Abnormal epithelial cell polarity and ectopic Epidermal Growth Factor Receptor (EGFR) expression induced in Emx2 KO Embryonic Gonads

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The gonadal primordium first emerges as a thickening of the embryonic coelomic epithelium, which has been thought to migrate mediodorsally to form the primitive gonad. However, the early gonadal development remains poorly understood. Mice lacking the paired-like homeobox gene *Emx2* display gonadal dysgenesis. Interestingly, the knockout (KO) embryonic gonads develop an unusual surface accompanied by aberrant tight junction assembly. Morphological and *in vitro* cell fate mapping studies showed an apparent decrease in the number of the gonadal epithelial cells migrated to mesenchymal compartment in the KO, suggesting that polarized cell division and subsequent cell migration are affected. Microarray analyses of the epithelial cells revealed significant up-regulation of *Egfr* in the KO, indicating that *Emx2* suppresses *Egfr* gene expression. This genetic correlation between the two genes was reproduced with cultured M15 cells derived from mesonephric epithelial cells. Epidermal growth factor receptor signaling was recently shown to regulate tight junction assembly through sarcoma viral oncogene homolog tyrosine phosphorylation. We show through *Emx2* KO analyses that sarcoma viral oncogene homolog tyrosine phosphorylation, epidermal growth factor receptor tyrosine phosphorylation, and *Egfr* expression are up-regulated in the embryonic gonad. Our results strongly suggest that *Emx2* is required for regulation of tight junction assembly and allowing migration of the gonadal epithelia to the mesenchyme, which are possibly mediated by suppression of *Egfr* expression. **(*Endocrinology* 151: 5893–5904, 2010)**

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Abbreviations: BrdU, Bromodeoxyuridine; CCFSE, 5-(and-6)-carboxy-2′,7′-dichlorofuluorescein diacetate, succinimidyl ester; c-Src, sarcoma viral oncogene homolog; c-Yes, Yamaguchi sarcoma viral oncogene homolog 1; E, embryonic day; EGF, epidermal growth factor; EGFR, EGF receptor; Emx2, empty spiracles homeobox 2; Emx2(HA), HA-tagged EMX2; GATA, globin transcription factor; GATA4, GATA binding protein-4; HA, hemaglutinin; KO, knockout; *Lhx9*, *LIM homeobox gene 9*; PAR, partitioning-defective protein; PI, propidium iodide; S, sense; SEM, scanning electron microscopy; SF-1, steroidogenic factor-1; siRNA, short interfering RNA; *Sry*, sex-determining region Y chromosome; TUNEL, terminal deoxynucleotidyltransferase-mediated 2′-deoxyuridine 5′-triphosphate nick end labeling; ts, tail somite; WT1, suppressor gene for Wilms' tumor; ZO, zonula occludens.

The gonadal primordium first appears as a thickening of the coelomic epithelium at the lateral sides of the mesentery. Histological observations suggested that these epithelial cells migrate mediodorsally to form the primitive urogenital ridge (1–3), which subsequently separates into the future gonad and mesonephros. Sexually dimorphic events occur thereafter in the genital ridge in response to transient expression of sex-determining region on the Y chromosome (*Sry*). A number of studies has attempted to elucidate the mechanisms underlying gonad development and sex differentiation. Disruption of some genes in mice and humans leads to hypoplastic gonads or complete dysgenesis, whereas disruption of other genes leads to sex reversal (4–6). Nevertheless, the early phase of gonad development is still poorly understood.

The paired-like homeobox gene *Emx2* is a mouse homologue of the *Drosophila empty spiracles* gene (7). Mouse knockout (KO) studies demonstrated that *Emx2* is implicated in the development of multiple tissues, including the kidney, gonad, reproductive tracts, and central nervous system. A detailed study of the kidney defect showed that ureteric bud extension is disrupted in the metanephric mesenchyme of *Emx2* KO mice, leading to the disappearance of the kidney (8). In addition, proliferation of neuronal progenitors and area specification of the neocortex are disrupted in *Emx2* KO animals (9–13). Some of the observed abnormalities in these animals appeared to be closely related to defects in the spatial expression of growth factors (14–17). Although these studies revealed important insights into how *Emx2* functions during kidney and central nervous system development, its role in gonad development is still unclear.

Epithelial cells are polarized by establishing functionally specialized apical, lateral, and basal surfaces and adhere tightly one another through tight and adherens junctions at their lateral interfaces. These junctions are comprised of transmembrane proteins, such as junctional adhesion molecules, cadherins, occludin, and claudin, and intracellular membrane-associated proteins, such as β-catenin and zonula occludens (ZO) (18, 19). Mammalian homologues of the *Caenorhabditis elegans* partitioning-defective proteins (PARs) PAR3 and PAR6 position tight junctions by forming a complex with atypical protein kinase C (aPKC). Multiple extracellular signals regulate the formation and localization of the PAR3/PAR6/aPKC complex via phosphorylation of the components (20, 21).

ErbB (erythroblastic leukemia viral oncogene homolog) receptors comprise a family of four structurally related tyrosine kinase receptors (22) that are activated by a variety of ligand molecules, including epidermal growth factor (EGF). Upon ligand binding, the receptor is dimerized and the kinase activity triggers numerous downstream signaling pathways (23). One family member, EGF receptor (EGFR), is expressed in the epithelial cells of a variety of tissues, where it plays fundamental roles in tissue development through regulating cell proliferation, cellular polarity formation, and epithelial cell migration (24). A number of studies revealed the presence of phospholipase C-γ, PKC-mediated cascades, mitogen-activated protein cascades, and small GTPase downstream of EGFR (23). Importantly, a recent study demonstrated that EGFR is implicated in the regulation of tight junction assembly via tyrosine phosphorylation of sarcoma viral oncogene homolog (c-Src)/Yamaguchi sarcoma viral oncogene homolog 1 (c-Yes), which subsequently phosphorylates PAR3 to regulate PAR3/PAR6/aPKC complex formation (25).

Here, we examine early stages of gonad development in *Emx2* KO embryos and show that tight junction assembly and migration of the epithelial cells of the gonad are significantly affected. Interestingly, microarray analysis of the epithelial cells of the embryonic gonad indicates that *Egfr* is dramatically up-regulated in *Emx2* KO mice. This ectopic *Egfr* expression is accompanied by aberrant c-Src tyrosine phosphorylation. Our data strongly suggested that *Emx2* is required for tight junction assembly and migration of epithelial cells at the early stage of gonadal development possibly through suppression of *Egfr* expression.

Materials and Methods

Experimental animals

Emx2 KO mice (accession no. CDB0018K; http://www.cdb.riken.jp/arg/mutant%20mice%20list.html) (8) were crossed to B6/J Jcl mice (Clea, Tokyo, Japan) for five generations. Genotypes were determined by PCR using the primers, empty spiracles homeobox 2 (Emx2)-sense (S) (5′-CCACCTTAGAGACCATTGCT-3′), Emx2-antisense (AS) (5′-TTCTCAAAAGCGTGCTCTAG-3′), and phosphoglycerate kinase-AS (5′-GCTACCGGTGGATGTGGAATG-3′). A wild-type allele is amplified with Emx2-S and Emx2-AS and a KO allele with Emx2-S and phosphoglycerate kinase-AS. The sex of the mice was determined by PCR with primers for *Sry*, Sry-M5 (5′-GTGGTGAGAGGCACAAGTTGGC-3′) and Sry-M3 (5′-CTGTGTAGGATCTTCAATCTCT-3′). Embryos were dissected between embryonic d (E)10.0 and E12.5. To stage the embryos accurately, tail somites (tss) were counted; ts stages are indicated in the figure legends. All protocols for animal experiments were approved by the Institutional Animal Care and Use Committee of the National Institute for Basic Biology.

Preparation of antibody for EMX2

Full-length mouse *Emx2* cDNA was amplified by PCR with primers, 5′-ACACACCTCGAGATGTTTCAGCCGGCGCCCAAG-3′ and 5′-ACACACACGCGGCCGCGCCTTAATCGTCTGAGGTCACATC-3′, and then cloned into pET-28a (Strat-

agene, La Jolla, CA) to produce His-tagged EMX2 recombinant protein. The recombinant EMX2 was purified with Ni-agarose (Invitrogen, Carlsbad, CA). Rabbits were immunized with the purified His-tagged EMX2 as described previously (26).

Scanning electron microscopy (SEM), histology, immunohistochemistry, and *in situ* hybridization

SEM of E11.5 embryos was performed using a Hitachi S-800 (Hitachi, Tokyo, Japan), as previously described (27). Histochemical and immunohistochemical analyses were performed as previously described (28). Rabbit antibodies to EMX2, Ad4BP/SF-1 [*Adrenal-4 Binding Protein* (29), *Steroidogenic Factor-1* (30), and *NR5A1* (31)] (26), aristaless-related homeobox (32), aPKC (PKCζ) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), suppressor gene for Wilms' tumor (WT1) (Santa Cruz Biotechnology, Inc.) (33, 34), laminin (Sigma, St Louis, MO), ZO-1 (Zymed, South San Francisco, CA), occludin (Zymed), Src phosphrylated at tyrosine 418 (Biosource, Camarillo, CA), EGFR phosphorylated at tyrosine 845 (Abcam, Cambridge, MA), and EGFR phosphrylated at tyrosine 1068 (Abcam), sheep antibody to EGFR (Upstate, Charlottesville, VA), goat antibody to globin transcription factor (GATA) binding protein-4 (GATA4) (Santa Cruz Biotechnology, Inc.) (35), mouse antibodies to bromodeoxyuridine (BrdU) (Roche, Indianapolis, IN) and hemaglutinin (HA) (Sigma), and guinea pig antibody to CBX2/M33 (Polycomb component) (36) were used. Biotinylated antirabbit, antigoat, antisheep, and antiguinea pig antibodies (Jackson ImmunoResearch, West Grove, PA), Alexa Fluor 488-labeled antirabbit and antigoat (Molecular Probes, Eugene, OR) antibodies, and Cy3-labeled and Cy5-labeled antimouse antibodies (Jackson ImmunoResearch) were used as secondary antibodies. Antigen-antibody complexes were detected using Histofine kit (Nichirei, Tokyo, Japan) or directly by fluorescence. *In situ* hybridization for *LIM homeobox gene 9* (*Lhx9*) (37) was performed as previously described (38).

Cell proliferation and apoptosis assays

Pregnant females received an ip injection of BrdU (Sigma) (50 mg/kg body weight) at E10.0, E10.5, and E11.0 (39) and were killed 2 h after injection. Paraffin sections of the embryos were double immunostained for BrdU and GATA4. In brief, after the sections were boiled in 10 mM citrate (pH 6.0) for 20 min (40), they were incubated with the mouse anti-BrdU antibody, and thereafter with the Cy3-labeled antimouse antibody. Subsequently, the sections were incubated with goat anti-GATA4 antibody and with Alexa Fluor 488-labeled antigoat antibody. Nuclei in the sections were stained with propidium iodide (PI) (Molecular Probes). The number of BrdU-immunoreactive gonadal epithelial cells was counted in more than 10 sections for every gonad. Apoptosis in E11.0 and E12.0 embryonic gonads was assayed using the ApopTag Plus Peroxidase kit (CHEMICON, Temecula, CA). After apoptotic cells were detected with rhodamine-labeled anti-digoxigenin antibody, the sections were stained by goat anti-GATA4 and Alexa Fluor 488-labeled antigoat antibodies.

Cell fate mapping with organ culture

After the abdominal tissues of E10.25 embryos were removed, the coelomic epithelial cells were labeled with 20 mM 5-(and-6)-carboxy-2′,7′-dichlorofuluorescein diacetate, succinimidyl ester (CCFSE) (Molecular Probes) in DMEM (Invitrogen) for 1 h. Subsequently, they were cultured for 5 or 24 h in DMEM containing 10% fetal bovine serum and antibiotics under a humidified atmosphere of 5% CO_2 in air at 37 C. After fixation in 4% paraformaldehyde for 5 min, the embryos were frozen sectioned and stained with Ad4BP/SF-1 or laminin antibody. The number of CCFSE-positive cells that migrated through the laminin layer was counted in more than 10 sections for every gonad.

Preparation of Emx2-expressing M15 cells and knockdown of Emx2 expression by short interfering RNA (siRNA)

Full-length cDNA for mouse *Emx2* was cloned into pOZ-FH retroviral vector (41). The construct, pOZ-FH-Emx2, encodes FLAG-HA-tagged EMX2 [Emx2(HA)] and IL-2 receptor with an internal ribosomal entry site. Recombinant viruses prepared with pOZ-FH-Emx2 were transfected into M15 cells derived from mesonephric epithelial cells (42). To prepare M15 cells expressing EMX2 [M15-Emx2(HA)], the infected cells were sorted by anti-IL-2 receptor monoclonal antibody (Upstate) conjugated with magnetic beads (Dynal Biotech, Oslo, Norway) (41, 43); 10^5 original M15 and M15-Emx2(HA) cells were plated on a six-well dish. After 24 h, cells were transfected with 100 pmol siRNA for *Emx2* (S, 5′-UUCGAAUCCGCUUUGGCUUUCUGGC-3′ and AS, 5′-GCCAGAAAGCCAAAGCGGAUUCGAA-3′) or control siRNA (Invitrogen) using lipofectamine 2000 (Invitrogen). The cells were collected for RT-PCR and Western blot analysis (26) after another 24-h incubation. For immunohistochemistry, the cells were grown on poly-L-lysine-coated glass (IWAKI, Tokyo, Japan), then fixed with 4% paraformaldehyde and incubated with anti-HA and anti-EGFR antibodies.

Microarray and quantitative RT-PCR analyses

Microarray analyses were performed essentially as described (44, 45). E10.5 wild-type and *Emx2* KO embryos were frozen in OCT compound (Sakura Finetechnical, Tokyo, Japan) without fixation. They were sectioned (30 μm), stained with hematoxylin, and air dried. Some were used for GATA4 immunostaining to locate gonadal primordia. The epithelial cells of the gonadal primordia were obtained using a Laser Microdissection System (Leica, Wetzlar, Germany). The specimens prepared from three individuals were combined into one group. Total RNA was prepared from three groups, and 15 ng total RNA was subjected to two-cycle amplification and biotin labeling using MessageAmp II aRNA Amplification and MessageAmp II-Biotin Enhanced kit (Ambion, Austin, TX), respectively. The labeled aRNA was fragmented and hybridized to GeneChip Mouse Genome 430 2.0 array according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Signals were scanned and scaled using Affymetrix GCOS 1.1 software. The scaled values were then analyzed by GeneSpring software (Silicon Genetics, Redwood City, CA). Pairwise comparison analysis was performed with Affymetrix GCOS 1.2 to identify differentially expressed transcripts. Each sample (n = 3) was compared with each reference samples (n = 3), resulting in nine pairwise comparisons. This approach, which is based on Mann-Whitney pairwise comparison test, allows ranking of differentially expressed genes as well as calculation of significance ($P < 0.05$) of each identified change. The microarray data have been deposited in the Gene Expression Omnibus of the National Center for Biotechnology Information (accession no. GSE10216; http://www.ncbi.nlm.nih.gov/geo/). Quantitative RT-PCR with TaqMan probes for mouse *Egfr* (Mm00433021_m1) and *Gapdh*

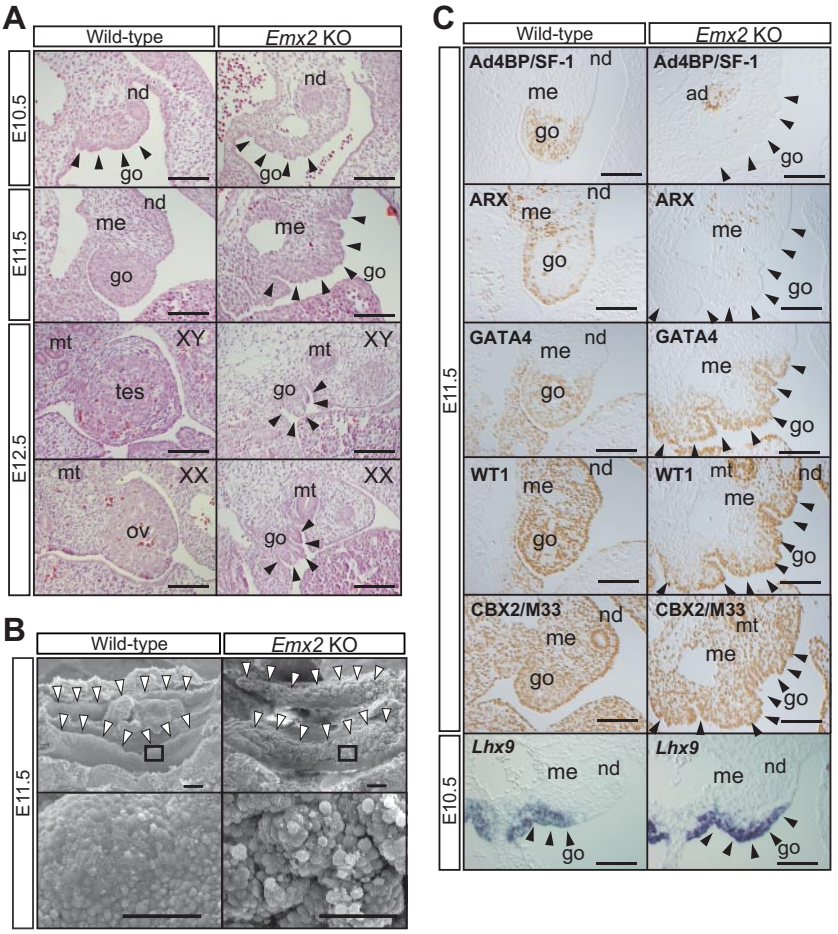

FIG. 1. Structural defects and altered marker gene expression in *Emx2 KO* gonads. A, Developing gonads of wild-type and *Emx2* KO embryos. Sections of wild-type and *Emx2* KO embryonic gonads at E10.5 (ts 8), E11.5 (ts 18–19), and E12.5 (ts 27–32) [male (XY) and female (XX)] were stained with hematoxylin and eosin. Developing and degenerating gonads are indicated by *closed arrowheads*. *Scale bars*, 100 μm. B, SEM of E11.5 (ts 19) wild-type and *Emx2* KO embryonic gonads (*open arrowheads*). The *regions enclosed by squares* are enlarged in the *lower panels*. *Scale bars*, 100 μm (*upper*) and 50 μm (*lower*). C, Gonadal marker gene expression in wild-type and *Emx2* KO gonads. The expression of gonadal markers was examined in wild-type and *Emx2* KO embryos by immunohistochemistry for Ad4BP/SF-1, ARA, GATA4, WT1, and CBX2/M33 at E11.5 (ts 18–21) and by *in situ* hybridization for *Lhx9* at E10.5 (ts 7). Gonadal regions are indicated by *closed arrowheads*. *Scale bars*, 100 μm. go, Gonad; nd, nephric duct (feature Wolffian duct); me, mesonephrose; mt, mesonephric tubule; ad, adrenal primordium; tes, testis; ov, ovary.

(TaqMan Rodent glyceraldehyde-3-phosphate dehydrogenase Control Regents; Applied Biosystems, Foster City, CA) was performed using QuantitectProbe RT-PCR kit (QIAGEN, Valencia, CA) and PM9000 cermal cycler (Applied Biosystems). Total RNAs prepared from M15 and M15-Emx2 cells were used for quantitative RT-PCR using Power SYBR Green PCR Master mix (Applied Biosystems). PCR primers for mouse *Egfr* are mEgfr-S (5′-CACAGCGCTACCTTGTTATCCA-3′) and mEgfr-AS (5′-CCTCCATGTCCTCTTCATCCA-3′). Primers for *β-actin* were as previously described (46).

Results

Abnormal surface of Emx2 KO embryonic gonad

Although the gonads of *Emx2* KO embryos disappear by E12.5 (8), the mechanism by which this defect arises was not analyzed. As shown in Fig. 1A, the urogenital primordium is observed as a thickening of eplithelia at E10.5 in both wild-type and *Emx2* KO embryos. By E11.5, the developing urogenital primordium separates into a sexually indifferent gonad and mesonephros and thereafter develops into either the testis or ovary. The gonads of *Emx2* KO embryos are underdeveloped at E11.5 and largely disappear by E12.5 in both sexes. We noted that during the degeneration process, the gonadal surface of *Emx2* KO embryos appears to be abnormal at E11.5. Thus, the gonadal surface was examined by SEM (Fig. 1B). The gonads of wild-type embryos exhibit smooth surfaces, whereas those of *Emx2* KO embryos show irregularly protruding cellular clusters.

To characterize the *Emx2* KO gonad, we examined the expression of various gonadal marker genes. The expressions of Ad4BP/SF-1 required for the gonadal and adrenal development (47, 48) and aristaless-related homeobox required for the development of testicular Leydig cells (32) disappeared from the *Emx2* KO gonads by E11.5, whereas the expression of Ad4BP/SF-1 in the adrenal primordium was unaffected (Fig. 1C). This loss of Ad4BP/SF-1 expression in the gonads but not the adrenal glands of *Emx2* KO mice is consistent with the observation that although *Ad4BP/SF-1* KO mice failed to develop both the gonads and adrenal glands (49), *Emx2* KO mice failed to develop the adrenal gland. The expression of GATA4, WT1, and CBX2/M33, all of which are expressed in the developing gonad of wild-type and involved in the gonadal development, was unaffected. Interestingly, the epithelial expression of *Lhx9* required for the gonadal development (37) was expanded laterally in *Emx2* KO.

Abnormal cell proliferation and apoptotic cell death induced in Emx2 KO gonads

The structural abnormalities in the gonadal surface of *Emx2* KO strongly suggested that *Emx2* is critical for the development of the epithelial cells of the gonad. Therefore, the expression of EMX2 in the developing gonad was examined. Immunohistochemical studies demonstrated that EMX2 is expressed in the gonadal epithelial and adjacent

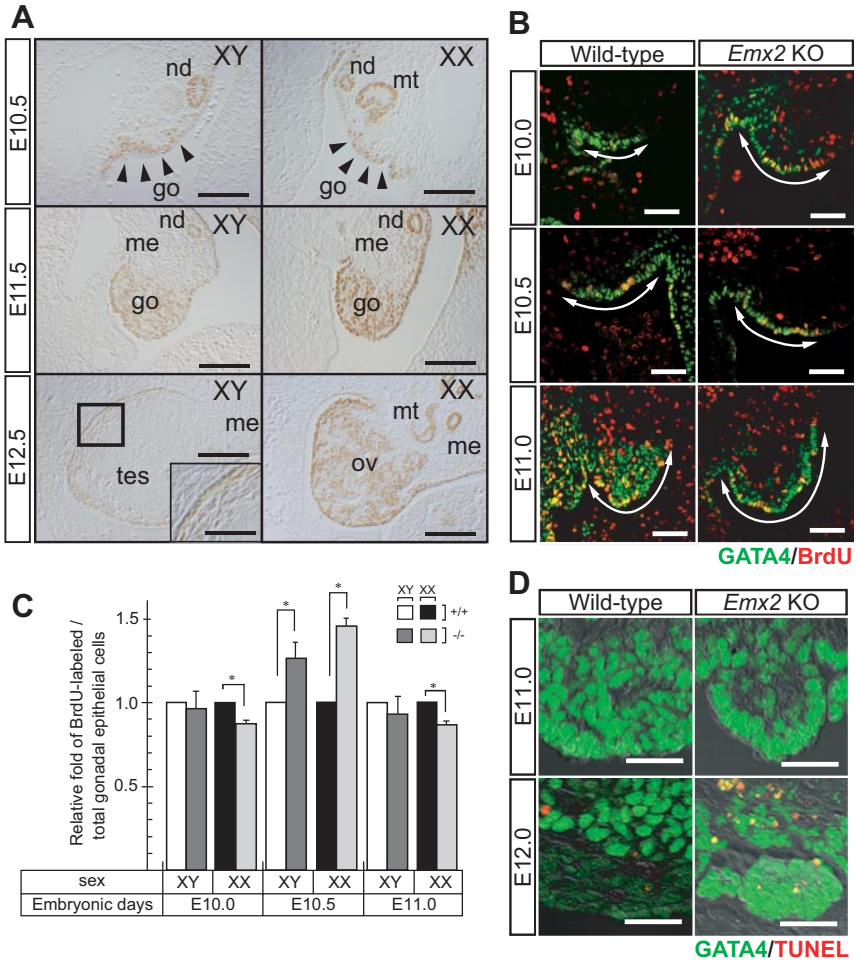

FIG. 2. Up-regulated epithelial cell proliferation and apoptosis in *Emx2 KO* embryonic gonads. A, Expression of EMX2 during gonadal development. The expression of EMX2 was examined immunohistochemically in male (XY) and female (XX) at E10.5 (ts 7–8), E11.5 (ts 18–19), and E12.5 (ts 32). *Arrowheads* in E10.5 indicate the early developing gonads. The *region enclosed by a square* in the E12.5 male gonad is enlarged as an *inset*. *Scale bars*, 100 and 50 μm (*inset*). go, Gonad; nd, nephric duct (feature Wolffian duct); me, mesonephrose; mt, mesonephric tubule; tes, testis; ov, ovary. B, BrdU labeling in wild-type and *Emx2* KO embryonic gonads. Cell proliferation was assessed by BrdU labeling as described in *Materials and Methods*. The BrdU-labeled gonads of wild-type and *Emx2* KO embryos were sectioned at E10.0 (ts 4–6), E10.5 (ts 9–11), and E11.0 (ts 13–17) and stained with anti-BrdU (*red*) and GATA4 (*green*) antibodies. As indicated by *arrows*, gonadal regions were determined by GATA4 staining and morphology. *Scale bars*, 50 μm. C, Transient up-regulation of BrdU incorporation into epithelial cells of *Emx2* KO embryonic gonads. The total number of BrdU-labeled gonadal epithelial cells in the *areas indicated by arrows* in A was counted at E10.0 (ts 4–6), E10.5 (ts 9–11), and E11.0 (ts 13–17). Data were obtained only when wild-type and *Emx2* KO embryos of the same sex were in the same litter. The relative fold changes of the number of BrdU-positive cells in the gonadal epithelial cells are plotted, with the number in wild-type embryos of each sex set at 1 for each stage. The number of gonads used in this study is as follows: two wild-type and 6 KO gonads for E10.0 males, two wild-type and 4 KO gonads for E10.0 females, five wild-type and 5 KO gonads for E10.5 males, five wild-type and five KO gonads for E10.5 females, four wild-type and 4 KO gonads for E11.0 males, and three wild-type and three KO gonads for E11.0 females. Values are the means $\pm$ SD; *, $P <$ 0.001. D, Ectopically increased apoptosis in *Emx2* KO gonads. A TUNEL assay (*red*) was used to detect apoptotic cells in the wild-type and *Emx2* KO gonads at E11.0 (ts 15) and E12.0 (ts 25). Gata4 immunostaining (*green*) was used to detect the developing gonads. Gonadal regions are indicated by *arrows*. *Scale bars*, 25 μm.

mescenchymal cells, nephric duct, and mesonephric tubule at E10.5 and expressed in all somatic cells in the gonad but not in the mesonephrose except tubular structure at E11.5 (Fig. 2A). Although the expression was similar between the two sexes before gonadal sex differentiation, the expression became different between the testis and ovary at E12.5. The expression was down-regulated in the testis except testicular tunica albuginea and underneath mesenchymal cells, whereas the expression was still evident in the whole ovary at E12.5. Because the testis initiates to synthesize testosterone as early as E12.5, the sexually dimorphic expression of *Emx2* established by E12.5 seems to be independent of gonadal sex steroid.

Next, we examined whether epithelial cell proliferation is affected in the *Emx2* KO gonad. After BrdU was injected into pregnant females, embryos were collected at E10.0, E10.5, and E11.0 and their gonads stained with an antibody against BrdU (Fig. 2B, *red*). Because the gonads at the stage are structurally primitive, it is difficult to discriminate between the future gonadal and mesonephric areas only by the morphology. For the gonad at the stages, Ad4BP/SF-1 and GATA4 are known to be potential gonadal markers. However, the expression of Ad4BP/SF-1 was affected significantly in the *Emx2* KO gonad, and thus GATA4 immunostaining was performed to evaluate the gonadal area (Fig. 2B, *green*) (35). In addition, considering that GATA4 is expressed in the mesentery, the gonadal area was eventually determined as GATA4 immunoreactive but not the mesentery cells. The number of BrdU-positive proliferating epithelial cells was similar between wild type and *Emx2* KO at E10.0 in both sexes (Fig. 2, B and C). By E10.5, the number of BrdU-positive cells in *Emx2* KO gonads was increased by approximately 1.3-fold in males and 1.5-fold in females compared with wild type. This increase in epithelial cell proliferation was not observed at E11.5.

Apoptosis was examined using terminal deoxynucleotidyltransferase-mediated 2′-deoxyuridine 5′-triphosphate

nick end labeling (TUNEL) labeling. Although the KO gonad showed obvious structural defects at E11.5, the number of TUNEL-positive apoptotic cells was not increased at E11.0. By E12.0, however, the number of TUNEL-positive cells was significantly increased (Fig. 2D).

Migration of gonadal epithelial cells affected in Emx2 KO

It has long been surmised that the coelomic epithelia at the both sides of the mesentery proliferate and migrate mediodorsally to give rise to the gonadal primordia. As described above, the BrdU incorporation study indicated that epithelial cell proliferation was transiently up-regulated in *Emx2* KO gonads. However, if migration of the epithelial cells is affected, it is assumed that the two BrdU-positive daughter cells remain in the epithelial compartment, thus causing an apparent increase in the number of the BrdU-positive epithelial cells. We therefore examined whether migration of epithelial cells to the mesenchymal compartment was affected in the KO.

Gonadal epithelial cells are thought to pass through the basement membrane during their migration into the mesenchymal compartment. The gonads of wild-type and *Emx2* KO embryos were sectioned and stained with laminin antibody to visualize the basement membrane (Fig. 3A, *green*), whereas nuclei were stained with PI (Fig. 3A, *red*). As expected, cells were frequently seen in the basement membrane in wild-type embryos (Fig. 3A, *arrowheads*) but approximately 6-fold less frequently in the KO gonads (Fig. 3B). Epithelial cells can undergo polarized cell division, with one daughter cell retaining epithelial cell features while the other cell losing them and migrating into the mesenchymal compartment through the basement membrane (21, 50, 51). The decreased number of cells localized to the basement membrane in the *Emx2* KO gonad strongly suggests that polarized cell division and cell migration are affected in these mutants. Moreover, we noticed that the basement membrane of the KO gonad is not tightly lining the epithelial cells when compared with wild type. This unusual basement membrane might affect the epithelial cell migration.

Therefore, we examined this migration defect using organ culture. Embryonic trunk tissue containing the developing gonads was prepared at E10.25, and whole coelomic epithelial cells, including the gonadal epithelia, were labeled with a fluorescent dye, CCFSE, to chase the epithelial cells (Fig. 3C, *green*). After the labeled trunks were cultured for 5 h, the gonads were sectioned and immunostained with antilaminin antibody (Fig. 3C, *red*). Expectedly, the gonadal epithelia of wild-type embryos migrated through the laminin layer, whereas those of *Emx2* KO

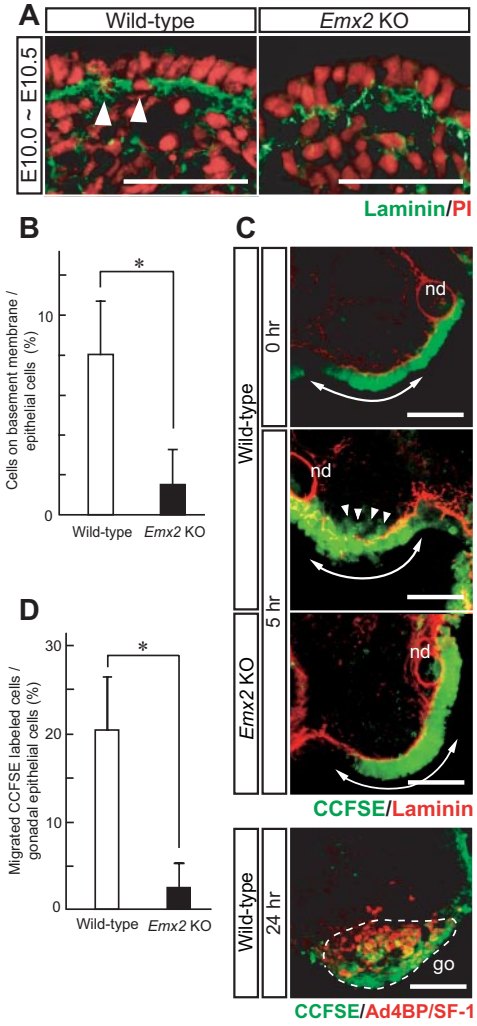

FIG. 3. Aberrant migration of gonadal epithelial cells to the mesenchymal compartment in *Emx2 KO*. A and B, A decreased number of cells was localized to the basement membrane in *Emx2* KO embryonic gonads. Gonadal sections at E-10.0–E10.5 (ts 4–7) were stained with laminin (*green*) and PI (*red*) to mark the basement membrane and nuclei, respectively. The cells localized near the basement membrane are indicated by *open arrowheads*. *Scale bars*, 50 μm. The number of cells near the basement membrane is shown relative to the total number of epithelial cells. Ten sections from six wild-type and 10 *Emx2* KO gonads were examined. Values are expressed as the means ± SD; *, $P < 0.01$. C and D, Aberrant migration of epithelial cells in the *Emx2* KO embryonic gonad. The abdominal epithelial cells of wild-type and *Emx2* KO embryos at E10.25 (ts 5–6) were labeled with CCFSE (*green*) (0 h), and thereafter cultured for 5 or 24 h (5 or 24 h). Specimens at 0 and 5 h were then sectioned and stained with an antibody against laminin (*red*), whereas those at 24 h were stained with an antibody against Ad4BP/SF-1 (*red*). The developing gonadal regions are indicated by *arrows*, which are determined through GATA4 staining (data not shown) and morphology. The migrated cells are indicated by *arrowheads* (5 h). As shown in the *lowermost panel* (24 h), the migrated cells became immunoreactive for Ad4BP/SF-1. The *area enclosed by a dotted line* is the developing gonad. *Scale bars*, 100 μm. go, Gonad; nd, nephric duct. The gonadal epithelial cells and cells that had migrated through the basement membrane after 5 h of incubation were counted. The number of migrated cells is shown relative to the total epithelial cell numbers. Six wild-type and six *Emx2* KO embryonic gonads were used in this study. Values are the means ± SD; *, $P < 0.0001$.

scarcely migrated. The number of migrating cells seen in wild-type gonads was approximately 9-fold higher than in *Emx2* KO (Fig. 3D). This migration defect possibly results in a substantial increase of epithelial cells and, at the same time, decrease of mesenchymal cells. Taken together, the observations above suggest that the loss of *Emx2* blocks gonadal development by restricting epithelial cell migration.

Based on the observation above, a question arose as to whether the migrating cells differentiate into gonadal somatic cells or not. Thus, the gonad sections were stained with anti-Ad4BP/SF-1 antibody after further incubation up to 24 h. Ad4BP/SF-1 was expressed in many CCFSE-positive migrating cells (Fig. 3C). These results demonstrate directly for the first time that the gonadal epithelial cells at around E10.25 have the potential to migrate and to form gonadal mesenchyme after migration.

Abnormal tight junction assembly of the gonadal epithelia

Because epithelial cells are characterized by specialized cellular junctions, we examined whether tight junction assembly is affected in the gonadal epithelia of *Emx2* KO. The intracellular component ZO-1 normally interacts with the homomeric tight junction protein occludin, and together, they localize to the apicolateral region in epithelial cells. aPKC forms a complex with PAR3 and PAR6 that localizes to tight junctions and regulates tight junction assembly (21, 22). These marker proteins were normally localized to the apicolateral region of the gonadal epithelia in wild-type embryos at E10.5 (Fig. 4) but were disrupted in *Emx2* KO. Occludin distribution is expanded deeply to the lateral domain, whereas ZO-1 and aPKC are localized irregularly to whole aspects of the cellular surface. These data suggest that the *Emx2* KO gonadal epithelia have lost their cell polarity.

Affected gene expression in Emx2 KO embryonic gonads

To assess the effect of *Emx2* gene disruption, gonadal epithelia were microdissected from wild-type and *Emx2* KO embryos at E10.5 to prepare total RNA. After biotinylation, these samples were used as probes for microarray analysis. Genes that satisfied a pairwise comparison test and displayed a more than 4-fold change in expression are summarized in Table 1. Because *Emx2* KO gonad showed affected cell polarity, genes encoding the components of tight and adherens junctions, as well as *Snail* and *Slug*, which regulate transcription of the junction component genes (21, 52), were expected to be affected in the KO gonad. However, none of these genes showed differential expression greater than 4-fold.

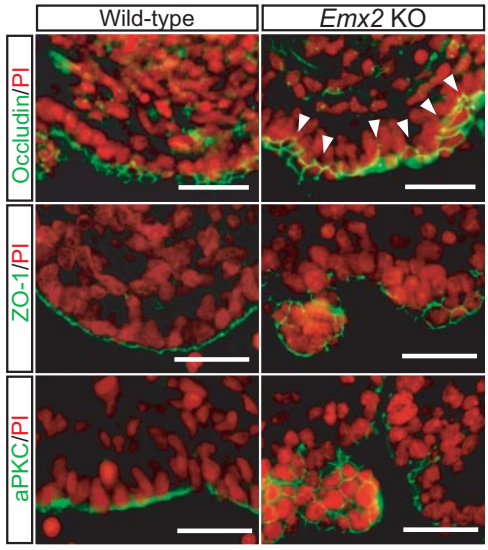

FIG. 4. Abnormal distribution of tight junction marker proteins. Wild-type (*left*) and *Emx2* KO (*right*) embryonic gonads at E10.5 (ts 8–9) were sectioned and stained with antibodies against occludin, ZO-1, and aPKC (*green*). The nuclei were stained with PI (*red*). The apicolateral distribution of marker proteins observed in wild-type embryos was disrupted in *Emx2* KO. *Arrowheads* indicate that occludin distribution expanded deeply to the lateral domain of cell surface. *Scale bars*, 25 μm.

EGFR ectopically induced in Emx2 KO embryonic gonads

Our microarray data listed *Egfr* as the top-scored gene induced in the *Emx2* KO gonad. Quantitative RT-PCR revealed an approximately 64-fold increase in *Egfr* expression in *Emx2* KO tissue compared with wild type (Fig. 5A). Furthermore, immunohistochemistry demonstrated that EGFR is expressed at a low level in the gonadal epithelia of wild-type embryos, whereas the expression is high in both the epithelial and mesenchymal cells of the *Emx2* KO gonad (Fig. 5B). In contrast, EGFR was not induced in the nephric duct and mesonephric tubules of KO.

The microarray study strongly suggested that *Egfr* gene is suppressed by EMX2. Therefore, we used M15 cells derived from mesonephric epithelial cells to test this hypothesis. Western blot analyses using an anti-EMX2 antibody showed that EMX2 is not expressed in M15 cells (Fig. 5D), whereas immunohistochemistry, Western blotting, and RT-PCR revealed that EGFR is expressed in the cells (Fig. 5, C–E). As expected, when Emx2(HA) was overexpressed, EGFR expression was reduced, as assayed by immunohistochemistry, Western blotting, and RT-PCR. Moreover, when the Emx2(HA)-overexpressing cells were treated with siRNA for *Emx2*, the expression of EGFR was significantly up-regulated. Such up-regulation was never observed with control siRNA.

TABLE 1. Down-regulated and up-regulated genes in *Emx2* KO gonadal epithelia

Gene symbol	Log2 fold change	P	Description	GenBank accesion no.
Down-regulated				
Cbln1	−4.42	0.00115262	Cerebellin 1 precursor protein	NM_019626
Inhbb	−3.47	0.00095948	Inhibin β-B	NM_008381
Dct	−3.34	0.00599337	Dopachrome tautomerase	NM_010024
Pnlip	−2.76	0.00564109	Pancreatic lipase	NM_026925
Enpep	−2.53	0.03860764	Glutamyl aminopeptidase	NM_007934
Myh6	−2.37	0.00233953	Myosin, heavy polypeptide 6, cardiac muscle, α	NM_010856
Sept4	−2.22	0.00394216	Septin 4	NM_011129
Hpgd	−2.12	0.00095948	Hydroxyprostaglandin dehydrogenase 15 (NAD)	NM_008278
Up-regulated				
Egfr	4.61	0.00003547	EGFR	NM_007912 NM_207655
Tbx18	3.73	0.00095948	T-box18	NM_023814
Crh	3.45	0.00643128	Corticotropin releasing hormone	NM_205769
Gpm6a	2.55	0.00156104	Glycoprotein m6a	NM_153581
Fut9	2.45	0.00115262	Fucosyltransferase 9	NM_010243
Slitrk6	2.20	0.00494608	SLIT and NTRK-like family, member 6	NM_175499
GA17	2.10	0.00886440	Dendritic cell protein GA17	NM_145380
Epha3	2.00	0.00864173	Eph receptor A3	NM_010140

Gene expression was compared between wild-type and *Emx2* KO gonadal epithelia at E10.5 (ts 7) by microarray analysis. Genes showing more than 4-fold change are listed ($P < 0.05$). All expression data have been deposited in the Gene Expression Omnibus of NCBI (accession no. GSE10216; http://www.ncbi.nlm.nih.gov/geo/). SLIT, *Drosophila* slit gene homolog; NTRK, neurotrophic tyrosine receptor kinase.

Ectopic activation of c-Src in Emx2 KO gonad

Recently, activated EGFR was shown to phosphorylate a tyrosine residue of c-Src/c-Yes, and phosphorylated c-Src/c-Yes in turn phosphorylates a tyrosine residue of PAR3. Through this successive tyrosine phosphorylation, EGFR signaling is thought to fine-tune tight junction assembly (25, 53). Therefore, we examined phosphorylation of EGFR and c-Src in the *Emx2* KO gonads. Interestingly, tyrosine phosphorylation of c-Src is clearly activated in the *Emx2* KO gonad in a pattern that overlaps with that of ectopically induced EGFR. A low level of c-Src phosphorylation is detected in the epithelial cells of wild-type gonads (Fig. 5F). It has been well established that EGFR is phosphorylated at the tyrosine 845 by activated c-Src and autophosphorylated at tyrosine 1068 upon ligand binding followed by dimerization (54). As indicated in Fig. 5G, phosphorylation of EGFR at the tyrosine 845 was clearly elevated in the *Emx2* KO gonad, whereas that at the tyrosine 1068 was unlikely elevated.

Discussion

Migration of coelomic epithelial cells to the mesenchymal compartment during development of the gonadal primordium

Based on histological observations, it has long been surmised that regions of the coelomic epithelia at the both sides of the mesentery proliferate and migrate mediodorsally to give rise to the gonadal primordium (1–3). However, this has not been addressed directly by cell fate mapping. Here, we chemically labeled coelomic epithelial cells at E10.25 and found that the labeled epithelial cells migrated through the basement membrane. After migration, they began to express *Ad4BP/SF-1*, a marker gene for Sertoli and Leydig cell lineages (47), strongly suggesting that the migrated epithelial cells differentiated into these gonadal somatic cells.

Transition of the gonadal epithelial cells to mesenchyme was previously demonstrated with mouse embryonic gonad at around E11.5 (55). Interestingly, the migrated cells at E11.2– E11.4 differentiated into Sertoli and interstitial cells, whereas those at E11.5– E11.7 no longer developed into Sertoli cells. Unfortunately, our study with E10.25 embryos failed to culture the gonads until Sertoli and Leydig cells differentiate, and thus it remains unsolved whether the epithelial cells at the earlier stage develop into Sertoli and Leydig cells. A new culture system, which enables to culture the early gonadal primordium for a longer period, is required to resolve the issue.

Emx2 implicated in the maintenance of epithelial polarity and the epithelial-to-mesenchyal transition

Many tissues are known to undergo epithelial-to-mesenchymal transition and/or mesenchymal-to-epithelial transition during the development. These transitions are closely correlated with the assembly and disassembly of tight junctions. In the present study, we have demonstrated that ectopic tight junctions are formed in *Emx2* KO gonadal epithelia, and thus it is assumed that the aberrant persistence of tight junctions inhibits the epithelial-

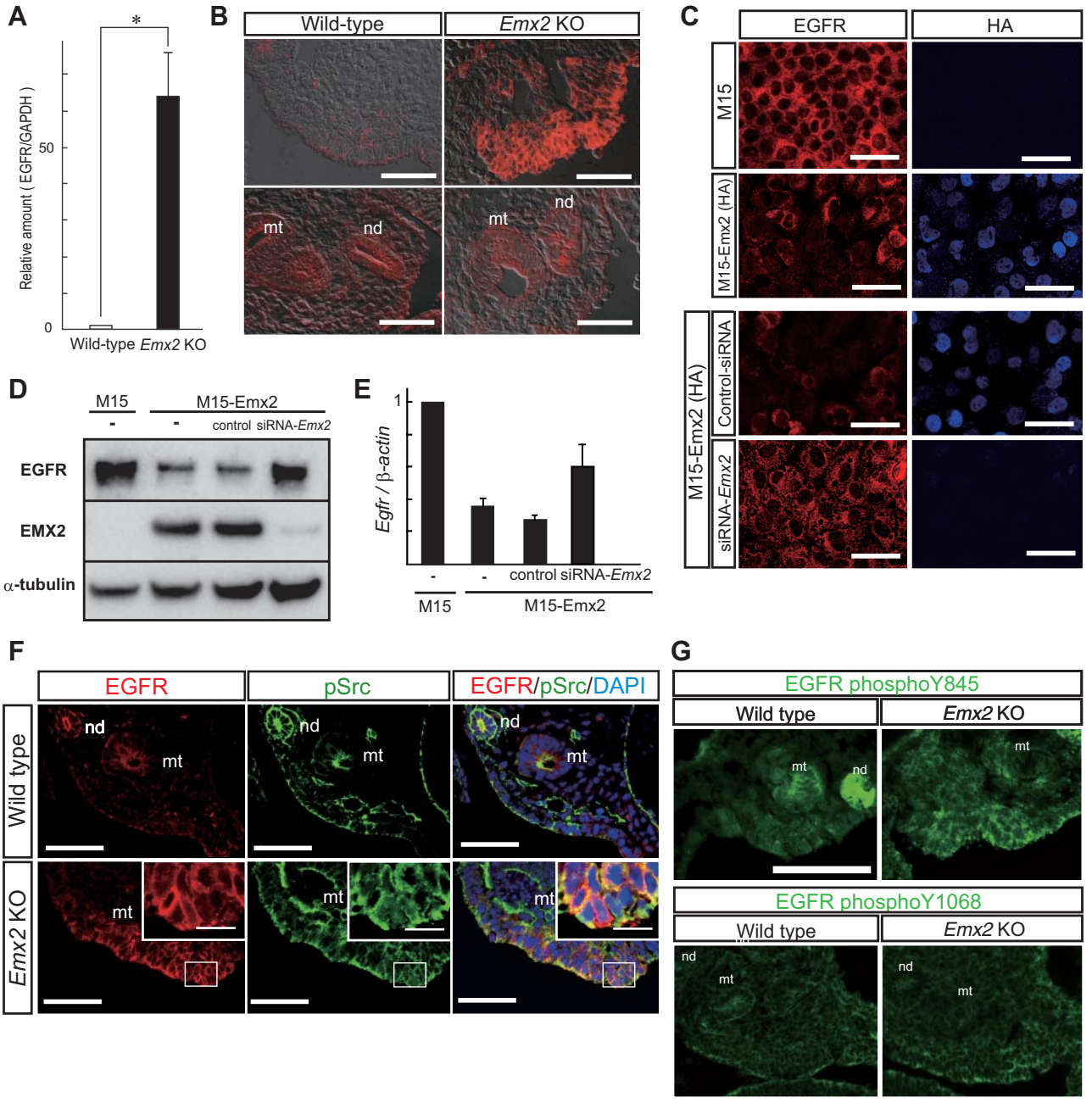

FIG. 5. EGFR gene expression suppressed by *Emx2*. A, Increased *Egfr* mRNA revealed by quantitative RT-PCR. Total RNA used in microarray analysis was subjected to RT-PCR for *Egfr*. *Gapdh* was used as a control. The amount of *Egfr* relative to that of *Gapdh* is shown, with wild-type levels set at 1. Values are the means ± SD; *, $P < 0.001$. B, Increased EGFR protein revealed by immunohistochemical staining. Sections prepared from wild-type and *Emx2* KO embryos at E10.5 (ts 9) were stained with EGFR antibody (*red*). Images for the gonadal (*upper*) and mesonephric regions (*lower*) are shown. *Scale bars*, 50 μm. nd, Nephric duct; mt, mesonephric tubule. C, Immunohistochemical examination of EMX2 and EGFR. After M15 cells overexpressing Emx2(HA) [M15-Emx2(HA)] were cultured for 48 h, they were immunostained with antibodies to HA-tag (*blue*) and EGFR (*red*). After M15-Emx2(HA) cells were cultured for 24 h, they were treated with siRNA for *Emx2* (siRNA-*Emx2*) or control siRNA (control siRNA) for 24 h. These cells were immunostained as above. *Scale bars*, 50 μm. D, Immunoblotting of M15 and M15-Emx2(HA) cells. Total cell extracts (15 μg) prepared from M15, M15-Emx2(HA), and M15-Emx2(HA) treated with siRNA-*Emx2* or control siRNA were used. EGFR, EMX2, and α-tubulin were detected with specific antibodies. E, Quantitative RT-PCR analysis of EGFR in M15 and M15-Emx2(HA) cells. Total RNA prepared from M15, M15-Emx2(HA), and M15-Emx2(HA) cells treated with siRNA-*Emx2* or control siRNA was used. The amount of *Egfr* mRNA relative to *β-actin* is plotted with levels in control M15 cells set at 1. Values are the means ± SD. F, Abnormal phosphorylation of c-Src in *Emx2* KO gonads. Sections prepared from wild-type and *Emx2* KO embryos at E10.5 (ts 7) were stained with EGFR antibody (*red*) and tyrosine-phosphorylated c-Src (pSrc) (*green*). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (*blue*). *Superimposed images* are shown at the *right*. The *regions enclosed by squares* are enlarged. G, Tyrosine phosphorylation of EGFR. Sections prepared from wild-type and *Emx2* KO embryos at E10.5 (ts 9–11) were stained with an antibody to EGFR phosphorylated at tyrosine 845 (Y845) or tyrosine 1068 (Y1068) (*green*). *Scale bars*, 50 μm. nd, Nephric duct; mt, mesonephric tubule.

to-mesenchymal transition during early gonadal development. As described below, this defect may correlate with ectopic EGFR expression in the developing *Emx2* KO gonad.

In addition to the gonad, *Emx2* is expressed in the epithelial cells of the ureteric bud, Wolffian duct, Müllerian duct, and mesonephric tubule (56). Interestingly, extension and branching of the ureteric bud are affected in *Emx2* KO. Similarly, Wolffian duct and mesonephric tubules degenerate after the structures initially develop, whereas Müllerian duct fails to develop (8). Given that these structures are formed via a mesenchymal-to-epithelial transition, *Emx2* is thought to be involved in both directions of transitions. However, because the expression of *Egfr* was unaffected in the tubular structures, *Emx2* may regulate two transition processes through differential target gene expression.

Potential function of EGFR in developing gonadal epithelial cells

Microarray studies clearly demonstrated that the *Egfr* expression was up-regulated in the *Emx2* KO gonads, and consequently, the question of why *Egfr* should be negatively regulated by *Emx2* arises from our studies. Given that EGFR is not required throughout gonad development, one would expect the *Egfr* locus to be silenced, possibly through inactivation by a suppressive chromatin state. However, considering that *Egfr* expression is activated by *Emx2* gene disruption, *Egfr* gene is not structurally silenced in the early gonad; instead, it is kept in a state that is ready to be activated.

Recently, Wang *et al.* (25) demonstrated that activated EGFR phosphorylates a tyrosine residue of c-Src/c-Yes, and subsequently, the phosphorylated c-Src/c-Yes phosphorylates a tyrosine residue of PAR3. Because tight junction assembly is delayed but not blocked with a phosphorylation-defective mutant of PAR3, EGFR signaling is thought to fine-tune tight junction assembly through successive tyrosine phosphorylation. Consistent with these observations, our data show that tyrosine-phosphorylated c-Src accumulates in the *Emx2* KO embryonic gonad, in which *Egfr* is ectopically up-regulated.

EGFR is phosphorylated at multiple tyrosine residues. Upon ligand binding and dimerization, autophosphorylation of EGFR occurs at several tyrosine residues, including Y1068. Likewise, activated c-Src phosphorylates several tyrosine residues, including Y845 (54). The present study showed that phosphorylation at Y1086 was not elevated in the *Emx2* KO gonad, whereas that at Y845 was elevated. This phosphorylation status strongly suggested that EGFR is phosphorylated by the activated c-Src but not EGFR itself. In fact, c-Src was phosphorylated at tyrosine residue and thus activated in the KO gonad. Taken together, the overexpressed EGFR was activated possibly by c-Src in the KO gonad, although it remains to be clarified how *Emx2* gene disruption causes c-Src activation.

Importantly, our immunohistochemical studies reproducibly detected EGFR and tyrosine-phosphorylated c-Src at low levels in the gonadal epithelial cells of wild type. Considering that the epithelial cells migrate to the mesenchymal compartment at an early stage of gonadal development, tight junctions of the epithelial cells should be disassembled sporadically before migration. EGFR signaling may act as the cue for this disassembly. Taken together, our data demonstrate that Emx2 guarantees proper gonadal development by regulating *Egfr* expression and thus modulating tight junction assembly.

Microarray analyses identified a number of genes whose expression was up- or down-regulated in the *Emx2* KO gonadal epithelial cells. Among the genes that showed up- and down-regulated expressions in the *Emx2* KO gonads, we highlighted the up-regulated expression of *Egfr* in the KO. However, this does not necessarily exclude the possible role of other genes described above in gonad development. Through its regulation of EGFR and possibly other genes, *Emx2* appears to play a crucial role in regulating epithelial cellular junctions of the early developing gonad.

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