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京都大学
Emergence of dorsal-ventral polarity in ESC-derived retinal tissue

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ABSTRACT
We previously demonstrated that mouse embryonic stem cell (mESC)-derived retinal epithelium self-forms an optic cup-like structure. In the developing retina, the dorsal and ventral sides differ in terms of local gene expression and morphological features. This aspect has not yet been shown in vitro. Here, we demonstrate that mESC-derived retinal tissue spontaneously acquires polarity reminiscent of the dorsal-ventral (D-V) patterning of the embryonic retina. Tbx5 and Vax2 were expressed in a mutually exclusive manner, as seen in vivo. Three-dimensional morphometric analysis showed that the in vitro-formed optic cup often contains cleft structures resembling the embryonic optic fissure. To elucidate the mechanisms underlying the spontaneous D-V polarization of mESC-derived retina, we examined the effects of patterning factors, and found that endogenous BMP signaling plays a predominant role in the dorsal specification. Further analysis revealed that canonical Wnt signaling, which was spontaneously activated at the proximal region, acts upstream of BMP signaling for dorsal specification. These observations suggest that D-V polarity could be established within the self-formed retinal neuroepithelium by intrinsic mechanisms involving the spatiotemporal regulation of canonical Wnt and BMP signals.

KEY WORDS: Morphogenesis, Organoid culture, Pattern formation, Retinal development

INTRODUCTION
Vertebrate eye development is a complex event that requires rigorous polarity regulation with specific gene expression patterns. In the murine eye, the optic primordium starts to evaginate laterally from the diencephalon at embryonic day (E)8.0 and forms the optic vesicle. In early mouse optic vesicles, Bmp4 is expressed on the distal portion and subsequently confined to the dorsal portion (Furuta and Hogan, 1998; Behesti et al., 2006). Ectopic expression of Bmp4 in the early optic cup induces the expansion of Tbx5 and Vax2 within the context of D-V polarization (reviewed by Yang, 2004; Zhao et al., 2010; Kobayashi et al., 2010). In early mouse optic vesicles, Bmp4 is expressed on the distal portion and subsequently confined to the dorsal portion (Furuta and Hogan, 1998; Behesti et al., 2006). Ectopic expression of Bmp4 in the early optic cup induces the expansion of Tbx5 and reduction of Vax2 (Koshiba-Takeuchi et al., 2000). Wnt signaling is also known to be involved in D-V polarization of retinal tissue (Veien et al., 2008; Hägglund et al., 2013). Wnt2b (Wnt13) is localized in dorsal RPE at an early optic vesicle stage in mice and chicks (Cho and Cepko, 2006; Steinfeld et al., 2013). In mice deficient in the Wnt receptor or its downstream component, dorsal retinal markers such as Bmp4 and Tbx5 are diminished (Zhou et al., 2008; Esteve et al., 2011; Hägglund et al., 2013). Although these previous reports suggest that both BMP signaling and canonical Wnt signaling profoundly affect the dorsal retinal specification, the underlying mechanisms of crosstalk between these signals are poorly understood.

We previously reported the formation of 3D optic tissue from mouse and human embryonic stem cells (mESCs and hESCs, respectively) and using a serum-free culture of embryoid body-like aggregates with a quick aggregation SFEBq method (Eiraku et al., 2011; Nakano et al., 2012). Addition of Matrigel to the initial medium induced the homogenous ESC aggregate to differentiate into a retinal structure with dynamic morphological changes such as formation of an optic vesicle-like structure and an optic cup-like structure. In this study, taking advantage of the simplicity and manipulability of this culture system, we focused on the spontaneous D-V polarity formation and its underlying mechanisms during optic morphogenesis. We found evidence that the mESC-derived retinal tissue spontaneously acquires D-V polarity with specific gene expression, and sequential signals of
Wnt and BMP cooperatively control the D-V polarization of the retinal neuroepithelium.

RESULTS

D-V regionalization in mESC-derived retinal tissue

To visualize the D-V polarity, we first raised antibodies against the dorsal retinal marker Tbx5 and the ventral retinal marker Vax2. Consistent with mRNA expression patterns as previously reported (Behesti et al., 2006; Mui et al., 2002), immunostaining using these antibodies showed that Tbx5 was expressed on the dorsal side of the embryonic optic cup, in particular, in the dorsal quadrant of the NR, whereas Vax2 was expressed in the ventral NR and RPE [Fig. 1A at E10.5, Fig. 1B at E9.75 (Fig. 1B included with a light-sheet microscope), Fig. S1A at E9.5].

We next examined the expression patterns of Tbx5 and Vax2 in the self-organized optic cup-like tissues derived from mESCs (Rx::EGFP) with these antibodies (Fig. 1C). In most cases, Chx10 is specifically expressed in the inner epithelium in which the expression level of Rx (retina and anterior neural fold homebox, also known as Rax) is higher than in RPE (Fig. 1D,E and Fig. S1B,C). In some cases, Chx10 is also expressed in the more proximal part rather than the hinge of the ESC-derived optic cup (Fig. S1D). We found that mESC-derived retinal tissue is regionalized into three domains by different marker expression patterns: Tbx5+/Vax2− (domain 1), Tbx5−/Vax2− (domain 2) and Tbx5−/Vax2+ (domain 3) (Fig. 1F-H). We observed that 21% of ESC-derived optic cups had all three domains, as seen in embryonic retina (Behesti et al., 2006), but optic cups without domain 1 or domain 2 were also observed (Fig. S1E,F). In addition, Coup-TFII (Nr2f1) and Coup-TFII (Nr2f2) are specifically expressed at the retina (Behesti et al., 2006), but optic cups without domain 1 or domain 2 were also observed (Fig. S1E,F). In addition, Coup-TFII (Nr2f1) and Coup-TFII (Nr2f2) are specifically expressed at the ventral and dorsal side of embryonic retina, respectively (Satoh et al., 2009). In mESC aggregates, Tbx5 expression was seen on the Coup-TFII+ and Coup-TFII− side, which is suggestive of the dorsal side, and Vax2 was expressed on the Coup-TFII− and Coup-TFII+ side, which is suggestive of the ventral side (Fig. 1I-L). These results suggest that D-V patterning in terms of marker expression was spontaneously generated in mESC-derived retinal tissue in a less-reproducible manner than in vivo.

To confirm the formation of D-V polarity in mESC-derived retinal tissue at later stages, we conducted long-term culture. In the embryonic optic cup, the ventral side of the NR is connected to the diencephalon through the optic disc and optic stalk, in which neurofilament (NF) optic nerves later pass (Fig. 1M,N). The optic disc is a small region marked with Vax2 and Pax2 co-expression (Fig. 1M,N). When mESC-derived NR was isolated and cultured in collagen gel until day 17, we observed that thick bundles of axons extended from a few points of the NR tissue. (Fig. 1O, Fig. S1G,H). Consistent with mRNA expression patterns as previously reported (Fuhrmann et al., 2000; Kobayashi et al., 2010; Steinfeld et al., 2013). However, in our ESC culture, interactions with these tissues are absent. To elucidate retinal epithelium-intrinsic mechanisms underlying the D-V polarization, we next examined roles of endogenous BMP signaling in mESC-derived retinal tissue.

Endogenous BMP signaling regulates D-V specification in mESC-derived retinal tissue

Previous studies have implicated the roles of BMP and Shh signals in the D-V patterning of the embryonic retina. In the developing eye, the optic cup is exposed to these external cues from surrounding non-retinal tissues such as periocular mesenchyme, diencephalic neuroepithelium and surface ectoderm (Furuta and Hogan, 1998; Fuhrmann et al., 2000; Kobayashi et al., 2010; Steinfeld et al., 2013). However, in our ESC culture, interactions with these tissues are absent. To elucidate retinal epithelium-intrinsic mechanisms underlying the D-V polarization, we next examined roles of endogenous BMP and Shh signaling on the expression of Tbx2, Tbx3, Tbx5 and Vax2 mRNA on day 7-8.5 (Fig. 3A). RT-qPCR analysis revealed that BMP4 treatment robustly elevated the expression levels of Tbx2, Tbx3 and Tbx5 mRNA, and substantially suppressed Vax2 expression. Conversely, dorsomorphin, an antagonist of the BMP ligand, strongly suppressed Tbx2, Tbx3 and Tbx5 expression and moderately increased Vax2 expression (Fig. S1B and S2A). On the other hand, neither a Shh signaling agonist nor an antagonist [Smoothened agonist (SAG) and cyclopamine-KAAD] had any effect on Vax2 expression. Furthermore, Tbx5 expression was suppressed by treatment with SAG (Fig. S2B). Collectively, these findings suggest that endogenous BMP signaling predominantly regulates D-V marker expression in mESC-derived retinal tissues, whereas Shh signaling has a minor function.

We next confirmed these results by immunohistochemistry. mESC-derived retinal tissues were treated with BMP4 or dorsomorphin from day 7 and analyzed with antibodies against phosphorylated Smad1/5/8 (pSmad) and Tbx5. In the mouse developing eye, the dorsal NR and RPE near the hinge region (pre-RPE) was positive for pSmad (Fig. S3D,E). In ESC culture, Bmp4 mRNA and pSmad were broadly detected at the distal portion of day 7 optic vesicle. By contrast, Tbx5 was more locally expressed structures reminiscent of embryonic optic fissure on one side (62% of optic cup-like structures, n=37, Fig. 2A-C; Movie 1). The 3D reconstruction of immunostained tissues demonstrated that the expression patterns of Tbx5 and Vax2 are mutually exclusive (Fig. 2D), and the fissure-like structure frequently formed in the Tbx5− domain of the mESC-derived optic cup-like structure (69%, n=13, Fig. 2E,F). These results suggest that a choroid fissure-like structure can also self-form in retinal tissues derived from mESCs.

We next sought to analyze how the fissure was folded during in vitro optic cup formation by time-lapse imaging using an incubator-docked multi-photon microscope (Eiraku et al., 2011; Movie 2). As we reported previously, before the onset of invagination, the distal portion of the optic vesicle thickens (Fig. 2I, red arrow). We noticed that the apical surface of the hinge region, a border between NR and RPE, became acutely angled at one side during cup formation (Fig. 2I, red circle, and M). At the opposite side, the apical surface remained gently angled with continuous curvature (Fig. 2I, yellow circle, and M). As invagination proceeds and the cup deepens, the NR grows laterally (Fig. 2K) and the marginal tissue between NR and RPE grows more distally except for the side with the gently angled apical surface (Fig. 2G,H, yellow arrows and Fig. 2L). As a result, the optic cup had a valley structure reminiscent of the optic fissure as seen in vivo. These observations suggest that the self-formed optic cup not only exhibits a D-V marker expression profile but also a morphology characteristic of D-V polarity, and the optic fissure-like structure could be formed in a self-organized manner in mESC culture.
On day 9, Tbx5 expression mostly overlapped with the region marked with Bmp4 mRNA and pSmad (Fig. 3C and Fig. S3J-N). We also found Tbx5/pSmad+ cells not only in dorsal NR but also in the pre-RPE region (Fig. 3C). Such dynamics of BMP signaling molecules are consistent with that seen during mouse optic cup formation (Yun et al., 2009). Treatment with exogenous BMP4 upregulated both Tbx5 and pSmad immunoreactivities, whereas treatment with BMP antagonist completely diminished both signals (Fig. 3D-F). Notably, when optic vesicles were treated with BMP4 or dorsomorphin, invagination was disturbed in both cases (Fig. 3C-E, Fig. S3A,B), but there was little effect on NR or RPE specification (Fig. S3C,D).
By whole mount immunostaining, we confirmed these results in a 3D context. Volumes of tissues expressing Tbx5 were increased by BMP4 treatment and decreased with dorsomorphin treatment. Conversely, the volume of Vax2-expressing tissue increased with dorsomorphin treatment and decreased with BMP4 (Fig. S2C). Together, these results suggest that BMP signaling is spontaneously activated in a spatially ordered manner and that it plays a crucial role in specifying the dorsal identity of mESC-derived retinal tissue.

Canonical Wnt signaling evokes dorsal specification through BMP signaling in early mESC-derived retinal tissue

To elucidate how BMP signaling is spatiotemporally regulated in mESC-derived retinal tissues, we next focused on canonical Wnt signaling.
signaling, which is another candidate for the dorsal regulator of optic vesicle at an earlier stage. To monitor canonical Wnt signaling dynamics during in vitro cup formation, we established transgenic cell lines in which fluorescent proteins are under control of a promoter with LEF1/Tcf binding domains (Rx::EGFP/7tcf::mCherry and Rx::EGFP/7tcf::H2BtdTomato; Takata et al., 2016). Using these cell lines, we performed live imaging analysis on day 2.5-7.5 and on day 7-8 (Movies 3 and 4). With maturation of NR, the intensity of Rx::EGFP strengthened throughout the retinal neuroepithelium, and at the same time, the canonical Wnt reporter was activated at a border between retinal neuroepithelium and non-retinal neuroepithelium by day 6 (Fig. 4A, arrowheads). On day 6.5, whereas Rx::EGFP expression was gradually attenuated at the proximal region of one side of the optic vesicle, Wnt signaling became augmented at this region (Fig. 4A). Importantly, the NR on the side with the stronger Wnt activation invaginated faster than the opposite side (Fig. 4B), consistent with the observation of non-axisymmetric morphogenesis with fissure formation (Fig. 2J). From these results, we hypothesized that locally activated canonical Wnt signal might be involved in BMP signal induction in a spatially-
Fig. 4. Canonical Wnt signaling induces dorsal specification in the early mESC-derived optic vesicle-like structure. (A) Live imaging for emergence of canonical Wnt signaling in Rx+ mESC-derived retinal tissue on day 2.5-7.5. Wnt activity was detected at the proximal region on one side of the optic vesicle on day 6.5 (arrowheads; see Movie 3). (B) Live imaging for visualization of asymmetrical expression of canonical Wnt signaling during invagination, using Rx::EGFP and 7tcf::H2BtdTomato cell lines. White dotted lines indicate apical side of retinal neuroepithelium. White arrows indicate presumptive dorsal hinge region, which started to invaginate (see also Movie 4). (C) Schematic of inhibitor assay to understand involvement of canonical Wnt signaling for D-V polarization at early differential stage. qPCR analysis was performed after treatment with 0.5 μM IWR1-endo (D) or in combination with 0.05 nM BMP4 (E) on day 5-6.5. (F) Model of sequential activities of canonical Wnt and BMP signaling for formation of D-V polarity. (G-I) BMP and Wnt affect later retinal development. (G) Schematic of experiment. After 0.1 nM BMP4 or 0.5 μM IWR1-endo treatment on day 6-8, Rx+ retinal neuroepithelium was cut and cultured until day 43. (H) Immunostaining of S-opsin and Rx::EGFP on day 43 in the matured mESC-derived retinal tissue. (I) qPCR analysis results on day 43. ***P<0.001, **P<0.01, *P<0.05; ns, not significant (Student’s t-test and one-way ANOVA with Tukey’s post hoc test). Data are mean±s.e.m. of n=3. Scale bar: 20 μm.
ordered manner and could regulate D-V polarization of mESC-derived retinal tissue.

We next examined the effects of a Wnt agonist and antagonist on D-V marker expression (Fig. 4C and Fig. S4B). In our mESC culture, Wnt3a treatment from day 5 resulted in upregulation of Tbx5 and downregulation of Vax2 (Fig. S4A). Bmp4 expression was also modestly increased by Wnt3a treatment (Fig. S3A). In contrast, when mESC aggregates were treated with the canonical Wnt antagonist IWR1-end on day 5-6.5, both Bmp4 and Tbx5 expression were significantly repressed, and Vax2 expression was increased, suggesting that endogenous canonical Wnt signaling is involved in the dorsal initiation through BMP4 induction (Fig. 4D).

Moreover, reduction of Tbx5 by inhibition of the canonical Wnt pathway was dramatically overturned by the addition of BMP4 (Fig. 4E). We also found that the level of Bmp4 mRNA was slightly upregulated by BMP4 treatment, and Bmp4 and Tbx5 were significantly reduced upon treatment with dorsomorphin, suggesting that Bmp4 signaling is activated by an autocrine effect (Fig. 4E and Fig. S4C). Collectively, these results suggest that the canonical Wnt pathway acts upstream of BMP signaling in dorsal retina initiation and specification in mESC culture (Fig. 4F).

We next examined whether these dorsalizing signals could affect D-V characteristics in the mature retinal tissue generated from mESCs. Previous studies have shown that short-wavelength (S)-opsin is more highly expressed on the ventral side of mouse retina (Applebury et al., 2000). After treatment with BMP4 or IWR1-end on day 6-8, the aggregates were transferred to long-culture medium and cultured until NR became fully mature on day 43 (Fig. 4G). Compared with controls, S-opsin and Vax2 expression were suppressed in BMP4-treated NR. However, IWR1-end treatment increased the expression of S-opsin and suppressed Tbx5 expression (Fig. 4H, I). The level of expression of Ephb2 (ephrin B2), a dorsal marker of mature NR, was slightly increased by BMP4 treatment, whereas expression of Ephb2 (Eph receptor B2), a ventral marker of mature NR, decreased (Peters and Cepko, 2002; Fig. S4D). Collectively, we confirmed that early canonical Wnt activation could regulate D-V polarization at both the early and later stages.

**Locally activated canonical Wnt signaling induced expression of Tbx5 in the neighboring retinal neuroepithelium**

To further elucidate the spatiotemporal regulation of Wnt and BMP signaling, we next examined the timing of Wnt activation and D-V marker onset. On day 6, neither 7ctf::H2BtdTomato nor Tbx5 neurites were densely accumulated, mimicking a ventral structure of the retina. Therefore, the Tbx5+ and Vax2− regions in our culture indeed represent dorsal and ventral retinal characteristics, respectively. In a previous study, we showed that optic cup self-forms from mESCs in the absence of other tissues, such as lens placode and periocular mesenchymes (Einiku et al., 2011). Therefore, the present results suggest that retinal neuroepithelium is capable of self-organizing to form an optic cup-like structure with D-V polarity.

**D-V regional specification emerges in self-formed retinal structure**

Several reports have shown that Tbx5 and Vax2 act as master regulators for dorsal and ventral specification of developing retina, respectively (reviewed by Yang, 2004 and McLaughlin et al., 2003; Zhang and Yang, 2001; Peters and Cepko, 2002; Behesti et al., 2006). In this report, we demonstrate that mESC-derived retinal tissues are spontaneously regionalized into several domains marked with each of dorsal (Tbx5 and Coup-TFII) and ventral (Vax2 and Coup-TFII) markers. Additionally, at a later stage of mESC-derived retinal culture, Vax2 expression was seen in a Pax2+ region in which Neurofllament+ neurites were densely accumulated, mimicking embryonic optic disc—a ventral structure of the retina. Therefore, the Tbx5+ and Vax2− regions in our culture indeed represent dorsal and ventral retinal characteristics, respectively. In a previous study, we showed that optic cup self-forms from mESCs in the absence of other tissues, such as lens placode and periocular mesenchymes (Einiku et al., 2011). Therefore, the present results suggest that the D-V patterning in mESC culture could be achieved by intrinsic mechanisms of the retinal neuroepithelium.

However, we also noticed that the D-V patterning was not as reproducible as the embryonic optic cup formation in our culture system. In the developing eye, the NR consists of three regions: Tbx5+Vax2− (most dorsal; domain 1), Tbx5+/Vax2− (domain 2) and Tbx5−/Vax2+ (most ventral; domain 3). Among mESC-derived retinal tissues, by contrast, only 21% were composed of all three domains. In other cases, the mESC-derived retinal tissue consisted of domains 1 and 3, or domains 2 and 3 (Fig. S1). Furthermore, it was observed that Chx10 occasionally extended its expression into the pre-RPE domain. Such variations in ESC culture, as well as...
Fig. 5. See next page for legend.
variations in the size of optic vesicles and the timing of the formation of optic cup-like structures, may be related to the absence of interactions with surrounding tissues such as surface ectoderm and pericellular mesenchyme. In embryonic retina, interactions with these surrounding tissues could be essential for the robust formation of D-V retinal patterning (Fuhrmann et al., 2000).

D-V morphogenesis in ESC-derived optic cup

We show that the mESC-derived optic cup-like structure is non-axisymmetric and has a cleft structure reminiscent of the optic fissure that is transiently observed in the ventral side of the embryonic optic cup. 3D reconstruction of Tbx5 expression in the retinal tissue revealed that the fissure tended to form at the Tbx5-negative region. These observations support the idea that optic fissure formation results from D-V retinal patterning. Live-imaging analysis revealed that fissure formation is achieved by the retinal tissue-intrinsic mechanism in a timely ordered manner. As seen in vivo, invagination started at the side opposite the fissure, and fissure formation was coupled with the polarized invagination processes. In vertebrate retinal development, it has been thought that the optic fissure is formed by folding of the ventral retinal epithelium (Heermann et al., 2015); however, there are no reports showing a detailed process of optic fissure formation by live imaging. Furthermore, it has previously been shown that increased cell proliferation and extensive apoptosis in the ventral optic vesicle might accompany optic fissure formation (Ozeki et al., 2000; Trousse et al., 2001; Morcillo et al., 2006), and in a Bmp7 mutant mouse, fissure formation was not initiated (Morcillo et al., 2006). However, the molecular mechanisms and tissue dynamics for optic fissure formation are not fully elucidated. With its simple culture methods and applicability to live imaging, our mESC culture system could provide a good platform for studying the cellular dynamics and underlying molecular mechanisms involved in optic fissure formation in future studies.

BMP signaling directly regulates D-V polarization and invagination of NR in vitro

We demonstrated that expression of Tbx2, Tbx3 and Tbx5 was upregulated and Vax2 expression was downregulated by BMP4 treatment in mESC-derived retinal tissue. In contrast, a BMP antagonist influenced Tbx5 and Vax2 expression in exactly the reverse manner. In addition, immunohistochemistry revealed that both pSmad signal and Tbx5 expression were upregulated by BMP4 treatment and downregulated by dorsomorphin. These results are consistent with previously reported mechanisms for retinal D-V patterning in which BMP signaling played a pivotal role in determining dorsal identities through Tbx5 (Koshiha-Takeuchi et al., 2000; Behesti et al., 2006).

Interestingly, BMP signaling was autonomously activated within mESC-derived neuroepithelium. This observation provides a novel view for induction of BMP signaling, which has been reported to be activated through interaction between optic vesicle and surface ectoderm in mouse and chick embryos (Furuta and Hogan, 1998; Trousse et al., 2001; Muller et al., 2007; Behesti et al., 2006; Steinfeld et al., 2013).

Meanwhile, in vitro manipulations of Shh signaling levels had only limited effects on Tbx5 and Vax2 expression (Fig. S2A-C). Previously, in a study of the optic cup of Smothened (Smo, a mediator of Shh signaling) conditional knockout mice, Vax2 expression was found to be downregulated (Zhao et al., 2010). However, in the optic cup of GlI (a transcription factor in the Shh pathway) mutant mice, Vax2 expression was not changed (Furimsky and Wallace, 2006). Further elucidation of the roles of Shh signaling in D-V polarization is required.

One intriguing aspect of the inhibitor assays was that neither the BMP nor the Shh inhibitor substantially suppressed Vax2 expression (Fig. 3A, Fig. S2A,B). Additionally, we observed that Vax2 expression preceded Tbx5 expression in mESC-derived optic vesicle (Fig. 5A,B). These data suggest that ventral specification is the default direction of NR tissue, and dorsal specification may require an active process by inductive signaling. In support of this, in Bmprr1a and Bmprr1b mutant mice, Vax2 expression is increased and Tbx5 expression is absent (Murali et al., 2005). In conclusion, we have shown that intrinsic BMP signaling is directly responsible for D-V polarization and invagination through regulation of Tbx5 and Vax2 in mESC-derived optic cup-like structures.

Canonical Wnt signaling locally activates the BMP signaling pathway

In our qPCR results, inhibition of the canonical Wnt signaling pathway at the early optic vesicle stage (before day 5) and at a later optic vesicle stage (before day 7) gave rise to a marked reduction of Tbx5 and Bmp4 expression (Fig. 4C,D), suggesting that canonical Wnt signaling is essential for dorsal induction and maintenance. The necessity of canonical Wnt signaling for maintenance of dorsal identity has already been reported (Veien et al., 2008; Zhou et al., 2008; Hägglund et al., 2013). In this study, we visualized canonical Wnt signaling dynamics. We found that Wnt activity started to spontaneously increase at the proximal portion of one side of the hemispherical vesicle. This pattern is in accord with sequential Wnt2b expression in vivo (Cho and Cepko, 2006). This biased expression may make a difference in the speed of maturation of RPE between the prospective dorsal and ventral sides to form...
morphologic D-V polarity (Fig. 4B). We also found that Vax2 is expressed until day 6 in the optic vesicle. At day 6.5, Tbx5 expression was first detected adjacent to the 7tcf::tdTomato + area and Vax2 expression was seen in a position opposite (Fig. 5A-G).

Additionally, downregulation of Tbx5 expression by treatment with an inhibitor of the Wnt pathway was rescued by additional BMP4 treatment. These results are consistent with the idea that Vax2 + tissue is a default state of retinal neuroepithelium and the dorsal tissue is induced by specific dorsalizing factors BMPs and Wnts that work upstream of BMP signaling.

When the Wnt agonist CHIR was applied locally to mESC-derived retinal tissue, 7tcf::H2Btdtomato expression appeared in a restricted area. Intriguingly, localized Tbx5 expression was induced by CHIR in the region adjacent to the 7tcf-activated area, which is different to the spatially direct induction by BMP signaling. Because Tbx5 is downstream of the BMP pathway, these results imply an intrinsic cross-talk between BMP and canonical Wnt signaling, which has been known to exist in several parts of the developing vertebrate embryo (reviewed by Guo and Wang, 2009). For example, in zebrafish dorsal retina, Wnt activity maintains BMP signaling (Veien et al., 2008). The question of how canonical Wnt signaling activated Tbx5 from a flanking portion still remains unclear. A possible explanation is that after the BMP ligand is locally activated by low-level canonical Wnt signaling, BMP expression may be strengthened by autocrine action. Tbx5 expression is induced and augmented in BMP + cells of Rx + retina, and then the Tbx5 +/BMP +/Rx + area and Wnt + area may be regionalized, because in retina, Wnt signaling is repressed by expression of Wnt inhibitor Dickkopf family proteins (DKKs) (Eiraku et al., 2011).

It is also unclear how the Wnt signal is locally activated in the proximal region of the optic vesicle. This may be achieved by the interaction with non-retinal tissues. As we previously described (Eiraku et al., 2011), when the optic vesicle is isolated from the main body of aggregate, the isolated tissue mainly differentiates into neural retina without RPE. This suggests that the non-retinal tissues provide extrinsic cues required for the RPE differentiation at the border between retinal and non-retinal tissue. It has also been demonstrated that Wnt3a treatment promotes RPE differentiation. Based on these previous experiments, BMP signal could be indirectly induced by interaction with non-retinal tissues through local Wnt activation.

Moreover, in our culture system, expression of S-opsin + photoreceptors in the fully matured retina was shifted with manipulation of the early D-V pattern. Further elucidation of the process for optic D-V regulation may lead to an optimized in vitro manipulation of photoreceptor subtypes for future clinical applications.

MATERIALS AND METHODS
Mouse ESC culture
Mouse ESCs (EB5, Rx::EGFP, Rx::EGFP, 7tcf::mCherry and Rx::EGFP, 7tcf::tdTomato) were maintained as previously described (Eiraku et al.,...
Materials and Methods.

Culture were prepared and maintained as described in supplementary materials. Hinge curvatures are calculated as the difference in curvature at the sampling point. The hinge curvatures are measured as the distance between fissure bottom and fissure surface using Imaris software (BitPlane). In Fig. 2B-M, black dot represents measurements of each time point of imaging data demonstrated in Movie 2. The tissue curvature is semi-automatically measured using the widely used three-point method. For preprocessing, the tissue contour was manually extracted from the image. Using our original program, the local curvature is measured as the inverse of radius of the fitting circle. The circle passes three points; one is the sampling point where the method was performed, others are at a distance of ±26 mm along the contour from the sampling point. The hinge curvatures are calculated as the maximum values around the dorsal and ventral hinge areas, respectively.

Recombinant proteins and small molecules

Signaling molecules were used as follows: SAG (R&D), cyclopamine, KAAD (Enzo), BMP4 (R&D), dorsomorphin (Tocris), mWnt3a (R&D), CHIR99021 (Tocris), IWR1-endo (Calbiochem). They were applied to the aggregates in each well on day 5-6.5 or day 7-8.5 prior to the RT-qPCR assay. Total final volume was 150 μl per well and the concentrations of compound are indicated in the text and legends.

Immunohistochemistry

Immunohistochemistry of sectioned samples was performed as described in Supplementary Materials and Methods. Primary antibodies used for immunohistochemistry are described in Table S1. Antibodies generated in this study were validated by sequential immunohistochemistry in mouse sections, confirming that the immunoreactivities are consistent with the results of in situ hybridization previously reported.

In situ hybridization

In situ hybridization was performed based on a previous report (Blackshaw, 2013) as described in supplementary Materials and Methods.

Quantitative PCR

Quantitative PCR was performed using the 7500 Fast Real Time PCR System (Applied Biosystems) using primers described in Table S2. Data were normalized to Gapdh expression. The values shown on graphs represent the mean±s.e.m. For inhibitor assay, 24-48 aggregates were examined, and for long-term culture assay 4-12 matured retinal tissues were examined, in duplicate in each experiment, which was repeated at least three times.

Statistical analysis

Statistical tests were performed using Prism software (GraphPad, v.6). Statistical significance was tested with Student’s t-test for two-group comparisons and one-way ANOVA with Tukey’s post hoc test for multi-group comparison.

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Competing interests

The authors declare that they have no competing or financial interests.

Author contributions

M.E and Y.S. designed the research; Y.H., M.K., N.T. and M.E. performed the experiments; Y.H., S.O. and M.E. analyzed the data; Y.H. and M.E. wrote the paper.

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Supplementary information

Supplementary information available online at http://dev.biologists.org/lookup doi:10.1242/dev.134601 supplemental

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