Gap junctional connexin mRNA expression in the ovine uterus and placenta:
Effects of estradiol-17 β -treatment, early pregnancy stages and embryo origin

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ABSTRACT

Gap junctions play a major role in direct, contact-dependent cell-cell communication, and they have been implicated in the regulation of cellular metabolism and the coordination of cellular functions during growth and differentiation of organs and tissues. Gap junctional channels, composed of connexin (Cx) proteins, have been detected and shown to be influenced by hormones (e.g. estrogen and /or progesterone) in uterine and placental tissues in several species. We hypothesized that 1) the mRNA for *Cx26*, *Cx32*, *Cx37* and *Cx43* is expressed in the uterus of ovariectomized sheep treated with estradiol-17 β (E2) and in ovine placenta during early pregnancy, 2) E2-treatment of OVX ewes would cause time-specific changes in *Cx26*, *Cx32*, *Cx37* and/or *Cx43* mRNA expression (Experiment 1), and 3) expression of these four Cx would vary across the days of early pregnancy (Experiment 2) and will be affected by embryo origin (i.e., after application of assisted reproductive technologies [ART]; Experiment 3). Thus we collected uterine tissues at 0 to 24 h after E2 treatments (Experiment 1), and placental tissues during days 14 to 30 of early pregnancy after natural (NAT) breeding (Experiment 2) and on day 22 of early pregnancy established after transfer of embryos generated through natural breeding (NAT-ET), in vitro fertilization (IVF) or in vitro activation (IVA, parthenotes; Experiment 3). In Experiment 1, expression of *Cx26*, *Cx37* and *Cx43* mRNA increased ($P < 0.05$) and *Cx32* mRNA decreased ($P < 0.06$) in both caruncular and intercaruncular tissues after E2 treatment. In Experiment 2, during early pregnancy, there were significant changes ($P < 0.01$) across days in expression of *Cx26*, *Cx37* and *Cx43* mRNA in the maternal placenta, accompanied by changes ($P < 0.001$) in *Cx37* and *Cx43* mRNA in the fetal placenta. In Experiment 3, in maternal placenta, *Cx32* mRNA expression was decreased ($P < 0.001$) in NAT-ET, IVF and IVA groups compared to the NAT group; but in fetal placenta, *Cx32* mRNA expression was increased ($P <$

0.05) in NAT-ET, IVF and IVF groups, and Cx26 mRNA expression was increased ($P < 0.05$) in IVA compared to NAT group. These data suggest that Cx26, Cx32, Cx37 and/or Cx43 play specific roles in E2-regulated uterine function and in placental development during early gestation both after natural mating and with application of ART.

Keywords: Gap junctions, connexins, placenta, early pregnancy, assisted reproductive technology, sheep

1. Introduction

Gap junctions are composed of two symmetrical structures called connexons, each on an adjacent cell and containing six proteins named connexins (Cx) that create an intercellular channel of communication which allows ions and molecules of less than ~1.5 kDa to pass freely from cell to cell [1,2]. Thus, gap junctions play a major role in direct, contact-dependent cell-cell communication. Cx have been implicated in the regulation of cellular metabolism and the coordination of cellular functions during growth and differentiation of organs and tissues with or without formation of the gap junctional channel [1-3]. Gap junctional channels and Cx mRNA and/or proteins have been detected in uterine and placental tissues in several species [4-14]. Thus, expression of Cx mRNA and/or proteins and cell-cell communication mediated through gap junctional channels are likely involved in the regulation of utero-placental and fetal function and growth.

Steroid regulation of cellular functions during growth and differentiation has been associated with expression of gap junctional Cx in humans and rats, which have hemochorial placentation [14-18]. For example, expression of *Cx43* mRNA and protein in human

endometrium changed in response to hormones during the menstrual cycle, and estradiol-17 β (E2) affected endometrial Cx37 expression [19,20]. Additionally, expression of endometrial gap junction proteins changed during the rat estrous cycle, and E2 treatment of ovariectomized (OVX) rats was shown to modulate expression of endometrial Cx proteins and influence the size, number, and distribution of gap junctional plaques [11,21-24]. The effects of E2 on Cx expression in ovaries and uterus in rats appeared to be cell-specific [25].

Much less is presently known about the expression and role of Cx in the uterus and synepitheliochorial placenta of ungulates. Bovine placental Cx26, Cx32, and Cx43 mRNA and proteins were detected in a cell-specific manner during pregnancy [26]. Additionally, a specific pattern of Cx26 and Cx43 protein expression in ovine placenta suggests that gap junctional communication is involved in the attachment/implantation process and the regulation of endometrial functions during pregnancy [12]. Gabriel and colleagues [12] also suggested that the pattern of Cx expression resembled that found in the more invasive type of implantation seen in humans, rats, and mice.

Early pregnancy is a critical period of gestation because of the major developmental events that take place, including embryonic organogenesis, and the processes of implantation and placentation [27,28]. In fact, most embryonic loss (30 to 50% of fertilized ova in those mammalian species studied including humans) occurs during early pregnancy rather than later in pregnancy [27,29]. Use of assisted reproductive technologies (ART), including embryo transfer, in vitro fertilization, and cloning, may lead to embryo loss or a compromised pregnancy [27,28]. We have previously demonstrated that compared to gestation after natural mating, application of ART resulted in smaller fetal size, decreased cell proliferation in maternal and fetal placenta, delayed vascular development in fetal placenta, and altered expression of several genes including

steroid receptors, during early pregnancy [30-32]. Additionally, we have reported epigenetic changes in ovine placenta during early pregnancy after the use of ART [30]. We postulated that these processes may be influenced by cell-cell communication through expression of Cx.

We therefore hypothesized that 1) mRNA for *Cx26*, *Cx32*, *Cx37* and *Cx43* is expressed in the uterus of OVX, E2-treated ewes and in ovine placenta during early pregnancy, 2) E2-treatment of OVX ewes would cause time-specific changes in *Cx26*, *Cx32*, *Cx37* and/or *Cx43* mRNA expression, and 3) expression of these four Cx would vary across the days of early pregnancy and will be affected by embryo origin (i.e., application of ART). Therefore, in this study, we quantified expression of the mRNA for these four Cx in the uterus of E2-treated, OVX ewes (Exp. 1), in fetal and maternal placenta from days 14 to 30 of early pregnancy after natural (NAT) breeding (Exp. 2), and on day 22 of pregnancy after transfer of embryos generated through natural breeding of FSH-treated donors (NAT-ET), in vitro fertilization (IVF) or in vitro activation (IVA, parthenotes; Exp. 3). We chose to evaluate expression of *Cx26*, *Cx32*, *Cx37* and *Cx43* mRNA, since all of them are expressed in uterine and placental tissues, and they have been demonstrated to play a role in the regulation of uterine and/or placental functions in several species, including sheep [9,14,17,33].

2. Materials and methods

2.1. Animals and tissue collection

The Institutional Animal Care and Use Committee at NDSU approved all animal procedures for this study. Western Range (primarily Rambouillet, Targhee, and Columbia crossbreeds) ewes, 3-6 years old, were used.

2.2. Experiment 1; Effects of E2

To examine the effects of E2 treatment on connexin mRNA expression, an OVX ewe model was used that we have previously validated and described in several studies [34-37]. Briefly, on days 10-12 after estrus, ewes (n=32) were OVX and allowed to recover for at least 30 days before steroid treatment was initiated. Two silicone elastomer implants each containing 100 mg of E2 were inserted subcutaneously into each ewe, and the uterus was collected at 0 h (controls), or at 2, 4, 8, 16 and 24 h after receiving the E2 implant (n=4-6 per time point) [34,36,37]. Then, caruncular and intercaruncular tissues were dissected from the endometrium, snap-frozen and stored at -70°C for isolation of total cellular RNA (tcRNA).

2.3. Experiment 2; Effects of early pregnancy stages

The design of this experiment was described in detail by Grazul-Bilska et al. [38,39]. Gravid uteri were obtained on days 14, 16, 18, 20, 22, 24, 26, 28, and 30 after mating (day of mating = day 0; n = 6 to 8 per day), and also from nonpregnant (NP) control ewes at the mid-luteal phase of the estrous cycle (day 10 after estrus; n = 8). For tcRNA extraction, maternal placenta (caruncular) tissues were obtained from the area of the uterus closest to the embryo and uterine body and fetal membranes (fetal placenta, chorion on day 16, chorioallantois thereafter) were collected, snap-frozen and stored at -70 C.

2.4. Experiment 3; Effects of embryo origin

The design of this experiment is described in detail by Grazul-Bilska et al. [30,31] and Reynolds et al. [32]. Estrus was synchronized for all ewes using a CIDR device (MWI, Boise,

ID) implanted for 14 days during breeding season. Twenty-four hours after CIDR removal, NAT ewes were exposed to a fertile ram, but for NAT-ET, IVF and IVA groups estrus was checked twice daily using a vasectomized ram. Beginning on day 13 of the estrous cycle, ewes from the NAT-ET group were treated twice daily with FSH for 3 days, whereas ewes from the IVF and IVA groups were treated with FSH for 2 days. On day 15 of the estrous cycle, ewes from the NAT-ET group were exposed to a fertile ram for 24-48 h, and for the IVF and IVA groups, ovaries were collected, oocytes isolated, matured, and then fertilized or activated in vitro. For the NAT-ET group, on day 5 post-mating, embryos were flushed and transferred to synchronized recipients (3 embryos/recipient). For the IVF and IVA groups, in vitro-generated embryos were transferred on day 5 after fertilization or activation to synchronized recipient ewes (3 embryos/recipient). On day 22 post-mating, fertilization or activation (day of mating, fertilization or activation=day 0 of pregnancy), gravid uteri were collected from NAT (n=8) NAT-ET (n=7), IVF (n=8) or IVA (n=7) ewes. For tcRNA extraction, tissues from maternal placenta from the area close to the embryo and fetal membranes were collected, snap-frozen, and stored at -70 C as described for Exp. 2.

2.5. *Quantitative real-time RT-PCR*

All procedures and sequences of Taqman probes and primers for determining the expression of mRNA for Cx in ovine tissues by RT-PCR have been reported previously [40,41]. Briefly, snap-frozen uterine and placental (caruncular and fetal membrane) tissues were homogenized in Tri-Reagent (Molecular Research Inc, Cincinnati, OH) according to the manufacturer's specifications. The quality and quantity of total cellular RNA (tcRNA) were determined via capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent

Technologies, Wilmington, DE). Real-time RT-PCR reagents, probes, and primers were purchased from and used as recommended by Applied Biosystems (Foster City, CA). Cx-specific sheep sequences used to develop the probes and primers have been reported and are shown in Table 1 [40,41]. For each sample, 30 ng of tcRNA was reverse transcribed in triplicate 20- μ L reactions using random hexamers. The ABI PRISM 7000 was used for detection of sequences amplified at 60°C typically for 40 or 45 cycles (Applied Biosystems). Expression of each Cx was normalized to expression of 18S 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. The multiplex reaction was also used to generate standard curves for quantification of 18S and the gene of interest based on dilutions of the cDNA generated from tcRNA collected from pooled placental tissues on day 130 of pregnancy [38,42] .

2.6. Statistical analysis

Data were analyzed using the general linear models (GLM) procedure of SAS (SAS, 2010) with the main effect of time after OVX (Exp. 1), day of pregnancy (Exp. 2) or pregnancy type due to different embryo origin (Exp. 3). For Exp. 1, data are expressed as a fold-change compared to the 0 h (control) group, which was arbitrarily set as 1. For Exp. 2, data for maternal placenta are compared to the NP (control, day 10 of the estrous cycle) value and data for fetal membranes are compared to Day 16 of pregnancy. For Exp. 3, data are compared to the NAT control group. The F-test was considered to be significant at $P \leq 0.05$ or showing a tendency to

be different at $P < 0.1$. Data are presented as means $\pm$ SEM; differences between specific means were evaluated by using the least significant differences test [43].

3. Results

3.1. Experiment 1; Effects of E2

Expression of mRNA for *Cx26*, *Cx32*, *Cx37* and *Cx43* was detected in the uterus of OVX ewes after E2 treatment. At the tissue level, expression of *Cx43* mRNA was greater ($P < 0.05$) in intercaruncular than in caruncular tissues, whereas *Cx26*, *Cx32*, and *Cx37* mRNA expression was similar in both caruncular and intercaruncular tissues after E2 treatment.

Compared with the 0 h controls, expression of *Cx26* mRNA increased in caruncular tissues by 16-fold ($P < 0.02$) at 4 h, 24-fold ($P < 0.01$) at 8 h, and greater than 30-fold ($P < 0.01$) at 16 through 24 h after E2 treatment (Fig. 1A). Expression of *Cx26* mRNA in intercaruncular tissues increased 36-fold ($P < 0.01$) at 4 h, greater than 40-fold ($P < 0.01$) at 8 h, 36-fold ($P < 0.01$) at 16 h and was maintained at a 30-fold ($P < 0.01$) increase at 24 h after E2 (Fig. 1A).

In contrast to expression of *Cx26* mRNA, *Cx32* mRNA in caruncular tissues tended to be decreased by approximately 1.7-fold ($P < 0.06$) at 2 h, and remained decreased by 2-3 fold ($P < 0.01$) at 4 through 24 h after E2 treatment compared to the 0 h controls (Fig. 1B). However, in intercaruncular tissues, there was tendency for an initial increase of *Cx32* mRNA by 1.7-fold at 2 h ($P < 0.08$) followed by a nearly 7-fold ($P < 0.06$) decrease at 8 h and a return to 0-h values at 16 through 24 h after E2 treatment (Fig. 1B).

Compared to the 0 h controls, expression of *Cx37* increased greater than 4-fold ($P < 0.05$) in caruncular tissues at 2 h, remained increased by 3- to 4-fold ($P < 0.05$) at 4 through 8 h, and

was maintained at a 2- to 3-fold ($P < 0.05$) increase at 16 through 24 h after E2 treatment (Fig. 1C). In intercaruncular tissues, *Cx37* mRNA increased by 4-fold at 4 to 8 h ($P < 0.05$) and was increased by 3-fold at 16 to 24 h after E2 treatment (Fig. 1C).

Compared to the 0 h controls, expression of *Cx43* mRNA in caruncular tissues tended to increase by 2.7-fold ($P < 0.06$) at 4 h, and was increased by 3-fold ($P < 0.05$) at 8 h and 4-fold ($P < 0.01$) at 16 h through 24 h after E2 treatment (Fig. 1D). Expression of *Cx43* mRNA in intercaruncular tissues increased by greater than 4-fold ($P < 0.01$) at 8 to 16 h and remained increased by 3-fold ($P = 0.09$) at 24 h after E2 treatment (Fig. 1D).

3.2. Experiment 2; Effects on early pregnancy stages

Expression of the mRNA for *Cx26*, *Cx32*, *Cx37* and *Cx43* was detected in uterine tissues from non-pregnant and placental tissues from pregnant ewes.

In the maternal placenta: Compared with the NP controls, expression of *Cx26* mRNA increased ($P < 0.001$) 3-fold on Day 14, 5- to 8-fold on Days 18 to 28 and greater than 12-fold on Days 16 and 30 (Fig. 2A); expression of *Cx32* mRNA tended to increase ($P < 0.09$) on Day 18 (Fig. 2C); expression of *Cx37* mRNA increased ($P < 0.001$) greater than 2-fold on Days 22 to 30 (Fig. 2E); and expression of *Cx43* mRNA increased ($P < 0.001$) by 5- to 6-fold on Days 20 and 22 of pregnancy (Fig. 2G).

In the fetal placenta: Expression of *Cx26* mRNA tended ($P = 0.1$) to be increased on Day 18 and 28 and *Cx32* mRNA tended ($P = 0.1$) to be increased on Days 18, 20, and 30 of pregnancy (Fig. 2B and 2D).

Expression of *Cx37* mRNA increased ($P < 0.0001$) greater than 3-fold on Day 18, greater than 15-fold on Day 20, and 30- to 40-fold on Days 22 through 30 of pregnancy (Fig. 2F).

Expression of *Cx43* mRNA increased ($P < 0.0001$) 3-fold on Day 18 and greater than 4-fold on Days 20 and 22 of pregnancy and then decreased to NP levels (Fig. 2H).

3.3. *Experiment 3; Effects of embryo origin*

In the maternal placenta: Expression of mRNA for *Cx32* was decreased ($P < 0.004$) 2-4-fold in NAT-ET, IVF and IVA groups compared to the NAT group (Fig. 3A). Expression of mRNA for *Cx26*, *Cx37*, and *Cx43* in CAR was similar in all groups (Fig. 3A).

In the fetal placenta: Compared to the NAT group, expression of mRNA for *Cx26* was increased ($P < 0.05$) 2.7-fold in the IVA compared to the NAT group, and was similar in NAT, NAT-ET and IVF groups (Fig. 3B). Expression of *Cx32* mRNA was increased ($P < 0.05$) ~2-fold in the NAT-ET, IVF and IVA compared to the NAT group, and expression of mRNA for *Cx37* and *Cx43* in FM was similar across all groups (Fig. 3B).

4. Discussion

In this study we have shown that the mRNA for four gap junctional Cx are expressed in the uterus of OVX, E2-treated ewes and in the maternal and fetal placenta during early pregnancy, and that their expression is regulated by E2, developmental stage, and embryo origin. We recognize the limitations of the data as we evaluated only mRNA expression of Cx in homogenates of whole uterine and placental tissues, but believe they lay a significant groundwork for future investigations into the role of Cx in uterine function and placentation. Additionally, to our knowledge, data regarding the expression of Cx in utero-placental tissues after application of ART have never been reported. Thus, these data will allow us to focus on specific Cx protein expression, localization and function in future studies.

Cellular interactions mediated through gap junctions as well as utero-placental secretory function and tissue growth before and after fertilization and implantation are probably critical for establishing and maintaining a successful pregnancy. Numerous factors, including E2 and Cx, affect uterine and placental functions through regulation of cell proliferation, angiogenesis, vascular tone, and other utero-placental processes in several species [14,17,44-48]. In sheep, humans and a few other mammalian species, embryonic loss is 30-50% during the first trimester of pregnancy [27-29]. Thus, investigation of regulatory processes, including the role of E2 and Cx expression, in utero-placental functions in the minimally invasive ovine synepitheliochorial placenta may contribute to establishing strategies for decreasing embryonic loss.

Connexin proteins are widely expressed in many cells of the reproductive system and are thought to be important for various functions. Cx26 protein has been found predominantly in the uterine epithelium of cows, sheep and rodents, whereas in the menstrual cycle of humans and baboons Cx26 and Cx32 are co-expressed in the basal regions of the uterine epithelial cells [5,7,14,17,49]. In rats, Cx26 protein was expressed in the epithelium of the implantation chamber before trophoblast invasion and subsequently in the decidual cells accompanying the invading trophoblast [5]. Cx37 protein has been localized to placental endothelial cells in humans and mice and may have a major role in blood vessel growth [13,33,50]. Additionally, Cx37 is expressed in endothelial cell-cell junctions in arterioles of the villous stroma in human first trimester placenta [6,51]. Cx32 and Cx43 proteins are expressed in the aggregated trophoblast cells of the placental bed of humans [17,52], and Cx43 is predominantly found in stromal tissue and in the decidua during implantation [14].

Although rodents and humans have hemochorial placentas, the function of specific Cx may differ across species. In mice and rats, the Cx26 gap junction facilitates the uptake of

glucose in the placental labyrinth by allowing transport of glucose and other molecules across placental barriers [13,53]. Mutations of Cx26 and Cx32 in women lead to impaired development of uterine epithelial cells, associated with failure of implantation and susceptibility to endometriosis [14]. Lack of Cx37 seems to affect primarily folliculogenesis, resulting in infertility in mice and other species [14,54]. Cx37 and Cx43 are expressed in vascular endothelial cells and may have a role in normal vascular development and/or maintaining vascular tone in placenta and other organs [48,50,55,56]. In humans, Cx43 mediates fusion of cytotrophoblastic cells to form the syncytiotrophoblast [13]. Subfertility and early embryonic loss are associated with aberrant or reduced expression of Cx43 during decidualization [14]. Mutations of Cx43 are associated with placental insufficiency resulting in intrauterine growth restriction and alterations in uterine blood flow as well as pre-eclampsia [14].

In our experiment, E2-treatment of OVX sheep increased mRNA for Cx26, Cx37 and Cx43, but decreased Cx32 expression in the endometrium. In OVX, E2-treated rats, Cx26 mRNA was detected by 3 h and Cx43 by 14 h after E2 treatment, but Cx32 mRNA was not detected [24]. During the preimplantation period of pregnancy in rats, expression of Cx26 and Cx43 was induced by E2 acting through the estrogen receptor 1 (known as alpha) [57]. In addition, endometrial Cx26 expression was more responsive to E2 treatment than was Cx43 in OVX rats [58]. Additionally, the effects of E2 on Cx expression in rat uterus appeared to be cell-specific [25]. In-vitro E2 treatment increased Cx37 mRNA expression in endometrial explants [20], and gap junctional communication and expression of Cx43 protein by human myometrial cells [59]. These studies clearly demonstrated that expression of Cx in uterus is regulated by E2 in several species. Moreover, we have confirmed the presence and changes in levels of these four specific Cx proteins in sheep uteroplacental tissues supporting the putative physiologic roles

of gap junctions in the function of these tissues during early gestation (unpublished observations). The functional significance of this Cx protein expression in uteroplacental tissues and its correlation with our mRNA expression data will be our focus for future studies.

In this study, significant changes in expression of four Cx mRNA in the maternal placenta, and expression of Cx37 and Cx43 mRNA in fetal placenta were observed. In cycling baboons, transcript levels of Cx32 were less than those of Cx26 and Cx43 in cycling baboons [7]. Similarly, we found a lower level of expression of mRNA for Cx32 and Cx37 in the sheep placenta during early pregnancy. Cx37 and Cx43 likely are involved in the intensive vasculogenesis and angiogenesis that occurs in placental villi during this time period [13,50]. In the human placenta during the first trimester of pregnancy, Cx32 and Cx43 mRNA levels were constant, but Cx37 mRNA increased by 1.5-fold at 14 weeks of gestation, a finding also attributed to blood vessel growth [33]. Additionally, Morschauser and coworkers [60] have demonstrated that Cx43 and Cx37 and eNOS proteins were increased in uterine artery endothelium and vascular smooth muscle during gestation. Using gap junctional blockage, they established that uterine artery endothelium Cx43 was responsible for the increased Ca^{2+} -mediated nitric oxide production that results in vasodilation to facilitate increased uterine blood flow. Thus, these changes in expression of Cx in maternal and fetal placenta suggest a specific role in regulation of placental development during early pregnancy [11].

During early pregnancy in sheep, Cx26 protein was expressed in the uterine epithelium and Cx43 in the uterine stroma, similar to observations in rats and several other species [5,12,15,21]. The process of Cx induction during the progression of implantation in sheep has been described as resembling that of the invasive type of implantation found in humans, baboons, rats and mice [5,12,21]. In rats, Cx26 expression in the uterine epithelium is induced

before placental invasion and then is found in the maternal decidual zone lining the invading trophoblast [5]. Thus, Cx26 may be involved in programmed cell death of the epithelium during implantation in rats. However, in the synepitheliochorial placenta of sheep, the maternal luminal epithelium participates in formation of a syncytial layer composed of feto-maternal hybrid cells which exhibit minimal invasiveness [61]. In the cow, Cx26 mRNA was found in maternal epithelial cells and trophoblast giant cells, whereas Cx26 protein was detected where the maternal epithelium contacted the fetal membranes and as cytoplasmic staining in syncytial cells [26]. Additionally, Cx32 protein was found in the bovine maternal epithelium at the tips of the chorionic plates, which is the site of interdigitation of fetal cotyledons with maternal caruncles, suggesting that Cx32 may regulate placental invasiveness in cows [26]. In the epitheliochorial placenta of horses and pigs, gap junctional Cx have not been detected during implantation, and this lack of Cx expression is likely associated with the minimal placental invasiveness of these species [14].

In this study, we found that Cx26, Cx37, and Cx43 mRNA expression were similar on day 22 of pregnancy in the maternal placenta, whereas Cx32 mRNA expression was increased in maternal, but decreased in the fetal placenta for NAT-ET, IVF and IVA groups compared with the NAT group. The changes in Cx32 mRNA expression may represent a compensatory reaction to rescue these embryos from developmental problems. Additionally, we found that Cx26 increased in fetal placenta of the IVA group, which is the group that never develops beyond early pregnancy. The reasons for these effects on specific Cx expression are unclear and require further investigations.

Application of ART to treat infertility and enhance reproductive efficiency in humans and livestock may have negative effects on placental and fetal development that may result in

embryo loss and/or delayed development. For example, it has been demonstrated that ART including embryo transfer and IVF may result in decreased birth-weight and/or placental weight, impaired placental function and decreased pregnancy duration in several species [30,31,27,28,62,63]. In recent studies, we found that NAT-ET, IVF, and IVF groups had reduced embryo length and placental cell proliferation compared to NAT accompanied by changes in gene methylation patterns in the maternal and fetal placenta [30,31]. Additionally, there were profound decreases in vascular proliferation, expression of angiogenic factors and the density of blood vessels in the maternal and fetal placenta after ART procedures [31]. Furthermore, our data suggest that these effects are linked to altered expression of estrogen and progesterone receptors [32]. The negative outcomes of ART may be influenced by gap junctional communication and other factors.

In summary, the results of present study have shown that Cx26, Cx32, Cx37 and/or Cx43 mRNA are expressed in uterine and placental tissues in sheep in a time-specific, hormonally responsive manner and also are influenced by embryo origin. Additionally, our data have clearly indicated that the greatest E2 effects were on Cx26 and Cx43 mRNA expression, suggesting that these are major Cx involved in the regulation of uterine and placental functions in sheep. These data also suggest that E2 does not affect Cx32 mRNA expression in the ovine uterus. Observed changes in Cx26, Cx32 Cx37 and/or Cx43 mRNA expression during early pregnancy indicate that Cx have specific roles in growth and development of the maternal and fetal placenta of sheep similar to those suggested for rodents and humans.

Future evaluations of Cx protein expression, localization and function, and Cx interactions with calcium signaling and the NO system will be performed to better understand the complex role of Cx in ovine utero-placental growth and development in physiological and

pathological conditions, especially since altered expression of Cx has been associated with some pathological conditions including endometriosis and preeclampsia [55,56,64]. Our future studies will include isolation of specific cells from uteroplacental tissues using laser microdissection techniques to define Cx expression in placental compartments. In addition, regulation of Cx expression in placental cells and tissues by hormones and growth factors will be examined in vitro and in vivo conditions. This knowledge can contribute to development of therapeutic strategies to treat impaired uterine functions, particularly those resulting from ART application and intrauterine growth restriction that are likely associated with Cx functions.

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Figure legends

Fig. 1. Expression of mRNA for Cx26 (A), Cx32 (B), Cx37 (C) and Cx43 (D) in the endometrium of OVX ewes at 0, 2, 4, 8, 16, and 24 h after treatment with E2. Data are expressed as a fold-change compared to the 0 h (control), which was arbitrarily set as 1. Bars: black = caruncular tissues, white = intercaruncular tissues. Means $\pm$ SEM with different superscripts differ. *P*-values are: *P* < 0.01 for A, B, and D; *P* < 0.05 for C.

Fig. 2. Expression of mRNA for Cx26, Cx32, Cx37 and Cx43 in the maternal (A, C, E and G; graphs in left column) and fetal (B, D, F and H; graphs in right column) placenta during early pregnancy in sheep. Means $\pm$ SEM with different superscripts differ. *P*-values are: *P* < 0.0001 for F and H, *P* < 0.001 for A and E, *P* < 0.01 for G, *P* = 0.09 for C, and *P* = 0.1 for B and D.

Fig. 3. Expression of mRNA for Cx26, Cx32, Cx37 and Cx43 mRNA in the maternal (A) and fetal (B) placenta in pregnancies achieved after natural breeding (NAT), or after transfer of embryos created in vivo (NAT-ET), or through in vitro fertilization (IVF) or in vitro activation (IVA) on day 22 after mating, fertilization or activation. . Means $\pm$ SEM with different superscripts differ; for A, *P* < 0.004, and for B, *P* < 0.05.

Table 1. Sequence of TaqMan primers and probes for Connexins.

Oligonucleotide ^a	Nucleotide Sequence	Accession number ^b
Sheep Cx43 FP	5'-TGT CTT CGA GGT GGC CTT CT-3'	AY074716
Sheep Cx43 RP	5'-AAA CGG CAC TCA AGC TGA ATC-3'	
Sheep Cx43 Probe	5'-(6FAM) TGA TCC AGT GGT ACA TCT - (MGBNFQ)-3'	
Sheep Cx37 FP	5'-CTG CAC CAA CGT CTG CTA TGA-3'	AY745977
Sheep Cx37 RP	5'-GCA CCC AGT AGC GGA TGT G-3'	
Sheep Cx37 Probe	5'-(6FAM) CAG GCC TTC CCC ATC- (MGBNFQ)-3'	
Sheep Cx32 FP	5'-CTC CTC GTG GCC ATG CA-3'	AY074717
Sheep Cx32 RP	5'-GCC CTC AAG TCG AAG CAT TTT-3'	
Sheep Cx32 Probe	5'-(6FAM) ACA CCA GCA GCA CAT - (MGBNFQ)-3'	
Sheep Cx26 FP	5'-AAC ACG GTG GAC TGC TTC GT-3'	AY074718
Sheep Cx26 RP	CGA TCA TGA AGA CCG TGA AGA C-3'	
SheepCx26 Probe	5'-(6FAM) CAG GCC CAC AGA GAA - (MGBNFQ)-3'	

^aFP, Forward primer and RP, Reverse primer.

^bNucleotide sequences for ovine-specific genes were obtained from the National Center for Biotechnology Information (NCBI, 2010) database.

Figure 1

Figure 2

Figure 3