Alteration of limb and brain patterning in early mouse embryos by ultrasound-guided injection of Shh-expressing cells

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Received 20 April 1998; revised version received 26 May 1998; accepted 27 May 1998

Abstract

A basic limitation of the study of development in the mouse is the inaccessibility of the embryos, which are encased in the maternal uterus. We demonstrate the first use of ultrasound backscatter microscopy for guiding injections of cells and other agents into early stage mouse embryos. Cells were injected into the mouse neural tube cavity as early as 9.5 days post coitus (E9.5), and into the developing limb buds as early as E10.5. Furthermore, a cell line engineered to express the secreted factor Sonic Hedgehog (Shh) was injected into early developing mouse brains or limbs. The Shh-expressing cells were found to induce ectopic expression of the Shh target genes Patched and Hnf3b in the dorsal brain, and to alter digit patterning in the anterior limb bud. These results show that gene misexpression studies can be performed in mouse embryos using ultrasound-guided injection of transfected cells or retroviruses. In combination with the many available mouse mutants, this method offers a new approach for analyzing genetic interactions through gain-of-function studies performed in mutant mouse backgrounds.

Keywords: Mouse; Sonic hedgehog; Gene misexpression studies

1. Introduction

The ability to manipulate embryos through the injection of cells, tracer dyes and retroviruses has provided powerful approaches for studying developmental processes in lower vertebrates such as chick, zebrafish and frog. Chick embryos, in particular have been used extensively for cell transplantation experiments and fate-mapping studies in the early developing nervous system and limbs. In contrast, the inaccessibility of mammalian embryos encased in the maternal uterus, has made in utero manipulations difficult or impossible at most stages. Injection of retroviruses (Jaenisch, 1980) and cells (Jaenisch, 1985) into the amnion of mouse embryos at gestational ages 8.5–9 days (E8.5–9) has been demonstrated, but remains a difficult technique, performed in a 'blind' manner using a-priori knowledge of embryonic orientation at this stage, and has been utilized by only a few laboratories. An image-guided method to inject cells and other agents into early-stage mouse embryos, for example to ectopically express specific genes, would provide the means to quickly and efficiently test genetic interactions in a system with extensive genetic information and many defined mutant strains.

We have previously described our development (Turnbull et al., 1995a) and use of high frequency (40–50 MHz) ultrasound imaging, also referred to as ultrasound backscatter microscopy (UBM), for high resolution in utero imaging of live mouse embryos (Turnbull et al., 1995b). More recently, we have used UBM-guided injections to transplant cells between specific parenchymal forebrain and mid–hindbrain target regions of midgestation...
(E13.5) mouse embryos (Olsson et al., 1997). In the present study, we demonstrate the first use of UBM-guided injections of cells into the neural tube cavity of much earlier-stage embryos (E9.5), as well as into another tissue, the limb buds at E10.5. E9.5 represents an embryonic stage at which neural patterning is occurring, at the onset of neurogenesis. For example, expression of Sonic hedgehog (Shh) in the ventral neural tube and notochord induces differentiation of ventral cell types in the mouse CNS at this stage (Echelard et al., 1993; reviewed in Ericson et al., 1995). E10.5 is an early stage in mouse limb development just prior to (hindlimb) or at the onset (forelimb) of the morphological development of the apical ectodermal ridge (AER), which is required for normal limb outgrowth and patterning. It is also the stage when Shh is first expressed in the hindlimbs in the zone of polarizing activity (ZPA) which regulates anterior–posterior (A/P) patterning (Riddle et al., 1993). To explore the utility of this novel approach for gene misexpression, we injected cells expressing Shh into the embryonic brain and limbs and analyzed the effects of the cells on brain and limb development. Shh provides an ideal test case since it is a secreted factor whose early embryonic expression has been shown to be both necessary and sufficient for normal dorsal–ventral patterning in the CNS and anterior–posterior limb specification (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Ericson et al., 1995; Chiang et al., 1996). A number of genes downstream of Hedgehog (Hh) signaling have been identified in flies and in vertebrates. In particular, the transmembrane protein Patched (Ptc) is both a Hh receptor and a target gene, since Ptc expression is augmented by Hh signaling in many developing tissues in mouse and chick (Goodrich et al., 1996; Marigo and Tabin, 1996). The floorplate gene Hnf3β has been shown to be induced by ectopic expression of Shh in the dorsal CNS, indicating the ventralizing effect of Shh signaling (Echelard et al., 1993). Ectopic expression of Shh in the anterior chick limb bud has been shown to alter anterior digit development and induce extra digit formation (Riddle et al., 1993; Yang et al., 1997). We show that Shh-expressing cells, injected into the E9.5 neural tube or E10.5 limb buds, can replicate all of these results in mouse embryos. Taken together, our results show that ultrasound-guided injections can be used as an effective method to introduce cells and other agents into a variety of developing embryonic tissues, allowing genes to be expressed ectopically for analysis of gene function through the use of transfected cell lines or viral vectors, in a manner which is more efficient and cost-effective than traditional transgenic mouse techniques. By combining this approach with the many available mouse mutants, gain-of-function studies can also be performed in mutant backgrounds to study genetic interactions.

2. Results

To investigate the feasibility of UBM-guided injections into early-stage mouse embryos, suspensions were prepared of a neonatal mouse cerebellar cell line C17 (Ryder et al., 1990; Snyder et al., 1992) which express lacZ and can be visualized in whole-mount embryos using X-Gal or Salmon-Gal histochemistry (see Section 4). Real-time UBM images were used to guide the injection needle to specific target sites and to visualize the cells as they were injected. The C17 cells were also transfected with an Shh expression vector in order to perform in utero gene misexpression studies in mouse embryos.

2.1. Injections into the early embryonic neural tube cavity

After performing a ventral midline abdominal incision on a pregnant mouse, part of the uterine horn containing a single embryo was positioned under the UBM transducer in order to image and inject the embryo (Fig. 1A; see Section 4). The neural tube cavity could be identified on UBM images from embryonic stages soon after closure of the cephalic neural folds (E9–9.5). Prior to this, E8.5 head folds could be identified on UBM images (data not shown). The high UBM contrast between the fluid-filled ventricular space and the surrounding embryonic tissue made this a relatively straightforward target region to identify and inject into from E9.5 onwards (Fig. 1B). The tip of the injection needle was visualized as a bright spot on the UBM images which could be viewed in real-time as it advanced through the uterine wall and into the neural tube (Fig. 1C).

After aligning the axis of the injection microcapillary in the plane of the UBM image, the entire length of the injection needle was evident in the 2D UBM image (Fig. 2A). It was not necessary for it to be perfectly aligned in the image plane since the tip of the injection needle appeared as a bright spot, displayed on the UBM image, larger than the true physical size of the tip (Figs. 1 and 2). By maximizing the UBM signal (image brightness) from this spot, we ensured that the needle tip was located in the image plane being monitored. The backscatter signals from the cell suspensions were sufficiently high that injected suspensions could be visualized filling the ventricular space (Fig. 2B).

C17 cells injected into an E9.5 forebrain ventricle were found to have widespread patterns of attachment to the ventricular wall and integration into brain parenchyma, even 2 days after injection (E11.5, Fig. 2C,D). Analysis of 26 injected embryos, performed to characterize the distribution pattern of the injected cells after 2 days (Table 1), showed a relatively random integration of the cells along the A/P axis of the neural tube. One consistent site of C17 cell integration was the choroid plexus of the fourth ventricle, positioned in the dorsal neural tube, just posterior to the midbrain–hindbrain junction.

2.2. Shh expressing cells induce ectopic Ptc and Hnf3β expression after injection into the E9.5 neural tube cavity

To explore the potential of UBM-guided injections in
gene misexpression studies, and the role of Shh in CNS and limb patterning, we made C17 cells expressing Shh. Mouse Shh expression in the CNS is normally restricted to the ventral midline, except at the zona limitans, and is known to be a powerful inducer of ventral cell fates (Echelard et al., 1993) and Ptc is expressed adjacent to Shh-expressing cells (Goodrich et al., 1996; Marigo and Tabin, 1996; Platt et al., 1997). Furthermore, ectopic expression of Shh in the dorsal CNS can induce transcription of Ptc and the floorplate gene Hnf3β in both chick and mouse embryos (Echelard et al., 1993; Goodrich et al., 1996; Marigo and Tabin, 1996). Thus Ptc induction is a good indicator of Shh signaling and Hnf3β of ventralization of the CNS.

C17 cells were transfected with an expression construct containing mouse Shh cDNA and selectable gene zeocin. Zeocin-resistant cell lines were isolated, and Northern blot hybridization demonstrated that most of the resulting cell lines expressed the transgene (Fig. 3A). The cell line expressing the highest level of Shh, C17-S4, was injected into E9.5 embryonic forebrain ventricles using the same method as described for the control C17 cells (see above and Section 4). The injected embryos were collected 3 days later (E12.5)

Fig. 1. Ultrasound-guided injections into the E9.5 neural tube cavity. (A) Schematic of the experimental setup. The anesthetized mouse was placed on the lower level of a two-level stage with a modified Petri dish pinned to the upper level, over her abdomen. Part of the uterus, accessed through a ventral midline incision, was gently pulled through a slit in a thin rubber membrane stretched across a hole in the bottom of the Petri dish into a bath of sterile PBS. Using real-time UBM images for guidance (B), the injection microcapillary (arrowhead) was inserted through the uterus and into the forebrain ventricle (C). Labeled structures: forebrain (f), midbrain (m) and hindbrain (h) cavities, and uterine wall (u). Scale bar (B), 500 μm.
and double-stained for both β-galactosidase activity and Ptc or Hnf3b expression (Table 2). To enhance the color contrast, Salmon-gal, a substrate which produces a red product after reacting with β-galactosidase, was used, rather than X-gal. C17-S4 cells in the choroid plexus induced Ptc expression in the roof of the fourth ventricle, a region which normally is Ptc-negative (Fig. 3B,C; n = 13/14). In contrast, the control C17 cells did not induce Ptc at the same site (data not shown; n = 11/11). Hnf3b was also induced by C17-S4 cell aggregates in the choroid plexus (Fig. 3D, E; n = 5/6) and ectopic Ptc induction was observed near C17-S4 aggregates in the dorsal diencephalon (Fig. 3B,F; n = 3/7). In addition to Ptc or Hnf3b induction adjacent to the C17-S4 aggregates in the choroid plexus, we also observed abnormal cell morphology, with thickening of the neuroepithelium adjacent to the Shh-expressing cells (Fig. 3C,E; n = 13/14).

Table 1
C17 cells integrate into different regions of the developing brain 2 days after UBM-guided injection into the forebrain ventricle of E9.5 embryos (26 embryos)

<table>
<thead>
<tr>
<th>No. aggregates</th>
<th>Telencephalon</th>
<th>Diencephalon</th>
<th>Mesencephalon</th>
<th>Metencephalon</th>
<th>Myelencephalon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>V</td>
<td>D</td>
<td>V</td>
<td>D</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>4</td>
<td>11</td>
<td>26</td>
<td>27</td>
</tr>
<tr>
<td>Large (≥0.5 mm)</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

D, dorsal; V, ventral.
*Cells in the dorsal myelencephalon were consistently found integrated into the choroid plexus.
Interestingly, two C17-S4 aggregates in the dorsal midbrain did not result in detectable levels of Ptc, but did induce epithelial thickening (data not shown). These results are summarized in Table 2.

2.3. Injections into early limb buds

Shh is normally expressed in a focal region of the early posterior distal limb mesenchyme, the zone of polarizing function.
activity (ZPA), starting at E9.75 in the forelimb and E10.5 in the hindlimb (Riddle et al., 1993). The ZPA is known to be crucial for anterior–posterior limb patterning, and chick-grafting experiments have shown that the fate of limb-bud cells (i.e. which digit they will form) depends on the distance from the ZPA (Tickle et al., 1975). More recently, a critical ZPA signal was shown to be Shh in mutant mice (Chiang et al., 1996) and in chick experiments, by grafting Shh-expressing cells or implanting Shh-coated beads. Shh beads induce digit posterior transformations and formation of extra digits in a manner dependent on dose and distance from the source of Shh signaling (Riddle et al., 1993; Yang et al., 1997).

We have performed experiments to determine the feasibility of transplanting Shh-expressing cells (C17-S4, see above) into E10.5 embryonic mouse limbs. Although it was not possible to precisely position the exact injection site in the limb buds, especially within E10.5 hindlimbs, analysis one day after injection (E11.5) showed that it was possible to place cell aggregates in the mesenchyme of both hindlimbs and forelimbs (Fig. 4). One cell aggregate in the tip of a forelimb appeared to be integrated into and expanding extensively along the AER (Fig. 4B). This is interesting in view of the recent evidence in chick that cell populations in the AER expand along the A/P axis of this structure and that there are limitations to cell movement across the AER (Altabef et al., 1997; Michaud et al., 1997; Vargesson et al., 1997). One of two cell aggregates in the anterior-distal limb appeared to induce a small outgrowth of cells (Fig. 4C). Even more striking was the observation, in an E13.5 forelimb in which C17-S4 cells were injected at E10.5 into the anterior-distal region, of a second digit forming beside digit 1 and an expansion of digit 1 (Fig. 4D). In eight additional limbs where the cell aggregates were located elsewhere (including the AER), no outgrowths were apparent. These results are similar to the transformations and extra digits reported to be induced by ectopic Shh expression in chick anterior limb buds (Riddle et al., 1993; Yang et al., 1997).

3. Discussion

These results show, for the first time, that in utero UBM-guided injections are possible into a variety of early mouse embryonic tissues. In particular, cells have been injected into the neural tube cavity as early as E9.5, and into developing limb buds at E10.5. The use of transfected cells has provided a means of misexpressing secreted factors in specific regions of the developing mouse embryo. In contrast to using traditional transgenic approaches for gene misexpression, the cells can be injected at specified times during development and positions in the embryonic tissue, to produce a range of misexpression patterns. Using this method, we have demonstrated that an Shh-expressing cell line can be used in mouse embryos to induce ectopic Ptc and Hnf3β expression and alter cell morphology in the CNS, and the development of extra digits in the limb. Other secreted factors could be similarly transfected into cell lines and injected into specific regions of the developing mouse embryo to study gene misexpression. While transfected cells were used in these studies, it should be noted that the UBM-guided injection system could also be used to introduce other agents such as drugs, retroviruses expressing marker genes for retrospective studies of cell lineage, and viruses expressing mouse cDNAs as an alternative method of misexpressing proteins.

Extensive genetic information available in the mouse has provided additional incentive to develop in utero injection methods in mouse embryos. Large numbers of spontaneous and induced mutations are known, and the recent development of gene targeting methods in the mouse have led to an increasing number of mutant mice and mouse models of human diseases. Injection of wildtype cells into mouse mutant embryos, or mutant cells into wildtype embryos can be used to compare cell developmental potential in normal and mutant environments. The availability of mouse mutants also opens up the possibility to study cell fate and the effects of altered gene expression using UBM-guided injections in mice lacking specific genes, as well as in normal animals at early developmental stages. Such studies would allow genetic interactions to be directly tested with a method that is quicker and less expensive than using transgenic mice. Mutant mice are also being used as model systems for human diseases, opening the possibility of using in utero injections to test the feasibility of rescuing defects in mutant embryos. In particular, it should be possible to test cell replacement and gene therapy strategies by injecting cells or retroviruses expressing specific genes into defined embryos.
mutant embryos. Our studies with Shh-expressing cells provide a basis for future studies of the requirement of genes proposed to be required downstream of Shh signaling by injecting C17-S4 cells into mutant mouse embryos.

4. Experimental procedures

4.1. Ultrasound-guided in utero injections

We have previously described the utility of high frequency (40–50 MHz) ultrasound imaging for in utero visualization of early mouse embryos (Turnbull et al., 1995b), and for transplantation of cells between distinct forebrain and mid-hindbrain regions of midgestation (E13.5) mouse embryos (Olsson et al., 1997). In this study, embryo visualization was provided with a custom-built UBM scanner (Turnbull et al., 1995a) as well as a commercial UBM (UBM Model 840, Humphrey-Zeiss, San Leandro, CA).

Animals used in these studies were maintained according to protocols approved by the Institutional Animal Care and Use Committee at New York University Medical Center. Timed pregnant Swiss Webster mice (Taconic, Germantown, NY) were anesthetized with sodium pentobarbital (0.5 mg/10 g body weight, injected intraperitoneally) mixed with magnesium sulfate (MgSO4·7H2O, 1 mg/10 g body weight) as a mild muscle relaxant. Embryos were staged in days post coitus, with E0.5 defined as noon of the day a vaginal plug was detected after overnight mating. In order to access the embryos, the abdomen of the pregnant female was wet shaved, and a 2-cm ventral midline incision was made through the skin and peritoneal muscle. The uterus was gently pulled out, allowing both sides of the uterine horn to be examined and one side selected for injection, with the number of embryos ranging between 4 and 10. The pregnant mouse was then placed in a two-level holding stage (Fig. 1A) and prepared for in utero injection.

The injection setup was similar to that described in our previous transplantation studies (Olsson et al., 1997). Petri dishes (100 mm diameter, 25 mm deep) were modified by punching a 25 mm diameter hole in the middle and then stretching a thin, 35 mm diameter rubber membrane (cast

Fig. 4. Shh-expressing cells injected into E10.5 limb buds can induce development of extra digits. (A) Horizontal UBM image of E10.5 mouse embryo (anterior to the left) demonstrating the appearance of forelimb (f) and hindlimb (h) buds. Injection needle (arrow) is shown with the tip (red arrowhead) in the hindlimb. a, amnion. Scale bar, 500 μm. (B,C) Examples of embryos injected at E10.5 and analyzed with Salmon-gal histochemistry to detect lacZ expression at E11.5. (B) An injected aggregate (arrowhead) in the distal tip of the forelimb (f), with cells expanding along the apical ectodermal ridge (arrow). (C) A C17-S4 cell aggregate (red, arrowhead) located anteriorly in the hindlimb (h) with outgrowth adjacent to it. (D) Forelimbs of an embryo injected at E10.5 and analyzed with X-gal histochemistry for lacZ expression at E13.5. The C17-S4 cell aggregate (blue, arrowhead) is located anteriorly in the distal left (L) forelimb. Note the extra digit (arrow) and elongation of digit 1 that developed near the Shh-expressing cells. The uninjected right forelimb (R) is shown for comparison. a, anterior; p, posterior.
from 2-component Silastic L RTV silicone rubber, Dow Corning, Midland, MI) over the hole. A 15–20 mm slit was cut in the rubber membrane in order to access the uterus after the Petri dish was mounted over the pregnant mouse (Fig. 1A). Part of the uterus containing one or two embryos was pulled through the slit in the membrane into the Petri dish filled with sterile phosphate-buffered saline (PBS with calcium- and magnesium-chloride, Sigma, St. Louis, MO) for injection through the uterine wall into selected embryonic target regions with UBM-guidance (Fig. 1B,C). After injecting 0.5–1.5 µl of cell suspension into each embryo, the injected embryos were pushed back into the abdominal cavity, and one or two new embryos were pulled into the PBS. In this way, it was possible to inject all the embryos on one side of the uterine horn (4–10) in 30–60 min, after which the mouse was sutured and allowed to recover in a warming/humidifying chamber.

Injection needles were made from glass micropipette pipettes (outer diameter = 1 mm, inner diameter = 0.5 mm, length = 100 mm; Sutter Instruments), pulled to produce a long taper (Flaming/Brown micropipette puller; Sutter Instruments) and broken under a microscope at an outer diameter of 60–70 µm (inner diameter 40–50 µm). The injection needles were beveled to an angle of 20° to produce a sharp tip (Microtipette Beveler EG-40; Narishige). Needles were held in a micropipette holder (MPH310; World Precision Instruments) and connected with 1-mm (inner diameter) tubing to an oil-filled manual microsyringe pump (Stoelting) to draw the cell suspension into the needle and to inject approximately 1 µl of cell suspension into each embryo. The micropipette holder was attached to a 3-axis micromanipulator (MO-155, Narishige), which was used to position the needle tip using UBM image-guidance during the injection procedure.

In order to characterize the integration pattern of C17 cells in the neural tube, 44 embryos were injected at E9.5, of which 26 (59%) survived to E11.5, when they were dissected, and lacZ-expressing cells were found in 26/26 embryos (100%) (Table 1). To study the effect of the Shh-expressing cells in the neural tube, 64 embryos were injected with C17-S4 cells at E9.5, of which 35 (55%) survived to E12.5, and lacZ-expressing cells were found in 29/35 embryos (83%). An additional 31 embryos were injected with control C17 cells, of which 21 (68%) survived to E12.5, and lacZ-expressing cells were detected in 15/21 embryos (71%). Finally, in a series of experiments injecting C17-S4 cells into E10.5 limb buds, 24 of 60 (40%) injected embryos survived to E11.5, of which 9/24 (38%) showed lacZ-expressing cells in the limbs, while 14 of 29 (48%) injected embryos survived to E13.5, and lacZ-expressing cells were found in the limbs of 3/14 (21%) embryos.

4.2. Establishment of Shh-expressing cell lines

C17 cells were cultured as described by Snyder et al. (1992). The 1.8 kb EcoRI–SpeI fragment of a mouse Shh cDNA (Echelard et al., 1993) was subcloned into pZeo-SV2(+) (Invitrogen). The BglII linearized expression vector was electroporated into C17 cells as described previously for embryonic stem cells (Wurst and Joyner, 1993). A total of 4 × 10^5 cells were electroporated with 20 µg DNA and selected in 250 µg/ml Zeocin. Twenty zeocin-resistant clones were picked and grown to confluency in 100-mm plates. Total RNA was prepared and Northern hybridization analysis (20 µg total RNA per lane) was performed using the EcoRI–SpeI Shh and a 1.2-kb β-actin cDNA fragments as probes. After hybridization, the blots were exposed to a phosphor screen and digitized on a Storm 820 scanner (Molecular Dynamics). Quantification of the signals was performed with Imagequant v1.11 on a Macintosh computer, the β-actin transcript was used to normalize the expression data (Fig. 3A).

Suspensions of C17 cells were made following the protocols described by Snyder et al. (1992). The cells were trypsinized and suspended in DMEM containing high glucose, with 0.05% DNAse, to generate cell suspensions of approximately 5 × 10^5 cells/µl. All cell suspensions were kept on ice prior to surgery.

4.3. Whole-mount preparation and LacZ staining

Embryos injected with C17 cells in the neural tube cavity at E9.5 were sacrificed 2 days (E11.5) after injection. Embryos were immersion-fixed in 4% paraformaldehyde in PBS at 4°C for 2 h after which whole brains were dissected. The resulting brains were then stained for β-galactosidase activity (Logan et al., 1993). Briefly, the brains were permeabilized in wash buffer containing 0.1 M phosphate buffer, 2 mM MgCl2 and 0.02% Nonidet P-40, rinsed for 5 min, three times, and then incubated in 5 mM K4Fe(CN)6·5mMK4Fe(CN)6·3H2O, 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, Sigma) in wash buffer at 37°C for 8–10 h. The brains were then rinsed in wash buffer (5 min) three times to stop the X-gal reaction, and fixed overnight in 4% paraformaldehyde in PBS. The X-gal reaction generally labeled most or all of the cytoplasm of the C17 cells, making it straightforward to identify the injected cells using a dissection microscope.

In the case of embryos injected in the limbs at E10.5, β-galactosidase activity was detected in whole embryos using either X-gal or Salmon-gal staining, which was performed as described above, except that Salmon-gal (6-Chloro-3-indoxyl β-D-galactopyranoside, Biosynth Ag) was substituted for X-gal. In addition, the staining time was increased to allow the reagents to penetrate the limb mesoderm. Embryos analyzed at E11.5 were stained at 37°C for 16–18 h, while E13.5 embryos were stained at 37°C for 36 h.
sing or control C17 cells at E9.5 were sacrificed 3 days after injection (E12.5). The heads of the embryos were fixed in 4% paraformaldehyde in PBS on ice for 2 h, washed with PBT (PBS containing 0.1% Tween20) three times (15 min each) and then stained for β-galactosidase activity as described above except that Salmon-gal was substituted for X-gal and the staining time was limited to 4 h at 37°C. The heads were then washed with PBT three times (5 min each) and fixed again for 4 h. Brains were dissected out and bisected along the mid-sagittal plane before whole-mount RNA in situ which was performed as described previously (Matise and Joyner, 1997).

Acknowledgements

This research was supported by grants from the Whitaker Foundation (DHT), and the National Institutes of Health (Ro1NS35876, Ro1HD35768; ALJ). We thank Martin Olson and Kenny Campbell for helpful discussions and assistance in the early stages of these experiments, Connie Cepko and Andy McMahon, Matt Scott and Janet Rossant for providing the mouse Shh, Ptc and Hnf3β cDNAs. We also thank Gerd Fishell for helpful discussions and critical review of the manuscript. ALJ is an Investigator of the Howard Hughes Medical Institute. DHT is an Investigator of the American Heart Association/New York City Affiliate.

References


