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# Gap junction communication between uterine stromal cells plays a critical role in pregnancy-associated neovascularization and embryo survival

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## Abstract

In the uterus, the formation of new maternal blood vessels in the stromal compartment at the time of embryonic implantation is critical for the establishment and maintenance of pregnancy. Although uterine angiogenesis is known to be influenced by the steroid hormones estrogen (E) and progesterone (P), the underlying molecular pathways remain poorly understood. Here, we report that the expression of connexin 43 (Cx43), a major gap junction protein, is markedly enhanced in response to E in uterine stromal cells surrounding the implanted embryo during the early phases of pregnancy. Conditional deletion of the *Cx43* gene in these stromal cells and the consequent disruption of their gap junctions led to a striking impairment in the development of new blood vessels within the stromal compartment, resulting in the arrest of embryo growth and early pregnancy loss. Further analysis of this phenotypical defect revealed that loss of Cx43 expression resulted in aberrant differentiation of uterine stromal cells and impaired production of several key angiogenic factors, including the vascular endothelial growth factor (Vegf). Ablation of CX43 expression in human endometrial stromal cells in vitro led to similar findings. Collectively, these results uncovered a unique link between steroid hormone-regulated cell-cell communication within the pregnant uterus and the development of an elaborate vascular network that supports embryonic growth. Our study presents the first evidence that Cx43-type gap junctions play a critical and conserved role in modulating stromal differentiation, and regulate the consequent production of crucial paracrine signals that control uterine neovascularization during implantation.

### Keywords

Implantation; Endometrium; Neovascularization; Estrogen; Connexin 43; Mouse

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## INTRODUCTION

During the early stages of pregnancy, the steroid hormones estrogen (E) and progesterone (P) orchestrate the structural and functional changes in the mammalian uterus that enable the blastocyst to attach to it, initiating the process of implantation (Psychoyos, 1973; Yoshinaga, 1988; Parr and Parr, 1989; Weitlauf, 1994). In the mouse and the human, as the embryo invades into the stromal compartment, the fibroblastic stromal cells undergo differentiation into a unique secretory tissue, known as the decidua. This transformation process, known as decidualization, is crucial for executing the extensive tissue remodeling that ensures proper maternal-fetal interactions, leading to the establishment of pregnancy. Decidualization is also accompanied by the creation of an extensive vascular network within the stromal bed that supports embryonic and placental growth and maintains early pregnancy (Cross et al., 1994; Irwin and Giudice, 1999; Carson et al., 2000).

During angiogenesis, new blood vessels are generated by the extension of pre-existing vessels into avascular space. This process involves the local degradation of the vascular basal membrane by proteases, proliferation and migration of endothelial cells, and assembly of these cells into new vessels (Folkman, 1995; Hyder and Stancel, 1999). In the female reproductive system, an active angiogenesis is required to support the cyclic remodeling of the uterus. In the human and the non-human primates, the spiral arteries that supply the functionalis layer of the endometrium increase in length, branching and coiling during each menstrual cycle as the endometrium is regenerated (Hyder and Stancel, 1999). In rodents, a link between the steroid-driven stromal differentiation program and active neovascularization within the pregnant uterus has long been speculated, although the underlying mechanisms are unknown. One of the earliest signs of a uterine response to an angiogenic stimulus is an increase in microvascular permeability at the sites of implantation (Chakraborty et al., 1995; Rockwell et al., 2002). E is recognized as a regulator of this phenomenon (Chakraborty et al., 1995; Rockwell et al., 2002). However, the precise nature of the hormone-regulated pathways that influence uterine angiogenesis remains unclear and controversial. Particularly intriguing is a previous report that E is an inhibitor and P is a stimulator of uterine angiogenesis (Ma et al., 2001). As this study was performed using ovariectomized non-pregnant mice following treatment with steroid hormones, the relevance of these findings under normal pregnancy conditions is questionable. A major challenge in reproductive medicine is, therefore, to gain a clear understanding of the steroid hormone-regulated pathways that control pregnancy-associated endometrial neovascularization in the stromal compartment. The present study was undertaken to uncover and functionally characterize these pathways.

## MATERIALS AND METHODS

### Animals and tissue collection

Mice were maintained in the designated animal care facility at the University of Illinois College of Veterinary Medicine according to the institutional guidelines for the care and use of laboratory animals. For mating studies, *Cx43*$^{fl/fl}$ and *Cx43*$^{d/d}$ female mice were housed with wild-type C57BL/6 male mice (Charles Rivers). The presence of a vaginal plug after mating was designated as day 1 of pregnancy.

In order to induce superovulation, 7- to 8-week-old female mice were injected intraperitoneally with 5 IU of pregnant mare serum gonadotrophin (PMSG) and 48 hours later with 5 IU of human chorionic gonadotropin (hCG). The mice were killed 16–18 hours post-hCG administration, and oocytes were flushed from the oviducts and counted.

To induce and maintain delayed implantation, mice were ovariectomized on day 4 (morning) of pregnancy and injected daily with P (2 mg) from days 5–7. To terminate delayed

implantation and induce blastocyst attachment, the P-primed delayed implanting mice were given an injection of E (50 ng) on the fourth day of the delay (day 8). Mice were killed at different time points after E injection and uteri were collected.

Decidualization was experimentally induced in non-pregnant mice as described previously (Cheon et al., 2004). Mice were first ovariectomized. Two weeks following ovariectomy, animals were injected with 100 ng of E in 0.1 ml of sesame oil for 3 consecutive days. This was followed by daily injections of 1 mg of P for 3 consecutive days. Decidualization was then initiated in one horn by injection of 50 μl oil. The other horn was left unstimulated. The animals were treated with P for an additional 6 days poststimulation and then killed to collect the uterine tissue.

### Immunohistochemistry

For Cx43, Pecam, Ki67 and Vegf immunostaining, uterine sections were obtained and flash frozen. Samples were embedded in OCT, cryosections were taken at 8 μm and subjected to immunostaining with antibodies against Cx43 (Zymed), Pecam1 (BD Biosciences), Ki67 (Santa Cruz Biotech) and Vegf (Santa Cruz Biotech). For double immunostaining against Pecam1 and Cx43, uterine tissues were collected from mice on day 8 of pregnancy and flash frozen in liquid nitrogen. Incubations with primary antibody were carried out overnight at 4° C (1:1000 dilution of rat anti-Pecam1 antibody, Pharmingen 557355; 1:500 dilution of a rabbit polyclonal antibody against Cx43, Zymed 71-0700), using frozen sections. Secondary antibody incubations were carried out for 2 hours at room temperature with a 1:200 dilution of fluorescently conjugated secondary antibodies: TRITC-conjugated goat anti-rabbit antibody (Sigma T6778) and FITC-conjugated goat anti-rat antibody (Sigma F6258). Sections were washed three times for 5 minutes each in PBS.

### Human endometrial stromal cells

Primary human endometrial stromal cells were isolated from endometrial biopsies of fertile women and immortalized by stable transfection of a gene coding for an essential catalytic protein subunit of human telomerase reverse transcriptase (Krikun et al., 2004). These telomerase-expressing stromal cells (termed HESC-T) were stably transfected with retroviral vectors expressing Cx43 small interfering RNA (siRNA) and a control Cx43 non-silencing sequence (Shao et al., 2005). The siRNA insert-containing retroviral vectors were first transduced into PT67 retro-packaging cells (BD Biosciences) to generate infectious viral particle-containing supernatant. Filtered supernatants were then used to infect HESC-T cells and the infected cells selected in media containing 50 μg/ml hygromycin. Cells transfected with a control non-silencing siRNA and Cx43 siRNA are designated as HESC-TC and HESC-T3, respectively. The cells were grown in DMEM/F-12 medium containing 5% charcoal-stripped FBS. To induce in vitro decidualization, the cells were treated with or without a hormone cocktail containing 1 nM E, 1 mM P and 0.5 mM 8-bromo-cAMP for 7–11 days. Cell culture supernatants were collected, and prolactin and VEGF were measured using standardized ELISA kits. Three independent experiments were performed to assure reproducibility and the data are presented as mean±s.d. Comparisons between HESC-TC and HESC-T3 cells were made using two-tailed Student's *t*-tests, with a significance threshold set at $P=0.05$.

### Dual label cell coupling assay

Donor cells were double labeled with the fluorescent dyes calcein (gap junction permeable dye) and Dil (gap junction impermeable dye). These cells were then placed in contact with unloaded cells in a monolayer. Dye transfer was visualized after 2 hours. The cells that fluoresce both green (calcein) and red (Dil) are the dual-loaded donor cells, whereas those fluorescing

only green were originally unlabeled in the monolayer and now demonstrate functional coupling.

## RESULTS

To identify steroid-regulated gene networks with functional relevance in implantation, we used a delayed implantation mouse model in which implantation is induced by an acute administration of E to ovariectomized pregnant mice maintained in the presence of P (Yoshinaga and Adams, 1966; Gidley-Baird, 1981). This hormonal profile mimics the transient preimplantation surge of E that is essential for implantation in the rodents. Gene expression profiling using Affymetrix mouse microarrays (430 2.0 Array) revealed that connexin 43 (Cx43), also known as gap junction protein alpha 1 (Gja1), is one of the many genes whose expression is altered in P-primed uteri in response to E (Mantena et al., 2006) (M.K.B. and I.C.B., unpublished). The connexins are a family of transmembrane proteins that form hexameric assemblies in the plasma membrane to create gap junctions, regulating intercellular communication. Cx43 is the principal and most well-studied component of the gap junctions (Kumar and Gilula, 1996; Bruzzone et al., 1996).

We first confirmed the hormonal regulation of Cx43 expression in pregnant uterus by performing immunohistochemical analysis. We observed that E treatment dramatically enhances the expression of Cx43 protein in the uterine stromal compartment during delayed implantation (Fig. 1A). Our findings are also in agreement with previous reports that Cx43 expression in the uterus is primarily under E regulation (Grummer et al., 2004). When we examined the profile of Cx43 protein expression in mouse uterus during normal pregnancy, it was undetectable in undifferentiated stromal cells during the preimplantation period (Fig. 1B, parts a,b). However, on day 5 of pregnancy, within 12 hours of the initiation of implantation, a marked induction in Cx43 protein expression was observed in the stromal cells in the primary decidual zone immediately surrounding the implanting embryo (Fig. 1B, parts c,d). As pregnancy progressed to day 7, Cx43 expression intensified and spread to the secondary decidual zone (Fig. 1B, parts e,f). The close spatio-temporal relationship between Cx43 expression and the progression of decidualization raised the possibility that stromal gap junctions harboring this protein may play an important role during the differentiation process.

To investigate the function of Cx43 during embryo implantation, we employed a loss-of-function approach using genetically engineered mice. *Cx43*-null mice exhibit a perinatal lethal phenotype due to impaired cardiovascular development (Reaume et al., 1995). To circumvent this problem, we created a conditional knockout of the *Cx43* gene in the uterus of adult mice by employing the Cre-LoxP strategy. Transgenic mice expressing Cre under the control of progesterone receptor (PR) promoter were used previously to ablate 'floxed' genes in the uterus (Lee et al., 2006; Mukherjee et al., 2006; Lee et al., 2007). We crossed these PR-Cre mice with those harboring the 'floxed' *Cx43* gene ($Cx43^{fl/fl}$) to create the $Cx43^{d/d}$ mice in which the *Cx43* gene is deleted in uterine cells expressing PR. The ablation of the *Cx43* gene in the uterine tissue of $Cx43^{d/d}$ mice during pregnancy was confirmed when uterine sections obtained from these mice failed to show any Cx43 protein expression in the stromal cells surrounding the implanted embryo (Fig. 2).

An 8-month breeding study demonstrated that female $Cx43^{d/d}$ mice exhibit severe fertility defects (Table 1). Our study revealed that ~50% of a cohort ($n$=13) of $Cx43^{d/d}$ female mice analyzed during this breeding experiment never gave birth, although they mated normally with wild-type males. Furthermore, the $Cx43^{d/d}$ mice that did give birth exhibited a more than 60% reduction in the number of pups per litter when compared with control $Cx43^{fl/fl}$ mice. Overall, these results indicated that the conditional excision of the *Cx43* gene led to an ~80% reduction in the total number of pups born per $Cx43^{d/d}$ female compared with a $Cx43^{fl/fl}$ female in the

breeding program (35/13 versus 419/37). Superovulation experiments indicated that *Cx43*$^{d/d}$ mice ovulate normally and release oocytes in quantities comparable to those of *Cx43*$^{fl/fl}$ mice (Table 1). In agreement with normal ovarian activity, serum P levels were normal in the *Cx43*$^{d/d}$ mice on day 8 of pregnancy (Table 1). Collectively, these data suggest that the observed fertility defect in *Cx43*$^{d/d}$ mice is not likely to be due to an impairment of the hypothalamic-pituitary-ovarian axis.

Further analysis indicated that the *Cx43*$^{d/d}$ mice are able to initiate embryo implantation and support pregnancy up to day 7 of gestation. Implanted embryos embedded in the stroma were observed in both *Cx43*$^{fl/fl}$ and *Cx43*$^{d/d}$ mice (Fig. 3A, parts a,c). However, starting on day 8 of pregnancy in *Cx43*$^{d/d}$ mice, we detected distinct signs of arrest of embryonic growth and noted embryo loss or resorption. Morphological analysis of uterine sections obtained from these mice on day 8 of pregnancy showed abnormally small embryos compared with those of *Cx43*$^{fl/fl}$ mice (compare parts b and d in Fig. 3A). An examination of the histological sections from day 8 pregnant uteri revealed a severe impairment in the development of an angiogenic network in the stromal bed of *Cx43*-deficient uteri (Fig. 3A, parts e,f). When the uterine sections of pregnant *Cx43*$^{fl/fl}$ mice were subjected to immunohistochemistry using an antibody against platelet/endothelial cell adhesion molecule (Pecam), a marker of endothelial cells, they displayed a well-developed vascular network spanning the endometrial bed that surrounds the implanted embryo on day 7 or day 8 of pregnancy (Fig. 3B, parts a–c). The Pecam immunostaining, however, was reduced drastically in uterine sections of *Cx43*$^{d/d}$ mice on day 7 or day 8 of pregnancy, indicating that only a rudimentary vasculature formed in the mutant uteri (Fig. 3B, parts d–f). It is important to mention here that immunofluorescence experiments using differently labeled antibodies against Pecam and Cx43 indicated that during early pregnancy Cx43 expression occurs solely in the uterine stromal cells and not in the endothelial cells (Fig. 3C). These results support the concept that the loss of Cx43 expression in the stromal gap junctions is responsible for the drastic reduction in the endothelial cell population in the pregnant uterus.

The lack of endothelial cell proliferation in *Cx43*-deficient uteri was further ascertained by immunostaining for Ki67, a marker for cell proliferation (Fig. 3D). Whereas the uterine sections obtained from *Cx43*$^{fl/fl}$ mice on day 8 of pregnancy exhibited robust Ki67 immunostaining in endothelial cells, consistent with microvascular proliferation (Fig. 3D, part a), those obtained from mutant animals showed greatly reduced Ki67 staining (Fig. 3D, part b), confirming a severely compromised endothelial cell proliferation in the absence of stromal Cx43 expression.

To gain further insight into the mechanisms underlying the phenotypical defects of *Cx43*$^{d/d}$ uteri, we examined whether the loss of Cx43 expression affected the stromal differentiation program. We analyzed the decidual response in *Cx43*$^{d/d}$ uteri by monitoring the expression of decidual prolactin-related protein (PRP) and prolactin-like protein (PLP), well-known biochemical markers of decidualization (Orwig et al., 1997; Rasmussen et al., 1997; Croze et al., 1990; Lin et al., 1997). As expected, uterine sections from *Cx43*$^{fl/fl}$ mice exhibited prominent PRP expression in both antimesometrial (AM) and mesometrial (M) areas on day 7 of pregnancy (Fig. 4A). A similar pattern of PRP expression was observed in *Cx43*-deficient pregnant uterus on this day (Fig. 4B). On day 8 of pregnancy in *Cx43*$^{fl/fl}$ mice, PRP expression was drastically reduced in the decidual cells at both antimesometrial and mesometrial regions. Only a few cells in the mesometrial region close to the ectoplacental cone retained strong PRP expression (Fig. 4C). By contrast, a strikingly different spatial expression of PRP was seen in *Cx43*-deficient uteri on day 8 of pregnancy (Fig. 4D). In these mutant uteri, expression of PRP, similar to that seen on day 7 of pregnancy, persisted in the majority of the decidual cells in the antimesometrial and mesometrial areas. A plausible explanation for the absence of timely downregulation of PRP expression in these cells is that they fail to reach a more advanced state of differentiation. When we analyzed PLP expression, it was prominent in the antimesometrial

region of uterine sections of *Cx43*$^{fl/fl}$ mice on day 8 of pregnancy and was markedly reduced in *Cx43*-deficient uteri (Fig. 4E,F). Collectively, the aberrant expression of both PRP and PLP indicated that the loss of Cx43 expression in the uterine stromal cells leads to an impairment in the proper progression of the decidualization program.

It is important to address whether Cx43 controls stromal decidualization and angiogenesis independently of embryonic development. We, therefore, subjected non-pregnant mice to experimentally induced decidualization in which a mechanical perturbation of the steroid-primed uteri triggers a decidual response in the absence of the implanting embryo (Cheon et al., 2004). Uteri of ovariectomized *Cx43*$^{fl/fl}$ and *Cx43*$^{d/d}$ mice were prepared by treating these animals with a well-established regimen of E and P, and then decidualization was initiated in the left uterine horn by injecting 50 μl oil while the right horn was left unstimulated. As shown in Fig. 5A, widespread expression of Cx43 is induced in stromal cells of *Cx43*$^{fl/fl}$ mice in response to the deciduogenic stimulus. We then examined the gross anatomy of the stimulated and unstimulated uterine horns of *Cx43*$^{fl/fl}$ and *Cx43*$^{d/d}$ mice. As expected, the uterine horn of *Cx43*$^{fl/fl}$ mice exhibited a robust decidual response within 48 hours of receiving the artificial stimulation (Fig. 5B, upper left panel). By contrast, the *Cx43*-deficient uteri under identical conditions showed significantly reduced decidualization (Fig. 5B, upper right panel). When the decidual response was assessed by measurement of uterine wet weight gain, the *Cx43*-deficient uteri exhibited a markedly reduced weight gain relative to that seen in the *Cx43*$^{fl/fl}$ uteri (Fig. 5B, middle panel). We further analyzed the decidualization response of *Cx43*$^{d/d}$ uteri by monitoring the expression of Hoxa10 and bone morphogenetic protein 2 (Bmp2), factors that are induced in stromal cells during decidualization and that play important regulatory roles during this process (Lim et al., 1999; Lee et al., 2007; Li et al., 2007). As shown in the lower panel of Fig. 5B, when *Cx43*$^{fl/fl}$ and *Cx43*-deficient uteri were subjected to artificial decidual stimulation, we observed a marked downregulation of mRNAs corresponding to Hoxa10 and Bmp2 in the uteri lacking Cx43, consistent with the other decidualization defects observed in the conditional mutant mice.

We next analyzed the angiogenic capacity of *Cx43*-deficient uteri during artificial decidualization. Immunohistochemical analysis of *Cx43*$^{fl/fl}$ and *Cx43*-deficient uteri with a Pecam antibody revealed that the artificially stimulated *Cx43*$^{fl/fl}$ horn displayed an extensive endothelial cell network spanning the endometrial bed (Fig. 5C, left panel). By contrast, similarly treated uterine horns of *Cx43*$^{d/d}$ mice displayed drastically reduced Pecam staining, indicating that only a rudimentary vasculature is formed (Fig. 5C, right panel). These studies clearly indicate that even in the absence of the conceptus, communication via Cx43 gap junctions plays a crucial role in stromal cell differentiation and angiogenesis in the steroid hormone-primed uterus.

We considered the possibility that the impaired decidualization of the mutant stromal cells might affect the timely production of paracrine regulators from these cells, thereby inhibiting endothelial proliferation or angiogenesis. Ample evidence indicates that Vegf is a potent paracrine stimulator of endothelial cell proliferation and is a crucial angiogenic factor during decidualization (Ferrara et al., 1996; Halder et al., 2000). We, therefore, examined the pattern of Vegf protein expression in *Cx43*$^{fl/fl}$ and *Cx43*$^{d/d}$ uteri during the decidualization phase. Widespread expression of Vegf was observed in *Cx43*$^{fl/fl}$ uteri on days 7 (Fig. 6A, part a) and 8 (Fig. 6A, part b) of pregnancy, particularly in the mesometrial area, which is the primary source of the growing implantation site vasculature. Its spatial expression pattern closely overlapped with that of Pecam, which marked the endothelial network (compare Fig. 3B and Fig. 6A). By contrast, a significant downregulation of Vegf expression, concomitant with the sharp reduction in Pecam immunostaining, was seen in *Cx43*-deficient uteri (Fig. 6A, part c,d; compare with Fig. 3B, parts d–f). The Vegf expression in *Cx43*$^{d/d}$ uteri was limited to only a few layers of cells surrounding the implanted embryo, and was markedly reduced in the

mesometrial region. We also observed a marked downregulation of mRNAs encoding angiopoietin 2 and angiopoietin 4 in *Cx43*-deficient uteri during the decidualization phase (Fig. 6B). As these factors are known to play important regulatory roles in endothelial cell proliferation, migration and new blood vessel formation, our findings provide mechanistic insights into the pathways via which stromal Cx43 gap junctions control angiogenesis during decidualization.

To further explore the relationship between Cx43 and Vegf expression, and to test whether this important functional link is conserved among the species, we extended our study to human endometrial stromal cells, which are known to produce these proteins during decidualization (Jahn et al., 1995; Shifren et al., 1996; Granot et al., 2000). We used primary human endometrial stromal cells (HESC-T) that have been immortalized by stable transfection of a gene encoding the catalytic subunit of human telomerase (Krikun et al., 2004). By using these cells, we established a low CX43-expressing human stromal cell line HESC-T3 that is stably transduced with a retroviral vector expressing small interfering RNA (siRNA) targeted to *CX43* mRNA, generously provided by Dr Dale Laird (Shao et al., 2005). A control cell line, HESC-TC, containing the same retroviral vector expressing a non-target sequence was also generated. Quantification by real-time RT-PCR indicated that *CX43* mRNA levels in HESC-T3 cells were drastically reduced (>90%) relative to those in control HESC-TC cells (data not shown). Correspondingly, western blot analysis demonstrated a marked reduction of CX43 protein in HESC-T3 cells (Fig. 7A). To examine whether the consequence of this forced suppression of CX43 is an inhibition of gap junctions, we used a double dye-labeling technique. As shown in Fig. 7B, gap junction-permeable green calcein dye diffused from injected control HESC-TC cells (yellow arrows, Fig. 7B) to adjacent cells, confirming that functional gap junctions exist between stromal cells. By contrast, the injected dye failed to diffuse from low CX43-expressing HESC-T3 stromal cells. The non-diffusible red DiI marker identifies the microinjected cells. Interestingly, the HESC-T3 cells also failed to undergo morphological decidualization in vitro following treatment with a hormonal cocktail containing E, P and cAMP, whereas the control HESC-TC cells, treated with this cocktail, exhibited distinct epithelioid morphological characteristics (Ryan et al., 1994) that were indicative of their differentiated status (Fig. 7C). These results correlate with the impaired progression through decidualization displayed by the uterine stromal cells of $Cx43^{d/d}$ mice.

We next examined whether this lack of stromal differentiation in the absence of CX43 resulted in differences in protein production from HESC-TC and HECS-T3 cells. Prolactin, a biomarker of decidualization in human endometrial stromal cells, similar to the expression of PRP in the mouse, was undetectable in untreated cells but was induced after 7–11 days incubation with E, P and cAMP. Prolactin production in hormone-treated HESC-T3 cells was reduced by 66 ±8% relative to control HESC-TC cells ($n$=3, $P$<0.02). In addition to a reduction in this classical marker of stromal differentiation, Fig. 7D indicates that basal and phorbol ester (TPA)-induced production of VEGF also was reduced in low CX43 expressing HESC-T3 cells compared with control HESC-TC cells. Quantification of VEGF synthesis was performed in three independent E, P and cAMP-treated cell cultures. In control HESC-TC cells, hormone treatment stimulated VEGF production 11.4±5.7 fold, whereas the hormone-induced augmentation in HESC-T3 cells was only 1.4±0.1-fold ($n$=3, $P$<0.04). Our findings indicate that CX43 gap junctions are directly involved in the regulation of VEGF production in human endometrial stromal cells during in vitro decidualization. Furthermore, conservation of the important functional link between CX43 and VEGF expression in both mouse and human stromal cells supports a fundamental role of gap junction communication during uterine angiogenesis.

## DISCUSSION

In rodents, a transient surge of E in the preimplantation phase is essential for initiating implantation of the blastocyst into the uterine epithelium (Psychoyos, 1973; Yoshinaga, 1988). The E-induced delayed implantation in rats and mice faithfully captures this hormonal effect. Although the implantation-inducing action of E in rodents has been known for a long time, the mechanism and mediators of this effect remain largely unknown. Using gene expression profiling, we have identified Cx43 as a target of E regulation in the uterus during delayed implantation. The connexins constitute a large family of gap junction proteins that regulate intercellular communications. Although other members of this family, including Cx26 and Cx31, are expressed in the pregnant mouse uterus (Grummer et al., 1996), our microarray data indicated that Cx43 is the sole member of this family whose uterine expression is induced by E during implantation. Our findings are in agreement with previous reports that Cx43 expression in the uterus is primarily under E regulation (Grummer et al., 2004).

The expression of Cx43 in uterine stromal cells is intimately associated with the decidualization phase of pregnancy. Following blastocyst attachment, the underlying stromal cells undergo extensive proliferation and differentiation that result in their transformation into the decidual cells. In mice and a few other species, decidualization can also be experimentally induced by a variety of artificial stimuli in steroid hormone-primed uteri. We found that the expression of Cx43 is robustly induced in the decidual tissue during both normal and artificial decidualization. This induction of Cx43 is likely to be regulated by E acting via ERα (Esr1 – Mouse Genome Informatics). In support of this view, our recent studies using conditional knockout mice harboring a null mutation of the gene encoding ERα in the uterus have shown that E-induced expression of Cx43 is absent in these mutant mice (M.J.L. and I.C.B., unpublished). A direct regulatory role for ERα in Cx43 expression, however, remains to be established, and would require a detailed analysis of the 5′-flanking regulatory region of the *Cx43* gene for the presence of functional ER-binding sites.

Analysis of the *Cx43*-deficient uteri revealed an impaired decidual response during both normal and artificial decidual reaction. A defect in decidualization could arise from either compromised stromal cell proliferation or an arrest in the differentiation program of these cells. Immunohistochemical studies using the cell proliferation marker Ki67 indicated that *Cx43*-deficiency did not significantly affect stromal cell proliferation, which occurs predominantly during days 5 and 6 of pregnancy (data not shown). However, in the absence of Cx43 gap junctions, the stromal cells failed to progress properly in the differentiation program initiated in response to a decidual stimulation. This was evidenced by a marked reduction in uterine wet weight gain, which is considered a hallmark of decidualization. Additionally, two well-known markers of uterine stromal differentiation, PRP and PLP, exhibited impaired or aberrant expression in *Cx43*-deficient uteri during days 7 and 8 of pregnancy. The altered expression of these biomarkers indicated that the loss of Cx43 expression in the uterine stromal cells leads to improper progression of the decidualization program. The defect in stromal differentiation in the absence of Cx43 was further substantiated when we analyzed the uterine expression of Hoxa10 and Bmp2, which are crucial regulators of decidualization. Previous studies have shown that mice harboring a targeted deletion of either the *Hoxa10* or the *Bmp2* gene exhibit impaired uterine stromal differentiation and are infertile (Lim et al., 1999; Lee et al., 2007). The expression of both of these factors was markedly reduced in *Cx43*-deficient uteri. Furthermore, we observed that the loss of Cx43 expression in human endometrial stromal cells blocked their differentiation into prolactin-producing decidual cells. Collectively, these results form the basis of the important concept that the formation of Cx43 gap junctions between stromal cells is critical for the efficient and timely progression of the decidualization program.

Our study also suggests that stromal differentiation and angiogenesis are intimately linked processes within the pregnant uterus. Paracrine factors secreted by the decidualizing stromal cells might influence the proliferation and function of uterine endothelial cells in the mesometrial region of the pregnant uterus where neovascularization mostly occurs. Impaired decidualization due to the loss of Cx43 gap junction communication might result in reduced expression and secretion of angiogenic factors by the uterine stromal cells, and might thereby lead to a concomitant impairment in angiogenesis. In strong support of this concept, we observed a markedly reduced expression of Vegf, a potent stimulator of endothelial cell proliferation and a well-known angiogenic molecule, particularly in the mesometrial region of the pregnant *Cx43*-deficient uterus. Previous studies have shown that the morphogen Bmp2 mediates the enhancement of Vegf expression and neovascularization in xenografts of breast tumors in mice (Raida et al., 2005). It was also demonstrated that Vegf expression in chondrocytes requires cooperative interactions between the Bmp2 and β-catenin signaling pathways (Chen et al., 2008). It is, therefore, conceivable that Bmp2 produced by the decidualizing stromal cells might regulate uterine Vegf expression during early pregnancy. Future studies will evaluate the role of Bmp2 and other stroma-derived factors in the regulation Vegf expression in the decidual uterus.

We also noted that the expression of two additional angiogenesis regulators, angiopoietins 2 and 4, was downregulated in the *Cx43*-deficient uterus during the decidualization phase. Whereas previous studies established that angiopoietin 2 is required for the sprouting of new blood vessels in adult tissues, recent reports implicated angiopoietin 4 in endothelial cell migration (Gale et al., 2002; Lee et al., 2004). The lack of expression of these crucial angiogenic factors was predictably associated with a dramatic impairment in the formation of neovasculature in pregnant *Cx43*-deficient uteri, and, consequently, led to poor embryo development and early pregnancy loss. Most importantly, the use of primary cultures of human endometrial stromal cells allowed us to establish that the interrelationship between stromal CX43 expression, the progression of decidualization, and the production of VEGF by the decidual cells also exists in the human. Our study, therefore, uncovered a conserved, fundamental role of Cx43 gap junctions in maintaining the stromal differentiation program and supporting angiogenesis in the uterus during early pregnancy. Another tantalizing possibility is that the Cx43 gap junctions additionally play a role in the regulation of communication between the maternal decidua and the conceptus. Further studies will be required to carefully examine whether Cx43 expression is linked to proper functioning of the extraembryonic tissues, such as the parietal endoderm, giant cells and the ectoplacental cone.

Gap junction channels allow direct exchange of ions, second messengers, metabolites, and other small molecules (up to ~1200 Da) between the cytoplasms of adjacent cells. This type of intercellular coupling is known to regulate cell proliferation and differentiation (Lo and Gilula, 1979; Simon and Goodenough, 1998; Evans and Martin, 2002). The identity of the intercellular signal(s) being exchanged via the stromal gap junction during decidualization needs to be established. Within the pregnant uterus, stromal differentiation proceeds in a strictly coordinated manner, spreading spatially from the antimesometrial to the mesometrial side as the pregnancy advances. It is conceivable that stromal cells that are more advanced in the differentiation program could facilitate the progression of their less differentiated neighbors by establishing direct contact with these cells. Once the gap junctions are formed, crucial signaling molecules may pass from the donor to the recipient cells via these connections. These regulatory molecules, which may include second messengers, such as cyclic nucleotides, calcium ions or prostaglandins, are likely to impact the gene expression program and differentiation fates of the recipient stromal cells, altering their ability to produce Vegf, angiopoetin 2, angiopoetin 4, and possibly other paracrine angiogenic effectors. In support of this concept, cAMP and phorbol ester-dependent signaling pathways were found to enhance VEGF expression by human endometrial stromal cells (Popovici et al., 1999; Fig. 7D). cAMP

was also implicated as the signaling molecule that passes through the gap junctions to influence the adrenocorticotropin-stimulated growth and steroidogenic function of adrenal cells (Murray and Fletcher, 1984; Shah and Murray, 2001). We postulate that a similar mode of intercellular coupling via Cx43 gap junctions may underlie the unique spatiotemporal appearance of the differentiation markers and the coordinated production of angiogenic regulators in the uterus during early pregnancy.

In summary, the present study revealed a central role for Cx43-containing stromal gap junctions in the establishment of an elaborate vascular network within the endometrial bed that is essential for successful implantation and subsequent embryonic growth. The ovarian hormone dependence of Cx43 functionally regulates gap junction communication among stromal cells, advancing the process of decidualization and stimulating these cells to produce crucial paracrine effectors, such as Vegf. Most importantly, a novel, linear molecular pathway has emerged providing yet another link between nuclear actions of steroid hormones and cellular signaling at the level of the plasma membrane. Ultimately, these molecules control stromal differentiation and angiogenesis during early pregnancy.

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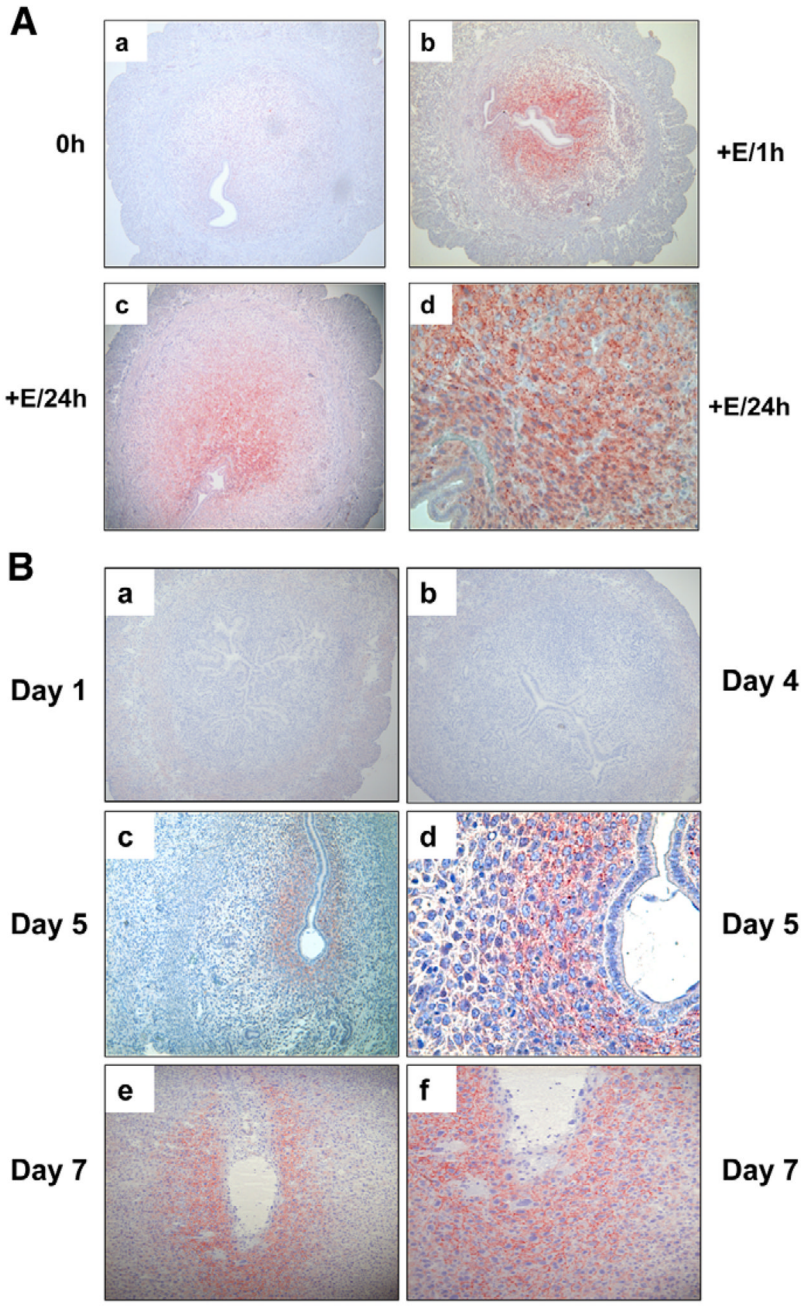


**Fig. 1. Induction of Cx43 expression in uterine stromal cells during implantation**

(**A**) Mice were subjected to delayed implantation as described in the Materials and methods. Uteri were collected 1 hour (b) and 24 hours (c,d) after E treatment and subjected to immunohistochemistry using anti-Cx43 antibody. (d) Higher magnification view of the 24-hour sample. The 0-hour sample (a) represents pregnant uterus obtained from a mouse treated with P alone for three days. Results are from two independent experiments. (**B**) Uteri were collected in the morning of day 1 (a), day 4 (b), day 5 (c,d) and day 7 (e,f) of pregnancy. Higher magnifications of Cx43 expression in stromal cells surrounding the implanted embryo on day 5 and day 7 are shown in d and f, respectively. Results are from two independent experiments.

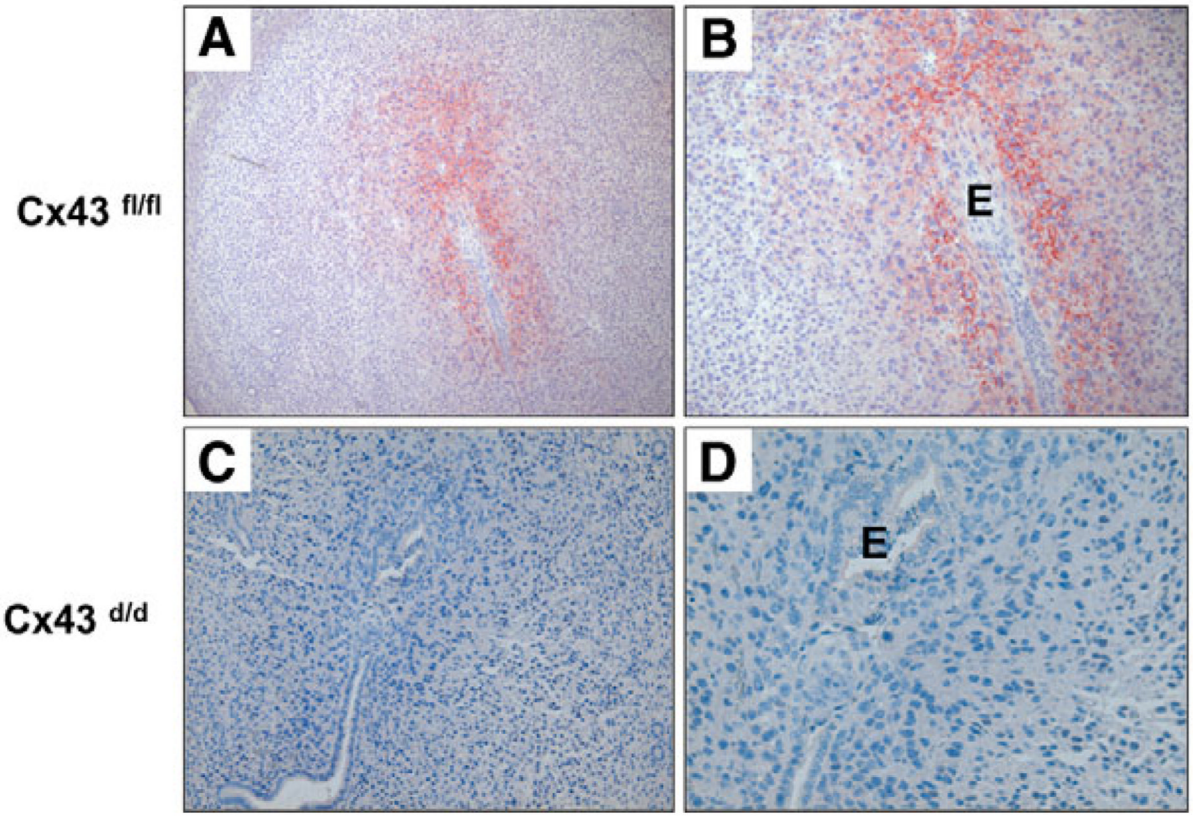


**Fig. 2. Loss of Cx43 expression in the uterus of the *Cx43* conditional-knockout mouse**

(**A,B**) Uterine sections from *Cx43*$^{fl/fl}$ (control) mice show prominent Cx43 expression in the stromal cells surrounding the embryo. (**C,D**) Uterine sections from *Cx43*$^{d/d}$ (conditional knockout) mice show efficient ablation of Cx43 in uterine stromal cells. E denotes implanted embryo.

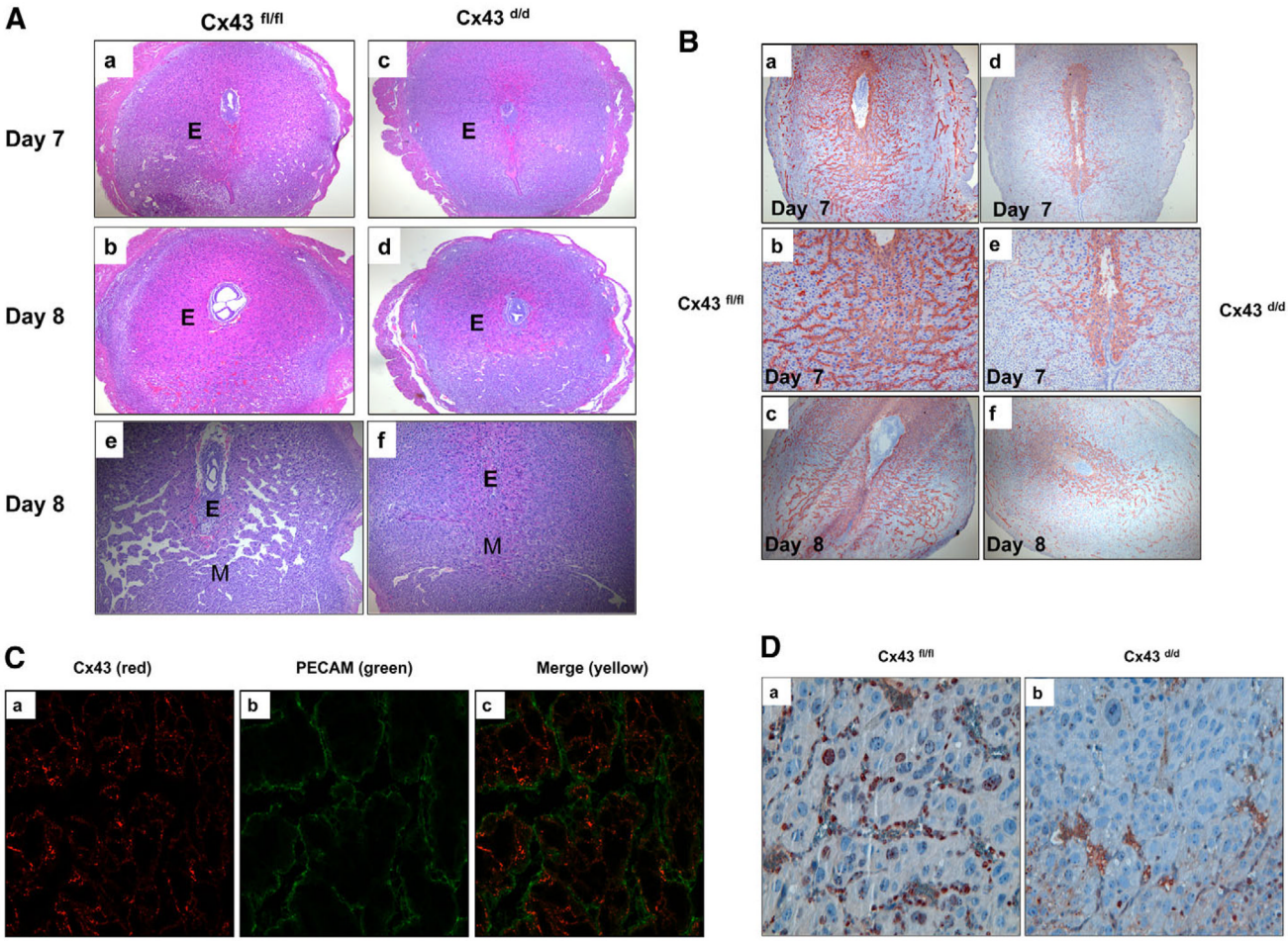


**Fig. 3. Impaired embryo development and lack of angiogenesis in uteri of *Cx43* conditional knockout mice on day 8 of gestation**

(**A**) Hematoxylin and Eosin staining of uterine sections obtained from *Cx43*$^{fl/fl}$ (a,b) and *Cx43*$^{d/d}$ (c,d) mice on days 7 and 8 of pregnancy. Note that implanted embryos (E) develop poorly in *Cx43*$^{d/d}$ mice compared with those in *Cx43*$^{fl/fl}$ mice. Histological analysis by Hematoxylin and Eosin staining of perfused uterine sections obtained from pregnant *Cx43*$^{fl/fl}$ (e) and *Cx43*$^{d/d}$ (f) mice on day 8 of pregnancy. E, implanted embryo; M, the mesometrial region. Note the extensive vasculature in the uteri of *Cx43*$^{fl/fl}$ mice and the lack of it in the uteri of *Cx43*$^{d/d}$ mice. (**B**) Immunohistochemical staining (indicated by red deposits) for Pecam in pregnant uteri. Uterine sections of *Cx43*$^{fl/fl}$ (a–c) and *Cx43*$^{d/d}$ (d–f) mice on day 7 (a,b,d,e) and day 8 (c,f) of pregnancy are shown ($n$=3). (**C**) Uterine sections collected from mice on day 8 of pregnancy were subjected to immunoflouroscence employing anti-connexin 43 (a) and anti-Pecam1 (b) antibodies. c represents a merge of both Cx43 and Pecam staining indicating the expression of Cx43 in the stromal cells but not in the endothelial cells ($n$=2). (**D**) Uterine sections from *Cx43*$^{fl/fl}$ (a) and *Cx43*$^{d/d}$ (b) mice on day 8 of gestation were subjected to immunohistochemistry using an antibody against Ki67, a marker of cell proliferation ($n$=2).

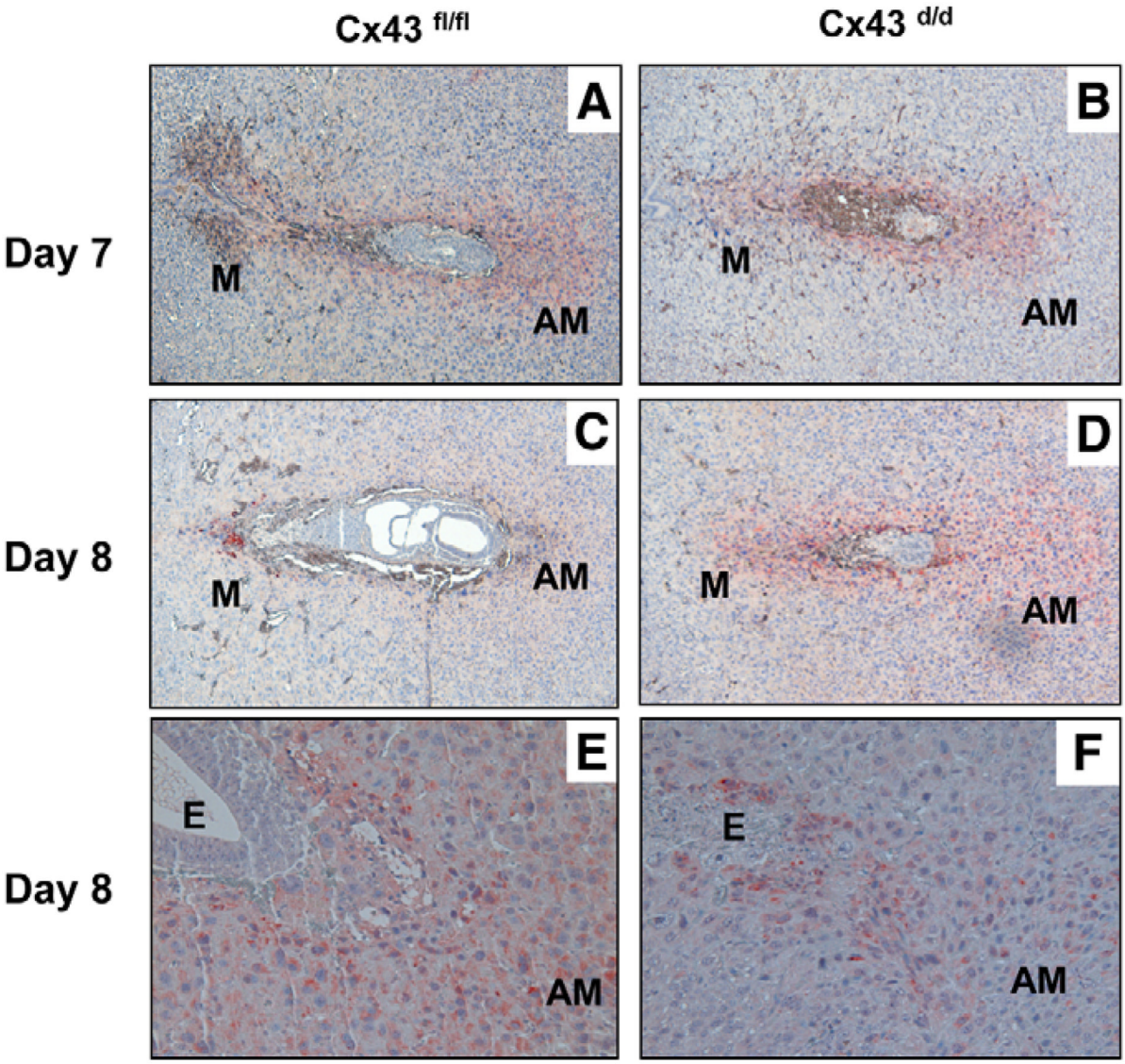


**Fig. 4. Cx43-deficient uteri exhibit impaired decidualization**

(**A**–**D**) Uterine sections from *Cx43*$^{fl/fl}$ and *Cx43*$^{d/d}$ mice on day 7 (A,B) and day 8 (C,D) of pregnancy were subjected to immunohistochemical staining using an antibody specific for the prolactin-related protein (PRP). (**E**,**F**) Uterine sections from *Cx43*$^{fl/fl}$ (E) and *Cx43*$^{d/d}$ (F) mice on day 8 of pregnancy were subjected to immunohistochemical staining using an antibody specific for the prolactin-like protein (PLP). AM and M denote antimesometrial and mesometrial regions, respectively. Results are from two independent experiments.

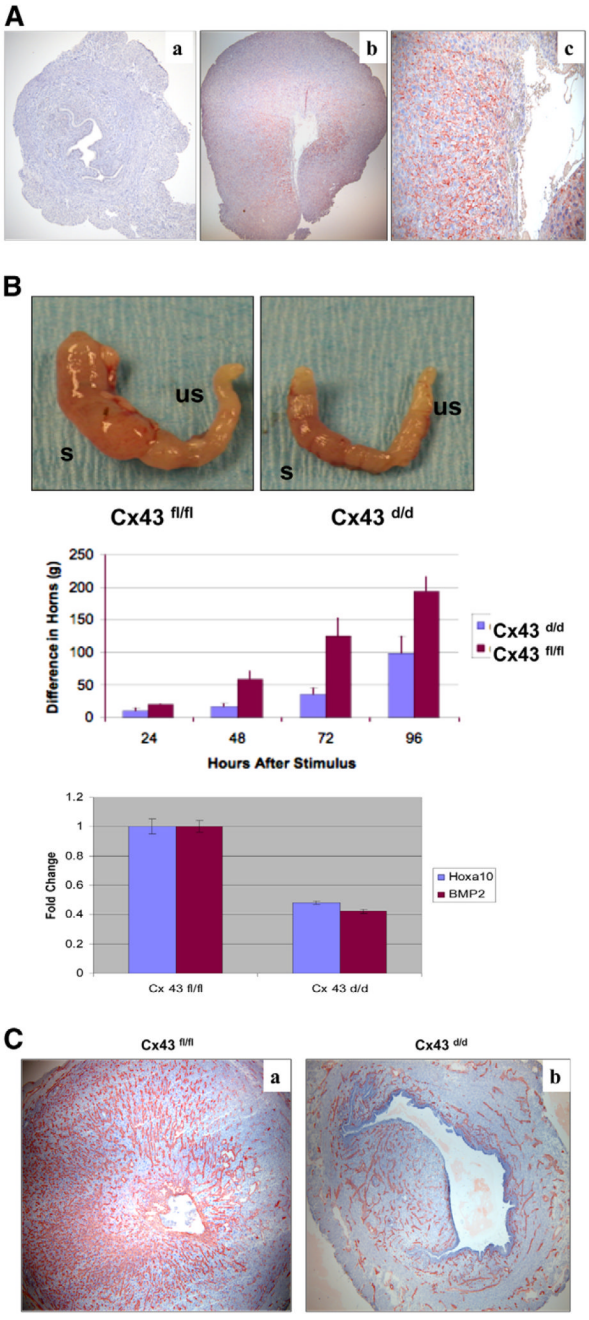


**Fig. 5. Cx43 is essential for decidual response and angiogenesis**

(**A**) Expression of Cx43 in artificially decidualized uterus. Mice were subjected to artificial decidual stimulation. Uteri were collected at 0 (a) and 72 (b,c) hours following the application of the stimulus. Uterine sections were analyzed for Cx43 expression by immunohistochemistry. c is a higher magnification of the 72-hour sample. (**B**, upper) Gross morphology. *Cx43*$^{fl/fl}$ (left) and *Cx43*$^{d/d}$ (right) mice were subjected to artificial decidual stimulation for 48 hours as described in the Materials and methods. For each mouse, one uterine horn was stimulated, while the other horn was left undisturbed. The stimulated horn is indicated as 's' and the unstimulated one as 'us'. (Middle) Comparative wet weight gains in uteri of *Cx43*$^{fl/fl}$ and *Cx43*$^{d/d}$ mice. Following artificial decidualization, stimulated and unstimulated horns were

assessed for wet weight gain. The histogram shows the ratios of average weights of stimulated over unstimulated horns from *Cx43*$^{fl/fl}$ and *Cx43*$^{d/d}$ mice. The data are represented as means ±s.e.m. ($n$=4). (Lower) Expression of *Hoxa10* and *Bmp2* mRNA in the uteri of *Cx43*$^{fl/fl}$ and *Cx43*$^{d/d}$ mice. Total RNA was isolated from uteri collected 96 hours after the application of the artificial decidual stimulus and qPCR analysis was performed using gene-specific primers. (**C**) Immunohistochemical staining for Pecam in artificially decidualized uteri. Uterine sections of *Cx43*$^{fl/fl}$ (a) and *Cx43*$^{d/d}$ (b) mice are shown. The results are representative of two independent experiments.

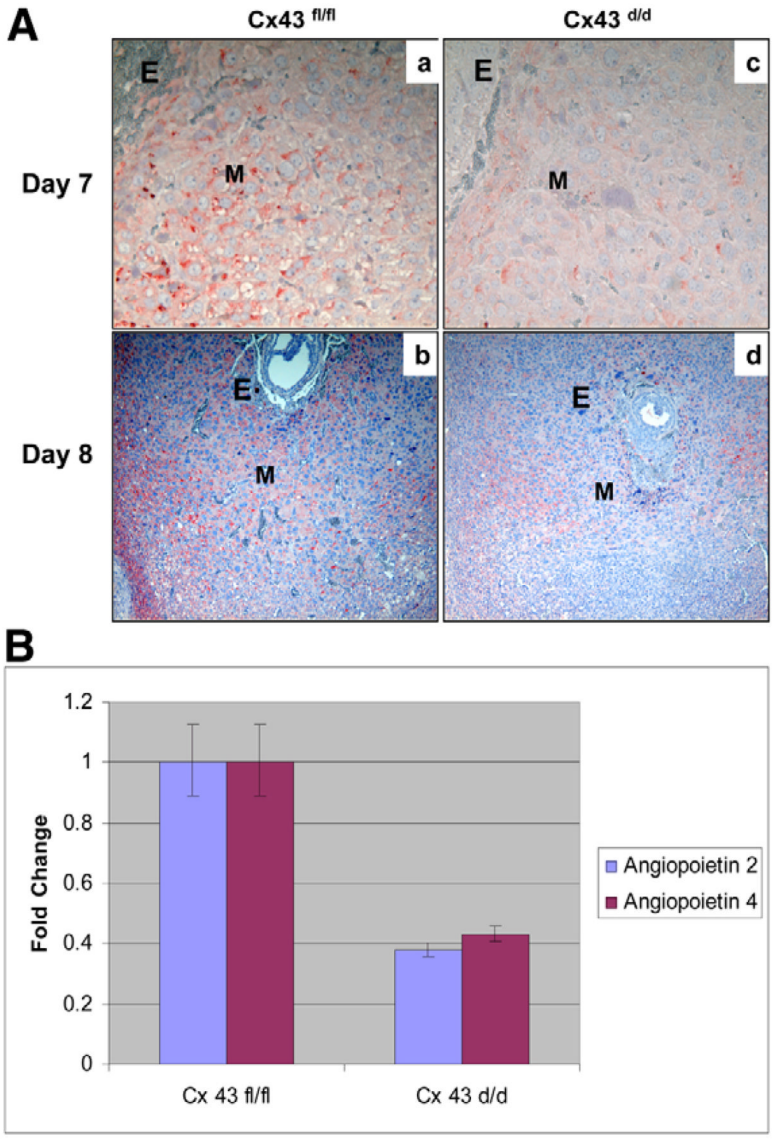


**Fig. 6. Reduced expression of angiogenic factors in *Cx43*-deficient uteri**

(**A**) Uterine sections from *Cx43*$^{fl/fl}$ mice on day 7 (a) and day 8 (b) and those from *Cx43*$^{d/d}$ mice on day 7 (c) and day 8 (d) were subjected to immunohistochemical analysis using anti-Vegf antibody. E, implanted embryo; M, the mesometrial region. (**B**) qPCR analysis was performed to monitor angiopoetin 2 and angiopoetin 4 mRNA expression in the uteri of *Cx43*$^{fl/fl}$ and *Cx43*$^{d/d}$ mice on day 8 of pregnancy (*n*=3).

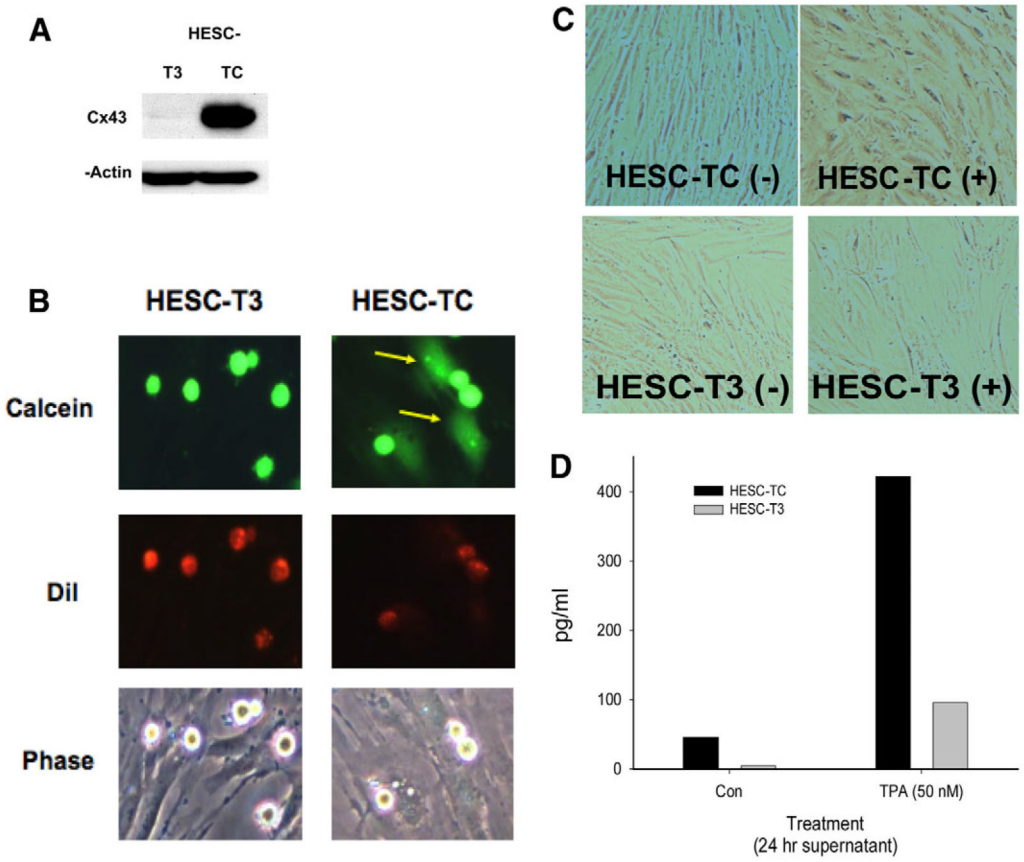


**Fig. 7. Attenuation of CX43 expression in human endometrial stromal cells leads to impaired gap junction communication, decidualization and a reduction in VEGF production**

(**A**) Western blotting was used to analyze the expression of CX43 in HESC-T cells stably transfected with a CX43 siRNA (HESC-T3) or a nonsilencing control vector (HESC-TC). Pronounced silencing of CX43 protein was observed in HESC-T3 cells, whereas the levels of beta actin protein remained unaltered. (**B**) Gap junction intercellular communication was determined using a double-labeling technique. Donor cells were double labeled with the fluorescent dyes calcein (gap junction permeable dye, green fluorescence) and DiI (gap junction impermeable dye, red fluorescence), and were then placed in contact with unloaded cells in the monolayer. Dye transfer was visualized after 2 hours. HESC-T3 cells with reduced CX43 expression exhibited lack of green dye diffusion. Arrows show examples of functional coupling between HESC-TC cells. (**C**) HESC-TC and HESC-T3 cells were grown in DMEM/F-12 medium containing 5% charcoal-stripped FBS. To induce in vitro decidualization, they were treated with or without a hormone cocktail containing 1 nM E, 1 mM P and 0.5 mM 8-bromo-cAMP for 7–11 days (Ryan et al., 1994). When the cells were examined morphologically, a distinct transition from fibroblastic to a plump, epitheloid phenotype, characteristic of decidual cells, was observed in control HESC-TC cells. Low CX43-expressing HESC-T3 cells failed to show this morphological transformation. (**D**) HESC-TC and HESC-T3 cells were grown for 24 hours in the absence (Con) or presence (TPA) of 50 nM phorbol ester. The VEGF in supernatant was measured in duplicate by ELISA.

**Table 1**

Impact of Cx43 ablation on female fertility

| Genotype | Number | Number of pups | Number of litters | Average pups/litter | Ovulation (ova/ovary)* | Serum $P_4$ (ng/ml)* |
|---|---|---|---|---|---|---|
| *Cx43fl/fl* | 37 | 419 | 65 | 6.4 | 19±2 | 84±12 |
| *Cx43*$^{d/d}$ | 7† | 35 | 13 | 2.6 | 15±1 | 95±3 |

Serum P concentrations were measured by radioimmunoassay (RIA) at the core facility of University of Virginia at Charlottesville. The values are expressed as mean±s.e.m.

*$n$=5.

†Thirteen female mice were placed in the breeding program; six mice did not give birth.