RESEARCH ARTICLE

DAN Directs Endolymphatic Sac and Duct Outgrowth in the Avian Inner Ear

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Bone morphogenetic proteins (BMPs) are expressed in the developing vertebrate inner ear and participate in inner ear axial patterning and the development of its sensory epithelium. BMP antagonists, such as noggin, chordin, gremlin, cerberus, and DAN (differential screening-selected gene aberrative in neuroblastoma) inhibit BMP activity and establish morphogenetic gradients during the patterning of many developing tissues and organs. In this study, the role of the BMP antagonist DAN in inner ear development was investigated. DAN-expressing cell pellets were implanted into the otocyst and the periotic mesenchyme to determine the effects of exogenous DAN on otic development. Similar to the effects on the inner ear seen after exposure of otocysts to the BMP4 antagonist noggin, semicircular canals were truncated or eliminated based upon the site of pellet implantation. Unique to the DAN implantations, however, were effects on the developing endolymphatic duct and sac. In DAN-treated inner ears, endolymphatic ducts and sacs were merged with the crus or grew into the superior semicircular canal. Both the canal and endolymphatic duct and sac effects were rescued by joint implantation of BMP4-expressing cells. Electroporation of *DAN* antisense morpholinos into the epithelium of stage 15–17 otocysts, blocking DAN protein synthesis, resulted in enlarged endolymphatic ducts and sacs as well as smaller semicircular canals in some cases. Taken together, these data suggest a role for DAN both in helping to regulate BMP activity spatially and temporally and in patterning and partitioning of the medial otic tissue between the endolymphatic duct/sac and medially derived inner ear structures. *Developmental Dynamics 229:219–230, 2004.* © 2003 Wiley-Liss, Inc.

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INTRODUCTION

In most vertebrates, including the chick, the complex process of inner ear formation begins with the specification of an epithelial placode that thickens and invaginates to form an otic cup. As the embryo matures, the otic cup closes to form the otic vesicle. The otic epithelium undergoes a series of poorly understood cellular movements to form the complex three-dimensional structure of the mature avian inner ear (Li et al., 1978; Bissonette and Fekete, 1996). The posterior semicircular canal (PSC) and the superior semicircular canal (SSC) develop from the superior dorsal region of the otocyst while the lateral semicircular canal (LSC) is derived from the more ventral region. The endolymphatic duct (ED) and sac (ES) emerge from the dorsomedial region of the otocyst. The cochlear duct elongates from the ventromedial region (Li et al., 1978; Bisonnette and Fekete, 1996).

Many genes have important roles in these processes, including transcription factors and growth factors. Bone morphogenetic proteins (BMP), members of the transforming growth factor-β superfamily of growth factors that includes *Drosophila decapentaplegic* (*dpp*), are expressed in the inner ear and are believed to direct

the axis formation of the developing otocyst (Wu and Oh, 1996; Gerlach et al., 2000; Gerlach-Bank et al., 2002; Thompson et al., 2003). BMP7 is expressed throughout the stage 17 otic vesicle except for a small medial–ventral patch. BMP5 and BMP4, expressed earlier in anterior and posterior placodal epithelial patches, are limited to an anterior and a posterior focus in the otic vesicle while at later stages BMP4 is expressed in the presumptive sensory epithelium (Oh et al., 1996; Wu and Oh, 1996).

Many BMP antagonists have been identified, including noggin, chordin, gremlin, cerberus, and DAN (differential screening-selected gene aberrative in neuroblastoma). We previously demonstrated that noggin was expressed in the periotic mesenchyme surrounding the otocyst (Gerlach et al., 2000). Exogenous noggin introduced at the BMP4 foci of the otocyst, blocking the activity of BMP4, produced specific canal abnormalities. Blocking BMP4 activity at the anterior focus generated abnormalities in the anteriorly derived canals, whereas interfering with BMP4 activity at the posterior focus prevented normal posterior semicircular canal development (Chang et al., 1999; Gerlach et al., 2000). Addition of exogenous BMP4 along with noggin prevented the noggin-mediated inner ear abnormalities as long as the two protein sources were implanted close to one another (Gerlach et al., 2000). This finding suggested that the noggin expressed in the periotic mesenchyme could act to restrict the activity of BMP4 to the otic epithelium. The question remained whether other BMP antagonists also acted to limit BMP4's patterning activity.

In this study, we investigated the BMP4 antagonist DAN, which we previously demonstrated was expressed in the medial otic epithelium throughout avian inner ear development (Fig. 1; see also Gerlach-Bank et al., 2002). DAN, first identified in transformed rat fibroblasts (Ozaki and Sakiyama, 1993), has been isolated subsequently in mouse, Xenopus, human, and chicken (Enomoto et al., 1994; Ozaki et al., 1996, 1997a; Hsu et al., 1998; Ogita et al., 2001; Gerlach-Bank et al., 2002). This se-
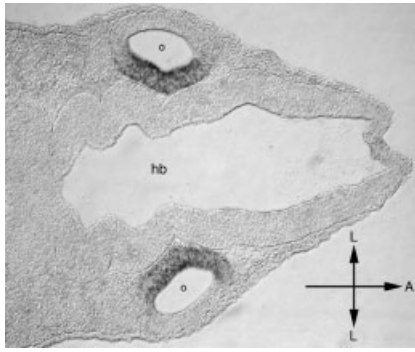

Fig. 1. DAN (differential screening-selected gene aberrative in neuroblastoma) expression in the medial otic epithelium of a stage 16 embryo. A, anterior; hb, hindbrain; L, lateral; o, otocyst. Scale bar = 100 μm.

creted protein is structurally related to cerberus and gremlin and is capable of antagonizing BMPs in embryonic explants (Stanley et al., 1998; Hsu et al., 1998).

Exogenous mouse DAN protein was introduced into avian inner ears by implantation of mouse DAN (mDAN) -producing Chinese hamster ovary (CHO) cells. The mouse and chick DAN homologues share 76% amino acid identity. Morphologic analysis of exposed ears indicates that semicircular canals are affected in an implantation site-specific manner, as was seen in the noggin implantation experiments (Gerlach et al., 2000). Unlike the noggin experiments, however, exposure to exogenous DAN also caused the endolymphatic duct to grow toward or fuse with the SSC or with the crus. In normal otic development, no connection between the endolymphatic duct and sac and the SSC is observed at any developmental stage. Joint implantation of mDAN-expressing cells with BMP4-expressing cells rescued this abnormal inner ear phenotype. Immunohistochemical analysis of the treated otocysts' hair cells demonstrated no obvious alteration in the shape or size of the sensory epithelium if the ampullae or basilar papilla were not abnormal.

Finally, in ovo electroporation of the otocyst, where DAN is expressed, with a chick DAN morpholino antisense oligonucleotide generated enlarged endolymphatic

sacs compared with the sacs that developed from otocysts electroporated with control morpholinos, including a sense morpholino, or not electroporated. The inner ear sensory patches in morpholino-treated inner ears appeared normal. Taken together with the DAN exposure data, these data suggest a role for DAN in partitioning the medial otic tissue between the endolymphatic duct and sac and the remainder of the medially derived inner ear structures, although the proteins with which DAN interacts to affect the development of these structures remain unknown.

RESULTS

Exogenous DAN Alters Gross Inner Ear Morphology

mDAN-producing CHO cells, BMP4-producing CHO cells, or untransfected CHO cells were implanted into or next to chick otocysts to determine their effects on inner ear morphology (Fig. 2). Cell pellets containing 1,000 cells were made of each cell type. After overnight culture, these pellets contained an average of 2,937 ± 41 (SEM) cells. Western blot quantification of V5-tagged mDAN demonstrated that each pellet produced approximately 52.7 pg of tagged protein per 24-hr period.

Five types of pellet implantations were performed. Pellets were inserted in the anterior, posterior, dorsal, or ventral regions of the periotic mesenchyme or in the center of the otocyst (Fig. 2A). The anterior and posterior implantation regions correspond to the anterior and posterior BMP4 expression foci in the developing otocyst (Wu and Oh, 1996; Gerlach et al., 2000). The implantations were performed between stages 11 and 19 (Hamburger and Hamilton, 1951; embryonic day (E) 1 and E2), and the inner ear morphology was analyzed between E5 and E10 by paint filling (Martin and Swanson, 1993). Implantations were performed on the right inner ear, with the left ear serving as an internal control.

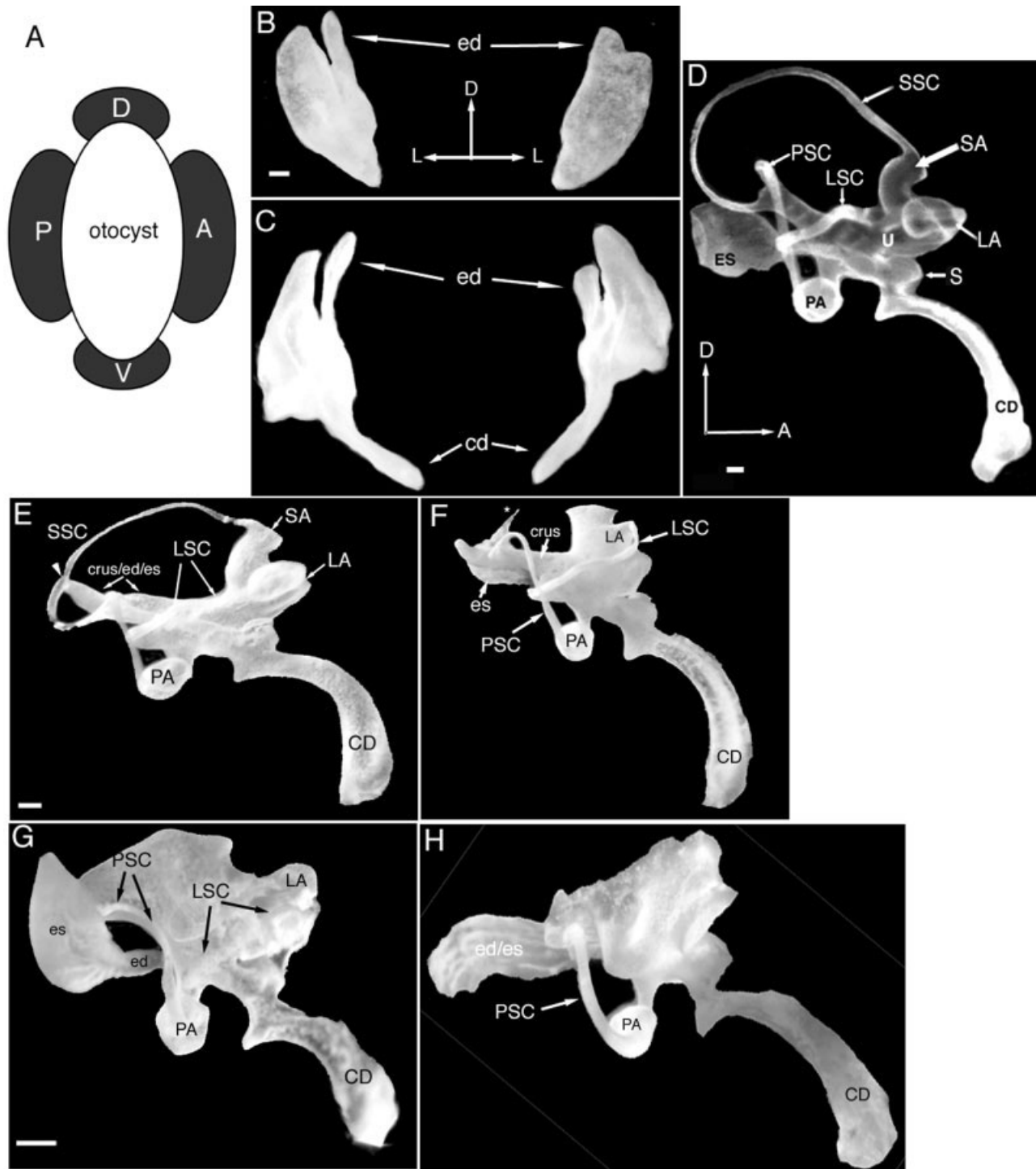Control implantations, which consisted of sham implantations (n = 7, Fig. 2D) or untransfected CHO cell

Fig. 2. Paint fill analysis of DAN (differential screening-selected gene aberrative in neuroblastoma) -exposed inner ears. **A:** Diagram of pellet implantation sites. Shaded regions represent the areas covered by each implantation category. **B:** Posterior view of stage 20 inner ears. The left ear was untouched. The right ear was exposed to a mouse DAN (mDAN) -expressing cell pellet. The endolymphatic duct (ed, ED) is shorter than the normal left ED and is partially merged with the main body of the otocyst. **C:** Posterior view of stage 26 inner ears. The mDAN-exposed right inner ear has a shorter and thicker ED than the left control ED. **D:** Normal stage 36 inner ear. **E:** A stage 36 mDAN-exposed inner ear with the ED joined to the superior semicircular canal (SSC, arrowhead). **F:** An mDAN-exposed stage 36 inner ear missing the SSC but with a nub (asterisk) growing from the endolymphatic sac (es, ES) toward the superior ampulla (SA). **G:** An mDAN-exposed stage 36 inner ear missing the SSC, having a reduced lateral semicircular canal (LSC) and an ES growing laterally toward the crus. **H:** A stage 36 mDAN-exposed inner ear with no SSC or LSC and an abnormally shaped ES/ED partially merged with the crus. A, anterior, CD, cochlea; D, dorsal; LA, lateral ampulla; P, posterior; PA, posterior ampulla; PSC, posterior semicircular canal; S, saccule; U, utricle; V, ventral. Scale bars = 100 μm in B (applies to B only), in D (applies to C,D), in E (applies to E,F), in G (applies to G,H).

	Ab PSC	Ab LSC	Ab SSC	PSC only	LSC only	SSC only	No canals	Ab ED/ES	Extra nubs	Normal	Total number	% Ab
Surgery												
A	1	2	6	3	0	0	0	13	7	21	42	50
C	1	2	2	0	0	0	3	19	2	13	34	62
P	5	1	3	0	0	3	1	15	2	14	40	65
D	0	1	0	0	0	0	1	0	0	1	3	66
V	0	1	1	0	0	0	0	4	1	4	9	55

TABLE 1. DAN Pellet Surgery Results[a]

[a]DAN pellets were implanted in either the periotic mesenchyme or the center of the otocyst, and the resulting abnormalities were characterized. A, anterior; Ab, abnormal; C, center; D, dorsal; ED/ES, endolymphatic duct/sac; LSC, lateral semicircular canal; P, posterior; PSC, posterior semicircular canal; SSC, superior semicircular canal; V, ventral; DAN, differential screening-selected gene aberrative in neuroblastoma.

pellet implantations (n = 15) resulted in normal inner ear phenotypes at all stages examined. The inability of CHO cells to alter inner ear morphology is also supported by the CHO cell bead implantations performed previously (Gerlach et al., 2000). No morphologic abnormalities were observed when cell pellets of two BMP4-expressing CHO cell lines, BMP4-6C and BMP4-3D, were implanted ($n_{BMP4-6C}$ = 11, $n_{BMP4-3D}$ = 19; data not shown).

mDAN Effects on Semicircular Canals

mDAN-expressing CHO cell pellet implantations (n = 153) altered inner ear semicircular canal morphology as early as stages 20 and 26 (n_{20} = 8, 87.5% abnormal; n_{26} = 17, 64.7% abnormal; Fig. 2B,C), the earliest stages analyzed. Fully developed inner ears, analyzed at stage 35 or higher, showed a range of abnormal inner ear phenotypes (Table 1). Canals were absent, severely reduced in size or present with an extra "nub." In Figure 2E, all the semicircular canals are present but the ED/ES is joined to the SSC. In some cases, a single canal would be eliminated (Fig. 2F), whereas in others, multiple canals were abnormal (Fig. 2G,H). The most severe SCC phenotype was the complete absence of all three canals and ampullae (not shown). No canals were present without their ampullae, whereas ampullae could be present without a canal. The canal phenotypes were similar to those observed in ears exposed to noggin (Gerlach et al., 2000).

The results from anterior (A), central (C), posterior (P), dorsal (D), and ventral (V) implantations are found in Table 1. Abnormalities were classified according to the structure(s) affected. For ease of comparison, partial canal truncations, abnormally shaped canals, and absent canals were grouped together. Anterior defects were those in which the SSC and/or the LSC were affected. These canals are derived from the anterior region of the developing otocyst. Posterior defects were those abnormalities that affected only the PSC, which develops from the posterior region of the developing otocyst. Anterior–posterior defects were those affecting the PSC and at least one of the anteriorly derived canals.

In general, posterior placement of an mDAN pellet generated posterior canal phenotypes (n = 5, 19%) and anterior–posterior defects (n = 4; 15%). Abnormalities affecting two canals always affected the PSC and the LSC, never the PSC and SSC. Anterior mDAN pellet implantation resulted in anterior defects (n = 11; 52%). Instances in which two canals were abnormal always involved SSC and the LSC. There were no instances of both the PSC and the SSC simultaneously affected either in anterior or posterior cell pellet implantations, corresponding with previous noggin-treated inner ear data (Gerlach et al., 2000). Central, ventral, and dorsal cell pellet implantations resulted in canal abnormalities as well. Central implantations affected each of the three SCC in the same number, with no double canal abnormalities. The LSC and SSC

were both affected by ventral pellet implantations (n = 2; 33%), whereas dorsal implantation resulted in one anterior defect and one inner ear with no canals (66%). Of all abnormal canals, 12% were structurally normal but reduced in size. Extra nubs were observed on some of the exposed utricles and ampullae as well (16%, Table 1).

mDAN Effects on Endolymphatic Ducts and Sacs

Unlike the noggin-exposed ears, however, DAN-exposed inner ears demonstrated endolymphatic duct and sac malformations. ED and ES abnormalities were observed in stage 20 and older embryos (65 of 153, 42.5%; Fig. 2; Table 1). Overall, 60.1% of DAN pellet implantations resulted in some sort of inner ear abnormality. Of these inner ear abnormalities, 81.3% involved ED and ES abnormalities. No ED or ES abnormalities were ever present in noggin-exposed inner ears (Gerlach et al., 2000). In some exposed inner ears, the ED was partially or completely merged with the crus (Fig. 2E). The ED and ES were sometimes smaller than the control, unexposed inner ear (data not shown). Other phenotypes in DAN-exposed inner ears included ES nubs that grew toward or connected to the superior semicircular canal (Fig. 2F). Such nubs are never found during the course of normal inner ear development. ED and ES morphologic abnormalities were found with or without SCC abnormalities. No connection was observed

TABLE 2. DAN Exposure Effects by Stage[a]			
Stage	Anterior % abnormal	Central % abnormal	Posterior % abnormal
11–13	40	NA	0
14–15	100	46	80
16–17	39	70.5	56
18–19	44	75	87

[a]The percentage of abnormalities observed at each stage of initial plantation were grouped according to implantation site. Total number of implantations = 128. DAN, differential screening-selected gene aberrative in neuroblastoma; NA, not available.

between an ED or ES abnormality and a particular SCC malformation.

The effects of exposure to mDAN in ovo on ED and ES development are reported in Table 1. In central mDAN cell pellet implantations, 90.5% of the abnormalities include ED/ES malformations. Anterior and posterior pellet implantations altered ED and/or ES morphology in 62% and 57% of the abnormal inner ears, respectively. ED/ES defects were also present in the ventral implantations (44%). No ED/ES effects were observed in the dorsal implantations. In these abnormalities, the EDs were merged with the crus or the ES grew toward or into the SCC. The defects observed did not correspond to the presence or absence of abnormalities in any particular canal.

Effectiveness of DAN Exposure Depends on the Stage Implanted

To determine the period during which the ear is most susceptible to DAN exposure, the surgeries were grouped by stage and site of implantation (Table 2). Implantations from stage 11 to stage 19 all generated at least one inner ear abnormality (n = 153). For posterior implantations, a biphasic trend was observed with ≥ 80% of surgeries between stages 14 and 15 and 18 and 19 exhibiting otic defects. Stages 14–15 were the most affected by anterior pellet implantations, whereas surgeries performed at stages 18–19 were the most effective for centrally placed pellet implantation-induced abnormalities. We did not include the

small numbers of ventral and dorsal DAN implants into the table. Nine ventral DAN implants were performed; of these, the three performed at stage 16–17 resulted in abnormal inner ears as did the one done at stage 18. The one ventral implantation done at stage 14–15 resulted in a normal inner ear. Only three (total) dorsal implants were performed. Those at stages 14/15 and 18 resulted in abnormal inner ears; that at stage 16/17 was normal.

Some DAN implantations (39.9%) resulted in normal inner ear phenotypes (Table 1). Although care was taken to ensure that the cell pellets were implanted intact and securely, the pellets could have been lost or moved during the normal mechanisms of otic morphogenesis. Furthermore, the inner ear is highly vascularized and some of the secreted DAN protein could have been washed away.

Rescue of the DAN Phenotype by Exogenous BMP4

To determine whether addition of BMP4-producing cells could rescue the effect of the DAN cell pellet implantations, BMP4-expressing CHO cell pellets were coimplanted with the DAN-expressing CHO cell pellets. By providing an exogenous source of BMP4, we sought to saturate the exogenous DAN activity. Inner ears were normal in 66% of those embryos coimplanted with CHO-BMP4 and DAN cells (n = 34). This is a frequency comparable to that previously seen for Noggin-expressing cell bead and BMP4-expressing cell bead rescue experiments (63%;

Gerlach et al., 2000). Only 42% of the DAN-expressing cell pellet and untransfected CHO cell pellet coimplantation controls (n = 10) resulted in normal inner ear phenotypes. This rate is comparable to the 40% of ears found to be normal when mDAN pellets are implanted alone. The abnormalities observed when BMP4-expressing cells and DAN-expressing cells were coimplanted, included both canal defects (50% of abnormal ears) and ED/ES defects (66% of abnormal ears).

Hair Cell Antigen Expression Is Not Affected by Exposure to Exogenous DAN

To analyze the effects of DAN on otic sensory epithelium, inner ears exposed to DAN were examined by using the hair cell-specific antibody HCA (Bartolami et al., 1991; Goodyear and Richardson, 1992). Hair cell antigen distribution was unaltered in unoperated control inner ears as previously described (Fig. 3A,B; Gerlach et al., 2000). The distribution of the hair cell patches and relative staining levels were altered in the DAN-exposed inner ears only if the structures housing the sensory epithelium were abnormal (Fig. 3C,D). If no inner ear sensory structures were affected, all vestibular and cochlear sensory epithelia expressed HCA in a pattern and at a level similar to control inner ears. No ectopic HCA expression was observed in any part of inner ears that were missing ampullae or within the endolymphatic sac outgrowths.

Blocking of Endogenous DAN Protein Expression by Antisense Oligo Morpholinos Alters Inner Ear Morphogenesis

The effect of eliminating functional DAN protein during the development of the inner ear was analyzed by using fluoresceinated antisense morpholinos designed against our published chick DAN sequence (Gerlach-Bank et al., 2002). The morpholinos (MOs) were first injected and then electroporated into the otocysts of stage 15, 16, and 17 chick embryos. All morpholino-in-
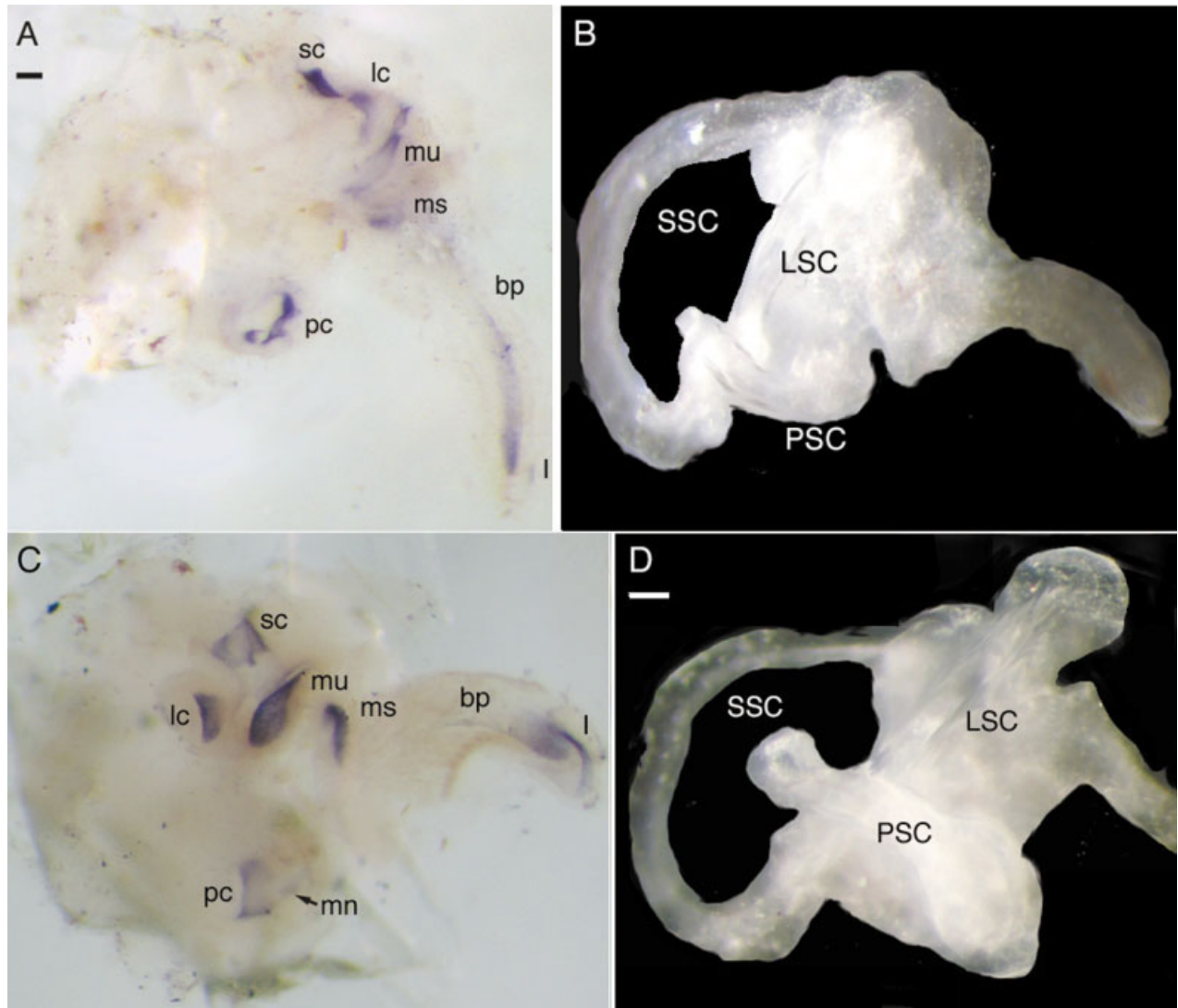
Fig. 3. Hair cell antigen (HCA) expression in normal and mDAN (mouse differential screening-selected gene aberrative in neuroblastoma) -exposed inner ears. **A:** Medial view of a normal stage 36 inner ear with normal HCA expression in the sensory epithelium. The macula neglecta is present but not visible. **B:** Paint fill of the ear in A demonstrating that all the canals and ampullae are present. **C:** A stage 36 mDAN-exposed inner ear with normal HCA expression. No canals were missing; however, the endolymphatic duct (severed during analysis) had an extraneous nub. **D:** Paint fill of the ear in C. bp, basilar papilla; l, lagena; lc, lateral crista; LSC, lateral semicircular canal; mn, macula neglecta; ms, macula saculi; mu, macula utriculi; pc, posterior crista; PSC, posterior semicircular canal; sc, superior crista; SSC, superior semicircular canal. Scale bars = 100 μm in A (applies to B,C).

jected otocysts were flushed gently with PBS before being viewed for fluorescein location 24 hr after electroporation and the embryo photographed (Fig. 4A). The extent of fluorescein within the otocyst varied from spotted expression in specific regions/individual cells of the otocyst (Fig. 4B,C) to label throughout one side or the other of the inner ear (Fig. 4A). Once photographed, the embryos were returned to the incubator until stage 35 at which time the inner ears were removed and processed for either paint fill analysis or HCA immunohistochemistry. To make sure that the MOs were indeed being taken up by cells and not retained in the lumen of the otocyst, in addition to flushing the otocyst before photography, in some experiments, cryostat sections of injected and electroporated inner ears were made of unfixed tissue (because retention of green fluorescent protein (GFP) is an issue in fixed tissue) and photographs were taken of these inner ears. Cell penetration in the otocyst was excellent at 2–4 hr postelectroporation, penetration was seen in as many as three to four cells in depth over 10 sections of the otocyst (Fig. 4B,C).

The morphology of inner ears electroporated with the control sense morpholino (n = 7), control *DAN*-inverted antisense morpholino (n = 6), or sham-injected and electroporated (n = 21) was completely normal (Fig. 4F). Inner ears electroporated with the antisense *DAN* morpholinos were abnormal (n = 9 per stage; 89% abnormal). The canals of these abnormal inner ears were frequently much smaller or
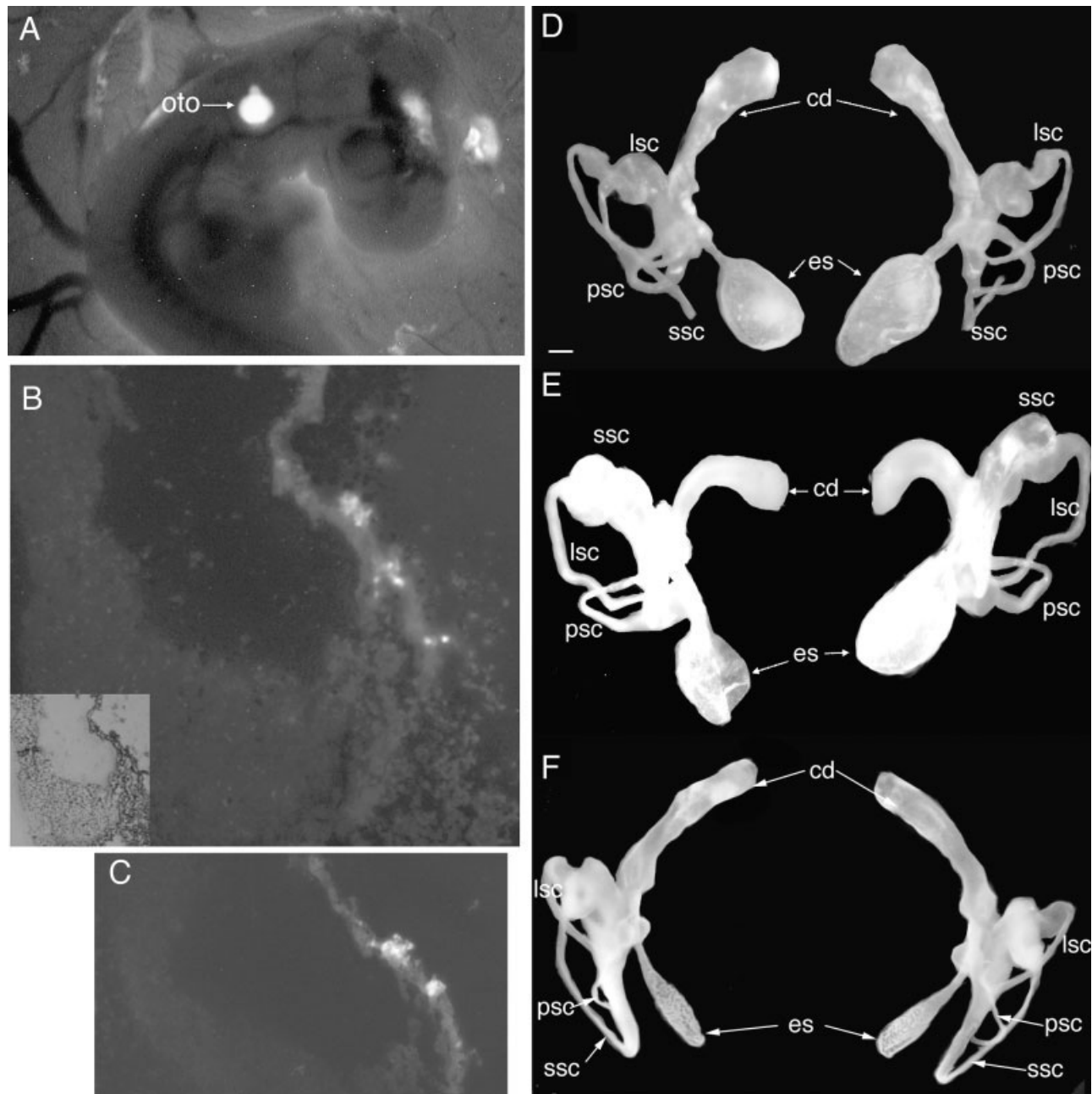
Fig. 4. DAN (differential screening-selected gene aberrative in neuroblastoma) morpholino-electroporated inner ear analyzed by paint fill. **A:** Stage 20 chick embryo 24 hr after in ovo electroporation with DAN antisense-fluoresceinated morpholino (MO). The entire inner ear demonstrates fluorescence. **B,C:** View of two adjacent sections of cryosectioned (but not fixed) stage 16 inner ear 4 hr after injection and electroporation of fluorescein-conjugated DAN sense MO. The electrodes were positioned so that the morpholino was electroporated into the lateral (outside) epithelium of the otocyst. Note that the fluorescent MO is located in different individual cells in the two adjacent sections, with those in (C) closer to the lumen. Medial is to the left, lateral is to the right, dorsal is to the top, and ventral to the bottom of the photographs in A–C, which were taken at ×20 original magnification. Insert in B: Brightfield view of the otocyst seen at ×4 original magnification. **D,E:** Ventral view of stage 36 paint-filled inner ears. Ventral is to the top. The right inner ear was electroporated with antisense DAN morpholinos. The superior semicircular canal (SSC) is truncated and the endolymphatic sac (es) is enlarged. **F:** Ventral view of stage 32 paint-filled inner ears. The right inner ear was electroporated with control inverted antisense DAN morpholinos. cd, cochlear duct; lsc, lateral semicircular canal; psc, posterior semicircular canal. Note that the ear morphology is normal in the electroporated inner ear. Scale bar = 100 μm in D (applies to D–F).

truncated (50% abnormal), whereas the endolymphatic sacs were grossly enlarged (62.5% abnormal; Fig. 4D,E). All of the inner ears included in the analysis demonstrate some number of fluorescent cells.

To determine whether the sensory epithelium was affected by electro-poration of the antisense *DAN* morpholinos, the electroporated ears were analyzed for HCA expression. In uninjected (n = 6) and control in-

ner ears (n = 3), HCA expression was normal in all of the sensory epithelia (data not shown, see Fig. 3A). The HCA pattern observed in antisense *DAN* morpholino-electroporated inner ears (n = 13) was unaltered (data not shown). No ectopic or expanded regions of HCA expression was observed in the sensory epithelium or in the enlarged ED/ES.

DISCUSSION

DAN is a member of a recently described cysteine knot protein family, including cerberus and gremlin (Hsu et al., 1998; Stanley et al., 1998; Pearce et al., 1999). DAN is a secreted protein with tumor suppressor activity that exhibits weak BMP antagonist activity in vitro and in vivo (Ozaki and Sakiyama, 1994; Ozaki et al., 1995; Hsu et al., 1998; Stanley et al., 1998; Dionne et al., 2001). Exposure to ectopic DAN protein in *Xenopus* animal cap assays induces anterior neural tissue and endoderm, suggesting a block in the BMP signaling cascade (Stanley et al., 1998). Biochemical analysis has demonstrated that DAN binds directly to BMP2 and interferes with BMP4 activity, although weakly compared with other antagonists (Hsu et al., 1998; Pearce et al., 1999). Direct binding of DAN with BMP4 or BMP2 under physiological conditions has not yet been shown (Dionne et al., 2001).

We recently have described DAN message expression in the developing chick embryo (Gerlach-Bank et al., 2002). Expression is found in the developing somites, intermediate mesoderm of the nephritic duct, eye, branchial arches, and limb buds. DAN is also expressed in the developing chick otocyst, where it is restricted to the medial otic epithelium through stage 25. As the vestibular and cochlear structures differentiate after this stage, DAN mRNA expression remains restricted to the endolymphatic duct and sac, the medial surface of the utricular–saccular–cochlear junction, and the cochlea. The medial otic expression of DAN at stage 16 overlaps to some degree with the expression of BMP7, which is located throughout the entire medial otic epithelium except for a small ventral region (Oh et al.,

1996; Wu and Oh, 1996). The potential interaction between BMP7 and DAN has not been investigated in this or other published studies. BMP4 and BMP5 focal expression is found at the anterior and posterior edges of the otocyst (Oh et al., 1996; Wu and Oh, 1996).

Many studies have shown that BMP4 is a secreted protein capable of acting over distances as a morphogen in multiple species (Harland, 1994; Re'em-Kalma et al., 1995; Dosch et al., 1997; Gerlach et al., 2000; for reviews of BMP4 activity, see Hogan, 1996; Hild et al., 2000). It is possible that DAN may act as an additional antagonist of BMP activity, complementing that of noggin, but that DAN has a specific functional role during inner ear development separate from that of its inhibition of BMPs. The experiments in this study were directed at ascertaining the role of DAN in chick otic development.

In an attempt to determine whether DAN could function in the developing otocyst as a BMP antagonist, mDAN-expressing cell pellets were implanted into the otocyst or the periotic mesenchyme, presenting the inner ear with an exogenous source of DAN protein. If DAN acts as an effective BMP antagonist in vivo, exogenous DAN would be expected to bind to any BMP present, thereby blocking the BMP's ability to bind its receptors. The resulting otic phenotype would be expected to be similar to that found previously when noggin-producing cells were implanted into the periotic mesenchyme (Gerlach et al., 2000). In general, treatment of the inner ear with DAN did mimic treatment of the inner ear with noggin. Posterior DAN pellet implantation resulted in PSC defects while anterior pellet implantation resulted in defects of the anteriorly derived canals, the LSC and SSC. As shown in Table 3, these canal phenotypes are similar to those previously reported when noggin-expressing cells were implanted into the posterior and anterior periotic mesenchyme, although the frequency of the DAN SCC abnormalities is lower (72% for noggin, Gerlach et al., 2000; 60.1% for DAN). Because DAN is reportedly a weaker BMP4

antagonist than Noggin, one would expect a lesser affect on BMP downstream events. This expectation is supported by the requirement for implantation of DAN cell pellets containing over 1,000 cells (at implant) to generate inner ear canal phenotypes similar to those seen when noggin beads carrying an average of 14 cells are implanted (Gerlach et al., 2000). Noggin beads produced 0.84 ± 0.11 pg of protein per 24 hr (Gerlach et al., 2000), whereas DAN pellets produced approximately 95 ng per 24 hr.

Implantation of DAN-expressing pellets produced canal abnormalities similar to those seen in noggin implantation studies and were rescued by coimplantation with BMP4-expressing cell pellets, supporting the idea that DAN functions at least in part to block BMP activity within the inner ear. However, central implantation of DAN pellets produced primarily anterior defects or complete elimination of the semicircular canals. This finding is not consistent with the data from noggin implantation experiments. Central implantation of noggin-producing cells did not generate any abnormal inner ear phenotype in previous work (Table 3; Gerlach et al., 2000). Abnormal cochlear duct morphology was not observed in the DAN-exposed inner ears, whereas shorter and thicker cochleae were observed in Noggin-exposed ears. The frequency of dorsal implant abnormalities was higher in DAN implants than in noggin implants (Table 3).

Furthermore, exogenous DAN exposure produced ED and ES abnormalities that were never observed in noggin-exposed inner ears. Exposure of inner ears to exogenous DAN resulted in a range of ED and ES abnormalities, from the growth of a nub toward the SSC and crus of the inner ear to the more severe merger of the ES/ED with the crus or SSC. Smaller ED and ES were also observed. The majority of ED/ES abnormalities resulted from central DAN pellet implantation within the otocyst, which may be a result of a larger portion of the otocyst being exposed to exogenous DAN in these implants or more DAN protein being presented to otic epithelial cells

	Number of					% ED/ES	
Surgery	implants	% A defect	% P defect	% AP defect	% CD defect	defect	% Abnormal
Anterior							
DAN	42	52	4.70	0.00	0	62	50
Noggin	39	83	8.50	8.50	4.30	0	59
Central							
DAN	34	19	4.70	14.30	0	90.50	62
Noggin	0	0	0	0	0	0	0
Posterior							
DAN	40	15	19	15	0	57.70	65
Noggin	39	0	57	43	25	0	71.80
Dorsal							
DAN	3	50	0	50	0	0	66
Noggin	18	57	0	43	0	0	38.90
Ventral							
DAN	9	40	0	0	0	80	55
Noggin	26	33	0	67	54.20	0	50

TABLE 3. Comparison of DAN and Noggin Implant Results[a]

[a]The abnormalities observed upon implantation of DAN pellets and Noggin beads were compared based upon implantation site. The Noggin implant data is from Gerlach et al. (2000). A, anterior; AP, anterior–posterior; CD, cochlear duct; ED, endolymphatic duct; ES, endolymphatic sac; P, posterior; DAN, differential screening-selected gene aberrative in neuroblastoma.

competent to interact with the protein.

The ability of DAN to affect ED/ES outgrowth and morphogenesis as seen in the DAN pellet assays was supported by the ED/ES phenotypes observed in the inner ears of chicks whose otocysts were electroporated in ovo with antisense DAN morpholinos. The resulting inner ears demonstrated grossly enlarged ED/ES (62.5%) compared with the left control and standard control morpholino-exposed inner ears. Furthermore, in the experimental ears, the semicircular canals were slightly reduced in size or truncated (50%). A combination of reduced SCC size and increased ED/ES growth was observed in some morpholino-injected ears (25% of those that were abnormal). This finding suggests a role for DAN in regulating the proportion of the medial otic epithelium into the future endolymphatic structures and the remainder of the inner ear. DAN may affect this result by disrupting the BMP feedback loop in the otocyst or by altering signaling pathways, which do not involve BMPs. For example, DAN is a member of the cerberus family, which is known to affect both Nodal and Wnt signaling activity. DAN may be acting in the inner ear to alter both BMP signaling as well as that of Nodal or Wnt. The antisense morpholino experiments provide an intriguing indication that DAN functions in the distribution of tissue or cells into the ED/ES and other medially derived structures, but there are certain caveats that require further exploration of this hypothesis. An antibody to DAN protein, which is presently unavailable, would allow us to ascertain to what extent the protein itself is being produced in MO-treated inner ear tissues. It is clear that only in the best of cases do we affect all DAN-producing cells (Fig. 4). A second series of rescue experiments, in which MO electroporation could be closely followed by DAN cell pellet implants in the same ear might also provide support for this hypothesis. However, we have found that an inordinately large number of embryos would have to be treated in this way, followed by assessment of MO distribution at 24 hr. Morbidity in such doubly operated embryos, which would require two successive and closely spaced invasive procedures is very high (on the order of 90%). However, that the MO treatments have effects only on the inner ear and not the surrounding tissues or hindbrain and that MO treatment produces the obverse of the gain-of-function phenotype seen when DAN cell pellets are implanted encourages us to persist in the exploration of DAN's role in medial epithelial partitioning.

BMP4 has been shown to presage sensory epithelium differentiation (Wu and Oh, 1996). To determine whether the potential DAN–BMP4 interaction affected this tissue, an anti-HCA antibody was used as a sensory epithelial marker and assayed in DAN-exposed and *DAN* morpholino-injected inner ears. HCA expression in the DAN-exposed inner ears was absent only if the otic structure housing the sensory epithelium was eliminated. This finding is consistent with HCA expression in noggin-exposed inner ears (Gerlach et al., 2000) and supports the hypothesis that DAN acts, at least partially, through BMPs. No ectopic or supernumerary cells with HCA staining were apparent. It is possible that DAN antagonism of BMP4, which is weaker than that of noggin, was insufficient to disrupt patterning of the sensory epithelium or that the DAN pellets were implanted after the sensory epithelium had already been patterned. Otocysts electroporated with the antisense *DAN* morpholino and, therefore, not able to translate DAN protein, also expressed HCA in

the appropriate locations as long as the structure housing the epithelium was present. No ectopic HCA expression was found in any of the abnormal ED/ES of the DAN-exposed or functionally DAN-negative inner ears. Therefore, DAN does not appear to affect sensory epithelium development in these inner ears.

Based on the data presented, we propose that DAN may have two functions in the development of the inner ear. First, DAN may act to inhibit BMP signaling by binding it and inhibiting its signaling. This possibility is supported by the similarities between DAN-exposed inner ear phenotypes, the noggin-exposed inner-ear phenotypes (Gerlach et al., 2000) and the chordin cell pellet implant phenotypes (unpublished data). The known differences in the strength of DAN and Noggin's antagonism of BMP4 can also explain why DAN treatment causes abnormalities in statistically fewer cases than does noggin (Table 3). Colocalization of BMP proteins and DAN has not been confirmed, because there are no antibodies available for either protein's immunohistochemical analysis; however, the mRNA expression patterns of DAN and the BMPs in the medial otocyst suggests that DAN may act with other antagonists in the otocyst to sequester BMP activity. Second, our data suggests that DAN functions to regulate the partitioning of the medial otic epithelium into the endolymphatic tissue and the remainder of the structures derived from the medial otic epithelium of the inner ear.

While the absence of or reduction in functional DAN protein increases the amount of tissue contained in the ED, the presence of exogenous DAN protein serves to prevent the normally observed complete separation of the ED/ES from the medial otocyst, resulting in connections with the crus and ventral portion of the SSC. DAN may be achieving this effect through interactions with BMPs in ovo. Alternatively, DAN may act through as yet unidentified factors to direct the separation of the ED/ES from the remainder of the medial otocyst. Such a mechanism is sug-gested by the absence of ED/ES abnormalities in noggin-exposed inner ears.

Factors, other than BMPs, with which DAN could interact to effect the ED/ES partitioning are presently unknown. Biochemical assays have demonstrated the ability of DAN to bind members of the GDF5/6/7 family (Dionne et al., 2001). Currently, it is unknown whether these factors are expressed in the developing inner ear. The protein DA41, a DAN binding protein that interacts with the EGF1-like factor S(1-5), may affect DAN signaling in the ED/ES but expression of DA41 has not yet been described in the otocyst (Ozaki et al., 1997b). Although Pax2 is known to be expressed in the developing ED/ES (Hutson et al., 1999), no interactions between Pax2 and DAN have been identified. As a member of the cerberus family, DAN might also function to alter Nodal and/ or Wnt signaling. Such an interaction has not yet been shown biochemically, however. Future studies require the identification of DAN-interacting proteins to elucidate the mechanism by which DAN is involved in the partitioning of the medial otocyst between the endolymphatic structures and the remainder of the inner ear.

EXPERIMENTAL PROCEDURES

Preparation of DNA Constructs

The mouse DAN sequence was amplified by using the following primers (forward: 5'-ACAATGCTTTGGGTC-CTGGT-3'; reverse: 5'-GTCCTCAGC-CCCCTCTTC-3') and subcloned in frame with the V5 epitope tag of the TopoTA pcDNA3.1(+)-V5 vector (Invitrogen). Orientation of the subcloned product was confirmed by sequencing. A full-length mouse BMP4 cDNA that was obtained from Drs. Deborah Thompson and Ron Koenig (University of Michigan) (Thompson et al., 2003) was subcloned into the pcDNA3.1(+) vector (Invitrogen), which contained an HA epitope tag and subsequently transfected into CHO cells for use in the "rescue" experiments.

In Situ Hybridization and Probe Syntheses

In situ hybridization and probe syntheses were performed as previously described (Gerlach-Bank et al., 2002). Embryos were collected and fixed in 4% paraformaldehyde (PFA), cryoprotected in 20% sucrose and embedded in OCT (Miles). The 12-μm sections were placed on Superfrost plus slides (VWR) and labeling was detected by alkaline phosphatase.

Cell Transfection and Culture

The mDAN and BMP4 constructs were individually transfected into CHO cells using the Transfast Transfection Kit (Promega). Stable clones were selected by using G418 (Gibco-BRL). Production of V5-tagged mDAN-expressing clones was determined and quantified by Western blotting (data not shown). Expression of mBMP4 was confirmed by Western blotting using antibodies against the HA epitope (Invitrogen). CHO and noggin-expressing CHO cells were grown as previously described (Smith et al., 1993, Gerlach et al., 2000), whereas the stable mDAN-expressing or mBMP4-expressing clones were cultured in CHO media containing 0.8 mg/ml G418. mDAN-expressing CHO cells, mBMP4-expressing CHO cells, and untransfected CHO cells were trypsinized, and 1,000-cell aliquots were used to make hanging drop-cultured cell pellets. The pellets were cultured overnight before implantation.

To determine the average number of mDAN-expressing CHO cells in each pellet after overnight culture, three groups of 48 cultured pellets were collected. The pooled sample was trypsinized with shaking for 20 min at 37°C. The cells were then counted in a Beckman Coulter Z1 Coulter Counter in triplicate (n = 9) to determine the number of cells per cultured pellet.

Western Blots

Western blots were used to quantify V5 epitope tagged mDAN expression in transfected CHO cells and

were performed according to the protocol suggested for use with the anti–V5-HRP antibody (Invitrogen). mDAN-transfected CHO cells were cultured for four days in serum-free CHO medium (CHO-SFII, GibcoBRL). The supernatants were collected, and serial dilutions were made for use in Western blot assays. The cells contributing to the supernatant were trypsinized and counted with a hemocytometer. Supernatants from untransformed CHO cells served as negative controls while the Positope peptide (Invitrogen), containing the V5 epitope, was used as the positive control. Chemiluminescent detection was performed by using an ECL kit (Amersham) and analyzed.

Surgical Pellet Implantations

Fertilized White Leghorn chicken eggs were obtained from the Bilbie Aviaries, Ann Arbor, MI, and incubated at $37 \pm 1°C$. All pellet implants were performed on embryos between stages 11 and 19 (all staging according to Hamburger and Hamilton, 1951). The eggs were windowed and prepared as previously described (Gardner and Barald, 1991; Gerlach et al., 2000). A slit was made in the otic mesenchyme or the center of the otocyst, and a cell pellet was mouth-pipetted into the opening. The cell pellets were dipped briefly in a neutral red solution before implantation to visualize the pellet as it was inserted. mDAN-expressing cells, BMP4-expressing cells, or untransfected CHO cells were implanted in dorsal, ventral, anterior, posterior, or central locations (see Fig. 2). For rescue or rescue control experiments, mDAN-expressing cell pellets were implanted with either BMP4-expressing or untransfected CHO cell pellets. All implantations were performed on the right inner ear. The left inner ear served as the internal, unmanipulated control. After implantation, the embryos were incubated until the desired stage. The embryos were then harvested and fixed in Bodian's fixative (75% 95% ETOH, 5% formaldehyde, 5% glacial acetic acid, 15% MilliQ water/v/v/v/v/) for paint fill analysis or in 4% paraformaldehyde for im-munohistochemistry as previously described (Gerlach et al., 2000).

Paint Injections

To more easily visualize the inner ear's morphology, a solution of 1% white semigloss paint (Benjamin Moore) in methyl salicylate was injected through the cochlear duct or utricle as previously described (Martin and Swanson, 1993; Bissonnette and Fekete, 1996; Gerlach et al., 2000).

Whole-Mount Immunohistochemistry

The inner ears from stage 35–36 implanted or electroporated embryos were excised and prepared for immunohistochemistry as previously described (Wu et al., 1998; Gerlach et al., 2000). The ears were stained in a 1:1,000 dilution of an antibody specific for a 275-kDa HCA kindly provided by Dr. Guy Richardson, Sussex University (Bartolami et al., 1991; Goodyear and Richardson, 1992) and post-fixed in 4% paraformaldehyde.

In Ovo Electroporation of Morpholino Oligonucleotides

In ovo electroporation was carried out as described by Kos et al. (2001). Briefly, eggs were windowed as for pellet implantation, and the vitelline membranes were removed. A 1-μg/ml solution of GFP–plasmid or a 500-μM solution of the DAN antisense morpholino, the control inverted DAN antisense morpholino, or the control sense morpholino standards obtained from GeneTools (Corvalis, OR) was mouth-pipetted into the otocyst. A GFP expression construct (Clontech) was initially used to demonstrate that the electroporation technology was working and that the cells could make GFP up to 48 hr later. GFP expression constructs were not used with morpholinos in the experiments described in this report. The fluoresceinated standard control sequence was 5'-CCTCTTACCTCAGTTACAATTATA-3'. The fluoresceinated DAN antisense morpholino was 5'-CACCCA-GGAGACGGCGGGCACATCC-3'. The fluoresceinated DAN inverted antisense control morpholino was 5'-CCTACACGGGCGGCAGAG-GACCCAC-3'. The plasmid and morpholinos were dissolved in vital dye ($1\times$ PBS with blue food coloring) to facilitate visualization. Each morpholino was fluorescein-conjugated to aid in confirming in ovo uptake. The 1-mm gold plated electrodes (BTX) were placed 1 mm away from each side of the embryo with the otocyst centered between them, and two drops of Locke's solution were placed over the embryos. Five 100-msec pulses of 12 V were applied (ECM 830, BTX) directing the morpholinos to the medial region of the otocyst. The inner ears were visualized 24 hr postelectroporation under a dissecting microscope, each MO-injected and electroporated ear was punctured and gently flushed with PBS to ensure that no trapping of MO/GFP had occurred and the fluorescein expression pattern subsequently recorded (Fig. 4A). The embryo was incubated until stage 35–36, at which point the ear was analyzed by paint injection (Fig. 4D–F) or immunohistochemistry. To determine whether the MOs were being successfully taken up by cells, we also performed a series of experiments in which the fluoresce-in-conjugated sense MO was injected into stage 16 otocysts and was then electroporated toward the lateral (external) otic wall. These embryos were cryosectioned without fixation 2–4 hr after electroporation to determine the cellular location of the labeled MO (Fig. 4B,C).

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