Dynamic remodeling of endometrial extracellular matrix regulates embryo receptivity in cattle

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Abstract

We aimed to evaluate in the bovine endometrium whether (1) key genes involved in endometrial extracellular matrix (ECM) remodeling are regulated by the endocrine peri-ovulatory milieu and (2) specific endometrial ECM-related transcriptome can be linked to pregnancy outcome. In Experiment 1, pre-ovulatory follicle growth of cows was manipulated to obtain two groups with specific endocrine peri-ovulatory profiles: the Large Follicle-Large CL group (LF-LCL) served as a paradigm for greater receptivity and fertility and showed greater plasma pre-ovulatory estradiol and post-ovulatory progesterone concentrations compared to the Small Follicle-Small CL group (SF-SCL). Endometrium was collected on days 4 and 7 of the estrous cycle. Histology revealed a greater abundance of total collagen content in SF-SCL on day 4 endometrium. In Experiment 2, cows were artificially inseminated and, six days later, endometrial biopsies were collected. Cows were retrospectively divided into pregnant and non-pregnant (P vs NP) groups after diagnosis on day 30. In both experiments, expression of genes related to ECM remodeling in the endometrium was studied by RNAseq and qPCR. Gene ontology analysis showed an inhibition in the expression of ECM-related genes in the high receptivity groups (LF-LCL and P). Specifically, there was downregulation of *TGFB2*, *ADAMTS2*, *5 and 14*, *TIMP3* and *COL1A2*, *COL3A1*, *COL7A1* and *COL3A3* in the LF-LCL and P groups. In summary, the overlapping set of genes differently expressed in both fertility models: (1) suggests that dysregulation of ECM remodeling can impair receptivity and (2) can be used as markers to predict pregnancy outcome in cattle.

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Introduction

The establishment and maintenance of pregnancy are complex events that require a fine-tuned synchrony between a receptive maternal uterus and the embryo's ability to develop in such an environment. The development of histologically and functionally normal endometrium is critical for endometrial receptivity and the initial embryo–maternal interactions. In this context, endometrial tissue remodeling might be a prerequisite to obtain the optimal uterine environment for the early embryo, as previously documented in mice and human studies. When focusing on endometrial morphology, the endometrial extracellular matrix (ECM) has crucial importance; it is the non-cellular component found in all tissues and organs providing physical support for the uterine cells. The ECM is a highly dynamic structure, under constant remodeling by enzymatic or non-enzymatic means to maintain tissue homeostasis (Jarvelainen *et al.* 2009, Schmidt & Friedl 2010).

Three major classes of biomolecules belong to the ECM: structural proteins (such as collagen and elastin),

specialized proteins (such as fibrilins, fibronectins and laminin) and proteoglycans (Alberts *et al.* 2002, Kim *et al.* 2011). The ECM's main three functions are: (1) structural, by filling the extracellular spaces of tissues; (2) functional, by mediating cell adhesion, growth, migration and differentiation and (3) regulatory, by controlling the release of growth factors and cytokines by the action of peptidases that can modulate cell physiology (Schenk & Quaranta 2003, Mott & Werb 2004, Jarvelainen *et al.* 2009).

The abundance of endometrial ECM proteins is finely controlled by synthesis and proteolytic degradation. Disturbance of such a critical balance may result in implantation abnormalities and, consequently, infertility (Skinner *et al.* 1999). Several proteases are involved in the proteolytic degradation of ECM proteins, but the most prominent are the members of the family of matrix metalloproteinases (MMP), disintegrins and metalloproteinases (ADAM), ADAMTS (a disintegrin and metalloprotease with a thrombospondin motif) and their inhibitors (TIMPs) (Reiss & Saftig 2009, Egeblad *et al.* 2010).

In cattle, the role of ECM remodeling in reproductive processes is poorly understood as only few studies were performed in ruminants at the implantation period (Guillomot 1999, Boos 2000, Yamada *et al.* 2002, Mishra *et al.* 2010). Most reports on the essence of ECM remodeling for endometrial receptivity and fertility originate from human and rodent studies (Farrar & Carson 1992, Clark *et al.* 1993, Das *et al.* 1997, Hurst & Palmay 1999). Mishra and coworkers (2010) studied the expression pattern of MMPs and described the bovine mechanism of ECM regulation pointing to MMPs and ECM metalloproteinase inducer (EMMPRIN) in the endometrium of cyclic and pregnant cows. They showed an attenuated expression of these molecules in day 19 pregnant in comparison to cyclic endometrium. Ulbrich and coworkers (2011) stressed the importance of high endometrial MMP2 enzymatic activity during the elongation phase of the blastocyst, which may increase the availability of growth factors supporting conceptus development. The latter researchers suggested that an increased TIMP2 abundance in the uterine fluid during trophoblast elongation points to a well-balanced ECM integrity, needed for the maintenance of pregnancy (Ulbrich *et al.* 2011).

However, clear information on how the ECM remodeling events can be linked to maternal receptivity in cattle is lacking. This highlights the need to gain more insights in the factors regulating the ECM remodeling in the endometrial tissue during early diestrus. In this regard, sexual steroid hormones are key regulatory factors of the endometrial tissue and abundance and distribution changes of specific collagen types across the estrous cycle was reported previously (Boos 2000). Indeed, greater pre-ovulatory estradiol and post-ovulatory progesterone concentrations are associated with increased bovine embryo survival (Perry *et al.* 2005, Meneghetti *et al.* 2009, Peres *et al.* 2009, Dadarwal *et al.* 2013). The endometrium expresses both progesterone and estradiol receptors and endometrial functions are modulated by changes in sex steroid concentrations to support conceptus growth and pregnancy maintenance (Robinson *et al.* 2001, Bazer & Slayden 2008). Reports by Klein and coworkers (2006), Forde and coworkers (2009), Bauersachs and coworkers (2006), Mamo and coworkers (2011), among others, used holistic transcriptomic approaches to understand changes in endometrial function in response to the growing conceptus. In general, conceptus-induced changes are initially observed after initial elongation (i.e., 12–13 days after estrus), and most clearly at maternal recognition of pregnancy (15–17 days after estrus). Despite their critical importance, specific biological processes and molecular pathways affected by peri-ovulatory variations in sex steroids (from proestrus to early diestrus; i.e., 7 days after estrus), are only now starting to be elucidated and have not been sufficiently studied and explored in the endometrium. Most importantly, at least in non-bovine species, ECM remodeling is regulated by steroid

hormones (Crawford & Matrisian 1996, Schroen & Brinckerhoff 1996, Zhang & Salamonsen 2002).

In this study, we hypothesize that the level of ECM remodeling events can be regulated by specific endocrine profiles associated with receptivity. Furthermore, we propose that the ECM transcript signature during early pregnancy has a direct relationship with the probability of pregnancy success in cows. Therefore, two bovine fertility models were used to study: (i) the effect of endocrine profiles during peri-ovulatory period on ECM remodeling events on endometrial tissue collected on days 4 and 7 of the estrous cycle and (ii) whether ECM characteristics on day 6 uterine biopsies can be associated to pregnancy outcome. To the best of our knowledge, these are the earliest time points after estrus that was investigated for endometrial events related to receptivity in beef cattle.

Materials and methods

Animals

Animal procedures were approved by the Ethics and Animal Handling Committee of the School of Veterinary Medicine and Animal Science of the University of São Paulo. The experiments were conducted at the University of São Paulo, campus Pirassununga, SP, using adult Nelore (*Bos indicus*) cows, pluriparous, cyclic, non-lactating, without reproductive disorders detectable by gynecological examination and body condition score between 3 and 4 (0 emaciated; 5 obese). All animals were kept in grazing conditions and supplemented with sugar cane and/or corn silage, concentrate and minerals to fulfill their maintenance requirements and water *ad libitum*.

Experiment 1. Reproductive management and experimental design

Study I

Ovulations were synchronized as described previously by Mesquita and coworkers (2014). The goal was to manipulate the peri-ovulatory endocrine milieu to obtain two groups of animals with distinct pre-ovulatory follicle (POF) and CL sizes and, consequently, contrasting peri-ovulatory endocrine milieus with distinct proestrus/estrus concentrations of E2 and diestrus concentrations of P4. This experimental model was used as a paradigm for lower (Small Follicle-Small CL group; SF-SCL) or greater (Large Follicle-Large CL group; LF-LCL) receptivity and fertility by our group (Perry *et al.* 2005, Peres *et al.* 2009, Meneghetti *et al.* 2009, Dadarwal *et al.* 2013, Pugliesi *et al.* 2016). Briefly, animals were pre-synchronized by two intramuscular (i.m.) injections of prostaglandin F2 α analogue (PGF2 α ; 0.5 mg of sodium cloprostenol; Sincrocio, Ourofino Saúde Animal, Cravinhos, Brazil), 14 days apart. At the second PGF2 α injection of Presynch (D-20), animals received an Estroject Heat detector patch (Rockway, Inc., Spring Valley, WI, USA) device, and estrus detection was performed twice daily from D-19 to D-16 and once daily from D-15 to D-10. Only animals that presented estrus after

Figure 1 Experimental design to obtain samples from groups with Large Follicles and CL (LF-LCL) or Small Follicles and CL (SF-SCL). Cows were pre-synchronized via two injections of prostaglandin F2α (PGF2α; 0.5 mg of sodium cloprostenol) given 14 days apart. On D-10, cows received a progesterone-releasing device (1 g; Sincrogest; Ourofino) and an injection of 2 mg estradiol benzoate (BE; Sincrodiol, Ourofino). On D-10, cows in the Large-Follicle and Large-CL group (LF-LCL) also received an injection of PGF2α. On the day the progesterone-releasing device was removed, all cows received PGF2α. On D0 ovulation was induced with 1 µg of gonadotropin-releasing hormone analog (GnRH; buserelin acetate; Sincroforte, Ourofino Saúde Animal). Cows were slaughtered on D4 (Study 1) or D7 (Study 2), reproductive tract was collected and dissected. An asterisk (*) indicates the moment of blood sampling.

the second PGF2α treatment and with a new PGF-responsive CL (at least 5 days old) on D-10 stayed in the experiment. Therefore, the remaining cows ($n=41$) received a new intravaginal P4-releasing device (1 g; Sincrogest, Ourofino) on D-10 along with an i.m. injection of 2 mg estradiol benzoate (Sincrodiol, Ourofino Saúde Animal; Fig. 1). Simultaneously on D-10, cows from the LF-LCL group received a single i.m. treatment of PGF2α. The P4 devices were removed eight days later and 42–60 h or 30–36 h before the gonadotropin-releasing hormone (GnRH) treatment in the LF-LCL ($n=20$) and SF-SCL ($n=21$) groups respectively. All animals received a PGF2α injection at P4 device removal. Ovulations were induced in

all cows on D0 using GnRH agonist (Buserelin acetate, 10 µg, i.m.; Sincroforte, Ourofino Saúde Animal) on D0. Expectation was that POF growth would be reduced in the presence of greater circulating P4 concentrations in cows from the SF-SCL group because of P4 from both exogenous (device) and endogenous (CL) sources.

To assess follicle growth and ovulation of the POF and CL area and blood flow, transrectal ultrasound examinations were carried out on D-10 and D-6, daily from D-2 to D0 and from D3 to D7, and every 12 h on D1 to D2. Ultrasound exams were performed with the aid of a duplex B-mode (gray-scale) and Color-Doppler instrument (MyLab30 Vet

Table 1 Characteristics of the primers used in the measurements of transcripts related to endometrial extracellular matrix remodeling.

Gene	Gene symbol	Representative ID	Sense primer	Antisense primer
ADAM metalloproteinase with thrombospondin type 1 motif, 1	ADAMTS1	NM_001101080.1	CAGGAAGCTGGAAGCCTAAAAA	GAGAACAAAGGTCAGAAGGAGTG
ADAM metalloproteinase with thrombospondin type 1 motif, 2	ADAMTS2	NM_174631.2	CTCCATCCCAGGCTACAATAAG	GCATTGGTGCTGGAAGTATG
ADAM metalloproteinase with thrombospondin type 1 motif, 5	ADAMTS5	NM_007038.3	GTGGTCGGAACCTTCAATAA	GGCTAAGTAGGCAGTGAATC
ADAM metalloproteinase with thrombospondin type 1 motif, 14	ADAMTS 14	NM_080722.3	GGGACAGGTCCATCTTCT	TCTAGGCTCAAGGTCTTCTT
Matrix metalloproteinase 2	MMP2	NM_174745.2	CCCAGACAGTGGATGATGC	TTGTCCTTCTCCAGGGTC
Matrix metalloproteinase 14	MMP14	NM_174390.2	TGCCGAGCCTTGGACTGT	GCCACCAGAAAGATGTCATTCC
Transforming growth factor, beta 1	TGFB1	NM_001166068.1	CCTGCTGAGGCTCAAGTTAAAG	GCCACTGCCGCAAACTCC
Transforming growth factor, beta 2	TGFB2	NM_001113252.1	AATTTGGTGAAGGCCGAGTTC	GGTTTTACGACTTTGCTCCA
Transforming growth factor, beta 3	TGFB3	NM_001101183.1	TTACTGCTTCCGCAATTTCA	TCTGAGCTGCGGAGGTATG
Metalloproteinase inhibitor 1	TIMP1	NM_174471.3	GAGTTTCTCATAGCTGGACAAT	GCATAGGTCTTGGTGAATCC
Metalloproteinase inhibitor 2	TIMP2	NM_174472.4	TGCAGACATAGTGATCAGGGCCA	AATCCGCTTGATGGGGTTGCCG
Metalloproteinase inhibitor 3	TIMP3	NM_174473.4	GCCCTTTGGCAGCATGGTCT	TAGACTCGGCTGTCACCAGGT
Collagen 1A2	COL1A2	NM_174520.2	GGATACGCGGACTTTGTTC	CTCCACTTGGGCCCTTTCT
Collagen 3A1	COL3A1	NM_001076831.1	ACTGGTGCTCCTGGATTAAAG	CTTCCTCGAGTCCATCATTAC
Collagen 4A1	COL4A1	NM_001166511.1	GGGTTCCGTAGGATTGAAAG	GAAGCCTATCTGCTCTTGTGTC
Collagen 4A2	COL4A2	XM_010810991.1	CAACGGTTACGTGCGAGAAG	CTTCAAGGAGACGCCTATTC
Collagen 4A5	COL4A5	XM_002699860.3	GCAGGAGAGAAAGGTAGTAAAG	CCTGCAACTCCAGGAATAC

Table 2 Functional enrichment of pathways related to ECM remodeling upregulated in the endometrium of SF-SCL cows ($n=3$ /group) on D7.

Category	Term	Count	$P > F$	Fold enrichment
GOTERM_CC_FAT	Collagen	9	<0.0001	11.61
GOTERM_CC_FAT	Fibrillar collagen	6	<0.0001	23.22
GOTERM_MF_FAT	Extracellular matrix structural constituent	7	<0.0001	8.9
GOTERM_BP_FAT	Collagen fibril organization	6	<0.0001	10.5
GOTERM_MF_FAT	Growth factor binding	6	0.040	3.14
GOTERM_BP_FAT	Skin development	3	0.107	5.28
GOTERM_BP_FAT	Epidermis development	5	0.134	2.51
GOTERM_BP_FAT	Ectoderm development	5	0.157	23.7
GOTERM_BP_FAT	Blood vessel development	6	0.521	12.5
GOTERM_BP_FAT	Vasculature development	6	0.544	1.22

P represents non-adjusted probabilities.

Gold; Esaote Healthcare, São Paulo, SP, Brazil) equipped with a multifrequency linear transducer. Blood samples were collected via jugular venipuncture in tubes containing heparin (BD, São Paulo, SP, Brazil) on D-1 and D4. Blood samples were immediately stored on ice after collection. To obtain plasma, blood was centrifuged at 1500g for 30 min at 4°C. The plasma was then aliquoted and stored at -20°C.

On day 4 of the estrous cycle (day 0=injection of GnRH to induce ovulation), cows from LF-LCL ($n=16$) and SF-SCL ($n=8$) groups were stunned by a captive bolt and slaughtered by jugular exsanguination. The reproductive tract was transported on ice to the laboratory, ovarian structures were measured and weighed and the uterine horn ipsilateral to the ovary bearing the CL was incised longitudinally. Tissue from a single strip of endometrial intercaruncular region was used for each cow. Strips measured approximately 2.5 cm long and 5 mm wide and were dissected from the mid third of the horn. Samples were snap-frozen in liquid nitrogen and then transferred to -80°C until processing for RNA extraction. Another portion of samples was fixed by immersion in 4% buffered formalin for 24 h at 4°C, followed by several washings in PBS. Subsequently, samples were embedded in Paraplast, sectioned and processed for histological analysis.

Concentrations of P4 were measured by radioimmunoassay (Coat-A-Count kit progesterone, Siemens Medical Solutions Diagnostics) validated previously for bovine plasma samples (Garbarino *et al.* 2004). Estradiol concentrations were measured using a commercial RIA kit (Double Antibody Estradiol, DPC, Los Angeles, CA, USA) validated previously for bovine plasma samples (Siddiqui *et al.* 2009). The intra-assay CV and sensitivity were 1.7% and 0.13 pg/mL for E2; and 0.8% and 0.05 ng/mL for P4, respectively.

Study II

Non-lactating, multiparous Nelore (*Bos indicus*) cows ($n=83$) were submitted to the same hormone protocol described in Study 1 (Fig. 1) to result in the SF-SCL ($n=39$) and LF-LCL ($n=35$) groups. Blood collections and collection of endometrial samples were performed as previously described for Study I; exception was that endometrium harvesting was seven days post-induction of ovulation (i.e., D7). The intra-assay CV and sensitivity were 1.7% and 0.13 pg/mL for E2; and 0.3% and 0.076 ng/mL for P4 respectively. Animals within each group were ranked according to responses to the following endocrine and ovarian variables: concentration of E2 on D-1, concentration of P4 at D7, CL size at D7, CL weight at D7, follicle size at

D-2, D-1 and D0 and pre-ovulatory follicle size. The eight top ranked animals of the LF-LCL group and the nine lowest ranked animals of the SF-SCL group were chosen for transcript analysis. Likewise, the top (LF-LCL) and lowest (SF-SCL) three animals were used for RNA-seq analyses. Histological analysis for collagen was performed on samples from Experiment 1 (LF-LCL, D4 $n=4$, D7 $n=5$; SF-SCL, D4 $n=3$, D7 $n=4$).

Figure 2 Collagen distribution in the endometrium of cows from SF-SCL and LF-LCL groups on D4 (A) and D7 (B). Sections were stained with Masson's trichrome stain, and images from the superficial and deep compartments of the endometrium at 40× magnification. A and B show histological sections stained with Masson's Trichrome stain. GL, gland; LE, luminal epithelium; ST, stroma. Arrows indicate collagen content colored in blue.

Table 3 Mean $\pm$ SEM for collagen abundance detected by Masson's Trichrome stain on days 4 ($n=3-5$ /group) and 7 ($n=4-5$ /group) of the estrous cycle from the superficial and deep compartments of the endometrium from cows from the Small Follicle-Small Corpus Luteum group (SF-SCL) and Large Follicle-Large Corpus Luteum group (LF-LCL).

End point	SF-SCL		LF-LCL		<i>P>F</i>		
	Superficial	Deep	Superficial	Deep	Group	Region	G $\times$ R ^a
Collagen abundance on Day 4 (%)	35.4 $\pm$ 1.7	40.9 $\pm$ 4.7	31.9 $\pm$ 2.9	37.7 $\pm$ 2.0	0.016	0.007	0.940
Collagen abundance on Day 7 (%)	36.0 $\pm$ 3.0	40.8 $\pm$ 1.4	35.1 $\pm$ 4.1	38.8 $\pm$ 3.7	0.755	0.045	0.768

^aG $\times$ R = interaction between group and region.

Experiment 2. Reproductive management and experimental design

As described previously by Binelli and coworkers (2015), cows were synchronized ($n=51$) to estrus by injecting two i.m. doses of PGF2 α analogue (0.5 mg sodium cloprostenol; Sincrocio) 14 days apart. At the time of the second PGF2 α injection, animals were equipped with an Estroject Heat detector patch (Rockway, Inc.) device and were observed twice daily for six days for estrous behavior. Ultrasound exams were performed 12 h after estrus to confirm the presence and location of a POF. Artificial insemination (AI) was performed in all cows that showed estrous signs ($n=33$) with cryopreserved semen from a single ejaculate. Six days after AI, an endometrial biopsy (~50 mg) was collected from the caudal third of the uterine horn contralateral to the ovary containing a CL and stored at -80°C . After the biopsy procedure, cows received two 1 g doses of the anti-inflammatory medication flunixin meglumine (Desflan, Ourofino) in a 24-h interval and one dose of penicillin/streptomycin base antibiotic (Penfort Reforçado, Ourofino). A blood sample was collected, and plasma P4 concentrations were measured as described for Experiment 1. Pregnancy diagnosis was performed on day 30 after AI via transrectal ultrasonography exam. RNA-Seq was performed on samples selected retrospectively based on the day 30 pregnancy diagnosis using six samples from each pregnant (P) and non-pregnant (NP) animals. Animals were selected based on plasma progesterone concentrations on day 6, that must have been within a 1.5 ng/mL interval among all animals. Abundance of specific transcripts was quantified by qPCR in ten P and sixteen NP randomly selected animals.

RNA isolation and cDNA synthesis

Endometrial tissue samples (approximately 30 mg) were snap frozen in liquid nitrogen, macerated in a stainless steel apparatus and immediately mixed with buffer RLT from the RNeasy Mini columns kit (Qiagen), to be processed according to the manufacturer's instructions. To maximize cell lysis, tissue suspension was passed at least ten times through a 21 G needle, and centrifuged at 13,000 g for 3 min for removal of debris, prior to supernatant loading and processing in RNeasy columns. Columns were eluted with 40 μL of Rnase-free water, and elution was repeated using the same 40 μL initially used to increase RNA concentration. Concentration and purity of total RNA were estimated using a spectrophotometer (NanoDrop, Thermo Fisher Scientific).

RNA integrity and quantity were assessed by Bioanalyzer (Agilent Technologies Brazil Ltda), and only samples with RNA integrity number (RIN) greater than 7 were processed further. Total RNA extracts were stored at -80°C until subsequent analysis. Immediately prior to cDNA synthesis, samples were treated with DNase I (Life Technologies) at room temperature for 15 min. DNase I activity was blocked using EDTA solution (25 mM) and heating at 65°C for 10 min. Reverse transcription was performed immediately after DNase I treatment using High-Capacity cDNA Reverse Transcription Kit (Life Technologies), according to manufacturer's instructions. Briefly, 9 μL of master mix containing reverse transcription buffer, deoxyribonucleotide triphosphate mix, random primers, RNase inhibitor and reverse transcriptase were added to the 11 μL DNase I treatment reaction. Immediately, samples were incubated at 25°C for 10 min, followed by incubation at 37°C for 2 h and reverse transcriptase inactivation at 85°C for 5 min and storage at 20°C .

Figure 3 Collagen type I distribution in the endometrium of cows from SF-SCL (A and B) and LF-LCL (C and D) groups on D4 and D7 respectively. Collagen type I is scarce in the stratum compactum (SC) and found predominantly within the stratum spongiosum (SS) in both groups, around endometrial glands (G) and blood vessels (arrows). (A) In the SF-SCL group, a dense sub-epithelial mesh was observed on day 4 (A, inset), but not on day 7, in which larger amounts and thicker fibers of collagen I were found in the stratum spongiosum (B, inset). IHC, counterstained with Harris' hematoxylin. (LE, luminal epithelium; A, B and D: 10 $\times$ objective; C and insets: 40 $\times$ objective).

Figure 4 Collagen type III distribution in the endometrium of cows from SF-SCL (A and B) and LF-LCL (C and D) groups on D4 and D7 respectively. Collagen type III is present in both the stratum compactum (SC) and stratum spongiosum (SS) in low quantities, as thin fibers in the interstitium (asterisk) and predominantly around blood vessels (arrows). IHC, counterstained with Harris' hematoxylin. (G, endometrial glands; LE, luminal epithelium; A, B and D: 10× objective; C and inset: 40× objective).

mRNA libraries, sequencing and bioinformatics

Methods and general results from transcriptomic analysis of tissues from Experiments 1 and 2 were published previously (Binelli *et al.* 2015, Mesquita *et al.* 2015). Briefly, cDNA was generated using a routine RNA library preparation TruSeq protocol developed by Illumina Technologies (San Diego, CA, USA) using 4 µg of total RNA as input. Preparation involved mRNA isolation from total RNA by a polyA selection step, followed by construction of paired-end sequencing libraries with an insert size of 300bp. Briefly, mRNA was selected by oligo-dT magnetic beads and fragmented into small pieces using divalent cations. Cleaved mRNA fragments were copied into first-strand cDNA using reverse transcriptase and random primers, which was followed by second-strand cDNA synthesis using DNA polymerase I. The cDNA fragments then underwent an end repair process, the addition of a single A base, and then

ligation of the adapters. Resulting products were amplified via PCR and cDNA libraries were then purified and validated using the Bioanalyzer 2100 (Agilent Technologies). Paired-end sequencing was performed using the Illumina HiScanSeq 2000 platform. The paired end (PE) reads were filtered using the package Seqclean v1.4.13, (<https://bitbucket.org/izhbannikov/seqclean>). The reads were mapped using the local alignment with Bowtie2 against the masked *Bos taurus* genome assembly (*Bos Taurus* UMD3.1). The mapping file was sorted using SAMTools v 0.1.18, and read counts were obtained using HTSeq-count v0.5.4p2 (<http://www-huber.embl.de/users/anders/HTSeq/doc/count.html>). The differential expression analysis was performed with package DESeq v1.12.1 (Andres & Huber 2010), from R (Gentleman *et al.* 2004) for samples of Experiment 1. For samples of Experiment 2, the differential expression analysis was performed with package DESeq2 v1.12.1 (Love *et al.* 2014) from R (Gentleman *et al.* 2004). The standard deviation along the baseMean values was also calculated for each transcript. To avoid artifacts caused by low expression profiles and high expression variance, only transcripts that had an average of baseMean >5, and the mean greater than the standard deviation were analyzed. The threshold for evaluating significance was obtained by applying a *P* value of 0.05 FDR-Benjamini-Hochber (Benjamini & Hochber 1995). Integrated analysis of different functional databases was done using the functional annotation tool of the Database for Annotation, Visualization, and Integrated Discovery (DAVID Bioinformatics Resources 6.7, NIAID/NIH) using as background the transcribed genes found (Dennis *et al.* 2003).

Design and validation of primers and quantitative real-time PCR

Quantitative PCR reactions were performed using the StepOnePlus apparatus (Life Technologies). Primer sequences were either obtained from the literature or designed by Primer Express software V3.0.1 (Life Technologies) (Table 1). Each newly designed primer pair was selected based on the Gibbs free energy (ΔG) values for hairpin, homodimers and heterodimers formation indicated by the Oligo Analyzer 3.1 software (IDT; <http://www.idtdna.com/analyzer/Applications/>

Table 4 Functional enrichment of pathways related to ECM remodeling upregulated in the endometrium of NP cows (*n* = 5–6 cows/group) on D6.

Category	Term	Count	<i>P</i> > <i>F</i>	Fold enrichment
GOTERM_CC_FAT	Proteinaceous extracellular matrix	14	<0.0001	77.7
GOTERM_CC_FAT	Extracellular region	30	<0.0001	3.0
GOTERM_CC_FAT	Extracellular matrix	14	<0.0001	7.1
GOTERM_CC_FAT	Basement membrane	8	<0.0001	20.1
GOTERM_CC_FAT	Extracellular matrix part	9	<0.0001	14.8
GOTERM_CC_FAT	Extracellular region part	19	<0.0001	4.0
GOTERM_CC_FAT	Basement membrane	8	<0.0001	20.1
GOTERM_CC_FAT	Extracellular matrix part	9	<0.0001	14.8
GOTERM_BP_FAT	Extracellular structure organization	6	<0.0001	839.5
GOTERM_BP_FAT	Extracellular matrix organization	5	0.002	896.0
GOTERM_BP_FAT	Regulation of cell adhesion	5	0.003	8.1
GOTERM_BP_FAT	Positive regulation of cell–substrate adhesion	3	0.014	16.1
GOTERM_BP_FAT	Regulation of cell–substrate adhesion	3	0.029	10.9
GOTERM_BP_FAT	Positive regulation of cell adhesion	3	0.035	9.8

P > *F* represents non-adjusted probabilities.

Table 5 Validation of RNAseq ($n=3$ cows/group) gene expression data by qPCR ($n=8-9$ cows/group). Fold change and probability value ($P>F$) of gene expression in SF-SCL vs LF-LCL uterine samples on D7.

Ensembl Id	Gene symbol	RNAseq			qPCR		
		Fold change	log2 fold change	$P>F$	Fold change	log2 fold change	$P>F$
ENSBTAG00000020457	<i>TGFB1</i>	1.25	0.32	0.627	2.05	1.04	0.551
ENSBTAG00000005359	<i>TGFB2</i>	1.77	0.82	0.053	1.86	0.90	0.043
ENSBTAG00000012004	<i>TGFB3</i>	1.23	0.30	0.932	1.43	0.52	0.487
ENSBTAG00000005043	<i>TIMP1</i>	1.49	0.57	0.018	2.04	1.03	0.043
ENSBTAG00000010899	<i>TIMP2</i>	0.86	-0.22	0.369	0.92	-0.12	0.326
ENSBTAG000000020638	<i>TIMP3</i>	0.68	-0.55	0.032	0.59	-0.76	0.019
ENSBTAG00000019267	<i>MMP2</i>	1.37	0.46	0.240	1.22	0.29	0.277
ENSBTAG00000014824	<i>MMP14</i>	1.44	0.53	0.134	1.38	0.46	0.215
ENSBTAG00000014665	<i>ADAMTS2</i>	1.46	0.55	0.054	1.53	0.61	0.042
ENSBTAG00000000648	<i>ADAMTS5</i>	1.71	0.77	0.000	1.24	0.31	0.003
ENSBTAG00000013103	<i>COL1A1</i>	1.50	0.58	0.058	1.80	0.85	0.046
ENSBTAG00000013472	<i>COL1A2</i>	1.56	0.64	0.001	1.87	0.90	0.014
ENSBTAG00000021466	<i>COL3A1</i>	1.62	0.70	0.000	1.32	0.40	0.002
ENSBTAG00000012849	<i>COL4A1</i>	1.38	0.46	0.032	1.92	0.94	0.023
ENSBTAG00000014575	<i>COL4A5</i>	0.64	-0.65	0.027	0.75	-0.42	0.021

OligoAnalyzer/). Primers were subsequently checked for specificity using the Basic Local Alignment Search Tool (Blast; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Selected primer pairs were evaluated to determine the optimal primer concentration by using a pool of endometrial cDNA, at a 1:80 dilution. Each primer pair was tested in reactions prepared with the following primer concentrations: 150, 300 or 600 nM. Optimal primer concentrations were determined based on the lowest primer concentration that obtained amplification efficiency between 85% and 100%, presented a single amplification peak and had no peaks detected on the negative control reactions, in the dissociation curve, and generated the lowest cycle quantification (Cq) value. Additionally, the standard curve amplification efficiency was determined based on a standard curve, generated by a serially diluted cDNA pool with at least 5 dilutions. Acceptable standard curve efficiencies were between 83% and 100% and r^2 close to 1.

Relative abundance of specific transcripts between LF-LCL and SF-SCL samples (Experiment 1) and pregnant and non-pregnant samples (Experiment 2) was assessed by 20 μ L reactions in a 96-well plate, sealed with MicroAmp optical adhesive cover (Life Technologies), in triplicates. Raw fluorescence data were extracted from the StepOne Plus apparatus with no baseline correction and submitted to LinRegPCR software for baseline correction, determination of PCR efficiency and cycle quantification (Cq) values per sample. Cq values were obtained from the LinReg PCR software. The log-linear portion of the amplification curve used for analysis on LinReg PCR software contained from four to six points with the highest R^2 value. Relative quantification for all tested genes was processed according to the $\Delta\Delta C_t$ method, using cyclophilin as a reference gene. Identity of each PCR amplification product was confirmed by sequencing.

Masson's trichrome staining

Paraplast embedded tissues were sectioned (4 μ m). Three histological sections from each animal were deparaffinized in xylene, rehydrated through a graded series of ethanol

(100%, 95% and 70%), rinsed in running tap water for 10 min and a final rinse in distilled water. Tissues were stained in Weigert's iron hematoxylin working solution for 10 min, rinsed in running tap water for 10 min and in distilled water. Then tissues were stained in Biebrich scarlet-acid fuchsin solution for 15 min and washed in distilled water placed in phosphomolybdic-phosphotungstic acid solution for 10 min and transferred directly (without rinse) to aniline blue solution and stained for 15 min. Next, tissues were washed in running tap water and rinsed briefly in distilled water, and then immersed in 1% acetic acid solution for 3 min. The tissues were washed in distilled water, dehydrated very quickly through 95% ethyl alcohol, absolute ethyl alcohol and cleared in xylene. Finally, tissues were mounted under a coverslip. From each section, ten random high-power fields (40 $\times$ objective) were digitized: five that were adjacent to the uterine lumen (denominated superficial glandular epithelium) and five that were adjacent to myometrium. This method was chosen to avoid superimposing of photomicrographs and, thus, to separate deep from superficial areas. Staining was analyzed using ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA). The area and corresponding pixels represented by a range of the color blue, representing collagen content was calculated and represented as percentage for the superficial and deep compartments of the endometrium. The samples were analyzed by a single pathologist, who was blinded to information about the animals. The final value was calculated as the average of five different evaluated fields in each compartment, for each tissue section.

Immunohistochemistry for collagens type I and III

Four cows were selected from each group on D4 and D7 for immunohistochemical analyses of collagen tissue distribution. From each cow, histological sections (4 μ m) were deparaffinized, rehydrated and subjected to enzymatic digestion with 0.4% pepsin (Sigma) diluted in 0.5N acetic acid for 30 min at 37°C. After blocking endogenous peroxidase with 6% H_2O_2 solution (Merck) for 30 min, the slides were incubated in a humidified chamber overnight at 4°C with the

Table 6 Validation of RNAseq ($n=5-6$ cows/group) gene expression data by qPCR ($n=10-16$ cows/group). Fold change and probability value ($P>F$) of gene expression in uterine biopsies from non-pregnant vs pregnant animals.

Ensembl Id	Gene symbol	RNAseq			qPCR		
		Fold change	log2 fold change	$P>F$	Fold change	log2 fold change	$P>F$
ENSBTAG000000020457	TGFB1	1.46	0.55	0.018	1.23	0.30	0.051
ENSBTAG00000005359	TGFB2	0.73	-0.46	0.016	1.20	0.26	0.032
ENSBTAG00000005043	TIMP1	0.87	-0.20	0.018	2.04	1.03	0.043
ENSBTAG00000010899	TIMP2	1.01	0.01	0.369	0.92	-0.12	0.326
ENSBTAG000000020638	TIMP3	1.40	0.49	0.033	0.59	-0.76	0.019
ENSBTAG00000019267	MMP2	1.56	0.64	0.240	1.22	0.29	0.277
ENSBTAG000000014824	MMP14	1.46	0.54	0.134	1.38	0.46	0.215
ENSBTAG00000000706	ADAMTS1	1.66	0.73	0.001	1.21	0.27	0.003
ENSBTAG000000014665	ADAMTS2	1.56	0.64	0.005	1.22	0.29	0.000
ENSBTAG000000013210	ADAMTS4	1.62	0.69	0.013	0.99	-0.01	0.230
ENSBTAG00000000648	ADAMTS5	1.37	0.45	0.083	1.22	0.29	0.055
ENSBTAG000000013472	COL1A2	1.40	0.49	0.050	1.49	0.57	0.033
ENSBTAG000000021466	COL3A1	1.44	0.52	0.041	1.22	0.29	0.023

following rabbit primary antibodies: collagen type I (#600-401-103, Rockland, USA; 1:3000) or collagen type III (#600-401-105, Rockland, USA; 1:5000). The slides were then incubated with the complex Super Picture Polymer Detection kit (Life Technologies) for 30 min at 37°C. The reaction was visualized with 3'3' diaminobenzidine chromogen and counterstained with Harris hematoxylin. The negative controls were performed replacing the primary antibodies with normal rabbit IgG (#sc-2027, Santa Cruz Biotechnology). Sections were finally observed by light microscopy using the 10× or the 40× objectives. One representative image from each group on each of days 4 or 7 was selected for visualization.

Statistical analyses

The data were tested for normality of residues using Shapiro-Wilk test. Data that were not normal were transformed by log10. Ovarian, endocrine and transcript abundance variables were analyzed by one-way ANOVA to test the effect of group (LF-LCL vs SF-SCL) using the PROC GLM procedure of the SAS software (Version 9.2; SAS Institute). Histology data were analyzed by two-way ANOVA to test the effect of group, region (superficial or deep compartments of the endometrium) and the interaction.

Results

Experiment 1: Influence of peri-ovulatory hormonal modulation on endometrial ECM remodeling characteristics on day 7 of the estrous cycle

Animal model

Results from the animal model used here were reported previously (Mesquita *et al.* 2015). Briefly, in Study I, follicle diameter on D-1, plasma concentration of E2 on D-1, CL area on D4 and plasma concentration of P4 on D4 were 38.81, 275.54, 65.68 and 75% greater for the LF-LCL compared to the SF-SCL group, respectively ($P<0.01$). Similarly, in Study II, follicle diameter on D0, plasma concentration of E2 on D-1, CL volume on D7 and plasma concentration of P4 on D7 were 23.99 ($P<0.01$), 360 ($P<0.01$), 61.67 ($P<0.04$) and 47.79%

($P<0.04$) greater for the LF-LCL compared to the SF-SCL group, respectively.

ECM-related gene expression

A comprehensive analysis of the RNA-seq data of Experiment 1 was reported previously (Mesquita *et al.* 2015). Briefly, the analysis of day 7 endometrial LF-LCL vs SF-SCL samples yielded a total of approximately 134 million reads and approximately 62% of the total reads uniquely mapped to the UMD 3.1 reference genome (<http://www.ncbi.nlm.nih.gov/genome/guide/cow/index.html>). After applying the filter for variance and minimal value of baseMean, a total of 562 genes showed differential expression (adjusted P value <0.1), of which 364 and 198 were upregulated in the endometrium of LF-LCL and SF-SCL animals, respectively. Functional enrichment analysis for the SF-SCL endometrial tissue revealed clusters with overrepresented ontology terms and activation of pathways associated with extracellular matrix region and organization (Table 2).

Histological and immunohistochemical evaluation of collagen content and tissue distribution

The Masson's trichrome staining method was used to determine the abundance of total collagen in the endometrium. There was increased collagen content in the low-receptivity SF-SCL group on day 4 ($P=0.02$; Fig. 2 and Table 3). On days 4 and 7, a greater abundance of collagen was observed in the deep compartment compared to the superficial in both treatment groups ($P<0.05$).

Immunohistochemistry analyses were performed to indicate cell-specific distribution of collagens I and III (Figs 3 and 4, respectively) in the endometrium. There were gradients of staining among animals and, sometimes, within a same animal. Collagen type I was scarce in the *stratum compactum* and found predominantly within the *stratum spongiosum*, in both groups. It appeared as thicker fibers around endometrial glands and blood vessels (Fig. 3). In the SF-SCL group, a

Table 7 ECM-related genes detected in the RNAseq data comparing cows with distinct peri-ovulatory milieus (SF-SCL vs LF-LCL; $n=3$ cows/group) and pregnancy status (NP vs P; $n=5-6$ cows/group). Fold changes were the ratio of the mean expression values of SF-SCL/LF-LCL and NP/P.

Ensembl Id	Gene symbol	SF-SCL vs LF-LCL			NP vs P		
		Fold change	log2 fold change	$P>F$	Fold change	log2 fold change	$P>F$
ENSBTAG00000020457	<i>TGFB1</i>	1.25	0.32	0.627	1.46	0.55	0.018
ENSBTAG00000005359	<i>TGFB2</i>	1.77	0.82	0.053	0.73	-0.46	0.016
ENSBTAG00000012004	<i>TGFB3</i>	1.23	0.30	0.932	1.03	0.04	0.870
ENSBTAG00000005043	<i>TIMP1</i>	1.49	0.57	0.018	0.87	-0.20	0.018
ENSBTAG00000010899	<i>TIMP2</i>	0.86	-0.22	0.369	1.01	0.01	0.369
ENSBTAG00000020638	<i>TIMP3</i>	0.68	-0.55	0.032	1.40	0.49	0.033
ENSBTAG00000019267	<i>MMP2</i>	1.37	0.46	0.240	1.56	0.64	0.240
ENSBTAG00000014824	<i>MMP14</i>	1.44	0.53	0.134	1.46	0.54	0.134
ENSBTAG00000014665	<i>ADAMTS2</i>	1.46	0.55	0.054	1.56	0.64	0.005
ENSBTAG00000000648	<i>ADAMTS5</i>	1.71	0.77	0.000	1.37	0.45	0.083
ENSBTAG00000013103	<i>COL1A1</i>	1.5	0.58	0.058	1.07	0.10	0.745
ENSBTAG00000013472	<i>COL1A2</i>	1.56	0.64	0.001	1.40	0.49	0.050
ENSBTAG00000021466	<i>COL3A1</i>	1.62	0.70	0.000	1.44	0.52	0.041
ENSBTAG00000012849	<i>COL4A1</i>	1.38	0.46	0.032	1.19	0.26	0.274
ENSBTAG00000014575	<i>COL4A5</i>	0.64	-0.65	0.027	–	–	–

dense sub-epithelial mesh was observed on day 4, but not on day 7, in which larger amounts of collagen I were found in the *stratum spongiosum*. Collagen type-III had ubiquitous distribution (Fig. 4). It was found in both the *stratum compactum* and *stratum spongiosum*, in lower quantities than collagen type I. The fibers were thin, homogeneously distributed in the interstitium, mainly around blood vessels.

Experiment 2 – ECM remodeling in endometrial biopsies collected 6 days after AI and its relation to pregnancy success

Animal model

Results from the animal model used here were reported previously (Binelli et al. 2015). A total of 51 cows were synchronized and 33 displayed estrus, ovulated and hence, received AI. Cows showing P4 concentrations on day 6 after AI between 4.3 and 5.8 ng/mL were selected for RNA sequencing P ($n=6$) and NP ($n=6$).

ECM-related gene expression

A comprehensive analysis of the RNA-seq data of Experiment 2 was reported previously (Binelli et al. 2015). Briefly, endometrial ECM-related gene expression levels in P vs NP day 6 endometrial tissue P and NP samples were analyzed in an Illumina single flow cell line. The 272,685,768 million filtered reads were mapped to the *Bos taurus* UMD3.1 reference genome, and 14,654 genes were effectively analyzed for differential expression between groups. From six biopsies collected in the NP cows, one sample did not align with the bovine genome and was omitted from the analysis. Transcriptome data showed that 216 genes were differently expressed when comparing P vs NP endometrial tissue ($P_{adj} < 0.1$). More specifically, 36 genes showed a significantly upregulated expression

for pregnant cows and 180 were upregulated for non-pregnant cows. Functional annotation clustering for the NP group revealed most significantly enriched pathways also associated with ECM remodeling (Table 4).

Experiments 1 and 2. Overlapping ECM-related gene expression

The RNA-seq data suggest that most enriched pathways in the SF-SCL and NP groups play a relevant role in endometrial extracellular matrix remodeling. Therefore, we focused on the expression of such genes and used quantitative real-time PCR (qRT-PCR) to compare samples from LF-LCL and SF-SCL animals on day 7 of the estrous cycle to detect changes due to a distinct peri-ovulatory endocrine milieu (Table 5). The same was done for samples from P and NP animals (Table 6). The mRNA expression of *TGFβ1* was increased in the NP group and *TGFβ2* was increased in both NP as well as in the SF-SCL group on day 7. A similar mRNA expression pattern was observed for *MMP2*, *MMP11* and *MMP14* for the NP group. As metalloproteinase inhibitors are a crucial factor to control MMP activity in endometrial ECM remodeling in humans (Irwin et al. 2001), we investigated their mRNA expression. *TIMP1* and *TIMP4* expression significantly increased in the SF-SCL and NP groups respectively, whereas *TIMP3* was highly expressed in both SF-SCL and NP groups.

Expression of *TIMP2* was neither influenced by steroid hormone levels nor the animal's receptivity status. A significant increase ($P < 0.05$) in several ADAM and ADAMTS mRNA expression level was observed in both SF-SCL and NP groups. In both experiments, the expression of mRNA was increased for several collagens including collagen type I (COL1A2), collagen type III (COL3A1), collagen type VII (COL7A1) and collagen type IX (COL9A3), although other types of collagens were independently overexpressed in each individual

experiment (Table 7). According to the gene ontological classification, such collagens, especially COL1A1, COL1A2, COL3A1, COL4A1 and COL4A2 are essentially involved in ECM receptor interaction pathway and structure organization, and hence, remodeling.

Discussion

Peri-ovulatory regulation of mice and human endometrial tissue remodeling has been considered a prerequisite for proper maternal receptivity. In comparison with the latter species, cows are expected to exhibit a much more limited degree of tissue remodeling during the reproductive cycle. However, recent studies identified ECM remodeling pathways as potential key for acquisition of maternal receptivity (Mesquita *et al.* 2015) and pregnancy success (Binelli *et al.* 2015). However, in cattle, clear insights in how, and to which extent, the ECM remodeling events are being regulated during early diestrus are lacking. Therefore, this study focused on the characterization of the ECM signature of the bovine endometrial tissue during early diestrus.

The first set of experiments revealed that specific peri-ovulatory endocrine profiles do impact on the ECM-related transcriptome signature of day 7 endometrial tissue. More specifically, higher mRNA levels for *MMP2*, *COL1A2* and *TIMP1*, that are genes encoding molecules involved in ECM remodeling processes, were observed in the SF-SCL endometrial tissue compared with the LF-LCL counterparts. The process of extracellular matrix remodeling is a dynamic process that requires the degradation of its components. In this context, collagenases, such as the MMPs, are considered as key mediators triggering degradation of the main ECM components (Nagase *et al.* 2006). More specifically, collagenases (MMP-1, -8, and -13) cleave the interstitial collagens I, II and III. The denatured collagen, or gelatin, is further degraded by gelatinases (MMP-2, and -9) (Snoek *et al.* 2005, Sluijter *et al.* 2006). Interestingly, based on the temporal fluctuations in collagen abundance between day 4 (SF-SCL > LF-LCL) and day 7 (SF-SCL ~ LF-LCL) endometrial tissue, a clear switch in sex steroid-mediated ECM remodeling activity can be reported during early diestrus. Thereby, the data seem to confirm that a physiological reorganization of the ECM during diestrus is required to promote a receptive endometrial state. Salilew-Wondim and coworkers (2010) also confirmed a higher expression of *COL1A1*, *COL1A2* and *COL27A1* in cows with a lower receptivity as seen in our animals with SF-SCL. Moreover, a study performed in cows revealed that genes related to ECM remodeling are more abundant at estrus, whereas at diestrus, the more prominent genes are related to immune response and metabolic pathways (Mitko *et al.* 2008).

Besides the gene expression data pointing to ECM remodeling events at transcriptome level, colorimetric assessments were used to evaluate potential phenotypic differences in endometrial ECM remodeling. Overall, the collagen visualization revealed a greater abundance of total collagen content in the SF-SCL group on day 4, but not at day 7. Remarkably, on day 4, pre-ovulatory follicle size appeared to have a greater influence on differences in collagen abundance in both epithelia, compared to data retrieved on day 7. At the latter timing, only a regional difference in collagen abundance was noted, being greater in the deep endometrial compartment (i.e., *stratum spongiosum*) compared to the superficial (i.e., *stratum compactum*). Such finding was consistent with the immunoreactive abundance of collagen I (Fig. 3). Main patterns of collagen I distribution were similar to the previous descriptions by Boos (2000). For example, a sub-epithelial felt was described by that author and also clearly visible in Fig. 3 (panel A). Regarding collagen III (Fig. 4), it was clearly associated with blood vessels and capillaries, which is also consistent with descriptions by Boos (2000). That author reported changes in distribution of both collagens I and III in the uterine wall across the estrous cycle. He suggested that the cycle dependent, sex steroid concentration changes probably regulate the abundance and distribution of these and other collagens. This concept is consistent with our gene expression data for Experiment 1, in that hormonal differences associated with each experimental group were associated with different patterns of ECM-related transcript abundance.

The bovine data documented in the present study match with human data available, represented by similar differences in ECM transcripts, as well as effective collagen abundance, when comparing receptive vs low-receptive endometrial samples during early diestrus (Jokimaa *et al.* 2002). Data from human studies also showed previously that hormonal profiles modulate changes in the ECM's composition and distribution (Marbaix *et al.* 1992, Rodgers *et al.* 1994, Curry & Kevin 2001). During the process of decidualization, the expression of MMPs is inhibited and TIMPs are stimulated; hence, endometrial MMP expression appears to be negatively regulated by P4. Expression of MMPs is suppressed during the secretory phase of the cycle and increased at menstruation (Osteen *et al.* 1999). In this context, women with unexplained infertility and recurrent miscarriages exhibit higher expression of collagen type I, MMP2 and TIMP1 (Jokimaa *et al.* 2002).

In the second experiment described in the present study, the transcriptome signature from day 6 uterine biopsies, all collected in the same cycle of the AI, was determined with particular focus on ECM remodeling pathways. This implies that, data on pregnancy outcome could be retrospectively linked with endometrial transcript characteristics. Samples were collected from contralateral uterine horn in relation to

the pregnancy without a negative effect on pregnancy outcome as reported previously (Pugliesi *et al.* 2014). Main pathways on day 6 P vs NP uterine tissue were related to ECM remodeling processes. Indeed, *COL7A1* and *ADAM12*, which are genes associated with extracellular matrix formation, belong to the top-20 list of genes with downregulated expression in the P uterus. In addition, *MMP11* also had a higher expression in the NP group, which has been linked to cell proliferation properties. This is in line with a study conducted by Bauersachs and coworkers (2006) that indicated that the expression of collagen type I (*COL1A1* and *COL1A2*) and collagen type VI (*COL6A1*) were reported to be higher in non-pregnant cows compared to pregnant cows. In accordance, *COL1A2* was also more expressed in NP cows in the present study. Other genes that were differently expressed between P and NP day 6 endometrial tissue included *ADAM12*, *ADAMTS1*, *TIMP3*, *BGN*, *EGFLAM*, *ANGPT4*, *AGRN*, *BMP4* and *MMP2*. Furthermore, also an increased Decorin (*DCN*) mRNA expression in the NP group and also concomitant upregulated levels of *TGFβ1* and *TGFβ2* were observed in the NP group compared to their P counterparts. Decorin is a leucine-rich proteoglycan associated with type I collagen fibrils in tissues to which TGFβ binds with high affinity in the ECM and in doing so neutralizes its activity. Thereby, Decorin acts as a reservoir of TGFβ in the extracellular milieu. TGFβ is a potent stimulator of collagen synthesis by increasing transcription and decreasing collagen degradation via reduced MMPs or enhanced TIMPs, thus favoring an accumulation of ECM and especially of collagen (Roberts *et al.* 1992, Brenner *et al.* 1994, Arici *et al.* 1996, Siwik & Colucci 2004). Interestingly, the presented data are in line with human studies, in which uterine tumor formations, such as leiomyoma, are characterized by an increased synthesis and deposition of ECM proteins, mainly collagens type I and III as well as an increased mRNA abundance of *MMP2* and *MMP11* (Wolańska *et al.* 2004, Malik *et al.* 2010, Iwahashi & Muragaki 2011). The increased expression of collagens, in combination with other differences observed in ECM related genes when comparing the low-fertile endometrial tissue, is suggestive for upregulated cell proliferation activities. In agreement, work performed by our group (Mesquita *et al.* 2015) suggests that early endometrial tissue of cows with lower probability for ongoing pregnancy are more permissive to proliferation and less favorable to activities related to cell differentiation.

When comparing the high-fertile (LF-LCL and P) and low-fertile (SF-SCL and NP) endometrial tissue from Experiment 1 and Experiment 2, respectively, a significant downregulated expression of genes involved of ECM remodeling pathways was noticed in both the high-receptive LF-LCL as well in the P endometrial tissue (shown in Table 7). These results suggest that ECM components should be appropriately

expressed or inhibited, and it leads to the proposition that ECM overexpression could possibly inhibit the initial embryonic contact with maternal endometrial tissue, thereby prompting pregnancy failure. Several authors have indeed shown the importance of pre-ovulatory follicle size on pregnancy outcome (Vasconcelos *et al.* 2001, Perry *et al.* 2005, Baruselli *et al.* 2012). Here, our analysis suggested that as follicle size increases, expression of ECM remodeling genes change, which may imply that there is a specific ECM phenotype that more adequately supports fertility. However, it is curious that a set of genes was commonly regulated between the two models despite the fact that cows in Experiment 2 were selected to show similar concentrations of progesterone among individuals. It is tempting to speculate that both sex steroid-dependent and -independent mechanisms are in place to control ECM remodeling and its effects on embryo receptivity.

Both fertility models used in the present study might provide a useful tool to modulate and fine-tune, within physiological limits, the uterine 'receptive' environment and thereby influence the probability for ongoing pregnancy. Our main focus was the characterization of specific ECM signatures in bovine endometrial tissue during the early diestrus period; a timing that coincides with the process of bovine embryo arrival in the uterus. This is a highly crucial moment that needs further investigation as several studies identify this receptivity period as the reason for the dramatic incidence in embryonic losses in cattle industry.

In conclusion, early diestrus ECM remodeling events can be considered as key factors determining pregnancy outcome. The first set of experiments showed that specific endocrine profiles, that drive endometrial receptivity, also control ECM-related gene expression profiles and even phenotypic characteristics of the ECM in the tissue. The second experiment determined the ECM-related gene expression as potential predictors for probability of further pregnancy success. The overlapping set of genes being differently expressed in both fertility-related models highlights the importance of this matter and the potential to use the ECM remodeling data as tools to modulate receptivity and predict pregnancy outcome.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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