Follicle-stimulating hormone regulates expression and activity of epidermal growth factor receptor in the murine ovarian follicle

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Edited by John J. Eppig, The Jackson Laboratory, Bar Harbor, ME, and approved October 21, 2014 (received for review August 4, 2014)

Fertility depends on the precise coordination of multiple events within the ovarian follicle to ensure ovulation of a fertilizable egg. FSH promotes late follicular development, including expression of luteinizing hormone (LH) receptor by the granulosa cells. Expression of its receptor permits the subsequent LH surge to trigger the release of ligands that activate EGF receptors (EGFR) on the granulosa, thereby initiating the ovulatory events. Here we identify a previously unknown role for FSH in this signaling cascade. We show that follicles of *Fshb*$^{-/-}$ mice, which cannot produce FSH, have a severely impaired ability to support two essential EGFR-regulated events: expansion of the cumulus granulosa cell layer that encloses the oocyte and meiotic maturation of the oocyte. These defects are not caused by an inability of *Fshb*$^{-/-}$ oocytes to produce essential oocyte-secreted factors or of *Fshb*$^{-/-}$ cumulus cells to respond. In contrast, although expression of both *Egfr* and EGFR increases during late folliculogenesis in *Fshb*$^{+/-}$ females, these increases fail to occur in *Fshb*$^{-/-}$ females. Remarkably, supplying a single dose of exogenous FSH activity to *Fshb*$^{-/-}$ females is sufficient to increase *Egfr* and EGFR expression and to restore EGFR-dependent cumulus expansion and oocyte maturation. These studies show that FSH induces an increase in EGFR expression during late folliculogenesis and provide evidence that the FSH-dependent increase is necessary for EGFR physiological function. Our results demonstrate an unanticipated role for FSH in establishing the signaling axis that coordinates ovulatory events and may contribute to the diagnosis and treatment of some types of human infertility.

ovary | follicle | FSH | EGFR | granulosa

Fertility in mammals depends on the coordinated execution of multiple events within the fully grown ovarian follicle at the time of ovulation (1, 2). The oocyte undergoes meiotic maturation, during which it progresses to metaphase II of meiosis and acquires the ability to begin embryonic development (3). Concomitantly, the layer of granulosa cells (GCs) immediately surrounding the oocyte, termed the "cumulus," undergoes a process termed "expansion," which is required for sperm to penetrate this layer and reach the oocyte (4–7). At the perimeter of the follicle, an inflammatory response associated with rupture of the follicular wall permits the cumulus–oocyte complex (COC) to escape from the follicle and enter the oviduct where fertilization will occur. These events are triggered by the preovulatory release of luteinizing hormone (LH), which acts on LH receptors (LHCGR) on the mural GCs that line the interior wall of the fully grown follicle (8).

Recent studies have identified a key downstream effector of LH activity at ovulation. Binding of LH to LHCGR triggers the release of the EGF-related peptides amphiregulin (AREG, betacellulin (BTC), and epiregulin (EREG) (9–11). These bind to EGF receptors (EGFRs) located on both the mural and cumulus GCs (12–19) and activate MAPK3/1 as well as other signaling networks (20–28). Considerable evidence supports the view that the EGFR signaling mediates many or most ovulatory events. First, the release of the EGFR ligands follows the LH surge but precedes the LH-dependent responses (9–11). Second, EGF and the EGFR ligands can induce cumulus expansion and oocyte maturation in vitro, independently of LH (9, 10, 20, 29). Third, these events are impaired in mice bearing a hypomorphic *Egfr* allele that reduces EGFR activity by about one-half and in mice in which *Egfr* has been selectively inactivated in GCs through a targeted mutation (22, 23). Thus, the activation of EGFR signaling in GCs of mature follicles appears to be a major effector of the ovulatory response to LH.

FSH binds to receptors located on GCs and induces the expression of numerous genes, including *Lhcgr* (8, 30). *Lhcgr* expression is impaired substantially in mice that lack either FSH, because of targeted mutation of the *Fshb* gene that encodes its β-subunit, or the FSH receptor and in humans bearing spontaneous mutations; these individuals fail to ovulate (31–34). Thus, the ovulatory response to LH depends strictly on the prior FSH-dependent expression of *Lhcgr*, and in this manner FSH indirectly controls the LHCGR-regulated release of the EGFR ligands. We report here that FSH also drives an increase in EGFR expression during late folliculogenesis and provide evidence that this increase is essential to enable the ovulatory response to EGF. By coordinating the expression of EGFR and the release of its ligands, FSH endows full-grown follicles with the capacity to activate EGFR signaling at ovulation.

Significance

Ovulation in mammals requires activation of EGF receptor (EGFR) signaling within the ovarian follicle, but the mechanisms responsible for implementing the EGFR network during follicular growth remain incompletely understood. The final phase of growth is driven by FSH. Here we show that during this phase EGFR expression increases sharply in follicular granulosa cells and that this increase requires FSH; we provide evidence that the FSH-dependent increase is essential for EGFR signaling. FSH also is known to induce expression of luteinizing hormone (LH) receptors in the granulosa, permitting them to release EGFR ligands in response to preovulatory LH. By coordinating receptor expression and ligand release, FSH endows fully grown follicles with the capacity to activate EGFR signaling at ovulation.

Author contributions: S.E.-H., I.D., and H.J.C. designed research; S.E.-H. and I.D. performed research; S.E.-H., I.D., and H.J.C. analyzed data; and S.E.-H. and H.J.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1414648111/-/DCSupplemental.

Results

COCs of *Fshb*$^{-/-}$ Females Do Not Expand or Up-Regulate Expansion-Related Transcripts in Response to EGF. To examine whether FSH was required for activation of follicular EGFR signaling, we analyzed *Fshb*$^{-/-}$ mice, which cannot produce FSH (35). Follicular development in *Fshb*$^{-/-}$ females is overtly normal until the early antral stage but then becomes arrested. These females fail to ovulate and hence are infertile (35, 36). Nonetheless, there is little or no effect on the expression of GC genes not regulated by FSH (31), and the follicles retain the ability to support the development of fully grown meiotically competent oocytes and to maintain them in prophase arrest (36). Although the oocytes can mature in vitro, they develop poorly after fertilization, indicating that some aspect of their development is abnormal (31, 36). *Fshb*$^{+/-}$ females are fertile (31, 36) and served as controls in our experiments. Because the absence of FSH eventually leads to follicular atresia (37), we used prepubertal *Fshb*$^{-/-}$ females so that we could study the large cohort of follicles that initiates growth shortly after birth in the mouse, reaching full size after about 3 wk. By using prepubertal *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ animals of the same age, we were able to compare follicles that had been growing for the same period in the presence or absence of FSH, before follicular atresia occurs in *Fshb*$^{-/-}$ females.

We first assessed cumulus expansion, which occurs downstream of EGFR signaling (9, 10). When we obtained COCs of *Fshb*$^{+/-}$ females at postnatal days (P) 21/23 and incubated them in the presence of EGF for 16 h, they expanded as expected (Fig. 1*A*). Although growing follicles of *Fshb*$^{-/-}$ females do not form large antra (35, 36), we were able to puncture the largest follicles and recover oocytes enclosed by GCs, which we provisionally term "COCs." In contrast to the COCs of *Fshb*$^{+/-}$ females, COCs from *Fshb*$^{-/-}$ females of the same age did not expand in response to EGF (Fig. 1*B*). Hence, this EGFR-dependent event failed to occur in COCs from follicles that had grown in the absence of FSH. We note that COCs from hypogonadal mice lacking FSH (on a different genetic background than the mice used here) can undergo expansion in vitro (38).

Cumulus expansion requires EGFR-dependent up-regulation of a subset of genes, including hyaluronan synthase 2 (*Has2*), prostaglandin-endoperoxide synthase 2 (*Ptgs2*), and tumor necrosis factor, alpha-induced protein (*Tnfaip6*) (6, 39–43). Therefore we isolated COCs from P21/23 *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females and examined basal and EGF-stimulated expression of these genes. Before EGF treatment, *Has2*, *Ptgs2*, and *Tnfaip6* mRNA levels were lower in *Fshb*$^{-/-}$ COCs than in heterozygotes (Fig. S1). Upon EGF stimulation, the quantity of all three transcripts increased significantly more in COCs of *Fshb*$^{+/-}$ females than in those of *Fshb*$^{-/-}$ females (Fig. 1*C*). Several lines of evidence indicate that the impaired *Fshb*$^{-/-}$ response is unlikely to reflect

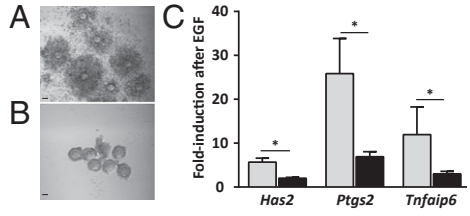

Fig. 1. COCs of *Fshb*$^{-/-}$ females do not undergo cumulus expansion or up-regulate expansion-related mRNAs in response to EGF. (*A* and *B*) COCs isolated from follicles of *Fshb*$^{+/-}$ females (*A*) or *Fshb*$^{-/-}$ females (*B*) at P21/23 and incubated for 16 h in the presence of EGF. Micrographs are representative of three independent experiments. (Scale bars: 50 μm.) (*C*) Fold-increase in *Has2*, *Ptgs2*, and *Tnfaip6* in COCs of *Fshb*$^{+/-}$ females (gray bars) and *Fshb*$^{-/-}$ females (black bars) following EGF stimulation relative to nonstimulated follicles of the same genotype. Data were analyzed using two-sample *t* test. **P* < 0.05.

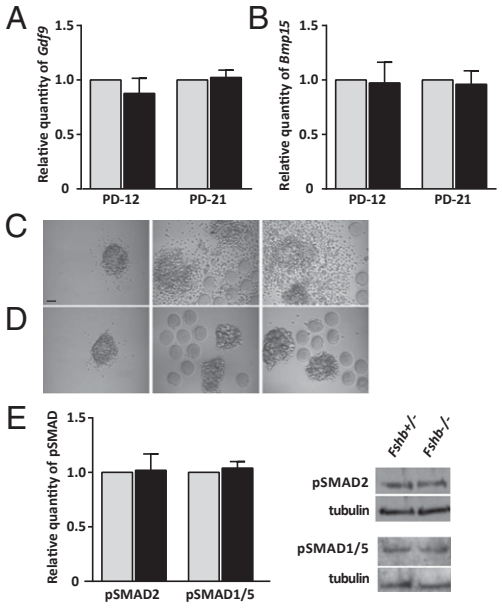

Fig. 2. *Fshb*$^{-/-}$ follicles produce OSFs required for response to EGF. (*A* and *B*) Relative quantity of *Gdf9* (*A*) and *Bmp15* (*B*) in growing (P12) and fully grown (P21/23) oocytes of *Fshb*$^{+/-}$ females (gray bars) and *Fshb*$^{-/-}$ females (black bars) (value set to 1 for each mRNA). (*C* and *D*) *Fshb*$^{+/-}$ (*C*) or *Fshb*$^{-/-}$ (*D*) OOX following incubation for 16 h in the presence of EGF with no oocytes (*Left*) or with oocytes of *Fshb*$^{+/-}$ females (*Center*) or *Fshb*$^{-/-}$ females (*Right*). Micrographs are representative of three independent experiments. (Scale bar: 50 μm.) (*E*) Basal levels of pSMAD2 and pSMAD1/5 in freshly collected COCs of *Fshb*$^{+/-}$ females (gray bars) and *Fshb*$^{-/-}$ females (black bars) at P21/23. Representative immunoblots are shown. Data in *A*, *B*, and *E* were analyzed using single-sample *t* test.

incipient atresia of the follicles (37). First, histological sections of *Fshb*$^{-/-}$ ovaries at P24 showed no evidence of increased pycnotic nuclei in *Fshb*$^{-/-}$ as compared with *Fshb*$^{+/-}$ ovaries (Fig. S2*A*). Second, GCs isolated from *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ follicles at P21/23 formed indistinguishable monolayers in tissue culture (Fig. S2*B*). Third, there was no difference between the two genotypes in the follicular expression of genes associated with atresia or apoptosis (Fig. S2*C*) (44, 45). We conclude that COCs of *Fshb*$^{-/-}$ females express lower constitutive levels of the three EGF-regulated transcripts and show an impaired functional and transcriptional response to EGF stimulation.

Oocyte-Secreted Factors Required for EGF Responses Are Functional in COCs of *Fshb*$^{-/-}$ Females. Oocyte-secreted factors (OSFs), including growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), are essential for EGF-induced *Has2*, *Ptgs2*, and *Tnfaip6* up-regulation and cumulus expansion (46–51). Their effects in GCs are transduced by activation of the Sma- and Mad-related proteins (SMAD) pathway. Therefore we tested whether the failure of *Fshb*$^{-/-}$ COCs to expand in response to EGF could be attributed to insufficient production of OSFs or their inability to activate SMAD signaling.

We first quantified *Gdf9* and *Bmp15* mRNAs and found no difference in transcript levels between *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females in either growing (P12) or fully grown (P21/23) oocytes (Fig. 2 *A* and *B*). To test directly whether the oocytes produced OSFs, we then performed a cross-fostering experiment. We removed the oocyte from complexes of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females at P21/23 and incubated the oocytectomized complexes (OOXs) with oocytes derived from the other genotype. As expected, neither *Fshb*$^{+/-}$ nor and *Fshb*$^{-/-}$ OOXs expanded in response to EGF when cultured in the absence of oocytes

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(Fig. 2 *C* and *D*, *Left*). Heterozygous OOXs underwent expansion in response to EGF when incubated with oocytes of *Fshb*$^{+/-}$ females (Fig. 2*C*, *Center*) or with oocytes of *Fshb*$^{-/-}$ females (Fig. 2*C*, *Right*). Thus, the oocytes of *Fshb*$^{-/-}$ females produce sufficient OSFs to permit cumulus expansion in response to EGF. In contrast, *Fshb*$^{-/-}$ OOXs failed to expand in response to EGF when incubated with oocytes of either *Fshb*$^{+/-}$ (Fig. 2*D*, *Center*) or *Fshb*$^{-/-}$ (Fig. 2*D*, *Right*) females. However, the levels of phosphorylated SMAD2/3 (pSMAD2/3) (a GDF9 effector) and pSMAD1/5 (a BMP15 effector) in COCs were similar in P21/23 *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females (Fig. 2*E*). This finding suggests that the *Fshb*$^{-/-}$ complexes activate SMAD signaling in response to OSFs. Together, these results indicate that the inability of the *Fshb*$^{-/-}$ complexes to expand in response to EGF is not caused by a failure of the oocytes to produce OSFs or by a failure of GCs to respond.

***Egfr* and EGFR Expression and Activity Are Reduced in the GCs of *Fshb*$^{-/-}$ Females.** In mice that bear the hypomorphic *Egfr*$^{wa/wa}$ allele, EGFR activity in GCs is reduced by about one half, and oocyte maturation and ovulation are severely impaired (22). This impairment demonstrates that a relatively modest reduction in EGFR activity can have a significant physiological effect and prompted us to examine whether the absence of cumulus expansion in response to EGF in the *Fshb*$^{-/-}$ females was associated with a reduced expression of *Egfr*. At P12, when the population of growing follicles is at the secondary preantral stage, we observed no difference in the quantity of *Egfr* mRNA in GCs of *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females (Fig. 3*A*). By P21/23, however, *Egfr* had increased significantly in GCs of early antral follicles of *Fshb*$^{+/-}$ females, but no increase was apparent in GCs of follicles of *Fshb*$^{-/-}$ females of the same age (Fig. 3*A*). Moreover, the increase in *Egfr* in the GCs of growing follicles of *Fshb*$^{+/-}$ females between P12 and P21/23 was accompanied by a quantitatively similar increase in EGFR protein (Fig. 3*B*).

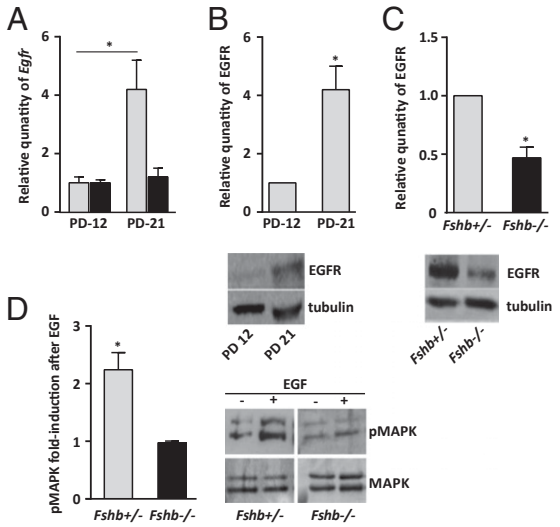

Fig. 3. FSH increases the expression and activity of EGFR in GCs. (*A*) Relative quantity of *Egfr* in GCs of *Fshb*$^{+/-}$ females (gray bars) and *Fshb*$^{-/-}$ females (black bars) at P12 and P21/23. (*B*) Relative quantity of EGFR in GCs of *Fshb*$^{+/-}$ females at P12 and P21/23. (*C*) Relative quantity of EGFR in GCs of *Fshb*$^{+/-}$ females (gray bars) and *Fshb*$^{-/-}$ females (black bars) at P21/23. (*D*) Fold-increase in pMAPK3/1 in follicles of *Fshb*$^{+/-}$ females (gray bars) and *Fshb*$^{-/-}$ females (black bars) at P21/23 following stimulation with EGF as compared with nonstimulated controls of the same genotype. Data were analyzed using a single-sample *t* test in *B*–*D* and a two-sample *t* test in *A*. **P* < 0.05. The asterisk in *D* denotes a significant difference between EGF-stimulated and nonstimulated follicles of the same genotype. Representative immunoblots are shown. (Note: The lanes shown in *C* are from the same blot but were not adjacent as shown here.)

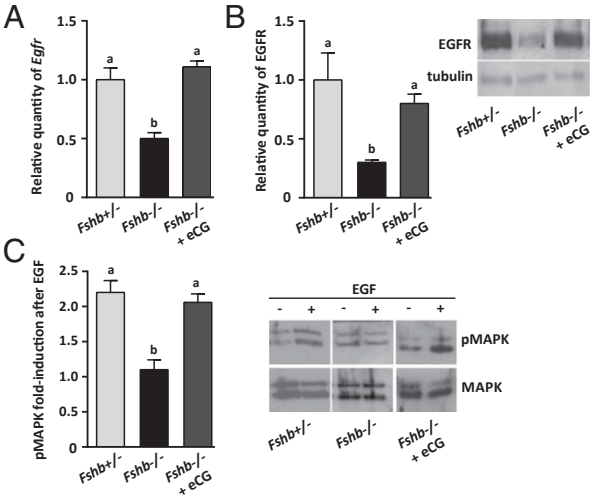

Fig. 4. Supplying FSH activity to follicles of *Fshb*$^{-/-}$ females in vivo restores EGFR expression and activity. All histograms show follicles of P18 *Fshb*$^{+/-}$ females (light gray bars), P18 *Fshb*$^{-/-}$ females (black bars), and P18 *Fshb*$^{-/-}$ females injected 48 h previously with eCG (dark gray bars). (*A*) Relative quantity of *Egfr*. (*B*) Relative quantity of EGFR. (*C*) pMAPK3/1 following EGF stimulation relative to nonstimulated controls. Data were analyzed using one-way ANOVA and Tukey HSD. Different letters above bars indicate *P* < 0.05. Representative immunoblots are shown.

However, as was consistent with the reduced quantity of *Egfr*, GCs of follicles of *Fshb*$^{-/-}$ females at P21/23 contained only about one half as much EGFR as those of the *Fshb*$^{+/-}$ females (Fig. 3*C*). Thus, *Egfr* mRNA and protein both accumulate during late follicular growth but fail to accumulate normally in follicles that grow in the absence of FSH. In contrast, the amounts of its ligand-encoding mRNAs *Areg*, *Btc*, and *Ereg* did not significantly differ between GCs of P21/23 *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females (Fig. S3).

Because GCs of *Fshb*$^{-/-}$ follicles contained detectable EGFR (although it was reduced compared with the heterozygotes), we tested whether EGFR signaling activity was impaired. MAPK3 and 1 are the principal effectors of EGFR signaling, and their activation by phosphorylation is necessary for cumulus expansion (21, 28, 39, 50). Upon the addition of EGF, MAPK3/1 phosphorylation increased significantly in *Fshb*$^{+/-}$ follicles but did not change detectably in *Fshb*$^{-/-}$ follicles (Fig. 3*D*). Thus, the reduced expression of EGFR was associated with impaired EGFR signaling in *Fshb*$^{-/-}$ follicles.

Exogenous FSH Activity Rescues EGFR Expression, Signaling, and Activity in GCs of *Fshb*$^{-/-}$ Females. To clarify further the link between FSH and EGFR expression in GCs, we then tested whether providing a brief pulse of FSH activity could restore EGFR expression and signaling in follicles that had grown in the absence of FSH. We injected equine chorionic gonadotropin (eCG), which exhibits FSH activity and is commonly used in vivo because of its long half-life (52), into *Fshb*$^{-/-}$ females at P16, After 48 h we measured *Egfr* and EGFR expression in follicles in these females and in the follicles of noninjected P18 *Fshb*$^{-/-}$ and *Fshb*$^{+/-}$ females. Both *Egfr* (Fig. 4*A*) and EGFR (Fig. 4*B*) levels were significantly higher in eCG-injected than in noninjected *Fshb*$^{-/-}$ females and were close to the amounts in *Fshb*$^{+/-}$ females. Two known FSH targets, *Cyp19a1* and *Lhcgr*, were reduced in *Fshb*$^{-/-}$ females, as previously reported (31), and were up-regulated after eCG priming (Fig. S4). In contrast with previous results (31), we found no change in the level of *Fshr* in GCs of *Fshb*$^{-/-}$ females, although it increased following eCG priming.

Coupled to the eCG-induced increase in *Egfr* expression was an increase in the phosphorylation of MAPK3/1 in response to

EGF (Fig. 4*C*). Moreover, EGF stimulation of follicles of eCG-primed *Fshb*$^{-/-}$ females caused an increase in the quantity of the three expansion-related mRNAs to levels similar to those observed in heterozygous females (Fig. 5*A*) and induced cumulus expansion in primed *Fshb*$^{-/-}$ females (Fig. 5*B*). Further confirming the link between FSH and *Egfr* expression, priming P16 *Fshb*$^{+/-}$ females with eCG similarly caused a significant increase in *Egfr* mRNA compared with nonprimed *Fshb*$^{+/-}$ females (Fig. S5*A*). The up-regulation of expansion-related mRNAs in response to EGF was not augmented in the eCG-primed *Fsh*$^{+/-}$ females (Fig. S5*B*), possibly indicating that endogenous EGFR is sufficient for maximal activation of its signaling pathway. Hence, providing exogenous FSH activity to *Fshb*$^{-/-}$ females increased follicular EGFR expression and signaling activity and restored the physiological responses to EGF.

EGF-Induced Oocyte Meiotic Maturation Is Impaired in *Fshb*$^{-/-}$ Follicles. To test whether follicular growth in the presence of FSH was more broadly required for EGFR-dependent signaling at ovulation, we examined meiotic maturation of the oocyte. EGFR ligands do not appear to initiate maturation by acting directly on the oocyte but instead reduce the transmission of inhibitory molecules to the oocyte from the EGFR-expressing GCs (9, 10, 23, 53). We isolated follicles from *Fshb*$^{+/-}$ and *Fshb*$^{-/-}$ females at P21/23, when developing oocytes of both genotypes have acquired meiotic competence (36). After incubating the follicles overnight in the presence or absence of EGF, we removed the oocytes and recorded the fraction that had undergone germinal vesicle breakdown (GVBD) indicative of maturation initiation (54). In the absence of EGF, only a small fraction of the oocytes in both *Fshb*$^{-/-}$ and *Fshb*$^{+/-}$ follicles underwent GVBD (Fig. 6). This result demonstrates that the follicles of P21/23 *Fshb*$^{-/-}$ females retain the ability to hold oocytes in meiotic arrest. The addition of EGF triggered maturation of most oocytes within *Fshb*$^{+/-}$ follicles, but this response was attenuated significantly in *Fshb*$^{-/-}$ follicles (Fig. 6). However, when *Fshb*$^{-/-}$ females were injected with eCG 48 h before follicle isolation, the fraction of oocytes that underwent GVBD in response to EGF was restored to that of the *Fshb*$^{+/-}$ females. Hence, FSH signaling promotes EGFR-dependent ovulation-associated events in the germ line as well as in the somatic compartment of the follicle.

Discussion

The EGFR signaling axis is a major effector of LH-dependent ovulatory events (9, 10, 20, 22, 25). We demonstrate here that follicles of *Fshb*$^{-/-}$ females suffer a severely impaired ability to undergo two key EGFR-regulated events, expansion of the cumulus cells and oocyte meiotic maturation, in response to EGF.

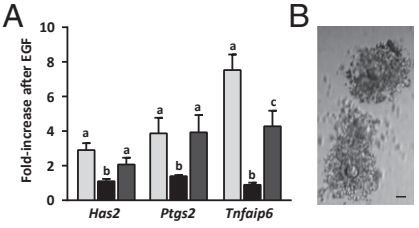

Fig. 5. Supplying FSH activity to *Fshb*$^{-/-}$ females restores the ability to undergo cumulus expansion in vitro. (*A*) Relative quantity of *Has2*, *Ptgs2*, and *Tnfaip6* in follicles of P18 *Fshb*$^{+/-}$ females (light gray bars), *Fshb*$^{-/-}$ females (black bars), and *Fshb*$^{-/-}$ females injected 48 h previously with eCG (dark gray bars) after stimulation with EGF as compared with nonstimulated follicles of the same genotype. (*B*) Cumulus expansion in COCs of eCG-injected *Fshb*$^{-/-}$ females following EGF stimulation. (Scale bar: 50 μm.) Data were analyzed using one-way ANOVA and Tukey HSD. Different letters above bars indicate $P < 0.05$.

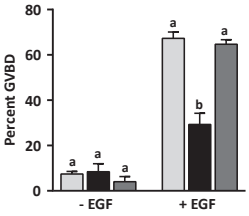

Fig. 6. Prior exposure to FSH promotes EGF-stimulated oocyte meiotic maturation. Follicles of *Fshb*$^{+/-}$ females (light gray bars), *Fshb*$^{-/-}$ females (black bars), or eCG-injected *Fshb*$^{-/-}$ females (dark gray bars) at P21/23 were incubated for 16 h in the presence or absence of EGF, and the percentage of oocytes that underwent GVBD was recorded. Data were analyzed using one-way ANOVA and Tukey HSD. Different letters above bars indicate $P < 0.05$.

In other respects, however, *Fshb*$^{-/-}$ follicles resemble wild-type follicles, at least during the time period studied here. For example, they show little change in the expression of numerous genes, apart from those known to be FSH-regulated (31), are able to support growth and the acquisition of meiotic competence, and are able to hold competent oocytes in meiotic arrest (36), which requires the production of cGMP by GCs and its transfer to the oocyte (55, 56). Our results also suggest that GCs of *Fshb*$^{-/-}$ females can activate SMAD signaling in response to OSFs. In addition, transcript levels of EGF-like peptides were normal. Thus, the inability of the *Fshb*$^{-/-}$ follicles to initiate cumulus expansion or oocyte maturation in response to EGF is unlikely to reflect a nonspecific loss of follicular function in the absence of FSH but rather indicates that FSH promotes specific events that enable the ovulatory response to EGF.

Several lines of evidence suggest that FSH promotes EGF responsiveness by increasing the expression of EGFR. First, the expression of both *Egfr* and EGFR increased during late folliculogenesis in *Fshb*$^{+/-}$ but not in *Fshb*$^{-/-}$ females. Second, an ∼50% decrease in EGFR activity in GCs, similar to that we observed in *Fshb*$^{-/-}$ females, is sufficient to impair the ovulatory response severely (22). Third, eCG injection into *Fshb*$^{-/-}$ females increased the expression of *Egfr* as well as that of known FSH targets. It also restored both cumulus expansion and oocyte maturation, which are independently regulated downstream of EGFR activation. We propose that FSH stimulates an increase in EGFR expression and activity in GCs and that this increase is essential to enable its ligands to trigger cumulus expansion, oocyte maturation, and perhaps other events of ovulation. However, notwithstanding the link between the increase in EGFR expression and response to EGF, other FSH-dependent events also may prepare GCs to respond to EGFR ligands.

Egfr expression increases during antral folliculogenesis in the hamster, and this increase is abolished when FSH is depleted by hypophysectomy (13). *Egfr* also is expressed in antral follicles of humans (19, 57). In the goat, stimulation with exogenous FSH increases the expression of *Egfr* in the cumulus GCs (58). These results are consistent with ours and suggest that FSH may play a key conserved role in regulating follicular EGFR expression in mammals. Although mutations in *Fshb* or in the FSH receptor are relatively rare in humans (33, 34, 59), women bearing these mutations would be candidates for assisted reproduction, including in vitro maturation, because of the probable absence of LHCGR on GCs. EGF is used increasingly during in vitro maturation, where it has been shown to increase its efficiency (60–62). Our results suggest that women lacking FSH activity might require a modified therapeutic intervention.

The mechanism by which FSH regulates the expression of *Egfr* remains to be established. Some effects of FSH are mediated through estradiol (63). However, although cumulus expansion was impaired in some follicles of mice lacking estrogen receptor-β, it

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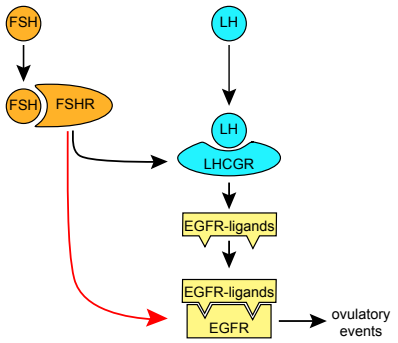

Fig. 7. Dual role of FSH in establishing the EGFR signaling axis in the ovarian follicle. FSH induces GCs to express both LHCGR, which enables them to release EGFR ligands in response to the preovulatory LH surge, and EGFR, which enables them to respond to these ligands. The two-step FSH-driven remodeling of the late-follicular environment may ensure an efficient and coordinated response to ovulatory signals.

was normal in others (64). These ovaries also showed normal induction of *Has2* in response to LH but reduced basal levels of *Ptgs2* and *Tnfaip6* (64, 65); these results suggest that EGFR signaling was partially functional in the absence of estrogen receptor-β. Alternatively, β-catenin and the transcription factor SP1, both of which are implicated in FSH-regulated gene expression (30, 66, 67), are important regulators of *Egfr* expression in other cell types (68–70).

Our results do not exclude a role for other factors in regulating *Egfr* expression, and previous work has shown that OSFs promote the expression of *Egfr* in the cumulus GCs (47–51, 71). However, the impaired *Egfr* expression we describe here is not likely to be caused by an absence of OSFs, because oocytes of $Fshb^{-/-}$ females express *Bmp15* and *Gdf9* and induce expansion of oocytectomized $Fshb^{+/-}$ complexes, indicating that they produce biologically active OSFs. It also is worth noting that, because OSFs typically generate differences between the cumulus and mural GCs (72), they might not be expected to regulate *Egfr* expression in the mural cells. FSH and OSFs may each contribute to establishing physiological levels of EGFR expression and activity.

FSH has long been known to play an indispensable role in preparing the mature follicle to respond to the preovulatory LH surge (8) by stimulating the expression of LHCGR by the mural GCs. Recent work has shown that LH induces the mural GCs to release ligands that bind to EGFR on the mural and cumulus GCs and that are the proximate trigger of LH-regulated ovulatory events (9, 10, 22, 25, 27). Our results reveal that prior exposure to FSH is required for follicles to respond to EGF and that this exposure is associated with an FSH-dependent increase in EGFR expression and activity. Thus, FSH appears to play a larger role than previously thought in the ovulatory cascade (Fig. 7): It not only promotes the expression of LHCGR on the mural GCs, enabling them to release EGFR ligands in response to LH, but also promotes the expression of EGFR itself on these cells. The FSH-dependent remodeling of the late-follicular environment may ensure an efficient and coordinated response to ovulatory signals.

Methods

Mice. All experiments were approved by the Animal Care Committee of the Royal Victoria Hospital and followed the regulations of the Canadian Council on Animal Care. Mice carrying a deletion in the gene encoding the β-subunit of FSH, *Fshb* (35), were obtained from Jackson Laboratories. $Fshb^{+/-}$ and $Fshb^{-/-}$ mice were generated by mating $Fshb^{+/-}$ females with $Fshb^{-/-}$ males, and offspring were genotyped as previously described (36). For some experiments, $Fshb^{-/-}$ females at P16 or P21/23 received an i.p. injection of 5 IU of eCG (Sigma) 48 h before sample collection.

Collection of Follicles, COCs, GCs, and Oocytes. Cells were obtained from females at different ages as described (36). Briefly, intact follicles were obtained by dissecting the ovary using fine needles, and COCs were obtained by puncturing follicles that protruded from the ovarian surface. Mechanical or enzymatic methods were used to obtain purified GCs and oocytes from follicles. To examine cumulus expansion, COCs were incubated for 16 h in serum-free Eagle's minimum essential medium (MEM; Gibco) in the presence of 10 ng/mL EGF (Becton Dickinson) at 37 °C in a humidified atmosphere of 5% CO_2 in air. To assess response to EGF, follicles or COCs were incubated as above for 10 min and then were processed. To assess oocyte maturation, follicles were incubated with EGF for 16 h, after which the oocytes were removed from the follicles and examined using light microscopy; the fraction that had undergone GVBD was recorded.

Oocytectomy. Oocytectomy was performed using fine glass needles. OOX complexes were cocultured with oocytes (five oocytes per complex) in 25-μL drops of MEM under paraffin oil under the conditions described above. After 16 h, the expansion of the COCs was assessed qualitatively.

RNA Purification and Quantitative PCR. RNA was purified, cDNA was generated, and quantitative PCR analysis performed as described (73). Primer sequences are given in Table S1. For each primer pair, a standard curve was generated using serial dilutions of cDNA prepared from ovarian RNA and used to determine the efficiency of amplification. Melting-curve analysis was used to verify product specificity. Data were analyzed using software provided by the manufacturer (Montréal Biotech). Relative quantities of amplified product were calculated according to $2^{-\Delta\Delta CT}$ method, using *Actb* (actin) for normalization.

Immunoblotting. Immunoblotting was performed as described (73). For EGFR and the pSMADs, each sample was loaded into one lane of a gel and was used to analyze both the protein of interest and tubulin. For MAPK3/1 analysis, each sample was suspended in loading buffer, heat-denatured, and then divided into two aliquots, one of which was used to detect total MAPK3/1 and the other to detect pMAPK3/1. Antibodies used were EGFR (sc-03; Santa Cruz Biotechnology); tubulin (T8203; Sigma); pSMAD2 (Ser465/467) (3108; Cell Signaling Technology); pSMAD1/5 (Ser463/465) (9516; Cell Signaling); MAPK3/1 (sc-94; Santa Cruz Biotechnology); and pMAPK3/1 (9106; Cell Signaling). Signals were quantified using Image J software (National Institutes of Health) and were normalized to the respective control (EGFR and pSMADs to tubulin; pMAPK3/1 to total MAPK3/1).

Statistical Analysis. Statistical analysis was performed using GraphPad Prism 6.0. A single-sample *t* test, a two-sample *t* test, or one-way ANOVA followed by a Tukey honestly significant difference (HSD) test was used, depending on the experiment. $P < 0.05$ was considered significant. All values represent the mean ± SEM of three or more independent experiments.

ACKNOWLEDGMENTS. This work was supported by a grant from the Canadian Institutes of Health Research (CIHR) (to H.J.C.; by the Royal Victoria Hospital Foundation). S.E.-H. received support from the Centre for the Study of Reproduction at McGill and the CIHR Training Program in Reproduction, Early Development, and the Impact on Health. I.D. was supported by les Fonds de la Recherche Scientifique (Belgium).

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