Epithelial expression of Gata4 and Sox2 regulates specification of the squamous–columnar junction via MAPK/ERK signaling in mice

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The squamous–columnar junction (SCJ) is a boundary consisting of precisely positioned transitional epithelium between the squamous and columnar epithelium. Transitional epithelium is a hotspot for precancerous lesions, and is therefore clinically important; however, the origins and physiological properties of transitional epithelium have not been fully elucidated. Here, by using mouse genetics, lineage tracing, and organoid culture, we examine the development of the SCJ in the mouse stomach, and thus define the unique features of transitional epithelium. We find that two transcription factors, encoded by Sox2 and Gata4, specify primitive transitional epithelium into squamous and columnar epithelium. The proximal–distal segregation of Sox2 and Gata4 expression establishes the boundary of the unspecified transitional epithelium between committed squamous and columnar epithelium. Mechanistically, Gata4-mediated expression of the morphogen Fgf10 in the distal stomach and Sox2-mediated Fgfr2 expression in the proximal stomach induce the intermediate regional activation of MAPK/ERK, which prevents the differentiation of transitional epithelial cells within the SCJ boundary. Our results have implications for tissue regeneration and tumorigenesis, which are related to the SCJ.

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Boundaries separating neighboring tissues with distinct functions are required for proper organogenesis and tissue homeostasis. The squamous-columnar junction (SCJ) in mammals, e.g., the esophageal–gastric junction and uterine cervix, is one of the boundaries that partition functionally distinct epithelial types. The squamous epithelium serves as a strong barrier against mechanical stress, whereas columnar epithelium produces mucus to protect epithelial cells from external stimuli such as acid (e.g., gastric and bile acid) and invading microorganisms. Because the SCJ is located between functionally distinct epithelial types, the cells adjacent to it are exposed to stresses from the two distinct environments. Indeed, esophageal ulcers caused by mechanical stress or gastroesophageal reflux often occur at the junction between the esophagus and stomach, and these lesions need to be repaired. Additionally, SCJs are hot spots for metaplastic lesions, e.g., intestinal metaplasia in the esophageal–gastric junction and squamous metaplasia in the uterine cervix. Metaplastic lesions are adaptive states that form in response to abnormal stimuli, and often give rise to carcinomas when the stimuli persist for long periods of time. Accordingly, stem-like cells have been implicated in the regeneration and tumorigenesis of damaged SCJs.

Recent studies showed that a KRT7-expressing transitional epithelium exists between the squamous and columnar epithelia at the esophageal–gastric junction and uterine cervix. Furthermore, genetic manipulation of the cells constituting the transitional epithelium in mouse models and human organoids suggests that these cells could be the origin of metaplasia at SCJs. Taken together, these observations raise the possibility that transitional epithelial cells have stem-like properties to maintain homeostasis of SCJs.

Although the involvement of the transitional epithelial cells in regeneration and tumorigenesis related to SCJs is proposed, the origins and physiological properties of transitional epithelial cells remain to be clear. During development, the SCJ is established at the later embryonic stage, when squamous and columnar epithelia are differentiated from a common pseudostratified epithelium. A previous study suggested that the embryonic pseudostratified epithelium resides in adult transitional epithelium, and also proposed the possibility that the residual embryonic cells are the origin of Barrett’s metaplasia. In addition, a key developmental gene regulating the regionalization of a gastro-intestinal tract are aberrantly expressed in the Barrett’s metaplasia. These observations prompted us to examine the development of transitional epithelium.

In this study, using mouse genetics, lineage tracing, and organoid culture, we define the unspecified feature of transitional epithelial cells that is mediated by MAPK/ERK activation. We find that two transcription factors, encoded by Sox2 and Gata4, specify primitive transitional epithelium into squamous and columnar epithelium while modulating the diagonal relationship between epithelial Fgf21 in the proximal stomach and mesenchymal Fgf10 in the distal stomach. Thus, the proximal–distal segregation of Gata4 and Sox2 expression levels confines the MAPK/ERK-activated transitional epithelial cells within the SCJ boundary during development.

**Results**

**Confinement of the primitive KRT7+ transitional epithelium within SCJ during stomach development.** In mouse stomach, the SCJ consists of precisely positioned KRT7+ transitional epithelium between P63+KRT14+LOR+ squamous epithelium in the proximal stomach and GATA4+CLDN18+ columnar epithelium in the distal stomach (Fig. 1a and Supplementary Fig. 1a). At embryonic day (E)18.5, the KRT7+ transitional epithelium can be further divided into KRT7+P63+KRT14+ transitional epithelium at the proximal side and KRT7+P63−KRT14− transitional epithelium at the distal side (Fig. 1a). To identify the development of KRT7+ transitional epithelium, we first observed the boundary between P63+ and GATA4+ stomach epithelium, where the SCJ is ultimately formed. Both P63+ proximal stomach epithelium and GATA4+ distal stomach epithelium at E13.5 shape pseudostratified structures with expressing KRT7 (Fig. 1b), histologically similar to KRT7+KRT14+ transitional epithelium at E18.5. In the proximal stomach, the KRT7+ epithelium lacks expression of P63 at E11.5, but starts to express P63 by E13.5 and KRT14 by E15.5 (Fig. 1b and Supplementary Fig. 1b, c). As P63 is required for the formation of a KRT14+ basal layer, KRT7+P63−KRT14+ epithelium is the primitive epithelial type of the proximal stomach. In the distal stomach, KRT7+ epithelium prominently expressed GATA4 at E11.5 and E13.5 (Fig. 1b). By contrast, the distal stomach epithelium was covered by the KRT7+GATA4+CLDN18+ columnar epithelium at E15.5 and E18.5 (Fig. 1a and Supplementary Fig. 1c), suggesting that KRT7+GATA4+ epithelium differentiates into columnar epithelium. Accordingly, KRT7+ transitional epithelium might be the primitive epithelial type harboring bidirectional differentiation potential into squamous and columnar epithelium in the developing stomach.

**Expression patterning of SOX2 and GATA4 in the stomach epithelium during development.** Sox2 and Gata4, which encode transcription factors, are co-expressed in a stomach primordium at E9.515,19. Both SOX2 and GATA4 are important for the boundary formation of a gastro-intestinal tract; SOX2 defines the boundary of the prospective stomach and intestine around E11.5 in conjunction with CDX2, while GATA4 regulates the formation of the posterior boundary between jejunum and ileum in combination with GATA61. It should be noted that SOX2 is predominantly expressed in the squamous epithelium, whereas GATA4 expression is restricted to the columnar epithelium in the newborn mouse15,19, raising the possibility that SOX2 and GATA4 are involved in the formation of the SCJ boundary. Hence, we examined the expression patterning of SOX2 and GATA4 in the stomach epithelium during development. For that purpose, we performed immunohistochemistry (IHC) together with lineage tracing experiments using Sox2-CreERT2+; RosaLacZ mice and Gata4-CreERT2+; RosaLacZ mice (Supplementary Fig. 2a, b). SOX2 was broadly expressed in the epithelial cells of the foregut from esophagus to pancreas at E8.5, and SOX2 expression was excluded from pancreas and duodenum by E11.5 (Supplementary Fig. 1d). SOX2 expression gradually formed a proximal–distal gradient in the stomach epithelium from E11.5 to E13.5 and was largely downregulated in the distal stomach at E18.5 (Fig. 1c). By contrast, GATA4 expression shaped the distal–proximal gradient from E9.5 to E11.5 and was subsequently restricted to the distal stomach after E15.5 (Fig. 1c, d). Notably, lineage tracing experiments using Gata4-CreERT2+; RosaLacZ embryos showed that GATA4-expressing cells retained the differentiation potential into P63+ basal cells at earlier stages (E9.5–E13.5) but not at E15.5 (Supplementary Fig. 1e).

**SOX2 specifies primitive KRT7+ transitional epithelium into squamous epithelium.** To investigate the roles of SOX2 and GATA4 in the specification of the primitive KRT7+ epithelium, we genetically depleted Sox2 and Gata4 in the stomach epithelium during development. We first crossed the Sox2CreERT2+; RosaLacZ mice with Sox2flx/flx mice to obtain Sox2CreERT2+; RosaLacZ mice, in which Sox2 can be depleted in the stomach epithelium and Cre-mediated recombination can be visualized upon tamoxifen (TAM) treatment. Pregnant females were treated with TAM at E11.5 or E13.5, and the stomach was analyzed at E18.5 (Fig. 2a). Genetic depletion of Sox2 at E11.5 or E13.5 resulted in the defect of LOR+ keratinized layers and the
Fig. 1 Expression patterning of SOX2 and GATA4 in the stomach epithelium during SCJ development. 

a Immunofluorescence (IF) analyses of P63 and LOR (top), KRT7 and KRT14 (middle), or GATA4 and CLDN18 (bottom) for the SCJs of wild-type stomachs at E18.5. All samples were counterstained with DAPI. SE squamous epithelium, TE transitional epithelium, CE columnar epithelium. Presented data are a representative image of \( n = 5 \). Scale bar, 100 \( \mu \)m.

b IF analyses of KRT7, P63, and GATA4 for wild-type stomachs at E11.5 and E13.5. PS proximal stomach epithelium, DS distal stomach epithelium. Presented data are a representative image of \( n = 5 \). Scale bar, 100 \( \mu \)m.

c IF analyses of SOX2, P63, and GATA4 for wild-type stomachs at E9.5, E11.5, E13.5, E16.5, and E18.5. Arrows indicate the P63\(^+\) cells on the epithelium at the most caudal side. Arrowheads indicate GATA4\(^+\) epithelial cells at the most rostral side. Dashed lines indicate the intermediate epithelial cells with low expression of both P63 and GATA4. Presented data are a representative image of \( n = 4 \). Scale bar, 100 \( \mu \)m.

d Left: whole-mount X-gal staining on the stomach of the Gata4\(^{CreERT2}\); Rosa26\(^{lacZ}\) embryos. Presented data are a representative image of \( n > 8 \) embryos out of three independent experiments. Scale bars, 1 mm. Right: quantification of the X-gal\(^+\) area in the proximal stomach. Data are presented as mean values \( \pm \) SD. \( n = 3 \) independent experiments, one-way ANOVA.
**Fig. 2** SOX2 specifies primitive transitional epithelium into squamous epithelium. **a** Left: the scheme for the Sox2 conditional knock out (Sox2 cKO) experiment in the embryonic stomach. Right: the whole-mount X-gal staining on the control, Sox2 cKO (E11.5), and Sox2 cKO (E13.5) stomachs at E18.5. Presented data are a representative image of \( n = 4 \) embryos out of three independent experiments. Scale bar, 1 mm. **b** Left: H&E staining and IF analyses of LOR, KRT7, and SOX2 (top), or P63, KRT14, and SOX2 (bottom) for the control, Sox2 cKO (E11.5), and Sox2 cKO (E13.5) stomachs at E18.5. Presented data are a representative image of \( n = 4 \). Scale bar, 100 \( \mu m \). Right: quantification of the number of P63\(^{+}\)KRT14\(^{+}\) basal cells in the proximal epithelial cells. Data are presented as mean values ± SD. \( n = 4 \) independent experiments, one-way ANOVA. **c** H&E staining and IHC analyses of SOX2, KRT14, and KRT7 for the ΔNp63 KO proximal stomach at E18.5. Presented data are a representative image of three independent experiments. Scale bar, 100 \( \mu m \). **d** A scheme illustrates the Sox2-mediated specification of the primitive transitional epithelium into the squamous epithelium. SE squamous epithelium, TE transitional epithelium.
replacement of squamous epithelium by KRT7+ transitional epithelium in the proximal stomachs at E18.5 (Fig. 2b). Close inspections identified that Sox2 depletion at E11.5 caused the partial expansion of the KRT7+KRT14+ transitional epithelium in the proximal stomach, whereas depletion at E13.5 caused the widespread expansion of KRT7+KRT14+ transitional epithelium (Fig. 2b and Supplementary Fig. 3a–c).

To elucidate the roles of Sox2 in the differentiation of the primitive transitional epithelium into squamous epithelium, we genetically ablated P63 (specifically the ΔNp63 isoform), which is the master transcription factor for the formation of the stratified squamous epithelium12,23. In the ΔNp63-deficient stomach at E18.5, the KRT7+KRT14+ transitional epithelium was persistent in the proximal stomach (Fig. 2c and Supplementary Fig. 3e). ΔNp63-deficient KRT7+ transitional epithelium expressed Sox2 (Fig. 2c and Supplementary Fig. 3d), indicating that P63 is dispensable for the maintenance of Sox2 expression. Notably, Sox2 depletion at E11.5 caused the defect of P63 expression (Fig. 2b and Supplementary Fig. 3a), whereas Sox2 depletion at E13.5 did not affect P63 expression (Fig. 2b), indicating that Sox2 is required for P63 induction but not for the maintenance of P63 expression. Together, our findings demonstrate that Sox2 plays a crucial role in the specification of the primitive KRT7+P63–KRT14+ transitional epithelium into KRT7–KRT14+ transitional epithelium, which eventually gives rise to squamous epithelium (Fig. 2d).

Even though Sox2 was also expressed in the distal stomach at E11.5, the expression levels of GATA4 and CLDN18 in the distal stomach at E18.5 were not affected by Sox2 depletion at E11.5 (Supplementary Fig. 3), implying that Sox2 does not have an impact on the specification of the primitive KRT7+ transitional epithelial cells into columnar epithelial cells.

**GATA4 specifies primitive KRT7+ transitional epithelium into columnar epithelium.** We next crossed Sox2CreERT2/+; Gata4fl/lox; RosalacZ males with Gata4tdTomato+ (Supplementary Fig. 2c) females to obtain Sox2CreERT2/+; Gata4tdTomato/lox; RosalacZ mice, in which Gata4 can be conditionally depleted in the stomach epithelial cells upon TAM treatment, and Gata4 expression as well as Cre-mediated recombination can be visualized. We treated the pregnant females with TAM at E9.5, and then the stomachs were analyzed at E18.5 (Fig. 3a). Genetic depletion of Gata4 in the stomach epithelial cells at E9.5 resulted in disruption of pit structures, characteristics of the mature glandular stomach (Fig. 3b). Histologically, GATA4-deficient epithelial cells at the distal stomach lost the columnar epithelial structure and CLDN18 expression, but exhibited a pseudostratified structure with mis-expression of KRT7 (Fig. 3c, d), suggesting that GATA4 specifies the primitive KRT7+ transitional epithelium into the columnar epithelium. Notably, some ectopic transitional epithelium contained a KRT14+ basal layer (Fig. 3e). Furthermore, GATA4-deficient KRT7+ cells in the distal stomach mis-expressed Sox2, and the P63+KRT14+ basal layer was preferentially observed in ectopic KRT7+ transitional epithelium that mis-expressed Sox2 (Fig. 3e and Supplementary Fig. 4a), supporting our conclusion that Sox2 specifies primitive KRT7+ transitional epithelium into KRT7–KRT14+ transitional epithelium. Ectopic KRT7+KRT14+ transitional epithelium at E18.5 was also detected following Gata4 depletion at E11.5, but was almost absent when Gata4 was depleted at E13.5 (Supplementary Fig. 4b, c). These findings imply that the proximal–distal patterning of the primitive KRT7+ transitional epithelium is completed around E13.5.

To examine the mechanisms underlying the proximal–distal patterning of the primitive KRT7+ transitional epithelium mediated by GATA4 and Sox2 expression levels, we performed a gain-of-function experiment. We injected Col::tetO-Gata4-HA-IRES-mCherry; Rosa26 CreERT2/+; Gata4fl/lox; Foxa2–/–; Foxn4–/–; Cckar–/–; Wnt7b–/– and Synec1–/– embryonic stem cells (ESCs) into wild-type blastocysts to generate chimeric mice. We treated pregnant females carrying chimeric embryos with doxycycline (DOX) starting at E11.5 and analyzed the embryos at E18.5 (Fig. 3f). The contribution of Gata4-overexpressing cells was visualized by mCherry fluorescence (Supplementary Fig. 4d). Overexpression of GATA4 resulted in ectopic emergence of CLDN18+ columnar epithelium-like cells in the proximal stomach (Fig. 3f). GATA4-overexpressing cells in the proximal stomach epithelium, marked by hemagglutinin (HA) expression, downregulated the expression of SOX2 and its downstream of P63, KRT14, and LOR (Fig. 3g and Supplementary Fig. 4e). Taken together, these observations indicate that GATA4 specifies the primitive KRT7+ transitional epithelium into the columnar epithelium with downregulating SOX2 expression, which promotes the specification of primitive KRT7+ transitional epithelium into squamous epithelium (Fig. 3h). In summary, primitive KRT7+ transitional epithelium is the unspecified epithelial type that is specified into columnar or squamous epithelium, dependent on the expression levels of GATA4 and SOX2.

**Isolation and characterization of the primitive KRT7+ transitional epithelial cells with different expression levels of Sox2 and Gata4.** Next, we asked how the KRT7+ transitional epithelium remains unspecified between committed squamous and columnar epithelium in the stomach. We generated dual-reporter mice (SGGT mice) harboring EGFp and td Tomato reporter alleles under the control of the Sox2 and Gata4 promoters by crossing Sox2EGFP+/– knock-in mice with Gata4tdTomato+– knock-in mice (Supplementary Fig. 2c). We confirmed the results of IHC analyses showing that Sox2 is equally expressed in the proximal and distal stomach at E11.5, but downregulated in the distal stomach at E18.5 (Fig. 4a). GATA4 was broadly expressed in the whole stomach at E11.5, but downregulated in the proximal stomach after E13.5 (Fig. 4a), consistent with the results of the lineage tracing experiment. Using fluorescence-activated cell sorting (FACS), we fractionated EpCAM+ epithelial cells in the stomach at E11.5, E13.5, and E18.5 into three populations according to their fluorescence intensities (gating strategies are described in Supplementary Fig. 5a), and designated the cells in the three populations as Sox2hi (Gata4lo), Sox2midGata4mid, and Sox2lowGata4hi (Fig. 4b).

To examine the molecular characteristics of Sox2hi, Sox2midGata4mid, and Gata4hi cells, we next performed RNA-sequencing (RNA-seq) analyses. We confirmed that SGGT fluorescence intensities reflected the expression levels of Sox2 and Gata4 (Fig. 4c). Remarkably, Krt7 was equally expressed in between Sox2hi, Sox2midGata4mid, and Gata4hi cells, while the expression levels of Krt14 and Cldn18 were lower than Krt7 at E13.5, when the stomach epithelium uniformly assumes the pseudostratified structures but is almost specified into squamous or columnar epithelium (Figs. 1b, d and 4c). Those cellular characteristics were also supported by our RNA-seq results that Sox2hi cells highly expressed key transcription factors involved in the development of the squamous epithelium, including p63 and Foxa2 (Fig. 4c), whereas Gata4hi cells expressed transcription factors associated with the development of the columnar epithelium, including Gata6hi, Pax6hi, and Hnf4a (Fig. 4c). Notably, Sox2midGata4mid cells expressed a negligible level of p63 relative to Sox2hi cells, as well as lower levels of Gata4, Gata6, and Hnf4a relative to Gata4hi cells (Fig. 4c), explicitly indicating their unspecified character. In addition, we found that the expression levels of Cckar, Wnt7b, and Synec1 were highest in Sox2midGata4mid cells (Fig. 4d). RNA in situ hybridization (RNA-ISH) analyses revealed that cells expressing CCKAR, Wnt7b, and Synec1 were localized in the boundary between the GATA4+ proximal stomach and the GATA4+ distal stomach at E13.5 and E15.5 (Fig. 4d and 2f).
Supplementary Fig. 5b), implying that Sox2\textsuperscript{mid} Gata4\textsuperscript{mid} cells have the unique characteristics and localizes within the boundary of the developing SCJ. Together, our transcriptome analyses suggested that Sox2\textsuperscript{mid} Gata4\textsuperscript{mid} cells at E13.5 may be the unspecified KRT7\textsuperscript{+} transitional epithelial cells. We also found that the proportion of the Sox2\textsuperscript{mid} Gata4\textsuperscript{mid} cells significantly decreased from E11.5 to E18.5 (28% at E11.5, 19% at E13.5, and 7% at E18.5) (Fig. 4b), presumably because the unspecified KRT7\textsuperscript{+} transitional epithelial cells are confined to the SCJ boundary during development.

Sorted Sox2\textsuperscript{mid} Gata4\textsuperscript{mid} cells give rise to both squamous and columnar epithelium in organoids. To evaluate the differentiation capacities of Sox2\textsuperscript{hi}, Sox2\textsuperscript{mid} Gata4\textsuperscript{mid}, and Gata4\textsuperscript{hi} cells, we performed organoid experiments. We isolated Sox2\textsuperscript{hi}, Sox2\textsuperscript{mid} Gata4\textsuperscript{mid}, and Gata4\textsuperscript{hi} cells from the stomach epithelium at E13.5 using FACS, and then cultured the single cells under three-dimensional culture conditions (Fig. 4e). After 10 days, each of the single cells autonomously formed organoids. These organoids could be subdivided into at least three distinct types according to the morphology and expression levels of Sox2 and Gata4 (Fig. 4f–h). Predominantly SOX2-expressing organoids with smaller surface areas consisted of P63\textsuperscript{+} KRT14\textsuperscript{+} basal layers and LOR\textsuperscript{+} cornified layers, which resembled the squamous epithelium (hereafter, squamous epithelium organoid: SEO).
Seo's were also less abundant in FGF10-treated + Activation of MAPK/ERK residualizes KRT7 and to the SCJ boundary at E18.5 (Fig. 5h). Together, these have the unspeci- CEOs and Sox2 not from + Intrinsic signaling pathway, we examined the expression of expression of Sox2, P63, and GAPA4 (bottom) for the stomach of the GA4 cK0 (E9.5) embryo at E185. Present data are a representative image of n=5 embryos out of three independent experiments. Scale bars, 100 μm. Top: the scheme for the GA4 overexpression (GA4 OE) experiment in the embryonic stomach. Bottom: IHC analysis of CLDN18 for the GA4 OE stomach at E18.5. Present data are a representative image of n=4 chimeric embryos out of three independent experiments. Scale bars, 100 μm. A scheme illustrates the GA4-mediated specification of the primitive transitional epithelium into the columnar epithelium. TE transitional epithelium, CE columnar epithelium.

Another type of predominantly SOX2-expressing organoids had larger surface areas that were composed of KRT7+ layers and KRT14+ basal layers, which resembled KRT7+KRT14+ transitional epithelium (hereafter, transitional epithelium organoid: TEO) (Fig. 4g). Predominantly GA4-expressing organoids consisted of GA4+CLDN18+ columnar cells, which resembled columnar epithelium (hereafter, columnar epithelium organoid: CEO). CEOs were further subdivided into PDX1+ organoids with larger surface areas and PDX1+ antirum-type organoids with smaller surface areas (Fig. 4h). Notably, Sox2hi cells mostly gave rise to SEOs, whereas GA4hi cells formed CEOs and Sox2midGA4mid cells formed all types of organoids (Fig. 4i). These findings demonstrate that Sox2midGA4mid cells have the unspeciﬁc properties with multi-lineage differentiation potential in organoids.

Activation of MAPK/ERK residualizes KRT7+ transitional epithelial cells within SCJ boundary during development. We next tested the effects of signaling molecules on the cellular differentiation in the organoid experiment and found that treatment with FGF10 signiﬁcantly changes the morphology and sizes of organoids generated from Sox2hi and Sox2midGA4mid cells, but not from GA4+ cells (Fig. 5a, b). Importantly, the percentage of TEOs significantly increased when Sox2hi cells were cultured with FGF10 (9% and 88% with no cytokine and FGF10 (100 ng/ml), respectively) (Fig. 5c). Consistent with this, the expression of KRT14 and Lor was signiﬁcantly decreased by FGF10 treatment (Fig. 5d). SEOs were also less abundant in FGF10-treated Sox2midGA4mid cells (Fig. 5e). To determine whether the increase in the abundance of TEOs was the result of the FGF10-mediated activation of the intrinsic signaling pathway, we examined the expression of phospho-ERK (pERK), an effector of MAPK/ERK, in each type of organoid. We found that pERK is predominantly expressed in the TEOs (Fig. 5f and Supplementary Fig. 5c). These ﬁndings suggest that MAPK/ERK activation blocks the differentiation of transitional epithelial cells into the squamous epithelium.

To assess the physiological activation of MAPK/ERK during development, we next performed IHC analyses of pERK in stomach epithelium. pERK expression was restricted to the KRT7+ transitional epithelium in the SCJ at E18.5 and postnatal day 56 (Fig. 5g). pERK was broadly expressed in the proximal stomach epithelium at E11.5 and E13.5 (Fig. 5h). Subsequently, pERK expression was gradually excluded from the proximal side, and eventually restricted to the SCJ boundary at E18.5 (Fig. 5i). Together, these ﬁndings suggest that MAPK/ERK activation residualizes KRT7+ transitional epithelial cells within the SCJ boundary during development.

Expression patterning of Fg10 and Fgr2 in the stomach dur- ing development. To examine which signal activates MAPK/ERK in vivo, we reevaluated our RNA-seq data to search the receptor that potentially activate MAPK/ERK during the SCJ development. Among the representative receptor tyrosine kinases27, only Fgfr2 was robustly expressed in the epithelial cells at 11.5 and E13.5 (Supplementary Fig. 6a). We also found that the expression level of Fgfr2 shapes the gradient, being highest in Sox2hi cells, moderate in Sox2midGA4mid cells, and lowest in Gata4hi cells, a pattern opposite to that of GA4 expression (Supplementary Fig. 6a). RNA-ISH analyses revealed that Fgfr2 expression in the proximal stomach epithelium was weak at E11.5, but became prominent at E13.5 and E15.5 (Fig. 6a). We next searched for FGF ligands expressed in the embryonic stomach by isolating PDGFRα-positive mesenchymal cells at E13.5, E15.5, and E17.5 (Supplementary Fig. 6b). We found that only Fg10 was highly expressed in the distal stomach mesenchyme at E13.5 and the Fg10 expression in the distal stomach mesenchyme decreased by E17.5 (Supplementary Fig. 6c). Notably, RNA-ISH analyses revealed that Fg10 expression in the distal mesenchyme was weak at E11.5, prominent at E13.5, and restricted to the distal end at E15.5 (Fig. 6a). Thus, Fg10/Fgfr2 signaling axis in the stomach is established after E11.5, and the distance between Fgfr2-expressing epithelial cells and Fg10-expressing mesenchymal cells increased from E13.5 to E15.5 (Fig. 6a). Based on these ﬁndings, we propose that the diagonal relationship between epithelial Fgfr2 and mesenchymal Fg10, along with their spatiotemporal patterning, is responsible for the regionally restricted activation of MAPK/ERK during the establishment of the SCJ in the stomach.

Epithelial expression levels of Gata4 and Sox2 regulate interme- diated regional activation of MAPK/ERK. We next examined whether the proximal–distal patterning of Sox2/GA4 expression interacts with MAPK/ERK activation mediated by the Fgfr2/Fg10 axis. First, we sought to determine why Sox2hi+ Sox2lox/GATA4- cells did not respond to Fg10 in the organoid experiment (Fig. 5a, b), even though Fg10 was released from the neighboring mesenchymal cells and Fgfr2 was partially expressed in Gata4hi cells (Supplementary Fig. 6a, c). From our RNA-seq data, we discovered that genes related to the negative regulation of the MAPK/ERK cascade, such as Spry2, Dusp4, and Dusp6, were highly expressed in the Gata4hi cells relative to Sox2hi and Sox2midGA4mid cells at E13.5 (Supplementary Fig. 6d). Depletion of Gata4 in the stomach epithelium at E11.5 using Sox2CreERT2/+; Gata4fl/fl embryos resulted in the mis-expression of pERK in the GATA4-deﬁcient epithelial cells at E13.5 (Fig. 6b), which persisted in the ectopic GATA4-deﬁcient KRT7+ transitional epithelium in the distal stomach at E18.5 (Fig. 6c), demonstrating that the negative regulation of MAPK/ERK in the stomach epithelium is dependent on epithelial GATA4 expression. Furthermore, when we blocked MAPK/ERK signaling by treating E11.5 embryonic stomach with SU5402 in ex vivo
culture, the number of Gata4hi cells in the stomach epithelium decreased (Supplementary Fig. 6e). This implies the existence of a negative-feedback loop between epithelial GATA4 and MAPK/ERK activation. By contrast, when we almost completely deleted Gata4 in the stomach epithelium from E11.5 to E13.5 using Gata4CreERT2/flx embryos (Fig. 6d), the activation of MAPK/ERK in the proximal stomach epithelium was significantly downregulated (pERK+/epithelial cells = 25% [control] vs. 7% [Gata4 cKO]) (Fig. 6h). Notably, expression of Fgf10 in the distal mesenchyme was downregulated in Gata4-depleted stomach at E13.5 (Fig. 6e), indicating that epithelial GATA4 expression regulates the mesenchymal Fgf10 expression.

We next examined the roles of epithelial Sox2 in MAPK/ERK activation. Pregnant females carrying Sox2CreERT2/flx embryos were treated with TAM at E11.5, and their stomachs were analyzed at E13.5 (Fig. 6f). The number of pERK+ epithelial cells in the Sox2-depleted stomachs decreased (pERK+/epithelial cells = 25% [control] vs. 5% [Sox2 cKO]) (Fig. 6h). Notably, expression of Fgfr2 was downregulated in
Sox2-deficient epithelial cells in the proximal stomach (Fig. 6g), suggesting that epithelial SOX2 expression regulates the epithelial Fgf2 expression. To confirm this idea, we performed experiments of GATA4 overexpression from E11.5 to E13.5 using KH2-Gata4 chimeric embryos, in which GATA4-overexpressing cells in the proximal stomach epithelium downregulated the expression levels of SOX2 and its target genes (Fig. 3g and Supplementary Fig. 4e). We found that expression levels of SOX2 and Fgf2 were decreased in GATA4-overexpressing cells in the proximal stomach epithelium at E13.5. In conclusion, epithelial expression levels of Gata4 and Sox2 orchestrate the intermediate regional activation of MAPK/ERK mediated by Fgf10/Fgf2 axis during development (Fig. 6i).

Activation of MAPK/ERK in human transitional epithelium and Barrett’s esophagus. Finally, we examined the activation of MAPK/ERK signaling at SCJs in humans. Different from mouse, the SCJ is not located in the stomach but at the esophageal–gastric junction in human. However, the human SCJs have common structures with the mouse SCJs in that KRT7+ transitional epithelial cells exist between squamous and columnar epithelium. We detected specific expression of pERK in KRT7+ transitional epithelium in the human esophageal–gastric junction, as well as KRT7+ transitional epithelium in the uterine cervix (Fig. 7a). Thus, the activation of MAPK/ERK cascade is a general feature of KRT7+ transitional epithelium at the SCJ boundary. Conversely, we showed that the epithelial deletion of Gata4 caused mesenchymal depletion of Fgf10 expression in distal stomach, highlighting the local crosstalk between epithelial cells and mesenchymal cells. We also found that Sox2 mediates epithelial expression of Fgf2 in the proximal stomach. Notably, the distance between Fgf2-expressing epithelial cells and Fgf10-expressing mesenchymal cells increased along with the growth of the stomach during development. Collectively, these results suggest that distant epithelial–mesenchymal cross-talk straddling the SCJ ensures the regional specificity of MAPK/ERK activation, leading to the persistence of pERK-positive transitional epithelial cells at the SCJ.

Given their unique localization, transitional epithelial cells have been implicated in tissue regeneration and maintaining homeostasis of SCJs. Here, by studying the SCJ development in mouse stomach, we showed that the activation of MAPK/ERK mediated by Fgf10–Fgf2 axis maintains the proliferative and unspecified properties of embryonic TE cells, which have the potential to differentiate into both squamous and columnar epithelial cells. Notably, activation of MAPK/ERK in the transitional epithelial cells was preserved after the establishment of the SCJ. Since the expression of Fgf10 in the mesenchyme decreased at the later developmental stage, it remains to be clear how MAPK/ERK is activated in adult transitional epithelial cells. Nevertheless, these results support the hypothesis that pERK-positive transitional epithelial cells might serve as undifferentiated stem cells capable of regenerating and maintaining the homeostasis of the adult SCJ. Future studies, using lineage tracing of the pERK-positive transitional epithelial cells and manipulating MAPK/ERK activation in the adult transitional epithelial cells, might verify this hypothesis.

Discussion
In this study, we identified the molecular mechanisms underlying the establishment of the SCJ boundary. We found that during development, pERK-positive primitive epithelial cells are confined to transitional epithelium at the SCJ boundary. Confinement of the pERK-positive cells is governed by the coordinated interplay between a concentration gradient of a signaling molecule (Fgf10–Fgf2 axis) and patterning of transcription factors (Gata4–Sox2). Moreover, transcription factor-mediated cell fate specification in epithelial cells affects mesenchymal expression of signaling molecules that play roles in the activation of the MAPK/ERK cascade.

Previous studies using tissue-swapping or explant culture experiments showed that mesenchymal directs epithelial cell fate in the stomach. Additionally, it was reported mesenchymal Fgf10 affects the cellular differentiation of the gastric gland in mice, supporting the notion that mesenchymal signals direct the epithelial cell fate. Conversely, we showed that the epithelial deletion of Gata4 caused mesenchymal depletion of Fgf10 expression in distal stomach, highlighting the local crosstalk between epithelial cells and mesenchymal cells. We also found that Sox2 mediates epithelial expression of Fgf2 in the proximal stomach. Notably, the distance between Fgf2-expressing epithelial cells and Fgf10-expressing mesenchymal cells increased along with the growth of the stomach during development. Collectively, these results suggest that distant epithelial–mesenchymal crosstalk straddling the SCJ ensures the regional specificity of MAPK/ERK activation, leading to the persistence of pERK-positive transitional epithelial cells at the SCJ.
Fig. 5 Activation of MAPK/ERK prevents the differentiation of KRT7⁺ transitional epithelial cells. a Fluorescent images of organoids generated from Sox2hi, Sox2midGata4mid, and Gata4hi cells in the culture condition with no cytokine (NC) or FGF10 (100 ng/mL). Arrows indicate the SEOs and arrowheads indicate CEOs. n = 5. Scale bar, 100 µm. b Surface areas of organoids generated from Sox2hi, Sox2midGata4mid, and Gata4hi in the culture condition with NC or FGF10 (100 ng/mL). n > 13 organoids of three independent experiments, two-sided t-test. c Quantification of organoid types generated from Sox2hi cells in the culture condition with NC or FGF10 (100 ng/mL). n = 14 independent experiments, two-sided t-test. d Quantitative (Q)-PCR analyses of Krt14 and Lor for organoids generated from Sox2hi cells in the culture condition with NC or FGF10 (100 ng/mL). The CT value of each gene is normalized by B2M. The average ΔCT value of the NC condition is set to 1. n = 6 independent experiments. Data are presented as mean values ± SD, two-sided t-test. e Quantification of organoid types generated from Sox2midGata4mid cells in the culture condition with no cytokine (NC) or FGF10 (100 ng/mL), n = 4 independent experiments. Data are presented as mean values ± SD. f Left: IF analysis of phospho-ERK (pERK) and β-catenin for TEOs. Presented data are a representative image of n > 10 organoids out of three independent experiments. Scale bar, 100 µm. Right: quantification of pERK⁺ cells in SEOs, TEOs, and CEOs. n = 3 independent experiments, one-way ANOVA. g IF analyses of pERK, KRT7, and GATA4 for wild-type stomachs at E18.5 and postnatal day 56. Arrows indicate the pERK⁺KRT7⁺ transitional epithelial cells in the SCJ. Presented data are a representative image of n = 5. Scale bar, 100 µm. h IF analyses of pERK, SOX2 and GATA4 for wild-type stomachs at E11.5, E13.5, E15.5, and E18.5. Arrows indicate the pERK⁺ epithelial cells in the boundary between proximal and distal stomachs. Presented data are a representative image of n = 5. Scale bar, 100 µm.
Notably, FGF10-mediated activation of MAPK/ERK is efficient for the long-term expansion of human Barrett’s metaplasia in organoids. Furthermore, activation of MAPK/ERK is frequently observed in adenocarcinomas associated with Barrett’s metaplasia. We found that a subset of proliferating cells in Barrett’s metaplasia expresses pERK, suggesting that these pERK-positive cells could be precursors of Barrett’s metaplasia-associated adenocarcinomas. Therefore, activation of MAPK/ERK signaling might play an important role not only in the pathogenesis of Barrett’s metaplasia, but also in development of esophageal adenocarcinomas from...
Barrett’s metaplasia, and thus may represent a potential therapeutic target.

In summary, we identified the unspecified features of transi-
tional epithelium in the SCJ, which is maintained by continuous
activation of MAPK/ERK. Our findings pave the way to an
improved understanding of pathogenesis and will facilitate the
development of strategies for cancer treatment, as well as
efficient regeneration, both of which are related to SCJs.

Methods

Vector constructions

Gata4-td Tomato-PGK-Puro-pA (2.7 kb) and CreERT2-pA-PGK-Bsd-pA (3.8kb) with 50-bp homo-
logy arms was established using KAPA HiFi HotStart ReadyMIX (KAPA
 Biosystems). This fragment was recombined into the first ATG of the exon 1 of the
Gata4 BAC (BACPAC Resources Center) using the Red/ET BAC recombination system.
The fragments of Gata4-td Tomato-pA-PGK-Puro-pA and Gata4-CreERT2-
pA-PGK-Bsd-pA sequence with 2.5-kb (5') and 4.2-kb (3') homology arms were
retrieved and used as targeting vectors.

Homologous recombination into ES cells

ES cell lines V6.5 and KH2 were used as previously described.20, 20 µg of each targeting vector was linearized using the
appropriate restriction enzymes (Gata4-td Tomato targeting vector: Scal, LSL-HA
tag-Krue),2,22 targeting vector: Prol, Gata4-CreERT2 targeting vector: Fbl, Sox2-
CreERT2 targeting vector: Sbf). All restriction enzymes were obtained from New
England Biolabs. After 37 °C overnight linearization, the products were purified and
collected by ethanol precipitation; the pellets were dissolved in 100 µL of high-glucose
DMEM (Nacalai Tesque) and resuspended in 500 µL of Tris-EDTA buffer. 0.11 mM mercaptoethanol (Gibco), and 1000 U/mL human LIF
(Sigma) and maintained in KSOM medium. Two days later, the blastocysts were
vested. Two-cell-stage fertilized eggs were collected by perfusion with M2 medium
into the uterus of each pseudo-pregnant ICR female mouse (Japan SLC). The membrane was washed twice with 2× SSC
buffer and 1× SDS, and twice with high-stringency buffer (containing 0.2 × SSC
buffer and 1× SDS). The DIG Labeling Kit for Nucleic Acids (Roche) was used for visualization, and an LAS4000 (GE Healthcare)
was used for detection. Uncrepted blots are found in Supplementary Fig. 7.

Blastocyst collection and microinjection

Blastocyst collection. Eight-week-old ICR female mice (Japan SLC) received 7.5 U
of serotonin (ASKA Animal Health) by intraperitoneal injection. Forty-eight
hours after of serotonin treatment, mice were injected with 7.5 U of gonadotropin
(LH, Sigma) and then mated with 5-8 males. These embryos were then obtained
from pregnant females for the times indicated.

Mice. Rosa26 LSL-LacZ mice36, ApN63-Cre mice23, Sox2-EGFP mice37, Sox2 flox
mice48, and Gata4 flox mice49 have been described previously. All mice were
maintained in a C57BL/6-129/c5v mixed background. For germline transmis-
sion, 8-week-old male chimeric mice were mated with 8-week-old C57BL/6 female mice
(Japan SLC) to obtain transgenic mice.

Mice genotyping. Tail tips of 3-week-old mice were collected and dissolved with 200 µL of DNA elution buffer (100 mM Tris-HCl, 5 mM EDTA, 0.2 % SDS, 200
mM NaCl, and 1% Protein kinase) at 65 °C. After centrifugation at 4 °C at 20,000 ×
g for 15 min, 3 µL of supernatant was dissolved into 100 µL of Tris-EDTA buffer.
Recombination was detected by PCR with GelEx polymerase (Takara Bio). Geno-
typing primers are shown in Supplementary Table 1.

Tamoxifen and doxycycline treatment. TAM (Sigma) was dissolved in corn oil.
For embryonic labeling and genetic manipulation, pregnant females were received a
single-dose intraperitoneal injection of TAM at the times indicated. DOX (Sigma)
was dissolved in drinking water at 0.2 mg/mL and was given to pregnant
females for the times indicated.

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**Fig. 7 Activation of MAPK/ERK in human SCJs and Barrett’s esophagus.**

**a** IF analyses of pERK and KRT7 for human esophageal-gastric junction and uterine cervix. Arrows indicate the pERK⁺KRT7⁺ transitional epithelial cells. SE squamous epithelium, TE transitional epithelium. Presented data are a representative image of n = 4. Scale bar, 100 µm. **b** Expression levels analyses of KRT7, SOX2, GATA4, and FGFR2 for esophageal squamous epithelium (SE) (n = 8), gastric columnar epithelium (CE) (n = 10), and Barrett’s metaplasia (n = 10). Raw data are extracted from GEO record GSE34619. Solid lines in each box indicate the median. Bottom and top of the box are lower and upper quartiles, respectively. **p < 0.01, one-way ANOVA.** **c** H&E staining (left) and IHC analyses of pERK (middle) and Ki67 (right) in human Barrett’s metaplasia. Arrows indicate pERK⁺Ki67⁺ cells. Top: ×10 magnifications. Bottom: ×20 magnifications. Presented data are a representative image of n = 4. Scale bar, 500 µm.
Mice experiments. All animal studies were conducted in accordance with ethical guidelines in Kyoto University and University of Tokyo and were approved by Center for iPSC Cell Research and Application, Kyoto University and Institute of University, University of Tokyo.

X-gal staining
Whole-mount staining. Pregnant females were sacrificed, and the dissected intestinal tracts of Gata4-CreERT2, Rosa LSL-LacZ and Sox2-CreERT2, Rosa LSL-LacZ embryos were collected and fixed with ice-cold 4% PFA for 2 h. After one wash with PBS, the stomach was poured into permeabilization solution (5 mM EGTA, 2 mM MgCl2, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 in PBS) and reacted with the X-gal solution (1 mg/mL X-gal, 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide in the permeabilization solution) at 4 °C for overnight.

Frozen tissue staining. After fixation of dissected tissues with PBS containing 30% sucrose, the tissues were embedded in Tissue-Tek OCT compound (Sakura). Frozen tissues were sectioned at 100-μm thickness, sliced serially into 7-μm-thick sections, and stained as described above. Counterstaining was performed using Contrast Red (KPL).

RNA extraction from tissues and organoids
RNA was extracted from tissues and organoids using the TRIzol reagent (Life Technologies) and RNeasy spin columns (Qiagen). RNA was quantitated using NanoDrop (Thermo Scientific). cDNA was synthesized from RNA using the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer’s instructions. Real-time quantitative PCR was performed using SYBR Green qPCR Master Mix (Roche). Transcript levels were normalized against the corresponding levels of B2-microglobulin mRNA.

Organoid culture. Epithelial cells were gathered from embryonic stomachs by FACs as described above. Four-well dishes (Nunc) were coated with 4% Matrigel (Corning) and cultured on 2.5% Matrigel with or without Fgf10 (R&D Systems). Medium was changed every 2 days.

RNA in situ hybridization
RNA-ISH was performed on paraffin-embedded tissues fixed in 4% PFA for 24 h following the procedure described above for paraffin-embedded specimen preparation. All probes and RNAseq 2.5HD assay-Duplex were purchased from Advanced Cell Diagnostics, and ISH was performed according to the manufacturer’s protocol. After sections were pre-treated with Preactet–3, probes were hybridized in the HybEZ oven for 2 h at 40 °C. Signals were amplified with AMP1–6, and then with alkaline phosphatase. After further amplification of the signals with AMP7–9, signals were detected by DAB and counterstained with Mayer’s hematoxylin. Probes used in this study are listed in Supplementary Table 2. Note that the probe for Gata4 is designed to bind the undeleted locus of Gata4 mRNA of Gata4-CreERT2/fox embryos that enabled to mark the GATA4-deficient epithelial cells in Fig. 6e.

Patients derived paraffin-embedded tissue samples. Patients derived paraffin-embedded tissue samples were used in accordance with ethical guidelines in Kyoto University Hospital. All patients were provided informed consent for use of the tissue samples in this research.

Statistics and reproducibility. All data are presented as means ± SD and represent a minimum of three independent experiments. Statistical parameters including statistical analysis, statistical significance, and n values are described in the figure legends. Statistical analyses were carried out using Prism 7 Software (GraphPad). Statistical comparisons of two groups were performed using the two-sided unpaired Student’s t-test, and comparisons of more than three groups were performed by one-way ANOVA. A value of p < 0.05 was considered significant. Presented data are a representative image of n = 3 out of more than three biologically independent experiments.

RNA sequencing.
Total RNA (50 ng) was purified by using the Truseq Stranded mRNA LT sample prep kit (illumina). PolyA-containing mRNA was purified using poly-T oligoattached magnetic beads, and the RNA was fragmented and primed for cDNA synthesis. Cleaved RNA fragments were reverse transcribed into first strand cDNA using transcriptase and random primers. Second strand cDNA was synthesized by the incorporation of dUTP, and ds cDNA was separated using AMPure XP beads (BECKMAN COULTER). A single “A” nucleotide was added to the 3’ ends of the blunt fragments, and then the indexed adapters were ligated to the ends of the ds cDNA. ds cDNA fragments were amplified by PCR with a PCR primer Cocktail. The number of PCR cycles was minimized (11–15 cycles) to avoid skewing the representation of the libraries. RNA-seq libraries were sequenced on NextSeq500 (75 bp single, Illumina).

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