Semaphorin 3E-Plexin-D1 pathway regulates ovulation, luteinization, and ovarian angiogenesis in mice

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Mammalian ovulation, triggered by the preovulatory surge of luteinizing hormone (LH), is accompanied by granulosa cell (GC) luteinization and ovarian angiogenesis. Various regulators of ovarian angiogenesis play crucial roles in ovulation and corpus luteum (CL) formation, but the picture of key regulators and the mechanisms governing their activities is far from complete. Semaphorin 3E (Sema3E), a secreted protein of the semaphorin family, regulates the guidance and migration of neurons and endothelial cells via binding to its receptor Plexin-D1; however, its role in the ovary has not been investigated. Elevated transcript levels for *Sema3e* in early luteal phase were reported in mice with GC-specific deletion of CCAAT/enhancer-binding proteins alpha and beta (*Cebpa/b*$^{gc-/-}$), which had blocked ovulation and impaired CL angiogenesis. We hypothesize that SEMA3E-PlexinD-1 pathway is active downstream of the preovulatory LH surge and regulates ovarian angiogenesis around the time of ovulation.

We first examined the expression patterns for *Sema3e* and *Plxnd1* in the mouse ovary following superovulation (time post-hCG is the biological equivalent of time post-LH surge). Utilizing real-time-qPCR on isolated GCs and ovarian stromal tissues, immunostaining, high-resolution spatial transcriptomics, and single-nucleus assay for transposase-accessible chromatin sequencing (snATAC-seq), we found that Sema3E and Plexin-D1 are induced by the LH surge in preovulatory follicles in dynamic spatial and temporal patterns. Sema3E is predominantly expressed in GCs. The expression of *Sema3e* mRNA is induced shortly after LH surge, peaks at 6 h post-hCG, and decreases to a lower level immediately before ovulation (12h post-hCG). Plexin-D1 is expressed in a sub-population of GCs and ovarian stromal tissues (primarily in ovarian vascular endothelial cells) with cell type-specific temporal dynamics. Fluorescence *in situ* hybridization revealed a dynamic shift of *Plxnd1*+ vascular endothelial cells from stromal tissues to the CL after ovulation.

We then asked whether C/EBPα and C/EBPβ regulate the expression of *Sema3e* and *Plxnd1* during the preovulatory stage. By immunostaining, snATAC-seq, and comparing *Sema3e* and *Plxnd1* mRNA levels in GCs and stromal tissues isolated from *Cebpa/b*$^{gc+/+}$ control and *Cebpa/b*$^{gc-/-}$ mutant mice, we found that mRNA levels of these two genes are

dysregulated in the mutants in a cell type-specific manner. SnATAC-seq further indicated that *Sema3e* and *Plxnd1* are expressed in sub-populations of preovulatory granulosa cells, and C/EBPα and C/EBPβ regulate their expression during the preovulatory stage by modulating chromatin accessibility. Specifically, deletion of *Cebpa/b* increased the expression of *Sema3e* during the late preovulatory stage.

To explore the functional impact of evaluated Sema3E in the mouse ovary, we conducted intraovarian injection of mouse recombinant Sema3E to one ovary and IgG control to the contralateral ovary shortly before hCG stimulation. The injection of Sema3E resulted in reduced ovulation, disrupted CL formation, and aberrant ovarian angiogenesis both in stromal tissues and in CL. Bulk RNA-seq analysis on isolated GCs and stromal tissues revealed altered transcripts of genes regulating GC luteinization, ovarian stromal angiogenesis, and inflammation in Sema3E-treated ovaries compared to controls. Furthermore, *in vitro* culturing of GCs and stromal tissues treated with Sema3E demonstrated a direct effect of Sema3E on GC luteinization and angiogenesis, with an indirect effect on stromal angiogenesis and inflammation primarily through its effects on GCs.

Taken together, our findings demonstrate the role of Sema3E-Plexin-D1 pathway in regulating ovulation, GC luteinization, and ovarian angiogenesis in mice. Sema3E-Plexin-D1 pathway may be a potential therapeutic target for infertility associated with impaired ovulation and ovarian angiogenesis.