Menin directs regionalized decidual transformation through epigenetically setting PTX3 to balance FGF and BMP signaling

Mengying Liu 1,2,4, Wenbo Deng 2,4, Lu Tang 2, Meng Liu 1, Haili Bao 2, Chuanhui Guo 2, Changxian Zhang 3, Jinhua Lu 2, Haibin Wang 2✉, Zhongxian Lu 1,2✉ & Shuangbo Kong 2✉

During decidualization in rodents, uterine stroma undergoes extensive reprogramming into distinct cells, forming the discrete regions defined as the primary decidual zone (PDZ), the secondary decidual zone (SDZ) and the layer of undifferentiated stromal cells respectively. Here we show that uterine deletion of Men1, a member of the histone H3K4 methyltransferase complex, disrupts the terminal differentiation of stroma, resulting in chaotic decidualization and pregnancy failure. Genome-wide epigenetic profile reveals that Men1 binding in chromatin recapitulates H3K4me3 distribution. Further transcriptomic investigation demonstrates that Men1 directly regulates the expression of PTX3, an extra-cellular trap for FGF2 in decidual cells. Decreased Ptx3 upon Men1 ablation leads to aberrant activation of ERK1/2 in the SDZ due to the unrestrained FGF2 signal emanated from undifferentiated stromal cells, which blunt BMP2 induction and decidualization. In brief, our study provides genetic and molecular mechanisms for epigenetic rewiring mediated decidual regionalization by Men1 and sheds new light on pregnancy maintenance.
Successful pregnancy requires rapid remodeling of the endometrium in a process termed decidualization, which requires the blastocyst attachment as a trigger and occurs in a region-specific manner in mice. At the initiation of decidualization, stromal cells surrounding the implanted blastocyst undergo proliferation and postmitotic differentiation extensively, forming the primary decidual zone (PDZ) between day 5 (day 1 = the day of positive of vaginal plug) and day 6 of pregnancy.

Subsequently, stromal cells adjacent to the PDZ continue to proliferate and differentiate into decidual cells to form the secondary decidual zone (SDZ) at the antimesometrial (AM) pole by day 8. A large portion of decidual cells experience polyploidization, characterized by large mono or bi-nucleated cells that undergo repeated DNA replication without cytokinesis. In addition, a thin layer of undifferentiated stromal cells lies between the myometrium and the SDZ. It is believed that the regionalized decidual structure is not only important for the supply of nutrition to the developing embryo but also acts as a barrier against excessive invasion of trophoblast cells. While only small incrementation has been made about the underlying mechanism directing programmed stromal cells differentiation in recent decades, several studies have demonstrated that a set of central genes in evolutionarily conserved signaling pathways during embryogenesis, such as BMP (bone morphogenetic protein), WNT (wingless-type MMTV integration site family), FGF (fibroblast growth factor) signaling, and HOX (homeobox) genes, possess the potential function in building the regional pattern of decidualization. BMP2, an indispensable factor for the differentiation of stromal cells into decidual cells, is intensely expressed in PDZ and SDZ. The expression of WNT ligand Wnt4, which is also critical for uterine stromal cell differentiation, is largely overlapped with Bmp2. Meanwhile, Sfrp4, a WNT antagonist, is observed in the layer of undecidualized stromal cells outside the SDZ. In addition to Sfrp4, Fgf2, a member of FGF family, also exhibits a similar expression pattern. The fine-tuned interplay of these regionalization determinants synergistically or antagonistically promotes tissue homeostasis during decidualization. However, how these genes are coordinated to specify the allocation of decidual cells remains largely unknown.

Menin, encoded by the Men1, is a multifunctional scaffold protein participating in a variety of cellular functions, whose deficiency contributes to embryonic lethality at E11.5-E13.5 with defects in multiple organs. Menin regulates target genes expression in a cell-type-specific manner, including Hox genes, Cdkn1b, and Cdkn2c, Esr1 and p35, by interacting with chromatin-modifying enzymes, in particular, the histone methyltransferase (HMT) MLL1/2 that catalyze histone 3 lysine 4 trimethylatation (H3K4me3) and HDAC1-5. Regarding that early pregnancy is a precisely regulated process, whether the target genes of Menin-MLL complex and how Menin and its interplay with H3K4me3 modification participate in embryo implantation and decidualization remain elusive.

In the current study, we utilize a combination of genetic, biochemical, and pharmacological approaches to investigate the role of Men1 in peri-implantation uteri and reveal that Men1 ablation disrupts the appropriated differentiation of decidual stromal cells, resulting in unordered regionalization of decidualization and compromised pregnancy success. The reduction of Bmp2 caused by aberrantly activated FGF2-ERK1/2 signaling pathway deranges the adequate development of the SDZ due to disorganized genomic H3K4me3 modification in the absence of Men1. PTX3, an extracellular protein trapping FGF2, is identified as a Menin directly regulated downstream gene and provides insightful information for the orchestration of decidualization and pregnancy maintenance in curbing the cross-talk between FGF2-ERK1/2 and BMP2.

### Results

**Men1** is expressed in the peri-implantation mouse uterus in a spatiotemporal manner. Highly heterogeneous uterine endometrium is comprised of different cell types that undergo tremendous changes during early pregnancy. To elucidate the physiological significance of Men1 during early pregnancy, we first examined the cell-specific and temporal expression of Men1 in peri-implantation uteri using in situ hybridization. There was weak Men1 expression in the pre-implantation uterus on days 1 and 4. After embryo implantation, Men1 transcript was moderately upregulated in proliferating stromal cells surrounding the blastocyst on day 5. Accompanied by the initiation of decidualization, stromal cells underlying the implantation chamber exit mitosis and differentiate into epithelioid cells to form the PDZ. The transcript of Men1 became undetectable in the PDZ but was intensely expressed in proliferating stromal cells outside the PDZ on day 6, which was further supported by the co-staining of Ki67 and Menin (Fig. 1a and Supplementary Fig. 1a, b). After the SDZ was established by day 8, Men1 expression was restricted in the mesometrial (M) pole (Fig. 1a). These observations indicated that Men1 expression was tightly correlated with the progress of decidualization. Consistent with its mRNA expression pattern, Menin protein was widely expressed in the uterus during decidualization and located mainly in nucleus (Fig. 1b). It was interesting to note that Menin protein possessed wider expression than its transcript which is perhaps due to the discrepant stability between mRNA and protein (Fig. 1b).

**Mice with uterine deletion of Men1 show severe subfertility.** To explore the functional role of uterine Men1 in pregnancy, we generated mice with uterine deletion of Men1 (Men1<sup>−/−</sup>) by crossing Men1 floxed mice (Men1<sup<flox</flox></sup>) with PR-Cre mice (Pgr<sup>Cre</sup>+)<sup>10,16</sup>. These mice show efficient deletion of Men1 in the pregnant uterus at both mRNA and protein levels (Fig. 1c–e). To interrogate the role of Men1 in female fertility, Men1<sup>−/−</sup> mice and their littermate controls (Men1<sup>+/+</sup>) were mated with wild type fertile males. Only 25% plug-positive Men1<sup>−/−</sup> mice gave birth to offspring and the litter size was significantly lower than those in littermate Men1<sup>+/+</sup> mice (Fig. 1f, g). These results demonstrated uterine Men1 was crucial for a successful pregnancy.

**Ablation of uterine Men1 causes defective decidualization and miscarriage at mid-gestation.** Early pregnancy is a highly programmed dynamic process involving blastocyst attachment, decidualization, and placentation. Defects at any stage will give rise to adverse pregnancy outcomes. To dissect out the defects causing subfertility in Men1<sup>−/−</sup> mice, we first assessed the early pregnancy events on day 5 with increased vascular permeability at the attachment site. The number of implantation sites and the litter size was significantly lower than those in littermate Men1<sup>+/+</sup> mice (Fig. 2a, b). The gross morphology and embryo attachment reaction as revealed by H&E staining and COX2 expression, respectively, were also similar between Men1<sup>−/−</sup> and Men1<sup>+/+</sup> (Supplementary Fig. 2a and Fig. 2c). Stromal cells surrounding the blastocyst undergo extensive proliferation and differentiation into decidual cells under the initiation of BMP2<sup>2,3</sup>. The semblable proliferation and expression pattern of Bmp2 and BMP antagonist Dan in Men1<sup>−/−</sup> and Men1<sup>+/+</sup> implantation sites confirmed that mice with uterine deletion of Men1 had normal initiation of
decidualization (Fig. 2d and Supplementary Fig. 1b–e). Moreover, IGFBP5, which provides a favorable environment for blastocyst growth by coupling with IGF118, had a similar subepithelial expression pattern in Men1f/f and Men1d/d implantation sites (Supplementary Fig. 1f).

Since the deficiency of Men1 has no severe defects in the blastocyst attachment reaction and the initiation of decidualization, we next examined the progress of decidualization. The morphology of the decidual bulge and the number of implantation sites were comparable between Men1f/f and Men1d/d mice on day 6 with the termination of stromal cell proliferation in the established PDZ (Fig. 2e, f and Supplementary Fig. 3a). Our results showed that there were comparable expressions of OCT4, Ki67, Dtppr, COX2, Bmp7, β-Catenin, and Wnt4 in day 6 uteri, implying normal embryo development, stromal cell proliferation, PDZ formation19–22 (Supplementary Fig. 3b–h). We also noticed that the glands distribution was abnormal with increased number in the lateral areas near the M pole in stroma of Men1d/d mice as characterized by FOXA223 from pregnant day 4–6 (Supplementary Fig. 4). Collectively, these results indicated that uterine Men1 was dispensable for blastocyst attachment, implantation, and PDZ formation during early pregnancy.

Fig. 1 Uterine-specific deletion of Men1 leads to female subfertility. a In situ hybridization of Men1 in WT uteri on days 1, 4, 5, 6, and 8 of pregnancy. Scale bars: 500 μm. b Immunohistochemical analysis of Menin in day 4 uteri and days 5, 6, and 8 implantation sites. Le luminal epithelium, Ge glandular epithelium, S stroma, Bl blastocyst, Em embryo. Scale bar: 200 μm. c Quantitative real-time PCR analysis of Men1 mRNA levels in Men1f/f and Men1d/d implantation sites on day 6. The values are normalized to Gapdh and indicated as the mean ± SEM (n = 8 biologically independent samples). Two-tailed unpaired Student’s t-test, ****p = 4.26e-7. d Immunoblotting analysis of Menin protein in Men1f/f and Men1d/d implantation sites on day 6. β-Actin was used as a loading control. e Immunohistochemical analysis of Menin in Men1f/f and Men1d/d implantation sites on day 6. S stroma, Em embryo. Scale bar: 200 μm. f Pregnancy rates in Men1f/f and Men1d/d female mice. The number within brackets indicates females with pups over total number of plug-positive females. g Average litter sizes in Men1f/f (n = 20 animals) and Men1d/d (n = 5 animals) mice. Data represent the mean ± SEM. Two-tailed unpaired Student’s t-test, ****p = 4.31e-8.
day 8. The reduced average weight of implantation sites with retarded embryos was emerged in Men1\textsuperscript{d/d} mice on day 8 in comparison to Men1\textsuperscript{f/f} mice (Fig. 2g–i). From day 9–12, more obvious defects appeared at the implantation sites, including abnormal placentation and severe embryo absorption (Fig. 2j–l). These data hinted that the development of SDZ was hindered from day 7–8. In order to further confirm the effect of Men1 deletion in the progress of decidualization, an oil-induced
artificial decidualization model was applied (Supplementary Fig. 5a). Men1 mice uteri exhibited a robust decidial response 96 h after intraluminal oil injection, while the Men1 uteri showed a remarkably weakened or disappeared decidual response (Supplementary Fig. 5b, c). Collectively, these results demonstrated that uterine-specific deletion of Men1 disturbed the progression of decidualization and ultimately resulted in embryo resorption and miscarriage at mid-gestation.

Uterine deletion of Men1 leads to inadequate differentiation of decidual cells in the SDZ. The observations of abnormal decidualization on day 8 led us to investigate the cellular defects in Men1 uteri. Polyolipidization, a hallmark of mature decidual cells in the SDZ, was marked by mono or bi-nucleated cells through endoduplication. Indeed, the ablation of Men1 in uteri resulted in decreased decidual cell polyplody and smaller nuclear size (Fig. 3a). H3K27me3 was critical epigenetic modification for X chromosome inactivation for dose complementary effect of female somatic cells. Our results showed that polyplody decidual cells in Men1 mice possessed >4 puncta H3K27me3 staining, indicating at least four sets of homologous chromosomes in polyplody decidual cells (Fig. 3b). While the number of polyplody decidual cells with four or more puncta H3K27me3 staining was obviously reduced in Men1 mice (Fig. 3b). Consistently, we found aberrantly increased PCNA (DNA replication in S-phase) and BrdU (DNA synthesis in S-phase) in Men1 decidual cells on day 8 of pregnancy (Fig. 3b). Further, flow cytometric analysis of DNA content revealed that 2 N cells were significantly increased with a concomitant decrease of >4 N cells in Men1 decidual tissues on day 8 (Fig. 3c, d and Supplementary Fig. 6). In Men1 mice, both Dtprp mRNA and protein were significantly reduced on day 8 (Fig. 3e, f), suggesting that the terminal differentiation of decidual cells was impaired. These results indicated Men1 was critical for the progression of polyplody during decidualization.

On day 8, the expression of COX2 in the presumptive site of placenta was disordered, foreboding impaired placenta in Men1 mice (Fig. 3g, h). Accompanied by normal decidualization, abundant uterine natural killer (uNK) cells accumulated at the mesometrial pole on day 8. In contrast to numerous DBA+ uNK cells in Men1 mice, Men1 null uteri exhibited dramatic decrease of uNK cells (Fig. 3h). Aberrant decidualization tends to give rise to abnormal trophoblast invasion and compromised placenta. Eccentric expression pattern of trophoblast giant cells marker PL1 in Men1 uteri signified shallow trophoblast invasion on day 8 (Fig. 3i). All these observations strongly proved the defective decidualization in the absence of uterine Men1.

The morphological and physiological changes of stromal cells during decidualization are mainly regulated by steroid hormones in the absence of uterine Men1. P450 cholesterol side-chain cleavage enzyme (P450scc) and 3 beta hydroxysteroid dehydrogenase (3β-HSD) in the ovary as well as comparable expression PR and its target genes, Hand2 and Hoxa10, in day 6 and day 8 uterus (Supplementary Fig. 7a, b and Supplementary Fig. 8a–i). These data suggested that the defect decidualization was primarily originated from local dysfunction of Men1 in stromal cells.

Men1-deficient uteri show diminished BMP responsiveness but enhanced FGF responsiveness. As aforementioned, the uterine

Procedures for decidualization dysregulated in the absence of uterine Men1. To further excavate the underlying molecular mechanism for the defective terminal differentiation of decidual cells in the SDZ observed in Men1 mice, decidual tissues were isolated from Men1 and Men1 uteri on day 8 and subjected to gene expression profiles analysis using RNA sequencing (RNA-seq). A total of 1669 genes were differentially expressed (RPKM > 0.5, p < 0.05) with 870 downregulated genes (Supplementary Data 1) and 789 up-regulated genes (Supplementary Data 2) in Men1 mice in comparison to wild type (Fig. 4a). Decidualization involves a series of biological changes including the proliferation and differentiation of stromal cells, angiogenesis, and immunological adaptation. Functional annotation revealed that downregulated genes upon Men1 deletion mainly enriched in the terms of cell proliferation inhibition, fatty acid metabolic process, multicellular organism development, and negative regulation of angiogenesis (Fig. 4b and Supplementary Data 3). In contrast, genes up-regulated in Men1 decidual tissues were enriched in categories that were resistant to cell differentiation such as apoptotic process and inflammatory response (Fig. 4b and Supplementary Data 4).

The downregulated genes in negative regulation of cell proliferation, including Bmp2, Ptn, Fgf10, and Igf1, possessed specific expression patterns and were important for decidualization. As aforementioned, the uterine angiopoietins (ANGPT1 and ANGPT2) and their receptor TIE2 directs angiogenesis during decidualization. ANGPT1 induces vessel maturation and maintains vessel leakiness, whereas ANGPT2, acting as a natural antagonist of ANGPT1, induces vessel destabilization required for further sprouting and regression of vessels. Our RNA-seq data indicated several genes in promoting angiogenesis (Angpt1, Edn1, Ednra) were upregulated, and genes in inducing destabilization of blood vessels and endothelial sprouting (Angpt2, Hif3a, Mmp2) were downregulated due to uterine Men1 deletion (Fig. 4c, e). Furthermore, NK-specific genes (Prf1, Klrk1, Ctsg, Il15r) and several granzyne genes (Gzmz, Gzmb, Gzmd, Gzme, Gzms) were significantly decreased in Men1 decidua (Fig. 4c), indicating the maturation of uNK was compromised. Moreover, Corin, functions in promoting trophoblast invasion and spiral artery remodeling, was decreased substantially in Men1 females (Fig. 4c).

Interestingly, loss of Men1 led to decreased Cdkn1b (encoding p27) in both mRNA and protein levels (Fig. 4c, f). Especially, the immunohistochemical analysis indicated that the expression of p27 was higher in polyplody cells (Fig. 4d). Previous studies manifest that increased p27 level in liver cells causes a failure to enter mitosis and thereby induces polyplody. Reduced polyplody decidual cells in Men1 decidual tissues might be accountable for the reduction of p27. In addition, gene set enrichment analysis (GSEA) analysis showed that cytokinesis-associated genes (Rhoc, Rhob, Dstrn) were significantly enriched by Men1 loss (Fig. 4g, h and Supplementary Data 5). Consistently, there were more stromal cells undergoing proliferating in Men1 knockout SDZ as evidenced by significant increase of mitotic marker pH3 (Fig. 4i, j). Above all, Men1 loss had a strong negative impact on normal physiological changes in decidualization.
with Men1 deletion had an insufficient terminal differentiation of stromal cells in the SDZ. Interestingly, the FGF signaling pathway that positively regulated cell proliferation was significantly enriched among the upregulated genes in Men1<sup>d/d</sup> uteri. In contrast, the gene set responding to BMP which motivated differentiation of stromal cells was significantly suppressed in Men1<sup>d/d</sup> uteri (Fig. 5a, b and Supplementary Data 5). During decidualization, the Fgf2 transcript was localized in the undifferentiated stromal cells underneath the myometrium<sup>6</sup>. FGF2 binds and activates the tyrosine kinase receptor complex FGFR1-FRS2 and recruits the
Fig. 3 Uterine Men1 deletion compromises the terminal differentiation of stromal cells during decidualization. a Histology of day 8 implantation sites and polyplody stromal cells in Men1+/f and Men1/d/d mice. Scale bars: 200 μm. SDZ, secondary decidual zone. b Immunofluorescence analysis of H3K27me3, PCNA, and BrdU in the SDZ of Men1+/f and Men1/f/f mice on day 8. Scale bars: 200 μm. c DNA content quantification of decidual cells by FACS from Men1+/f and Men1/d/d mice on day 8. D Cell distribution for 2 N, 4 N, and >4 N populations of stromal cells in Men1+/f (n = 3 animals) and Men1/d/d (n = 3 animals) implantation sites on day 8. Data represent the mean ± SEM. Two-tailed unpaired Student’s t-test, *p = 0.021 (2 N), ***p = 9.97e-4 (>4 N). d The expression of decidual marker Dtprp in Men1+/f and Men1/d/d implantation sites on day 8. In situ hybridization indicates the expression of Fox5b2 mRNA (top panel). Immunohistochemical staining indicates the expression of protein Dtprp (bottom panel). Scale bar: 200 μm. e Immunoblotting analysis of Dtprp in Men1+/f and Men1/d/d implantation sites on day 8. Actin was used as a loading control. f Quantitative real-time PCR analysis of Ptg2 in Men1+/f and Men1/d/d implantation sites on day 6 and day 8. The values are normalized to Gsdph and indicated as the mean ± SEM (n = 3 biologically independent samples). Two-tailed unpaired Student’s t-test, ***p = 1.82e-4. g Immunohistochemical analysis of COX2 and DBA staining in Men1+/f and Men1/d/d implantation sites on day 8. Scale bar: 200 μm. i Immunohistochemical analysis of PL1 in Men1+/f and Men1/d/d implantation sites on day 8. Scale bar: 200 μm.

Menin positively regulates the expression of Ptx3 in an H3K4me3-dependent manner. Menin is an indispensable component of MLL1/2 HMT complex that catalyzes H3K4me3 in a locus-specific manner, which is usually associated with transcriptional activation of gene.9,36 We speculated that Menin activated gene expression through H3K4me3 during decidualization. Total H3K4me3 protein level was not affected by Men1 ablation as previously reported14,37,38 (Supplementary Fig. 10a). Although deficiency of Men1 does not disrupt global H3K4me3, Menin regulates the H3K4me3 levels across regions proximal to TSSs of a limited number of cell-specific genes.38,39 In order to define its relevance to specific gene expression in decidual cells, we performed ChiP-seq for Menin and H3K4me3 in decidual tissues on day 8. Consistent with the published literature, Menin and H3K4me3 is predominantly positioned at gene promoter (Menin peaks: n = 13,347, 76.65% at promoters; H3K4me3 peaks: n = 29,040, 70.62% at promoters) (Fig. 6a). Almost 80% of peaks bound by Menin were also H3K4me3 modified (Supplementary Fig. 10b). Combined with RNA-seq data, we found that Menin and H3K4me3 were significantly enriched at the transcription start site (TSS) of expressed genes (RPKM > 1) (Fig. 6b and Supplementary Fig. 10c). A direct comparison of Menin and H3K4me3 ChiP-seq peaks at TSSs showed a strong positive correlation (R² = 0.5888291) (Fig. 6c).

downstream GRB2-SOS1 complex to incite ERK1/2 signaling cascade which is a major pathway controlling cell proliferation.34,40 Fgrf1 and Frs2 but not the ligand Fgf2 were significantly increased by Men1 deletion (Fig. 5b, c). BMP2 is a well-known decidual marker critical for the differentiation of stromal cells during decidualization.6,8 The expression of Bmp2 and its target Id1 was substantially lower in Men1/d/d mice uteri on day 8 of pregnancy (Fig. 5b, d). BMPs execute their effects by inducing the phosphorylation of SMAD1/5/8 proteins. Accordingly, the protein (Fig. 5b, d). Immunohistochemical staining indicates the expression of protein Dtprp (bottom panel).

Analysis of CHIP-seq data revealed that although Menin-H3K4me3 typical target genes identified in other cell types (e.g. Cdkn1b, Cdkn2c, Esr1, Hoxa cluster, and Meis1) were also bound by Menin in decidual tissue, none of them had H3K4me3 reduction after Men1 deletion at the promoters (Fig. 6e). Correspondingly, except for the decreased expression of Cdkn1b, the mRNA levels of other Menin target genes had no significant difference in the absence of Men1 (Figs. 4c, 6e). There might be an alternative mechanism different from the H3K4me3 dependent mechanism as previously reported participated in transcripational regulation of Cdkn1b by Menin35. Researches investigating genome-wide function of Menin yielded cell-specific results in terms of the expression of H3K4me3 dependent target genes.35,38,40 According to these results, we hypothesized that Men1 regulates the transcription of a subset of decidual-specific target genes through H3K4me3 modification. H3K4me3 peaks at regions proximal to TSSs were classified into two clusters depending on the changes after Men1 deletion (Fig. 6f and Supplementary Fig. 10d). Cluster 1 contains loci where H3K4me3 ChiP-seq signal was decreased following Men1 deletion, representing 6469 associated genes (Fig. 6f). While loci in cluster 2 have no significant change of H3K4me3 levels, representing 5644 associated genes (Supplementary Fig. 10d). To identify the direct Menin-H3K4me3 target genes responsible for the decidualization defects observed in Men1/d/d uteri, we intersected the RNA-seq data with genes bound by Menin as well as 6469 genes in cluster 1 and found 2995 potential Menin-H3K4me3 direct target genes. Among the 348 genes that were differentially expressed after Men1 deletion, only 217 genes had significantly reduced mRNA levels (Fig. 6g). We listed the top-ranked downregulated genes that are most likely to be the targets of Menin-H3K4me3 in decidual cells (Fig. 6h, i). We also determined the H3K4me3 accumulation in promoters of Menin target genes (e.g. Sfrp2, Twit2, Smx10, and 378 Adora2b) by ChiP-qPCR assays, and it displayed a reduced H3K4me3 modification in decidual cells upon uterine Men1 deletion (Supplementary Fig. 11a–d). These results suggested that loss of Men1 resulted in reduction of H3K4me3 at regions proximal to TSSs of a very limited number of genes expressed in decidual cells.

In these potential target genes, Ptx3 attracted our attention since the deletion of Ptx3 resulted in compromised decidualization.41 Indeed, the peak of H3K4me3 was significantly reduced at the Ptx3 locus, and RNA-seq tracks for

Specifically, read coverage of H3K4me3 around TSSs was modestly reduced in the absence of Men1 (Fig. 6b, d). These results suggested that Menin is involved in H3K4me3 modification across regions proximal to TSSs during decidualization. We, therefore, focused on the potential function of Menin-dependent H3K4me3 in transcriptional activation of specific genes during decidualization.
Ptx3 were profoundly decreased in Men1
d/d uteri following Men1 deletion (Fig. 7a). Men1 loss decreased Ptx3 expression in decidual tissues on day 8 of pregnancy (Fig. 7b). Through Menin ChIP assays using a pair of specific primers targeting the Ptx3 promoter locus, we further confirmed that Ptx3 was a direct target gene of Menin in decidual cells (Fig. 7c). Meanwhile, H3K4me3 occupancy at the Ptx3 promoter locus was dramatically decreased in Men1-knockout stromal cells (Fig. 7d). These results suggest that Menin upregulates Ptx3 expression in an H3K4me3 dependent manner.
Menin inhibits the cross-talk between FGF2-ERK1/2 and BMP2 by regulating the transcription of Ptx3. Ptx3 has been shown to bind with selected FGFs, including FGF2 and FGF8b, through its N-terminal domain, and sequester them in the extracellular matrix, thus inhibiting their biological effects. As described above, Men1 loss upregulated FGF response. We found Ptx3 specifically expressed in the SDZ close to the thin layer of undifferentiated stromal cells where Fgf2 located and significantly decreased following Men1 deletion (Fig. 7e). During embryogenesis, FGF2 is considered to be a paracrine factor and is essential for tissue patterning and organogenesis. Based on these results, we considered that the decrease of Ptx3 caused by Men1 deficiency contributed to excessive ERK1/2 activation induced by paracrine FGF2 produced by the layer of undifferentiated stromal cells. To confirm this conjecture, primary mouse endometrial stromal cells (mESC) isolated from day 4 uterus of pseudopregnancy were cultured with or without recombinant mouse PTX3 in the presence of FGF2. PTX3 treatment for 72 h significantly inhibited the phosphorylation of ERK1/2 induced by FGF2 in mESC compared with cells without PTX3 treatment (Fig. 7f). Consistent with previous studies, the inhibitory effect of PTX3 on FGF2 was conserved during mouse decidualization. Thus, we concluded Menin suppressed the activation of FGF2-ERK1/2 signaling pathway in the SDZ by modulating Ptx3 transcription through epigenetic H3K4me3 modification.

In the preceding results, Men1 loss decreased BMP response program (Fig. 5). Indeed, suppression of BMP signaling by FGF2 sustained undifferentiated proliferation of human ES cells. Next, we explored whether the reduction of BMP2 was caused by abnormal highly activated FGF2-ERK1/2 signal in the absence of Men1. During in vitro decidualization of mESC induced by E2 and P4, the expression pattern of Menin was almost consistent with that of BMP2 in mice during regionalization of decidualization. Histone modifi cation rewiring, guaranteeing the expansion of the SDZ boundary during regionalization of decidualization.

Discussion

The regulatory machinery of endometrial heterogeneity and decidua regionalization after embryo implantation has disturbed scientists for a long time due to the scarcity of a suitable mouse model. Developmentally associated transcription factors HOXA10, whose expression expanded throughout the PDZ and SDZ, have been reported to play an important role in establishing region-specific decidual tissue during decidualization. While, other potential participants in decidua regionalization remained largely unknown. Here, we demonstrated that uterine Men1, which was spatiotemporally expressed during decidualization, guided the stromal cells towards the fate of terminal differentiation by directing histone modification rewiring, guaranteeing the expansion of the SDZ boundary during regionalization of decidualization.

Previous studies indicated that spatiotemporal expression pattern of Bmp2 with the progression of decidualization contributed to highly heterogeneous decidual cells. At the initiation of decidualization, distinct accumulation of Bmp2 in the subepithelial stroma was essential for blastocyst attachment reaction at the implantation site. With the spatiotemporal progression of decidualization, expanded Bmp2 expression in the PDZ and SDZ was critical for the terminal differentiation of stromal cells. A significant decrease of Bmp2 in the SDZ on day 8 caused by Men1 deficiency disturbed the terminal differentiation of stromal cells during the expansion of SDZ without affecting the blastocyst attachment reaction and the PDZ formation.

Menin has yielded cell-specific results in terms of H3K4me3 regulation and the expression of target genes including Hoxa genes, Cdkn1b and Cdkn2c, Esr1, and p35 through connecting with histone methyltransferases MLL1/211–15. There was a dramatic whole genomic chromatin alteration in human endometrium decidualization marked by histone modification and chromatin accessibility. However, the role of H3K4me3 modification in decidual regionalization remained elusive. Distinctive histone methyltransferases in large COMPASS-like complexes performed non-redundant histone modifications in various cell types. The SET1A/B family is responsible for genome-wide bulk H3K4me2/H3K4me3 modification and the...
MLL3/4 complex family mediates H3K4me1 modification at enhancers and super-enhancers. MLL1/2 complex, containing unique subunit Menin, is responsible for H3K4me3 modification at certain genes in cell-specific manner. In this study, we depicted the H3K4me3 profiling in regionalized decidualization during early pregnancy and unraveled its regulatory apparatus by Menin. Among the direct targets marked by Menin-H3K4me3 during stroma decidualization, Ptx3 involved in decidualization regulation possesses specific expression pattern in the SDZ, but its physiological significance in stromal cell differentiation remains intangible. Several pieces of evidence showed that PTX3 acted as a natural FGF2 trap through its N-terminal domain to sequester FGF2-ERK1/2 signaling from promoting cell proliferation.
Interestingly, our investigation demonstrated that Men1-deficient uteri showed augmented FGF2-FGFR1-ERK1/2 signaling activity in the SDZ in the absence of PTX3. This abnormality subsequently reduced Bmp2 and blocked cell differentiation in the SDZ. Consistent with previous studies, the inhibitory effect of PTX3 on FGF2 was conserved during mouse decidualization. Indeed, the homeostasis between BMP and FGF has been described as “constituting a common module” throughout embryogenesis. FGF signaling inhibited the expression of Bmps in dorsal mesoderm, thus limiting their expression to ventral mesoderm for proper dorsoventral axis specification. Inhibition of BMP activity by the FGF signal promoted posterior neural development. Furthermore, suppression of BMP signaling by FGF2 sustained undifferentiated proliferation of human ES cells. Consistently, we evidenced that the discouraged cross-talk between BMP2 and FGF2 coordinated by Menin regulated downstream gene in decidual regionalization and pregnancy maintenance by curbing the cross-talk between FGF2-ERK1/2 and BMP2. Our findings will remarkably advance our knowledge on current model of decidualization and inspire future studies for human fertility improvement.

Methods

Animals. Two-month-old C57BL/6 male and female mice were used in the present study. Men1 floxed mouse line (Men1) was constructed as previously described. Uterine-specific knockout mice (Men1) were generated by mating Men1 females with Pgrcre/+ males. Mice were housed in the animal care facility of Xiamen University with a controlled environment (22 ± 2 °C, 50–60% humidity, 12-h light/dark cycle, lights on at 7 AM) and free access to food and water according to the guidelines for the care and use of laboratory animals. All experimental procedures were approved by the Animal Welfare Committee of Xiamen University (XUE202811), Xiamen University.

Analysis of pregnancy events. Two-month-old female mice were mated with fertile wild-type males to induce pregnancy (vaginal plug = day 1 of pregnancy). Plug-positive females were kept separately for pregnant experiments. Pregnancy rate and litter size were monitored for the whole pregnancy process. For day 5 and day 6 pregnant mice, implantation sites were visualized by an intravenous injection of 100 μL of 1% Chicago blue in saline. The number and average weight of implantation sites demarcated by distinct blue bands was recorded. Uterine horns were flushed to check for the presence of embryos if no blue band was observed. Mouse blood samples were collected on day 6 and serum E2, as well as P4 levels, were measured by radioimmunoassay. At least three mice were used for every individual experiment in each mouse model.

Artificial decidualization model. Pseudopregnant mice were obtained by mating 2-month-old females with vasectomized males of the same strain. To induce artificial decidualization, one uterine horn of pseudopregnant mice was infused with sesame oil (25 μL) on day 4; the non-infused contralateral horn was taken as a control. The weight of infused and non-infused uterine horns was recorded 4 days after the oil infusion and the fold increase in uterine weights was served as an index of decidualization.

In situ hybridization. In situ hybridization with isopeptides or digoxigenin (DIG) was modified according to the previously described method. Frozen sections (10 μm) were mounted onto poly-L-lysine coated slides and stored at −80 °C until used. After removal from −80 °C, the slides were placed on a slide warmer (37 °C) for 3 min and then fixed in 4% paraformaldehyde in PBS for 15 min at 4 °C. Following prehybridization, uterine sections were hybridized to specific cRNA probes overnight at 65 °C. After hybridization, the slides were incubated with RNase A (10 mg/ml) at 37 °C for 30 min. RNase A-resistant hybrids were detected by autoradiography using liquid emulsion or Anti-Digoxigenin-AP, Fab fragments (Roche). Mouse-specific cRNA probes labeled with isotope or digoxin for Men1.
Bmp2, Dan, Igfbp5, Bmp7, Wnt4, Prl8a2, Hoxa10, Ptn, Sfrp4, Ptx3, and Fgfr1 were used for hybridization.

**Immunostaining.** The tissue specimens were fixed overnight in 10% neutral buffered formalin and then dehydrated in increasing concentrations of ethyl alcohol, followed by clearing of alcohol by xylene. Uterine slices were deparaffinized and incubated in citrate buffer for antigen retrieval by hyperbaric heating and then incubated overnight at 4 °C with the primary antibodies including Menin (Bethyl, 1:1000), COX2 (Santa Cruz, 1:200), Dtprp (homemade, 1:200), Ki67 (Service-bio, 1:200), PL1 (Santa Cruz, 1:200), 3β-HSD (Santa Cruz, 1:200), p450scc (Santa Cruz, 1:200), PR (Cell Signaling Technology, 1:200), HAND2 (Santa Cruz, 1:200), p27 (Abcam, 1:100), pH3 (Cell Signaling Technology, 1:200), ERK1/2 (Cell Signaling Technology, 1:200) and p-ERK1/2 (Cell Signaling Technology, 1:200).
Histostain-SP Kit (Zhongshan Golden Bridge Biotechnology) was applied to visualize the antigen. Immunofluorescence staining for OCT4 (Cell Signaling Technology, 1:200), β-Catenin (Abcam, 1:200), H3K27me3 (Cell Signaling Technology, 1:200), BrDU (Abcam, 1:500), PCNA (Santa Cruz, 1:200) and FOXA2 (Abcam, 1:500) was performed in paraffin-embedded sections and secondary antibody Cy3TM AffiniPure Goat Anti-Rabbit IgG(H + L) (Jackson Immunoresearch,1:200) were used. The images were captured by Leica DM2500 light microscope. Antibodies with detailed information are listed in Supplementary Table 1.

**Western blot.** Decidual cells were isolated as described previously. The proteins were separated by using 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Then the membranes with proteins were blocked with 5% skim milk in TBST for 1 h at room temperature (RT) and incubated overnight at 4°C with primary antibody for Menin (Bethyl, 1:2000), β-Actin (Bioland, 1:1000), Dprop (homemade, 1:2000), ERK1/2 (Cell Signaling Technology, 1:1000), p-ERK1/2 (Cell Signaling Technology, 1:1000), JNK1/2 (Bethyl, 1:500), SMAD1/5/8 (Cell Signaling Technology, 1:1000), H3K4me1 (Abcam, 1:2000) and H3 (Abcam, 1:2000). After incubation with primary antibody, membranes were incubated with specific secondary antibodies (Zhongshan Golden Bridge Biotechnology, 1:5000) for 2 h at RT. Bands were visualized using Supersignal West Pico (Thermo Scientific) according to the manufacturer instructions. Uncropped and unprocessed scans of the blots are presented in the Supplementary Fig. 13. Antibodies with detailed information are listed in Supplementary Table 1.

**Quantitative real-time PCR.** Total RNA was extracted from uterine tissues or cells using TRIzol reagent (Invitrogen) following the manufacturer’s protocol. About 1 μg RNA was used to synthesize cDNA. Expression levels of the genes were validated by quantitative real-time PCR analysis with SYBR Green (Takara). All assays were performed at least three times. All PCR primers are listed in Supplementary Table 2.

**Flow cytometry.** Day 8 decidual cells were digested and harvested. The cell pellet was suspended in 0.25 ml PBS after centrifugation; 1 ml of cold 80% ethanol was added dropwise under constant and gentle vortexing. Samples were incubated for 30 min on ice and subsequently overnight at −20°C before being subjected to staining. Cell sediment were suspended in staining solution (PBS containing 5 mg/ml Propidium Iodide and 2 mg/ml DNase-free RNase A) the next day. Samples were incubated for 30 min at 37°C in the dark. They were then returned to RT and subjected to flow cytometry using BD FACS. Flow cytometry data were analyzed using FlowJo software (v10, Tree Star). The intensity value of SSC-A and FCS-A were incubated for 30 min at 37 °C in the dark. They were then returned to RT and subjected to flow cytometry using BD FACS. Flow cytometry data were analyzed using FlowJo software (v10, Tree Star). The intensity value of SSC-A and FCS-A were incubated for 30 min at 37 °C in the dark. They were then returned to RT and subjected to flow cytometry using BD FACS. Flow cytometry data were analyzed using FlowJo software (v10, Tree Star). The intensity value of SSC-A and FCS-A were incubated for 30 min at 37 °C in the dark. They were then returned to RT and subjected to flow cytometry using BD FACS. Flow cytometry data were analyzed using FlowJo software (v10, Tree Star). The intensity value of SSC-A and FCS-A were incubated for 30 min at 37 °C in the dark. They were then returned to RT and subjected to flow cytometry using BD FACS. Flow cytometry data were analyzed using FlowJo software (v10, Tree Star). The intensity value of SSC-A and FCS-A.
Fig. 7 Menin inhibits the cross-talk between FGF2 and BMP2 by promoting Ptx3 expression in an H3K4me3 dependent manner. 

a Genome browser view of normalized Menin and H3K4me3 ChIP-seq signals at Ptx3 promoter locus and RNA-seq tracks for Ptx3 in Men1<sup>f/f</sup> and Men1<sup>d/d</sup> decidual tissues. 
b Quantitative real-time PCR analysis of Ptx3 in Men1<sup>f/f</sup> and Men1<sup>d/d</sup> uteri on day 8. The values are normalized to Gapdh and indicated as the mean ± SEM (n = 3 biologically independent samples). Two-tailed unpaired Student’s t-test, ***p = 0.0025. 
c Quantitative ChIP analysis of Menin at Ptx3 promoter in uterine decidual tissue. Data represent the mean ± SEM (n = 3 biologically independent samples). Two-tailed unpaired Student’s t-test, ***p = 0.0025. 
d Quantitative ChIP analysis of H3K4me3 at Ptx3 promoter in Men1<sup>f/f</sup> and Men1<sup>d/d</sup> uterine decidual tissue. Data represent the mean ± SEM (n = 3 biologically independent samples). Two-tailed unpaired Student’s t-test, **p = 0.0025. 
e In situ hybridization of Ptx3 in Men1<sup>f/f</sup> and Men1<sup>d/d</sup> uteri on day 8. Scale bar: 200 μm. 
f Immunoblotting analysis of the phosphorylation of ERK1/2 in mouse primary endometrial stromal cells treatment with FGF2 (20 ng/ml) in the absence or presence of PTX3. Total ERK1/2 and β-Actin were used as loading controls. 
g Quantitative real-time PCR analysis of Ptx3 in primary mESC treated with DMSO or MI-503 (2 μM) in vitro. The values are normalized to Gapdh and indicated as the mean ± SEM (n = 3 biologically independent samples). Two-tailed unpaired Student’s t-test, **p = 0.0053. 
h Immunoblotting analysis of Menin, BMP2, and phosphorylated ERK1/2 after the addition of PTX3 (100 ng/ml) or PD0325901 (5 μM) in mESC treatment with MI-503 (2 μM) in the presence of FGF2 (20 ng/ml). Total ERK1/2 and β-Actin were used as loading controls.
**Fig. 8** A proposed schematic diagram showing the function of uterine Men1 during decidualization. **a** Uterine deletion of Men1 leads to abnormal polyploidization of decidual cells in the SDZ, accompanied by impeded maturation of uNK cells, increased inflammatory response, and shallow trophoblast invasion. **b** Menin positively regulates the expression of Ptx3 in an H3K4me3 dependent manner. Ptx3, trapping FGF2 from undifferentiated stromal cells, ensures the expression of BMP2 in the SDZ by inhibiting the activation of FGF2-ERK1/2.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
The sequencing data generated in this study have been deposited in the Gene Expression Omnibus database under accession code GSE182525, GSE191327, and GSE182539. Source data are provided with this paper.

**Code availability**
The data analysis pipeline used in this paper is deposited at [https://github.com/dwb0211/Menin](https://github.com/dwb0211/Menin).

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