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Binding of FGF2 to FGFR2 in an autocrine mode in trophectoderm cells is indispensable for mouse blastocyst formation through PKC-p38 pathway

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Fibroblast growth factors (FGF1, FGF2 and FGF4) and fibroblast growth factor receptors (FGFR1, FGFR2, FGFR3 and FGFR4) have been reported to be expressed in preimplantation embryos and be required for their development. However, the functions of these molecules in trophectoderm cells (TEs) that lead to the formation of the blastocyst as well as the underlying mechanism have not been elucidated. The present study has demonstrated for the first time that endogenous FGF2 secreted by TEs can regulate protein expression and distribution in TEs via the FGFR2-mediated activation of PKC and p38, which are important for the development of expanded blastocysts. This finding provides the first explanation for the long-observed phenomenon that only high concentrations of exogenous FGFs have effects on embryonic development, but *in vivo* the amount of endogenous FGFs are trace. Besides, the present results suggest that FGF2/FGFR2 may act in an autocrine fashion and activate the downstream PKC/p38 pathway in TEs during expanded blastocyst formation.

Introduction

After fertilization, mammalian preimplantation embryos undergo cleavage and differentiation to form a hollow-shaped embryo called the blastocyst, which is crucial for all stages of subsequent embryonic development.[1] At the end of the blastocyst stage, the embryo establishes 3 spatially and molecularly distinct cell lineages [the trophoblast, the trophectoderm (TE) and the inner cell mass (ICM)]. The TE forms a polarized epithelial cell layer that encloses a fluid-filled cavity termed the blastocoel. The ICM, an undifferentiated mass of cells, is composed of 2 distinct layers: an inner population of epiblast (EPI) cells and a superficial layer of primitive endoderm (PrE) adjacent to the blastocyst cavity. The EPI will give rise to the embryo proper, the PrE forms some of the extraembryonic membranes, and the TE contributes to the placenta.[2,3] Functional tight junctions form a seal between the cells of the TE, which is essential for fluid accumulation and formation of the blastocyst cavity.[4,5] The Na-K ATPase is also a critical mediator of blastocyst formation as it establishes a trans-trophectoderm ionic gradient that directs fluid movement across the epithelium of the TE.[4,6,7] Aquaporins (AQP3 and AQP9) in the TE membrane facilitate the movement of fluids across the TE from the 'outside' to the 'inside' of the blastocyst cavity, along the ionic gradient established by the Na-K ATPase localized on the baso-lateral membrane.[4,8] After segregation of the early lineages is complete, the blastocyst emerges from the zona pellucida to invade the maternal uterine endometrium, where it implants.[9,10,11]

Generally, the development of the blastocyst is guided by highly organized interactions of multiple growth factors.[12,13,14] Growth factors perform their roles in embryonic development and blastocyst function in an autocrine/paracrine manner.[15] Fibroblast growth factors (FGFs) encompass a large family of autocrine, paracrine, endocrine, and intracrine factors. The FGFs affect target cells via the activation of cell-surface tyrosine kinase receptors that are encoded by 4 genes in mammals (designated *fgf1/2/3/4*).[16,17] Evidence has shown that FGFs play important roles in the regulation of pluripotency and lineage segregation in both the early mouse embryo and pluripotent mammalian stem cells.[18] In addition to the indispensability of correct lineage segregation, FGFs activated FGF signaling pathways, characterized by the expression of adherens junctions (AJ), tight junctions (TJ), ion channels and AQPs, are essential for fluid accumulation and formation of the blastocyst cavity in the development of a proper blastocyst.[4,18,19]

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We found that some human embryos could develop to expanded blastocysts without ICM in our *in vitro* fertilization (IVF) laboratory. This phenomenon may infer that blastocyst formation is independent of ICM. Therefore, the FGFs that have effects on blastocyst formation are from TE or outside of embryos. Rappolee's research showed that FGF3 mRNA was not detected in mouse preimplantation embryos.[20] Besides, some researchers observed that the expression of FGF4 polypeptide as well as mRNA was limited to the ICM cells in the blastocyst.[17,20] One of central interest is FGF2, which is produced by luminal and glandular epithelium and is detectable in the uterine lumen throughout early pregnancy in animals.[21,22] But previous studies found FGF2 improved blastocyst formation during bovine embryo culture *in vitro* unless large amounts of recombinant protein were provided (500–1000 ng/ml.[23-25] The previous research found that FGF2 performs its function in an autocrine manner, which is physiologically significant for FGF2 to bind its high-affinity receptor.[26,27] So we explored the possibility that FGF2 might bind to FGFRs in TE in an autocrine model to modulate blastocyst formation in early stage embryos.

Results

Endogenous FGF2 from TEs is required for expanded blastocyst formation

In this study, the micromanipulation system was used to microinject RNA into the cavity gap between the zona pellucida and the trophectoderm to transfect siRNA into trophectoderm (**Fig. 1A**) and microinject antibodies into blastocoels to eliminate the specific growth factors that may be from ICM (**Fig. 1B**) (See details in Materials and Methods). As shown in **Figure 1C**, the formation of murine expanded blastocysts was apparently not affected when exogenous FGF1, FGF2 or FGF4 were eliminated in the medium, respectively, by the corresponding antibodies. In addition, no significant differences were observed between the control and test embryos when endogenous FGF1, FGF2 or FGF4, which may originate from the ICM, was eliminated in the blastocoels by microinjection of the corresponding antibodies (**Fig. 1D**). The FGF1, FGF2 or FGF4 knockdown (siRNA*fgf1*, siRNA*fgf2* or siRNA*fgf4* transfection) was performed to confirm the effect of the 3 FGFs secreted by TEs on expanded blastocyst formation. Quantitative real-time PCR showed that the expression of FGF1, FGF2 and FGF4 sharply decreased 80–90% in the TEs after transfection with siRNA (**Fig. 1E–G**). We found that the knockdown of FGF1 and FGF4 in the TEs of early blastocysts had no effect on the formation of expanded blastocysts (**Fig. 1H**). However, the rate of blastocyst expansion significantly decreased after FGF2 knockdown (**Fig. 1H**). Similarly, FGF2 knockdown also suppressed expanded blastocyst development (**Fig. 1I**). When exogenous FGF2 (1000 ng/ml) was added to the medium and maintained for 12 h, the inhibition of blastocyst formation was reversed (**Fig. 1I**). However, this reversal was significantly affected by the addition of 200 ng/ml of FGF2 antibody to the medium (**Fig. 1I**). These results indicate that endogenous FGF2 from TEs is important for expanded blastocyst formation.

FGFR2 in TEs is required for expanded blastocyst formation

The FGFR1, FGFR2, FGFR3 or FGFR4 knockdown (siRNA*fgfr1*, siRNA*fgfr2*, siRNA*fgfr3* or siRNA*fgfr4* transfection) was performed to examine the function of each of the 4 FGFRs in expanded blastocyst formation. Quantitative real-time PCR showed that FGFR1, FGFR2, FGFR3 and FGFR4 expression also sharply decreased by 80-90% of the control in TEs after the transfection with the corresponding siRNA (**Fig. 2A–D**). Twenty-four hours after the knockdown of the FGFRs in the TEs of early blastocysts, we found that the knockdown of FGFR1, FGFR3 or FGFR4 had no effect on the development of expanded blastocysts (**Fig. 2E**). However, the rate of expanded blastocyst formation significantly decreased after FGFR2 knockdown (**Fig. 2E**). In order to investigate whether FGF2 knockdown affect FGFR2 expression or the *vice versa*, we examined the mRNA levels of FGFR2 and/or FGF2 when they were knockdown respectively. The results showed that FGF2 knockdown suppressed FGF2 expression and up regulated FGFR2 expression meanwhile (**Fig. 2F**). On the other side, FGFR2 knockdown suppressed FGFR2 expression and increased FGF2 expression as well. (**Fig. 2G**). In addition, confocal analysis of double-immunofluorescence staining showed that the co-expression of FGF2 and FGFR2 was detected in the cell membrane and cytoplasm of the TEs (**Fig. 2H**). The results show that FGF2 acted upon FGFR2 in an autocrine manner in the regulation of expanded blastocyst development.

Expression of E-cadherin, ZO-1, Na-K ATPase, ENaCα, AQP3 and AQP9 mRNAs in TEs after the knockdown of FGF2/FGFR2

Quantitative real-time PCR showed that FGF2 or FGFR2 knockdown sharply decreased the mRNA expression of E-cadherin, Zonula occluden-1 (ZO-1), Na-K ATPase, Epithelial sodium channel α (ENaCα), AQP3 and AQP9 in the TEs after transfection with their respective siRNAs.(**Fig. 3A–F**)

The alteration of E-cadherin, ZO-1, Na-K ATPase, ENaCα, AQP3 and AQP9 protein expression in TEs after knockdown of FGF2 or FGFR2

The formation and distribution of E-cadherin and ZO-1 in TE was observed by immunofluorescence staining after the knockdown of FGF2 or FGFR2. As shown in **Figure 4A**, E-cadherin was not expressed in some regions of the TE after FGF2 or FGFR2 knockdown. At the same time, the control embryos demonstrated continuous and well-organized ZO-1 expression only at the cell junction, whereas the FGF2 or FGFR2 knockdown embryos showed disrupted expression of ZO-1 at the cell junction (arrow head) and diffuse localization in the cytoplasm (**Fig. 4B**). In addition, confocal analysis of immunofluorescence staining showed that the expression of Na-K ATPase on the inner side of the TEs and the expression of ENaCα on the outer side of the TEs were both significantly reduced in the TE after knockdown of FGF2 or FGFR2 (**Fig. 4C and D**). Moreover, confocal analysis of double-immunofluorescence staining showed that both FGF2 knockdown and FGFR2 knockdown significantly reduced the expression of AQP3 and AQP9 in the TE (**Fig. 4E**).

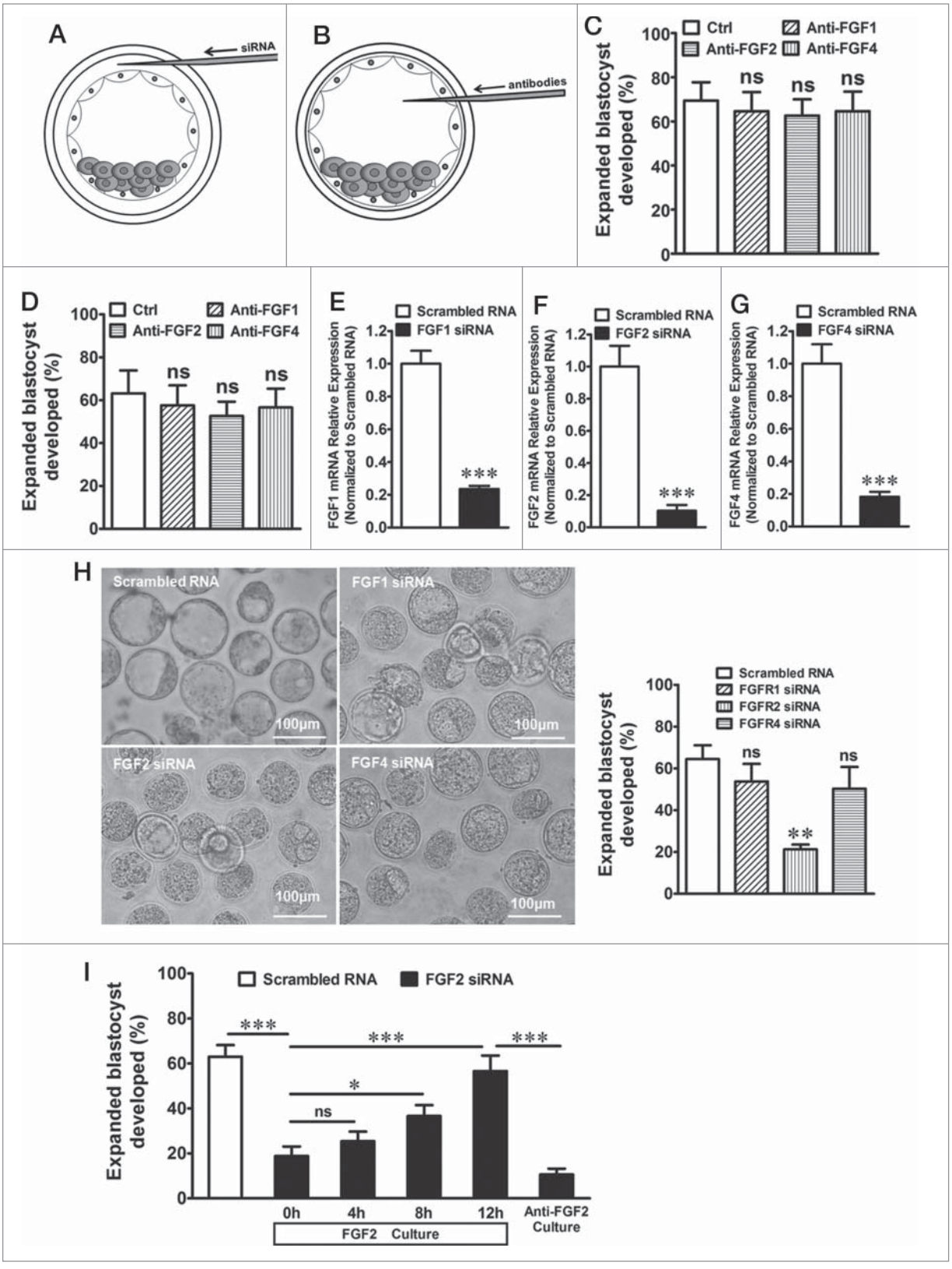

Figure 1. For figure legend, see page 3321.

The down regulation of E-cadherin, ZO-1, Na-K ATPase, ENaCα, AQP3 and AQP9 proteins by FGF2 or FGFR2 knockdown was confirmed by western blots (**Fig. 4F**). Taken together, these results suggest that FGF2/FGFR2 interaction is involved in mediating the expression and localization of cavitation formation markers in TE in the development of expanded blastocysts.

FGF2 binding to FGFR2 in TEs is required for the regulation of the formation of expanded blastocysts by activation of the PKC-p38 cascade

We have established that FGF2 binding to FGFR2 in the TEs is a key event in the regulation of expanded blastocyst formation. Next, we wanted to explore the signaling mechanism involved in this regulation. The expression of certain protein markers in the development of expanded blastocysts has been shown to be mediated by a PKC-dependent pathway.[28] The addition of the PKC inhibitor Gö6983 to the culture medium of embryos drastically inhibited the transformation of early blastocysts to expanded blastocysts compared with the DMSO-treated vehicle control (**Fig. 5A**). The stimulation of PKC-activated p38 mitogen-activated protein kinase (MAPK) or ERK1/2 MAPK, is linked to the induction of blastocyst development.[4,29,30] To test this link, we studied the phosphorylated (p-) of p38 or ERK1/2 and their transport between the cytoplasm and nucleus using an immunofluorescence confocal technique. As shown in **Figure 5B**, p-p38 translocated to the nucleus of TEs in blastocysts that were transfected with scrambled (control) RNA. The p-p38 and p-ERK1/2 levels were examined by protein gel blots in TEs in response to FGF2 or FGFR2 knockdown (**Fig. 5C**). This translocation and phosphorylation of p38 was inhibited by the knockdown of either FGF2 or FGFR2 or by treatment with the p38 MAPK inhibitor SB202190 (50 μM) (**Fig. 5B–E**). However, the translocation and phosphorylation of ERK1/2 was not affected obviously by the above treatments (**Fig. 5B and C**). These results suggest that the translocation or activation of p38 in the TEs of mouse blastocysts depends on both FGF2 and FGFR2, which are known to activate the PKC/p38 pathway. If the activation of PKC or p38 is blocked by the inhibitors Gö6983 or SB202190, respectively, the downregulation of E-cadherin, ZO-1, Na-K ATPase, ENaCα, AQP3 and AQP9 mRNA is observed in the TEs of blastocysts in the presence of FGF2, as demonstrated by quantitative real-time PCR (**Fig. 5F**). These results indicate that the FGF2/FGFR2-dependent expression of the above proteins, which are important to the development of the expanded blastocyst, is mediated by the PKC-dependent activation of p38 MAPK.

Discussion

FGFs (FGF1, FGF2 and FGF4) and FGFRs (FGFR1, FGFR2, FGFR3 and FGFR4) have been reported to be expressed in preimplantation embryos and be required for their development.[18,20,31-35] However, the functions of these molecules in TEs that lead to the formation of the blastocyst as well as the underlying mechanism have not been elucidated. The present study has demonstrated for the first time that endogenous FGF2 secreted by TEs can regulate protein expression and distribution in TEs via the FGFR2-mediated activation of PKC and p38, which are important for the development of expanded blastocysts (**Fig. 6**). This finding provides the first explanation for the long-observed phenomenon that only high concentrations of FGFs have effects on embryonic development and suggests that FGF2 acts as an endogenous factor for the regulation of TE function during expanded blastocyst development.[23]

The results of the present study show that the development of expanded blastocysts was not affected when the activities of exogenous FGF1, FGF2 or FGF4 in the medium were eliminated by the corresponding antibodies. The same phenomenon was also observed when the activities of FGF1, FGF2 or FGF4 were eliminated by the microinjection of the corresponding antibodies into the blastocoels. This finding suggests that neither exogenous FGFs from the oviduct and endometrium nor endogenous FGFs from the ICM affect the development of expanded blastocysts under normal physiological conditions. These results may provide a useful explanation of why an abnormal ICM was found in some blastocysts that were cultured *in vitro* while the TE developed normally. FGF1, FGF2 and FGF4 have been reported to be expressed in preimplantation embryos.[20,31,32] Here, we found that the formation of expanded blastocysts was arrested only after

Figure 1 (See previous page). The microinjection technique used in the study and the effect of FGFs on expanded blastocyst formation. (**A**) Working models for RNA microinjection of the embryo. After the blastocysts were dehydrated in drops of 1 M mannitol, each embryo was injected with an RNA solution into the cavity gap between the zona pellucida and trophectoderm using the micromanipulation system. (**B**) Working models for antibody microinjection of the embryo. Blastocoels of the blastocyst were injected with antibodies against FGFs using the micromanipulation system. Negative control blastocysts were injected with the same volume of normal saline that was substituted for the antibodies. (**C**) Formation of expanded blastocysts when exogenous FGF1, FGF2 or FGF4 were eliminated in the medium by their respective antibodies. ns indicates $P > 0.05$ (by one-way ANOVA, n = 5) compared with the control. (**D**) Formation of expanded blastocysts when endogenous FGF1, FGF2 or FGF4 was eliminated in the blastocoels by the blastocyst microinjection of the respective antibodies. ns indicates $P > 0.05$ (by one-way ANOVA, n = 5) compared with the control. (**E**) Expression of FGF1 mRNA in TEs after transfection with FGF1 siRNA. *** indicates $P < 0.001$ (by unpaired *t*-test, n = 4) compared with the control (transfection with scrambled RNA). (**F**) Expression of FGF2 mRNA in TEs after transfection with FGF2 siRNA. *** indicates $P < 0.001$ (by unpaired *t*-test, n = 4) compared with the control (transfection with scrambled RNA). (**G**) Expression of FGF4 mRNA in TEs after transfection with FGF4 siRNA. *** indicates $P < 0.001$ (by unpaired *t*-test, n = 4) compared with the control (transfection with scrambled RNA). (**H**) Left, effect of the knockdown of FGF1, FGF2 or FGF4 in TEs on the formation of expanded blastocysts. Scale bar: 100 μm. Right, summary of the results. ns indicates $P > 0.05$ and ** indicates $P < 0.01$ (by one-way ANOVA, n = 4) compared with the control (transfection with scrambled RNA). (**I**) Effect of exogenous FGF2 on expanded blastocyst formation after the knockdown of FGF2 in TEs. ns indicates $P > 0.05$, * indicates $P < 0.05$, and *** indicates $P < 0.001$ (by one-way ANOVA, n = 4) compared with the corresponding controls.

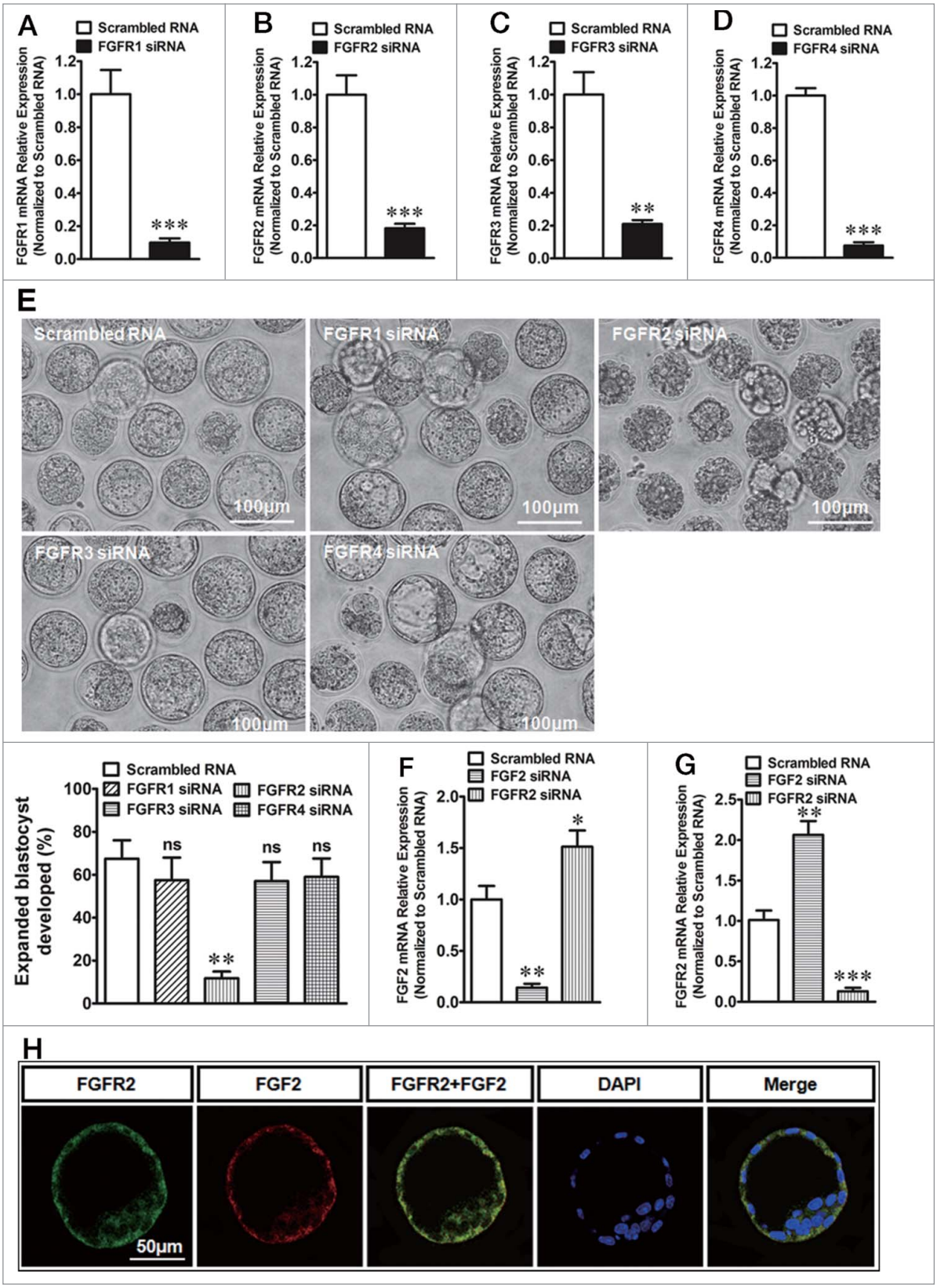

Figure 2. For figure legend, see page 3323.

the knockdown of FGF2 in TEs *in vitro*, which suggests that FGF2 is required in TEs for the development of expanded blastocysts. The knockdown of FGF2 in TEs affected the growth of expanded blastocysts, which could be reversed when the embryos were cultured with 1000 ng/ml exogenous FGF2 for 12 h. However, this rescue was completely abolished when the activation of FGF2 was eliminated by the application of an FGF2 antibody. This finding suggests that exogenous FGF2 can also perform its function in the formation of expanded blastocysts after the knockdown of FGF2 in TEs.

Some research has suggested that the 4 FGFRs, including FGFR1, FGFR2, FGFR3 and FGFR4, are all expressed in preimplantation embryos.[31,32] The development of expanded blastocysts was not affected when the expression of FGFR1, FGFR3 or FGFR4 was suppressed by transfection with specific siRNAs. In addition, using the double-immunofluorescence confocal technique, we found that FGF2 and FGFR2 were co-expressed in the membrane and the cytoplasm of TEs. We also found that the development of expanded blastocysts was inhibited by the addition of FGF2 antibodies to the culture medium, which presumably blocks the extracellular ligand-receptor interaction. This finding implies that FGF2 acts in an autocrine manner when it binds to FGFR2 in TEs to promote the formation of expanded blastocysts. Our results are consistent with the previous report that FGF2 performs its function in an autocrine manner.[26] FGF2 was previously demonstrated to bind to the cell membrane via heparin or heparin-like molecules, which is physiologically significant because heparin is required for the binding of FGF2 to its high-affinity cell surface receptor.[27,36]

The ion channels (including Na-K ATPase and ENaC), tight junctions (including E-cadherin and ZO-1) and AQPs (including AQP3 and AQP9) contribute directly to the mechanism that enables the TEs to regulate cavitation and blastocyst formation.[6,37] Functional adheren junctions and tight junctions form a seal between the cells of the TE, which is essential for fluid accumulation and the formation of the blastocyst cavity during the process of expanded blastocyst formation.[5,38] In this study, we found that the knockdown of either FGF2 or FGFR2 reduced the expression of E-cadherin and ZO-1 and perturbed their distribution within the TE as determined by quantitative real-time PCR and immunofluorescence staining. The Na-K ATPase plays an important role in the establishment of a trans-trophectoderm ionic gradient that directs fluid movement across the TE epithelium during expanded blastocyst formation.[7,39] During the process of Na^+ transport, Na^+ crosses the apical membrane via ENaC into TEs and is extruded into the blastocoels by Na-K ATPase on the basal side of the membrane.[7,39,40] Thus, the activities of ENaCα and Na-K ATPase must be highly coordinated to accommodate variations in Na^+ transport during expanded blastocyst formation. In this study, the knockdown of FGF2 or FGFR2 in the TE was accompanied by the down-regulation of the expression of Na-K ATPase and ENaCα in TEs. These outcomes implicate FGF2 and FGFR2 as mediators that coordinate the function of proteins that are involved in the establishment of the trans-TE ionic gradient during expanded blastocyst formation. Additionally, fluid movement across the TE is facilitated by the presence of AQP3 and AQP9 in the TE membrane.[8] AQP9 is localized to the apical surface of the TE, whereas AQP 3 is localized to the baso-lateral surface of the TE. Together, they facilitate fluid movement across TEs into the blastocyst cavity along the ionic gradient established by the Na-K ATPase.[8] We have demonstrated that the knockdown of FGF2 or FGFR2 in TEs resulted in the downregulation of AQP3 and AQP9 mRNA levels, the reduction of AQP3 and AQP9 localization and the suppression of expanded blastocyst formation.

We have established that the FGF2/FGFR2 interaction plays an important role in the regulation of E-cadherin, ZO-1, Na-K ATPase, ENaCα, AQP3 and AQP9 expression and in the localization in the development of expanded blastocysts. We also explored the signaling mechanism involved in this process. The activation of FGFR2 has been shown to regulate cell differentiation via PKC.[41,42] Interestingly, the activation of PKC has been shown to be involved in the regulation of blastocoel formation during mouse preimplantation development,[28,43] which is in accordance with our observation that the inhibition of PKC significantly suppressed the formation of expanded blastocysts. More importantly, p38 regulates cavitation and the function of tight junctions, which affects the expanded formation of the blastocyst.[4] As PKC is an activator of p38/ERK1/2,[19,44,45] the effect of the PKC-mediated pathway on the formation of expanded blastocysts may be mediated by the p38/ERK1/2 pathway. To test this relationship, we studied the inhibition of p38 by examining the shuttling of p-p38 between the cytoplasm and nucleus using double-immunofluorescence confocal techniques. As shown in **Figure 5B**, the translocation of p-p38 to the nucleus in TEs was inhibited by the knockdown of FGF2 or FGFR2. In

Figure 2 (See previous page). The effect of FGFRs in TEs on expanded blastocyst formation. (**A**) Expression of FGFR1 mRNA in TEs after transfection with FGFR1 siRNA. *** indicates $P < 0.001$ (by unpaired *t*-test, n = 4) compared with the control (transfection with scrambled RNA). (**B**) Expression of FGFR2 mRNA in TEs after transfection with FGFR2 siRNA. *** indicates $P < 0.001$ (by unpaired *t*-test, n = 4) compared with the control (transfection with scrambled RNA). (**C**) Expression of FGFR3 mRNA in TEs after transfection with FGFR3 siRNA. ** indicates $P < 0.01$ (by unpaired *t*-test, n = 4) compared with the control (transfection with scrambled RNA). (**D**) Expression of FGFR4 mRNA in TEs after transfection with FGFR4 siRNA. *** indicates $P < 0.001$ (by unpaired *t*-test, n = 4) compared with the control (transfection with scrambled RNA). (**E**) Up, effect of FGFR1, FGFR2, FGFR3 or FGFR4 knockdown in TEs on the formation of expanded blastocysts. Scale bar: 100 μm. Low-right, summary of the results. ns indicates $P > 0.05$ and ** indicates $P < 0.01$ (by one-way ANOVA, n = 4) compared with the control (transfection with scrambled RNA). (**F**) Expression of FGF2 mRNA in TEs after transfection with FGF2 or FGFR2 siRNA. * indicates $P < 0.05$ and ** indicates $P < 0.01$ (by one-way ANOVA, n = 4) compared with the control (transfection with scrambled RNA). (**G**) Expression of FGFR2 mRNA in TEs after transfection with FGF2 or FGFR2 siRNA. ** indicates $P < 0.01$ and *** indicates $P < 0.001$ (by one-way ANOVA, n = 4) compared with the control (transfection with scrambled RNA). (**H**) Double-immunofluorescence by confocal microscopy that shows the co-expression of FGF2 and FGFR2 in TEs. Total 19 normal blastocysts were used. And all the 19 blastocysts were labeled successfully (19/19 blastocysts). Scale bar: 50 μm.

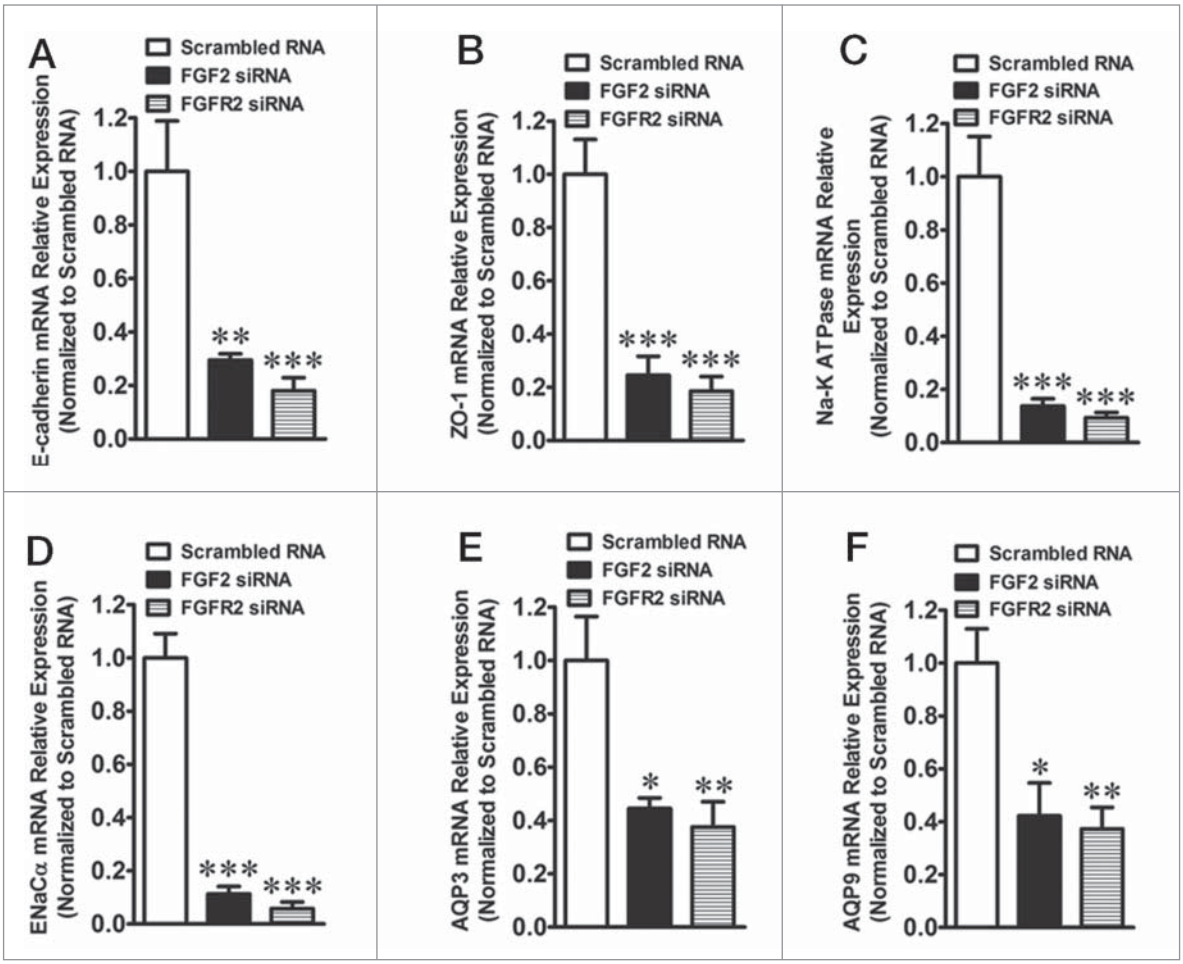

Figure 3. Quantitative real-time PCR results show the effect of the knockdown of FGF2 or FGFR2 on the expression of protein markers of blastocyst in TEs. (**A**) Expression of E-cadherin mRNA in TEs after transfection with FGF2 or FGFR2 siRNA. ** indicates $P < 0.01$ and *** indicates $P < 0.001$ (by one-way ANOVA, n = 4) compared with the control (transfection with scrambled RNA). (**B**) Expression of ZO-1 mRNA in TEs after transfection with FGF2 or FGFR2 siRNA. *** indicates $P < 0.001$ (by one-way ANOVA, n = 4) compared with the control (transfection with scrambled RNA). (**C**) Expression of Na-K ATPase mRNA in TEs after transfection with FGF2 or FGFR2 siRNA. *** indicates $P < 0.001$ (by one-way ANOVA, n = 4) compared with the control (transfection with scrambled RNA). (**D**) Expression of ENaCα mRNA in TEs after transfection with FGF2 or FGFR2 siRNA. *** indicates $P < 0.001$ (by one-way ANOVA, n = 4) compared with the control (transfection with scrambled RNA). (**E**) Expression of AQP3 mRNA in TEs after transfection with FGF2 or FGFR2 siRNA. * indicates $P < 0.05$ and ** indicates $P < 0.01$ (by one-way ANOVA, n = 4) compared with the control (transfection with scrambled RNA). (**F**) Expression of AQP9 mRNA in TEs after transfection with FGF2 or FGFR2 siRNA. * indicates $P < 0.05$ and ** indicates $P < 0.01$ (by one-way ANOVA, n = 4) compared with the control (transfection with scrambled RNA).

contrast, the translocation of p-ERK1/2 remained unchanged. Blocking the activation of p38 by an inhibitor, SB202190, also resulted in the inhibition of its translocation within TEs. These results suggest that the translocation or activation of p38 in mouse TEs depends on FGF2/FGFR2, which are known to activate the PKC/p38 pathway. The inhibition of PKC or the activation of p38 also resulted in the down-regulation of the expression of E-cadherin, ZO-1, Na-K ATPase, ENaCα, AQP3 and AQP9 in the TE. These results indicate that the FGF2/FGFR2-dependent formation of expanded blastocysts is mediated by the PKC/p38-dependent pathway.

The present study has demonstrated a critical role for FGF2/FGFR2 in the mediation of expanded blastocyst formation. The expression and localization of E-cadherin, ZO-1, Na-K ATPase, ENaCα, AQP3 and AQP9 were altered when the expression of FGF2/FGFR2 changed in the TEs of blastocysts. In addition, the autocrine interaction of FGF2/FGFR2 and the activation of the downstream PKC/p38 pathway in TEs suggest that the progression of expanded blastocyst formation is a self-regulatory process under physiological conditions.

Materials and Methods

Animals

The care and use procedures for the ICR mice were performed in accordance with the Institutional Guide for Laboratory Animals established by the Animal Care and Use Committee (ACUC). All animal handling protocols were approved by the ACUC of the School of Medicine of Zhejiang University. The

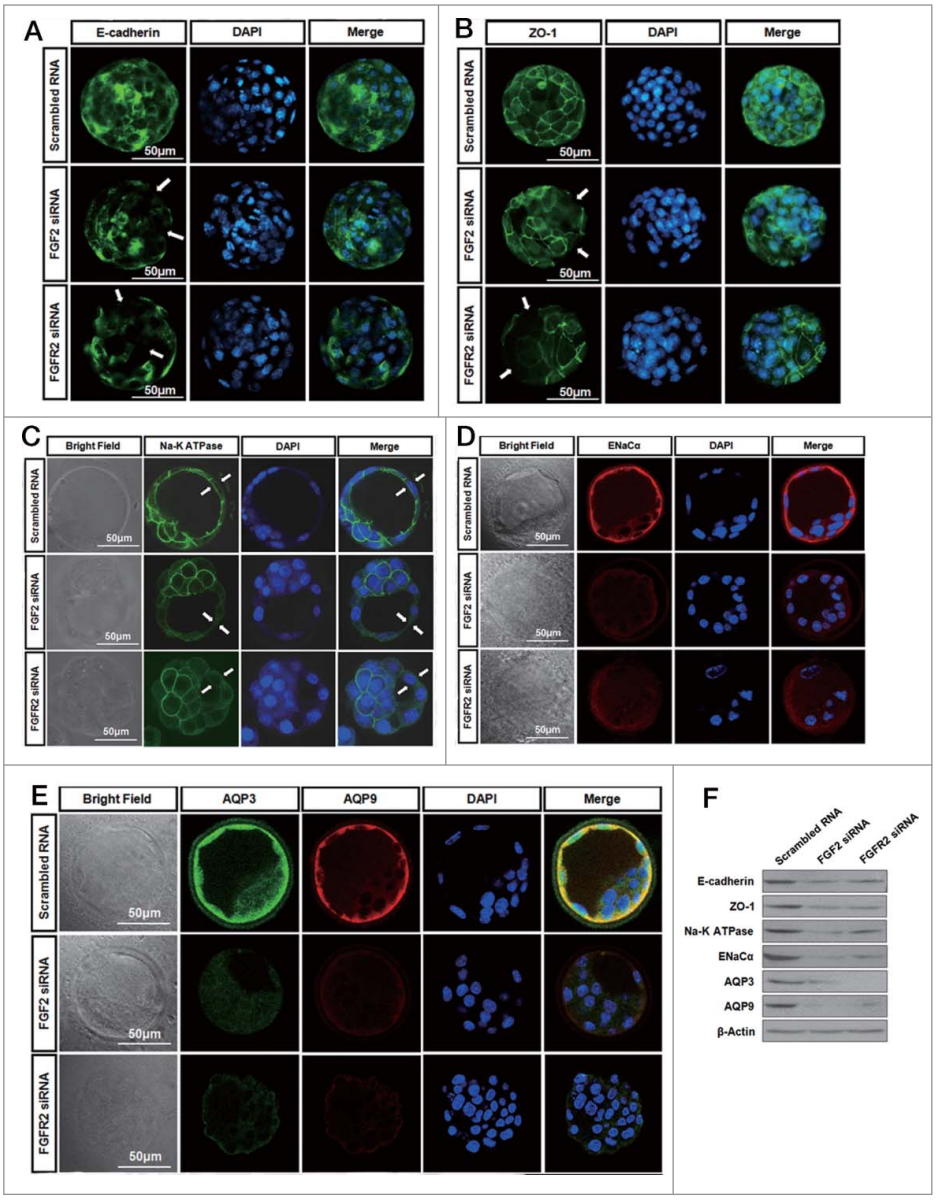

Figure 4. Immunofluorescence microscopy illustrates the effect of the knockdown of FGF2 or FGFR2 on the distribution or expression of protein markers of blastocyst in the TEs. (**A**) Effect of the knockdown of FGF2 or FGFR2 on the distribution of E-cadherin within the TEs by immunofluorescence. Phenotype as labeled by scrambled RNA (13/15 embryos). Phenotype as labeled by FGF2 siRNA (11/14 embryos). Phenotype as labeled by FGFR2 siRNA (13/17 embryos). Scale bar: 50 μm. (**B**) Effect of the knockdown of FGF2 or FGFR2 on the distribution of ZO-1 within the TEs by immunofluorescence. Phenotype as labeled by scrambled RNA (12/14 embryos). Phenotype as labeled by FGF2 siRNA (12/16 embryos). Phenotype as labeled by FGFR2 siRNA (17/21 embryos). Scale bar: 50 μm. (**C**) Effect of the knockdown of FGF2 or FGFR2 on the expression and localization of Na-K ATPase in the TEs by confocal immunofluorescence. Phenotype as labeled by scrambled RNA (21/23 embryos). Phenotype as labeled by FGF2 siRNA (16/19 embryos). Phenotype as labeled by FGFR2 siRNA (16/20 embryos). Scale bar: 50 μm. (**D**) Effect of the knockdown of FGF2 or FGFR2 on the expression of ENaCα in the TE by confocal immunofluorescence. Phenotype as labeled by scrambled RNA (22/25 embryos). Phenotype as labeled by FGF2 siRNA (15/20 embryos). Phenotype as labeled by FGFR2 siRNA (14/18 embryos). Scale bar: 50 μm. (**E**) Effect of the knockdown of FGF2 or FGFR2 on the expression of AQP3 and AQP9 in the TE by confocal double-immunofluorescence. Phenotype as labeled by scrambled RNA (26/30 embryos). Phenotype as labeled by FGF2 siRNA (19/25 embryos). Phenotype as labeled by FGFR2 siRNA (23/28 embryos). Scale bar: 50 μm. (**F**) Western blots showing E-cadherin, ZO-1, Na-K ATPase, ENaCα, AQP3 and AQP9 in TEs after FGF2 or FGFR2 knockdown.

mice were housed under a 12/12-h light/dark cycle at 25 ± 0.5°C and 50 to 60% humidity and were fed *ad libitum* with a standard diet and water.

Induction of ovulation and collection of mouse embryos

Female ICR mice (7–8 weeks old) were superovulated via intraperitoneal injections of 10 IU of pregnant mare's serum gonadotrophin (PMSG; Hangzhou Animal Pharmaceutical Factory, Hangzhou, Zhejiang, China), followed by 10 IU of human chorionic gonadotrophin (hCG; Hangzhou Animal Pharmaceutical Factory) after 48 h; they were then mated with ICR males (10-11 weeks old). Successful mating was confirmed the following morning by the appearance of a vaginal plug. The early-stage blastocysts were obtained after the sacrifice of the mice at 89 h after the hCG injection. Blastocysts were collected by flushing the uterus with human tubal fluid HEPES (HTF-HEPES, Irvine Scientific, Irvine, CA, USA) medium.

Culture and treatment of embryos

The collected embryos were transferred to human tubal fluid (HTF; Irvine Scientific) medium, cultured in 5% CO_2 at 37°C and then prepared for further experiments. The embryos were washed 3 to 4 times in HTF-HEPES medium and transferred to medium containing the following: 20-μl drops of HTF + 0.2% dimethylsulfoxide (DMSO, D2650; Sigma, St. Louis, MO, USA), HTF + 1 μM of the PKC inhibitor Gö6983 (sc-203432; Santa Cruz Biotechnology, Santa Cruz, CA, USA), HTF + 50 μM of the p38 MAP kinase inhibitor SB 202190 (ab120238; Abcam, Cambridge, MA, USA), HTF + 100 ng of FGF1 antibody, HTF + 100 ng of FGF2 antibody, 100 ng of FGF4 antibody or 100 ng of FGF2 (PMG0033; Invitrogen, Gibco, Carlsbad, CA, USA) under light paraffin oil. The information of the antibodies used is given in **Table 1**. The embryos were maintained in culture in 5% CO_2 and atmospheric air at 37°C. After 24 h of treatment, all embryos from each group were imaged at time point equivalent to

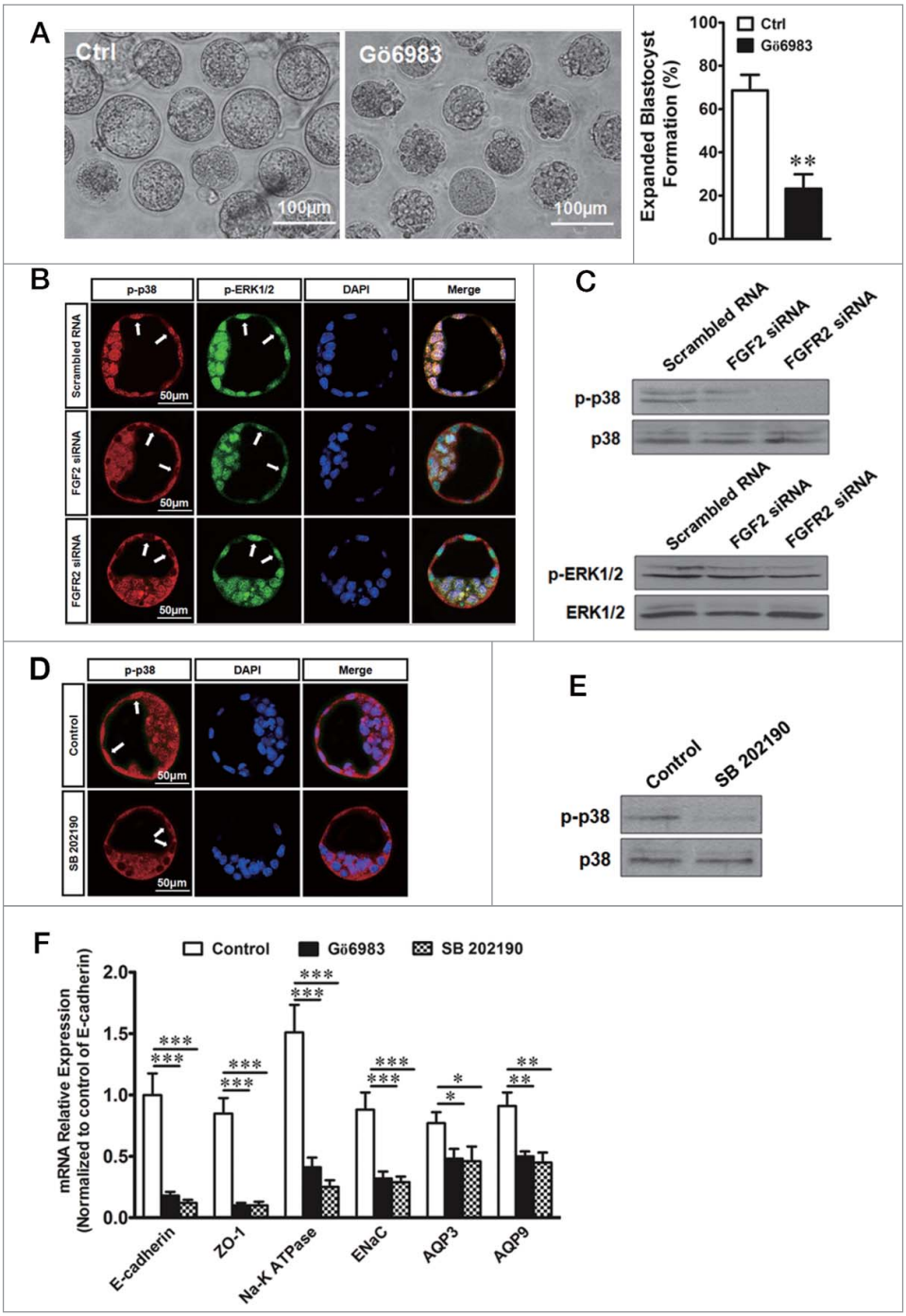

Figure 5. Effect of FGF/FGFR2 on the PKC-p38 pathway during expanded blastocyst formation. (**A**) Left, effect of the PKC inhibitor Gö6983 (1μM) on expanded blastocyst formation. Scale bar: 100 μm. Right, summary of the results. ** indicates $P < 0.01$ (by unpaired *t*-test, n = 5) compared with the control. (**B**) Effect of FGF2 or FGFR2 knockdown on the nuclear translocation of p-p38 MAPK and p-ERK1/2 MAPK in TEs by confocal double-immunofluorescence. Phenotype as labeled by scrambled RNA (24/30 embryos). Phenotype as labeled by FGF2 siRNA (22/29 embryos). Phenotype as labeled by FGFR2 siRNA (18/24 embryos). Scale bar: 50 μm. (**C**) Western blots of phosphorylated (p-) p38 (up) or ERK1/2 (down) levels in TEs in response to FGF2 or FGFR2 knockdown with siRNA transfection. (**D**) Effect of the p38 MAPK inhibitor SB202190 (50 μM) on the nuclear translocation of p-p38 MAPK in TEs by confocal immunofluorescence. The embryos were treated with SB202190 for 10 min. Phenotype as labeled by control (31/35 embryos). Phenotype as labeled by SB202190 treatment (27/32 embryos). Scale bar: 50 μm. (**E**) Western blots showing p-p38 in response to SB202190 (50 μM). (**F**) Effect of Gö6983 (1 μM) or SB202190 (50 μM) on the mRNA expression of protein markers of blastocysts in TEs by quantitative real-time PCR. * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$ (by one-way ANOVA, n = 4) compared with the corresponding controls.

113 h post-hCG injection. The diameter of the embryos was used to assess blastocyst expansion. The diameter of each blastocyst was measured in 2 different directions (using Image Pro analysis 6.2 software) and then averaged. After treatment, some embryos were fixed in 4% paraformaldehyde for immunofluorescence staining or frozen at −80°C for quantitative real-time PCR analysis.

Transfection of embryos with siRNA

Five microliters of Lipofectamine 2000 (Invitrogen) and 20 pmole of scrambled RNA (4390843; Ambion, Carlsbad, CA, USA), siRNA*fgf1*, siRNA*fgf2*, siRNA*fgf4*, siRNA*fgfr1*, siRNA*fgfr2*, siRNA*fgfr3* or siRNA*fgfr4* were diluted separately in 250 μl of serum-free Opti-MEM (Invitrogen) and incubated at room temperature for 5 min. The specific siRNA duplexes were synthesized by Sangon (Shanghai, China). The siRNA sequences are listed in **Table 3**. The diluted scrambled RNA/siRNA pools were then gently mixed with the diluted Lipofectamine 2000 reagent and incubated at room temperature for 20 min. The embryos were washed in HTF-HEPES medium 3 times and transferred to HTF-HEPES drops containing 1 M mannitol (M9647; Sigma) under light paraffin oil. After the blastocysts were dehydrated, each embryo was injected with 200 fmol of RNA solution into the cavity gap between the zona pellucida and the trophectoderm using the micromanipulation system

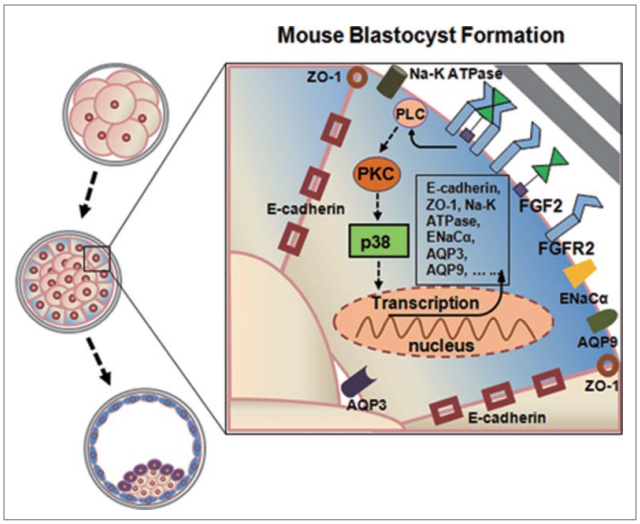

Figure 6. Working model for the regulation of blastocyst formation by the autocrine interaction of FGF2/FGFR2 activated PKC/p38 pathway in mouse TEs. Binding of FGF to cell surface heparin-like molecules in an autocrine fashion, FGF2 activates FGFR2, which stimulates phospholipase C (PLC) to generate diacylglycerol (DAG). In turn, DAG activates protein kinase C (PKC), which stimulates p38 activation, triggering transcription factor to regulate the expression of E-cadherin, ZO-1, Na-K ATPase, ENaCα, AQP3 and AQP9, which is required for blastocyst formation.

(Olympus Corporation, Tokyo, Japan) (**Fig. 1A**). After injection, the embryos were transferred to HTF (Irvine Scientific, Santa Ana, CA, USA) medium and allowed to recover in 5% CO_2 and at 37°C. Eight hours after injection, the expression of the FGFs and FGFRs was evaluated by quantitative real-time PCR. The embryos were imaged 24 h after RNA injection to assess blastocyst expansion.

Isolation of TEs from transfected embryos

The TEs were isolated from blastocysts (110-113 h after hCG injection; 24 h after siRNA transfection) as previously described with modifications.[43,46] After removal of the zona pellucida with Tyrode's solution, the ICMs were shelled out mechanically with a thinly pulled and polished micropipette.[43] The freshly isolated TEs were intensively washed in phosphate-buffered saline (PBS), frozen in liquid nitrogen and stored at −80°C for quantitative real-time PCR analysis.

Quantitative real-time PCR

Total RNA extraction and reverse transcription (RT) from 100 pools of TE were performed using the Cells-to-cDNA™ II Kit according to the manufacturer's instructions (Ambion, Austin, TX, USA). Quantitative real-time PCR was performed with the ABI Prism 7900HT detection system (Applied Biosystems, Carlsbad, CA, USA). The specific primers were provided by

Table 1. Primary antibodies

Poly/monoclonal	Host species	Against	Catalog number	Company	Application
Polyclonal	Rabbit	FGF1	sc-7910	Santa Cruz	$_{EC}$ (100ng in 20μl)
Polyclonal	Goat	FGF2	sc-1360	Santa Cruz	IF (1:200), $_{EC}$ (100ng in 20μl)
Polyclonal	Rabbit	FGF4	sc-1361	Santa Cruz	$_{EC}$ (100ng in 20μl)
Polyclonal	Rabbit	FGFR2	Ab10648	Abcam	IF(1:500)
Polyclonal	Rabbit	E-cadherin	sc-7870	Santa Cruz	IF(1:500),WB(1:1000)
Polyclonal	Rabbit	ZO-1	sc-10804	Santa Cruz	IF(1:200),WB(1:1000)
Polyclonal Polyclonal	Rabbit Goat	Na-K ATPase ENaCα	sc-10804 sc-21012	Santa Cruz Santa Cruz	IF(1:100), WB(1:1000) IF(1:200), WB(1:1000)
Polyclonal	Rabbit	AQP3	sc-20811	Santa Cruz	IF(1:100), WB(1:1000)
Polyclonal	goat	AQP9	sc-14988	Santa Cruz	IF(1:100), WB(1:1000)
Polyclonal	rabbit	β-Actin	sc-7210	Santa Cruz	WB(1:5000)
Polyclonal	rabbit	p38	sc-535	Santa Cruz	WB(1:1000)
Monoclonal	Mouse	p-p38	Ab45381	Abcam	IF(1:300), WB(1:1000)
Polyclona	rabbit	ERK1/2	#9102	Cell Signaling	WB(1:1000)
Polyclonal	rabbit	p-ERK1/2	#9101	Cell Signaling	IF(1:200), WB(1:1000)

IF: Immunofluorescence labeling, WB: Western-blots, $_{EC}$: Embryo culture

Table 2. Secondary antibodies

Host species	Against	Conjugated to	Catalog	Company	Application
Goat	rabbit IgG	Alexa Fluor 488	A31627	Invitrogen (Carlsbad, CA, USA)	IF (1:500)
Rabbit	goat IgG	Alexa Fluor 594	A11080	Invitrogen (Carlsbad, CA, USA)	IF (1:500)
Rabbit	mouse IgG	Alexa Fluor 594	A11005	Invitrogen (Carlsbad, CA, USA)	IF (1:500)
Goat	Mouse IgG	Alexa Fluor® 790	A11375	Invitrogen (Carlsbad, CA, USA)	WB (1:2000)
Goat	Rabbit IgG	Alexa Fluor® 790	A11367	Invitrogen (Carlsbad, CA, USA)	WB (1:2000)
Donkey	Goat IgG	Alexa Fluor® 790	A11370	Invitrogen (Carlsbad, CA, USA)	WB (1:2000)

IF: Immunofluorescence labeling, WB: Western-blots

Table 3. Nucleotide sequences of siRNA used for knockdown

Target RNA(mouse)	Guide (5′–3′)	Passenger (5′–3′)
FGF1	AAGUGUUAUAAUGGUUUUCUU	GAAAACCAUUAUAACACUUAC
FGF2	UCGUUCAAAGAAGAAACACUC	GUGUUUCUUCUUUGAACGACU
FGF4	UUUACACUCGUCGGUAAAGAA	CUUUACCGACGAGUGUAAAUU
FGFR1	AAAAACCACUAUGAUACACGG	GUGUAUCAUAGUGGUUUUUUU
FGFR2	UUAUGUCAAGUUAGGAAACAA	GUUUCCUAACUUGACAUAAAA
FGFR3	AUUCUUUGCCAUUCUUCAGCC	CUGAAGAAUGGCAAAGAAUUC
FGFR4	ACAUACAGGAUGAUAUCUGUG	CAGAUAUCAUCCUGUAUGUAU

Table 4. Nucleotide sequences of primers used for quantitative real-time PCR (SYBR Green)

Target RNA(mouse)	Forward (5′–3′)	Reverse (5′–3′)	Product size (bp)	Accession No.
ENaCα	AACTCCTGGTGACCCTGACAA	GAAGGGACAGGAAATGATGGG	131	NM_011324.2
Na-K ATPase	CTCGGCAAGGACAGAACAAAA	AGGCTTTCCCAGAGAAGGTG	119	NM_144900.2
ZO-1	GTTCCGGGGAAGTTACGTGC	AAGTGGGACAAAAGTCCGGG	187	NM_001163574.1
E-cadherin	ATGTGGAGGGTCCTGACTCG	CCTCCGGATCCCAACTTTCTT	101	NM_009864.2
AQP3	TAACCCTTGCCTTACTGGGC	AACGGTCTCCCTTCACTCCT	164	NM_016689.2
AQP9	CATTACCGTCCTGGACTTCCC	TGCCCAAGTGCCATGTTCTC	120	NM_001271843.1
GAPDH	CCCCAGCAAGGACACTGAGCAAGAG	GCCCCTCCTGTTATTATGGGGGTC	107	NM_008084.2

Sangon. The full list of primer sequences for quantitative real-time PCR (mouse E-cadherin, ZO-1, ENaCα, Na-K ATPase, AQP3, AQP9 and GAPDH) is given in **Table 4**. Three to 6 replicates were performed.

Immunofluorescence labeling and confocal microscopy

The embryos were fixed in 4% paraformaldehyde for 1 h and then permeabilized with 0.1% Triton X-100 in PBS for 30 min. After incubation with 5% goat serum and rabbit serum for 1 h to block nonspecific antigens, the embryos were grouped and incubated with the primary antibodies at 4°C overnight. Then, the embryos were washed with 0.1% Triton X-100 in PBS (PBST) and were incubated with the secondary antibodies for 30 min, followed by nuclear counterstaining with 4′, 6-diamidino-2-phenylindole (DAPI, 1:1000; Sigma) for 20 min. Antibodies used in the present study are listed in supplementary material **Table 1** (primary antibodies) and **Table 2** (secondary antibodies). For the negative controls, we incubated the embryos in PBS without the addition of the primary antibody to determine the levels of non-specific fluorescence. Finally, the fluorescent images were analyzed using a Zeiss LSM 510 META laser scanning confocal microscope (Carl Zeiss, Thornwood, NY, USA).

Western blot analysis

After isolation of TEs from blastocysts (200 embryos/group) were pooled, lysed by loading buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. After blocking with 4% milk, the blots were probed overnight at 4 °C with the primary antibodies (**Table 1**). The signal was detected with HRP-conjugated secondary antibody (**Table 2**) and visualized using ECL Western Blot Detection Reagent (GE Healthcare).

Microinjection of FGF antibodies

The blastocoel of the blastocyst was injected with 100 ng of FGF1 polyclonal antibody, FGF2 polyclonal antibody or FGF4 polyclonal antibody using the micromanipulation system (**Fig 1B**). The information of the antibodies used is given in **Table 1**. The negative control was injected with the same volume of saline, which substituted for the antibodies. After the injection, the embryos were cultured in HTF (Irvine Scientific) medium in 5% CO_2 at 37°C. The expansion rate of the blastocyst was assessed 24 h after the injection.

Statistical analysis

Each experiment was repeated at least 3 times. The data are presented as the means ± standard error (SEM). Student's unpaired *t*-test was used for comparisons between 2 groups. One-way analysis of variance (ANOVA) was used for comparisons among 3 or more groups. A probability of $P < 0.05$ was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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