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Original Article

Transforming growth factor beta2 promotes the formation of the mouse cochleovestibular ganglion in organ culture

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ABSTRACT The inner ear structures are derived from the otic vesicle (OV) which is formed by thickening and invagination of the otic placode of the surface ectoderm. A number of neuroblasts, which arise from epithelial cells of the otic vesicle, delaminate and differentiate into neurons of the cochleovestibular ganglion (CVG). We have found that *transforming growth factor-β2* (*Tgfβ2*) was expressed in the otic epithelium at the OV stages between embryonic days (E) 9.5 and 11.5 and that anteroventrolateral localization of its expression in the OV overlapped with that of *NeuroD,* which is a marker of delaminating CVG precursors. The expression of *TGFβ type I* and *type II receptors* in the otic epithelium and the nuclear localization of phosphorylated-Smad2 in both the otic epithelium and CVG suggested that TGFβ2 signaling plays some roles in CVG formation. In order to examine the roles of TGFβ2 in differentiation of the inner ear, otic vesicle explants of E10.5 mouse embryos were treated *in vitro* with TGFβ2 or the TGFβ type I receptor kinase inhibitor, SB431542. Addition of TGFβ2 peptide to the culture led to enlargement of the CVG, while the inhibitor reduced its size. These findings strongly imply that TGFβ2 contributes to the development of the CVG in mouse embryos.

KEY WORDS: *TGFβ, epithelial-mesenchymal transformation, delamination, neuronal differentiation*

Introduction

The mammalian ear is a delicate sensory organ for hearing and balance and is one of the most complex structures in the body. The inner ear primordium, the otic placode, first appears as a thickening of the surface ectoderm on which the neural plate neighbors. The otic placode invaginates to form the otic vesicle, which subsequently detaches from the surface ectoderm and form the inner ear structures such as the semicircular canals, vestibule and cochlea (Rossant, 2002).

The cochleovestibular ganglion (CVG) is formed by the cells which delaminate and migrate from the anteroventral portion of the otic vesicle (Carney *et al.*, 1983) and the neural crest-derived cells (D'Amico-Martel and Noden, 1983; Rubel and Fritzsch, 2002). It has been shown that a large area of the anteroventrolateral part of the OV gives rise to the CVG neuroblasts in mice (Fritzsch, 2003) and these cells are among the first cell types to be specified during the early OV stages (Fekete and Wu, 2002; Hemond and Morest, 1991; Hossain *et al.*, 2000). Recently, several genes have been identified that play crucial roles in development of the CVG. For example, the differentiation of the CVG depends on two basic helix-loop-helix (bHLH) factors, Neurogenin1 (NGN1) and NeuroD. Ma *et al.* (1998) reported that the CVG was not formed in *Ngn1* null mutants. In *NeuroD* mutant mice, migration of CVG precursors was compromised and there was considerable size reduction of the CVG (Liu *et al.*, 2000; Kim *et al.*, 2001). The roles of some growth factors have also been implicated in inner ear development. Double mutants of *fibroblast growth factor* (*Fgf*) *3* and *Fgf10* lacked the CVG (Alvarez *et al.*, 2003). It has also been shown that two neurotrophins, brain-derived neurotrophic factor (BDNF) and neurotrophin (NT)-3, are important for survival of the neuroblasts since the size of the CVG was decreased in single and double homozygous mutant mice (Ernfors *et al.*, 1995; Bianchi *et al.*, 1996; Farinal *et al.*, 1994; Jones *et al.*, 1994). Further, insulin-like growth

Abbreviations used in this paper: BDNF, brain derived neurotrophic factor; BMP, bone morphogenetic protein; CVG, cochleovestibular ganglion; E, embryonic day; Fgf, fibroblast growth factor; IGF, insulin-like growth factor; NGN, neurogenin; NT, neurotrophin; OV, otic vesicle; TGF, transforming growth factor.

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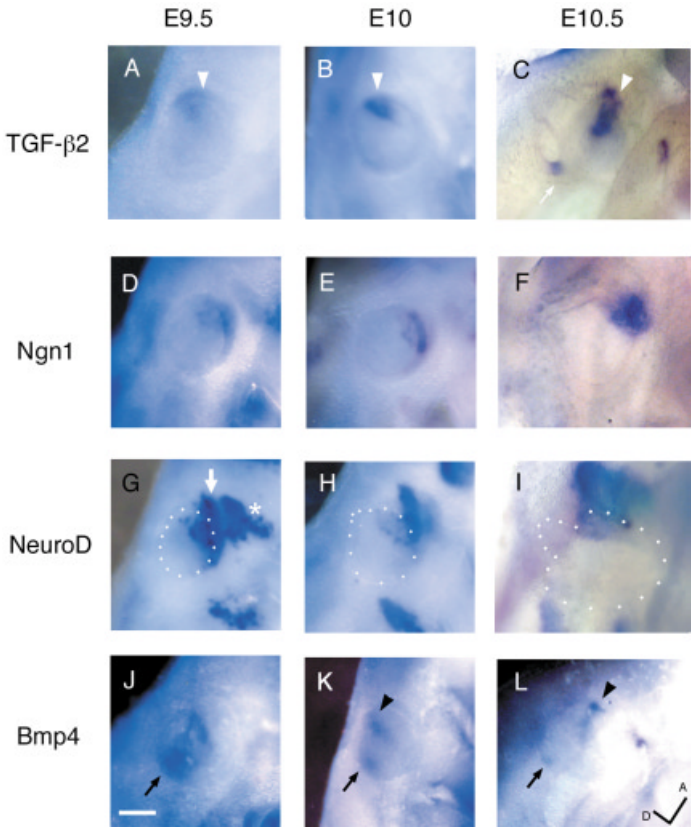

Fig. 1. Expression patterns of *Tgfβ2, Neurogenin1(Ngn1), NeuroD* and *Bmp4* in the mouse developing OV. Tgfβ2 *expression was detected in the anterior wall (arrowhead) of the otic vesicle at E9.5* **(A)** *and became more intense at E10.0* **(B)**. *At E10.5,* Tgfβ2 *transcripts were also detected in the posterior part of the vesicle as well as in the anterior wall* **(C)**. Neurogenin1 *was also expressed in the anterior region between E9.5 and 10.5* **(D,E,F)**. *Note that its expression domain partially overlapped with that of* Tgfβ2. NeuroD *was expressed in the same region as* Neurogenin1 *in the epithelium as well as in the neuronal precursors of the CVG between E9.5 and E10.5* **(G,H,I)**. Bmp4 *was expressed in the posterior wall (arrow) of the OV at E9.5* **(J)**, *E10.0* **(K)** *and E10.5* **(L)** *and also in the anterior wall (arrowhead) at E10.0 (K) and E10.5 (L). Dotted circles indicate the contour of the OV. The thick arrow and the asterisk in (G) show the developing CVG and the facial ganglion, respectively. Direction is indicated in (L) for all panels (A, anterior; D, dorsal). Scale bar, 100 μm.*

factor-1 (IGF-1) has been suggested to be necessary for CVG differentiation (Camarero *et al.*, 2001; Camarero *et al.*, 2002; Camarero *et al.*, 2003).

Transforming growth factor-β (TGFβ) is a secreted peptide which promotes cell survival during the development of the central and peripheral nervous systems (Sporn *et al.*, 1987; McFarlane and Cooper, 1993). Three isoforms of TGFβ (TGFβ1, 2 and 3) show distinct spatial and temporal expression patterns in mouse embryos, suggesting that they play various roles during embryogenesis (Flanders *et al.*, 1991; Johnson *et al.*, 1993). Blottner *et al.* (1996) reported that implantation of gelform soaked with TGFβ2 rescued all the injured neurons of the preganglionic sympathetic neurons in the spinal cord, though the mechanisms remain to be clarified. It has been shown that TGFβ1 is a potent survival factor for motor neurons of rat embryos (Martinou *et al.*, 1990) and it has also been shown that the TGFβ2/3 promote the survival of midbrain dopaminergic neurons (Poulsen *et al.*, 1994). Recently, Farkas *et al.* (2003) showed that both TGFβ2 and TGFβ3 are required for induction of midbrain dopaminergic neurons as well. They showed that TGFβ2/3 were expressed in the notochord and floor plate and that neutralization of TGFβ2/3 abolished induction of dopaminergic neurons in rat primary cultures and E2 chick embryos.

Although the pattern of *Tgfβ2* expression during inner ear development has been reported previously (Pelton *et al.*, 1990; Millan *et al.*, 1991; Schmid *et al.*, 1991), their description was not conferred at the OV stages. In this study, we examined the

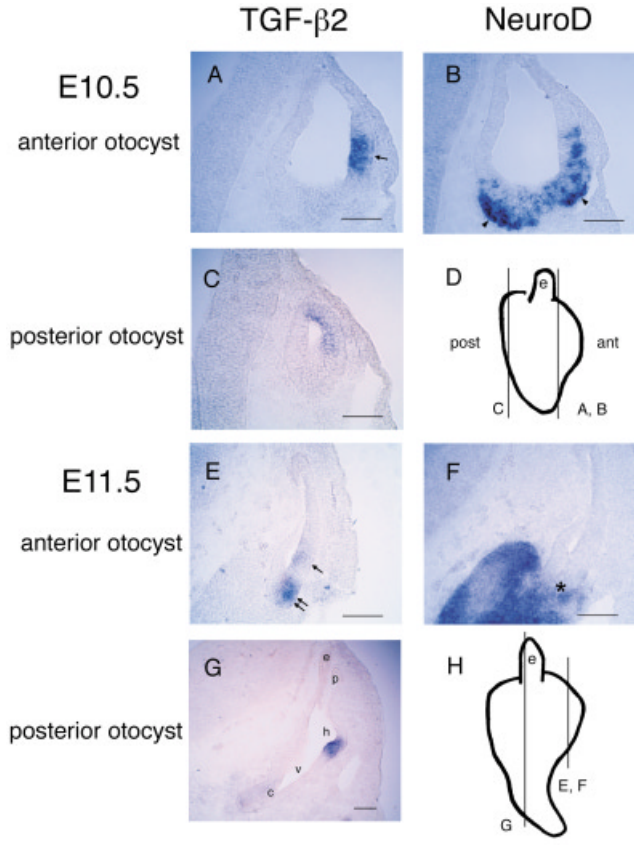

Fig. 2. Detailed expression patterns of *Tgfβ2* and *NeuroD* by section RNA *in situ* hybridization. *Sections of the anterior (A,B,E,F) and posterior (C,G) parts of the OV were stained with* Tgfβ2 *(A,C,E,G) and* NeuroD *(B,F) probes. (D) and (H) indicate the plane of each section. At E10.5,* Tgfβ2 *was detected in the ventrolateral region of the anterior OV* **(A)** *(see arrow), while* NeuroD *was expressed in both ventrolateral and ventral regions* **(B)** *(see arrowheads).* NeuroD *was also detected in the delaminated precursors (B). In the posterior OV,* Tgfβ2 *was observed in the dorsolateral region where* NeuroD *was not expressed* **(C)**. *At E11.5,* Tgfβ2 *was expressed in the ventral region (* **(E)**, *double arrow) in addition to the ventrolateral region (E, arrow) of the anterior OV. On the other hand,* NeuroD *expression was observed in a small number of the ventral epithelial cells of the anterior OV* **(F)** *(see asterisk) and in the delaminated CVG cells. In the posterior OV,* Tgfβ2 *was expressed around the boundary between the horizontal semicircular canal and the vestibule. ant, anterior; c, cochlear duct; e, endolymphatic duct and sac; h, horizontal semicircular canal; p, posterior semicircular canal; post, posterior; v, vestibule. In all panels dorsal is towards the top. In A, B, C, E, F, G, lateral is towards the right. Scale bar, 100 μm.*

expression patterns of *Tgfβ2* in the developing OV of mouse embryos by RNA *in situ* hybridization. We also treated cultured mouse embryonic OVs with TGFβ2 or TGFβ type I receptor kinase inhibitor and analyzed their effects on the developing OV. Our data suggest that TGFβ2 may contribute to inner ear development, especially to the formation of the CVG.

Results

The expression of Tgfβ2 *overlaps that of* Neurogenin *and* NeuroD *in the early otic vesicle*

Tgfβ2 expression in the otic epithelium was examined at OV stages (E9.5-E11.5) by RNA *in situ* hybridization. At E8.5-9, *Tgfβ2* expression was not detected in the otic placode or pit. At E9.5, when the otic vesicle formed, *Tgfβ2* transcripts were detected in the anteroventrolateral region of the otic vesicle (Fig. 1A). At E10.0 and E10.5, *Tgfβ2* was more intensely expressed than at E9.5 in the anteroventrolateral wall and also came to be detectable in the posterodorsolateral wall (Fig. 1 B,C). Transverse sections revealed that its expression was confined to the epithelium of the otic vesicle, but not in the surrounding mesenchyme (Fig. 2 A,C).

The neuronal precursors for the CVG delaminate from the anteroventral wall of the OV between E9.5 and E11.5 with its peak at E10.5 (Carney *et al*., 1983). We compared the expression patterns of *Tgfβ2* with those of the neural bHLH genes *Ngn1* and *NeuroD*, which have essential roles in CVG development (Ma *et al*., 1998; Liu *et al*., 2000). *Ngn1* expression was first detected in the anteroventrolateral region of the otic epithelium at E9.0 when the otic placode began to invaginate (Raft *et al*., 2004). At E9.5 and E10.0, *Ngn1* was expressed in the anteroventrolateral wall of the OV and its expression domain partially overlapped with that of *Tgfβ2* (compare Fig. 1 A,B and D,E).

NeuroD was first faintly expressed in the same region as *Ngn1* at E9.0 (Liu *et al*., 2000; Raft *et al*., 2004). Its expression became intense in the delaminating precursors and CVG cells by E9.5 (Fig. 1G). The epithelial domains of *Ngn1* and *NeuroD* expression overlapped with each other up to E11.5 (Fig. 1 D-I). The expression domain of *NeuroD* in the otic vesicle also partially overlapped with that of *Tgfβ2* (compare Fig. 1 A,B,C and G,H,I). To analyze their expression patterns in detail, we performed section RNA *in situ* hybridization. At E10.5, both *Tgfβ2* and *NeuroD* transcripts were found in the anteroventrolateral regions of the otic vesicle (Fig. 2 A,B), although their expression patterns were not exactly identical. *Tgfβ2* was expressed more intensely in the luminal and intermediate layers than in the outer layers of the otic epithelium (Fig. 2A), whereas *NeuroD* was more intensely expressed in the otic epithelial cells near the mesenchyme (Fig. 2B). *Tgfβ2* expression was also observed in the posterodorsolateral otic epithelium (Fig. 2C) where *NeuroD* was not detectable (data not shown). At E11.5, when the endolymphatic duct became more overt and the cochlear anlage expanded ventrally, *Tgfβ2* expression still remained on the anteroventral wall of the OV, although the expression domain slightly shifted to the outer layers in the ventral region (Fig. 2E). *NeuroD* was also detected in a few cells of the same domain (Fig. 2F). Robust expression of *Tgfβ2* was also detected in the posteroventrolateral wall at E11.5 (Fig. 2G).

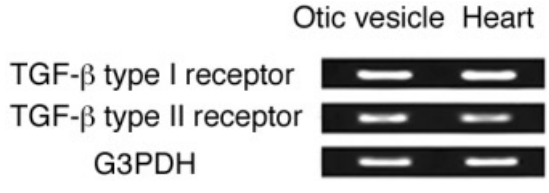

Fig. 3. Expression of *TGFβ* type I and II receptors in the developing OV. *One μg of RNA extracted from E10.5 otic vesicles was subjected to RT-PCR. The heart was used as a positive control. Robust expression of both receptors was observed in the otic vesicle (27 cycles). Three independent experiments were performed and the representative data are shown.*

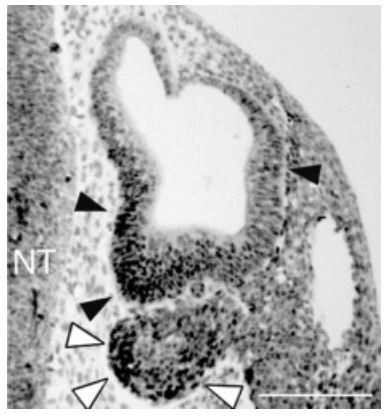

Fig. 4. Immunohistochemistry of phosphorylated Smad2 (P-Smad2) at E10.5. *Intense nuclear staining of P-Smad2 was detected in the anteroventral regions of the otic vesicle (arrowheads) and CVG (open arrowheads). Note that the staining was more intense in the outer parts of both structures than in the inner parts. A number of cells were positive for nuclear P-Smad2 in the adjacent neural tube (NT). Scale bar, 100 μm.*

Bone morphogenetic protein (BMP) 4 is another member of TGFβ superfamily which has been shown to be expressed in the developing OV of both chick and mice (Wu and Oh, 1996; Morsli *et al*., 1998; Raft *et al*., 2004). To further clarify the expression domain of *Tgfβ2,* we compared its expression with that of *Bmp4*. At E9.5, *Bmp4* was expressed in the posterodorsal wall of the OV (Fig. 1J). At E10 and later, it was also detectable as a streak domain in the anterior wall (Fig. 1 K,L). These two loci correspond to the anterior and posterior cristae (Wu *et al*., 1996). The expression domains of *Tgfβ2* and *Bmp4* seemed to be very close to each other but not to overlap.

In summary, *Tgfβ2* transcripts were localized in the antreoventrolateral wall and in the posterolateral region of the otic vesicle at E9.5-11.5. *Tgfβ2* expression overlapped with that of *NeuroD* in the anteroventolateral compartment.

TGFβ type I *and* type II receptors *and phosphorylated Smad2 are present in the developing OV*

To examine whether *TGFβ type I* and *type II receptors* are expressed in the developing OV, we used RNA *in situ* hybridization. Since their expression levels were too low to be detected with digoxigenin-labeled probes, we performed RT-PCR, which is much more sensitive than RNA *in situ* hybridization, using OVs of E10.5 (35-39 somites) embryos. The embryonic heart tissue, which expresses those receptors most strongly at this stage (Mariano *et al*., 1998), was used as a positive control. The OVs showed comparable expression levels of both *TGFβ type I* and *type II receptors* to the embryonic hearts (Fig. 3).

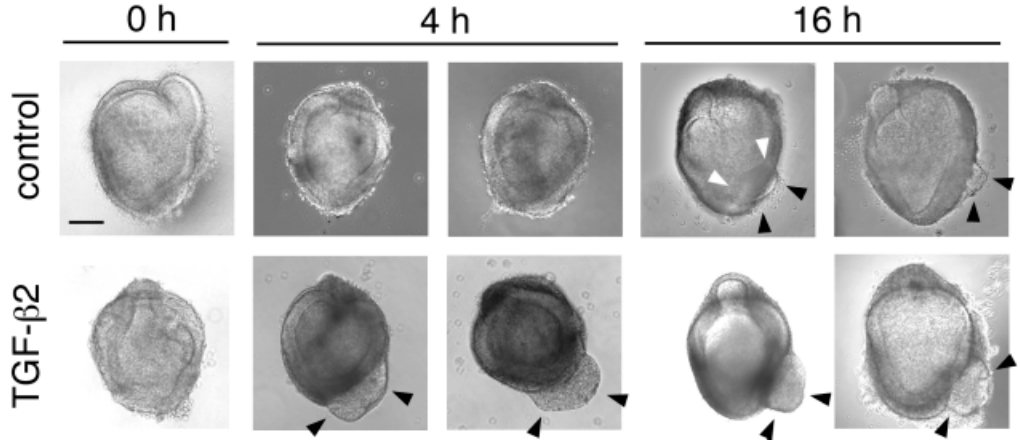

Fig. 5. Organ culture of otic vesicles from E10.5 embryos. *Top and bottom panels show controls and the culture treated with TGFβ2 (10 ng/ml), respectively. Incubation periods are indicated at the top. 0 h represents the beginning of the culture. The already formed CVG was removed from the explants before culture. After 4 hours, there was no CVG in controls, while several vesicles already produced the CVG (arrowheads) when treated with TGFβ2 protein (see Table 1). After 16 h, larger CVGs (arrowheads) were formed with TGFβ2 than in controls. Scale bar, 100 μm.*

Next, to confirm that TGFβ signaling is actually active in the OV, we carried out an immunohistochemical study using an anti-phosphorylated Smad2 antibody. Phosphorylation of Ser465 and Ser467 of Smad2 is required for the Smad2/Smad4 complex formation and TGFβ signal transduction (Nakao *et al*., 1997; Abodollah *et al*., 1997; Souchelnytskyi *et al*., 1997; de Caestecker *et al*., 1998). Nuclear localization of phosphorylated Smad2 indicates that TGFβ signaling is conveyed to the nucleus in the cells (Heldin *et al*., 1997; Massague *et al*., 1998; Massague and Wotton, 2000). Intense nuclear staining was observed in the anteroventolateral wall of the otic epithelium and the outer cells of the CVG (Fig. 4), indicating that TGFβ may signal in these regions. There were phosphorylated Smad2-positive cells in the neural tube (Fig. 4) as previously reported by Farkas *et al*. (2003).

TGFβ2 increases the size of the cochleovestibular ganglion in cultured otic vesicles

Since *Tgfβ2* was expressed in the anteroventrolateral wall of the OV, we assumed that TGFβ2 might play some role in CVG development. To test this hypothesis, we cultured the otic vesicles of E10.5 mouse embryos in which CVG precursors delaminate most actively from the otic epithelium (Carney *et al*., 1983) and treated them with TGFβ2. Within 16 hours in culture a mass of cells migrated out of the anteroventrolateral region of the OV and formed a CVG-like structure (Fig. 5). Many of the cells forming this structure were positive for Tuj1, an early neuronal marker (Fig. 6). Our observation along with the study of Camarero *et al*. (2003) suggests that this structure was likely a newly-formed CVG in the culture.

To examine the effects of TGFβ2 on the differentiation of OVs, we treated cultured OVs with recombinant TGFβ2 protein. With TGFβ2, 6 of 14 OV explants (42.9%) produced the CVG after 4 hours incubation whereas no CVG was formed in the control cultures (Fig. 5; Table 1). Likewise, when the explants were incubated for 6 hours, 15 of 24 cultured vesicles (62.5%) produced the CVG with TGFβ2 whereas only 4 of 25 (16.0%) formed without TGFβ2 (Table 1). Moreover, the size of the CVG was increased with TGFβ2 (compare the top and bottom panels in Figs. 5,6) when compared to the control samples (Figs. 5,6), suggesting that TGFβ2 accelerates CVG formation.

To quantitate the effects of TGFβ2 on CVG development the area of the CVG on photographs was measured using the technique of Camarero *et al*. (2003). The area of the CVG treated with TGFβ2 was increased by 1.4-fold (P< 0.001, Fig. 7A). We also measured the area of the remaining epithelium of the OV and found that the average area of the treated group was significantly smaller than that of the control group (P< 0.001, Fig. 7B), suggesting that TGFβ2 promotes CVG formation at the expense of the epithelial cells of the OV.

To determine whether TGFβ2 increases the number of CVG precursors or cell proliferation of neuroblasts, we performed immunostaining with anti-phosphorylated histone3, a proliferation marker. There was no clear difference between the control and TGFβ2-treated tissues (data not shown), suggesting that TGFβ2 increases the number of CVG precursors instead of promoting cell proliferation.

Next, we treated OVs with the TGFβ type I receptor kinase inhibitor SB431542 (5μM) in order to block the endogenous TGFβ signaling. The area of the CVG treated with SB431542 was

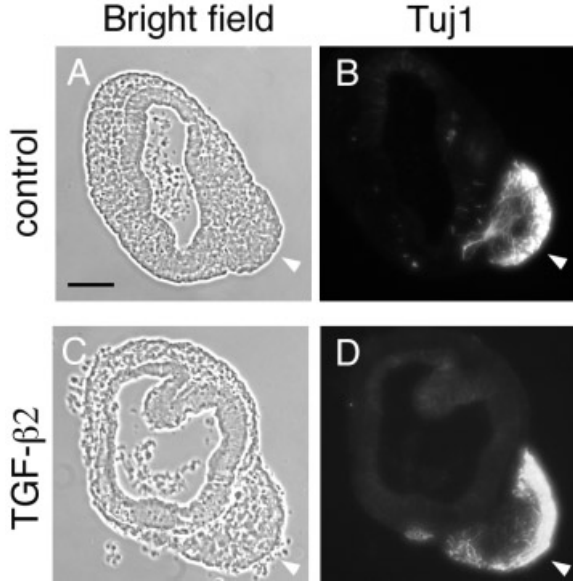

Fig. 6. Tuj1 immunohistochemistry of OV culture. (A,B) *Controls.* **(C,D)** *Explants treated with TGFβ2. After 16 hours, OV explants were harvested and subjected to immunostaining for Tuj1, an early neuronal marker. (A,C) Phase contrast photographs. Tuj1 immunoreactivity was detected in the CVG (arrowhead) in both control and TGFβ-positive cultures. Note that the CVG in TGFβ2-treated culture (C,D) is larger than in the control culture (A,B). Scale bar, 100 μm.*

significantly reduced when compared to the control vesicles (P< 0.01, Fig. 7C).

Slug *expression is downregulated in the TGFβ2-treated OV*

It was previously reported that TGFβ2 upregulates the expression of *Slug*, a member of the *Snail* family that is involved in delamination of endothelial cells in the embryonic heart (Romano and Runyan, 2000). We have found that *Slug* was expressed in the developing OV of the mouse embryo (unpublished data). Since the delamination of CVG precursors from the OV is a similar phenomenon to epithelial-mesenchymal transformation (EMT), we examined by semi-quantitative RT-PCR whether the expression of *Slug* is affected with TGFβ2 in the OV. mRNA was extracted from the whole culture including both the vesicle and CVG after various incubation intervals (Fig. 8). The intensity of the PCR bands was measured and normalized by that of EF1 products. The relative intensity of the bands from TGFβ2-treated OVs to the control was 0.624 (1.5h), 0.409 (2h), 0.569 (4h), 0.631 (6h) and 0.886 (16h), indicating that *Slug* mRNA was substantially reduced in culture treated with TGFβ2.

Discussion

In this study we found that *Tgfβ2* is expressed in the epithelium of the mouse OV in a distinct pattern. *TGFβ type I* and *type II receptors* and phosphorylated Smad2 were also detected in the otic vesicle, suggesting that TGFβ signaling is underway during inner ear development.

Brigande *et al.* (2000) proposed that the spherical OV can be divided into eight compartments by the anterior-posterior, dorsal-ventral and medial-lateral boundaries and that different parts of the inner ear are derived from different compartments. Alsina *et al.* (2004) designated the anteroventral domain as "proneural sensory territory" (proNS) because proneural and neurogenic genes, including *Neurogenin1* and *NeuroD*, are expressed in this domain. They observed that CVG precursors delaminated around the boundary between proNS and its posterior region.

During inner ear development, numerous otic epithelial cells delaminate from the anteroventrolateral region, a part of the proNS, to form the CVG. This delamination is similar to EMT as seen in neural crest cell differentiation in terms of the loss of epithelial characters, migration and neuronal differentiation. In addition, TGFβ signaling is known to be involved in neural crest cell development. TGFβs have been shown to induce EMT during cardiac, palatal and hair follicle development and in cutaneous wound repair (Sanford *et al.*, 1997; Sun *et al.*, 1998; Romano and Runyan, 2000; Camenisch *et al.*, 2002). During heart development TGFβ2 promotes EMT of the endothelial cells to produce the endocardial cushions (Romano and Runyan, 1999). It has also been reported that cytokeratin was downregulated when the otic epithelial cells delaminate (Lawoko-Kerali *et al.*, 2004). When the mouse mammary epithelial cell line was treated with TGFβ1 or TGFβ2, its characteristic epithelial pattern was disrupted and the regular meshwork pattern of cytokeratin was disturbed in these cells (Miettinen *et al.*, 1994).

TABLE 1

FORMATION OF CVG IN EXPLANTS CULTURED WITH TGFβ2

Interval	TGFβ2	Total explants	CVG(+) explants	(%) CVG(-) explants(%)
4 hours	-	14	0 (0.0)	14(100)
	+	14	6(42.9)*	8(57.1)
6 hours	-	25	4(16.0)	21(84.0)
	+	24	15(62.5)*	9(37.5)

*Significantly different from controls by Chi-square test (P<0.01)

In our present study *Tgfβ2* showed an intense and dynamic expression pattern in the developing OV. It was expressed in the anteroventrolateral otic epithelium in the proNS compartment at E9.5 and then also detected in the posterodorsolateral wall by E10.5. At E11.5, the latter *Tgfβ2* expression domain shifted to the posteroventrolateral wall. It was noteworthy that the expression domain of *Tgfβ2*, at least partially, overlapped with those of nuclear phosphorylated Smad2 and *NeuroD*. *NeuroD* is a marker of delaminating CVG precursors in the mouse OV (Ma *et al.*, 1998; Raft *et al.*, 2004). This result suggests that TGFβ2 is likely to signal to *NeuroD* -positive cells in paracrine and/or autocrine fashions.

NGN1 is another neurogenic bHLH transcription factor which is expressed in the same population of the otic epithelium. Both *Ngn1* and *NeuroD* knockouts showed CVG defects (Ma *et al.*,

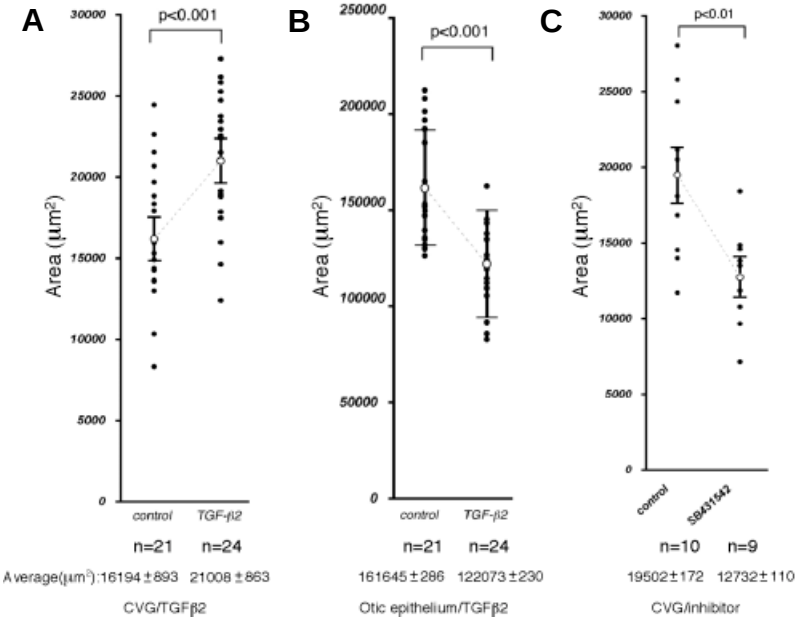

Fig. 7. The size of the CVG and otic epithelium in culture. *The areas of the CVG* **(A)** *and otic epithelium* **(B)** *were estimated on microphotographs by NIH Image program (http://rsb.info.nih.gov/nih-image/). The vertical axis indicates areas (μm²). Dots represent individual values. Open circles indicate the average areas with standard errors. The total numbers of the explants (n) and the average areas are indicated at the bottom of each panel. The area of the CVG with TGFβ2 was significantly larger than control (P<0.001). In contrast, the area of the epithelium with TGFβ2 was significantly smaller than control (P<0.001).* **(C)** *A TGFβ signaling inhibitor, SB431542 (5 μM), significantly decreased the size of the CVG (P<0.01).*

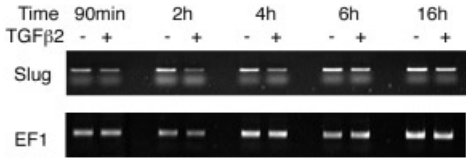

Fig. 8. Downregulation of *Slug* with TGFβ2 in culture *To examine whether Slug is involved in size increase of the CVG,* Slug *expression levels were analyzed by semi-quantitative RT-PCR. Otic vesicle explants from E10.5 embryos were cultured with or without TGFβ2 for 1.5, 2, 4, 6 and 16 hours. After culture, mRNA was extracted from the whole culture including both the otic epithelium and CVG and subjected to RT-PCR with* Slug *primers (top panel). At each time point, the left lane is control (-) and the right lane is with TGFβ2 (+).* Slug *was substantially downregulated with TGFβ2 at 2 hours. The extent of downregulation was less at other time points. Elongation factor 1 primers were used as a control (bottom panel).*

1998; Liu *et al.*, 2000; Kim *et al.*, 2001) and downregulation of *NeuroD* in *Ngn1* mutants implied the requirement of *Ngn1* for *NeuroD* expression. According to Sun *et al.* (2001), Smad1, a mediator of TGFβ signaling, forms a transcriptional complex with NGN1. Therefore, it may be interesting to determine whether TGFβ2 signals to *NeuroD* -positive cells through *Ngn1.*

The *Ngn1* and *NeuroD* expression domains seemed to be restricted to the anterior compartment by TBX1. *Tbx1* is one of T-box genes and is expressed in the developing OV (Raft *et al.*, 2004). Its expression showed a posterior-anterior gradient first at E9.5, suggesting that TBX1 specifies the posterior compartment of the OV. Indeed, the *Ngn1* and *NeuroD* expression domains expanded in *Tbx1* knockouts, leading to the duplication of the CVG rudiment (Raft *et al.*, 2004). Therefore, it is intriguing to examine whether *Tbx1* expression is downregulated by TGFβ2 treatment.

It has been reported that *Bmp4* is also expressed in the developing OV. Wu and Oh (1996) suggested that BMP4 plays important roles in specification of sensory primordia of the otic epithelium. The previous and present studies demonstrated that the expression domains of *Bmp4* and *Tgfβ2* were very close to each other but not overlapped in the anterior and posterior parts of the OV. Since addition of TGFβ2 to OV culture led to increased size of the CVG (a mass of sensory neurons), it may be possible that BMP4 and TGFβ2 cooperatively specify sensory neuron precursors in the developing OV.

We showed that exogenous TGFβ2 increases the size of the CVG and that inhibition of the endogenous signaling suppresses the CVG formation. The data suggest that TGFβ2 may have increased the number of cells which are to become delaminating CVG precursors and/or their proliferation during CVG development. Although TGFβ has been shown to stimulate the proliferation of spiral ganglion cells (Hansen *et al.*, 2001), we observed that the size of otic vesicles treated with TGFβ2 was significantly reduced after 16 hours in culture (Fig. 6C) while the size of the CVG was increased. It is possible that more cells are specified as delaminating CVG precursors with exogenous TGFβ2 at the expense of the epithelial cells, though the possibility cannot be excluded that TGFβ2 accelerates proliferation of the neuronal precursors while it decreases proliferation of non-neuronal precursors.

Recently Lawoko-Kerali *et al.* (2004) reported that the *NeuroD-positive* domain of the OV might be further divided into two regions. The medial region was positive for GATA3, a zinc finger transcription factor and thought to produce the cochlear part of the CVG. The GATA3-negative region was thought to produce the vestibular part. Although these two parts of the ganglion are morphologically separated *in vivo*, we cannot distinguish the two parts in organ culture. Still, it is intriguing to determine which part of the CVG is increased in size after TGFβ2 treatment by examining GATA3 expression.

In spite of defects in other parts of the inner ear (Sanford *et al.*, 1997), the CVG phenotype in *Tgfβ2* mutants has not been reported to date. The inner ear phenotypes of the mutants included the defects of the spiral limbus in the basal cochlear turn and Rosenthal's canal. The primary auditory neurons were normally formed in the mutants, but the interdental cells overlying the spiral limbus were undifferentiated. It should be noted that such anomalies of the inner ear in *Tgfβ2* mutants were restricted to nonsensory areas, which is not consistent with our *in vitro* data. It is likely that the phenotype of the sensory area was rescued *in vivo* by other members of the *Tgfβ* family while that of the nonsensory area was not. This posterior domain did not express the proneural gene *NeuroD*. According to Fekete and Wu (2002), the nonsensory area of the cochlear duct arises from the posteroventrolateral compartment. Therefore, the inner ear defects in null mutants may be related to the posterior domain of *Tgfβ2* expression.

It is not known whether *Tgfβ1/3* rescue the CVG phenotype though they are expressed in or near the OV. TGFβ1 begins to be immunolabeled in the periotic mesenchymal cells surrounding the developing OV, which form the capsule of the inner ear (Frenz *et al.* 1992). Other factors might also compensate the *Tgfβ2* function, since TGFβ2 works synergistically with other factors such as BDNF and glial cell line-derived neutrophic factor (GDNF) in the nervous system (Sometani *et al.*, 2001; Krieglstein *et al.*, 2002; Sometani *et al.*, 2002).

Slug, a zinc finger-type transcription factor, has been reported to play a crucial role in EMT during neural crest cell differentiation (Sefton *et al.*, 1998; del Barrio and Nieto, 2002). Romano and Runyan (2000) suggested that *Slug* is an essential target of TGFβ2 signaling during EMT of endocardial cushion formation. Delamination of CVG precursors from the otic epithelium is similar to EMT in terms of loss of epithelial character. However, in our RT-PCR study, *Slug* mRNA was downregulated when cultured OVs were treated with TGFβ2, which is not consistent with the previous report (Romano and Runyan, 2000). One possible explanation for the discrepancy between the studies is as follows: Slug functions in the epithelial cells to cause transformation and is quickly downregulated in the transformed cells. Although addition of TGFβ2 led to increase of CVG cells and decrease of epithelial cells, the analysis of the culture explants showed that *Slug* mRNA was seemingly downregulated in our experiments. Another possibility is that the delamination of CVG precursors is an unrelated phenomenon to EMT. Delaminated precursors do not seem to go through mesenchymal cell stages. It is rather similar to migration of neuroblasts from the ventricular zone to the mantle zone in the developing neural tube. Therefore, it is possible that Slug plays a different role from that in neural crest development and is differently regulated by TGFβ2 signaling in the OV. Further study is required to elucidate how TGFβ2 regulates *Slug* expression and transformation of the otic epithelial cells and whether Slug is involved in transformation of the otic epithelial cells.

Materials and Methods

RNA in situ *hybridaization*

Embryos were collected between E9.0 and E11.5. The noon on the day of finding a vaginal plug was designated as E0.5. Whole mount and section RNA *in situ* hybridization was carried out as previously described (Wilkinson, 1992; Ishii *et al.*, 1997). Embryos were fixed overnight in 4% paraformaldehyde at 4ºC, rinsed in PBS and dehydrated in 25% sucrose for section or in methanol for whole mount. Transverse sections (20μm) were mounted on APS-coated slides (Matsunami, Japan). Murine *Tgfβ2* probe (Pelton *et al.*, 1991) was kindly provided by Dr. Moses. *NeuroD* (Lee *et al.* 1995) and *Ngn1* probes (Cau *et al.*1997) were gifts from Dr. Kageyama. Digoxigenin-labeled sense and antisense probes were synthesized by digoxigenin RNA labeling kit (Roche Diagnostics Corporation, Indianapolis, IN, USA). Whole mount embryos were cleared in 80% glycerol.

Immnohistochemistry

Embryos at E10.5 were fixed for 1 h in 4% paraformaldehyde at 4ºC, followed by routine procedures for embedding in paraffin. Transverse sections (6μm) were mounted in a serial order on slides. Sections were treated in 10mM sodium citrate buffer at 95ºC for 20 min to unmask antigens. After incubation with the blocking reagent (Nacalai tesque, Japan) for 20 min, sections were incubated with primary antibodies overnight at 4ºC. The anti-phospho-Smad2 antibody and anti-phosphorylated histone 3 antibody were purchased from Cell Signaling (Beverly, MA) and Upstate (Lake Placid, NY), respectively. Unimmuned rabbit IgG was used as a negative control. Immunoreactivity was visualized by ABC kit (Vector Laboratories, USA) with 0.04 % $NiCl_2$. Cultured otic vesicles were fixed for 30 min in 4% (w/v) paraformaldehyde at room temperature. Mouse anti-Tuj1 mAb (Covance, Berkeley, CA, USA) was diluted at 1:1000 with blocking solution. Alexa 568-conjugated goat anti-mouse antibody (Molecular Probes, USA) was used at 1:200. Stained samples were embedded in OCT compound and sectioned at 8μm. Microphotographs were taken on AxioPlan (Zeiss, Germany) or VB-7010 (Keyence, Japan). Adobe Photoshop was used to process the photographs.

Semi-quantitative RT-PCR

Otic vesicles with surrounding mesenchyme were dissected out from embryos at E10.5. They were treated in 1 unit/ml dispase for 10 minutes at 37ºC. After rinsing in PBS briefly, the surrounding mesenchyme was removed from otic vesicles. Hearts were also dissected out as a positive control. RNA from otic vesicles or heart tissues was isolated using TRIZOL (Invitrogen, Tokyo, Japan). The first strand complementary DNA (cDNA) was synthesized with random primers by Superscript First-Strand Synthesis System (Invitrogen, Tokyo, Japan). PCR was performed with HotStarTaq (QIAGEN, Tokyo, Japan). PCR conditions were: 30sec at 94ºC, 30sec at 58ºC and 1 min at 72ºC for 27, 30 or 33 cycles. Products were electrophoresed on 1.5% agarose gel. Primers were as follows: For TGFβ typeI receptor (511bp product),

Forward: 5'-GCCATAACCGCACTGTCA-3',
Reverse: 5'-ATGGGCAATAGCTGGTTTTC-3';

For TGFβ typeII receptor (437bp product),

Forward: 5'-CCCGGGGCATCGCTCATCTC-3',
Reverse: 5'-AATTTCTGGGCGCCCTCGGTCTCT-3';

For Slug (166bp product),

Forward: 5'-CCAAGGATCACAGTGGTTCA-3',
Reverse: 5'-CAGTGCAGCTGCTTGTGTTT-3'

Organ culture of otic vesicles

Otic vesicles were dissected out as described above. CVGs which had already developed were removed and then vesicles were transferred into four-well plates (NUNC, Roskilde, Denmark). They were incubated at 37ºC in a water-saturated atmosphere containing 5% CO_2 (Leon *et al.*, 1995). The standard culture medium consisted of M199 medium with Earle's salts (Gibco, Tokyo, Japan) supplemented with 2mM glutamine (Nacalai tesque, Kyoto, Japan), 1% antibiotic-antimycotic (10,000 units/ml penicillinG, 10,000 μg/ml streptomycin and 25 μg/ml amphotericinB; Gibco, Tokyo, Japan) and 0.1% BSA (Wako, Tokyo, Japan). Recombinant human TGFβ2 protein (Peprotech EC Ltd, London, UK) was added in culture at 10 ng/ml. The TGFβ inhibitor, SB431542, was purchased from Tocris Cookson Ltd (Bristol, UK) and dissolved at a concentration of 10 mM in DMSO as a 2000 x stock solution. As SB431542 was dissolved in DMSO, 0.05% DMSO was used as a negative control in each experiment.

Statistical analysis of otic vesicle and CVG areas

Areas of otic vesicles or CVGs were measured on their photomicrographs using Adobe Photoshop and NIH Image. Mann-Whitney U-test was used to assess significance of the data. For Table 1, 2◊2 Chi square test and Fisher's test was used.

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