LysM-Type Mycorrhizal Receptor Recruited for Rhizobium Symbiosis in Nonlegume *Parasponia*

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show long torpor bouts interrupted by regular arousal episodes, black bears in Alaska exhibit distinct cyclic non-diurnal T_b patterns. Bear metabolism is reduced by 53% from BMR, even when T_b has returned to normothermic levels. These observations expand the phenotype of mammalian hibernation that occurs in diverse animals over body mass ranges from 0.005 to 200 kg. Insights into how hibernating bears achieve and cope with these reductions in energy need and T_b, as well as conservation of muscle (*27*, *28*) and bone mass (*29*) despite prolonged seasonal inactivity and disuse, could lead to the development of novel clinical therapies. Current molecular and genetic approaches (*28*, *30*) in combination with better physiological knowledge can increase our understanding of the regulation of hibernation in small and large hibernators and their evolution.

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LysM-Type Mycorrhizal Receptor Recruited for Rhizobium Symbiosis in Nonlegume *Parasponia*

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Rhizobium–root nodule symbiosis is generally considered to be unique for legumes. However, there is one exception, and that is *Parasponia*. In this nonlegume, the rhizobial nodule symbiosis evolved independently and is, as in legumes, induced by rhizobium Nod factors. We used *Parasponia andersonii* to identify genetic constraints underlying evolution of Nod factor signaling. Part of the signaling cascade, downstream of Nod factor perception, has been recruited from the more-ancient arbuscular endomycorrhizal symbiosis. However, legume Nod factor receptors that activate this common signaling pathway are not essential for arbuscular endomycorrhizae. Here, we show that in *Parasponia* a single Nod factor–like receptor is indispensable for both symbiotic interactions. Therefore, we conclude that the Nod factor perception mechanism also is recruited from the widespread endomycorrhizal symbiosis.

The rhizobial nodule symbiosis is widespread in the legume family (Fabaceae). Although this nitrogen-fixing symbiosis provides the plant with a major advantage, it is in principle restricted to a single family, and it is a major challenge for future agriculture to transfer this symbiosis to nonlegumes (*1*). The genus *Parasponia* could provide a key to this, because it encompasses the only nonlegume species that acquired also the rhizobium symbiosis (*2*, *3*), where "rhizobium" refers to all species and genera that form nodules on legumes. *Parasponia* comprises several tropical tree species and belongs to Celtidaceae (*4*). Celtidaceae (order Rosales) and Fabaceae (order Fabales) are only remotely related. Further, not a single species phylogenetically positioned between *Parasponia* and Fabaceae is able to establish such rhizobium symbiosis. Hence, in all probability the common ancestor of present *Parasponia* species gained the rhizobium-nodule symbiosis independent from legumes. Therefore, a legume-*Parasponia* comparison provides a key to identifying genetic constraints underlying this symbiosis. In this study, we focused on parallel evolution of the recognition of the rhizobial signal that starts the symbiotic interaction, the Nod factor.

Parasponia makes lateral rootlike nodules that are associated with cell divisions in the root cortex (*5*). Rhizobium enters the *Parasponia* root intercellularly and becomes imbedded in a dense matrix. Rhizobium obtains an intracellular lifestyle when it reaches a nodule primordium. There, cortical cells are infected via threadlike structures that remain connected to the plasma membrane. These so-called fixation threads branch, fill up the cells, and provide a niche to rhizobia to fix nitrogen (*5*). This is illustrated by the expression, in these threads, of the rhizobium *nifH* gene that encodes one of the subunits of nitrogenase (fig. S1). In contrast, rhizobia enter most legume roots via root hair–based intracellular infection threads, and the bacteria are released in nodule cells as membrane-surrounded nitrogen-fixing organelle-like structures (symbiosomes) that harbor a single or only a few bacteria. Legume nodules are considered to be genuine organs with a unique ontogeny (*6*). The fact that the *Rhizobium* symbiosis is very common in 65-million-year-old Fabaceae led to the conclusion that the symbiotic

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interaction emerged as early as 60 million years ago (*7*). In contrast, the lateral rootlike nodule structure and more primitive rhizobium infections in *Parasponia* (*5*), together with the very close relation with the nonnodulating genus *Trema* (*2*–*4*), strongly suggest that *Parasponia* gained the *Rhizobium* symbiosis more recently than legumes.

A key step in rhizobium symbiosis is the recognition by the host of bacterial Nod factors, which are specific lipochito-oligosaccharides. This holds for (almost) all nodulated legumes but also for *Parasponia* (*8*). This implies that a nonlegume species evolved independently from legumes, a Nod factor perception mechanism. In legumes, Nod factors are perceived by specific LysM receptor kinases that coevolved with the Nod factor structure of their host-specific rhizobium species (*9*–*12*). Legume Nod factor receptors activate a common signaling cascade that is shared with and recruited from the more common and far more ancient arbuscular mycorrhizal symbiosis (*13*, *14*). This common signaling pathway comprises an additional plasma membrane receptor kinase, several components in the nuclear envelope including a cation ion channel and subunits of nuclear pores, and a nuclear-localized calcium/calmodulin-dependent kinase (CCaMK) (*13*, *14*). Rhizobium- and mycorrhizae-induced signaling diverge downstream of CCaMK, possibly because of a different nature of the induced calcium spiking (*14*, *15*). Because legume Nod factor receptors are not essential for mycorrhization, it is generally assumed that mycorrhizal symbiosis is controlled by other receptors specific for mycorrhizal signals (i.e., Myc factor). Such Myc factor receptors, like Nod factor receptors, are presumed to activate the common symbiotic signaling pathway (*13*–*17*). Two scenarios can be envisioned for how Nod factor receptors could have evolved. The complete mycorrhizal signaling pathway, including the Myc receptor, has been recruited by legumes, resulting in a common signaling pathway. In such a case, present Nod factor receptors have emerged upon gene duplication events and subsequently neofunctionalized during coevolution with specific rhizobium species. In this scenario, Myc receptors would be close homologs of known Nod factor receptors, as was argued previously (*16*, *17*). However, such a scenario also implies that early in rhizobium symbiosis evolution a single receptor fulfilled a dual function, namely in mycorrhization as well as in *Rhizobium* symbiosis. A second scenario is that only the common signaling pathway devoid of a fungal-specific Myc receptor was recruited, and a novel receptor obtained the ability to activate this common signaling pathway upon Nod factor recognition. We favor the first hypothesis, because it is more simple and finds support in the fact that the chitooligosaccharide backbone of Nod factors is a "fungal" characteristic; chitin is a major component in fungal cell walls. The occurrence of Nod factor signaling in *Parasponia* provides a possibility to investigate this hypothesis.

First, we confirmed and extended the idea that *Parasponia*-rhizobium symbiosis is induced by Nod factors. To this end, we used *Parasponia andersonii*, a species that can be nodulated by the broad host strain *Sinorhizobium* sp. NGR234 (*18*). A mutant of *Sinorhizobium* sp. NGR234 (NGR234ΔnodABC) that does not produce Nod factors was unable to trigger nodule formation or to infect roots of *P. andersonii* plantlets (0 of 30), whereas wild-type NGR234 does form nodules on ~40% of the plantlets [12 of 30; 8 weeks post inoculation (wpi)], similar as reported previously (*5*). Furthermore, root cortical cell divisions could be induced by local application of Nod factors (16 of 19; fig. S2). Next, we obtained evidence that, also in *P. andersonii*, the common symbiotic pathway is recruited to facilitate rhizobium symbiosis. A dominant active form of *Medicago truncatula MtCCaMK* was introduced in *P. andersonii* roots (*19*). In legumes, CCaMK is a key element in the common symbiotic pathway, and dominant active forms of this kinase result in spontaneous nodulation in absence of rhizobia (*20*, *21*). In *P. andersonii*, we also observed spontaneous formation of nodule-like structures (6 of 30, Fig. 1), indicating that activation of the common signaling pathway is sufficient to induce nodule organogenesis. These data suggest that in *P. andersonii* the common signaling pathway is activated upon Nod factor perception.

In legumes, two different Nod factor receptor types are involved. One of these, *MtLYK3/LjNFR1* in *M. truncatula/Lotus japonicus*, has several paralogous genes that resulted from recent duplication events (*9*, *11*, *16*, *17*, *22*, *23*). In contrast, the second Nod factor receptor (*MtNFP/*

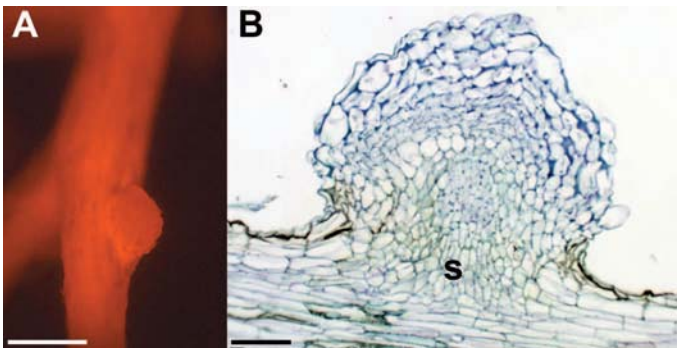

Fig. 1. *P. andersonii* spontaneous nodule-like structure triggered by dominant active MtCCaMK. Scale bars indicate 50 μm. (**A**) Nodule-like structure on a transgenic *P. andersonii* root (selected on the basis of red fluorescence resulting from *DsRED1* expression). Scale bar indicates 0.5 mm. (**B**) Longitudinal section of spontaneous nodule-like structure. Nodule-like structure originates from cortical and pericycle cell layers and has a rudimentary stele (s), reflecting the lateral rootlike origin of *P. andersonii* nodules.

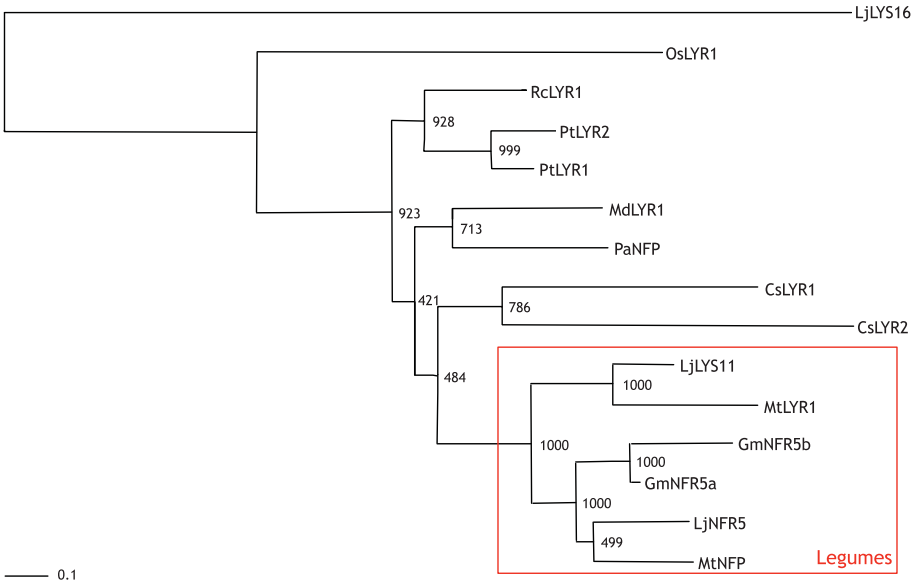

Fig. 2. Maximum-likelihood phylogeny of *MtNFP/LjNFR5*-like genes in the Rosid I (Fabidae) clade. *P. andersonii* (Pa), apple (Md), and castor bean (Rc) contain only a single gene, whereas in poplar (Pt), cucumber (Cs), and legumes (Gm/Lj/Mt) lineage-specific duplications have occurred. In legumes, LjNFR5, MtNFP, GmNFR5a, and GmNFR5b are *Rhizobium* Nod factor receptors. Branch lengths are proportional to the number of amino acid substitutions per site. Branch support was obtained from 1000 bootstrap repetitions. LjLYS16 and the closest *MtNFP/LjNFR5* homolog in *Oryza sativa* (*OsLYR1*) were used as outgroups.

LjNFR5) has only one paralog in *M. truncatula* and *L. japonicus* (*11*, *23*), and a putative orthologous gene is absent in *Arabidopsis*, a species that is unable to establish mycorrhizal symbiosis (*11*, *16*, *17*). Interestingly, in *M. truncatula* this paralog, *MtLYR1*, is transcriptionally up-regulated during mycorrhization (*24*). Therefore, we focused on the putative *MtNFP/LjNFR5* orthologous gene in *P. andersonii*. To clone *P. andersonii* homologs a bacterial artificial chromosome (BAC) library was constructed and screened with *MtNFP* as probe. All eight positive BACs came from a single locus and shared the region containing one *MtNFP/LjNFR5*-like LysM receptor that we named *PaNFP* (*Parasponia andersonii NOD FACTOR PERCEPTION*). Southern blotting as well as sequencing of *P. andersonii MtNFP/LjNFR5*–like sequences generated by polymerase chain reaction (PCR) using degenerated primers and genomic DNA, as well as nodule and root cDNA, confirmed that *P. andersonii* has a single *NFP*-like gene. Next we searched for *MtNFP/LjNFR5*-like genes in available genome sequences of other Fabidae (Rosid I) species (*19*). Apple (*Malus x domestica*), a close relative of *P. andersonii*, also has only a single *MtNFP/LjNFR5*-like gene that we named *MdLYR1* [*Malus x domestica LYK-RELATED1* (*11*)]. Subsequent phylogenetic analysis revealed that *PaNFP* and *MdLYR1* are close homologs of legume *MtNFP/LjNFR5* and *MtLYR1/LjLYS11* (Fig. 2). On the basis of this result, we conclude that, in contrast to legumes, *P. andersonii* contains only a single *MtNFP/LjNFR5*-like gene. The legume-specific nature of the gene duplications is supported by the presence of two conserved deletions in the legume genes (fig. S3). Also a substantial level of microsynteny in paralogous regions as well as a low level of nucleotide substitutions in paralogous gene pairs supports the recent nature of the duplication (*17*, *23*). To determine whether this duplication predates the Fabaceae, we searched for *MtNFP/LjNFR5*-like sequences in a collection of cDNA clones from the basal legume *Chamaecrista fasciculata* (*25*). We identified a single clone (named *CfNFP1*) that is phylogenetically ancestral to the duplication observed in *M. truncatula* and *L. japonicus* (fig. S4). Therefore, we conclude that the duplication of *MtNFP/LjNFR5* in the legume lineage was not essential to gain symbiosis with *Rhizobium*.

Reverse transcription PCR studies revealed that *PaNFP* is expressed in roots (fig. S5). To study whether *PaNFP* has a symbiotic function, we performed RNA interference (RNAi) knockdown experiments (*19*). *P. andersonii* roots transformed with the empty vector (control roots) could be nodulated effectively with *Sinorhizobium* sp. NGR234 (Fig. 3A; 11 out of 30 plants formed nodules, and in total 55 nodules were formed 8 wpi). Transgenic *P. andersonii* roots that express a PaNFP RNAi construct have markedly reduced *PaNFP* expression levels (often below detection level, and, in cases where it is detected, it is ≥50% reduced; fig. S5). Inoculation of such RNAi roots with *Sinorhizobium* sp. NGR234 resulted only in a few nodules (PaNFP RNAi roots had 13 nodules on 30 plants, 8 wpi), and these were much smaller than nodules on control roots (Fig. 3, A and D). Sectioning of NFP RNAi nodules showed that they harbored rhizobia intercellularly, but fixation thread formation was completely blocked in all nodules investigated ($n = 10$) (Fig. 3, B and E). This demonstrated that PaNFP is involved in nodule formation and is essential for the switch to an intracellular lifestyle of rhizobia. Also in legumes, *MtNFP/LjNFR5* is essential for nodule formation as well as intracellular accommodation of rhizobia (*11*, *12*). On the basis of these results, we conclude that *P. andersonii* has recruited a gene orthologous to the *MtNFP/LjNFR5* Nod factor receptor in legumes to control rhizobium symbiosis. This points to constraints in evolution of Nod factor perception mechanisms. As hypothesized above, a Nod factor receptor could have been recruited from the mycorrhizal signaling pathway. Because *P. andersonii* has only a single *MtNFP/LjNFR5*-like gene, we determined whether *PaNFP* is also essential for endomycorrhization. *PaNFP* RNAi knockdown and control roots were inoculated with *Glomus intraradices*. This experiment showed that both are equally well infected by fungal hyphae. However, arbuscle formation is blocked in *PaNFP* RNAi roots, whereas in control roots arbuscules were effectively formed (Fig. 3, C and F, and fig. S6). PaNFP therefore is also essential for successful intracellular infection during arbuscle formation by mycorrhizal fungi. We conclude that in *P. andersonii* a single *MtNFP/LjNFR5*-like receptor, *PaNFP*, fulfills a dual symbiotic function and controls the intracellular life style of both arbuscular mycorrhizae fungi and rhizobium.

Our findings in *P. andersonii* provide strong support for the hypothesis that during evolution a Myc factor receptor, as part of the common signaling cascade, was recruited to serve as Nod factor receptor in the rhizobial-plant symbiosis. Because in *P. andersonii PaNFP* fulfills a dual function, we suggest that only a few adaptions, if any at all, will have occurred to enable perception of a new ligand, rhizobium Nod factors. Also this result suggests that the Myc factor will have structural characteristics similar to those of Nod factors. In most legumes, *MtNFP/LjNFR5* underwent at least one round of gene duplication (Fig. 2). However, our data suggest that this duplication occurred within the Papilionoideae subfamily of the Fabaceae (e.g., *Medicago*, *Lotus*, and *Glycine*), because CfNFP of *Chamaecrista*, as part of the basal Caesalpinioideae subfamily, is ancestral to the duplication events (fig. S4). Therefore it is likely that in *Chamaecrista* mycorrhization and *Rhizobium* symbiosis are controlled by just a single receptor, CfNFP. In more recent legumes like *M. truncatula* and *L. japonicus*, a duplication of this receptor has occurred, and only one of these has evolved as a Nod factor receptor. It seems very probable that the second copy functions as a Myc factor receptor.

The bacterial genera collectively named rhizobium that evolved the ability to establish a nodule symbiosis, in general, acquired this by horizontal transfer of *nod* genes (*26*). This event allowed them to produce fungal-like molecules, namely Nod factors, by which they could use the ancient mechanism by which endomycorrhizal

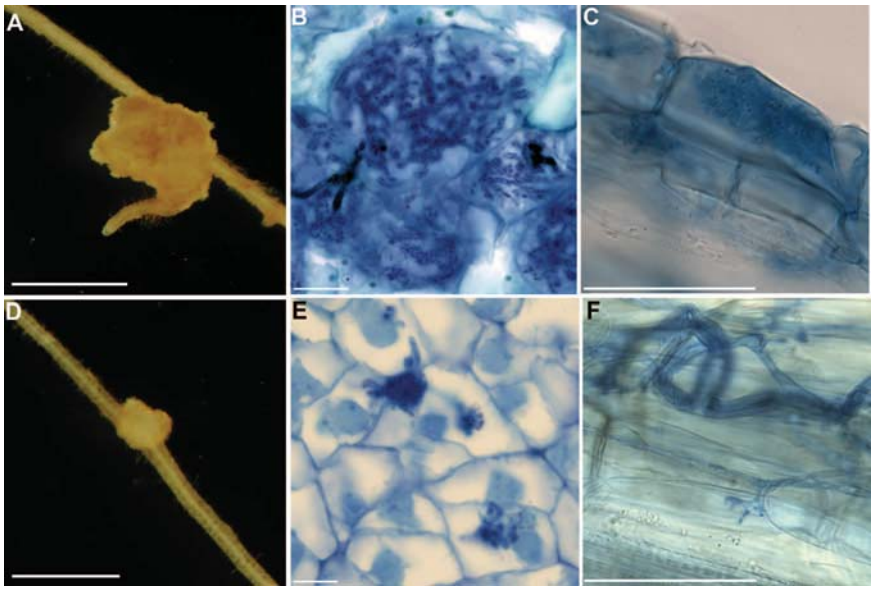

Fig. 3. *Rhizobium* nodulation and mycorrhization on *P. andersonii* control (**A** to **C**) and *PaNFP* RNAi knockdown (**D** to **F**) roots. (A) Control nodule. Scale bar, 1.0 mm. (B) *Rhizobium* fixation threads in control nodule. Scale bar, 10 μm. (C) Arbuscle in inner root cortical cell of (slightly squashed) control roots. Scale bar, 50 μm. (D) *PaNFP* RNAi nodule. Scale bar, 1.0 mm. (E) Aborted fixation threads in *PaNFP* RNAi nodule. Scale bar, 10 μm. (F) Aborted intracellular infection of *Glomus intraradices* in *PaNFP* RNAi root. Scale bar, 50 μm.

fungi establish an intracellular life style and turned these rhizobia from free-living bacteria into nitrogen-fixing endosymbionts. However, although the endomycorrhizal symbiosis is widespread in the plant kingdom only very few plant lineages, namely legumes and *Parasponia*, have recruited this mechanism for the rhizobial nodule symbiosis. Studies on the constraints underlying this evolutionary event in *Parasponia* can provide insight into whether and how this nitrogen-fixing symbiosis can be transferred to other nonlegumes.

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The Antiproliferative Action of Progesterone in Uterine Epithelium Is Mediated by Hand2

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During pregnancy, progesterone inhibits the growth-promoting actions of estrogen in the uterus. However, the mechanism for this is not clear. The attenuation of estrogen-mediated proliferation of the uterine epithelium by progesterone is a prerequisite for successful implantation. Our study reveals that progesterone-induced expression of the basic helix-loop-helix transcription factor Hand2 in the uterine stroma suppresses the production of several fibroblast growth factors (FGFs) that act as paracrine mediators of mitogenic effects of estrogen on the epithelium. In mouse uteri lacking Hand2, continued induction of these FGFs in the stroma maintains epithelial proliferation and stimulates estrogen-induced pathways, resulting in impaired implantation. Thus, Hand2 is a critical regulator of the uterine stromal-epithelial communication that directs proper steroid regulation conducive for the establishment of pregnancy.

A sequential and timely interplay of the steroid hormones 17β-estradiol (E) and progesterone (P) regulates critical uterine functions during the reproductive cycle and pregnancy (*1*–*3*). Whereas E drives uterine epithelial proliferation in cycling females, P counteracts E-induced endometrial hyperplasia. In mice, preovulatory ovarian E stimulates uterine epithelial growth and proliferation on days 1 and 2 of pregnancy (*1*). However, starting on day 3, P produced by the corpora lutea terminates E-mediated epithelial proliferation. In response to P, epithelial cells exit from the cell cycle and enter a differentiation pathway to acquire the receptive state that supports embryo implantation on day 4 of pregnancy (*4*–*6*). To identify the P-regulated pathways that underlie the implantation process, we had previously examined alterations in mouse uterine mRNA expression profiles in the periimplantation period in response to RU-486 (mifepristone), a well-characterized progesterone receptor (PR) antagonist (*7*). Our results identified *Hand2*, a critical regulator of morphogenesis in a variety of tissues (*8*, *9*), as a potential PR-regulated gene. Real-time polymerase chain reaction (PCR) confirmed that the expression of Hand2 mRNA was greatly reduced in the uteri of RU-486–treated mice (*10*) (fig. S1A). The expression of Hand2 protein, localized exclusively in the uterine stroma, was also abolished after RU-486 treatment (fig. S1B), which indicated that PR controls Hand2 expression in the mouse uterus during early pregnancy.

To further confirm P regulation of *Hand2*, ovaries were removed from nonpregnant mice, and then these animals were injected with either vehicle or P. We observed intense nuclear expression of Hand2 protein in uterine stromal cells after P treatment. Similar treatment of PR-null females showed no induction of Hand2 protein (Fig. 1A). These results established that P induces Hand2 expression in the uterine stroma. Consistent with its regulation by P, Hand2 expression was observed in the stromal cells underlying the luminal epithelium on days 3 and 4 of pregnancy (Fig. 1B).

To investigate the function of *Hand2* in the uterus, we created a conditional knockout of this gene in the adult uterine tissue. Crossing of mice harboring the "floxed" *Hand2* gene ($Hand2^{f/f}$) with PR-Cre mice (in which Cre recombinase was inserted into the PR gene) generated $Hand2^{d/d}$ mice in which the *Hand2* gene is deleted selectively in cells expressing PR. As shown in fig. S2, *Hand2* expression was successfully abrogated in uteri of $Hand2^{d/d}$ mice. A breeding study demonstrated that $Hand2^{d/d}$ females are infertile (table S1). An analysis of the ovulation and fertilization in $Hand2^{f/f}$ and $Hand2^{d/d}$ females revealed no significant difference in either the number or the morphology of the embryos recovered from their uteri (fig. S3, A and B). The serum levels of P and E were comparable in $Hand2^{f/f}$ and $Hand2^{d/d}$ females on day 4 of pregnancy, which indicated normal ovarian function (fig. S3, C and D).

We next examined embryo attachment to the uterine epithelium by using the blue dye assay, which assesses increased vascular permeability at implantation sites. $Hand2^{f/f}$ mice displayed distinct blue bands, indicative of implantation sites on day 5 of pregnancy (fig. S4). In contrast, none of the $Hand2^{d/d}$ females showed any sign of implantation. Implanted embryos with decidual swellings were also absent in $Hand2^{d/d}$ uteri on days 6 and 7 of pregnancy. Histological analysis of $Hand2^{f/f}$ females on day 5 of pregnancy

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