SLIT1 and -2 Act Redundantly Through an Akt/FOXO1 Pathway in Mouse Follicular Granulosa Cells

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Previous studies conducted in our laboratory have demonstrated the involvement of SLIT1 in female fertility in mice. SLIT1-deficient mice display increased fertility, characterized by higher rates of ovulation and a greater abundance of healthy antral follicles compared to wild-type (WT) mice. Our findings identified SLIT1 as an inhibitor of AKT, a crucial effector known for its roles in gonadotropin signaling. One of the well-known downstream targets of AKT is FOXO1, a transcription factor involved in regulating various cellular processes, including cell survival and apoptosis. The Slit/Robo pathway comprises three ligands: SLIT1/2/3 and their four receptors ROBO1/2/3/4. While SLIT1 and SLIT2 share similar structures and properties, SLIT3 diverges structurally and seems more associated with angiogenesis. The current study seeks to clarify the potential redundancies among SLIT ligands and their mechanisms of action in mouse ovarian follicles. To investigate the ovarian expression of Slit/Robo genes, granulosa cells from WT mice were isolated. mRNA levels of Slit/Robo genes were assessed using RT-qPCR, which revealed the expression of all effectors in mouse granulosa cells. Western blotting analyses were conducted on cultured granulosa cells treated with exogenous SLIT1, -2, or -3 alone or in combination with FSH to investigate the potential redundant effects of SLIT ligands on the AKT pathway. The results showed that SLIT1 and -2 significantly inhibit the induction of AKT and FOXO1 phosphorylation by FSH. In line with expectations, cells treated with SLIT3 showed no alteration in AKT and FOXO1 phosphorylation levels. To explain the increased number of healthy follicles observed in SLIT1-deficient mice, apoptosis was evaluated in cultures granulosa cells treated with SLIT1. Our analysis revealed that SLIT1 treatment induces an elevation in cleaved-caspase 3 levels, as well as the proportion of TUNEL-positive cells. Furthermore, granulosa cells derived from *Slit1*$^{-/-}$ mice exhibited elevated mRNA levels of *Cyp11a1*, *Cyp19a1*, *Inha*, *Nr5a2*, and *Star* compared to wild-type mice. These genes have been previously identified in the literature as targets of FOXO1. To elucidate the genes regulated by SLIT1, we conducted RNA sequencing on granulosa cells from both SLIT1-deficient and WT mice. Our analysis revealed a marked impact of SLIT1 on the Renin/Angiotensin system (RAS) pathway. Subsequent qPCR analysis confirmed significant downregulation of *Agt*, *Agtr2*, *Clca3a*, and *Mme* in SLIT1-deficient mice compared to WT counterparts. Notably, RAS is also known to be regulated by FOXO1 in other cell types. To identify the receptor responsible for SLIT1 action in mouse ovarian granulosa cells, we utilized a *Robo1*$^{-/-}$;*Robo2*$^{f/f}$ mutant model, in which the *Robo2* allele is highly hypopomorphic. Granulosa cells isolated from these mutants and WT mice were cultured with SLIT1 and/or FSH, followed by Western blot analyses. Interestingly, SLIT1 treatment led to a comparable decrease in AKT phosphorylation in the granulosa cells from WT or mutant mice, indicating that ROBO1 is not the exclusive receptor for SLIT1 in granulosa cells, and that neither ROBO1 nor ROBO2 may be required for SLIT1 to exert its effects. Together, these data suggest that SLIT1 induces an

increase in nuclear FOXO1 levels by inhibiting AKT phosphorylation, resulting in suppression of the transcription of its target genes and the induction of apoptosis. To validate this hypothesis, ChIP-sequencing experiments will be conducted. Further investigations will be required to elucidate the mechanisms of action of SLIT2 and SLIT3 in mouse ovarian granulosa cells.