase (MMP-12) and membrane type-MMPs.

Intrauterine infection (IUI), which accounts for 30% of all premature labour (*Goldenberg et al., 2008*), leads to the production of MMPs through inflammatory mediators. Activation of the MMPs leads to ECM degradation and consequently membrane rupture and cervix ripening (*Cockle et al.,2007*; *Goldenberg et al., 2000*). A number of studies have shown that preterm labour is associated with elevated MMP-9 and MMP-3 in amniotic fluid and fetal membranes (*Maymon et al.,2000*; *Fortunato et al.,1999b*). Moreover, placental MMP-2 and MMP-3 levels are reportedly higher in women delivering preterm compared with term (*Sundrani et al., 2013*).

MMPs are inhibited by specific endogenous tissue inhibitors of metalloproteinases (TIMPs), which comprise a family of four protease inhibitors: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. MMPs and TIMPs are secreted by a variety of connective tissues and pro-inflammatory cells, including fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils and lymphocytes (*Visse and Nagase, 2003*). Parturition is associated with an increase in pro-inflammatory cytokine- and chemokine- initiated responses. For example, leukocytes infiltrate the pregnant uterus utilizing MMPs and release cytokines and chemokines to produce an inflammatory environment and initiate labor (*Geng et al.,2016*; *Gomez-Lopez et al.,2014*).

Previous studies have evaluated the expression of uterine MMPs and TIMPs throughout pregnancy and during parturition in different species. An induction of Mmp-2, Mmp-9, Timp-1 and Timp-*2* in the rat uterus has been demonstrated at early pregnancy (*Zhao et al., 2002*). On the other hand, MMP-

9 and MMP-3 expression was significantly higher in the human myometrium during active labour at term (*O' Brien et al., 2007*; *Ror et al., 2000*). In the rat myometrium, the availability of Mmp-2, Mmp-9, Mmp3 and Mmp-10 is low throughout gestation, in keeping with the very high concentrations and stable expression of Timp-1, Timp-3, Timp-4 throughout gestation (*Nguyen et al., 2016*).

We hypothesised that an imbalance in the MMPs/TIMPs activity ratio in the uterine microenvironment may result in aberrant degradation of ECM and account for premature onset of labor.

The objective of the present study was to evaluate the expression of MMP-2, MMP-9, MMP-3, MMP-10, TIMP-1 and TIMP-2 in the mouse uterus during different stages of pregnancy and at labour. In addition we have investigated the expression of MMPs and TIMPs in a mouse model of inflammation-induced preterm labour to better understand the role of uterine inflammatory cascades in the regulation of MMPs action.

No data have been reported on the expression of MMP-10 in human myometrium, during pregnancy, labour and preterm labour therefore we further characterized the localization and expression of MMP-10 and TIMP-1 in the human myometrium at term and preterm, both non-labouring and labouring.

Material and Methods

Human Myometrium

A biopsy of myometrium was obtained from the upper margin of the lower segment of myometrium of women undergoing elective or emergency caesarean section at the Simpson's Centre for Reproductive Health at the Royal Infirmary of Edinburgh, following informed written consent. The ethical approval for recruitment of all pregnant women was granted by the West of Scotland Research Ethics Committee. All biopsies were collected from lean women (BMI>19 -< 25) delivering at term (>37 weeks of gestation) prior to the onset of labour (TNL, n=7) or during active labour (TL, n=8) and preterm (<37 weeks of gestation) prior to the onset of labour (PTNL, n=7) or during active labour (PTL, n=8). The indication for elective caesarean section in TNL women was breech presentation or previous caesarean section. In TL deliveries, emergency caesarean sections were performed due to fetal distress and failure to progress. The biopsies were collected in 10% NFB for histology processing or they were snap-frozen immediately after collection for RNA and protein extraction.

Mouse uterus

Uterus was obtained from C57BL/6 female 6-8 weeks old mice purchased from Charles River Laboratories. All animal care and experimental protocols were performed according to United Kingdom Home Office guidelines under the project license 60/4241. Mice were time-mated with plug day considered to be day 1. Mice were sacrificed and uterus was collected on gestational day 5 (d5), day 8 (d8), day 12 (d12), day 15 (d15), day 17 (d17), day 18 (d18) and during active labour (LB) usually on day 19/20 of gestation (n=6/group).

Mouse model of preterm birth

As previously described (*Rinaldi et al., 2015*) on D17 of gestation, mice were anesthetized with isoflurane (5% for induction, 1.5% for maintenance) in oxygen and were positioned supine on the ultrasound stage and 20 μg of lipopolysaccharide (LPS) (from E. coli 0111:B4; n=7) or phosphate-buffered saline (PBS) (n = 6), each in a volume of 25 μL, was injected directly into the intrauterine space between two gestational sacs under ultrasound guidance.

Immunohistochemistry

Tissue sections were dewaxed in xylene and rehydrated in ethanol. Slides were blocked with 3% hydrogen peroxidase and 5% normal goat serum (NGS) and incubated overnight at 4°C with primary

antibodies (detailed in *Table 1*). In negative controls primary antibody was absent. Following incubation with secondary antibodies (detailed in *Table 1*) positive staining was detected with 3,3'-diaminobenzidine (DAB, Vector, UK) and sections were mounted. Images were obtained with a PROVIS microscope (Olympus Optical, Hamburg, Germany) and AxioVision Rel software version 4.8 (Zeiss, Cambridge, UK).

qRT-PCR

Total RNA was extracted from mouse uterus and human myometrium collected using the RNeasy mini kit (Qiagen, Crawley, U.K.) as per the manufacturer's guidelines. Total RNA (300 ng) was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Life Technologies). qRT-PCR was carried out to quantify the mRNA expression of specific genes of interest. Predesigned gene expression assays from Applied Biosystems were used to examine the expression of Mmp-2 (Mm00439498_m1), Mmp-9 (Mm00442991_m1), Mmp-3 (Mm00440295_m1), Mmp-10 (Mm01168399_m1), Timp-1 (Mm01341361_m1), Timp-2 (Mm00441825_m1), Actb (Mm00607939_s1), MMP-10 (Hs00233987_m1), TIMP-1 (Hs00171558_m1) and ACTB (Hs99999903_m1). Target gene expression was normalized for RNA loading using Actb, and the relative expression in each sample was calculated relative to a calibrator sample (untreated day 5 uterus), which was included in all reactions, using the $2^{-\Delta\Delta Ct}$ threshold cycle method of analysis. All qRT-PCR analysis was performed on an Applied Biosystems 7900HT instrument (Applied Biosystems).

Western blotting

Western immunoblotting was used to quantify and compare the concentration of total Mmp-3 protein from uterine tissues collected 6h and 12h hours after treatment from mice administered LPS (n=6) or PBS (n=6) on D17 of gestation. Briefly, 20 µg of reduced protein was separated on 4-12% Bis-Tris precast NuPAGE gels at 180 V using the XCell SureLock™ Mini-Cell Electrophoresis System (Thermo Scientific, UK). The protein was transferred onto an Immobilon-FL PVDF membrane (Milipore, UK) pre-activated with 10% methanol, using a Mini-PROTEAN II electrophoresis cell system (Biorad) at 100 V. The membrane was blocked with Odyssey Blocking Buffer (OBB), incubated overnight at 4 °C with primary antibodies (detailed in *Table 1*) diluted in 5% milk /TBST, secondary antibodies (IRDye, Licor) and scanned using the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences).

Table 1. Antibodies used for Immunohistochemistry and Western Blotting

Antibody	Supplier
Primary: Monoclonal Rabbit Anti-MMP-3	Abcam, Cambridge,UK
Primary: Polyclonal Rabbit Anti-MMP-10	Abcam, Cambridge,UK
Primary: Polyclonal Rabbit Anti-TIMP-1	Santa Cruz Biotechnology, USA
Primary: Monoclonal Mouse anti-α-Tubulin	Sigma-Aldrich
Secondary: ImmPRESS anti-rat IgG reagent	Vector Laboratories, Peterborough, UK

Statistical analysis

Data are expressed as mean ± SEM. Normally distributed data were then analyzed by one-way ANOVA followed by Turkey multiple comparison when more than two groups were present or unpaired t tests when only comparing between two groups. All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA). A p value < 0.05 was considered statistically significant.

Results

Mmps and Timps mRNA expression in the pregnant and labouring mouse uterus

The expression of 4 MMPs and 2 TIMPs was evaluated in the pregnant and labouring mouse uterus. Mmp-2, Mmp-3 and Mmp-9 mRNA level concentration was higher in early gestation (d5-d8) compared to late gestation and labour (*figure 1A, B,C*). Specifically, the concentration of Mmp-3 mRNA was 1,5 fold higher on d8 compared to labour ($p<0,05$, *figure 1C*) and the concentration of Mmp-2 (*figure 1A*) and Mmp-9 (*figure 1B*) mRNA was significantly higher on d5 ($p<0,001$) compared to labour. On the contrary, Mmp-10 mRNA concentration was 53-fold higher ($p<0,001$) during labour compared to d18 and d8 *(figure 1D*). Interestingly, the selective inhibitor of Mmp-9, Mmp-3, Mmp-10, Timp-1 was 2,9-fold higher on d8 compared to day of labour ($p<0,001$, *figure 1E*) whereas Timp-2 mRNA concentration remained relatively stable throughout gestation (*figure 1F*).

The localization of Mmp-3, Mmp-10 and Timp-1 proteins was assessed in the pregnant mouse uterus from early gestation to labour by immunohistochemistry (*figure 2*). All Mmps and Timp-1 were expressed in the uterine epithelium and stroma/myometrium. The immunostaining for Mmp-3 was stronger at mid-gestation on d15 (*Figure 2A ii*) compared to labour (LB) (*figure 2A iv*) and, as expected, a strong Mmp-10 staining was evident on day of labour (*figure 2B iv*). Consistent with the low mRNA concentration, Mmp-10 protein was absent in the uterus on d8 (*figure 2B i*) and Timp-1 immunostaining was stronger on d8 compared to late gestation (*figure 2C iii*) and labour (*figure 2C iv*).

MMPs and TIMPs expression in a mouse model of inflammation-induced preterm birth

We assessed the expression of Mmp-2, Mmp-9, Mmp-3, Mmp-10, Timp-1 and Timp-2 in the uterus of mice that received LPS intrauterine injection on day 17 of pregnancy to induce preterm birth. The uterus was collected after 6 hours and the expression of Mmps and Timps was compared between the LPS and the control (PBS) groups.

The mRNA concentration of Mmp-3 (*figure 3C*) and Timp-1 (*figure 3E*) was 11-fold and 6-fold respectively increased after LPS treatment ($p<0,001$) but no differences at the protein level were observed (data not shown). In addition, a 0.7 fold decrease in the expression of Timp-2 ($p<0,05$) mRNA occured after treatment with LPS (*figure 3F*). There was no significant difference between LPS and control treated groups in the mRNA expression of Mmp-2, Mmp-9 and Mmp-10 (*figure 3A, B, D*). Mmp-3, Mmp-10 and Timp-1 proteins were detected with immunohistochemistry in the mouse uterus and localised to both the endometrium and myometrium. Mmp-3 staining was stronger in the

LPS group (*figure 4A ii*) compared to the control (*figure 4A i*) but Mmp-10 (*figure 4B*) and Timp-1 *(figure 4C*) did not displayed display visible differences between the two groups.

MMP-10 and TIMP-1 expression in human myometrium during pregnancy, labour and preterm labour.

The expression and localization of MMP-10 and TIMP-1 were investigated in the human myometrium at term and preterm pregnancy, both non-labouring and labouring.

MMP-10 mRNA was in 2,3 fold higher concentration in the TNL ($p<0,05$) group compared to TL group and in low abundance in the PTL and PTNL groups (*figure 5A*). TIMP-1 mRNA was 3,2 fold higher in TL compared to TNL ($p<0,001$) and 2,8 fold lower in PTL compared to TL ($p<0,05$) *(figure 5B*). MMP-10 (*figure 6A*) and TIMP-1 (*figure 6B*) were localized to the myometrial smooth muscle cells, interstitial fibroblasts and inflammatory cells around the myometrial vasculature. The intensity of immunostaining for MMP-10 and TIMP-1 was stronger in TL (*figure 6A, B ii*) compared to TNL (*figure 6A,B iii*) and PTL (*figure 6A,B iv*).

FIGURE 1

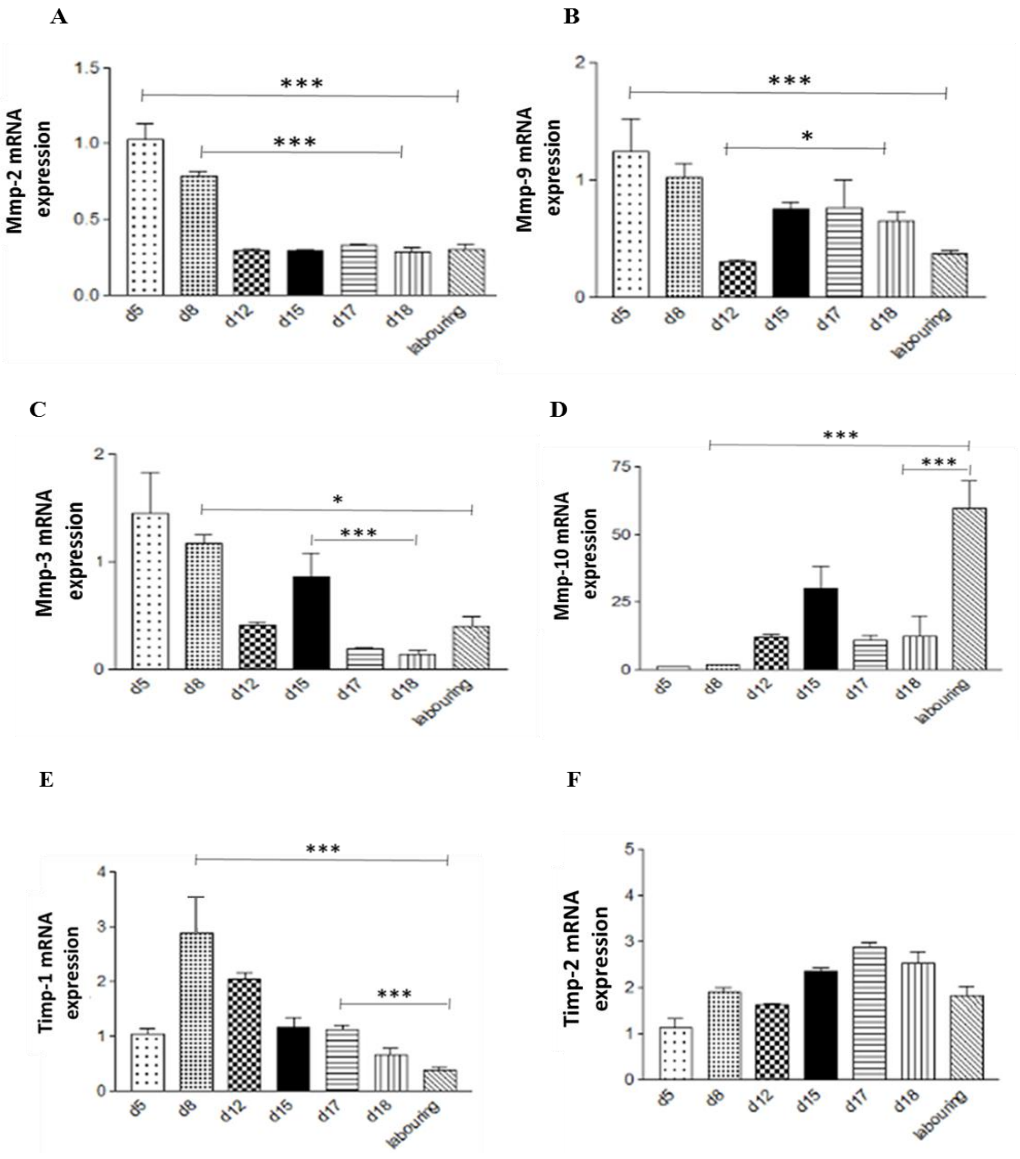
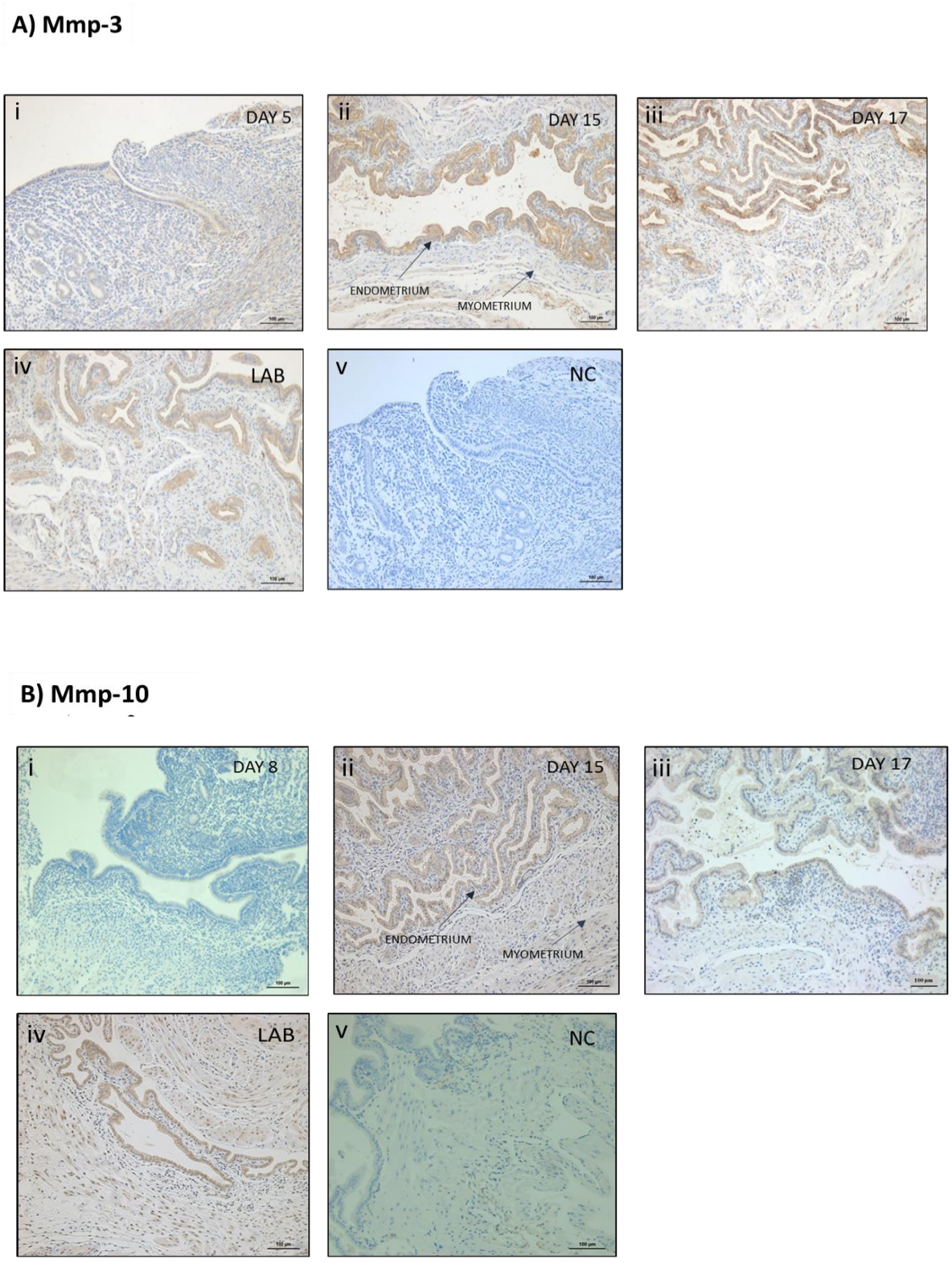


Figure 1. Expression of MMPs and TIMPs mRNA in pregnant and laboring mouse uterus. Mice uterus were collected at day 5, 8, 12, 15, 17, 18 and labour. mRNA expression of matrix metalloproteinases Mmp-2 (A), Mmp-9 (B), Mmp-3 (C), Mmp-10 (D), Timp-1 (E), Timp-2 (F) was quantified with qRT-PCR. Data were analysed using one-way ANOVA with Tukey's post-hoc test. (n=6/group) (*$p< 0.05$; ***$p< 0.001$).

FIGURE 2


A) Mmp-3
i
DAY 5
ii
DAY 15
ENDOMETRIUM
MYOMETRIUM
iii
DAY 17
iv
LAB
v
NC
B) Mmp-10
i
DAY 8
ii
DAY 15
ENDOMETRIUM
MYOMETRIUM
iii
DAY 17
iv
LAB
v
NC

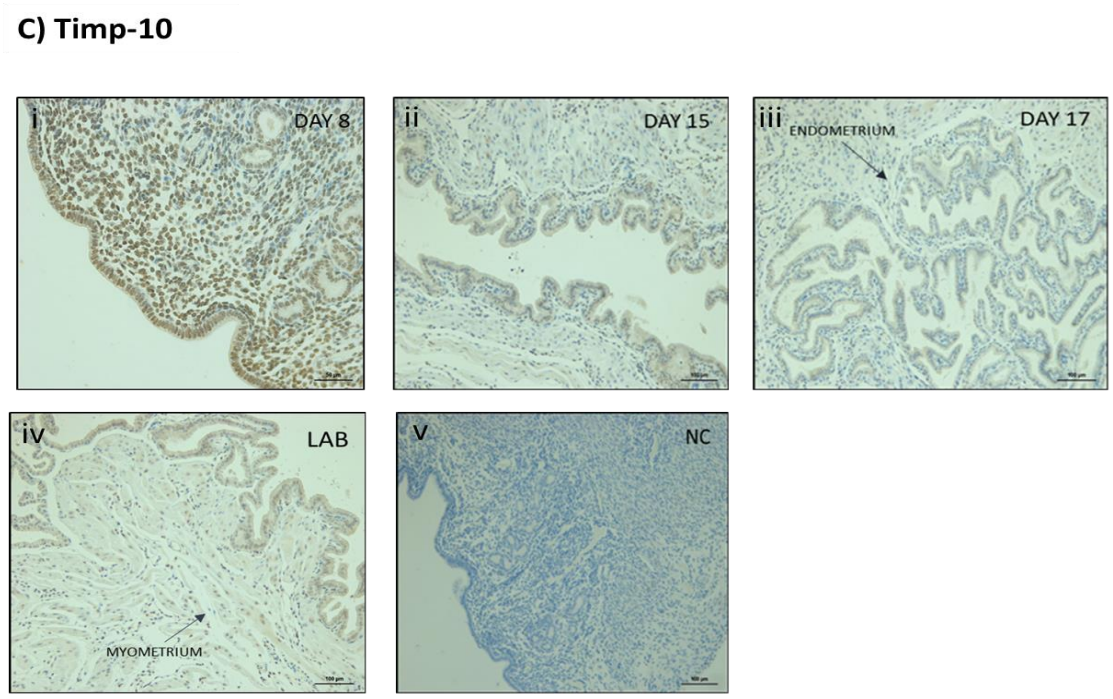


Figure 2. Localization of Mmp-3, Mmp-10 and Timp-1 during pregnancy and at labour in the mouse uterus. Mmp-3 (A), Mmp-10 (B) and Timp-1 (C) were detected in both endometrium and myometrium in different stages of pregnancy and labour. (i) day 5; (ii) day 15; (iii) day 17; (iv) labour; (v) negative control. (n=4) Original magnification, X10.

FIGURE 3

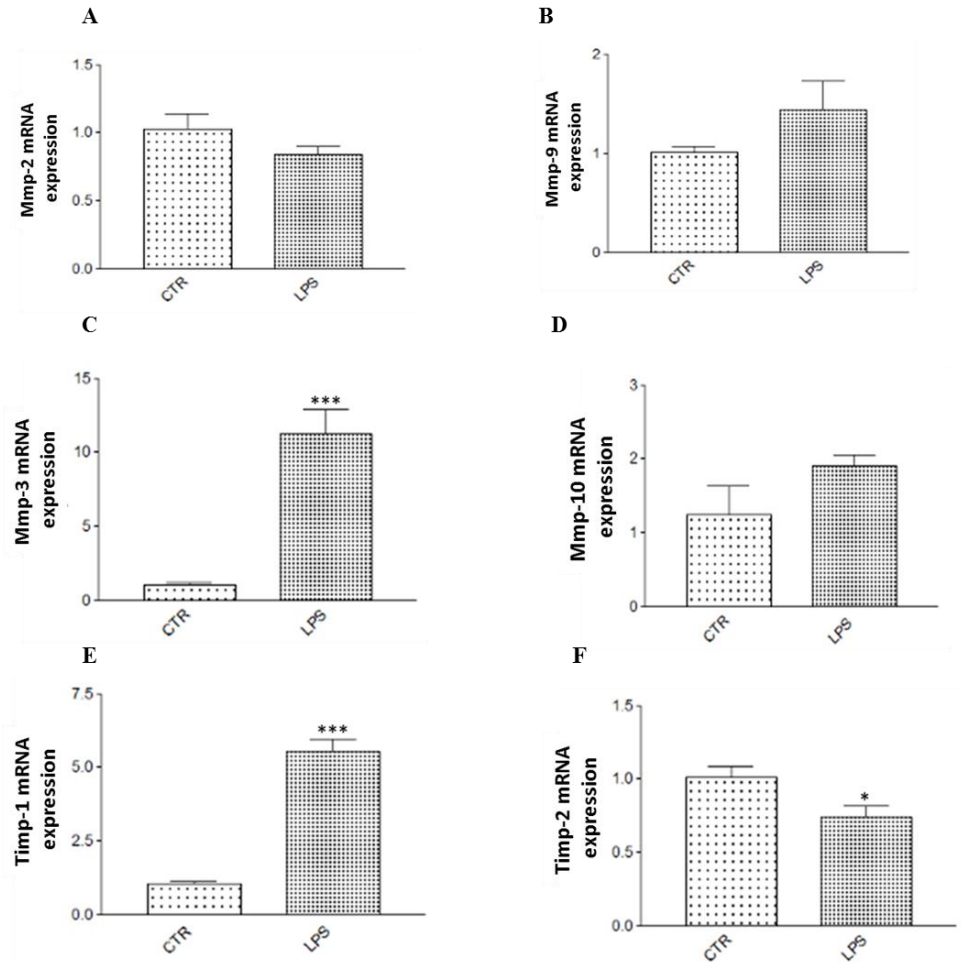


Figure 3. Expression of Mmp-2, Mmp-9, Mmp-10, Timp-1 and Timp-2 mRNA in a uterus of the mouse in the model of preterm birth. The expression of Mmp-3, Mmp-10, Timp-1 mRNAs were assessed 6 hours after treatment with intrauterine LPS or PBS on day 17 of gestation. The expression of Mmp-3 (C)and Timp-1 (E) mRNAs was significantly upregulated in LPS uterine treated mice compared to the PBS control. ***p<0,001, n=6. Timp-2 (F) mRNA levels were significantly downregulated. (*p<0,05) and no difference in Mmp-2 (A), Mmp-9 (B), Mmp-10 (D) mRNA expression between LPS treated group compared with the control. Data were analysed using two-tailed unpaired t-test.

FIGURE 4

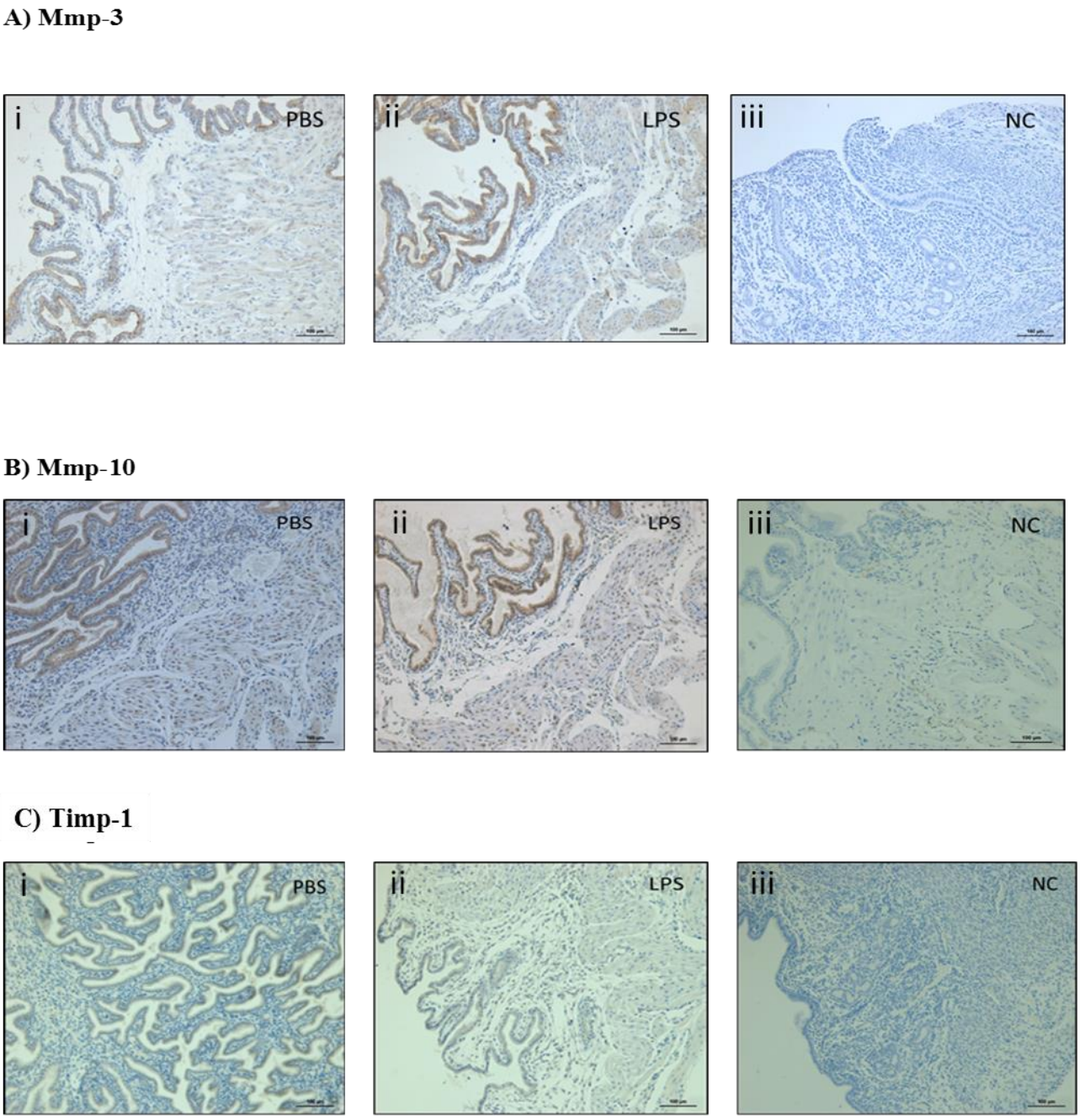


Figure 4. Localization of Mmp-3, Mmp-10 and Timp-1 in the mouse uterus. Representative images of Mmp-3 (A), Mmp-10 (B), Timp-1 (C) by immunohistochemistry in uterine tissue collected 6 hours after treatment from mice administered intrauterine LPS or PBS on day 17 of gestation. Mmp-3 (A), Mmp-10 (B) and Timp-1 (C) was detected in both endometrium and myometrium. Only Mmp-3 showed difference between LPS (i) treated mouse uterus compared to PBS control (ii). Negative control (iii). (n=4) Original magnification, X10.

FIGURE 5

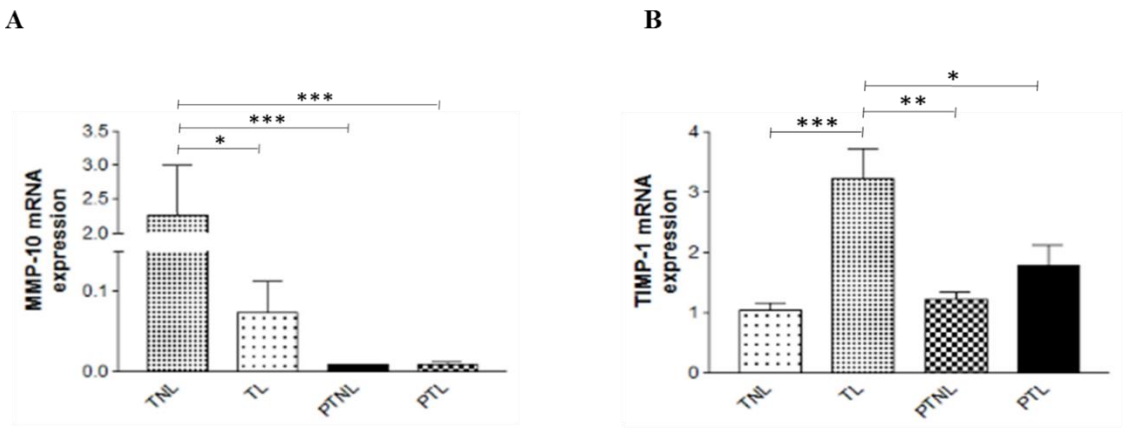


Figure 5. Expression of MMP-10 and TIMP-1 mRNA in the human myometrium. The expression of MMP-10 mRNAs was significantly downregulated in TL myometrium. *p<0,05, n=7 (A). TIMP-1 mRNA was significantly upregulated in TL samples (***p<0,001, n=8) and there was a significant difference between TL and PTL (*p<0,05 n=8) (B). No difference between PTL and PTNL of both MMP-10 and TIMP-1. Data were analysed using one-way ANOVA with Tukey's post-hoc test.

FIGURE 6

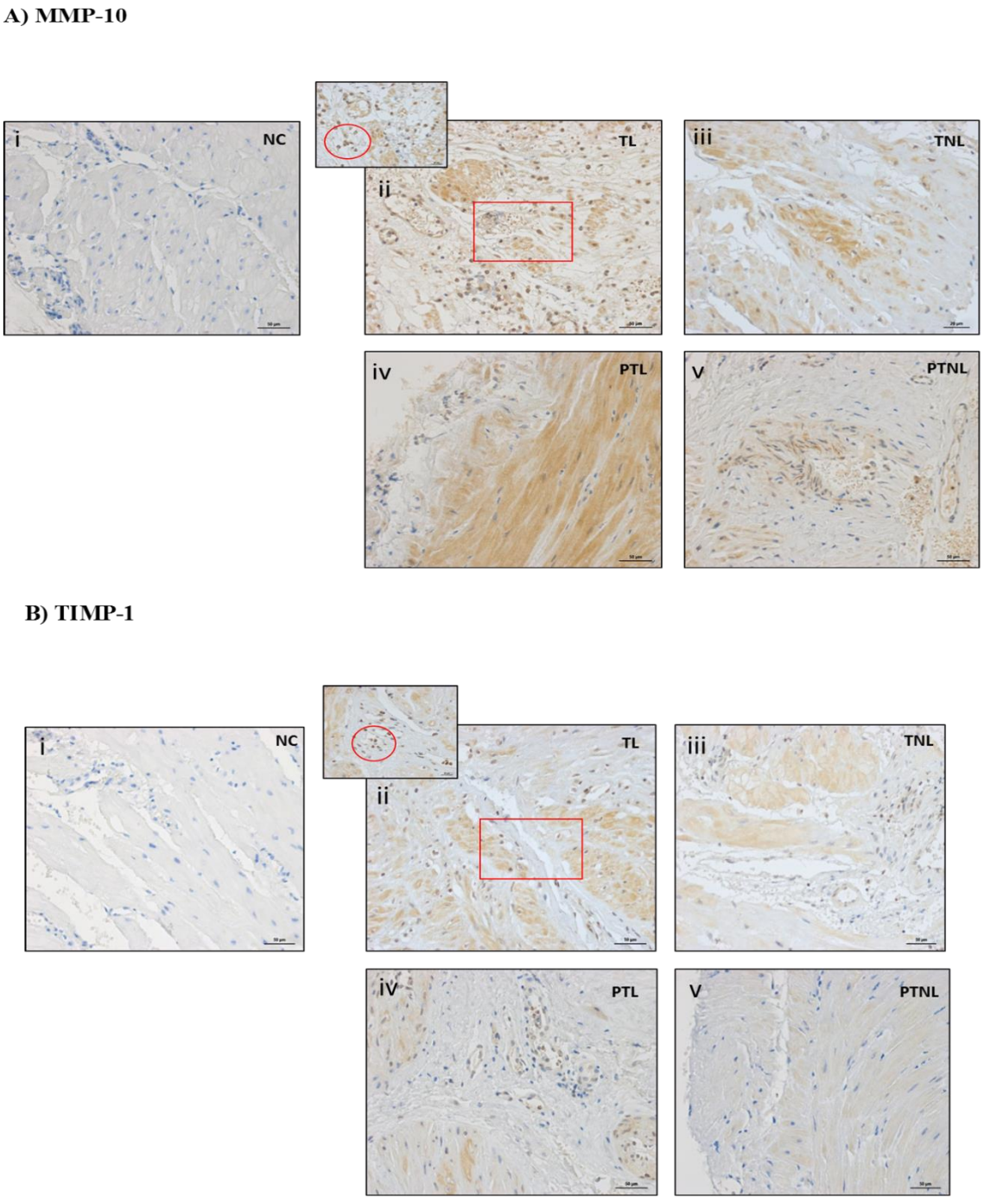


Figure 6. Localization of MMP-10 and TIMP-1 mRNA in human myometrium. MMP-10 (A) and TIMP-1 (B) were detected mainly in myometrial smooth muscle cells, around myometrial vasculature and in inflammatory cells. The intensity of the immunostaining for MMP-10 and TIMP-1 was stronger during TL (ii) than TNL (iii) and no difference between PTL (iv) and PTNL (v). Negative control (i). (n=4) Original magnification, X20.

Discussion

The present study provides the first comprehensive view of gene-expression changes of the two major groups of MMPs (stromelysins and gelatinases) and their inhibitors TIMP-1 and TIMP-2 in the mouse uterus from early pregnancy to parturition, and in a mouse model of inflammation induced preterm birth. Moreover, to our knowledge, this is the first report to demonstrate the expression of MMP-10 and TIMP-1 in human myometrium at term and preterm, both non-labouring and labouring.

The role of MMPs and TIMPs has been widely investigated in different reproductive tissues of several species during pregnancy, labour and preterm labour over the past decades. MMPs are important enzymes that are involved in ECM degradation but also contribute to cell proliferation, migration, differentiation, apoptosis, and angiogenesis (*Yang et al.,2009*; *Menon et al., 2005*;*Shan B et al., 2007*). They are almost undetectable in normal adult tissue, but their expression is induced in response to injury, disease, or pregnancy (*Paul et al., 2008*; *Vu et al.,2008*; *Choi et al.,2009*).

Altered expression of MMPs and their inhibitors in amniotic fluids and fetal membranes resulting in disruption of physiological balance has been previously linked to preterm labour (*Geng et al., 2016*).

Little is known about the potential role of MMPs, especially MMP-10, within the uterus during the different stages of pregnancy and labour. Our results show higher expression levels of Mmp-3, Mmp-2 and Mmp-9 in the mouse uterus during early gestation compared to late gestation and labour. Only Mmp-10 transcription was significantly upregulated at labour suggesting a role for Mmp-10 in parturition.

MMP-10 (stromelysin-2) and MMP-3 (stromelysin-1) belong to the MMPs family of stromelysins. MMP-10 and 3 have similar substrate specificities, but MMP-3 has a proteolytic efficiency higher than that of MMP-10. Besides digesting ECM components such as IV and IX collagens, laminin, fibronectin, elastin, and proteoglycans, MMP-3 and MMP-10 are involved in the activation of a number of proMMPs (*Visse and Nagase.,2003*).

The ECM molecules surrounding myometrial SMCs include structural proteins (fibrillar collagens and elastin), substrate adhesion molecules (fibronectin, laminin and collagen IV) and proteoglycans (*Shynlova et al., 2004*), all of which are MMP-10 substrates.

Previous studies have suggested a role of sex steroid hormones in the regulation of MMPs in the uterine tissue. For example, progesterone has been shown to inhibit the expression of most MMPs, probably through transcriptional repression (*Schroen DJ et al., 1996*). Progesterone plays a central role in the maintenance of pregnancy by promoting myometrial quiescence. In most mammals,

including rodents, the end of pregnancy correlates with a rapid decline in circulating maternal progesterone concentration to promote slower uterine growth and passive mechanical stretch of the uterine walls (*Shynlova O et al., 2003*; *Zakar T et al.,2007*).

One of the MMP targets of progesterone is MMP-10. MMP-10 is dowregulated by progesterone (*Lei et al., 2011*) but upregulated by proinflammatory cytokines (*Rechardt et al.,2000*), in keeping with the hypothesis that the activation of myometrial inflammation, or more specifically of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway in the myometrium, is key to the onset of parturition (*Keski-Nisula et al., 2003*). Our finding of the labour-stimulated induction of MMP-10 expression in the mouse uterus may reflect the cease of MMP-10 transcriptional suppression due to progesterone withdrawal and myometrial inflammation.

The activity of secreted MMP enzymes is under the strict control of locally produced TIMPs (*Hulboy et al. 1997*). In particular, TIMPs inhibit MMPs activity via the formation of a tight, noncovalent enzyme/inhibitor complex in a 1:1 molar ratio. Thus, an increase in metalloproteinase activity in labour may represent either an increased secretion of MMPs or a decreased secretion of TIMPs (*Riley et al., 1999*). Herein we observed a constant expression of TIMPs during pregnancy, which suggests a well-controlled MMP activity by the formation of inhibitory complex.

It is known that TIMP–2 inhibits the activity of most MMPs, except MMP–9 while TIMP-1 inhibits MMP-9, stromelysins MMP-3 and MMP-10 with higher affinity for MMP-3 (*Brew et al.,2010*).

Surprisingly, we found that Timp-1 transcription followed a similar expression pattern to that of gelatinases and Mmp-3 with Timp-1 expression being elevated during early gestation and dropping towards the end of pregnancy.

The role of TIMPs in uterine physiology is uncertain due to the fact that these proteins are multifunctional and TIMPs have been shown to regulate a variety of physiologic events within the uterus. Although TIMPs were first identified based upon their ability to regulate MMP activity, several evidences suggest that they can act as growth factors, antiangiogenic agents, modulators of steroidogenesis and apoptosis (*Hayakawa et al.,1994*; *Stetler-Stevenson et al.,2008*).

Our work reveals that MMP and TIMP are present in both the endometrium and stroma throughout gestation and during labour. We report for the first time that Mmp-3 and Timp-1 increase in the uterus of mice destined to undergo premature labour due to LPS-induced intrauterine inflammation, in contrast to Timp-2, which decreases. Our findings are in line with the hypothesis that MMP-3 increases and TIMP-2 reduces in the host response to intrauterine infection to contribute to parturition (*Park et al., 2003*; *Maymon et al., 2000*).

MMP-3 production is greatly enhanced by a number of factors including lipopolysaccharide, tumor necrosis factor (TNF), and interleukin-1 (IL-1) (*Keller et al., 2000*; *So et al., 1992*; *Watari et al., 2000*). Therefore, in the context of intrauterine infection, bacterial and host products (pro-inflammatory cytokines), which were produced in the resident macrophage, might up-regulate MMP-3 gene expression in fetal membranes and decidua (*Fortunato et al., 1999b*).

In addition, numerous studies suggested the implication of MMP-3 in the process of physiological parturition, premature parturition and premature rupture of fetal membranes (*Park et al., 2003*; *Sundrani et al., 2013*).

Consistently, women with preterm labor have reportedly elevated MMPs and decreased TIMPs. Indeed, premature rupture of fetal membranes (PPROM) has been associated with increased expression and activity of multiple MMPs coupled with reduced levels of TIMP-1 or TIMP-2 in the amniotic fluid (*Vadillo-Ortega et al.,1996*). The literature concerning TIMP-1 is conflicting because other studies have demonstrated that TIMP-1 concentrations in amniotic fluid were increased in the presence of IUI (*Locksmith et al., 2001*; *Athayde et al., 1998*) and in patients with rupture of the membranes either term or preterm (*Fortunato et al.,1999a*).

In our study, we evidenced an increase of Timp-1 in the preterm labouring uterus. However, TIMP-1 is a multifunctional protein and MMP inhibition is just one of its many properties (*Hayakawa et al.,1994*).

It is likely that the increased uterine Mmp-3 levels in our mouse model of preterm labour is a manifestation of increased infection/inflammation in the uterus. Large quantities of Timp-1 in our mouse model of preterm labour may indicate an host response designed to minimize the biodegradation initiated by MMP-3 and other MMPs that are active during preterm labour. Our observations may suggest a role of MMP-3 and TIMP-1 in preterm labor.

The role of MMP-10 in the human myometrium is not well defined. In this study we observed a decrease in the expression of MMP-10 in the myometrium with the onset of term, but notably not preterm labour. A recent study showed that the expression of Mmp-10 follows a similar regulation in the rat myometrium before and during labour (*Nguyen et al.,2016*). On the other hand, human TIMP-1 is dramatically increased with the onset of term, and preterm, labour. It is possible that MMP-10 expression is counterbalanced by TIMP-1 and thus, TIMP-1 may be a selective inhibitor of MMP-10 in the human myometrium during physiological labour and premature labour. The specificity of TIMP-1/MMP-10 regulation to term labour in our study is another yet example of the different mechanisms underlying physiological and pathological labour.

We found MMP-10 and TIMP-1 localised to the myometrial smooth muscle cells, interstitial fibroblasts and inflammatory cells around the myometrial vasculature. These latter cells, different from myocytes, may be infiltrating leukocytes. Leukocytes, especially macrophages, are able to secrete several MMPs and TIMPs. Macrophages have been identified as the predominant immune cells in the myometrium and decidua during gestation and term labour. Monocytes have been suggested to support active labor contractions by amplifying the inflammatory signal through the secretion of cytokines including IL-1β, IL-6, TNFα and MMPs (*Bollapragada et al.,2009*; *Gomez-Lopez et al., 2014*). In macrophages MMP10 expression is an important moderator of cell migration and invasion (*Murray et al., 2013*).

We speculate that the increase of Mmp-10 in the labouring mouse uterus may be attributed to progesterone withdrawal. Although, we did not expect a similar response in the human myometrium due to the absence of progesterone withdrawal in the human (*Mesiano et al.,2002*), a decrease of MMP-10 with labour was surprising. We believe that this decrease may be correlated to circulating progesterone levels during parturition and to an opposite regulation by proinflammatory cytokines.

The novelty of our study is the indication that 1) the majority of MMPs studied in the mouse uterus displayed higher expression in early gestation compared to late gestation and labour; only MMP-10 exhibited increased expression at labour suggesting a role in parturition; 2) the significantly increased expression of Mmp-3 and Timp-1 in a mouse model of inflammation-induced preterm labour suggesting an involvement of MMP-3 and TIMP-1 in preterm labour; 3) MMP-10 in human myometrium show a gestational profile expression differently than the inhibitor TIMP-1 that seems to be involved in parturition and preterm labour.

MMP-10 and TIMP-1 uterine kinetics deserve further investigation to understand how they facilitate ECM remodelling during pregnancy and stimulate preterm or term labour. Our study highlights the important involvement of MMPs and TIMPs in uterine remodeling demostrating the expression profile and localization of gelatinases, stromelysins and TIMPs during pregnancy, labour and preterm labour supporting that the appropriate level of MMPs and their balance by inhibitors of MMP activity is needed for normal pregnancy.

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3 DISCUSSION

The role of glucocorticoids in early pregnancy has been highlighted in numerous studies suggesting their importance in several key aspects of early pregnancy including effects on the maternal immune response, embryo attachment and fetal development. The extensive distribution of glucocorticoid receptor (GR) across the feto-maternal interface also suggests the engagement of glucocorticoids in the actions on both maternal and fetal sides. A tight control of GC levels at the fetal-maternal interface is essential for establishment and maintenance of a healthy pregnancy (*Makrigiannakis et al., 2011*; *Michael et al., 2008).* Moreover, a large body of evidences suggest that matrix metalloproteinases and their tissue inhibitors have key function in uterine extracellular matrix (ECM) remodeling occurs throughout pregnancy and at parturition and an imbalance in the MMPs/TIMPs activity ratio in the uterine microenvironment may result in aberrant degradation of ECM and account for premature onset of labor (*Geng et al., 2016*)

This study represents the first report evaluating GR transcripts expression in first trimester human decidua cells and their regulation by inflammatory citokines. Furthermore, the present work provides the first comprehensive view of gene-expression changes of the two major groups of MMPs (stromelysins and gelatinases) and their inhibitors TIMP-1 and TIMP-2 in the mouse uterus from early pregnancy to parturition, and in a mouse model of inflammation-induced preterm birth. Additionally, to our knowledge, this is the first report to demonstrate the expression of MMP-10 and TIMP-1 in human myometrium at term and preterm, both non-labouring and labouring.

Prior to the current study, several isoforms of GR have been identified in term human placenta and placental trophoblast (*Johnson et al., 2008*; *Zaif et al., 2014*). This study demonstrated that there are six isoforms of the glucocorticoid receptor present in cultured first trimester decidual cells. Of the mature mRNA variants, GRα GRβ, GR P and GRγ, are all detectable in first trimester decidual cells with GRα mRNA represented at the highest level. Among the five untranslated exon 1 regions, mRNAs for exon 1C and 1B were observed. Taken together these findings, with the previous results obtained in human placenta, contribute to provide a picture of GR transcript expression at the fetal-maternal interface.

These findings have been extended by examining the role of inflammatory cytokines on the control of GR mRNA transcripts expression in cultured first trimester decidual cells demonstrating a

significant increased expression in GRα, GRβ, GR P and GRγ mRNA levels and an increased protein level expression of GRβ and GRα D in response to IL-1β and TNF-α.

Interestingly, several studies demonstrated that these GR isoforms, GRβ, GR P, GRγ and GRα D are correlated with glucocorticoid resistance (*de Lange et al.,2001; Oakley et al., 2013*) therefore we speculated that in FT-DCs, inflammatory cytokines might drive GC resistance by increasing the expression of specific resistance-related GR isoforms.

In a previous study, we reported a clear dominance of 11β-HSD1 over 11β-HSD2 in first trimester human decidua and an enhancement of decidual 11β-HSD1 levels by IL-1β in cultured first trimester decidual cells. This findings led us to hypothesize that it may represent a novel mechanisms by which inflammatory mediators can affect implantation by increasing the availability of biologically active GCs at the fetal-maternal interface (*Funghi et al., 2016*)

Many evidence suggests that prolonged exposure to high levels of glucocorticoids in pregnancy is dangerous for both placental and fetal development indicating that tight control of GC levels at the fetal-maternal interface is essential for establishment and maintenance of a healthy pregnancy (*Michael and Papageorghiou, 2008*) (*Singh et al.,2012*).

Taking together these findings suggest that in the inflammatory milieu of early pregnancy, an increased expression of GC resistance-related GR isoforms could represent a novel mechanism to protect decidua cells against GC overexposure driven by proinflammatory cytokine-dependent 11β-HSD1 induction.

In the second part of this work, we demonstrated that the majority of MMPs (Mmp-3, Mmp-2, Mmp-9, Mmp-10) studied in the mouse uterus displayed higher expression in early gestation compared to late gestation and labour; only Mmp-10 exhibited increased expression at labour suggesting a role in parturition. MMP-10 is dowregulated by progesterone (*Lei et al., 2011*) but upregulated by proinflammatory cytokines (*Rechardt et al.,2000*), in keeping with the hypothesis that the activation of myometrial inflammation, or more specifically of the NF-kB pathway in the myometrium, is key to the onset of parturition (*Keski-Nisula et al., 2003*).

Our finding of the labour-stimulated induction of Mmp-10 expression in the mouse uterus may reflect the cease of Mmp-10 transcriptional suppression due to progesterone withdrawal and myometrial inflammation. Surprisingly, we found that Timp-1 transcription followed a similar expression pattern to that of gelatinases and Mmp-3, with Timp-1 expression being elevated during early gestation and dropping towards the end of pregnancy. Our work reveals that Mmp-3, Mmp-10 and Timp-1 are present in both the endometrium and stroma throughout gestation and during labour.

Furthermore, we report for the first time that Mmp-3 and Timp-1 increase in the uterus of mice destined to undergo premature labour due to LPS-induced intrauterine inflammation, in contrast to Timp-2, which decreases. Our findings are in line with the hypothesis that MMP-3 increases and TIMP-2 reduces in the host response to intrauterine infection to contribute to parturition (*Park et al., 2003; Maymon et al., 2000*). In addition, numerous studies suggested the implication of MMP-3 in the process of physiological parturition, premature parturition and premature rupture of fetal membranes (*Park et al., 2003; Sundrani et al., 2013*).

The literature concerning TIMP-1 is conflicting because some studies have demonstrated that TIMP-1 concentrations in amniotic fluid were increased in the presence of IUI (*Locksmith et al., 2001; Athayde et al., 1998*) and in patients with rupture of the membranes either term or preterm (*Fortunato et al.,1999a*), in contrast other studies have demonstrated that premature rupture of fetal membranes is associated with a decreased expression of TIMP-1 in the amniotic fluid (*Vadillo-Ortega et al., 1996*).

It is likely that the increased uterine MMP-3 levels in our mouse model of preterm labour is a manifestation of increased infection/inflammation in the uterus. Large quantities of TIMP-1 in our mouse model of preterm labour may indicate a host response designed to minimize the biodegradation initiated by MMP-3 and other MMPs that are active during preterm labour. Our observations may suggest a role of MMP-3 and TIMP-1 in preterm labor.

Little is known about the potential role of MMP-10, within the human uterus during pregnancy, labour and preterm labour. We demonstrated a decreased expression of MMP-10 in the human myometrium with the onset of term, but notably not preterm labour. A recent study showed that the expression of Mmp-10 follows a similar regulation in the rat myometrium before and during labour (*Nguyen et al., 2016*). We speculate that the increase of Mmp-10 in the labouring mouse uterus may be attributed to progesterone withdrawal. Although, we did not expect a similar response in the human myometrium due to the absence of progesterone withdrawal in the human, a decrease of MMP-10 with labour was surprising. We believe that this decrease may be correlated to circulating progesterone levels during parturition and to an opposite regulation by proinflammatory cytokines. On the other hand, human TIMP-1 is dramatically increased with the onset of term, and preterm, labour. It is possible that MMP-10 expression is counterbalanced by TIMP-1 and thus, TIMP-1 may be a selective inhibitor of MMP-10 in the human myometrium during physiological labour and premature labour. Moreover, we found MMP-10 and TIMP-1 localised to the myometrial smooth muscle cells, interstitial fibroblasts and inflammatory cells around the myometrial vasculature. These cells, different from myocytes, may be infiltrating leukocytes that secrete several MMPs and TIMPs.

Conclusions

This present work highlights a) the importance of GCs and their receptors GRs in the establishing of pregnancy and that the identification, characterization and regulation of the several GR isoforms involved in the modulation of glucocorticoid action may lead to potential strategies for the treatment or prevention of a variety of pregnancy-related disorders. b) the important involvement of MMPs and TIMPs in uterine remodeling demostrating the expression profile and localization of gelatinases, stromelysins and TIMPs during pregnancy, labour and preterm labour supporting that the appropriate level of MMPs and their balance by inhibitors of MMP activity is needed for normal pregnancy.

Taken together these observations provide key novel findings in the pathophysiology of pregnancy and parturition.

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5 ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor Prof. Felice Petraglia for the support of my Ph.D study and related research, for his patience, motivation, and immense knowledge.

I would also like to thank Dr. Felice Arcuri for all his help, precious advices and support during my PhD studies.

Massive thanks to my fellow labmates Lucia Funghi, Patrizia Carrarelli and Francesco Damiani for their encouragement, help, suggestions and enjoyable moments together.

I am grateful to Prof. Jane Norman, who provided me the opportunity to join her team at the University of Edimburgh and for her valuable guidance towards this project. I would also like to thank her research team members: Sofia Makieva, Sara Rinaldi, Caterina Fazzi, Ashley Boyle, Ioannis Pavlidis, Heather Macpherson, Marian Aldhous for their help and support.

Last but not the least, I would like to thank my family: my parents and my sister for their unfailing support and continuous encouragement.