Craniofacial Anomalies of the Cultured Mouse Embryo Induced by Inhibition of Sonic Hedgehog Signaling: An Animal Model of Holoprosencephaly

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The pathogenesis of holoprosencephaly is multifactorial, and blockage of Sonic hedgehog signaling is one of the most important causative factors in animal models and human cases. In this study, the authors analyzed facial anomalies of mouse embryos, which were cultured in vitro and exposed to cyclopamine, an alkaloid blocker of Sonic hedgehog signaling. When cultured with cyclopamine for embryonic day 8.5 to 10.5, the whole body size was smaller than normal, and the distance and angle between the nasal placodes were remarkably reduced. Extension of the cranial surface vessels also was noted. No cyclopia was observed. Migration of the cranial neural crest cells seemed to be intact. Expressions of *Patched-1* and *Gli-1*, downstream genes of Sonic hedgehog signaling, also were down-regulated in in situ hybridization and real-time reverse transcriptase-polymerase chain reaction analyses. The authors consider that these facial anomalies represent milder phenotypes of holoprosencephaly.

Key Words: Holoprosencephaly, mouse embryo, neural crest cells, Sonic hedgehog, whole embryo culture

Recent advances in developmental biology have been gradually unraveling enigmas regarding the molecular pathogenesis of craniofacial anomalies.[1,2] Holoprosencephaly is a congenital anomaly characterized by hypotelorism, false median cleft, and developmental deficiencies in the forebrain.[3,4] It is well known that holoprosencephaly shows a wide spectrum of phenotypic severity, from cyclopia to a single central incisor.[2,5] Holoprosencephaly is a multifactorial disease caused by various environmental and genetic factors,[4] among which Sonic hedgehog (Shh) signaling is one of the most important.

Shh is a representative among morphogens functioning in early development, mediating epithelial-mesenchymal interaction and morphogenetic patterning of various tissues and organs.[6] For example, *Shh* is expressed in the notochord and floor plate of the neural tube, regulating positional information within the neural tube and induction of the motor neurons.[7] *Shh* is also expressed in the posterior portion of the limb bud, conferring anterior-posterior positional information within the limb bud.[8] In these senses, Shh signaling can be regarded as one of factors involved in organizing centers of the morphogenetic fields.[9] Shh is a diffusible protein attached to the receptor Patched (Ptc) and a co-receptor Smoothened (Smo), and the signal is transduced to a transcription factor Gli, which up-regulates expressions

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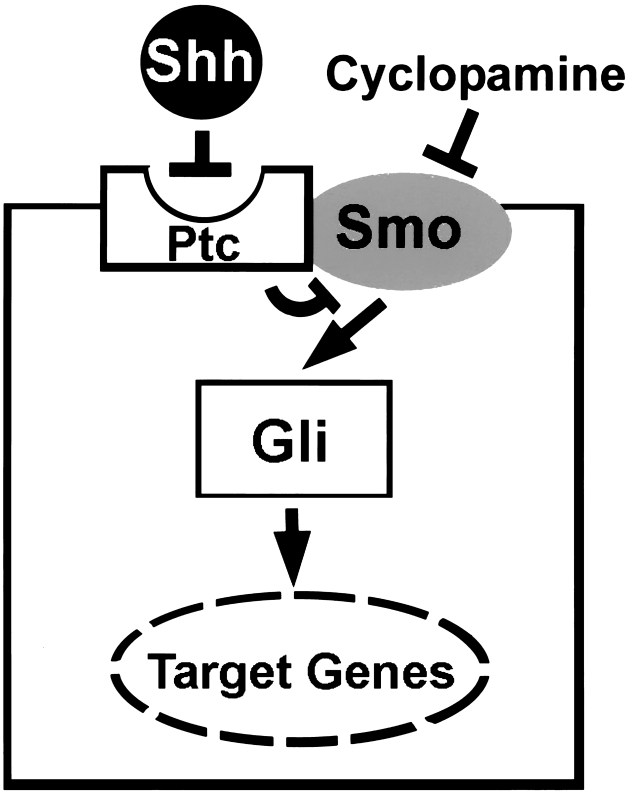

Fig 1 Sonic hedgehog signaling cascade. Sonic hedgehog (Shh) is a diffusible molecule and inhibits functions of the cell surface receptor Patched (Ptc). Ptc again inhibits functions of the membrane-bound co-receptor Smoothened (Smo), which activates functions of a transcription factor Gli. Thus, activity of Gli is eventually rescued when Shh binds to Ptc. Gli is transported to the nucleus and promotes transcription of the target genes. Cyclopamine is an inhibitor of Shh signaling, and recently it has been reported that cyclopamine targets Smo.[15]

of downstream genes[6] (Fig 1). There are two subtypes of Ptc and three subtypes of Gli in mammals, thus forming a complex signaling network, and the most representative are Ptc-1 and Gli-1.[10] Interestingly, expression of *Ptc* and *Gli* themselves are upregulated by Shh signaling,[10,11] thereby forming a positive feedback loop. Therefore, blockage of Shh signaling can be monitored by reduced expressions of *Ptc* or *Gli*.

It has been reported that mouse gene targeting of *Shh* revealed embryonic lethality with severe craniofacial anomalies including cyclopia.[12] *Shh* mutation also was reported in human holoprosencephaly cases,[5,13] and it is known that *Shh* mutation is responsible for about 17% of familial cases of holoprosencephaly.[2,4] Shh signaling is also important as an environmental factor in holoprosencephaly. Exposing chick embryos to ethanol causes holoprosencephaly, and expressions of Shh signaling genes are inhibited in these embryos.[14] This phenomenon may be related to the molecular pathogenesis of fetal alcohol syndrome, which is characterized by craniofacial anomalies and mental retardation in the infants born to mothers with excess alcohol intake.[15] Another interesting example is a cyclopic lamb born to a mother who had eaten the plant *Veratrum californicum*.[16] This plant contains two types of teratogens (jervine and cyclopamine), both of which block Shh signaling. Cyclopamine is a steroidal alkaloid targeting Smo,[17] and it frequently is used in experiments as a blocker of Shh signaling. Indeed, cyclopamine causes holoprosencephaly-like craniofacial anomalies in chick embryos,[18] and maternal administration of cyclopamine also results in similar craniofacial anomalies in murine embryos.[19]

Previous studies regarding mammalian experimental holoprosencephaly by Shh signaling inhibition have several shortcomings. Functions of the genes are blocked for all the period of embryonic development in knockout mouse analysis, and the phenotype should be a complicated end result of the multiple functional defects of the genes. Shh signaling has multiple roles in development, so analysis of the facial anomalies in Shh knockout mouse may be obscured by the multiple developmental defects of the surrounding tissues and organs. A possible approach to minimize this problem is to administer cyclopamine to the embryo in the narrow time window specific to the craniofacial development. However, maternal administration of cyclopamine is not sufficient to specify the time window because even littermates from the same mother usually have considerable differences in their developmental stages–as much as half a day.

To overcome these shortcomings, we used the whole embryo culture system[20,21] in the current study (Fig 2A, B). Murine embryos included within the yolk sac can be taken out of the mothers and cultured in vitro for several days using this system. Cyclopamine can be directly applied to the embryos by simply adding it to culture medium in vials. We can estimate the developmental stage of each embryo by direct observation, so the time window of cyclopamine exposure can be precisely controlled. In the current study, we cultured mouse embryos from embryonic day (E) 8.5 until E 10.5 with or without cyclopamine in the medium. The cultured embryos were collected at E 10.5, and the craniofacial anoma-

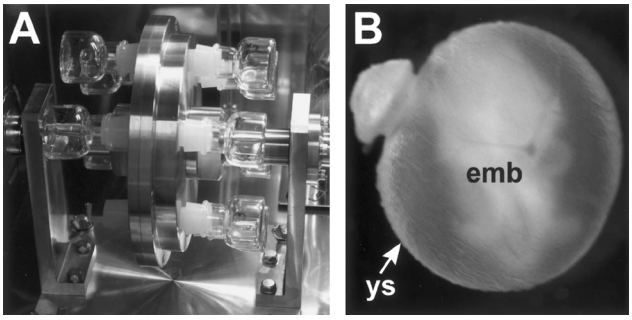

Fig 2 Whole embryo culture system. (A) An incubator of whole embryo culture. Murine embryos are cultured in vials containing medium (rat serum with glucose) attached to the rotator. The optimum concentration of oxygen is supplied to the vials, according to developmental stages. (B) A cultured mouse embryo of embryonic day 10.5. An embryo (emb) is included within the yolk sac (ys) and can be maintained in this condition for a few days.

lies were analyzed at the morphologic and molecular levels.

Materials and Methods

Mouse Whole Embryo Culture and Cyclopamine Exposure

Embryos were cultured in vitro as described previously.[22,23] Twenty millimolers cyclopamine (TRC, Toronto, Canada) dissolved in ethanol was stored at –30°C as a stock solution. Culture medium was prepared as usual, adding 2 mg/mL of glucose to rat serum (specially purchased from Charles River Japan, Yokohama, Japan). Just before the embryo culture, 2 μL of the stock solution was added to the 2 mL medium in each culture vial, yielding 20 μM of cyclopamine. Two microliters of 100% ethanol was added to the 2 mL medium in the control group.

E 8.5 mouse embryos were dissected from pregnant ICR mice (Clea Japan, Tokyo, Japan), anesthetized with ether, and killed by cervical dislocation, taking care not to damage the yolk sacs surrounding the embryos. Embryos around 15 somites stage were selected for the experiment, and littermates with excessively advanced or delayed stages were eliminated. Embryos within the yolk sacs were cultured for about 2 days, until the stage corresponding to E 10.5 in utero embryo, using the whole embryo incubator (Ikemoto Rika, Tokyo, Japan). The embryos were collected after incubation, and the yolk sacs were removed. The embryos were inspected under the dissecting microscope, and digital images of the craniofacial region were recorded. The embryos were processed for further investigation as described below.

Scanning Electron Microscope

The collected embryos were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in phosphate buffered saline (PBS) and stored at 4°C until further processing. The samples were postfixed with 1% osmium tetroxide in PBS, dehydrated in a graded series of ethanol, critical point dried using liquid carbon dioxide, and sputter-coated with platinum-palladium using E-1010 sputter coater (Hitachi, Tokyo, Japan). The specimens were examined and photographed with a S-3500N (Hitachi) scanning electron microscope at an accelerating voltage of 20 kV.

Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from head regions of the control and cyclopamine-treated embryos using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The isolated RNA was treated with DNase I (Invitrogen, Carlsbad, CA) to remove contaminating genomic DNA.

Gene expression was analyzed by real-time quantitative RT-PCR using an ABI PRISM 7700 (Applied Biosystems, Foster City, CA). SYBER Green chemistry was used for mouse *Ptc-1*, *Gli-1*, and β-*actin*. PCR primers were as follows: *Ptc-1* forward, CTCCAAGTGTCGTCCGGTTT; *Ptc-1* reverse, CTGTACTCCGAGTCGGAGGAA; *Gli-1* forward, TGGATATGATGGTTGGCAAGTG; Gli-1 reverse, ACAGACTCAGGCTCAGGCTTCT; β-*actin* forward, ACGGCCAGGTCATCACTATTG; β-*actin* reverse, ATGGATGCCACAGGATTCCAT.

cDNA was synthesized from 1 μg of total RNA at 42°C for 50 minutes with oligo(dT)12-18 primer and Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR was carried out on ABI PRISM 7700 with an initial activation of AmpliTaq Gold DNA polymerase at 95°C for 10 minutes, then 40 cycles of denaturation at 95°C for 15 seconds, and annealing and extension at 60°C for 1 minute. All assays were performed in triplicate. Relative quantification was accomplished with measurement of the threshold cycle and use of the standard curve. Gene expression of the target sequence was normalized to that of the housekeeping gene β-*actin*.

In Situ Hybridization

The collected embryos were fixed at 4°C for overnight in 4% paraformaldehyde in PBS. For cryosectioning, embryos were embedded in optimal cutting temperature compound, sectioned by cryostat in 14 μm thickness and thaw-mounted onto VECTABOND (Vector Laboratories, Burlingame, CA) coated glass slides. The horizontal sections were made in this study, which include the forebrain, midbrain, two eyes, and two nasal placodes in the same plane.

RNA probes for mouse *Shh*, *Ptc-1*, and *Gli-1* genes were gifts from Dr. J. Motoyama.[24] An RNA probe for mouse cellular retinoic acid binding protein-I (CRABP-I) gene was a kind gift from Dr. Pierre Chambon.[25] In situ hybridization on sections was performed as described previously.[23,26] Briefly, the sections were rehydrated in PBS with 0.1% Tween 20 and postfixed in 4% paraformaldehyde for 10 minutes. Then the sections were treated with proteinase K (0.3 μg/mL, Roche, Basel, Switzerland) at 37°C for 3 minutes, postfixed again in 4% paraformaldehyde for 20 minutes, and hybridized with digoxigenin-labeled probes (1 μg/mL) overnight at 60°C. After hybridization, the sections were washed in 50% formamide, 5× SSC, and 1% sodium dodecyl sulfate for 30 minutes at 60°C, then twice in 50% formamide and 2× SSC for 45 minutes at 60°C, and finally in Tris-buffered saline containing 0.1% Tween 20 (TBST). The slides were then immersed in blocking solution (Roche) and incubated overnight at 4°C with anti-digoxigenin Fab-alkaline phosphatase conjugate (Roche) diluted to 1:2000 by blocking solution. The hybrids were visualized by an alkaline phosphatase reaction with nitro blue tetrazolium (Wako, Osaka, Japan) and 5-bromo-4-chloro-3-indolyl phosphate substrate (Wako).

Immunohistochemistry

Immunostaining using anti-Pax6[27] and anti-Pax7 (Hybridoma Bank of University of Iowa) antibodies was performed basically as described previously.[23,26] Frozen sections were prepared as described, rehydrated in TBST, and boiled in 0.01 M sodium citrate for antigen enhancement. After preincubation with the blocking solution for 30 minutes, the sections were incubated with anti-Pax6 or anti-Pax7 antibodies diluted to 1/500 by the blocking solution overnight at 4°C. Biotinylated antirabbit IgG (Vector Laboratories) was used as a secondary antibody at a dilution of 1/200, and immunoreactivity was detected using an ABC kit (Vector Laboratories) and a metal-enhanced diaminobenzidine kit (Pierce, Rockford, IL).

RESULTS

Craniofacial Morphology of the Cyclopamine-Treated Embryos

Mouse E 8.5 embryos were cultured in vitro for about 2 days with or without cyclopamine, and the craniofacial morphology was investigated after the cultures were finished and the embryos were collected. Craniofacial development was almost normal in the control group without cyclopamine (Fig 3A, B). Both of the nasal placodes in the control group were obliquely situated, and their axes were inferior-laterally directed (N = 34, Fig 3B). In contrast, the whole body size of the embryos in the cyclopamine-treated groups was slightly smaller (Fig 3C). The nasal placodes in the cyclopamine-treated embryos were directed vertically in parallel, and the angle between the nasal placodes was reduced compared with that in the control embryos (Fig 3D). This morphologic change was observed with considerable consistency, in 43 of the 56 cyclopamine-treated embryos (79.6%). This phenotype in craniofacial mor-

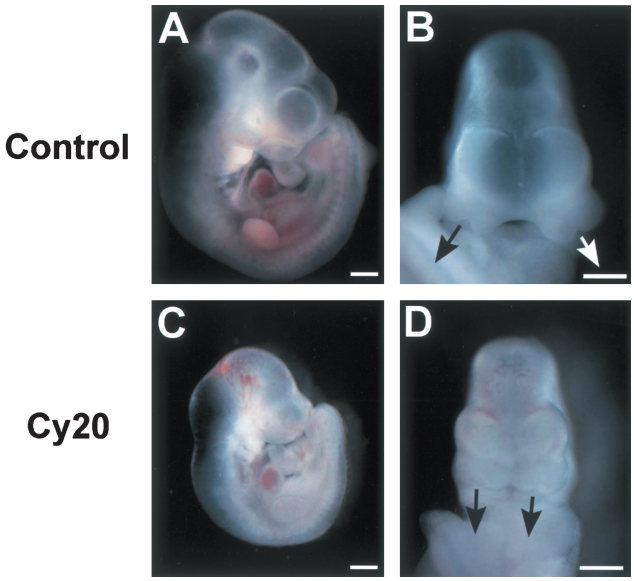

Fig 3 Morphologic changes of the control and the cyclopamine-treated embryos cultured in vitro. (A, B) The control group cultured without cyclopamine. The size and development of the embryos are nearly normal. The nasal placodes are obliquely situated and infero-laterally directed (arrows). (C, D) The group treated with 20 μM cyclopamine (Cy20). Overall size of the embryos is smaller than that of the control embryos. The nasal placodes are vertically directed (arrows) with reduced distance between them. No cyclopic change is observed. Bars: 500 μm.

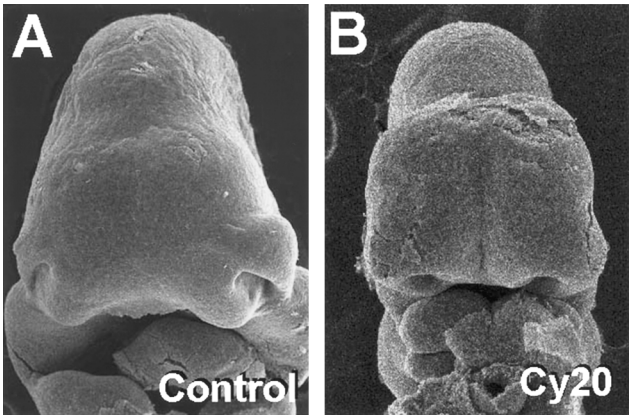

Fig 4 Craniofacial morphology of the (A) control and (B) the cyclopamine-treated embryos viewed by scanning electron microscope.

phology was more clearly observed by the scanning electron microscope (Fig 4A, B).

External cardinal veins in the craniofacial region were extended, and venous networks were more clearly observed in the cyclopamine-treated embryos (Fig 3C). We found no cyclopic embryos in the cyclopamine group, possibly because formation of cyclopic phenotype in *Shh* −/− embryos is attributable to lack of Shh signaling from earlier development.[12,18] However, we could not obtain cyclopic phenotype when the same concentration of cyclopamine was administered to E 7.5 cultured embryos (data not shown). In this case, morphology of the embryos was so severely and drastically affected in the whole body that we did not recognize detailed structures of the craniofacial region, possibly because of inhibition of the wide spectrum of the Shh functions in development.

In conclusion, by blocking Shh signal within the time window of E 8.5 to E 10.5, we could consistently observe holoprosencephaly-like malformations, which are considerably subtle compared with the drastic cyclopic phenotype seen in the *Shh* −/− embryos. We consider that the malformation seen in the current study may correspond to clinically milder symptoms of holoprosencephaly, such as false median cleft or mild hypotelorism.

Reduced Expression of Shh Signaling Genes in the Cyclopamine-Treated Embryos

We next examined whether expressions of Shh signaling genes such as *Ptc-1* and *Gli-1* were changed by cyclopamine treatment. We extracted total RNA from the head region of the control and cyclopamine-treated embryos, and expression amounts of *Ptc-1* and *Gli-1* were quantitatively examined by real-time RT-PCR (Fig 5). In the cyclopamine-treated group, expressions of both *Ptc-1* and *Gli-1* were remarkably reduced. *Ptc-1* and *Gli-1* expressions were approximately 38% and 9%, respectively, of those in the control group.

To further examine the changes of expression patterns of these genes, we next performed in situ hybridization of the cranial regions of the control and cyclopamine-treated embryos with RNA probes of *Shh*, *Ptc-1*, and *Gli-1*. In the control group, Shh was expressed in the midline neuroepithelium in the forebrain and the midbrain (Fig 6A). *Ptc-1* and *Gli-1* were expressed in the neuroepithelium and the mesenchyme along the midline around the *Shh*-positive area (Fig 6C, E), suggesting positive regulation of *Ptc-1* and *Gli-1* expression by Shh signaling itself. Although *Shh* expression was maintained in the midline neuroepithelium in the cyclopamine-treated group (Fig 6B), expressions of *Ptc-1* and *Gli-1* genes were dramatically inhibited in this group (Fig 6D, F).

Expression Patterns of Other Molecular Markers in the Cyclopamine-Treated Embryos

To characterize developmental events downstream of Shh signaling at the molecular level, we next performed immunostaining using an antibody against Pax6, a molecular marker of the eye and nose primordial,[26,28] in the control and the cyclopamine-treated embryos. We also examined expression patterns of *CRABP-I* and Pax7, molecular markers of the

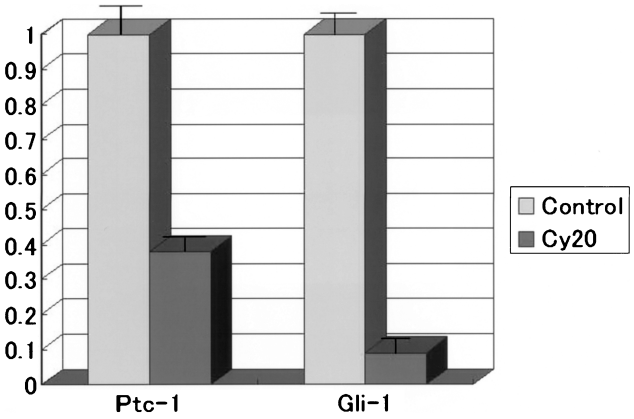

Fig 5 Reduced expression of Ptc-1 and Gli-1 genes in the cranial region of the cyclopamine-treated embryo, examined by real-time quantitative RT-PCR. *Ptc-1* and *Gli-1* expressions are approximately 38% and 9% of those in the control group, respectively.

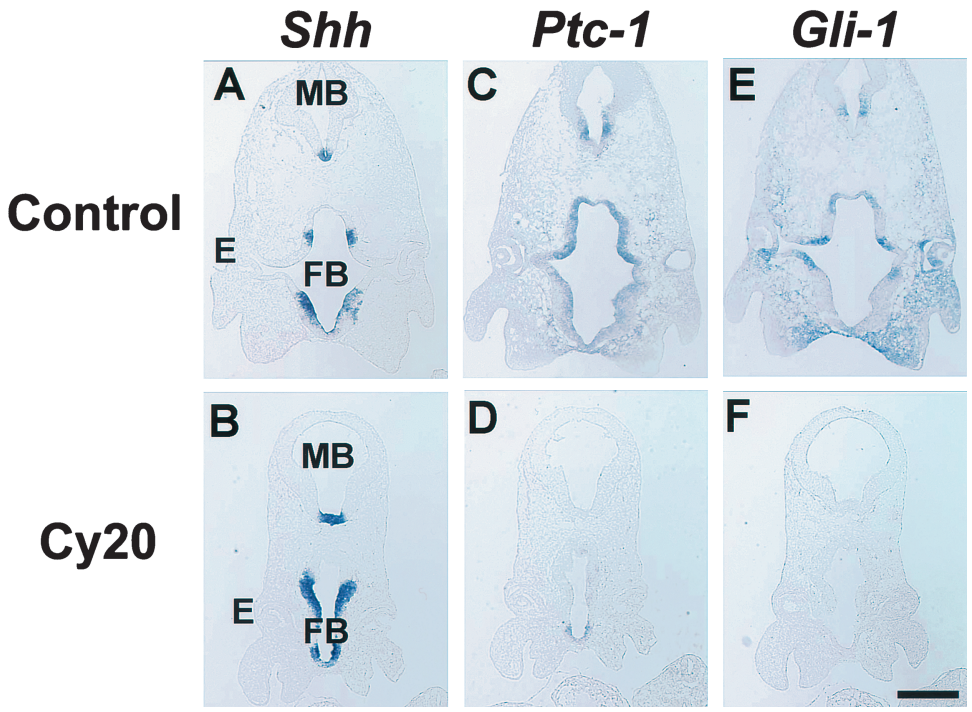

Fig 6 Expression pattern of the *Shh, Ptc-1*, and *Gli-1* genes in (A, C, E) the control and (B, D, F) the cyclopamine-treated embryos. (A) *Shh* is expressed in the medial neuroepithelium of the forebrain (FB) and midbrain (MB) in the control embryo. (B) *Shh* expression pattern is not changed in the cyclopamine-treated group. (C) *Ptc-1* is expressed in the neuroepithelium and the mesenchyme around the midline in the control group. (D) *Ptc-1* expression is faintly observed only in the midline neuroepithelium in the cyclopamine-treated embryo. (E) *Gli-1* mRNA is found in the midline neuroepithelium and the surrounding mesenchyme. (F) *Gli-1* expression is mostly inhibited in the cyclopamine group. Bar: 400 μm.

neural crest cells (NCCs),[26] in these embryos by in situ hybridization and immunohistochemistry.

Pax6 is a transcription factor playing a pivotal role in development of the eyes, nose, and the central nervous system.[29] We previously reported that Pax6 immunostaining clearly detected the eyes and nose primordium of the rat embryo in the corresponding stage.[26,28] In the current study, Pax6 was again expressed in the eyes and the nasal placodes in the control group (Fig 7A). The distance between the Pax6-positive areas in the nasal placodes was reduced in the cyclopamine-treated group (Fig 7B).

NCCs are a major component of the mesenchyme in the craniofacial region.[1,15,30–32] We reported previously that *CRABP-I* is expressed in the NCCs in both of the medial and lateral nasal prominences (MNP and LNP), whereas Pax7 is specifically expressed in the NCCs of the LNP, as seen in the control group in the current study (Fig 7C, E).[26] These expression patterns were basically unchanged in the cyclopamine-treated embryo; *CRABP-I* expression was again observed in the mesenchyme of the MNP and LNP (Fig 7D), and Pax7 expression was positive in the LNP mesenchyme (Fig 7F). However, the *CRABP-I*-positive or Pax7-positive area around the noses was reduced in the cyclopamine group. These results suggest that migration of these NCCs from the midbrain toward the nasal region is intact, but the number of the NCCs is reduced by cyclopamine.

Discussion

In this study, we administered Shh signaling inhibitor cyclopamine to cultured mouse embryos and examined changes in the craniofacial morphology. We found that cyclopamine-treated embryos exhibited craniofacial anomalies with reduced angles between the nasal placodes, which we consider a milder phenotype of holoprosencephaly. In the molecular analysis, we noticed that expressions of Shh signaling genes *Ptc-1* and *Gli-1* were remarkably reduced in the cyclopamine-treated group. It seemed that the midbrain NCCs migrate normally toward

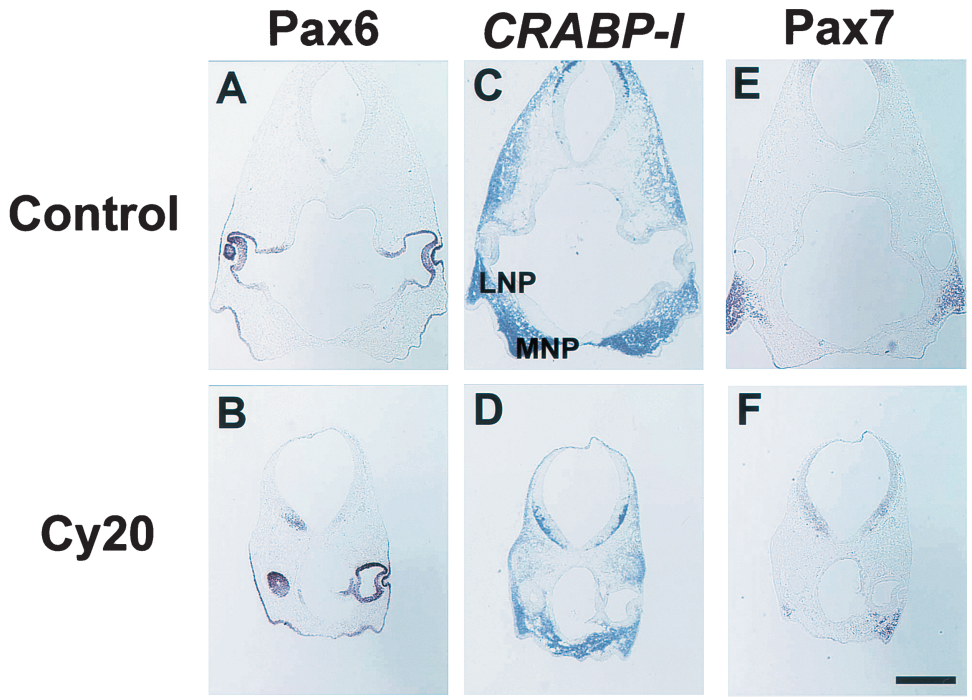

Fig 7 Localization of the Pax6 protein, *CRABP-I* mRNA and Pax7 protein in (A, C, E) the control and (B, D, F) the cyclopamine-treated embryos. (A) Pax6 protein is localized in the eyes, nasal placodes, and surrounding epithelium in the control embryo. (B) In the cyclopamine-treated group, the distance between the Pax6-positive area in the nasal placodes is reduced. (C) *CRABP-I* transcripts are localized in the neural crest cells in the medial and lateral nasal prominences (MNP and LNP) in the control embryo. (D) The *CRABP-I* expression pattern is not changed, but the positive area is reduced in the cyclopamine-treated embryo. (E) Pax7 is specifically localized in the LNP neural crest cells. (F) Pax7 localization is again unchanged, but the positive area is reduced in the cyclopamine-treated embryo. (The right side of the Pax7-positive area seems to be more reduced than the left side, but this is just because of asymmetry in the section plane.) Bar: 400 μm.

the nasal mesenchyme in the cyclopamine-treated embryo.

There have been a considerable number of reports that Shh has multiple roles in craniofacial morphogenesis according to the developmental stages. To facilitate our understanding, we divide craniofacial development into three stages. The first is a stage of neurulation and antero-posterior patterning of the central nervous system. The second is a period of formation, proliferation, migration, and differentiation of the NCCs. The third is a stage of local patterning of facial structures, such as the eyes and nose, through epithelial-mesenchymal interactions. In the first stage (< E7.5 in mice), *Shh* is expressed in the prechordal plate mediating overall morphogenesis of the forehead.[33] Shh-positive prechordal plate downregulates *Pax6* expression in frog embryos in this stage, so the *Pax6* -positive single eye field is divided in two in midline, probably by Shh signaling.[34] Therefore, it is thought that inhibition of Shh signaling in this stage results in drastic craniofacial anomalies, including cyclopia. The second stage (E 7.5–9.5) is characterized by the NCC induction, migration, and differentiation. Shh signaling inhibition in this stage results in apoptosis of migrating NCCs in the chick embryos, thus forming holoprosencephaly-like anomalies.[35] In the third stage in development (< E9.5), *Shh* is expressed in the nasal placodes in the chick embryos, acting as a signaling center mediating epithelial-mesenchymal interaction around the nose.[9] Enhanced Shh signaling in this stage can cause overgrowth or duplication of the nasal structure, thus forming hypertelorism.[36]

In the current study, we inhibited Shh signaling with cyclopamine during E 8.5 to 10.5 in the mouse embryos. This period roughly corresponds to the aforementioned second and third stages in craniofacial development, characterized by NCC migration/differentiation and later epithelial-mesenchymal interaction. We could not observe cyclopia which is seen in *Shh* −/− mice,[12] possibly because such a drastic phenotype is found when Shh signaling is

inhibited from the first stage of the development. We have obtained holoprosencephaly-like phenotype in the craniofacial region, including reduced distance and angle between the two nasal placodes. Our phenotype is less severe than *Shh* −/− mice or most of the previously reported animal models, and highly reproducible, with an occurrence rate of nearly 80%. We consider that our study may provide a good animal model corresponding to minor manifestations of holoprosencephaly, such as median facial dysplasia or a single central incisor. As far as we know, there have been no previous reports of animal models for these conditions.

There have been limited reports regarding downstream genes regulated by Shh signaling in craniofacial development. *Ptc* and *Gli* are directly regulated by Shh signaling itself.[11] Aoto et al[37] reported detailed analysis of craniofacial anomalies in *Gli3* knockout mice, and expressions of several genes such as *Fgf8* were changed, suggesting that these genes were regulated by Shh signaling. Our real time RT-PCR analysis and in situ hybridization confirmed that *Ptc-1* and *Gli-1* expressions were down-regulated by cyclopamine. As stated, *Pax6* is negatively regulated by the Shh-positive prechordal plate in the forebrain of *Xenopus* embryo in the earlier developmental stage.[34] We have also noticed that the distance between the Pax6-positive areas in the nasal placodes was reduced by cyclopamine administration. Although these facts suggest a possibility that the reduced distance between the nasal placodes in the cyclopamine-treated embryos is mediated by the Shh-dependent change of the Pax6 expression pattern, it awaits further investigation whether Shh signaling directly controls *Pax6* expression in our experimental model.

NCCs are induced at the edge of the neuroepithelium, migrate throughout the embryo, and differentiate into broad spectrum of tissues, including peripheral nerves, Schwann cells, pigment cells, and most of the craniofacial skeleton.[31,32] Migratory patterns of the cranial NCCs have been extensively investigated in the rodent embryo, and the mesenchyme of the medial and lateral nasal prominences (MNP and LNP) are derived from the NCCs migrated from the forebrain and midbrain, respectively.[30,38] Carstens[1] suggested that the false median cleft in holoprosencephaly is caused by the migratory defect of the midbrain NCCs. We also reported that the facial cleft seen in Small eye rat embryos possibly is caused by defective NCC migration in the craniofacial region.[26] These facts suggest a possibility that the anomaly seen in the current study is also caused by defective migration of the NCCs. To clarify this possibility, we examined localization of the cranial NCCs in the control and cyclopamine-treated embryos using two NCC markers, *CRABP-I* and Pax7. *CRABP-I* in situ hybridization can detect all the population of the cranial NCCs, and Pax7 immunostaining can specifically detect NCCs in the LNPs.[26] We found that *CRABP-I* transcripts and Pax7 protein were localized in the nasal mesenchyme in the cyclopamine-treated embryo, in a pattern similar to the localization in the control embryos. This finding suggests that the migration of the cranial NCCs is not perturbed in the cyclopamine-treated embryo. However, the number of the NCCs seemed to be reduced in the cyclopamine group, suggesting that proliferation or survival of the NCCs is impaired by cyclopamine. Ahlgren and Bronner-Fraser[35] reported that blockage of Shh signaling by grafting hybridoma cells producing neutralizing antibodies results in extensive cell death of the NCCs, as well as the neural tube, in chick embryos. It is to be elucidated whether this is also the case in our model.

The pathogenesis of human holoprosencephaly is polygenic and multifactorial, and several reports indicate that other genes, such as *Zic2* and *TGIF*, are also involved.[2,4] The severity of its phenotype has a broad spectrum, reflecting the complexity of its multifactorial pathogenesis. This complexity is, at least in part, based on the complicated nature of the craniofacial development in which Shh plays multiple roles. We should focus on a specific time window for analyzing a specific role of Shh in a molecular level, avoiding the confounding effects of other roles of Shh. One of the advantages of the whole embryo culture system is to detect the precise developmental stage by direct observation. In mouse embryos, there is usually a difference, of as much as half a day, of developmental stages within single littermates. We cannot adjust this time difference if cyclopamine is maternally administrated. Our strategy of cyclopamine treatment in the cultured mouse embryo, in this sense, enabled us to elucidate molecular mechanisms of minor types of holoprosencephaly. The methodology of investigating craniofacial malformations is being drastically changed by advances in modern developmental biology. We believe that our data in this study will promote our understanding toward the pathogenesis of holoprosencephaly.

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