Placental development during early pregnancy in sheep: cell proliferation, global methylation, and angiogenesis in the fetal placenta

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Abstract

To characterize early fetal placental development, gravid uterine tissues were collected from pregnant ewes every other day from day 16 to 30 after mating. Determination of 1) cell proliferation was based on Ki67 protein immunodetection; 2) global methylation was based on 5-methyl-cytosine (5mC) expression and mRNA expression for DNA methyltransferases (DNMTs) 1, 3a, and 3b; and 3) vascular development was based on smooth muscle cell actin immunolocalization and on mRNA expression of several factors involved in the regulation of angiogenesis in fetal membranes (FMs). Throughout early pregnancy, the labeling index (proportion of proliferating cells) was very high (21%) and did not change. Expression of 5mC and mRNA for DNMT3b decreased, but mRNA for DNMT1 and 3a increased. Blood vessels were detected in FM on days 18–30 of pregnancy, and their number per tissue area did not change. The patterns of mRNA expression for placental growth factor, vascular endothelial growth factor, and their receptors FLT1 and KDR; angiopoietins 1 and 2 and their receptor TEK; endothelial nitric oxide synthase and the NO receptor GUCY13B; and hypoxia inducing factor 1α changed in FM during early pregnancy. These data demonstrate high cellular proliferation rates, and changes in global methylation and mRNA expression of factors involved in the regulation of DNA methylation and angiogenesis in FM during early pregnancy. This description of cellular and molecular changes in FM during early pregnancy will provide the foundation for determining the basis of altered placental development in pregnancies compromised by environmental, genetic, or other factors.

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Introduction


The placenta represents a type of organ, which expresses a high rate of growth in order to fulfill the metabolic demands of the growing fetus (Reynolds et al. 2002, 2006, 2010). Although the role of hypertrophy and hyperplasia in placental growth has been recognized (Boos et al. 2006, Murphy et al. 2006), very limited data are available concerning the rates and pattern of cell proliferation in fetal membranes (FMs) during early pregnancy. However, high proliferation rates have been reported for placenta during early pregnancy in several species (Blankenship & King 1994, Correia-da-Silva et al. 2004, Wei et al. 2005, Kar et al. 2007, Grazul-Bilska et al. 2010). In addition, it has been demonstrated using transcriptome analysis that genes which regulate trophoblast cell proliferation, cell differentiation and angiogenesis and numerous other genes that facilitate mother–fetus interactions are upregulated in the fetal placenta during early pregnancy in ruminants (Blomberg et al. 2008).

DNA methylation, which is catalyzed by DNA methyltransferases (DNMTs), is generally associated with transcriptional silencing and imprinting, principally
occuring at cytosine residues located in dinucleotide CpG sites and is the most extensively characterized epigenetic mark in mammals (Hiendleder et al. 2004, Wilson et al. 2007, Beck & Rakyan 2008). In fact, DNMTs are required for cell differentiation during embryonic development to regulate gene expression through methylation mechanisms (Gopalakrishnan et al. 2008). DNMT1 is primarily considered the maintenance methyltransferase (Bird 2002, Gopalakrishnan et al. 2008, Kim et al. 2009); however other functions of DNMT1, such as methylation of non-CpG sites in DNA bubbles have recently been discovered (Ross et al. 2010). Other methyltransferases, DNMT3A and DNMT3B are responsible for establishing de novo DNA methylation patterns (Gopalakrishnan et al. 2008).

Although DNA methylation is the most commonly studied mode of epigenetic regulation, the process of methylation/demethylation or the expression of the enzymes that promote methylation has not been investigated in detail in the placenta. It has been demonstrated that around the time of gastrulation and implantation, de novo methylation reestablishes the developing organism’s methylation patterns both in the embryo and in the extraembryonic tissues (Maccani & Marsit 2009). However, the pattern of methylation in the embryo differs from the extraembryonic tissues (Monk et al. 1987, Katari et al. 2009). In human placenta collected from several stages of pregnancy and at term, low expression of 5-methyl-cytosine (5mC) and relative hypomethylation have been reported (Kokalj-Vokac et al. 1998, Katari et al. 2009). Many genes expressed in extraembryonic tissues are imprinted (Reik et al. 2001, Myatt 2006, Jansson & Powell 2007), and several of these imprinted genes are involved in regulating the fetal and placental growth (Reik et al. 2001, 2003, Myatt 2006, Wagschal et al. 2008). Thus, DNA methylation plays a significant role during embryonic and placental development in physiological and pathological conditions (Kim et al. 2009). However, little is known about global methylation and expression of DNMTs in placental tissues during early pregnancy in any species.

Vascularization of both the fetal and maternal placenta is a critical factor in pregnancy maintenance (Zygmunt et al. 2003, Huppertz & Peeters 2005, Reynolds et al. 2006, 2010, Arroyo & Winn 2008). Fetal placental vasculogenesis, which is a result of de novo formation of blood vessels, is initiated very early in pregnancy (e.g. in humans about 21 days, in rhesus monkey about 19 days post-conception; Kaufmann et al. 2004). Vasculogenesis is very tightly regulated by angiogenic and other factors (Flamme et al. 1997, Patan 2000, Kaufmann et al. 2004, Demir et al. 2007). Although the expression of several factors involved in the control of angiogenesis has been studied in the placenta of several species, limited information concerning the expression of these factors is available for FM during early pregnancy.

We hypothesized that the patterns of cellular proliferation, global methylation of DNA, expression of several DNMTs, vascular development, and expression of factors involved in the regulation of angiogenesis in FM will change as early pregnancy progresses. Therefore, the objective of this study was to determine 1) labeling index (LI); a proportion of proliferating cells), 2) global methylation based on the expression of 5mC in DNA and expression of mRNA for DNMT1, 3a, and 3b, 3) development of blood vessels based on immunodetection of smooth muscle cell actin (SMCA; a marker of pericytes and smooth muscle cells, and thus blood vessels), and 4) expression of 12 factors involved in the regulation of angiogenesis and their receptors including placental growth factor (PGF), vascular endothelial growth factor (VEGF), and their receptors FLT1 and KDR; fibroblast growth factor (FGF) 2 and its receptor 211c; angiopoietins (ANGPT) 1 and 2 and their receptors TEK; endothelial NO synthase (NOS3) and its receptor soluble guanylate cyclase (GUCY1B3); and hypoxia inducing factor 1 α (HIF1A) in FM during early pregnancy in sheep.

**Results**

The length of the fetus increased (P<0.0001) approximately threefold from day 20 to 30 of pregnancy (Fig. 1A). Labeling index (a proportion of proliferating cells, based on Ki67 protein detection) did not change significantly (P>0.2) from day 16 to 30 of pregnancy (Fig. 1B). Overall, LI was 20.7 ± 1.5% in FM, and ranged from 17 to 26%; regression analysis demonstrated a linear decrease (R² = 0.110; P<0.055; Y = −0.63X + 36.3) from day 16 to 30 of pregnancy. Ki67, 5mC, and SMCA proteins were immunodetected in the FM throughout early pregnancy (Fig. 2A, B, and C). Ki67 and 5mC were localized to the cell nuclei (Fig. 2A and B), but SMCA was localized to cytoplasm of blood vessel cells (Fig. 2C).

Image analysis demonstrated that positive 5mC staining occupied 10.5 ± 1.0% of cell nuclei in FM (range 9–13%), and significant changes were not observed throughout early pregnancy. However, DNA dot blot analysis demonstrated an approximately twofold decrease (P<0.003) in 5mC expression in FM on days 16–20 compared with that on days 28–30, and regression analysis demonstrated a cubic decrease (R² = 0.355; P<0.0003; Y = −12.07 + 1.89X − 0.09X² + 0.001X³) throughout early pregnancy (Fig. 3A). Expression of DNMT1 mRNA tended (P<0.11) to increase approximately twofold from day 16 to 30 (Fig. 3B), and regression analysis demonstrated a linear increase (R² = 0.173; P<0.002; Y = 0.18 + 0.03X) throughout early pregnancy. Expression of DNMT3a mRNA increased (P<0.004) approximately twofold from day 16 compared with days 24–30 (Fig. 3C), but DNMT3b mRNA decreased (P<0.0001) approximately
threefold from day 16 to 18 compared with days 20–22 and decreased by fivefold on day 30 (Fig. 3D) of pregnancy. Regression analysis of mRNA expression for DNMT3a demonstrated a linear increase ($R^2 = 0.301$; $P < 0.0001$; $y = -0.06 + 0.04x$) but for DNMT3b a cubic pattern ($R^2 = 0.624$, $P < 0.0001$; $Y = 11.57 - 0.97X + 0.02X^2 - 0.0002X^3$) of decrease throughout early pregnancy.

Blood vessels marked with SMCA were detected in FM on days 18–30 of pregnancy (Fig. 2C). Overall, the number of blood vessels per FM tissue area was $1.7 \pm 0.4/10,000 \, \mu m^2$, ranging 0–7/10,000 $\mu m^2$, and did not change throughout early pregnancy.

Expression of mRNA for factors involved in the regulation of angiogenesis including $PGF$, $VEGF$, $FLT1$, $KDR$, $ANGPT1$, $ANGPT2$, ANGPT receptor TEK, $FGF2$, $NOS3$, $GUCY1B3$, and $HIF1A$ (Fig. 4A–H), but not for $FGFR2IIIc$ (data not shown) in FM changed ($P < 0.0001$–0.06) during early pregnancy (Fig. 4A–K). $PGF$ mRNA expression increased ($P < 0.0001$) approximately twofold on days 28 and 30 compared with days 16–20 (Fig. 4B). $FLT1$ mRNA expression increased ($P < 0.0001$) ~5- to 50-fold on days 28 and 30 compared with days 16–24 (Fig. 4C). $KDR$ mRNA expression was 2- to 11-fold greater ($P < 0.0001$) on days 20–24 than on days 16–18 and 26–30 (Fig. 4D).

$ANGPT1$ mRNA expression was low on days 16–24 of pregnancy and then increased ($P < 0.0001$) ~2- to 50-fold on days 26–30 of pregnancy (Fig. 4E). Expression of $ANGPT2$ mRNA was not detectable on day 16 of pregnancy but increased ($P < 0.0001$) ~3.5- to 5-fold from day 18 to days 22–30 of pregnancy (Fig. 4F). $TEK$ mRNA expression increased ($P < 0.0001$) approximately seven- to ninefold from day 16 to days 20–24, and then decreased on days 26–30 (Fig. 4G).

$FGF2$ mRNA expression increased ($P < 0.06$) approximately four- to fivefold from day 16 to days 20–24, and then decreased on days 26–28 (Fig. 4H), whereas $NOS3$ mRNA expression increased ($P < 0.001$) ~5- to 16-fold from days 16–18 to days 22–30 of pregnancy (Fig. 4I). $GUCY1B3$ mRNA expression was approximately two- to sevenfold greater ($P < 0.01$) on day 18 than on any other day of pregnancy (Fig. 4J). $HIF1A$ mRNA expression was approximately one- to twofold greater ($P < 0.02$) on days 18, 20, and 30 than on days 16 and 24 of pregnancy (Fig. 4K).

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![Figure 1](image1.png)

**Figure 1** Crown-to-rump length of fetuses from day 20 to 30 of pregnancy (A) and labeling index (percentage of proliferating cells; B) in fetal membranes on days 16–30 of pregnancy. Fetuses from days 16 and 18 were not collected and measured due to their small size (<2 mm) and tissue transparency. a,b,c,d$P < 0.0001$; values ± S.E.M. with different superscripts differ within a specific measurement.

![Figure 2](image2.png)

**Figure 2** Representative photomicrographs of immunohistochemical staining for Ki67 (A), 5-methyl-cytosine (5mC) (B), and smooth muscle cell actin (SMCA) (C) in uterine tissues from day 24 of early pregnancy. Dark color represents positive staining, and pink color (nuclear fast red staining) indicates unlabeled cell nuclei. In (A), note nuclear staining of Ki67 (arrows) in fetal membranes (FM) and endometrium (E). In (B), note punctate staining of 5mC in the nuclei of the majority of cells (arrows) in FM and E, and a lack of staining in some cells (arrowheads) in FM. In (C), note SMCA cytoplasmic staining in blood vessels in FM (arrows) and E (arrowheads). In (D), note a lack of positive staining in the controls in which mouse IgG was used in place of the primary antibody.

$VEGF$ mRNA expression increased ($P < 0.0001$) approximately two- to sevenfold (data not shown) in FM changed ($P < 0.0001$–0.06) during early pregnancy (Fig. 4A–K). $PGF$ mRNA expression increased ($P < 0.0001$) ~3.5- to 34-fold from days 16–22 to days 24–30 of pregnancy (Fig. 4A).
Results of regression analysis demonstrating a pattern of change in mRNA expression for all 12 investigated genes involved in the regulation of angiogenesis are presented in Table 1. Correlation coefficients for mRNA expression of evaluated genes involved in the regulation of angiogenesis are presented in Table 2. Expression of mRNA for the majority of these genes was significantly (P<0.0001–0.08) correlated (Table 2).

Discussion

Early pregnancy is characterized by dramatic uterine and embryonic/fetal tissue growth, differentiation, and remodeling, and it is the critical period for establishing a healthy pregnancy. During this critical period, maternal recognition of pregnancy, initial attachment/implantation of FM to uterine epithelium, and initiation of placental growth and development take place (Bowen & Burghardt 2000, Spencer et al. 2007, 2008). In addition, most embryonic loss occurs in early pregnancy with rates of pregnancy losses reported as ≥30% in most mammalian species and possibly >50% in humans (Reynolds & Redmer 2001, Miri & Varmuza 2009). Thus, investigation of the fetal and maternal placental growth during early pregnancy is needed to establish the mechanisms that contribute to pregnancy maintenance or loss.

This study demonstrated a rapid increase in fetal size, decrease in 5mC expression (as determined by DNA dot blot), and dramatic changes in the mRNA expression in FM of several factors involved in the regulation of DNA methylation, angiogenesis, and tissue growth during early pregnancy. However, the rates of cellular proliferation were maintained at a high level but not significantly changed. In addition, image analysis did not show any differences in 5mC expression throughout pregnancy. The discrepancies between the results of 5mC evaluation by DNA dot blot and image analyses were likely due to the lower sensitivity of immunohistochemistry and image analysis than dot blot analysis.

Early embryonic development is tightly regulated and includes control of cell growth, proliferation and differentiation, morphogenesis, and protein synthesis and trafficking (Blomberg et al. 2008, Igwebuike 2009). In this study, growth of the fetal placenta was reflected by rapid increase in embryonic size and very high rates of cellular proliferation in FM. High rates of cell proliferation were also observed in the fetal placenta during early pregnancy in humans and monkeys (Wei et al. 2005, Korgun et al. 2006, Kar et al. 2007). Interestingly, cell proliferation in the fetal and maternal placenta obtained after transfer of embryos created in vitro or through parthenogenetic activation was less than in pregnancies after natural breeding in sheep (Borowicz et al. 2009; AT Grazul-Bilska, PP Borowicz, DA Redmer & LP Reynolds 2010, unpublished observations). In addition, altered placental cell proliferation or turnover was observed in several pathological conditions including diabetes and trophoblastic diseases at several pregnancy stages in humans (Burleigh et al. 2004, Zhang et al. 2009). These data suggest that altered cellular

![Figure 3](https://example.com/image.png)

**Figure 3** Expression of 5mC as determined by DNA dot blot assay (A) and mRNA for DNA methyltransferases (DNMTs) 1 (B), 3a (C), and 3b (D) in fetal membranes (FM) on days 16–30 of pregnancy. a,b,c,dP<0.0001–0.11; values±s.e.m. with different superscripts differ within each specific gene.
proliferation in the fetal placenta is a feature of compromised pregnancies. However, the mechanism of regulation of cell proliferation in FM has not been elucidated, and this subject requires additional investigation.

Since epigenetic modifications of the genome include methylation of DNA at cytosine residues and histone modifications through methylation catalyzed by DNMTs, we choose to use 5mC and DNMT1, 3a, and 3b as markers of global methylation in our study. In this study, expression of these markers was detected in FM, and the pattern of changes differed during early pregnancy. Interestingly, expression of 5mC decreased during early pregnancy indicating that demethylation was occurring in the FM. However, in our study, only one of the enzymes catalyzing methylation and/or demethylation, $DNMT3b$ (Ooi & Bestor 2008), had decreased mRNA expression; whereas expression of $DNMT3a$ mRNA increased during early pregnancy. Therefore, we hypothesize that a specific balance exists between

### Table 1 Regression analysis of angiogenic genes in fetal membranes (FM) from early pregnancy.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Regression type</th>
<th>$P$ value</th>
<th>$R^2$</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGF</td>
<td>Exponential sigmoidal</td>
<td>$&lt;0.0001$</td>
<td>0.7904</td>
<td>$Y = 5.284 \times 10^2 \exp(0.996X - 0.016X^2)$</td>
</tr>
<tr>
<td>VEGF</td>
<td>Exponential sigmoidal</td>
<td>$&lt;0.0001$</td>
<td>0.5432</td>
<td>$Y = 0.249 - 0.021X + 0.001X^2$</td>
</tr>
<tr>
<td>FLT1</td>
<td>Exponential sigmoidal</td>
<td>$&lt;0.0001$</td>
<td>0.7788</td>
<td>$Y = 0.0007 \exp(0.231X)$</td>
</tr>
<tr>
<td>KDR</td>
<td>Cubic</td>
<td>$&lt;0.0001$</td>
<td>0.4568</td>
<td>$Y = 13.535 + 1.724X - 0.069X^2 + 0.0009X^3$</td>
</tr>
<tr>
<td>ANGPT1</td>
<td>Exponential sigmoidal</td>
<td>$&lt;0.0001$</td>
<td>0.7860</td>
<td>$Y = 4.453 \times 10^{-12} \exp(1.621X - 0.027X^2)$</td>
</tr>
<tr>
<td>ANGPT2</td>
<td>Exponential sigmoidal</td>
<td>$&lt;0.0001$</td>
<td>0.7741</td>
<td>$Y = 4.083 \times 10^{-19} \exp(3.138X - 0.061X^2)$</td>
</tr>
<tr>
<td>TEK</td>
<td>Exponential sigmoidal</td>
<td>$&lt;0.0001$</td>
<td>0.4610</td>
<td>$Y = 2.823 \times 10^{-8} \exp(1.367X - 0.028X^2)$</td>
</tr>
<tr>
<td>FG2F</td>
<td>Exponential sigmoidal</td>
<td>0.0002</td>
<td>0.3033</td>
<td>$Y = 1.267 \times 10^{-11} \exp(1.855X - 0.036X^2)$</td>
</tr>
<tr>
<td>FGFR2</td>
<td>NS</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>NO3S</td>
<td>Cubic</td>
<td>0.0002</td>
<td>0.3393</td>
<td>$Y = 1.542 - 0.537X + 0.039X^2 - 0.0007X^3$</td>
</tr>
<tr>
<td>GUCY1B3</td>
<td>NS</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>HIF</td>
<td>Cubic</td>
<td>0.007</td>
<td>0.2250</td>
<td>$Y = -11.594 + 1.666X - 0.075X^2 + 0.001X^3$</td>
</tr>
</tbody>
</table>

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expression and/or function of DNMTs, and likely other enzymes involved in methylation and/or demethylation (e.g. DNA glycosylase; Zhu 2009) are present in the tissue to further regulate methylation processes. It is believed that genomic imprinting, regulated by methylation mechanisms, may play a critical role in placental biology (Coan et al. 2005, Maccani & Marsit 2009, Miri & Varmuza 2009). In fact, alterations in imprinting have been linked to placental pathologies (Tycko 2006, Wagshal & Feil 2006). Therefore, correction of the DNA methylation may offer new strategies for preventing pregnancy complications. However, more research is required to gain a better understanding of the mechanisms of imprinting and methylation in the placenta in order to establish a strategy for successful pregnancy outcomes.

Very few studies have evaluated global methylation during early placental development, but several studies investigated methylation in the placenta at specific time points. For human placenta, it has been demonstrated that methylation levels measured by 5mC content increased in a gestational stage-dependent manner (Fuke et al. 2004), that DNA methylation measured by the mean CpG methylation status of genes probed in a microarray analysis was decreased after in vitro versus in vivo conception (Katari et al. 2009), and that the decrease in X chromosome-linked placental methylation was greater in pregnancies carrying female than male babies Cotton et al. (2009). Studies of embryonic and/or extraembryonic tissues during mouse development demonstrated that global methylation and demethylation and expression of DNMT were stage and tissue specific (Monk et al. 1987, Trasler et al. 1996, Watanabe et al. 2002). For cows, global methylation in the fetal placenta on day 80 was similar for pregnancies established after transfer of embryos created through artificial insemination, in vitro fertilization, or somatic cell nuclear transfer (Hiendleder et al. 2004). In our study, significant changes in global methylation, measured by 5mC and DNMTs mRNA expression, indicate that the pattern of methylation in FM is changing throughout early pregnancy. Since data concerning the methylation process in developing and growing placenta are extremely limited, further studies should be undertaken to study this process in detail.

In this study, blood vessels marked with SMCA were detected in FM as early as on day 18 of pregnancy. For human placenta, it has been demonstrated that angiogenesis, manifested by vascular tube formation, and presence of hemangiogenic cell cords, was evident 21–27 days post-conception; on day 32 post-conception, erythrocytes were observed within blood vessels lumen, and between days 35 and 42, the networks of cords were heavily connected with each other without any interruption (Demir et al. 1989, 2004, Zygmunt et al. 2003, Torry et al. 2004, Arroyo & Winn 2008, Burton et al. 2009, van Oppenraaij et al. 2009). Thus, vasculogenesis is initiated very early in pregnancy in order to support dramatic fetal growth.

FM growth and vascular development have to be tightly regulated to coordinate the development of the fetal and maternal placenta and embryonic tissues. Therefore, vasculogenesis, angiogenesis, and tissue growth within the fetal placenta are regulated by numerous growth factors (Patan 2000, Zygmunt et al. 2003, Demir et al. 2007, Herr et al. 2009, Burton et al. 2009). In this study, increased mRNA expression of several factors and their receptors involved in the regulation of angiogenesis and growth in FM was observed as pregnancy progressed. In fact, changes in

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### Table 2: Correlation coefficients for mRNA expression of angiogenic factors in fetal membranes.

<table>
<thead>
<tr>
<th>Factor</th>
<th>VEGF</th>
<th>FLT1</th>
<th>KDR</th>
<th>ANGPT1</th>
<th>ANGPT2</th>
<th>TEK</th>
<th>FGFR2</th>
<th>FGFR2IIc</th>
<th>NOS3</th>
<th>GUCY1B3</th>
<th>HIF1A</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PGF</strong></td>
<td>0.735</td>
<td>0.750</td>
<td>0.783</td>
<td>0.544</td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td>0.453</td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
</tr>
<tr>
<td><strong>VEGF</strong></td>
<td>0.779</td>
<td>0.775</td>
<td>0.458</td>
<td><strong>NS</strong></td>
<td>0.290</td>
<td>0.274</td>
<td>0.510</td>
<td><strong>P&lt;0.0009</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
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<tr>
<td><strong>FLT1</strong></td>
<td>–</td>
<td><strong>NS</strong></td>
<td>0.080</td>
<td>0.485</td>
<td><strong>NS</strong></td>
<td>0.237</td>
<td>0.248</td>
<td>0.367</td>
<td><strong>P&lt;0.008</strong></td>
<td><strong>NS</strong></td>
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<tr>
<td><strong>KDR</strong></td>
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<td><strong>NS</strong></td>
<td>0.034</td>
<td><strong>NS</strong></td>
<td>0.358</td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
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<tr>
<td><strong>ANGPT1</strong></td>
<td>–</td>
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<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td>0.472</td>
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<td><strong>NS</strong></td>
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<tr>
<td><strong>ANGPT2</strong></td>
<td>–</td>
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<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
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<tr>
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<td><strong>GUCY1B3</strong></td>
<td>–</td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
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<td><strong>NS</strong></td>
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*NS, not statistically significant. $P \geq 0.1$. 

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mRNA expression of several growth/angiogenic factors and/or their receptors including PGF, FLT1, ANGPT1, TEK, NOS3, and HIF1A in FM paralleled the expression of these factors in maternal placenta in sheep (Grazul-Bilska et al. 2010), indicating a similar role of these factors in the regulation of fetal and maternal placental growth and function.

Although protein and/or mRNA expression of VEGF, PGF and receptors, and/or FGF2 and receptor was detected in extraembryonic tissues at specific stages of early pregnancy in monkeys, humans, and cows (Vuorela et al. 1997, Ghosh et al. 2000, Hildebrandt et al. 2001, Wang et al. 2003, Demir et al. 2004, Wei et al. 2004, Pfarrer et al. 2006), the changes during placential development have not been evaluated for these or other species. In this study, dramatic changes in the expression of mRNA for members of the VEGF and ANGPT systems were observed. Since during early pregnancy first vasculogenesis and then angiogenesis are initiated, it seems that high expression of VEGF and ANGPT systems is required to regulate these processes. In fact, members of the VEGF family and ANGPTs are recognized as the major regulators of vasculogenesis and angiogenesis in the placenta (Reynolds et al. 2002, 2006, Zygmunt et al. 2003, Demir et al. 2007, Seval et al. 2008). In the primate placenta, protein and/or mRNA expression of VEGF, FLT1, KDR, ANGPT1, ANGPT2, and/or receptor TEK was detected during early pregnancy (Demir et al. 2004, Wei et al. 2004, Seval et al. 2008, Demir 2009). These factors appeared to be spatio- and temporal-regulated during early pregnancy in primates (Demir et al. 2004, Wei et al. 2004, Kayisli et al. 2006, Seval et al. 2008). The increased expression of several angiogenic factors during early pregnancy indicates that these factors are involved in the regulation of vascular development, remodeling, and trophoblast function. However, functional studies should be undertaken to verify the specific roles of VEGF and ANGPT systems in placental growth and function.

Expression of mRNA for FGF2 but not for FGFR2IIIc in FM increased during early pregnancy in this study. Although the expression of FGF2 and its receptor was detected in the fetal placenta in sheep and other species (Kaufmann et al. 2004, Wei et al. 2004, Liu et al. 2005), little is known about the specific role of FGF system in early placental development. However, it has been demonstrated that FGFs stimulate differentiation of the embryonic germ layers, and it has been suggested that the FGF system is involved in the regulation of growth and differentiation of vascular and non-vascular compartments of the placenta (Reynolds et al. 2002). In addition, FGF2 is a potent stimulator of cell proliferation (Reynolds & Redmer 2001); therefore, it is reasonable to postulate that FGF2 and its receptor are involved in the regulation of cell proliferation in FM.

Expression of NOS3 mRNA gradually increased from day 16 to 22 and remained at a similar level until day 30, but the NOS3 receptor GUCY1B3 mRNA expression was enhanced only on day 18 of pregnancy in FM in our study. Endothelial NOS is expressed in the fetal placenta from early pregnancy in several species (Sladek et al. 1997, Ariel et al. 1998, Gagiotti et al. 2000, Al-Hijji et al. 2003). NOS are recognized as regulators of implantation and pregnancy maintenance, and angiogenesis in the fetal and maternal placenta (Gagiotti et al. 2000, Maul et al. 2003); however, the mechanism of NOS effects on these processes remains to be elucidated. Furthermore, it has been demonstrated that NOS3 expression is regulated by FGF2 and VEGF in ovine placental artery endothelial cells (Mata-Greenwood et al. 2008). These interactions seem to be reflected in our study by significant correlations between the mRNA expression of NOS3 and expression of members of the VEGF and FGF2 systems.

An increased HIF1A mRNA expression in FM was observed on days 18–20 of pregnancy in our study. This transient high expression of HIF1A mRNA may be associated with low oxygen levels observed during early pregnancy in several species (Rodesch et al. 1992, Rajakumar & Conrad 2000, Fryer & Simon 2006, Letta et al. 2006, Pringle et al. 2007). It seems that HIF1A expression is decreasing after delivery of oxygen is well established through developing blood vessel network. A decrease in HIF1A mRNA expression from day 50 to the end of pregnancy in the fetal placenta was observed in sheep (Borowicz et al. 2007). It has clearly been demonstrated using the knockout and other models that HIF activity is necessary for placental development, since HIF1A is involved in the regulation of placental morphogenesis, cell migration, angiogenesis, erythropoiesis, and cell metabolism, and is critical for adaptive responses to hypoxia (Cowden Dahl et al. 2005, Fryer & Simon 2006). In fact, HIF1A expression is altered in preeclampsia and IUGR placentas (Rajakumar et al. 2007, Zamudio et al. 2007). Therefore, HIF1A may be used as a marker of compromised pregnancies.

In summary, this study demonstrates a dramatic increase in fetal size, high cellular proliferation rates, decrease in 5mC expression (as determined by DNA dot blot), and lack of changes in vasculation measured as the number of blood vessels per tissue area, but significant changes in mRNA expression of factors involved in the regulation of methylation, angiogenesis, and tissue growth in FM during early pregnancy. Positive correlations among mRNA expression of several growth/angiogenic factors and/or their receptors indicate interactions among these factors in the regulation of development of fetal placenta. However, since we have evaluated the expression for the factors mentioned above at the mRNA level only, additional studies should be undertaken to determine the pattern of protein expression and its relation to mRNA expression in
order to better understand the process of placental growth and function. This description of cellular and molecular changes in FM during early pregnancy will provide a foundation for determining whether and how placental development is altered in compromised pregnancies. Furthermore, it will help to establish a baseline that can be used to design therapeutic treatments to restore normal fetal development in compromised pregnancies.

**Material and Methods**

**Animals**

The NDSU Institutional Animal Care and Use Committee approved all animal procedures in this study. Gravid uteri were obtained from crossbred Western Range (primarily Rambouillet, Targhee, and Columbia) ewes (n = 5 to 8 per day) on days 16, 18, 20, 22, 24, 26, 28, and 30 after mating (day of mating = day 0). At tissue collection for immunohistochemical staining, specimen pins were inserted completely through the uterus and FM at the level of the external intercornual bifurcation to maintain specimen morphology; cross sections of the entire gravid uterus (~0.5 cm thick) were obtained using a Stadie–Riggs microtome knife followed by immersion in formalin or Carnoy’s solution and embedding in paraffin. For total cellular RNA extraction, chorioallantoic FM were stored at −80°C. Purified DNA (0.5 µg) was denatured by adding NaOH and EDTA to final concentrations of 0.4 M NaOH and 10 mM EDTA, heated to 100°C for 10 min, followed by cooling to 4°C, and then neutralized with an equal volume of cold (4°C) 2 M ammonium acetate. Denatured DNA was spotted onto Ambion BrightStar-Plus Nylon Membrane (Ambion/Applied Biosystems, CA, USA), 5mC (a marker of global DNA methylation; 1:500; mouse monoclonal; Eurogentec North America, San Diego, CA, USA), or SMCA (a marker of pericytes and smooth muscle cells and thus blood vessels; 1:150; mouse monoclonal; Oncogene Research Products; San Diego, CA, USA) overnight at 4°C. Primary antibodies were detected by using secondary anti-mouse antibody coupled to peroxidase (ImPress Kit; Vector Laboratories). The sections were then counterstained with nuclear fast red (Sigma) to visualize cell nuclei. Control sections were incubated with normal mouse IgG (4 µg/ml) in place of primary antibody. Fetal placental cell types were not identified in this study due to methodological difficulties, such as a lack of specific markers for these cell types in sheep or absence of some cell types in individual tissue slides; thus we used the entire fetal placenta for immunohistochemical and other evaluations, which of course has some limitations that the reader should keep in mind.

**Image analysis**

For each tissue section, images were taken at 400× (Ki67 staining), 600× (5mC staining), or 200× (SMCA staining) magnification, using an Eclipse E600 Nikon microscope and digital camera for 5–40 randomly chosen fields (0.025 mm² per field) from areas containing FM. To determine LI, the percentage of 5mC positive staining in cell nucleus or the number of blood vessels per FM tissue area, an image analysis system (Image-Pro Plus, Media Cybernetics, Inc., Bethesda, MD, USA) was used as described previously (Grazul-Bilska et al. 2010). The LI was calculated as the percentage (%) of proliferating Ki67-positive cells out of the total number of cells within an FM tissue area.

**DNA dot blot assay**

DNA dot blot analysis of 5mC was based on modifications of previously described methods (Tao et al. 2004, Park et al. 2005). DNA was isolated from FM tissues homogenized in TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). Purified DNA (0.5 µg) was denatured by adding NaOH and EDTA to final concentrations of 0.4 M NaOH and 10 mM EDTA, heated to 100°C for 10 min, followed by cooling to 4°C, and then neutralized with an equal volume of cold (4°C) 2 M ammonium acetate. Denatured DNA was spotted onto Ambion BrightStar-Plus Nylon Membrane (Ambion/Applied Biosystems, CA, USA), 5mC (a marker of global DNA methylation; 1:500; mouse monoclonal; Eurogentec North America, San Diego, CA, USA), or SMCA (a marker of pericytes and smooth muscle cells and thus blood vessels; 1:150; mouse monoclonal; Oncogene Research Products; San Diego, CA, USA) overnight at 4°C. Primary antibodies were detected by using secondary anti-mouse antibody coupled to peroxidase (ImPress Kit; Vector Laboratories). The sections were then counterstained with nuclear fast red (Sigma) to visualize cell nuclei. Control sections were incubated with normal mouse IgG (4 µg/ml) in place of primary antibody. Fetal placental cell types were not identified in this study due to methodological difficulties, such as a lack of specific markers for these cell types in sheep or absence of some cell types in individual tissue slides; thus we used the entire fetal placenta for immunohistochemical and other evaluations, which of course has some limitations that the reader should keep in mind.

**Immunohistochemistry**

Immunohistochemical procedures were used as described before (Grazul-Bilska et al. 2010). Paraffin-embedded uterine tissues containing FM were sectioned at 4 µm and mounted onto slides. Sections were rinsed several times in PBS containing Triton X-100 (0.3%, v/v) and then were treated for 20 min with blocking buffer (PBS containing normal horse serum (2%, vol/vol)) followed by incubation with specific primary antibody for Ki67 (a marker of proliferating cells; 1:500; mouse monoclonal; Vector Laboratories, Burlingame, CA, USA), 5mC (a marker of global DNA methylation; 1:500; mouse monoclonal; Eurogentec North America, San Diego, CA, USA), or SMCA (a marker of pericytes and smooth muscle cells and thus blood vessels; 1:150; mouse monoclonal; Oncogene Research Products; San Diego, CA, USA) overnight at 4°C. Primary antibodies were detected by using secondary anti-mouse antibody coupled to peroxidase (ImPress Kit; Vector Laboratories). The sections were then counterstained with nuclear fast red (Sigma) to visualize cell nuclei. Control sections were incubated with normal mouse IgG (4 µg/ml) in place of primary antibody. Fetal placental cell types were not identified in this study due to methodological difficulties, such as a lack of specific markers for these cell types in sheep or absence of some cell types in individual tissue slides; thus we used the entire fetal placenta for immunohistochemical and other evaluations, which of course has some limitations that the reader should keep in mind.

**Table 3** Sequence of TaqMan primers and probes for DNMT1, DNMT3a, and DNMT3b.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Nucleotide sequence</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep DNMT1 FP</td>
<td>5'- CCT GGG TCC ACG GTG TTC -3'</td>
<td>NM_001009473</td>
</tr>
<tr>
<td>Sheep DNMT1 RP</td>
<td>5'- CCA CCC ATG ACC AGC TIC A -3'</td>
<td></td>
</tr>
<tr>
<td>Sheep DNMT1 probe</td>
<td>5'- (6FAM) AGA GTA CTG CAA CGT CCT -(MGBNFQ)-3'</td>
<td></td>
</tr>
<tr>
<td>Sheep DNMT3a FP</td>
<td>5'- TGC ACG AGG TAC GGC AGT G -3'</td>
<td>HQ203740</td>
</tr>
<tr>
<td>Sheep DNMT3a RP</td>
<td>5'- GGC TCC CAC AAG AGA TGC A -3'</td>
<td></td>
</tr>
<tr>
<td>Sheep DNMT3a probe</td>
<td>5'- (6FAM) ATG TCC TCG ATG TTC CC -(MGBNFQ)-3'</td>
<td></td>
</tr>
<tr>
<td>Sheep DNMT3b FP</td>
<td>5'- AGC GGC AGG CGA TGT CT -3'</td>
<td>HQ203741</td>
</tr>
<tr>
<td>Sheep DNMT3b RP</td>
<td>5'- GAG AAC TTG CCA TCA CCA AAC C -3'</td>
<td></td>
</tr>
<tr>
<td>Sheep DNMT3b probe</td>
<td>5'- (6FAM) CTG GAC CCA CCG CAT -(MGBNFQ)-3'</td>
<td></td>
</tr>
</tbody>
</table>

FP, forward primer; RP, reverse primer.

*Nucleotide sequences for ovine-specific genes were obtained from the National Center for Biotechnology Information (NCBI, 2010) database.*
Biosystems, Austin, TX, USA) using the BRL HYBRI-DOT Manifold (Bethesda Research Laboratories, Gaithersburg, MD, USA). The DNA was cross-linked to the membrane for 2 min with the CL-1000 Ultraviolet Crosslinker (UVP, Upland, CA, USA) and then dried. After wetting in dH2O, the membrane was blocked with 5% skim milk in PBS+0.1% Tween 20 (PBST) by rocking for 3 h at room temperature. The membrane was probed with a 1:2000 dilution in 2% milk–PBST of (PBST) by rocking for 3 h at room temperature. The membrane was washed three times for 10 min each in PBST before incubation with a 1:5000 dilution of HRP-conjugated anti-mouse secondary antibody in 2% milk–PBST with rocking for 1 h at room temperature. After three washes in PBST, the membrane was incubated with ECL Plus Western blotting reagent (GE Healthcare; Piscataway, NJ, USA), and the chemiluminescence of 5mC was detected and quantified using the AlphaEaseFC imager (Alpha Innotech, San Leandro, CA, USA). After detection of 5mC, the membrane was stained with 0.02% methylene blue for DNA quantification, and the relative dot intensity was measured with the AlphaEaseFC imager. Each sample was normalized to its DNA concentration by dividing the 5mC signal intensity of the sample by the dot intensity of methylene blue.

Quantitative real-time RT-PCR

All procedures for determining the expression of mRNA of several genes in ovine tissues by RT-PCR have previously been reported (Redmer et al. 2005, Johnson et al. 2006, Grazul-Bilska et al. 2010). Briefly, snap-frozen FM tissues were homogenized in TRI Reagent (Molecular Research Center) according to the manufacturer’s specifications. The quality and quantity of total RNA were determined via capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE, USA). Real-time RT-PCR reagents, probes, and primers were purchased from and used as recommended by Applied Biosystems. For each sample, 30 ng total RNA were reverse transcribed in triplicate 20 μl reaction volume using random hexamers. Sequence-specific TaqMan probes and primers were designed using the Primer Express Software from Applied Biosystems, and sequences for 12 factors involved in the regulation of angiogenesis have been published before (Redmer et al. 2005, Johnson et al. 2006, Grazul-Bilska et al. 2010). The sequences of probes and primers for DNMT1, 3a, and 3b are presented in Table 3. The ABI PRISM 7000 was used for detection of sequences amplified at 60 °C typically for 40 or 45 cycles (Applied Biosystems). Quantification was determined from a relative standard curve of dilutions of the cDNA generated from tcRNA pooled from placentomes collected on day 130 of pregnancy. Expression of each gene was normalized to the expression of 18S rRNA in a multiplex reaction using the human 18S pre-developed assay reagent (PDAR) from Applied Biosystems. The PDAR solution, which is primer limited and contains a VIC-labeled probe, was further adjusted by using one-fourth the normal amount, so that it would not interfere with amplification of the FAM-labeled gene of interest. Standard curves were also generated with the multiplex solution, and the quantity of 18S rRNA and the gene of interest were determined using each specific standard curve. The concentrations of mRNA were then normalized to 18S rRNA by dividing each of the mRNA values by their corresponding 18S rRNA value (Grazul-Bilska et al. 2010).

Statistical analysis

Data were analyzed using the general linear models procedure of SAS and presented as means ± s.e.m. with the main effect of day of pregnancy (SAS Institute 2010). When the F-test was significant (P<0.05), differences between specific means were evaluated by using the least significant differences test (Kirk 1982). The SAS procedure PROC REG was used for regression analysis, and PROC CORR was used to calculate simple linear correlations between specific variables.

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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References


Burleigh DW, Stewart K, Grindle KM, Kay HH & Golos TG 2003.11.007

Cotton AM, Avila L, Penaherrera MS, Affleck JG, Robinson WP & Brown CJ 2004 Patterns of hypoxia-inducible factors (HIFs) and temporal distribution of Tie-1 and Tie-2 during very early pregnancy in sheep: vascular growth and expression of angiogenic factor messenger ribonucleic acid levels in and vascularization of the villous placenta during baboon pregnancy. Endocrinology 142 2050–2057. (doi:10.1210/en.1.2.5.2050)


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