

ology. However, it indicates that an inflammatory disturbance may influence the etiology.

MT-3 is a neuron-specific member of the zinc- and copper-binding metallothionein family with a role in metal homeostasis and in scavenging free radicals [19,20]. The elevation of MT-3 expression in G93A SOD1 transgenic mice was reported [21,22], and reduction of MT-3 promotes the onset of disease and death in G93A SOD1 transgenic mice [23]. In sporadic Alzheimer's disease brains, MT-3 is significantly down-regulated [19,24]. In the present study we demonstrated that MT-3 mRNA is decreased in the SALS spinal cords, but another report failed to show a significant difference in the MT-3 mRNA expression level in SALS [25]. The fact that MT-3 was up-regulated in FALS but down-regulated in SALS leads one to speculate that this molecule may work for protection in FALS but at the same time may have a causative role in the disease progression of SALS pathogenesis. Although the reduction of its expression might be only due to the loss of neurons in the SALS spinal cords, we did not investigate its expression level in single neurons.

UBL5 is one of the ubiquitin-like proteins. It may possibly organize another ubiquitin-like system, but its function remains unknown [26].

In summary, we identified six differentially expressed genes in SALS spinal cords, including two novel genes, using molecular indexing, subsequent microarray analysis, and real-time RT-PCR for verification. These molecules are involved in cellular mechanisms known to be associated with SALS pathophysiology, including oxidative toxicity, inflammation, transcription and the ubiquitin-proteasome system.

Thus, our screening method using molecular indexing for preliminary screening was useful to uncover unknown genes and genes with rather low expression levels that might have pivotal roles in the pathogenesis of SALS.

Extensive analysis of these genes is necessary to understand how they participate in the disease course and if they are key molecules to explore treatments for ALS.

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Suppression of Immune Induction of Collagen-Induced Arthritis in IL-17-Deficient Mice¹

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Interleukin-17 is a T cell-derived proinflammatory cytokine. This cytokine is suspected to be involved in the development of rheumatoid arthritis (RA) because this cytokine expression is augmented in synovial tissues of RA patients. The pathogenic roles of IL-17 in the development of RA, however, still remain to be elucidated. In this study, effects of IL-17 deficiency on collagen-induced arthritis (CIA) model were examined using IL-17-deficient mice (IL-17^{-/-} mice). We found that CIA was markedly suppressed in IL-17^{-/-} mice. IL-17 was responsible for the priming of collagen-specific T cells and collagen-specific IgG2a production. Thus, these observations suggest that IL-17 plays a crucial role in the development of CIA by activating autoantigen-specific cellular and humoral immune responses. *The Journal of Immunology*, 2003, 171: 6173–6177.

Rheumatoid arthritis (RA)⁴ is one of the most serious medical problems, affecting ~1% of all people worldwide, irrespective of race. The disease is autoimmune in nature and characterized by chronic inflammation of the synovial tissues in multiple joints that leads to joint destruction, but the etiopathogenesis has not been elucidated completely (1).

Various disease models for RA have been developed, and collagen-induced arthritis (CIA) is one of the well-established models (2). CIA can be induced in susceptible rodents by intradermal injections of homologous or heterologous native type II collagen (IIC), a major component of cartilage Ags. Susceptibility for the disease is dependent on MHC class II haplotypes, and only mice with H-2^a and H-2^b haplotypes respond to immunization with IIC and develop arthritis (3). Recently, however, it is reported that CIA can also be induced on C57BL/6 (H-2^b) mice by repeated administration with IIC intradermally (4–6). The development of CIA is dependent on both cellular and humoral immune responses to IIC (6), and various cytokines are thought to play crucial roles in the pathogenesis through the activation of immune system (6, 7).

A T cell-derived proinflammatory cytokine, IL-17 is produced by TCR α / β ⁺CD4⁻CD8⁻ thymocytes, as well as activated CD4⁺ and CD4⁺CD45RO⁺ memory T cells (8). The producer cells of IL-17 also express TNF- α , but not Th1 or Th2 cytokines, in mice (9). IL-17 has pleiotropic activities, including induction of TNF- α , IL-1 β , IL-6, IL-8, G-CSF, and monocyte chemoattractant protein-1 on various types of cells (8).

Involvement of IL-17 in the development of RA is suggested, because IL-17 is found in the synovial fluid of RA patients (10) and is produced by T cell clones established from RA patients (11). Actually, the incidence of arthritis reduced partially by the administration of an extracellular domain of IL-17R-Fc fusion protein (IL-17R:Fc), which inhibits IL-17-IL-17R binding, in the elicitation phase during CIA (12). The precise role for IL-17 in the pathogenesis of RA, however, still remains to be elucidated.

In this study, using IL-17^{-/-} mice, we assessed the role of IL-17 in the development of CIA. The incidence of CIA was markedly suppressed in IL-17^{-/-} mice, and a crucial role for IL-17 was suggested in activating collagen-specific T and B cells during CIA.

Materials and Methods

Mice

IL-17^{-/-} mice were generated, as described previously, using E14.1 ES cells (13). For CIA induction, IL-17^{-/-} mice on (129/Sv \times C57BL/6)F₁ hybrid background were used, and the IL-17^{+/+} littermates were used as the controls. These mice were kept under specific pathogen-free conditions in an environmentally controlled clean room in the Center for Experimental Medicine, Institute of Medical Science, University of Tokyo. The experiments were conducted according to the institutional ethical guidelines for animal experiments and the safety guideline for gene manipulation experiments.

Collagen-induced arthritis

CIA in mice on 129 \times B6 F₁ hybrid background was performed, as described elsewhere (5). Briefly, mice were immunized with 100 μ l of 1 mg/ml chicken IIC (Sigma-Aldrich, St. Louis, MO) emulsified with CFA intradermally at several sites into the base of the tail. CFA was prepared by the mixture of 100 mg heat-killed *Mycobacterium tuberculosis* (H37Ra; Difco Laboratories, Detroit, MI) and 20 ml IFA (Difco). At 21 days after the first immunization, mice were again challenged with collagen/CFA nearby the primary injection site intradermally.

Clinical and histological assessment of arthritis

Development of arthritis by macroscopic evaluation was determined, as described elsewhere (14). At 60 days after the first immunization during CIA, limbs were fixed with 10% neutral Formalin and were decalcified with 5% formic acid. They were embedded in paraffin, and 5- μ m slices were prepared. Sections were stained with H&E.

T cell culture

For IIC-specific T cell proliferative response, inguinal lymph nodes (LNs) were harvested from mice at 1 wk after the primary immunization with IIC/CFA. Single cell suspension was prepared, and LN cells (3 \times 10⁵ cells/well on 96-well flat-bottom plate) were cultured in the absence or presence of 50 μ g/ml denatured chicken IIC for 72 h, followed by incorporation of [³H]thymidine (0.25 μ Ci/ml) (Amersham, Chalfont St. Giles,

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⁴ Abbreviations used in this paper: RA, rheumatoid arthritis; CHS, contact hypersensitivity response; CIA, collagen-induced arthritis; EAE, experimental autoimmune encephalomyelitis; IIC, type II collagen; LN, lymph node.

Buckinghamshire, U.K.) for 6 h. Then cells were harvested with a Micro 96 cell harvester (Skatron, Lier, Norway), and radioactivity was measured with Micro Beta (Pharmacia Biotech, Piscataway, NJ).

Detection of cytokine by ELISA

IL-17 levels were measured by ELISA, as described previously (13). Monoclonal rat anti-mouse IL-17 and polyclonal biotinylated goat anti-mouse IL-17 Abs (DAKO, Carpinteria, CA) were used as a capture and detection Ab, respectively. HRP-avidin was obtained from BD Pharmingen (San Diego, CA), and a tetramethylbenzidine One-Step Substrate System was obtained from DAKO. rIL-17 as a standard reagent was obtained from Sigma-Aldrich. To measure IL-4 and IFN- γ levels, BD OptiEIA ELISA Sets were purchased from BD Pharmingen.

Measurement of collagen-specific Ig titers

Sixty days after the first and second immunizations with IIC/CFA, serum was collected. A total of 10 μ g/ml IIC in PBS was coated on Falcon 3912 Micro Test III Flexible Assay Plates (BD Biosciences, Oxnard, CA) at 4°C overnight. After washing with PBS, serially diluted serum samples were applied and incubated at room temperature for 1 h. Then the wells were washed with PBS + 0.05% Tween 20, followed by the addition of alkaline phosphatase-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (Zymed, San Francisco, CA). Alkaline phosphatase activity was measured using Substrate Phosphatase SIGMA104 (Sigma-Aldrich) as the substrate. Results are expressed by the absorbancy at 415 nm.

Results

Suppression of development of CIA in IL-17^{-/-} mice

To assess the role of IL-17 in the development of arthritis, the effect of IL-17 deficiency on the development of CIA was examined. As shown in Fig. 1A, the incidence of the disease in IL-17^{-/-} mice was markedly suppressed compared with that in IL-17^{+/+} mice. Likewise, the severity score in IL-17^{-/-} mice was also milder than that in IL-17^{+/+} mice (Fig. 1B). A histological analysis of the joints of IL-17^{+/+} mice immunized with IIC showed typical features of arthritis, which was characterized by marked synovial and periarticular inflammation with extensive polymorphonuclear cell infiltration, synovial hyperplasia, and bone erosion (Fig. 2, C and E). The articular bone and cartilage were invaded with granulation tissues forming a pannus, and degenerative products of the bone structure were found in the articular cavity. In contrast, the joint pathology of IL-17^{-/-} mice revealed much milder inflammation with profoundly reduced cell infiltration, and synovial hyperplasia and bone erosion were also suppressed, indicating both inflammatory and destructive features of the joints were suppressed in IL-17^{-/-} mice (Fig. 2, D and F). These results indicate that IL-17 is involved in the development of CIA.

Reduced Ab production against IIC in IL-17^{-/-} mice

Because Ab levels against IIC correlate well with the development of arthritis (15), we examined the development of IIC-specific Abs in IL-17^{-/-} mice. Sera were collected 60 days after the first immunization with chicken IIC/CFA, and IIC-specific Ab levels were measured by ELISA. Collagen-specific IgG2a levels in sera from IL-17^{-/-} mice were significantly lower than those from IL-17^{+/+} mice, whereas the levels of other IgG subclass Abs were not different between IL-17^{+/+} and IL-17^{-/-} mice (Fig. 3). These results indicate that IL-17 is involved in collagen-specific Ab production during CIA.

Reduced IIC-specific T cell responses in IL-17^{-/-} mice

To elucidate the role of IL-17 in T cell function, we examined T cell response in IL-17^{-/-} mice. A T cell Ag-recalling assay against IIC was conducted 1 wk after the first immunization with chicken IIC/CFA. Proliferative response against IIC of LN cells from IL-17^{-/-} mice was significantly reduced compared with that from IL-17^{+/+} mice (Fig. 4A), showing that IL-17-deficient T cells are sensitized incompletely. During the incubation, a significant

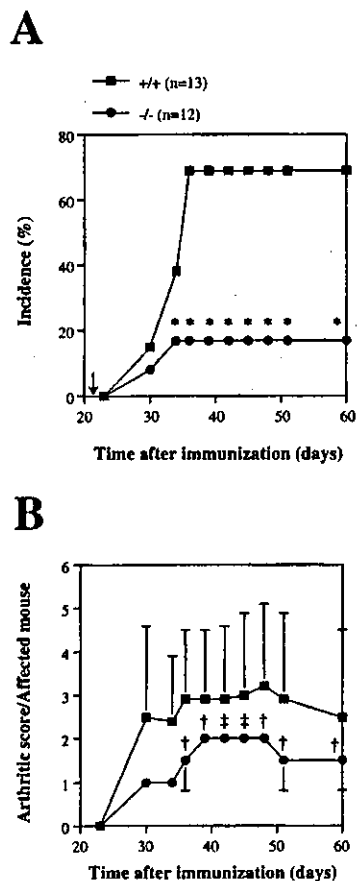


FIGURE 1. The reduced development of CIA in IL-17^{-/-} mice. Mice were immunized with chicken IIC emulsified with CFA intradermally at several sites into the base of the tail on days 0 and 21 (arrow). Incidence (A) and severity (B) of CIA are shown. IL-17^{+/+} mice, ■ (n = 13) and IL-17^{-/-} mice, ● (n = 12). Data obtained from two independent experiments were combined and shown. Average and SD are indicated. *, $p < 0.005$ vs IL-17^{+/+} mice by χ^2 test. †, $p < 0.01$, and ‡, $p < 0.05$ vs IL-17^{+/+} mice by Mann-Whitney U test.

amount of IL-17 was secreted in the supernatants of IL-17^{+/+} LN cell cultures, but no production was detected in IL-17^{-/-} LN cell cultures (Fig. 4B). IFN- γ production after stimulation with IIC was markedly reduced in IL-17^{-/-} LN cell culture, while IL-4 production was almost undetectable both in the IL-17^{+/+} LN cell culture and IL-17^{-/-} T cell culture (Fig. 4B). These results indicate that IL-17 plays an important role in the Ag-specific T cell activation during priming phase of CIA.

Discussion

In this study, we showed that, by using IL-17^{-/-} mice, IL-17 is crucial for the activation of autoantigen-specific T and B cells in the sensitization phase of CIA. Various inflammatory cells including T cells are found to infiltrate the local inflammatory site of RA patients, and it has been believed that the T cell-derived Th1 cytokine is involved in the pathogenesis of RA. However, IFN- γ , a major Th1 cytokine, has been considered to play rather beneficial roles (16–18). In contrast, inflammatory cytokines, TNF- α and GM-CSF, which are also produced by CD4⁺ T cells (9), are suggested to have an important role in the RA pathogenesis. Indeed, the development of CIA in TNF- α ^{-/-} mice (C57BL/6 background) was milder than that of wild-type mice, and markedly suppressed in GM-CSF^{-/-} mice (backcrossed to C57BL/6 for 11

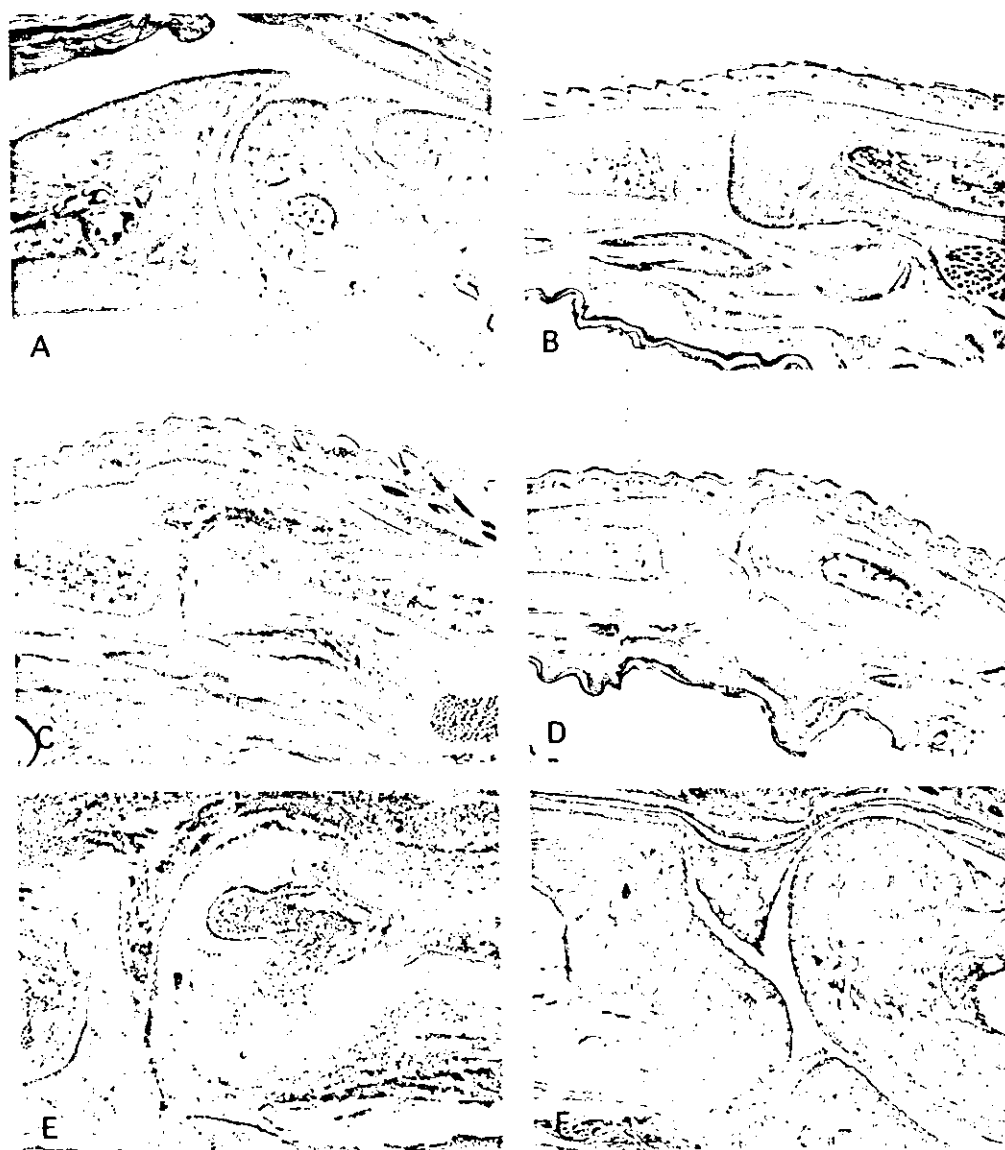


FIGURE 2. Histological analysis in the joint of hind limbs during CIA. Sections of the joint of hind at 60 days after the first immunization during CIA were stained with H&E. The joint of nonimmunized IL-17^{+/+} (A) and IL-17^{-/-} (B) mice and that of IIC-immunized IL-17^{+/+} (C and E) and IL-17^{-/-} (D and F) mice. A–D, $\times 40$; E and F, $\times 100$.

generations) (6). Because both TNF- α and GM-CSF are produced by various cell types including fibroblasts, the suppressive effect of CIA may not solely be a result of their production by CD4⁺ T cells. However, in a recent study, it was reported that IL-17 is produced by CD4⁺ T cells that are coexpressing TNF- α and/or GM-CSF, but not by Th1 and Th2 cells (9). Thus, a certain T cell subset, coexpressing IL-17, TNF- α , and/or GM-CSF rather than Th1-producing IFN- γ , may have an important role in the pathogenesis of RA. Although it was believed that contact hypersensitivity response (CHS) is mediated by IFN- γ -producing Tc1 and Th1 cells (19), we showed that CHS was normal in IFN- γ ^{-/-} mice (20), while it was markedly suppressed in IL-17^{-/-} mice (13), indicating that CHS as well as CIA are mediated by CD4⁺ T cells producing IL-17, but not IFN- γ . Moreover, experimental autoimmune encephalomyelitis (EAE) is also considered to be an IFN- γ -producing Th1 cell-mediated inflammatory autoimmune disease. However, many investigators have shown that IFN- γ ^{-/-} and IFN- γ R^{-/-} mice are highly susceptible to EAE compared with control mice, indicating that IFN- γ -producing Th1 cells are required for

the protection of this disease (21–24). In contrast, IL-17 mRNA was increased in multiple sclerosis, suggesting that IL-17 is involved in the pathogenesis of multiple sclerosis (25, 26). In fact, in our unpublished observation, EAE in IL-17^{-/-} mice was significantly suppressed (Y. Komiyama, S. Nakae, and Y. Iwakura, in preparation). Thus, IL-17-producing T cells rather than IFN- γ -producing Th1 cells play critical roles in the pathogenesis of T cell-dependent autoimmune disease.

Recently, it was reported that the administration of IL-17R:Fc fusion protein to DBA/1 mice after secondary immunization with IIC/CFA suppresses the disease by blocking the IL-17/IL-17R binding, suggesting that IL-17 plays an important role in the inflammatory phase (12). In the present study, we have shown that IL-17 is involved in autoantigen-specific T cell priming and expansion (Fig. 4), although the roles of IL-17 in the inflammatory phase are not known in this experiment. Taken together, these observations indicate that IL-17 plays important roles not only in the induction of local inflammation of joints, but also in autoantigen-specific T cell activation in the priming phase.

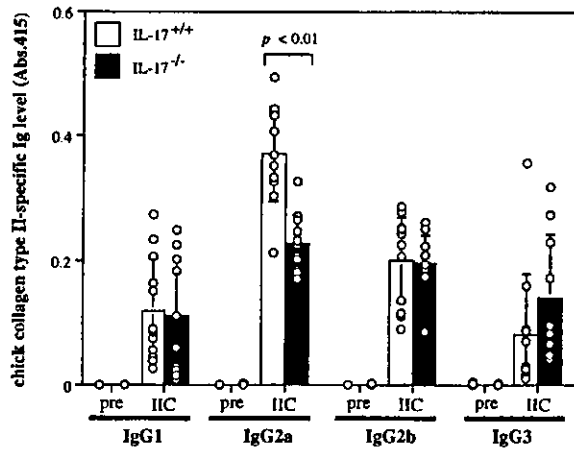


FIGURE 3. Impaired collagen-specific IgG2a production in IL-17^{-/-} mice during CIA. Before or 60 days after the first immunization during CIA, sera were collected and collagen-specific Ig levels in sera were determined by ELISA. Serum samples were diluted 100-fold. Each circle represents an individual mouse, and an average and SD are shown. IL-17^{+/+} mice are open columns ($n = 13$), and IL-17^{-/-} mice are closed columns ($n = 12$).

As mentioned above, IL-17 is required for autoantigen- and allergen-specific T cell priming and Ab production (Figs. 3 and 4) (13). Yao et al. (27) also reported that T cell proliferation and IL-2 production induced by PHA, Con A, and anti-TCR mAb were inhibited by soluble IL-17R, indicating that IL-17 is involved in T cell activation. To elucidate the molecular mechanism of T cell activation, we analyzed compartmentalization of T cell subset in lymphoid tissues and DC functions in IL-17^{-/-} mice, and found they are normal (13). Microarchitecture and germinal center formation of the spleen and LNs were also normal in IL-17^{-/-} mice before and after IIC immunization (data not shown). Thus, the precise molecular mechanism of T cell activation by IL-17 still remains to be elucidated.

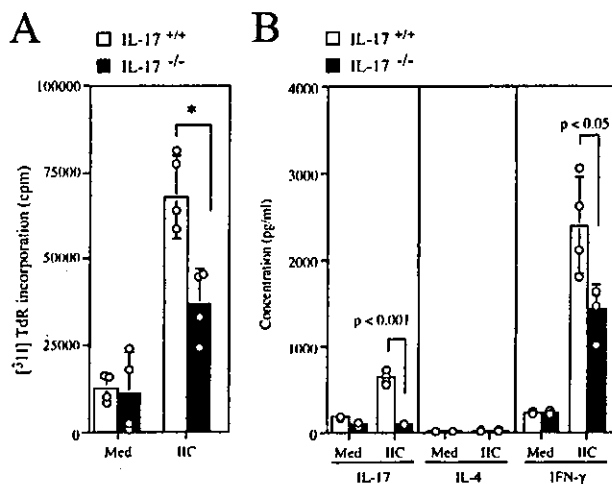


FIGURE 4. Impaired collagen-specific T cell responses in IL-17^{-/-} mice during CIA. One week after immunization with chicken IIC/CFA intradermally, inguinal LN cells from IL-17^{+/+} or IL-17^{-/-} mice were cultured in the absence or presence of 50 μ g/ml denatured chicken IIC for 72 h. **A**, Proliferative response was measured by [³H]thymidine incorporation. **B**, IL-17, IL-4, and IFN- γ levels in supernatants of **A** were determined by ELISA. Each circle represents an individual mouse, and an average and SD are shown. These results were reproducible two independent experiments. *, $p < 0.05$, and #, $p < 0.01$.

It was reported that C57BL/6 mice produce IgG2c instead of IgG2a, while 129/Sv mice produce IgG2a (5, 28). Although it was suggested that collagen-specific IgG2a contributes to the development of CIA (29), C57BL/6 mice and (C57BL/6 \times 129/Sv) F_1 mice, but not 129/Sv mice, developed CIA under our experimental conditions (5). Thus, IgGs other than IgG2a might also contribute to the development of arthritis.

We showed that collagen-specific IgG2a levels in sera of collagen-immunized IL-17^{-/-} mice were significantly reduced compared with those of wild-type mice (Fig. 3). However, because secondary anti-IgG2a Ab that we used may cross-react with IgG2c, although the efficiency for IgG2c should be much reduced (28), the IgG2a fraction may contain a small fraction of IgG2c in addition to IgG2a.

In summary, IL-17 plays a critical role in the development of CIA. These findings may provide a clue to develop novel therapeutics.

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Six1 controls patterning of the mouse otic vesicle

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Summary

Six1 is a member of the Six family homeobox genes, which function as components of the Pax-Six-Eya-Dach gene network to control organ development. *Six1* is expressed in otic vesicles, nasal epithelia, branchial arches/pouches, nephrogenic cords, somites and a limited set of ganglia. In this study, we established *Six1*-deficient mice and found that development of the inner ear, nose, thymus, kidney and skeletal muscle was severely affected. *Six1*-deficient embryos were devoid of inner ear structures, including cochlea and vestibule, while their endolymphatic sac was enlarged. The inner ear anomaly began at around E10.5 and *Six1* was expressed in the ventral region of the otic vesicle in the wild-type embryos at this stage. In the otic vesicle of *Six1*-deficient embryos, expressions of *Otx1*, *Otx2*, *Lfng* and *Fgf3*, which were expressed ventrally in the wild-type otic vesicles, were abolished, while the expression

domains of *Dlx5*, *Hmx3*, *Dach1* and *Dach2*, which were expressed dorsally in the wild-type otic vesicles, expanded ventrally. Our results indicate that *Six1* functions as a key regulator of otic vesicle patterning at early embryogenesis and controls the expression domains of downstream otic genes responsible for respective inner ear structures. In addition, cell proliferation was reduced and apoptotic cell death was enhanced in the ventral region of the otic vesicle, suggesting the involvement of *Six1* in cell proliferation and survival. In spite of the similarity of otic phenotypes of *Six1*- and *Shh*-deficient mice, expressions of *Six1* and *Shh* were mutually independent.

Key words: *Six1*, Otic vesicle, Inner ear, Pattern formation, Cell proliferation, *Shh*, Mouse

Introduction

The Six gene family was identified as a homologue of the *Drosophila sine oculis* (*so*) and is conserved in various species (Seo et al., 1999; Kawakami et al., 2000). Six gene products are characterized by the Six domain and Six-type homeodomain, which are required for specific DNA binding activity and function as transcription factors (Kawakami et al., 1996; Spitz et al., 1998; Ohto et al., 1999; Li et al., 2002; Lagutin et al., 2003). At present, six members of the family have been identified in mammals, and all members show a spatiotemporally regulated pattern of expression during embryogenesis, suggesting their involvement in embryonic development (Seo et al., 1999; Kawakami et al., 2000). The Six gene family is known to function as a component of the Pax-Six-Eya-Dach gene network. This property was originally identified in genetic studies using *Drosophila*. Compound eye formation has been extensively examined as a model system of organ development, and the important eye-forming genes, *eyeless* (*ey*, a *Pax6* homologue), *twin of eyeless* (*toy*, another *Pax6* homologue), *sine oculis* (*so*, a Six homologue), *eyes absent* (*eya*, an Eya homologue) and *dachshund* (*dac*, a Dach

homologue), have been identified. Genetic and biochemical studies have revealed the hierarchy, cooperative relationships and physical interactions among these genes and their encoded proteins; *toy* activates *ey* (Czerny et al., 1999), and *ey* and/or *toy* activate *so* and *eya* (Halder et al., 1998; Niimi et al., 1999; Zimmerman et al., 2000), then *so* and *eya* cooperate to activate *dac* (Pignoni et al., 1997; Chen et al., 1997). In addition to such a hierarchy, reciprocal feedback loops operate to form complex regulatory gene networks (Chen et al., 1997; Pignoni et al., 1997). Of note is that the vertebrate homologues of these *Drosophila* genes, *Pax6* (Walther and Gruss, 1991), *Six3/Six6* (Oliver et al., 1995a; Toy et al., 1998), *Eya1/Eya2/Eya3* (Xu et al., 1997) and *Dach1/Dach2* (Caubit et al., 1999; Davis et al., 2001), are expressed in the developing eyes, and some of them were shown to be involved in eye development (Hill et al., 1991; Ton et al., 1991; Glaser et al., 1992; Oliver et al., 1996; Kobayashi et al., 1998; Loosli et al., 1999; Lagutin et al., 2001; Carl et al., 2002; Li et al., 2002; Lagutin et al., 2003). A similar gene network was found to control chick myogenesis, in which *Six1*, *Eya2* and *Dach2* synergistically regulate the expression of myogenic genes such as myogenin and *MyoD* (Heanue et

al., 1999). In addition, *Pax3* induces the expression of *Six1* and *Eya2* before induction of *MyoD* and myogenin expression (Ridgeway and Skerjanc, 2001). *Pax3* is involved in myogenesis also by activating *Dach2* expression and is reciprocally activated by *Dach2* (Heanue et al., 1999; Kardon et al., 2002). Furthermore, homologues of these gene families are expressed in various developing organs in a spatially and temporally overlapping manner during embryogenesis, suggesting that similar gene networks regulate the development of various organs in addition to the eye and skeletal muscles. In fact, an increasing number of loss-of-function mutations in *Pax*, *Eya* and *Six* genes have been reported to cause defects in various organs. *Pax2*-deficient mice show defects in eyes, ears and the urogenital system (Favor et al., 1996; Torres et al., 1996). Loss of *Eya1* in mice results in the absence or anomalies in the ear, thymus, parathyroid gland, kidney, thyroid and skeleton (Xu et al., 1999; Xu et al., 2002). For *Six* genes, inactivation of mouse *Six6* is associated with hypogenesis of the pituitary gland and retina (Li et al., 2002). *SIX3* mutations in humans cause holoprosencephaly, and *Six3* inactivation in mice results in a lack of anterior head structures, including eyes and nose (Wallis et al., 1999; Lagutin et al., 2003).

Six1 is expressed in otic vesicles, nasal epithelia, branchial arches/pouches, nephrogenic cords, somites and a limited set of ganglia (Oliver et al., 1995b). However, it is unknown whether or how *Six1* is involved in the development of the inner ear, nose, branchial arch/pouch-derived organs, kidney, ganglia and skeletal muscles. To address this question, we generated and analyzed the organ development of *Six1*-deficient mice. The inner ear, nose, thymus, kidney and skeletal muscles are severely affected in *Six1*-deficient mice, suggesting crucial roles for *Six1* in the development of these organs. Among these phenotypes, the defects in inner ear development in the mutant mice are intriguing because inner ears develop elaborate structures with precise disposition and orientation in normal embryogenesis. They are derived from the otic vesicle by successive transformation and compartmentalization, but it is poorly understood how the patterning of the otic vesicle is established and what are the key factors for such complex processes. Thus, this paper focused on the analysis of inner ear development and identified the essential roles of *Six1* in otic vesicle patterning.

Materials and methods

Construction of the *Six1* targeting vector

The entire coding region of the murine *Six1* gene was isolated from a 129/SvJ mouse genomic library (Stratagene, La Jolla, California) using a *Six1* cDNA (Oliver et al., 1995b) as a probe, and the exon-intron organization was determined. An *NcoI* site was generated at the initiation codon by PCR mutagenesis to allow the insertion of an in-frame enhanced green fluorescent protein (EGFP) gene. The targeting vector was constructed in pBluescript KS(+) (Stratagene) and the organization is shown in Fig. 1A. In this construct the entire coding region, including exons 1 and 2, the intervening intron and the short stretch of the 3' untranslated region of exon 2, were replaced with an EGFP fragment (*NcoI-SspI*, 1.0 kb) from pEGFP-N3 (Clontech, Palo Alto, California) and an *hph* cassette (*EcoRV-PvuII*, 2 kb) from pPGK-*hph-bpA* (Horai et al., 1998). The diphtheria toxin A cassette (dt) (*XhoI-NotI*, 1.4 kb) from pMC1DTPa (Yagi et al., 1993) was added at the 3' terminus for the

negative selection. The resulting plasmid was linearized with *SaII* at the 5' end of the insert.

ES cell screening and chimeric mouse production

The linearized targeting vector (80 µg) was electroporated (250 V, 500 µF) into 1×10^7 E14.1 ES cells (Kuhn et al., 1991) and transformants were selected with hygromycin B (230 µg/ml; Invitrogen Japan K.K., Tokyo) for 5-9 days. Homologous recombinants were screened by Southern blot hybridization. Genomic DNA from each resistant clone was digested with *NcoI*, analyzed by Southern blotting using the probes *NcoI-SaI*, 1.2 kb fragment upstream of 5' homology (5' probe), and *XbaI-EcoRI*, 2.0 kb fragment downstream of 3' homology (3' probe), to confirm the correct homologous recombination at 5' and 3' sides, respectively (Fig. 1A). Chimeric mice were produced by the aggregation method (Horai et al., 1998). Male chimeras were bred with C57BL/6 female mice to check germline transmission. Heterozygous mice were intercrossed to produce *Six1*-deficient mice. Genotyping was carried out by Southern blot analysis (Fig. 1B) or PCR (data not shown) in combination with morphological analyses. In the PCR analysis, the targeted allele was detected with primers WtmSix1F (GCG CCC GGG CCC GTG CGC CCC) and K0mSix1R (TGC CCC AGG ATG TTG CCG TCC), and the wild-type allele with primers WtmSix1F and WtmSix1R (GCT TTC AGC CAC AGC TGC TGC).

In this study, *Shh* mutant mice with a targeted deletion of exon 2 of the gene were also used (Chiang et al., 1996) (kindly supplied by C. Chiang and C. C. Hui).

Mice were kept under specific pathogen-free conditions in environmentally controlled clean rooms at the Center for Experimental Medicine, Jichi Medical School, and at the Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo. All mice used in this study were sacrificed by cervical translocation or anesthetization with diethyl ether. The experiments were conducted according to the institutional ethical guidelines for animal experiments and safety guidelines for gene manipulation experiments.

Histological examinations

Embryos and neonates were fixed in 10% formalin or 4% PFA in PBS, embedded in paraffin wax and then cut into 5-µm thick serial sections. De-waxed sections were stained with hematoxylin and eosin as described previously (Ozaki et al., 2001). Alcian Blue/Alizarin Red staining of neonatal skeletons was performed as described previously (Wallin et al., 1994).

RNA in situ hybridization

In situ hybridization was performed using digoxigenin (DIG)-labeled antisense riboprobes as described previously (Xu and Wilkinson, 1998). *Eya1* riboprobe was synthesized from a 528 bp *HindIII* fragment of pHM6Eya1 (Ohto et al., 1999) subcloned into pBluescript KS(+). *Six4* riboprobe was synthesized from a 630 bp *PstI* fragment (ntd 1545-2175 of *Six4* SM type cDNA) subcloned into pBluescript KS(+). The following cDNAs were also used for in situ hybridization probes: *Six1* (Oliver et al., 1995b), *Otx1* and *Otx2* (Matsuo et al., 1995), *Fgf3* (Wilkinson et al., 1988), *Lfng* (Morsli et al., 1998), *Dlx5* (Miyama et al., 1999), *Dach1* (Caubit et al., 1999), *Dach2* (Davis et al., 2001), *Pax2* (Nishinakamura et al., 2001), *Bmp4* (a kind gift from N. Ueno), *Hmx3* (Wang et al., 1998), *Shh* (Uruse et al., 1996), *Ptch* (Goodrich et al., 1996), *Gli1* (Hui et al., 1994), *Wnt2b* (Riccomagno et al., 2002).

TUNEL analysis

For terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling (TUNEL), embryos were fixed in 4% PFA in PBS, embedded in OCT compound, and frozen and sectioned into serial cryosections. Apoptotic cells were detected with the In Situ Cell Death Detection Kit, POD (Roche Diagnostics Mannheim, Germany). Briefly,

fragmented DNA in apoptotic cells was end-labeled with fluorescein and the labeled DNA was detected with anti-fluorescein antibody conjugated with peroxidase and a chromogenic substrate.

BrdU Incorporation

Pregnant female mice of gestation day 10.5 and 11.5 were intraperitoneally injected with 100 mg 5-bromo-2'-deoxyuridine (BrdU) per kg body weight. Embryos were collected 1.5 hours later and processed for preparation of 8- μ m thick paraffin sections as described above. De-waxed serial sections crossing otic vesicles were treated with 2 N HCl/0.5% Triton X-100 in PBS for 30 minutes at room temperature and rinsed with 0.1 M borate buffer (pH 8.5), followed by incubation in 0.6% H₂O₂ in PBS for 30 minutes at room temperature. Subsequently, the sections were incubated overnight in peroxidase-labeled anti-BrdU (Roche) at 4°C. After washing, sections were stained in 0.4 mg/ml diaminobenzidine, 0.68 mg/ml imidazole, 0.01% H₂O₂, and 50 mM Tris-HCl (pH 7.4).

Paint-fill analysis

Paint-fill was performed as described previously (Bissonnette and Fekete, 1996). In brief, embryos were fixed in Bodian's fixative, dehydrated through graded ethanol solutions, then cleared in methyl salicylate and injected into the lumen of the membranous labyrinth with white paint diluted 1 to 100 in methyl salicylate.

ABR threshold measurements

The auditory evoked response was recorded with stainless steel needle electrodes inserted subcutaneously into the vertex (active), left and right of the retro-auricular regions (inactive) and the opposite thigh (ground). The stimulus sound in peak equivalent sound pressure level (peSPL) of a tone pips of 0.1 millisecond slopes, 1 millisecond duration, 70 millisecond repeat interval with 5.6, 8.0, 12.0, 18.0, 24.0, 32.0 kHz frequencies was given by free field in an electrically shielded room. A tweeter (PT-R111, Pioneer) was placed 10 cm in front of the external acoustic foramen. The stimulus sound pressure was corrected by a Bruel & Kjaer-type 2636 noise meter. A microcomputer (ER-2104, GE Marquet) was used to analyze the response. Auditory

thresholds were obtained for each stimulus by varying at 10 dB steps up and down to identify the lowest level at which an auditory brain response (ABR) pattern could be recognized. These experiments were conducted in five wild-type and six heterozygous mice at 5 to 6 weeks of age.

Results

Generation of *Six1*-deficient mice

To explore the developmental roles of *Six1*, we inactivated *Six1* in ES cells by replacing the entire coding region with EGFP gene (Fig. 1A). Two independent ES clones were confirmed as homologous recombinants (data not shown), and both of them gave germline chimeric mice. These chimeric mice were crossed with C57BL/6 to obtain F1 heterozygous mutant mice. Heterozygotes appeared normal in appearance and grew up to adulthood as wild type (data not shown). The concentrations of Na⁺, K⁺, Cl⁻, Ca²⁺, Mg²⁺, inorganic phosphate, urea nitrogen, creatinine, albumin and uric acid in serum and urine were measured with an auto-analyzer, and we observed no significant difference in the concentrations of all these parameters between the wild-type and heterozygous mutant mice (data not shown). We also performed ABR testing for hearing impairment in the heterozygous mutant mice. The ABR thresholds were 25.0 \pm 5.3, 14.0 \pm 5.2, 14.0 \pm 5.2, 11.0 \pm 3.2, 10.0 \pm 0.0 and 14.0 \pm 5.2 dBpeSPL for the wild-type mice and 23.3 \pm 4.9, 15.0 \pm 5.2, 10.8 \pm 2.9, 10.0 \pm 0.0, 10.8 \pm 2.9 and 14.2 \pm 5.1 dBpeSPL for the heterozygotes at frequencies of 5.6, 8.0, 12.0, 18.0, 24.0 and 32.0 kHz, respectively, and there were no significant differences in these values between the wild-type and heterozygous mutant mice.

Homozygous mutants were born at Mendelian frequency and showed few body movements but were apnoeic and died immediately after birth. They had micrognathia, and the eyelids were sometimes open (data not shown). No *Six1* mRNA was detected in homozygotes (Fig. 1C), confirming that the entire coding region of *Six1* was replaced by EGFP gene in this mutant. In the following analyses, we used neonates and embryos from F1 heterozygous matings.

Defects in ears, nose, thymus, kidneys and skeletal muscles of *Six1*-deficient neonates

Dissection analyses and hematoxylin and eosin (H-E) staining of sections of the neonates revealed defects in the ears, nose, thymus, kidneys and skeletal muscles in

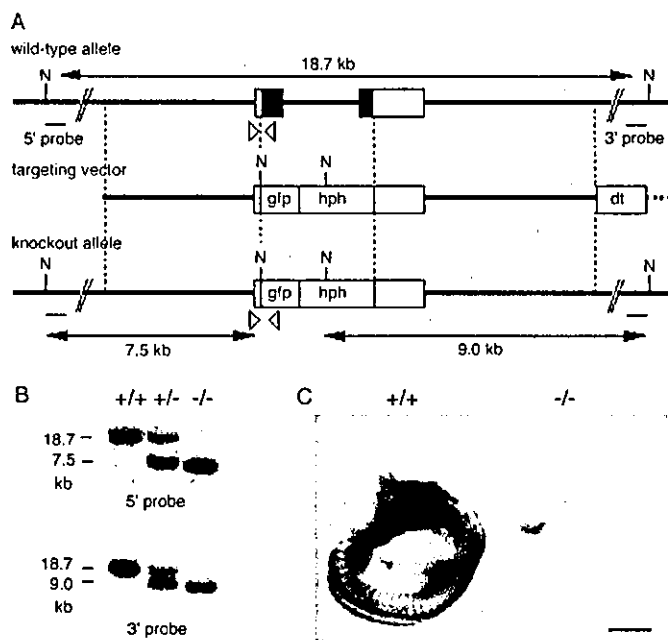


Fig. 1. Generation of *Six1*-deficient mice. (A) Targeting strategy of *Six1*. The *Six1* gene consists of two exons (indicated by boxes), and the coding regions are marked in black. The entire coding regions were replaced with the EGFP gene (*gfp*) and the *hygromycin-B-phosphotransferase* gene (*hph*). Open arrowheads indicate the positions of PCR primers for genotyping. (B) Southern blot analyses of wild-type (+/+), heterozygous (+/-), and homozygous (-/-) mutant neonates. Tail DNA was digested with *NcoI* and hybridized to 5' probe (upper panel) and 3' probe (lower panel). The size of each band is indicated on the left side. (C) In situ hybridization to *Six1* in E10.5 wild-type and homozygous mutant embryos. Absence of *Six1* mRNA was confirmed in the *Six1*-deficient embryo. dt, diphtheria toxin A gene; N, *NcoI* site. Scale bar: 1 mm.

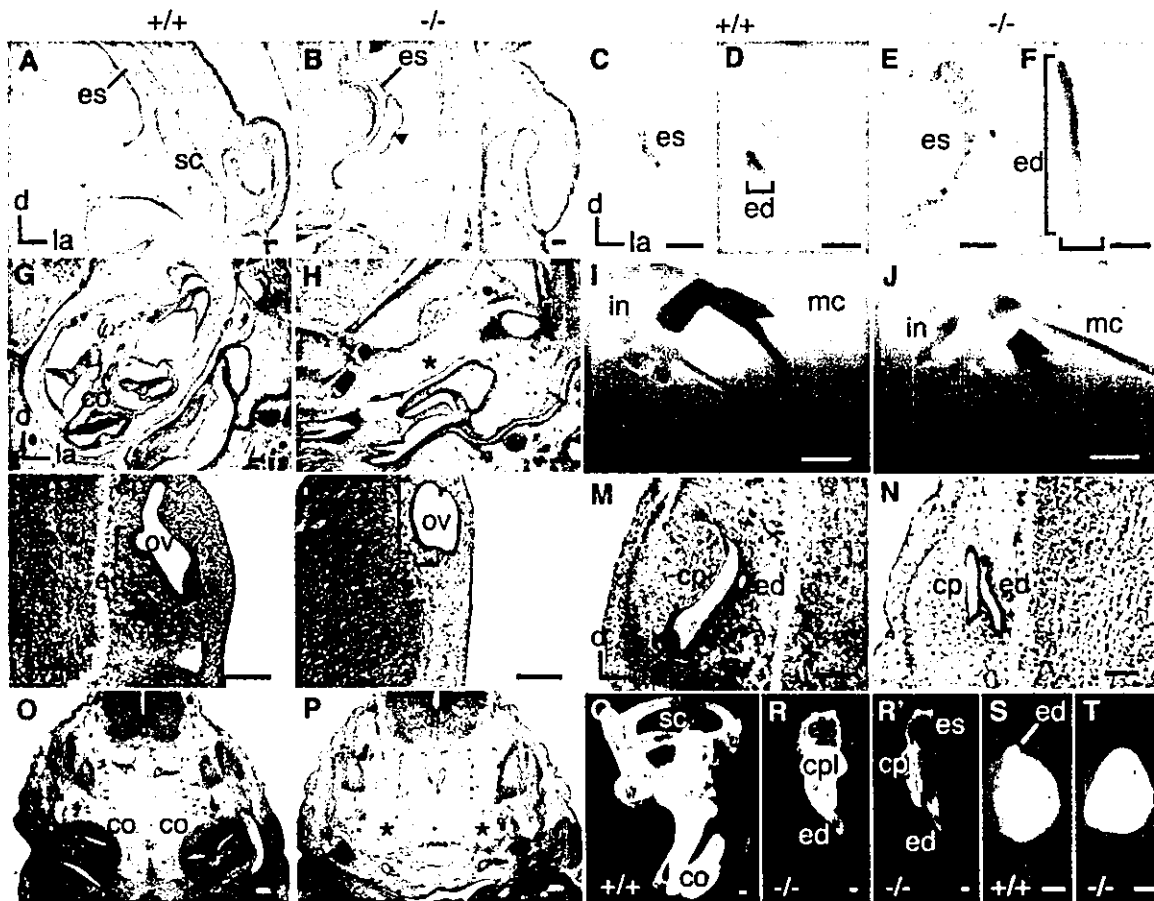


Fig. 2. Defects in the inner and middle ear in *Six1*-deficient mice. (A,B) Transverse sections at the level of the pinna of the neonates. The semicircular canals and the endolymphatic sac are irregularly formed in the *Six1*^{-/-} neonates. The semicircular canals and the common crus are fused, forming a large cavity (B, arrowhead). (C-F) *Wnt2b* expression analysis by in situ hybridization. Expansion of the *Wnt2b* expression domain in the *Six1*^{-/-} embryos (E,F) compared with wild type (C,D) indicates that the enlarged region is the endolymphatic sac at E17.5 (E) and endolymphatic duct at E11.5 (F). In F, *Wnt2b* was expressed in the medial half of the otic vesicle, which corresponds to the enlarged endolymphatic duct as depicted in L (otic vesicle shown in F was flattened during the hybridization process). (G,H) Transverse sections at the cochlea level show complete loss of the cochlea in *Six1*^{-/-} neonates (asterisk). (I,J) Alcian blue/Alizarin red staining of neonatal skeletons revealed malformations of ossicles. (K,L) Transverse sections of wild-type (K) and *Six1*^{-/-} (L) embryos at E11.5. The cochlear region does not extend ventrally and the endolymphatic duct is dilated in *Six1*^{-/-} embryos. (M-P) Transverse sections of wild-type (M,O) and *Six1*^{-/-} (N,P) embryos at E12.5. The endolymphatic duct and canal plate are formed, but the morphology is abnormal in the *Six1*^{-/-} embryo (N). The cochlea is completely absent in *Six1*^{-/-} embryos (P, asterisks). (Q-T) Lateral views of the paint-filled inner ear of wild-type (Q) and *Six1*^{-/-} (R) E18.5 embryos and otic vesicles of wild-type (S) and *Six1*^{-/-} (T) E10.5 embryos. (R') Posterior view of the same inner ear as (R). Relative positions are aligned between wild type and *Six1*^{-/-}. The two ventrally protruding structures observed in (R and R') are the ventral ends of residual cavities of the canal plate-like structure and the endolymphatic duct. More than five *Six1*^{-/-} neonates or embryos at each stage were analyzed, and virtually the same results were obtained. co, cochlea; cp, canal plate; cpl, canal plate-like structure; d, dorsal; ed, endolymphatic duct; es, endolymphatic sac; in, incus; la, lateral; m, medial; ma, malleus; mc, Meckel's cartilage; ov, otic vesicle; sc, semicircular canals; st, stapes; tr, tympanic ring. Scale bars: 100 μ m.

the *Six1*-deficient mice. In the inner ear, the dorsalmost parts of semicircular canals and common crus remained as a common fused space. The endolymphatic sac was present but was irregularly larger in size than that of wild-type littermates (Fig. 2A,B). The enlargement was confirmed by comparing the diameter of the paint-filled endolymphatic sacs of the *Six1*-deficient and the wild-type embryos (data not shown). The

expansion of the expression domain of *Wnt2b*, an expression marker for the endolymphatic sac and duct, also supports the enlargement of the endolymphatic sac (Fig. 2C,E). Other parts of the inner ear were completely absent, including the cochlea, vestibule and accompanying vestibulo-acoustic ganglia (Fig. 2G,H, data not shown). These structural defects were also demonstrated by paint-fill analyses (Fig. 2Q,R,R'). Because *Six1*

expression was evident in the branchial arch and periotic mesenchymes (Fig. 1C, Fig. 4C), we examined the middle ear defects in the *Six1*-deficient neonates and found malformations of the malleus and the incus and the absence of the stapes (Fig. 2I,J). In the nose, *Six1*-deficient mice manifested a hollowed nasal region with traces of nasal bleeding (data not shown). A pair of mere simple, rounded nostrils was present with no nasal epithelium, by contrast to the well-branched cavities with thick layers of nasal epithelia in the wild-type littermates (Fig. 3A,B). Both nasal cavities did not connect with the oral cavity or the nasopharynx, and the vomeronasal organs were absent in *Six1*-deficient mice (data not shown). The surrounding ossified region was abnormally enlarged (Fig. 3B), as observed in the inner ear (Fig. 2B,H). *Six1*-deficient mice also lacked a thymus (Fig. 3C,D). Kidneys were severely affected to variable degrees (Fig. 3E,F). Small kidneys with normal structure were found in mild cases (data not shown), while both kidneys were absent in extreme cases, although the ureters were always formed but were occasionally shorter (Fig. 3F). We also found markedly reduced skeletal muscle mass of the trunk, limbs, diaphragm and tongue (Fig. 3G,H, data not shown). The thymus, kidney, ear, nose and skeletal muscle defects are consistent with the *Six1*-deficient mice with different targeting strategy (Laclef et al., 2003a; Laclef et al., 2003b; Xu et al., 2003). These affected organs correlated well with the expression sites of *Six1* during development, such as otic vesicles, nasal pits, branchial arches/pouches, nephrogenic cords and somites (Oliver et al., 1995b). These results indicate that *Six1* is required for the formation of the ear, nose, thymus, kidneys and skeletal muscles.

Defects in inner ear appear at mid-gestation in *Six1*-deficient embryos

To determine the developmental stages at which the inner ear defects start to appear, *Six1*-deficient mice of several embryonic stages were sectioned and analyzed by H-E staining. At E9.5, otic vesicles were morphologically normal in *Six1*-deficient embryos, but the vestibulo-acoustic ganglia were missing (data not shown). At E10.5 and E11.5, the otic vesicles began to compartmentalize into saccular and utricular regions. The saccular region extended to the ventral side as a thin bulge in the wild type (Fig. 2K). By contrast, the extension of the saccular region to the ventral side did not occur in *Six1*-deficient embryos (Fig. 2L). The endolymphatic duct was observed as a thin outpocketing from the medial side of the otic vesicle in wild-type embryos, while the endolymphatic duct was observed as a large swelling in *Six1*-deficient embryos (Fig. 2K,L). The thin outpocketing and the large swelling region coincided with the expression domain of *Wnt2b* (Fig. 2D,F). We also used paint-fill analyses to compare otic vesicle structures at E10.5 with those of the wild type and confirmed the absence of the thin outpocketing and the dilatation of the endolymphatic duct in *Six1*-deficient otic vesicles (Fig. 2S,T, data not shown). At E12.5, the main structures of the inner ear (cochlea, saccule, utricle, endolymphatic duct and canal plates, from which three semicircular canals and common crus are formed) were distinguishable in the wild type (Fig. 2M,O, data not shown). By contrast, the dorsal extremity of the semicircular canals and common crus was observed as a fused cavity, and abnormally large endolymphatic duct was present (Fig. 2N), while other parts were completely absent in *Six1*-deficient embryos (Fig. 2P, data not shown).

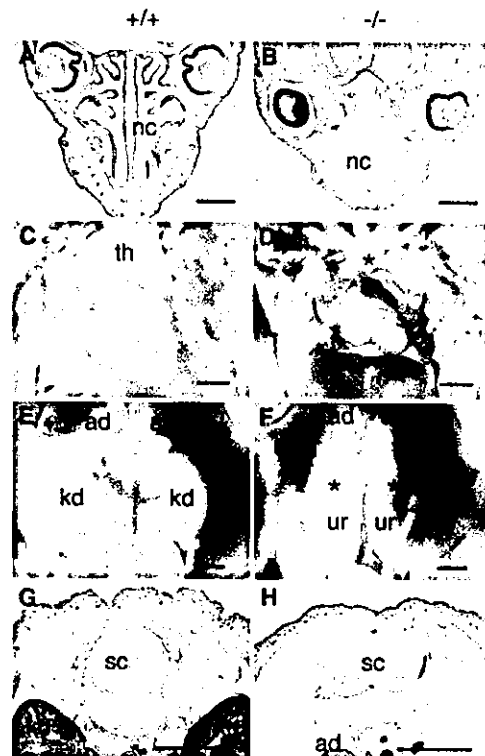


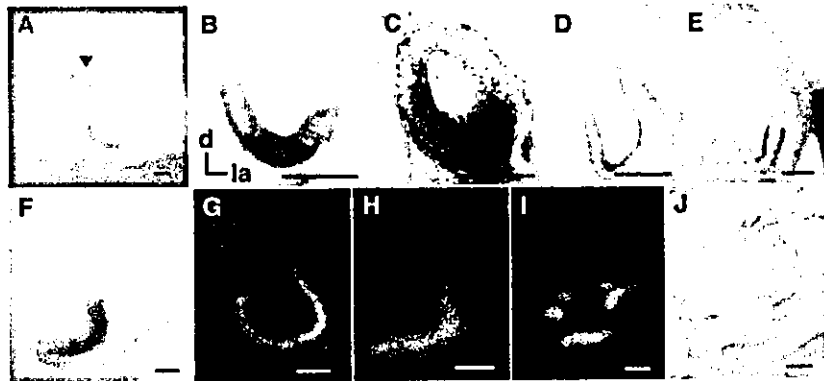
Fig. 3. Defects in the formation of the nose, thymus, kidney and skeletal muscles. Histological analyses of the wild-type (A,C,E,G) and the *Six1*^{-/-} (B,D,F,H) neonates. (A,B) Transverse sections of the nasal region. Nasal cavities form complex, branched structures with nasal epithelia in the wild type (A), and a pair of simple round cavities with no nasal epithelia is seen in the *Six1*^{-/-} neonates (B). (C,D) The thymus is prominent anterior to the heart in the wild type (C) but completely absent in the *Six1*^{-/-} neonate (D, asterisk). (E,F) Kidney defects in *Six1*^{-/-} mice. Note the bilateral renal aplasia (F, asterisks). (G,H) Abdominal transverse sections. Note severe reduction of skeletal muscle mass in *Six1*^{-/-} neonates (H). More than five pairs of wild-type and *Six1*^{-/-} neonates were analyzed and virtually the same results obtained except for the kidney (see text). ad, adrenal gland; kd, kidney; nc, nasal cavity; sc, spinal cord; th, thymus; ur, ureter. Scale bars: 1 mm.

In summary, the development of the inner ear was defective at mid-gestation around E10.5-12.5.

Expression of *Six1* in the developing inner ear

To gain insight into the function of *Six1* during inner ear development, we first examined the expression pattern of *Six1* by in situ hybridization in the wild type (Fig. 4A-F) and GFP fluorescence in heterozygous embryos (Fig. 4G-I). *Six1* mRNA was first detected in the otic placode and the surrounding surface ectoderm at E8.5 (Fig. 4A). *Six1* expression became prominent at the invaginating otic pit and the nascent otic vesicle at E9.5 (Fig. 4B,C), consistent with previous observations (Oliver et al., 1995b). Notably, the expression level was considerably lower in the dorsalmost region than in the other region of the otic vesicle (Fig. 4C). At E10.5, *Six1*

Fig. 4. *Six1* expression pattern during inner ear development detected by in situ hybridization in the wild type (A-F) and by GFP luminescence in the heterozygotes (G-I) viewed laterally (A) and in transverse sections (B-I). (A) At E8.5, *Six1* is weakly expressed in the otic placode (arrowhead) and the surrounding surface ectoderm. (B) At E9.5, *Six1* is expressed in the invaginating otic pit and (C) in the whole region of the otic vesicle except the dorsalmost region. (D,G) At E10.5, *Six1* is expressed in the ventral half of the otic vesicle. (E) At E11.5 and (F,H) E12.5, *Six1* is expressed exclusively in the cochlea. (I) Expression of *Six1* in the cochlea is maintained at E14.5 embryos. (J) A bright field image of the section in (I) stained with hematoxylin and eosin. More than three embryos at each stage were analyzed and virtually the same results obtained. d, dorsal; la, lateral. Scale bars: 100 μ m.



expression was limited to the ventral half of the otic vesicle (Fig. 4D,G). Subsequently, the expression domain of *Six1* became gradually restricted to the cochlear region at E11.5 (Fig. 4E) and E12.5 (Fig. 4F,H). At later stages, *Six1* transcripts were detected exclusively in the cochlea at E14.5 (Fig. 4I), and the expression of *Six1* in the cochlear duct persisted in the neonate (data not shown).

***Six1* is required for correct patterning of the otic vesicle**

The morphological defects in *Six1*-deficient mice were not restricted to the cochlea but extended to all regions of the inner ear except the dorsal extremity of the semicircular canals (Fig. 2). The missing ventral structures of the mutant mouse inner ear appeared to be related to the expression domain of *Six1* in the ventral otic vesicle at E9.5-10.5 (Fig. 4C,D). The absence of cochlea and vestibule and the enlargement of the endolymphatic sac prompted us to examine the following three possibilities: that the specification along the dorsoventral axis within the otic vesicle is altered in *Six1*-deficient embryos, that the cells within the ventral region of the *Six1*-deficient otic vesicle undergo enhanced apoptotic cell death, and that the cells within the ventral region of the *Six1*-deficient otic vesicle proliferate at a lower rate than those of the wild type. We assessed the first possibility by comparing the expression pattern of genes differentially expressed within the otic vesicle at E9.5-10.5. The ventralmost cells of the otic vesicle are marked by the co-expression of *Otx1* and *Otx2* (Morsli et al., 1999). *Otx1* and *Otx2* were not expressed in the *Six1*-deficient otic vesicle, by contrast to the wild type, although an ectopic faint expression of *Otx1* was reproducibly detected in the dorsalmost region (Fig. 5A-D). *Lunatic Fringe* (*Lfng*), a component of the Notch signaling pathway, is known as a molecular marker for inner ear sensory structures (Morsli et al., 1998). *Lfng* was expressed in the rostroventral region in the wild type (Fig. 5E), but no such expression was noted in the *Six1*-deficient otic vesicle (Fig. 5F). *Fgf3*, which is required for normal morphogenesis of the inner ear (Mansour et al., 1993), was expressed in the rostroventral region of the wild-type otic vesicle as *Lfng* (Fig. 5G), while the expression of *Fgf3* was absent in the *Six1*-deficient otic vesicle (Fig. 5H). *Bmp4* is an

early marker for the superior, lateral and posterior cristae. It was expressed in the restricted regions of the otic vesicle in the wild type (Fig. 5I), but no such expression was noted in *Six1*-deficient mice (Fig. 5J).

The dorsal side of the otic vesicle gives rise to the semicircular canals and endolymphatic duct/sac and is well marked by the expression of *Dlx5*, which is required for the normal development of the semicircular canals and endolymphatic duct/sac (Fig. 5K) (Acampora et al., 1999; Depew et al., 1999). In *Six1*-deficient embryos, the expression domain of *Dlx5* expanded to the entire otic vesicle (Fig. 5L). The expression domains of *Hmx2* and *Hmx3*, both of which are required for the formation of the vestibular structures (Wang et al., 1998; Wang et al., 2001), expanded ventrally from the dorsolateral side in the *Six1*-deficient otic vesicle (Fig. 5M,N, data not shown). *Dach1* is a member of the Dach family genes, which constitute the Pax-Six-Eya-Dach gene network. It was also expressed at the dorsal edge of the otic vesicle in the wild-type embryos (Fig. 5O). *Dach1* expression expanded ventrally along the medial and lateral sides almost down to the ventral end in *Six1*-deficient embryos (Fig. 5P). *Dach2*, another member of Dach family genes, was expressed mainly in the dorsal end of the otic vesicle in wild-type embryos, but *Dach2* expression domain was expanded ventrally along the lateral side of the otic vesicles (Fig. 5Q,R).

We also examined the expression pattern of *Pax2*, *Eya1* and *Six4* to clarify whether the expression of these genes is dependent on *Six1*. These genes are components of the Pax-Six-Eya-Dach gene network and are co-expressed in the otic vesicle. *Pax2* was expressed in the medial side of the otic vesicle of the wild-type and *Six1*-deficient embryos (Fig. 5S,T). *Eya1* expression in the ventral side of the wild-type otic vesicle was maintained in the *Six1*-deficient otic vesicle (Fig. 5U,V). *Six4* was expressed in the ventral side of the otic vesicle in wild-type embryos, and this expression pattern was almost the same in the *Six1*-deficient embryo (Fig. 5W,X). However, the most abundantly expressed regions of *Eya1* and *Six4* appeared slightly shifted from the ventromedial (wild-type) to the ventrolateral (*Six1*-deficient) side of the otic vesicle. These results suggest that the expression of *Pax2*, *Eya1* and *Six4* in the otic vesicle is not dependent on *Six1*.

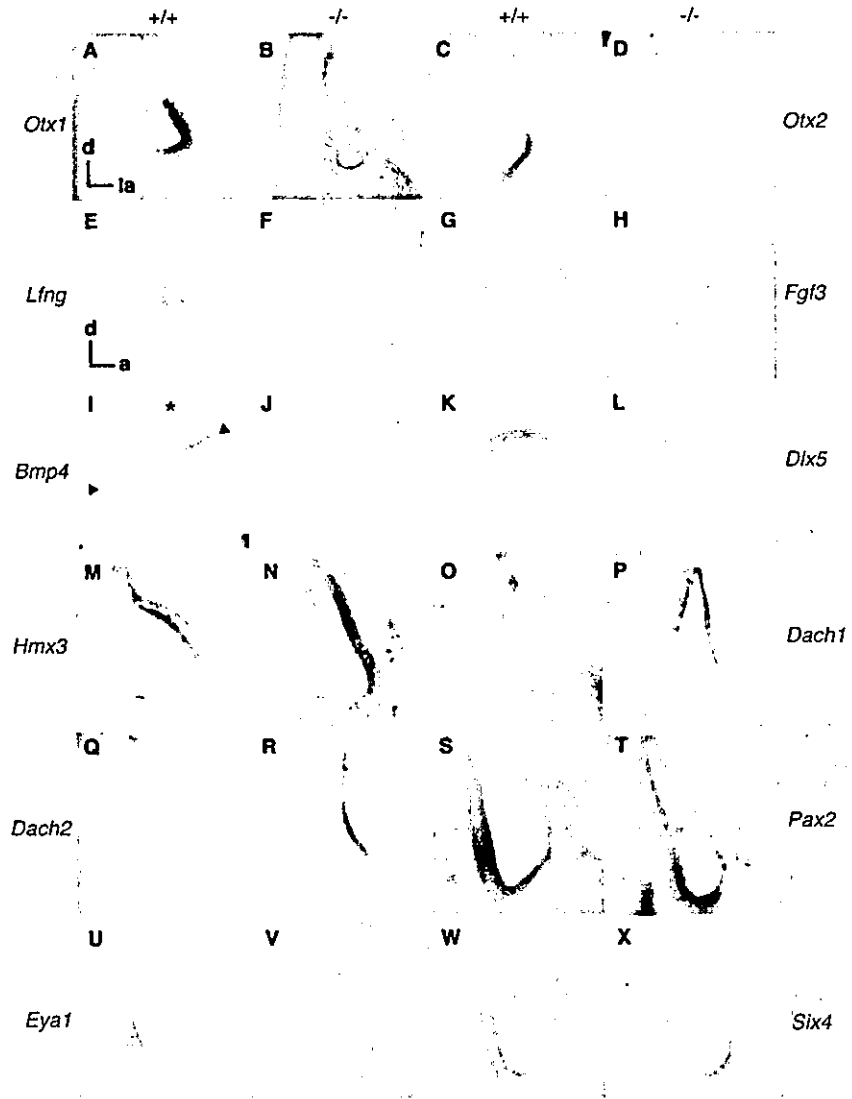
Fig. 5. *Six1* specifies the expression domains of differentially expressed genes in the otic vesicle. Transverse section or whole-mount view of the otic vesicle of in situ hybridized wild type (A,C,E,G,I,K,M,O,Q,S,U,W) and *Six1*^{-/-} embryos (B,D,F,H,J,L,N,P,R,T,V,X) of E10.5 (A-J,M-X) and E9.5 (K,L). (A,B) No *Otx1* expression in the *Six1*^{-/-} otic vesicle except for ectopic faint expression at the dorsal end.

(C,D) Absence of *Otx2* transcripts in the *Six1*^{-/-} otic vesicle. (E,F) *Lfng* is expressed in the rostroventral region of the otic vesicle in the wild-type embryo but not in the *Six1*^{-/-} embryo.

(G,H) *Fgf3* expression in the rostroventral region of the otic vesicle in the wild type is lost in the *Six1*^{-/-} embryo. (I,J) *Bmp4* expression in the wild-type otic vesicle (arrowheads) is lost in the *Six1*^{-/-} otic vesicle. Staining in the ectoderm over the dorsal region of the otic vesicle (asterisk) has also disappeared. (K,L) *Dlx5* is expressed dorsally in the wild type but in the whole region of the otic vesicle in the *Six1*^{-/-} embryo. (M,N) *Hmx3* expression domain is located only in the dorsolateral region in the wild type but is expanded ventrally in the *Six1*^{-/-} embryo. (O,P) *Dach1* expression is restricted to the dorsalmost region in the wild type, but the expression domain extends ventrally in the *Six1*^{-/-} otic vesicle. Signals in the neighboring mesenchyme are also observed in the lower right side of the otic vesicle.

(Q,R) *Dach2* is expressed at the dorsal end of the otic vesicle in the wild type, but the expression domain of *Dach2* is expanded ventrally along the lateral side in *Six1*^{-/-}. (S,T) *Pax2* is expressed in medial and ventral sides of the otic vesicle of both wild-type and *Six1*^{-/-} embryos. (U,V) *Eya1* is expressed in the ventral side of the wild-type and the *Six1*^{-/-} otic vesicle. (W,X) *Six4* expression in the ventral side of the otic vesicle is maintained in the *Six1*^{-/-} embryo. More than three pairs of wild-type and *Six1*^{-/-} embryos were analyzed and virtually the same results obtained. A-D and M-X: top, dorsal side (d); right, lateral side (la).

E-L: top, dorsal side (d); right, anterior side (a).



In conclusion, loss of *Six1* expression leads to marked changes in the expression domains of many genes in the otic vesicle, suggesting that the first possibility listed above is the case: i.e. the specification along the dorsoventral axis within the otic vesicle is altered in *Six1*-deficient embryos. Next, we assessed the second and third possibilities by TUNEL method and BrdU incorporation.

Enhanced apoptosis and reduced cell proliferation in the ventral otic vesicle

We examined whether enhanced apoptotic cell death or reduced cell proliferation within the ventral region of the otic

vesicle contributes to the inner ear phenotype. TUNEL method was used to detect apoptotic cells in the otic vesicle at E10.5 and E11.5, just before the extensive morphological changes. Several apoptotic cells were detected in the wild type, while enhanced apoptotic cell death was observed in the ventral and medial sides of the otic vesicles of *Six1*-deficient embryos at E11.5 (Fig. 6A,B). Statistical analysis revealed significant augmentation of apoptosis at E10.5 and E11.5 (Fig. 6C). We also examined BrdU incorporation in the otic vesicle at the same developmental stages. In the wild type and in the *Six1*-deficient embryos at E11.5, BrdU incorporation was abundant in the ventral region of the otic vesicle (Fig. 6D). By contrast,

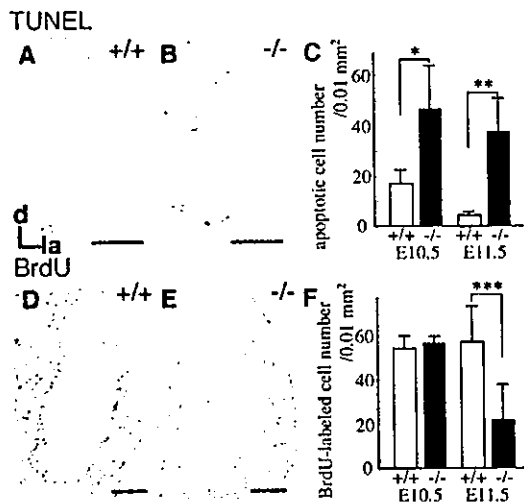


Fig. 6. Apoptosis and cell proliferation in the otic vesicle. (A-C) TUNEL method was used to examine apoptotic cell death in the otic vesicle of wild-type and *Six1*^{-/-} embryos at E10.5 and E11.5. The results of TUNEL analysis for E11.5 otic vesicles of the wild type (A) and *Six1*^{-/-} (B) are shown. For quantitative analysis, four to five pairs of wild-type and *Six1*^{-/-} embryos were examined. For each embryo, apoptotic cell number was measured on three transverse sections passing through the central region of the otic vesicle and converted into the apoptotic cell number per 0.01 mm². Their mean value for the three sections was adopted as the datum point for the otic vesicle of each embryo. The mean value of four or five embryos is shown with the standard deviation (C). Enhanced apoptosis (C) was seen in the ventral and medial regions of the *Six1*^{-/-} otic vesicle in comparison with the wild type in these stages. Statistical analysis was performed by Student's *t*-test. (D-F) Cell proliferation in the wild-type and the *Six1*^{-/-} otic vesicle was assessed by BrdU incorporation. The results of immunohistochemistry for BrdU at E11.5 otic vesicles of wild-type (D) and *Six1*^{-/-} (E) are shown. Quantitative analysis for BrdU-incorporated cell number was performed as described in TUNEL analysis (F). BrdU incorporation was considerably reduced in the *Six1*^{-/-} ventral otic vesicle at E11.5. d, dorsal; la, lateral. Scale bars: 100 μ m. **P*<0.05; ***P*<0.005; ****P*<0.01.

the incorporation was profoundly reduced in the ventral side of the otic vesicles of *Six1*-deficient embryos at E11.5 (Fig. 6E). A significant decrease in the number of BrdU-incorporated cells was observed at E11.5 but not at E10.5 (Fig. 6F). The reduced cell proliferation observed in *Six1*-deficient otic vesicles may be in line with the roles of *Six1* in cell cycle control (Ford et al., 1998). These results suggest that the lack of ventral structures of the inner ear in the *Six1*-deficient mice is partly due to enhanced apoptosis and reduced cell proliferation, as well as altered patterning of the otic vesicle.

Sonic hedgehog (Shh) signaling pathway is independent of *Six1*

We noticed that the inner ear phenotype of *Six1*-deficient mice is similar to that of *Shh*-deficient mice, which is characterized by the absence of the cochlear duct and vestibulocochlear ganglia, ventral expansion of the expression domains of *Dlx5*, loss of the expression domain of *Otx2*, and ventral restriction

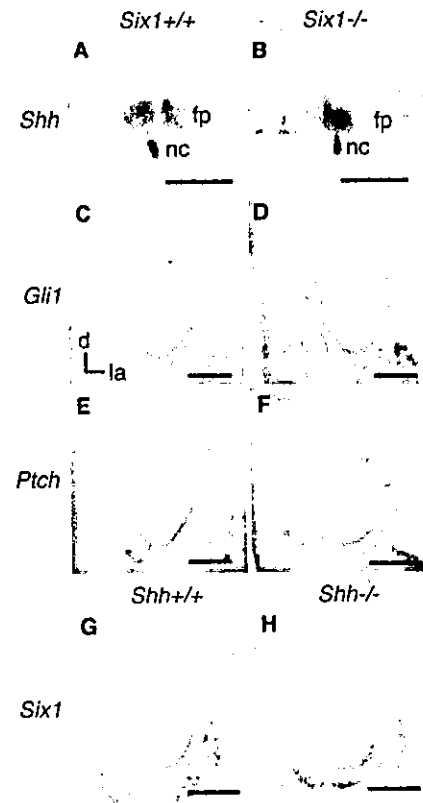


Fig. 7. Expression patterns of *Shh*, *Gli1*, *Ptch* and *Six1*. (A-F) Expressions of *Shh* (A,B), *Gli1* (C,D) and *Ptch* (E,F) in wild-type (A,C,E) and *Six1*^{-/-} (B,D,F) embryos at E10.5. (G,H) Expression of *Six1* in wild-type (G) and *Shh*^{-/-} (H) otic vesicles at E10.5. Three pairs of wild-type and *Six1*^{-/-} embryos and three pairs of wild-type and *Shh*^{-/-} embryos were analyzed and virtually the same results obtained. d, dorsal; la, lateral; fp, floor plate; nc, notochord. Scale bars: 100 μ m.

of *Otx1* expression (Riccomagno et al., 2002). The similarity of the phenotypes could be explained by the assumption that the Shh signaling pathway is dependent on *Six1* expression or vice versa. To test this possibility, we first examined the expression of *Shh* and the Shh-inducible genes, *Ptch* and *Gli1* in *Six1*-deficient embryos. *Shh* was expressed in the notochord and the floor plate near the otic vesicles in the wild type (Fig. 7A), and this expression pattern was virtually unchanged in *Six1*-deficient embryos (Fig. 7B). *Ptch* and *Gli1* were expressed in the otic vesicle and periotic mesenchyme in the wild-type embryo, and these expression patterns were similar in *Six1*-deficient embryos (Fig. 7C-F). Next, we examined *Six1* expression in *Shh*-deficient embryos (Chiang et al., 1996). *Six1* expression was abundant in the ventral region of the otic vesicles in *Shh*-deficient embryos, as observed in the wild type (Fig. 7G,H). These results indicate that the Shh signaling pathway is independent of *Six1* and that the expression of *Six1* in the otic vesicle is also independent of the Shh signaling pathway.

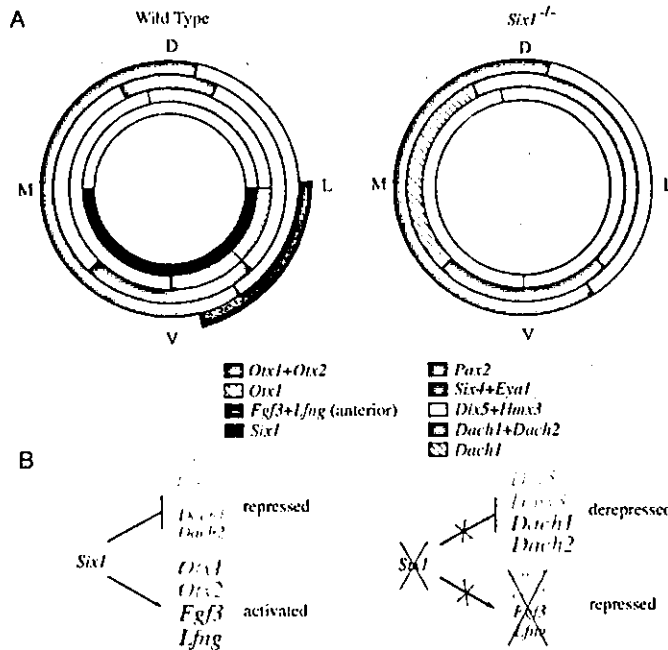


Fig. 8. (A) Schematic representation of expression of genes in otic vesicles of the wild-type (left) and *Six1*^{-/-} mice (right) at E10.5. In the otic vesicle of the wild-type, otic genes are expressed in the specified regions represented in different colors. In the wild-type otic vesicle, *Six1* is expressed in the ventral half of the otic vesicle. In the otic vesicle of *Six1*^{-/-}, the expression domains of *Dlx5*, *Hmx3*, *Dach1* and *Dach2* are expanded ventrally, and the expressions of the ventral marker genes (*Otx1*, *Otx2*, *Fgf3* and *Lfn3*) are lacking due to the absence of *Six1*. (B) Regulation of gene expression by *Six1* in the ventral otic vesicle. *Six1* activates the expression of ventral marker genes, *Otx1*, *Otx2*, *Lfn3* and *Fgf3*, but represses dorsal marker genes, *Dlx5*, *Hmx3*, *Dach1* and *Dach2*, and contributes to the patterning of the otic vesicle. D, dorsal; L, lateral; M, medial; V, ventral.

directly by *Six1*, it is concluded that *Six1* plays a key role in establishing otic vesicle patterning.

In addition to the patterning of the otic vesicle along the dorsoventral axis, *Six1* may play roles in the otic vesicle patterning along the anteroposterior and/or mediolateral axes, because anteroposteriorly and/or mediolaterally asymmetrical expression patterns of *Otx1*, *Otx2*, *Lfn3*, *Fgf3* and *Bmp4* were also affected (Fig. 5). For these issues, further histological examinations and analyses of molecular marker expression will be required.

Furthermore, our results showed a marked reduction of cell proliferation and enhanced apoptosis in the ventral otic vesicle in *Six1*-deficient embryos (Fig. 6). This may contribute to the inner ear phenotype lacking most of the ventral structures. Thus, *Six1* controls inner ear development by regulating cell death and proliferation as well as by establishing otic vesicle patterning.

Phenotypic similarity of the inner ear compared with *Shh*-deficient mice

Previous and present studies indicated that specification of the cochlea is dependent on *Shh* signaling and that perturbation of otic vesicle patterning in *Shh*-deficient mice (Riccomagno et al., 2002) is similar to that of *Six1*-deficient mice. Considering these phenotypic similarities of inner ear formation between *Six1*- and *Shh*-deficient mice, we assumed a genetic interaction between *Six1* and *Shh*. However, the expression patterns of *Shh*, *Gli1* and *Ptch* in *Six1*-deficient mice and that of *Six1* in *Shh*-deficient mice (Chiang et al., 1996) indicate that the expressions of *Shh*, *Gli1* and *Ptch* are not dependent on *Six1*, and that the expression of *Six1* is not dependent on the *Shh* signaling pathway in and around the otic vesicle at E10.5 (Fig. 7). Another possible mode of genetic interaction is through functional cooperation between *Six1* and the components of *Shh* signaling cascades. *Shh* protein is emanated from the notochord and/or the floor plate, probably giving a gradient of *Shh* across the otic vesicle with a high concentration in the ventral side and a low concentration in the dorsal side. This *Shh* gradient would enhance putative collaborative interaction between downstream components of *Shh* signaling cascades and *Six1* in the ventral otic vesicle. Modulation of the transactivating function of *Six1* by *Shh* signaling would be one of the plausible mechanisms. However, we cannot exclude

Discussion

Establishment of otic vesicle patterning by *Six1*

The inner ear originates from a transient embryonic structure, the otic vesicle. Successive transformations and compartmentalization of the otic vesicle give rise to the entire membranous region of inner ear structures. The fate of cells in the otic vesicle is dependent on the gene expression specific to each compartment of the otic vesicle (Fekete, 1999). For example, *Dlx5*, which is expressed in the dorsal side of the otic vesicle, is required for the formation of the semicircular canals (Acampora et al., 1999; Depew et al., 1999). *Otx1*, which is expressed in the ventral side, is needed for the correct morphogenesis of the cochlea (Acampora et al., 1996). The establishment of such a gene expression profile in the otic vesicle, orchestrated by networks or cascades of transcription factors, is essential for inner ear development. However, the genes involved in these networks and components of the cascades are largely unknown. Our study showed for the first time that *Six1* functions to establish the correct expression pattern of many otic genes and contributes to the formation of the majority of inner ear structures. Altered expression pattern of many otic genes (Fig. 5) results in the loss of specifications of the ventral region of the otic vesicle, with resultant expansion of the dorsally specified domains at E9.5-10.5, and consequently in the absence of the cochlea and most of the vestibule, together with dysgenesis of residual region of semicircular canals and enlargement of the endolymphatic sac in *Six1*-deficient neonates (Fig. 2). The ventral expression of *Six1* in the otic vesicle at E9.5-10.5 suggests that *Six1* activates the expression of *Otx1*, *Otx2*, *Lfn3* and *Fgf3* and represses the expression of *Dlx5*, *Hmx3*, *Dach1* and *Dach2* (Fig. 8). Although it is unknown whether these genes are regulated

independent actions of *Six1* and components of *Shh* signaling cascades in the otic vesicle. For example, expression of *Pax2* in the medialventral otic vesicle is maintained in *Six1*-deficient mice (Fig. 5S,T), but is downregulated in *Shh*-deficient mice (Riccomagno et al., 2002). To determine whether *Six1* and *Shh* interact genetically, it would be important to examine the phenotypes of the *Six1/Shh* double mutant.

Roles of *Six1* In Pax-Six-Eya-Dach gene network

Six genes function as components of the Pax-Six-Eya-Dach gene network in organ development. In the ventral otic vesicle, *Six1* is co-expressed with *Pax2*, *Pax8*, *Six4* and *Eya1* to control inner ear development. Outside the otic vesicles, various combinations of Pax, Six, Eya and Dach genes are co-expressed in the primordia of the organs affected in *Six1*-deficient mice: the olfactory placode (*Pax6*, *Six1*, *Six2*, *Six3*, *Six4*, *Six6*, *Eya1*, *Eya2*, *Eya4*); the thymus (*Pax9*, *Six1*, *Six4*, *Eya1*); the metanephros (*Pax2*, *Pax8*, *Six1*, *Six2*, *Six4*, *Eya1*); and the somite/myotome (*Pax3*, *Six1*, *Six4*, *Eya1*, *Eya2*, *Eya4*, *Dach1*). *Six1* plays important roles in the development of these organs, probably through the control of patterning and/or cell proliferation, as observed in the otic vesicle. Notably, Dach genes are not co-expressed with Six and Eya genes in the ventral otic vesicle, nose or kidney (Figs 4, 5, data not shown). Furthermore, Dach expression domains were expanded ventrally in the *Six1*-deficient otic vesicle, indicating that *Six1* represses the expression of Dach genes in the ventral otic vesicle. Likewise, augmentation of Dach expression was observed in the nasal pit of *Six1*-deficient embryos (data not shown), indicating that expression of the Dach gene is repressed by *Six1* also in the nasal pit. These findings are in contrast to *Drosophila* compound eye formation and chick myogenesis. In both those cases, Pax, Six and Eya are co-expressed with Dach, cooperatively to execute the developmental programs. Thus, the Pax-Six-Eya gene network lacking Dach may demarcate the two placode-derived sensory organs, the inner ear and the nose, and the kidney from other organs such as the eye and the skeletal muscles. In addition, hierarchy among Pax, Six, Eya and Dach genes in the otic vesicle has been revealed in this study. That is, the expression patterns and levels of *Eya1* and *Pax2* were not affected but expression domains of *Dach1* and *Dach2* were expanded ventrally in the *Six1*-deficient otic vesicle (Fig. 5). Conversely, *Six1* expression is lost but *Pax2* expression is not disturbed in the *Eya1*-deficient otic vesicle (Xu et al., 1999). Thus, in the otic vesicle, expression of *Eya1* and *Pax2* is independent of *Six1*, expression of *Six1* depends on *Eya1*, and *Six1* controls *Dach1* and *Dach2* expression. In the myotome, *Six4* expression is not dependent on *Six1*, as observed in the otic vesicle (Laclef et al., 2003a), but *Pax2* expression is dependent on *Six1* in metanephric mesenchyme (Xu et al., 2003). The similarities among these organ primordia in the context of the Pax-Six-Eya(-Dach) network and the diversity in selecting members from respective gene hierarchies among them raise interesting issues regarding the ontogeny of these organs during evolution.

In conclusion, our study identified the essential role of *Six1* in the regulation of otic vesicle patterning. Together with mice homozygous for other Pax, Six, Eya and Dach genes, *Six1*-deficient mice should allow a comprehensive understanding of the roles of the Pax-Six-Eya-Dach gene network in various organogenesis.

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Interleukin (IL)-6, But Not IL-1, Induction in the Brain Downstream of Cyclooxygenase-2 Is Essential for the Induction of Febrile Response against Peripheral IL-1 α

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IL-1 is an endogenous pyrogen produced upon inflammation or infection. Previously, we showed that, upon injection with turpentine, IL-1 is induced in the brain in association with the development of fever. The role of endogenous IL-1 in the brain and the signaling cascade to activate thermosensitive neurons, however, remain to be elucidated. In this report, febrile response was analyzed after peripheral injection of IL-1 α . We found that a normal febrile response was induced even in IL-1 α / β -deficient mice, indicating that production of IL-1 in the brain is not necessarily required for the response. In contrast, IL-6-deficient mice did not exhibit a febrile response.

Cyclooxygenase (Cox)-2 expression in the brain was strongly induced 1.5 h after injection of IL-1 α , whereas IL-6 expression was observed 3 h after the injection. Cox-2 expression in the brain was not influenced by IL-6 deficiency, whereas indomethacin, an inhibitor of cyclooxygenases, completely inhibited induction of IL-6. These observations suggest a mechanism of IL-1-induced febrile response in which IL-1 in the blood activates Cox-2, with the resulting prostaglandin E₂ inducing IL-6 in the brain, leading to the development of fever. (Endocrinology 145: 5044–5048, 2004)

IL-1 IS A MAJOR mediator of inflammation, performing numerous functions related to host defense mechanisms by regulating not only the immune system but also the neuronal and endocrine systems (1). Two molecular species, IL-1 α and IL-1 β , are known as agonists and an antagonist, IL-1 receptor antagonist (IL-1Ra), which binds to the same receptors, is also known. Two IL-1 receptors, the type I IL-1 receptor (IL-1RI) and type II (IL-1RII), exist; only the former transduces IL-1 signaling and the latter rather acts as a decoy. IL-1 is produced by a large variety of cells, including monocytes and macrophages, and IL-1Rs are expressed on a wide range of cells in the immune, neural, and endocrine systems. Because IL-1Rs are induced upon peripheral inflammation in the brain, particularly the hypothalamus, hippocampus, and choroid plexus (2–4), a role for IL-1 has been suggested in the neuronal system.

Fever is a common response of the body to various stresses such as infection and inflammation. Such peripheral stimuli are transmitted to the brain through the nervous system and also by “endogenous pyrogens” (5). Although it is well known that circulating cytokines such as IL-1 and tumor necrosis factor (TNF) α are important endogenous pyrogens, the precise mechanism by which these cytokines induce fever through activation of the thermoregulatory neurons in the hypothalamus remains to be elucidated. It is especially interesting to elucidate how these cytokines stimulate relevant

thermoregulatory brain structures, because these large hydrophilic polypeptides hardly penetrate the blood-brain barrier (BBB) (6, 7).

We showed previously that IL-1 expression was induced in the diencephalon of the brain upon injection with turpentine, and that the febrile response to turpentine was abolished in IL-1 α / β -deficient mice, suggesting involvement of IL-1 in the brain in the development of fever (8). Consistently, IL-1RI-deficient mice also failed to respond to turpentine (9). On the other hand, it has been demonstrated that endogenous hypothalamic IL-1 β is not necessary for the development of IL-1 α -, IL-1 β - or lipopolysaccharide (LPS)-induced fever (10). It is not known, however, whether or not a febrile response can be induced in the complete absence of both IL-1 α and IL-1 β in the brain.

It is known that prostaglandin (PG)E₂ is involved in the development of fever during inflammation, because inhibitors of cyclooxygenases, which catalyze synthesis of PGH₂, a precursor of PGE₂, can suppress febrile response (11), and mice lacking the EP₃ receptor, one of the receptors for PGE₂, showed an impaired febrile response during the first hour after IL-1 β injection (12). Although two types of cyclooxygenases (Cox) are known, it was suggested that only Cox-2 is involved in the febrile response upon inflammation (8, 13–15). Furthermore, endogenously induced IL-6 has also been suggested to be involved in the febrile response induced by IL-1 (16). However, the relationship among IL-1, IL-6, and Cox-2 in the brain has not been established conclusively.

In this report, to elucidate the roles of IL-1, which is endogenously induced in the brain during fever, we examined the febrile response in IL-1 α / β -deficient mice upon peripheral administration of IL-1 α . Furthermore, we analyze the

Abbreviations: BBB, Blood-brain barrier; BW, body weight; Cox, cyclooxygenase; IL-1R, IL-1 receptor; IL-1Ra, IL-1R antagonist; LPS, lipopolysaccharide; PG, prostaglandin.

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signaling cascade in the brain using *IL-1 α / β* - and *IL-6*-deficient mice.

Materials and Methods

Reagents

Recombinant murine *IL-1 α* was obtained from Pepro Tech EC LTD (London, UK). The lyophilized protein was dissolved in pyrogen-free 0.9% NaCl (saline) containing 0.1% BSA (A9306; Sigma, St. Louis, MO). Indomethacin was obtained from Sigma.

Animals

IL-1 α / β -doubly deficient mice were produced as described (8), and *IL-6*-deficient mice were kindly provided by Dr. Manfred Kopf (17). These mice were backcrossed to C57BL/6J mice for eight generations, and C57BL/6J mice were used as controls. Mice were housed individually from weaning at 4 wk of age, and sex- and age-matched adult (9–15 wk of age) male mice were used for each experiment. Mice were kept under specific, pathogen-free conditions in an environmentally controlled clean room at the Center for Experimental Medicine, Institute of Medical Science, University of Tokyo. They were housed at an ambient temperature of 24 C and a daily cycle of 12-h light/dark (0800–2000 h light). All experiments were carried out both according to the institutional ethical guidelines for animal experiments and the safety guidelines for gene manipulation experiments.

Measurement of body temperature

Intraperitoneal temperature of mice was measured using an electric thermometer and tips (ELAMS system; BioMedic Data System, Inc., Maywood, NJ) with an accuracy of 0.1 C. All the tips were tested and adjusted before use. Mice were anesthetized with Nembutal, and the tip was implanted chronically into their peritoneal cavity and ligated to the peritoneum. The position of the tips was verified by postmortem examination. These mice were used for experiments 18 d after the thermometer implantation. *IL-1 α* [1 μ g/kg body weight (BW)] was injected iv at 1100 h, and the temperature was measured every 15 min by a person who was accustomed to using the system.

Northern blot hybridization analysis

Northern blot hybridization was performed as described previously using mouse *IL-1 α* , *IL-1 β* , *Cox-2*, *IL-6*, and *β -actin* cDNA as probes (8). Wild-type, *IL-1 α / β* -deficient, and *IL-6*-deficient mice were injected with *IL-1 α* (1 μ g/kg BW). Mice were killed 1.5 and 3 h after injection, and poly-A⁺ RNA was isolated from the diencephalons. Samples from four mice were pooled for each genotype. Poly-A⁺ RNA (8–11 μ g) was electrophoresed on a denatured agarose gel and hybridized with specific probes; *β -actin* was used as a control. Relative radioactivities of the *IL-1 α* , *IL-1 β* , *IL-6*, and *Cox-2* bands were compared after normalization with the intensity of the *β -actin* band.

Indomethacin treatment

Mice were injected with indomethacin (10 mg/kg BW, ip injection) dissolved in a buffer containing 0.9% NaCl, 5% ethanol, and 4% sodium hydrogen carbonate, or the buffer only 30 min before *IL-1* (1 μ g/kg BW, iv) injection or saline injection, and *IL-6* and *Cox-2* mRNA levels were measured 3 h after the treatment by Northern blot hybridization.

Statistical analysis

Averages \pm SD are shown. In Fig. 1, Student's *t* test was used to evaluate statistical significance. Animals with consecutive missing temperature recordings, due to failure of the telemetry system, were excluded from the statistical analysis. In Fig. 2, Student's paired *t* test was performed to compare before (basal) and after (1.5 h or 3 h after *IL-1* injection) data for each genotype.

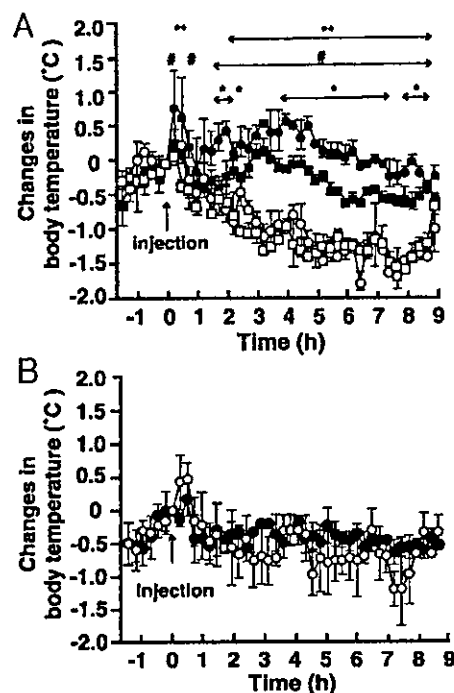


FIG. 1. Effects of *IL-1 α / β* or *IL-6* deficiency on fever development after injection with *IL-1 α* (1 μ g/kg BW, iv injection). The body temperatures relative to that at the time of *IL-1 α* injection are shown. A, *IL-1 α* -injected wild-type mice (n = 4; \blacksquare); saline-injected wild-type mice (n = 4; \square); *IL-1 α* -injected *IL-1 α / β* -deficient mice (n = 3; \bullet); and saline-injected *IL-1 α / β* -deficient mice (n = 3; \circ). B, *IL-1 α* -injected *IL-6*-deficient mice (n = 3; \bullet); and saline-injected *IL-6*-deficient mice (n = 3; \circ). Averages \pm SD are shown. *, $P < 0.05$, *IL-1 α* -injected wild-type mice vs. *IL-1 α* -injected *IL-1 α / β* -deficient mice; #, $P < 0.05$, *IL-1 α* -injected *IL-1 α / β* -deficient mice vs. saline-injected *IL-1 α / β* -deficient mice; **, $P < 0.05$, *IL-1 α* -injected wild-type mice vs. saline-injected wild-type mice.

Results

Febrile response to *IL-1 α* in *IL-1 α / β* -deficient and *IL-6*-deficient mice

Wild-type, *IL-1 α / β* -deficient, and *IL-6*-deficient mice were injected iv with *IL-1 α* , and the resulting febrile responses were examined. The measurement of the temperature started at 0930 h, and *IL-1* was injected at 1100 h. The body temperature of untreated mice gradually decreased from 0930 to 1900 h and again rose after that, reflecting the circadian temperature rhythm. The preinjection body temperatures for each experimental group were the same: *IL-1*-injected wild-type mice, 37.6 ± 0.2 ; saline-injected wild-type mice, 37.5 ± 0.4 ; *IL-1*-injected *IL-1 α / β* -deficient mice, 37.6 ± 0.1 ; saline-injected *IL-1 α / β* -deficient mice, 37.3 ± 0.3 ; *IL-1*-injected *IL-6*-deficient mice, 37.4 ± 0.3 ; and saline-injected *IL-6*-deficient mice, 37.6 ± 0.3 . As shown in Fig. 1A, when *IL-1 α* was administered, wild-type mice showed significantly elevated body temperatures (at 30 min and from 2 h 15 min to 8 h 45 min after injection), compared with saline-injected wild-type mouse controls (**, $P < 0.05$). *IL-1*-injected *IL-1 α / β* -deficient mice also showed a febrile response similar to wild-type mice: significantly elevated temperature was observed at 15,