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Nodal Expression in the Uterus of the Mouse Is Regulated by the Embryo and Correlates with Implantation[1]

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ABSTRACT

Nodal, a transforming growth factor beta (TGFB) superfamily member, plays a critical role during early embryonic development. Recently, components of the Nodal signaling pathway were characterized in the human uterus and implicated in the tissue remodeling events during menstruation. Furthermore, the Nodal inhibitor, Lefty, was identified in the mouse endometrium during pregnancy, and its overexpression led to implantation failure. Nonetheless, the precise function and mechanism of Nodal signaling during pregnancy remains unclear. In order to elucidate the potential roles Nodal plays in these processes, we have generated a detailed profile of maternal Nodal expression in the mouse uterus throughout pregnancy. NODAL, although undetectable during the nonpregnant estrus cycle, was localized throughout the glandular epithelium of the endometrium during the peri-implantation period. Interestingly, Nodal expression generated a banding pattern along the proximal-distal axis of the uterine horn on Day 4.5 that directly correlated with blastocyst implantation. Embryo transfer experiments indicate the embryo regulates Nodal expression in the uterus and directs its expression at the time of implantation, restricting NODAL to the sites between implantation crypts. During the later stages of pregnancy, Nodal exhibits a dynamic expression profile that suggests a role in regulating the endometrial response to decidualization and associated trophoblast invasion.

blastocyst, decidualization, embryo, embryo-uterine crosstalk, female reproductive tract, glandular epithelium, growth factors, implantation, placenta, trophectoderm, trophoblast invasion, uterus

INTRODUCTION

Nodal, a member of the transforming growth factor beta (TGFB) superfamily, plays an integral role in many processes of vertebrate development. During embryogenesis, NODAL activates a signaling pathway that is essential to primary germ layer induction and axis specification [1, 2]. Insertional mutation of the Nodal gene in mice results in failed mesoderm formation, hyperplasia of the embryonic and extraembryonic ectoderm, and lethality on Day 10.5 [3]. Furthermore, Nodal acts to direct left-right development, and misexpression studies affect tissue and organ laterality [4, 5]. In addition to these established roles, Nodal has also been implicated in neural patterning and anterior-posterior axis specification [6–8].

Like other members of the TGFB superfamily, the Nodal signaling pathway is activated when NODAL ligand binds to a cell surface complex comprised of a type I and type II serine-threonine kinase receptor belonging to the activin receptor family [9]. Type I activin receptor-like kinase 4 or 7 (ALK4 or ALK7) and type II ACVR2A or ACVR2B are the only known receptors that facilitate Nodal signaling [10]. Unlike other TGFB superfamily members, such as Activin, NODAL requires the presence of an extracellular, membrane-associated EGF-CFC co-receptor (TDGF1/Cripto or CFC1/Cryptic) to activate the signaling pathway despite its ability to bind the receptors in its absence [11]. Although the precise mechanics of co-receptor binding remain unclear, it is currently hypothesized that the EGF domain recruits NODAL to the cell membrane while the CFC domain mediates binding to the ALK receptor [9]. Formation of the cell surface complex results in phosphorylation of the type I ALK by the type II receptor kinase, which in turn phosphorylates and activates the downstream SMAD2 and/or SMAD3 proteins [10]. Activation of SMAD2 and SMAD3 permits the binding of SMAD4, forming a complex that is imported into the nucleus and regulates the expression of target genes. NODAL ligand can signal both locally or as a morphogen, diffusing over long distances and in a concentration-dependent manner [12].

Nodal signaling is regulated by complex autoregulatory interactions that propagate and restrict activity, as NODAL binding results in the expression of more NODAL ligand and its diffusible inhibitor, LEFTY [9, 12]. LEFTY proteins are divergent TGFB molecules that antagonize Nodal signaling by both interacting with NODAL ligand directly to prevent activin receptor binding and interacting with the EGF-CFC co-receptor to block complex formation [13, 14]. As a result, NODAL and LEFTY create interesting spatial and temporal patterns of expression as the proteins diffuse over long distances and compete for receptor binding, thereby limiting their range of influence [13]. Lefty has several established roles in Nodal-dependent processes during early embryonic development [15]. LEFTY2, initially designated endometrial bleeding associated factor (*EBAF*), was also previously detected in the human uterus and implicated in tissue remodeling events associated with the menstrual cycle [16]. Furthermore, endometrial LEFTY was significantly increased during the receptive phase of patients with unexplained infertility [17]. Recently, LEFTY protein was observed in the mouse endometrium during the peri-implantation period, and in vivo gene delivery led to implantation failure; however, the precise role of Lefty in facilitating successful implantation remains unclear [18, 19].

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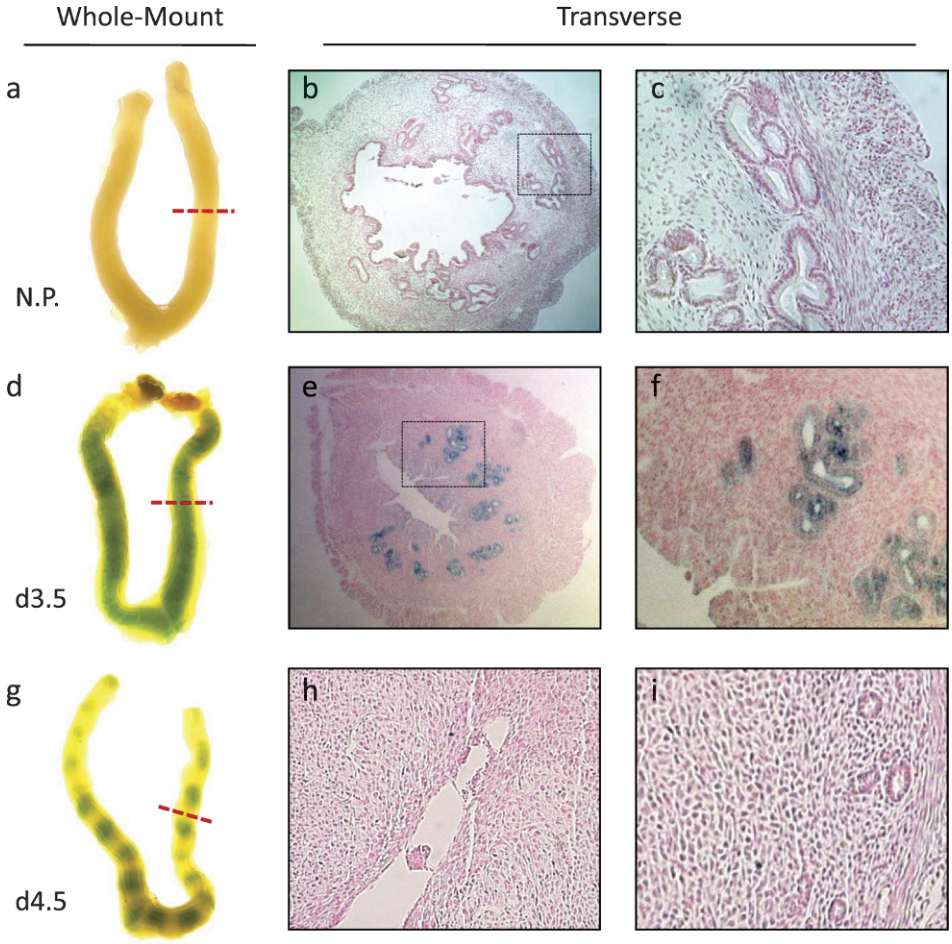


FIG. 1. Nodal-lacZ expression profile in the mouse uterus during the peri-implantation period. **a**) Whole-mount staining and (**b**–**c**) transverse sectioning of nonpregnant (N.P.) uteri did not reveal β-galactosidase activity at any point in the estrus cycle. **d**) However, staining was readily detectable throughout the uterus after whole-mount staining from Day 0.5 to 3.5 postcoitum (representative d3.5 shown). **e**–**f**) Transverse sectioning indicates expression was restricted to the glandular epithelium within the uterine endometrium. **g**) On Day 4.5 postcoitum (d4.5), whole-mount staining generated a banding pattern along the proximal-distal axis of the uterine horn. **h**) Serial transverse sections showed embryos undergoing implantation exclusively within the nonstained uterine bands, and (**i**) glandular epithelium at the implantation site was unstained, indicating Nodal expression was restricted to the sites between implantation crypts. Original magnification ×4 (**b**, **e**) and ×10 (**c**, **f**, **h**, **i**).

In spite of the evidence that indicates Lefty is expressed within the vertebrate endometrium and that overexpression leads to implantation failure in mice, the nature of Nodal expression in the uterus during pregnancy has not been explored. As the primary target of Lefty antagonism, this critical aspect of the Nodal signaling pathway must be evaluated to fully understand the molecular basis underlying these critical uterine functions. Here, we provide a detailed profile of Nodal expression in the mouse uterus throughout pregnancy. Furthermore, we consider the role of the embryo in directing uterine Nodal expression at sites of implantation.

MATERIALS AND METHODS

Mating and Manipulation of Transgenic Mice

All animal care and experimental procedures were approved by the Animal Care Committee of the Royal Victoria Hospital and were in accordance with the regulations established by the Canadian Council on Animal Care. Nodal-lacZ mice on a CD1 background were previously generated and donated by E.J. Robertson [20]. Transgenic females were mated with either fertile or vasectomized CD1 males, and the day of vaginal plug was assigned as Day 0.5. Nodal-lacZ females used for embryo transfer experiments were mated with vasectomized males, and the indicated number of wild-type CD1 blastocysts was transferred into the experimental uterine horn on Day 3.5. Contralateral uterine horns were used as a nontransferred control by injecting an equivalent volume of potassium simplex optimized media (KSOM) [21]. Uteri were recovered on Day 4.5 and stained for β-galactosidase activity.

Detection of β-Galactosidase Activity

Isolated uteri were dissected in PBS and fixed in 4% paraformaldehyde (PFA)/PBS for 30 min at 4°C. Uteri were then washed three times in wash buffer (0.02% Nonidet P-40, 0.01% deoxycholate, 2 mM $MgCl_2$, and 100 mM sodium phosphate; pH 7.3) for 15 min at room temperature. β-Galactosidase activity was revealed by incubating uteri overnight at 37°C in wash buffer with 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6$. Uteri were then rinsed in wash buffer and fixed with 4% PFA/PBS at 4°C prior to whole-mount photography and histological sectioning. β-Galactosidase activity was also assessed directly on cryosections prepared for immunohistochemistry as described below.

Paraffin Embedding and Sectioning

Uteri were dehydrated with increasing ethanol concentrations (25%, 50%, 75%, and 100%, 20 min each) and treated with two 15-min xylenes washes. The tissue was placed in melted paraffin wax overnight and embedded at room temperature, and the blocks were solidified at −80°C. Seven-micrometer sections were cut with the Leica RM2145 microtome and dried overnight. Slides were then washed in xylenes, rehydrated with a decreasing ethanol gradient (100%, 95%, 85%, 75%, 50%, and 20%, 2 min each), and counterstained with Nuclear Fast Red (Sigma) for 5 min. The stained sections were dehydrated, cleared in xylenes, and mounted with paramount glue.

Immunohistochemistry

Wild-type CD1 or Nodal-lacZ uteri were dissected in cold PBS and fixed overnight at 4°C in 4% PFA/PBS. Uteri were treated with 15% sucrose for 3 h and 30% sucrose overnight at 4°C before embedding in Shandon Cryomatrix (Thermo). Ten-micrometer sections were cut with a cryostat, mounted on Super Frost Plus slides (VWR International), and stored at −80°C. Slides were rehydrated in PBS, post-fixed in 4% PFA/PBS for 10 min at 4°C, and treated with proteinaseK (30 μg/ml) for 12 min at room temperature, with interspersed washes in PBS + 0.1% Tween-20 (PBT). Sections were blocked in 20% heat-inactivated goat or rabbit serum + PBS for 1 h and incubated overnight at 4°C with rabbit anti-Nodal (sc-28913; 0.2 mg/ml; Santa Cruz), rabbit anti-β-galactosidase (3 mg/ml; 55976; MP Biomedicals), or goat anti-Lefty antibody (AF746; 0.1 mg/ml; R&D Systems) diluted 1:100 in 2% goat or rabbit serum +

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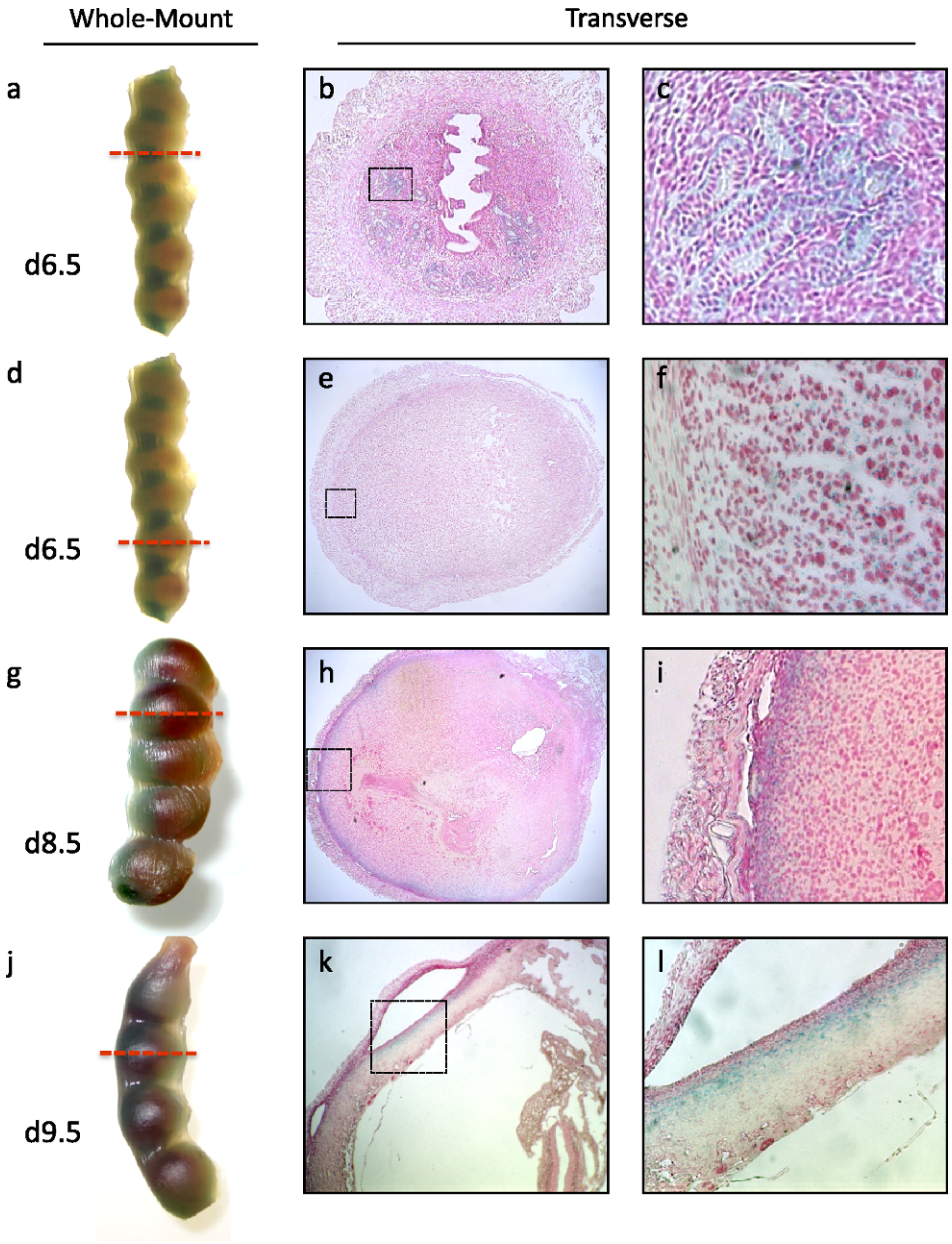


FIG. 2. Postimplantation Nodal-lacZ expression profile in the mouse uterus. **a**) Whole-mount staining and (**b**–**c**) transverse sectioning of Day 6.5 uteri displayed significant β-galactosidase activity between decidua swellings within the glandular epithelium. **d**–**f**) Within the implantation crypts, faint staining was observed in the stroma immediately adjacent the antimesometrial (oriented left) myometrium with decreasing β-galactosidase activity toward the central decidua. **g**) By Day 8.5, increased staining was observed along the antimesometrial surface of the whole-mount uterus that was primarily localized within the endometrial stromal cells on the periphery of the zone of decidualization (**h**–**i**). **j**–**l**) On Day 9.5, expression was observed within the stroma on the antimesometrial and lateral surfaces of the conceptus site. Original magnification ×2.5 (**e**, **h**), ×4 (**b**, **k**), ×10 (**l**), ×15 (**i**), and ×20 (**c**, **f**).

PBS. After PBT washes, slides were incubated in the dark with Alexa-488 goat anti-rabbit (AC11008; 1:500, 1 mg/ml; Invitrogen) or Cy-2 rabbit anti-goat antibody (305-225-003; 1:500, 1.5 mg/ml; Jackson Laboratories) for 2 h, washed, counterstained with propidium iodide (0.2 μg/ml), and mounted in Mowiol. Uterus sections were analyzed with the Zeiss LSM 510 Meta confocal microscope and associated software.

RESULTS

Nodal Exhibits Dynamic Expression in the Uterus Throughout Pregnancy

Using a Nodal-lacZ reporter mouse strain, which was previously demonstrated to faithfully recapitulate *Nodal* transcripts [20], the pattern of Nodal expression in the adult uterus was characterized throughout the full duration of pregnancy. Expression of the β-galactosidase reporter was not detected in uteri isolated from nonmated females at any point in the estrus cycle (Fig. 1, a–c). Conversely, Nodal-lacZ females mated with CD1 wild-type males produced positive staining throughout the full whole-mount uterus that remained prominent from Days 0.5 to 3.5 of the peri-implantation period (Fig. 1d). Transverse sections of positively stained uteri indicated that Nodal expression was restricted to the glandular epithelium, with no staining detected in the luminal epithelium or stromal cells (Fig. 1, e and f). Interestingly, on Day 4.5 of pregnancy, whole-mount staining for β-galactosidase activity generated a banding pattern along the proximal-distal axis of the uterine horn (Fig. 1g). As Day 4.5 coincides with the time of blastocyst attachment to the uterine wall, complete serial transverse sectioning through the uterus was performed to locate the sites of implantation. Embryos within implantation crypts were identified exclusively in sections cut through the negatively stained bands of late Day 4.5 uteri (Fig. 1h), and glandular epithelium at the implantation site was unstained (Fig. 1i). As a result, the Nodal-lacZ banding pattern directly correlates with the sites of implantation, with nodal expression restricted to the inter-implantation nodes.

Following implantation, Nodal expression remained exclusively within the glandular epithelium between conceptus sites until Day 6.5, when faint expression could also be detected

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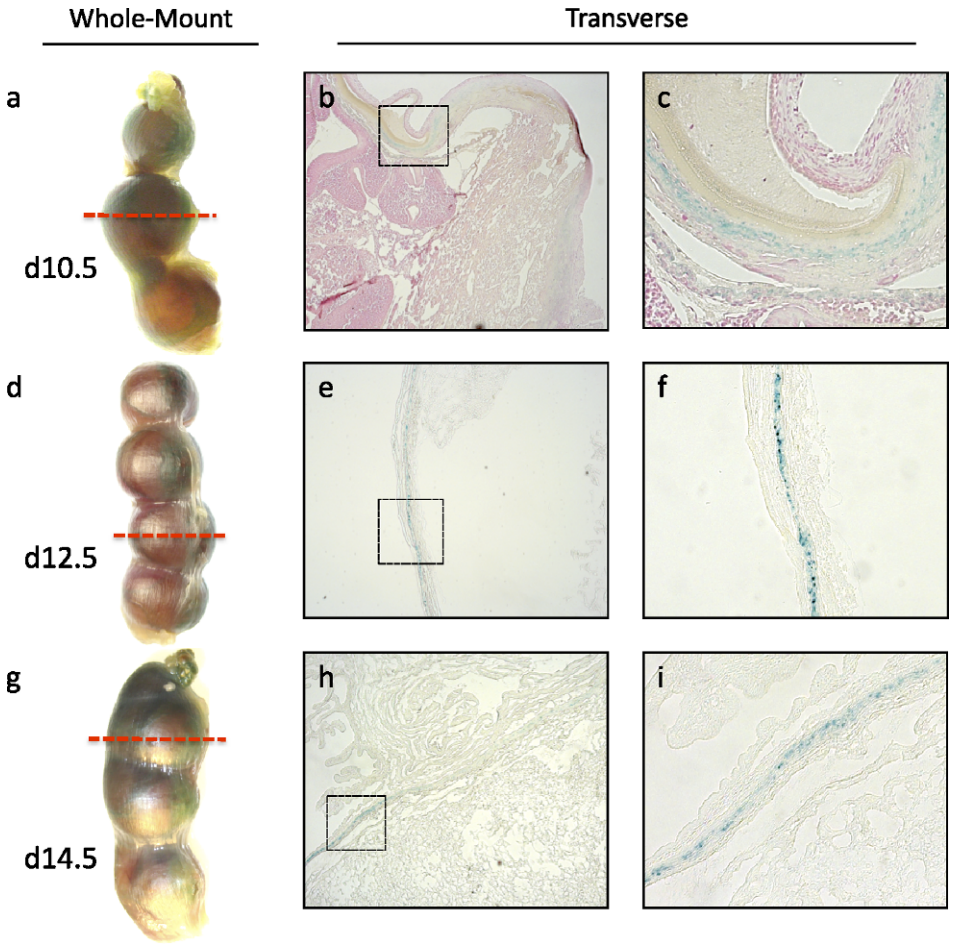


FIG. 3. Nodal-lacZ expression profile in the mouse uterus during late pregnancy. **a**) Whole-mount staining on Day 10.5 was restricted to the mesometrial aspect (oriented right) of the uterus. **b**–**c**) Transverse sectioning displayed prominent expression within a distinct layer of the decidua parietalis on the lateral and mesometrial surface of the conceptus. β-Galactosidase activity was readily detectable in the lateral (**d**–**f**) and mesometrial (**g**–**i**) decidua parietalis throughout the remainder of pregnancy as depicted in Day 12.5 and 14.5 representative samples. Original magnification ×4 (**b**, **e**, **h**) and ×10 (**c**, **f**, **i**).

along the anti-mesometrial surface of decidua swellings at the implantation sites in the whole-mount uterus (Fig. 2, a–d). Transverse sectioning indicated that the faint staining at the implantation site was limited to the anti-mesometrial uterine stroma, with expression predominately adjacent to the myometrium and exhibiting lower intensity toward the decidua (Fig. 2, e and f). By Day 8.5, pronounced expression encompassed the entire antimesometrial surface of the whole-mount conceptus site (Fig. 2g), and increased staining was observed in the undifferentiated uterine stromal cells immediately adjacent to the myometrium, creating a crescent along the antimesometrial periphery of the decidual zone (Fig. 2, h and i). Subsequently, Nodal expression underwent a dramatic shift to the mesometrial surface as the placenta was established. Following a transitional period on Day 9.5 that produced increased staining in the lateral stroma (Fig. 2, j–l), expression ultimately became restricted to a thin, distinct layer of the uterine decidua parietalis by Day 10.5 that tracked up the lateral surfaces alongside the conceptus into the placenta on the mesometrial periphery (Fig. 3, a–c). Within the placenta, the positively stained Nodal-lacZ layer was situated immediately between the maternal decidua basalis and uterine wall. Once established on Day 10.5, expression within the mesometrial decidua parietalis remained easily detectable throughout the full duration of pregnancy (Fig. 3, d–i).

Immunolocalization of Maternal NODAL in Uterine Sections

In order to fully characterize NODAL protein localization in the uterus and verify that the Nodal-lacZ reporter adequately reflects the sites of expression, immunohistochemistry was performed on tissue from CD1 wild-type females. Uteri were isolated from CD1 females at several critical time points throughout pregnancy and compared with the Nodal-lacZ profile. As expected, no protein was detected in uteri collected from nonpregnant females (Fig. 4a). However, NODAL was readily detectable around the glandular epithelium and lining the apical surface of the luminal epithelium during the periimplantation period (Fig. 4b). Although NODAL is apparent on both surfaces of glandular epithelial cells, the protein is primarily located on the apical surface that leads into the uterine lumen.

On Day 8.5, maternal NODAL protein was localized exclusively on the antimesometrial aspect of the conceptus, primarily within the stromal and myometrial compartments (Fig. 4c). By Day 10.5 and continuing throughout the remainder of pregnancy, NODAL was again evident exclusively within a distinctive layer of the uterine decidua parietalis on the mesometrial aspect of the conceptus, paralleling the Nodal-lacZ data (Fig. 4d). Additional NODAL protein, not reported by the transgenic mouse line, was faintly observed in the spongiotrophoblast cells of the developing placenta on Day 10.5 and appeared to increase intensity by Day 14.5. To differentiate maternal NODAL from protein produced in the extraembryonic tissues that invade the endometrium, immunohistochemistry with anti-β-galactosidase on Nodal-lacZ uteri was also performed (Fig. 4, e–h). Although β-galactosidase was consistently detectable in the decidua parietalis, indicating a maternal origin, placental β-galactosidase was only observed if the embryo inherited the Nodal-lacZ allele, suggesting an extraembryonic source.

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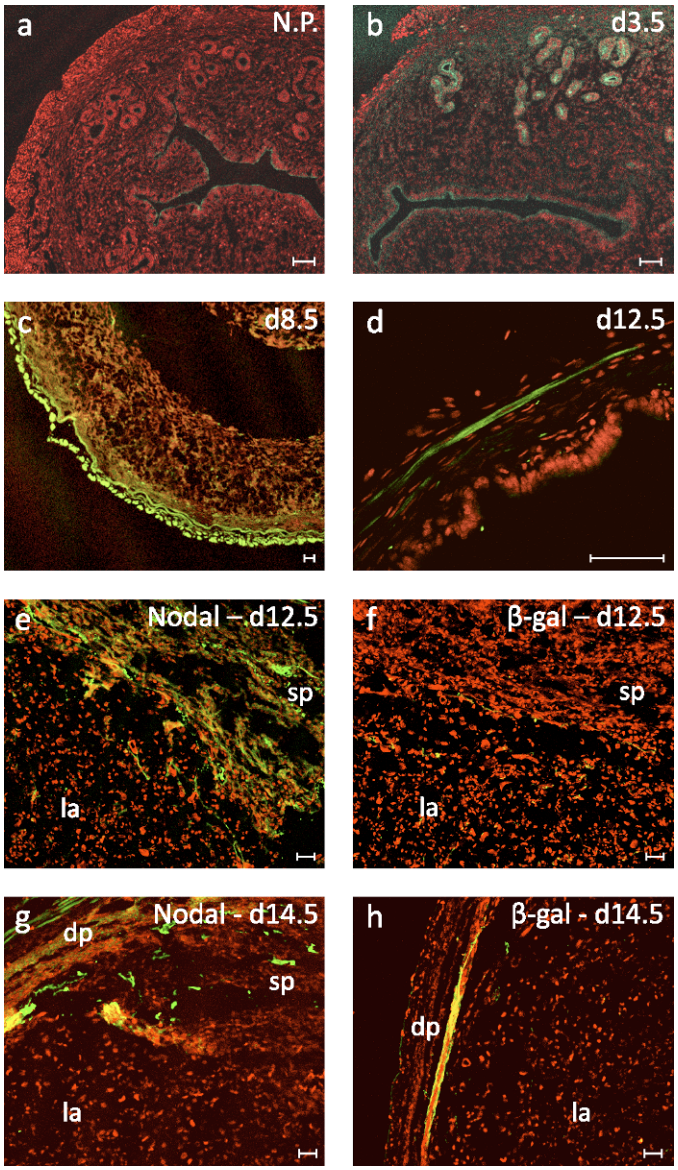


FIG. 4. Immunohistochemical localization of NODAL in the mouse uterus throughout pregnancy. **a**) Immunolocalization did not detect NODAL protein in the endometrium of nonpregnant (N.P.) females. **b**) NODAL protein was observed within the glandular epithelium and lining the uterine lumen from Day 0.5 to 3.5 postcoitum (representative d3.5 shown). **c**) On Day 8.5, NODAL was localized within the stromal and myometrial compartments exclusively on the antimesometrial aspect of the uterus. **d**) By Day 12.5, NODAL protein was readily detectable in a distinct layer of the decidua parietalis on the lateral (shown) and mesometrial poles of the conceptus. **e**, **g**) NODAL protein was also localized in spongiotrophoblast layer of the placenta during late pregnancy (Days 12.5 and 14.5 shown). **f**, **h**) Although the β-galactosidase enzyme was consistently detectable in the decidua parietalis, spongiotrophoblast β-galactosidase was not observed in placentas of embryos that did not inherit the Nodal-lacZ allele, suggesting an extraembryonic origin. Bars = 50 μm. dp, decidua parietalis; la, labyrinth; sp, spongiotrophoblast.

Immunolocalization of NODAL and LEFTY in the Endometrium During Embryo Implantation

It has previously been reported that Lefty is expressed in the mouse endometrium during implantation; however, differentiation between the sites of implantation and the inter-implantation space was not provided [18]. In order to investigate a potential correlation between NODAL and LEFTY at the time of implantation, immunohistochemistry was performed on uterine sections isolated from wild-type CD1 females on Day 4.5. Within the inter-implantation space, NODAL protein was localized in the glandular epithelium and lining the luminal surface, mirroring the pattern observed during the peri-implantation period (Fig. 5a). Similarly, LEFTY protein was readily detectable on the apical surface of the glands, and significant immunoreactivity was exhibited on the luminal epithelial surface (Fig. 5b). In addition to the overlapping localization with NODAL, LEFTY protein was also observed in the stromal compartment of the endometrium. The sites of implantation, as determined by decidualized stroma and a closed lumen, contained minimal NODAL immunoreactivity (Fig. 5d). However, LEFTY protein was observed within the glandular epithelium and stromal cells while faintly detected around the closed lumen, indicating Lefty is expressed throughout the full proximal-distal axis of the uterine horn at the time of implantation (Fig. 5e).

The Embryo Facilitates Nodal Expression at the Time of Implantation

Based upon the cross-talk model of implantation, uterine gene expression during the peri-implantation period is regulated by ovarian steroid hormones and complex reciprocal signaling with the blastocyst [22]. As Nodal expression in the uterus is restricted exclusively to the nodes between implantation sites at the time of blastocyst attachment, the role of the embryo in directing this banding pattern was investigated further. Using vasectomized CD1 males, pseudopregnancy was induced in Nodal-lacZ females, and a peri-implantation period expression profile was generated. As in natural pregnancy, positive staining was detected throughout the full whole-mount uterus from Days 0.5 to 3.5 (Fig. 6a). Alternatively, no staining was observed in the pseudopregnant Day 4.5 uterus (Fig. 6b). These results suggest fertilized embryos, and not the ovarian hormones, are required to maintain and direct uterine Nodal expression at the time of implantation during pregnancy.

To verify embryo-dependent Nodal expression on Day 4.5 of pregnancy, fertilized Day 3.5 embryos were acquired from naturally mated CD1 females and transferred into a single uterine horn of pseudopregnant Nodal-lacZ females on Day 3.5. The contralateral horn was used as a mock transfer control. Nodal-lacZ females were allowed to recover for 24 h, and uteri were isolated on Day 4.5. Whole-mount staining generated the banding pattern in the embryo-transferred experimental horn, whereas nontransferred control horns were completely stained (Fig. 6, c and d). Furthermore, the number of nonstaining bands in experimental uterine horns correlated with the number of embryos introduced. Transfer of 2, 6, and 10 embryos resulted in two (Fig. 6c), five (Fig. 6d), and seven (data not shown) nonstained bands representing successful implantation sites. Taken together, these results indicate the embryo acts to both maintain Nodal expression throughout the entire uterus on Day 4.5 while also inhibiting expression at the implanting site.

DISCUSSION

The results presented in this study raise many interesting questions regarding Nodal regulation and function in the uterus. Although NODAL was not observed during the cyclic phases of estrus, successful copulation resulted in pronounced expression within the glandular epithelium as early as Day 0.5. This expression was also observed after mating with vasectomized males and is therefore independent of ovum

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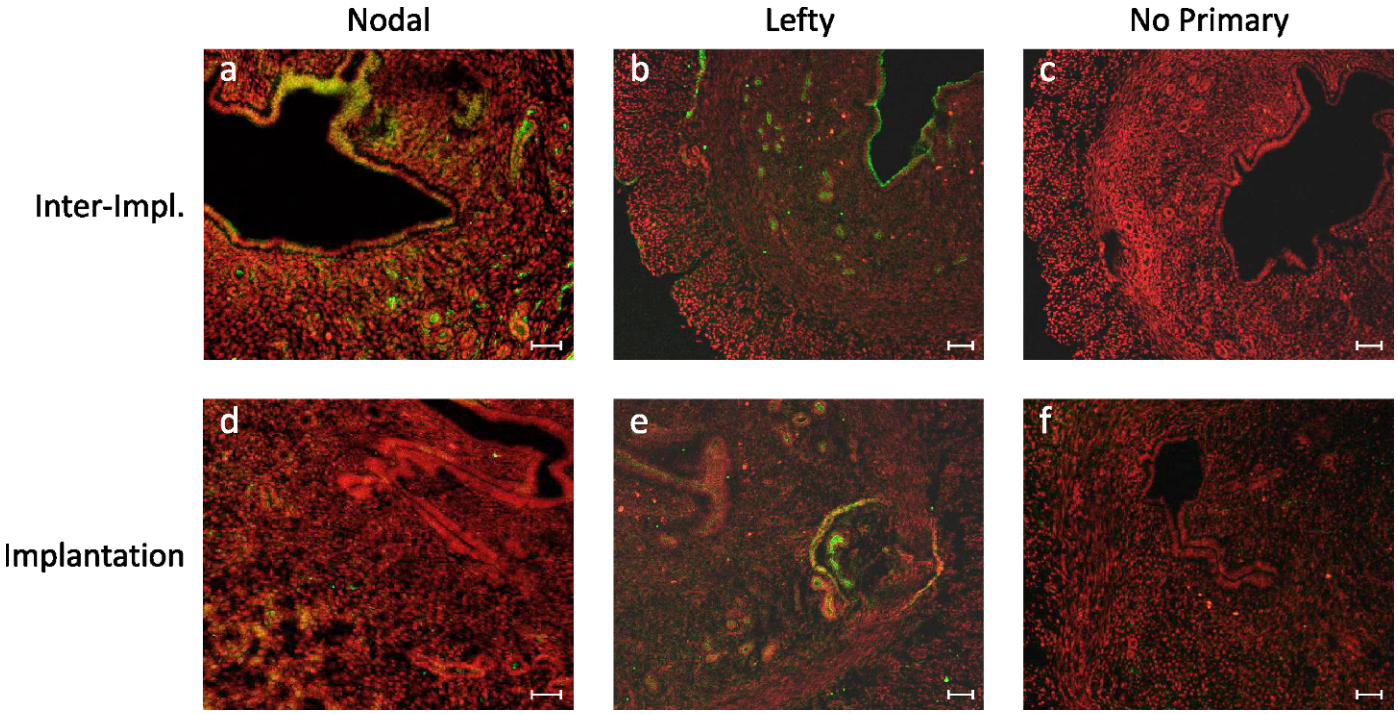


FIG. 5. Immunolocalization of NODAL and LEFTY in the mouse uterus during embryo implantation. **a**) Within inter-implantation spaces, NODAL immunoreactivity was evident around the glandular epithelium and lining the apical surface of the luminal epithelium. **b**) LEFTY protein was localized around the uterine glands, luminal epithelium, and stroma in the endometrium of the inter-implantation space. **d**) Minimal NODAL immunoreactivity was observed in sections cut through the sites of implantation; however, (**e**) LEFTY was evident in similar structures of the endometrium regardless of position along the proximal-distal axis of the uterine horn in relation to implantation. **c**, **f**) Negative control sections subjected to dilution buffer with no primary antibody displayed no positive staining. Bars = 50 μm.

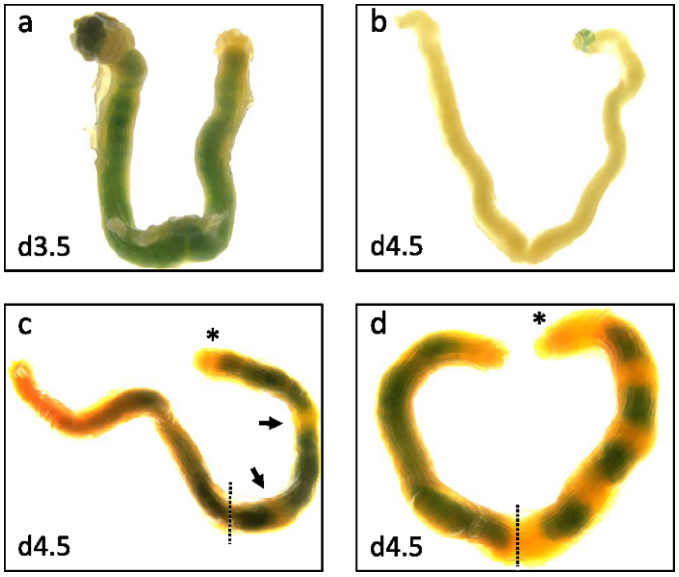


FIG. 6. The embryo directs Nodal expression at the time of implantation. **a**) Whole-mount staining revealed β-galactosidase activity throughout the entire uterus isolated from Day 0.5 to 3.5 pseudopregnant females (representative d3.5 shown). **b**) In contrast to natural mating, Day 4.5 pseudopregnant uteri were devoid of staining. Embryo transfer of two (**c**) or six (**d**) blastocysts into a single uterine horn (*) restored the banding pattern on Day 4.5, and the number of nonstaining sites (arrows) correlated with the number of embryos introduced. Control horns were completely stained, indicating the embryo acts to maintain expression at the time of implantation, but inhibits Nodal expression at the implanting site.

fertilization, suggesting the hormonal changes associated with copulation activate Nodal in the uterus long before maternal recognition of successful fertilization. Based on comparative studies in pseudopregnant mice, the mating-induced neuroendocrine response that stimulates prolactin secretion is a potential candidate of upstream Nodal regulation in the uterus during early pregnancy [23]. It is not inconceivable that the function of preimplantation Nodal expression is related to early endometrial modifications and/or lumen fluid conditioning prior to blastocyst arrival in the uterus on Day 3.5. The majority of uterine gland products are secreted into the uterine lumen [24], and human NODAL proprotein was recently identified in lavage fluid samples from women [25].

Embryo implantation is an intricate and highly orchestrated process that is directed by reciprocal signaling between the maternal uterus and free-floating blastocyst [22]. Here we present data indicating that the embryo coordinates Nodal expression at the time of implantation, ultimately generating a banding pattern along the uterine horn that correlates with the sites of implantation. Based on the pseudopregnant embryo transfer experiments, it appears the blastocyst directs this pattern via a two-stage process that involves both positive and negative regulation. In the first stage, Nodal expression is maintained throughout the uterus on Day 4.5 and is likely instructed by factors that are secreted from the blastocyst prior to attachment. It is an intriguing observation that even a limited number of embryos in a single uterine horn can maintain Nodal expression throughout the uterus, indicating a sensitive maternal detection of the blastocyst that can alter the composition of global uterine expression. In the second stage, the embryo acts to inhibit Nodal expression at the underlying site of implantation and restrict it to the inter-implantation spaces by either emitting factors that act locally during

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apposition or a direct, physical interaction with the uterine luminal epithelium during attachment. As such, the second inhibitory stage might proceed very shortly after Nodal maintenance. Based on the positive and negative regulators associated with Nodal signaling, it is possible that NODAL, or related TGFBs, emanating from the embryo initiate the restriction process by increasing Lefty expression in the endometrium at the underlying implantation site. NODAL is produced in the pre-implantation embryo, and TGFBs secreted by the blastocyst have previously been shown to influence uterine gene expression during implantation [26, 27]. Moreover, endometrial *Lefty* mRNA is relatively low during early pregnancy before undergoing a significant increase in expression at the implanting site between Days 3 and 5 [18]. It remains to be seen if the restriction of Nodal expression is mediated through the glandular network or via the stromal cells; however, the positive identification of LEFTY and upregulation of its processing convertases (PC5/6) in the decidualized stroma suggests it may be transmitted through the stromal compartment [18, 28].

The results presented here and by Tang et al. [18] indicate that LEFTY is localized throughout the endometrium during implantation, whereas Nodal is expressed in alternating sites along the uterine horn that directly correlate with implantation. It has previously been reported that inducing a state of Lefty overexpression by in vivo gene transfer reduced implantation rates in mice and that Lefty expression is increased in the endometrium of human patients who experience unexplained infertility [17, 19]. Although implicated in the process, the exact mechanism of how Lefty overexpression disrupts implantation remains unclear. As the methods used to overexpress Lefty in vivo encompassed the whole uterine horn, the site-specific expression of Nodal between implantation sites would undoubtedly be dysregulated. It is possible that an alternating pattern of NODAL along the proximal-distal axis of the uterine horn is critical to directing implantation, and disrupting this balance significantly reduces the efficiency of this process.

As mice are a polytoccus litter bearing species, the potential role of maternal NODAL in proper embryo spacing also remains an interesting possibility when one considers the banding pattern observed. In fact, it has been shown that *Bmp5*/*Nodal* double mutant litters generated from double heterozygous crosses often contain two to four embryos of differing genotypes within the same deciduum, leading the authors to speculate that signals produced by and/or interpreted by the embryo to indicate an implantation site is occupied have been disrupted [29]. Nodal-dependent signaling between the embryo and uterus may underlie the spacing phenomenon that ensures an adequate distance between implantation crypts.

Following implantation, uterine Nodal exhibits a dynamic pattern of expression that includes a dramatic antimesometrial to mesometrial shift. The related TGFB superfamily member activin βA becomes polarized to the primary decidua zone before undergoing a similar antimesometrial to mesometrial shift during secondary decidualization in the days immediately following implantation [26]. Eventually, activin βA is restricted to the decidua basalis by midpregnancy. These expression patterns have suggested a role for activins, and now Nodal, in regulating the endometrial response to decidualization and associated trophoblast invasion.

Activin βA, expressed in the endometrium at the onset of decidualization, likely prepares the endometrium for decidualization or directly promotes the decidual reaction. Conversely, Nodal signaling may provide inhibitory signals that provide a spatial and temporal limit along the decidualization front. Nodal signaling components have also been implicated in human decidualization that occurs with each cycle, independent of copulation or successful fertilization. The human uterus does not contain multiple sites of implantation along a uterine horn; however, an overall decrease in *Nodal* mRNA expression during the midsecretory to menstrual phase was observed. This coincides with the initiation of decidualization and a dramatic increase (155-fold) in *Lefty* mRNA [25, 30]. As a result, Nodal downregulation appears to be conserved during the onset of decidualization in both rodent and human. Although the co-receptor Cripto has yet to be characterized in the mouse uterus, it is consistently expressed in the human endometrium throughout the menstrual cycle, and it exhibited a similar pattern of localization as that of the NODAL ligand [25].

It is an interesting observation that maternal NODAL ultimately becomes restricted to a thin, distinct layer of the decidua parietalis at the maternal-fetal interface. The decidua parietalis is the outer portion of the decidua that lines the uterine membranes; however, a unique role for the parietalis has not been documented. Previously, a role for Nodal in restricting trophoblast invasion was suggested by Munir et al. [31], who demonstrated decreased proliferation and increased apoptosis after overexpression of Nodal in human trophoblast cells. Although the precise mechanism behind any Nodal-mediated trophoblast invasion remains uncertain, maternal Nodal expressed in the ''Nodal layer'' may provide a morphogenic barrier to overexpansion of extraembryonic tissues during the later stages of pregnancy.

In summary, we have characterized the complete spatial and temporal expression patterns of Nodal throughout pregnancy in the mouse uterus. We have also provided evidence suggesting the embryo plays an essential regulatory role in directing endometrial expression of Nodal that directly correlates with implantation. Taken together, the results presented here support previously implicated roles for Nodal signaling components in various reproductive processes and also highlight some potentially new roles during pregnancy that require further investigation.

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