—Original—

Regulation of Preimplantation Embryo Development in Mice by FMS-like Tyrosine Kinase 3 Ligand

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Abstract: Successful development of preimplantation embryos is essential for reproduction. Growth factors secreted by reproductive tracts are important for the development of preimplantation embryos. FMS-like tyrosine kinase 3 (FLT3) is a tyrosine kinase receptor related to colony-stimulating factor-1 receptor and c-KIT, promoting preimplantation embryo development following their ligand binding. We found expression of FLT3 ligand transcripts in the oviducts and uteri of pregnant mice. The transcripts for its receptor, FLT3, were detectable throughout the early embryonic stages with an increase after the eight-cell stage. In contrast, the expression of FLT3 ligand was negligible after the morula stage, suggesting potential paracrine actions of FLT3 ligand produced by the reproductive tracts. Treatment with FLT3 ligand promoted the development of two-cell embryos to the morula and blastocyst stages in a dose-dependent manner with increases in cell proliferation, but not inhibition of cell survival. The effects of FLT3 ligand were blocked by a FLT3 receptor inhibitor, TCS359. Studies using specific inhibitors demonstrated the potential involvement of the PI3K pathway in mediating FLT3 ligand actions. Our findings suggest that the FLT3 ligand/FLT3 signaling system plays important paracrine roles during the development of preimplantation embryos.
Key words: FLT3 ligand, FLT3, Preimplantation embryo, Oviduct, Uterus

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Introduction

Accumulating evidence demonstrates that a number of growth factors and cytokines act as paracrine and/or autocrine factors during early embryo development and implantation in mammals [1–3]. Brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, leptin, ghrelin, gonadotropin-releasing hormone I (GnRH-I), insulin-like growth factor-I, epidermal growth factor, colony-stimulating factor-1 (CSF1), kit ligand and other hormonal factors secreted by the maternal reproductive tract regulate the development of pre- and periimplantation embryos through specific receptors [2–12]. In addition, developing early embryos produce diverse growth factors that act in an autocrine manner to regulate their own growth and differentiation, or to serve as paracrine factors by regulating uterine endometrial receptivity for blastocyst implantation.

FMS-like tyrosine kinase 3 (FLT3) is a tyrosine kinase receptor structurally related to CSF1 receptor and c-KIT. FLT3 is reported to be expressed in early hematopoietic progenitor cells that regulate hematopoiesis [13–16]. The FLT3 ligand acts in synergy with other cytokines to promote hematopoietic expansion [13, 17]. Upon stimulation with FLT3 ligand, FLT3 dimerizes and undergoes autophosphorylation, leading to the upregulation of its tyrosine kinase activity. This increase in activity triggers intracellular signaling through an array of downstream pathways, including the PI3 K cascade, resulting in promotion of cell proliferation and survival [14, 15]. Although

CSF1 and kit ligand have been shown to regulate the development of preimplantation embryo upon binding to their cognate receptors, the roles of the FLT3 ligand/FLT3 signaling system in early embryonic growth are unknown.

Here, we show the expression of FLT3 ligand and FLT3 in preimplantation embryos and pregnant oviducts and uteri of mice. We demonstrate the paracrine role of the FLT3 ligand/FLT3 signaling system in the development of preimplantation embryos using FLT3 ligand and FLT3 inhibitors, and involvement of PI3 K in FLT3 ligand signaling.

Materials and Methods

Animals

To obtain preimplantation embryos, B6D2F1 mice at 25 days of age (CLEA Japan, Tokyo, Japan) were treated sequentially with 7 IU of pregnant mare serum gonadotropin (PMSG; Calbiochem, Cambridge, MA) and 10 IU of human chorionic gonadotropin (hCG; ASKA Pharmaceutical, Tokyo, Japan), and allowed to mate immediately after hCG treatment. At 46–47 h after hCG injection, two-cell embryos were obtained by flushing the oviducts of mated mice for *in vitro* culture, whereas unfertilized mature oocytes were obtained from oviducts of unmated mice at 14–16 h after hCG injection as previously described [9]. After mechanical removal of cumulus cells, mature oocytes were subjected to real-time RT-PCR analyses. Oviducts and uteri were obtained from immature untreated mice, mice treated with PMSG (7 IU), mice treated with PMSG followed 12 h later by hCG (10 IU) treatment, and from animals at 2 and 4 days after mating. The care and use of animals was approved by the Animal Research Committee, Akita University School of Medicine and St. Marianna University School of Medicine.

Embryo cultures and apoptosis detection

Two-cell embryos were obtained and groups of 10–12 embryos were placed in 50 µl drops of modified M16 medium (Sigma. St. Louis, MO) with or without FLT3 ligand (PeproTech, Rocky Hill, NJ) and covered with mineral oil. The embryos were cultured for 96 h, up to the hatched blastocyst stage with fresh medium replacement every 24 h. Some embryos were cultured with FLT3 ligand with or without a FLT3 inhibitor, TCS359 (Tocris, Bristol, UK). To analyze the involvement of the PI3 K pathway in the FLT3 ligand induction of early embryonic development, groups of 10–12 two-cell embryos were cultured with FLT3 ligand with or without a PI3 K inhibitor, LY294002 (Sigma) or its inactive analog, LY303511 (Calbiochem, La Jolla, CA). Embryonic development was monitored after 24, 72, and 96 h of culture to determine the proportion of embryos at the morula, expanded blastocyst, and hatched blastocyst stages, respectively. At the end of culture, some embryos were subjected to the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay to detect apoptosis as previously described [20]. Briefly, zona pellucida-intact embryos were washed, fixed in 4% paraformaldehyde for 30 min at room temperature, and permeabilized in 0.5% Triton X-100 (Sigma) for 1 h at room temperature. Embryos were incubated with TUNEL reagent (Roche Applied Science, Indianapolis, IN) for 1 h at 37°C in the dark. For positive controls, embryos were treated with 50 µg/ml Ribonuclease A (Sigma) for 20 min at 37°C before the TUNEL reaction. For negative controls, embryos were incubated with TUNEL reagent in the absence of the enzyme terminal deoxynucleotidyl transferase. Counterstaining was performed by incubating embryos with 100 µg/ml propidium iodide and 50 µg/ml RNase A (Ambion, Inc., Austin, TX) for 20 min at room temperature. Embryos were washed and mounted with Slowfade light antifade solution (Invitrogen, Carlsbad, CA). The fluorescence signals of the embryos were visualized using a confocal laser scanning microscope (LSM 410; Carl Zeiss, Oberkochen, Germany).

RT-PCR

For quantitative real-time RT-PCR, two-cell embryos were allowed to continue development to different stages and were then collected after culturing in individual microdrops for 50–52 (four-cell), 59–60 (eight-cell), 70–72 (morula), 94–96 (early blastocyst), 119–120 (expanded blastocyst), and 142–144 (hatched blastocyst) h after hCG injection. Quantitative real-time RT-PCR of transcript levels in preimplantation embryos, oviducts and uteri was performed using a SmartCycler (Takara, Tokyo, Japan) as previously described [18]. The primers and hybridization probes for real-time RT-PCR of FLT3 ligand, FLT3, and histone H2a are as follows: FLT3 ligand: sense 5′-GAGGACGTCAACACCGAGAT-3′, antisense 5′-AGGTGGGAGATGTTGGTCTG-3′, probe 5′–6-carboxy-fluorescein (FAM)- TTGTCACCTCATGTACCTTCCAGCC-6-carboxy-tetramethyl-rhodamine (TAMRA)-3′; FLT3: sense 5′-GCACCAAGCTGTTCACCATA-3′, antisense 5′-ATGGCCTTACACCTGATCCA-3′, probe 5′-FAM-AACCAGGCTCCTCAGAGCACACTG-3′; histone H2a: sense 5′-ACGAGGAGCTCAACAAGCTG-3′, antisense 5′-TATGGTGGCTCTCCGTCTTC-3′, probe 5′-FAM-AACATCCAGGCCGTGCTGCT-TAMRA-3′.

To determine the absolute copy number of target transcripts, cloned plasmid cDNAs for individual genes were

used to generate a calibration curve. Purified plasmid cDNA templates were measured, and copy numbers were calculated based on absorbance at 260 nm. A calibration curve was created by plotting the threshold cycle against the known copy number of each plasmid template diluted in log steps from 10^5 to 10^1 copies. Each run included standards of diluted plasmids to generate a calibration curve, a negative control without a template, and samples with unknown mRNA concentrations. Data were normalized based on histone H2a transcript levels [19].

Statistical analysis

One-way ANOVA, followed by Fisher's protected least significant difference test, was used for multiple group comparison. Results are presented as mean ± SEM of at least three separate experiments.

Results

Temporal expression of FLT3 ligand and FLT3 in preimplantation embryos

To examine the potential roles of FLT3 ligand and its receptor during preimplantation embryo development, we evaluated the expression of these genes in early mouse embryos. Quantitative real-time RT-PCR was performed to determine temporal expression of FLT3 ligand and FLT3 in preimplantation embryos. Levels of FLT3 ligand transcripts were high in the unfertilized oocytes and two-cell embryos and showed a decrease at in the four-cell stage, reaching negligible levels at the morula stage (Fig. 1A). In contrast, levels of FLT3 mRNA were low in unfertilized oocytes and embryos at the two-cell and four-cell stages, but increased at the eight-cell stage, reaching its highest levels at the morula to hatched blastocyst stage (Fig. 1B).

Expression of FLT3 ligand in preimplantation oviducts and uteri

To address the paracrine action of FLT3 ligand during preimplantation embryo development, the expression of FLT3 ligand in mouse oviducts and uteri was examined during the preimplantation period. In the oviducts, quantitative real-time RT-PCR analyses revealed that FLT3 ligand transcript levels were high in immature mice at 25 days of age (Fig. 2A, 0 h), then decreased after PMSG treatment, and stayed at low levels until 12 h after hCG treatment (Fig. 2A). At days 2 and 4 of pregnancy, FLT3 ligand transcript levels increased (Fig. 2A). Similar changes in the uterine FLT3 ligand were detected (Fig. 2B).

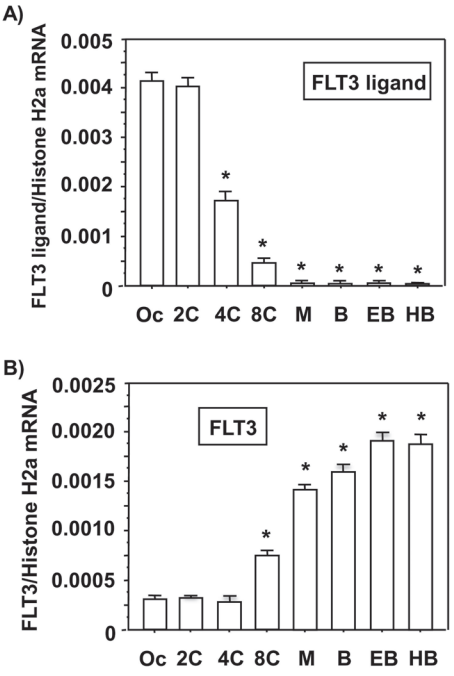

Fig. 1. Temporal expression of the transcripts of FLT3 ligand and FLT3 in mouse preimplantation embryos. Transcript levels of FLT3 ligand (A) and FLT3 (B) in unfertilized mature oocytes and developing preimplantation embryos were determined using real-time RT-PCR. Levels of all mRNAs were normalized to those of histone H2a in the same sample (mean ± SEM, n=3). *, $P<0.05$ vs. Oc. Oc: oocyte; 2C: 2-cell; 4C: 4-cell; 8C: 8-cell; M: morula; B: early blastocyst; EB: expanded blastocyst; HB: hatched blastocyst.

Effects of FLT3 ligand on the growth of preimplantation embryos in vitro

The expression of FLT3 ligand and FLT3 in preimplantation embryos and the expression of FLT3 ligand in oviducts and uteri suggest that the FLT3 ligand/FLT3 signaling system plays a role in early embryonic growth. We further examined the effect of FLT3 ligand treatment on cultured mouse preimplantation embryos. Although FLT3 ligand treatment did not affect the development of two-cell embryos to the eight-cell stage (data not shown), FLT3 ligand promoted the development of embryos to the morula, expanded and hatched blastocyst stages, evaluated at 24, 72 and 96 h of culture, respectively, in a dose-dependent manner (Fig. 3A). The specificity of the effects of FLT3 ligand on preimplantation embryos was examined using a FLT3 inhibitor, TCS359. The ability of FLT3 ligand to promote the development of two-cell embryos to the hatched blastocyst stage was blocked by

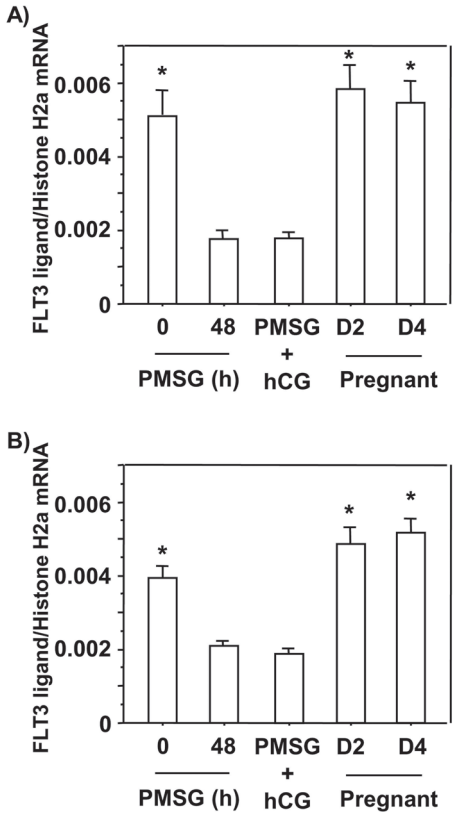

Fig. 2. Expression of FLT3 ligand in mouse oviducts and uteri during the preimplantation period. Quantification of FLT3 ligand transcripts in oviducts (A) and uteri (B) was done using real-time RT-PCR. Levels of FLT3 ligand mRNA were obtained at different times after PMSG and hCG treatment. Some samples were obtained from animals at days 2 or 4 of pregnancy (mean ± SEM, n=3). At each stage, three samples prepared from individual animals were used. Levels of FLT3 ligand mRNA were normalized using transcript levels of histone H2a in the same sample. *P<0.05 vs. samples from animals treated with PMSG followed by hCG treatment after 12 h.

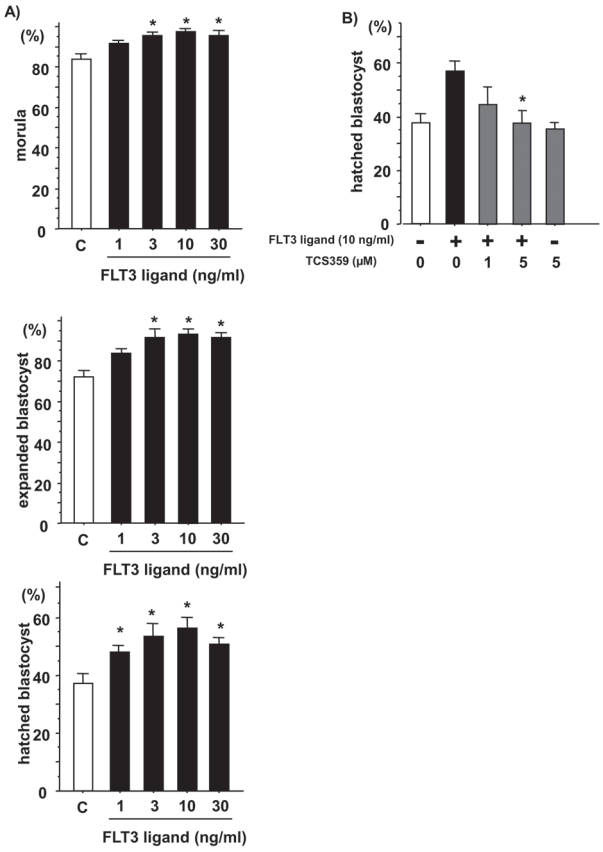

Fig. 3. FLT3 ligand stimulation of the development of cultured preimplantation embryos. (A) Effects of FLT3 ligand treatment on early embryonic development. Two-cell embryos (10–12 embryos/50 μl culture drop) were cultured without (control, C) or with FLT3 ligand for 24, 72 and 96 h to evaluate the proportions of embryos at the morula, expanded and hatched blastocyst stages, respectively (Mean ± SEM, n≥6, 80–96 embryos per group). *, P<0.05 vs. control group. (B) Antagonistic effects of the FLT3 inhibitor, TCS359, on the FLT3 ligand promotion of embryo development. Two-cell embryos were cultured for 96 h with FLT3 ligand (10 ng/ml) with or without TCS359 (1 or 5 μM) (Mean ± SEM, n ≥5, 60–76 embryos per group). *, P<0.05 vs. FLT3 ligand group.

co-treatment with the TCS359 (Fig. 3B). Consistent with the decrease of FLT3 ligand expression in early embryos after the four-cell stage, inhibition of endogenous FLT3 ligand by treatment with the TCS359 in the absence of exogenous FLT3 ligand did not affect the development of embryos (Fig. 3B).

Effects of FLT3 ligand treatment on apoptosis of cultured blastocysts

To determine whether FLT3 ligand acts as a survival factor for early embryos, we evaluated apoptosis in embryos treated with FLT3 ligand. Because hatched blastocysts are difficult to retrieve due to their extreme viscosity, expanded blastocysts were subjected to the TUNEL assay. Cell counts indicated that FLT3 ligand treatment increased the total number of cells in blastocysts (Fig. 4A). However, the proportion of TUNEL positive nuclei was similar in the control and FLT3 ligand-treated groups (Fig. 4B).

Involvement of the PI3 K pathway in the FLT3 ligand promotion of preimplantation embryo development

We further analyzed the roles of the PI3 K signaling

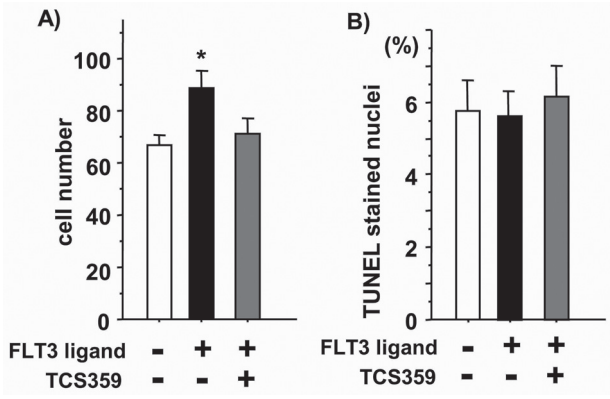

Fig. 4. Effect of FLT3 ligand on cell numbers (A) and apoptosis (B) in cultured blastocysts. Two-cell embryos were cultured for 72 h with or without FLT3 ligand (10 ng/ml) and the specificity of FLT3 ligand actions was evaluated by cotreatment with the FLT3 inhibitor, TCS359 (5 μM). Apoptosis in embryos was assayed by detecting apoptotic nuclei using the TUNEL assay (mean ± SEM, total n=15 embryos per group). *, $P<0.05$ vs. control group.

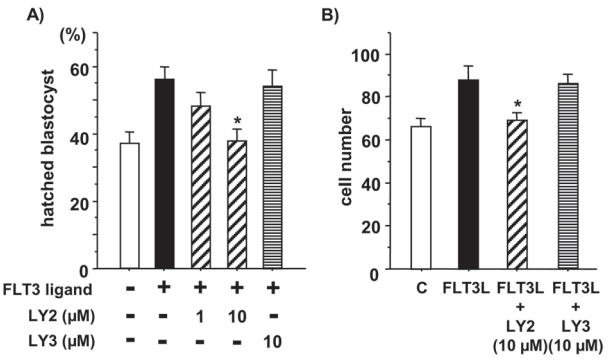

Fig. 5. Involvement of the PI3 K pathway in the FLT3 ligand stimulation of early embryonic development. Effects of PI3 K inhibitors on the FLT3 ligand promotion of preimplantation embryo development (A), stimulation of cell numbers in blastocysts (B). Two-cell embryos were cultured without (control, C) or with FLT3 ligand (10 ng/ml) with or without the inhibitor of PI3 K, LY294002 (LY2), or its inactive analog, LY303511 (LY3). At 96 h after culture, the proportion of embryos at the hatched blastocyst stage was evaluated (Mean ± SEM, n=5, 48–62 embryos per group). Total cell numbers of the expanded blastocysts were determined (Mean ± SEM, total n=15 embryos per group) at 72 h of culture. *, $P<0.05$ vs. FLT3 ligand group. FLT3L: FLT3 ligand.

pathway as a downstream mediator of the FLT3 ligand/FLT3 signaling system in embryo development. The ability of FLT3 ligand to promote the development of two-cell embryos to the hatched blastocyst stage was suppressed by co-treatment with a PI3 K inhibitor, LY294002, but not with its inactive analog, LY303511, in a dose-dependent manner (Fig. 5A). Furthermore, treatment with LY294002 inhibited the FLT3 ligand stimulation of cell numbers (Fig. 5B) in blastocysts.

Discussion

The present study demonstrated the ability of FLT3 ligand to promote early embryonic development, suggesting the inclusion of this factor could be important for *in vitro* embryo cultures. The expression of FLT3 ligand mRNA was high in oocytes and two-cell embryos but decreased after the four-cell stage, indicating the maternal origin of the mRNA.

In contrast, because the expression of FLT3 mRNA in oocytes and embryos at the two- to four-cell stages was low, transcription of *Flt3* may be triggered by zygotic genome activation. The high expression of FLT3 in eight-cell embryos to hatched blastocysts was likely responsible for the observed ability of FLT3 ligand to promote early embryonic development. Because FLT3 ligand is also expressed in the oviducts and uteri, the observed actions of FLT3 ligand on blastocyst development could involve paracrine actions.

Treatment with FLT3 ligand promoted the development of cultured preimplantation embryos from the two-cell stage to the morula, expanded and hatched blastocyst stages, and the observed effects were suppressed by the FLT3 inhibitor, TCS359. Although FLT3 ligand did not stimulate early embryonic development prior to the morula stage, FLT3 ligand may regulate cellular functions or condition early embryos in preparation for development. However, the possibility cannot be ruled out that the downstream pathways of the FLT3 ligand /FLT3 signaling system are not operational before the morula stage. The effect of FLT3 ligand on blastocyst development was correlated with its ability to increase total cell numbers in blastocysts without inhibition of apoptosis. Using specific inhibitors, we also demonstrated the contribution of the PI3 K signaling pathway in the FLT3 ligand promotion of early embryonic development. These actions of FLT3 ligand on cell proliferation are consistent with the result of earlier studies, in whitch stimulation of cell proliferation of hematopoietic cells, including stem cells, progenitor cells, dendritic cells, and natural killer cells in concert with other growth factors was observed [14, 15].

We detected FLT3 ligand in the oviductal and uterine epitheliums with RT-PCR, due to the unavailability of suitable antibodies for immunoassays. Because preimplantation embryos develop in the oviduct until early on day

4 of pregnancy, when they reach the late morula/blastocyst stage and then migrate to the uterus, we observed increases in the levels of FLT3 ligand in the oviduct and uterus during the preimplantation period, suggesting its paracrine role during early embryonic development. Because of FLT3 expressed in both the oviduct and uterus (data not shown), future studies of autocrine actions in the oviduct and uterus might reveal the roles of FLT3 ligand/FLT3 signaling in these reproductive tracts, where it has the potential to affect the development and implantation of preimplantation embryos.

Although there is no available data on the fertility of *Flt3* ligand-null mice [21], an earlier study demonstrated the normal fertility of *Flt3*-null mice [22]. Because diverse growth factors have been shown to promote preimplantation embryo growth [2, 3, 12], these factors could compensate for the action of FLT3 ligand/FLT3 signaling in embryo growth *in vivo*.

In conclusion, the present study demonstrated a role of the FLT3 ligand/FLT3 signaling system in the development of preimplantation embryos. Accumulated evidence indicates the importance of autocrine/paracrine factors secreted by the reproductive tract for embryonic development and implantation [2, 3, 12]. The present study demonstraed that the FLT3 ligand/FLT3 signaling pathway augmented early embryo development, underscoring the importance of diverse autocrine/paracrine systems for early embryonic development. Because paracrine factors derived from the reproductive tract are lacking in *in vitro* culture, the development of embryo culture media based on ligand/receptor expressions of the individual development stages may facilitate the future formulation of optimal culture conditions for preimplantation embryos in *in vitro* fertilization-embryo transfer.

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