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Expression pattern of the FGF-related proto-oncogene *int*-2 suggests multiple roles in fetal development

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Summary

The FGF-related proto-oncogene *int*-2 is implicated in mouse embryogenesis, since it is expressed in specific tissues during gastrulation and neurulation (Wilkinson *et al.* 1988). Here, we describe the expression of this gene during subsequent fetal development. *int*-2 transcripts are restricted to Purkinje cells in the cerebellum and to regions of the developing retina containing early-stage differentiating cells. This high level expression is not detected in the mature cerebellum or retina. In addition, *int*-2 RNA is detected in the mesenchyme of the developing teeth and in sensory regions of the inner ear. This complex and dynamic pattern suggests multiple roles of this proto-oncogene during fetal development of the mouse.

Key words: proto-oncogene, *int*-2, growth factors, mouse development, cerebellum, retina, tooth, inner ear.

Introduction

There is increasing evidence that growth factors and their receptors have important roles in development (Adamson, 1987; Mercola & Stiles, 1988). Although these roles may include the control of cell proliferation in certain tissues, a number of growth factors have also been implicated in other biological processes (Sporn & Roberts, 1988; Gospodarowicz *et al.* 1986). This is exemplified by the apparent involvement of the endothelial cell mitogen, basic fibroblast growth factor (FGF), in cell migration (Folkman & Klagsbrun, 1987), neuronal cell differentiation and survival (Morrison *et al.* 1986; Walicke *et al.* 1986; Anderson *et al.* 1988; Hatten *et al.* 1988) and inductive tissue interactions (Slack *et al.* 1987; Kimelman & Kirschner, 1987). Recently, we have presented evidence that an FGF-related gene, *int*-2 (Dickson & Peters, 1987), may have roles in the development of the gastrulating and early-somite-stage mouse embryo (Wilkinson *et al.* 1988).

int-2 was first identified as a locus associated with mammary tumour formation in the mouse (Peters *et al.* 1983). In many of these tumours, activation of *int*-2 transcription has occurred due to insertion of mouse mammary tumour virus in the vicinity of the *int*-2 gene (Dickson *et al.* 1984; Moore *et al.* 1986; Peters *et al.* 1986). However, normal expression of *int*-2 thus far reported is restricted to the postimplantation mouse embryo (Jakobovits *et al.* 1986; Wilkinson *et al.* 1988). Between 7·5 and 9·5 days of development, *int*-2 is expressed in parietal endoderm, early-migrating mesoderm, a portion of the pharyngeal pouches and the neuroepithelium of the hindbrain adjacent to the developing otocysts (Wilkinson *et al.* 1988). Based on these observations and the properties of other FGF-related proteins, we proposed that *int*-2 may have multiple roles, influencing cell migration and perhaps acting as a morphogen in the development of the otocyst.

In the present report, we describe the pattern of *int*-2 expression throughout the subsequent period of gestation. *int*-2 transcripts are restricted to sensory regions of the inner ear, the neuroblastic layer of the retina, Purkinje cells in the cerebellum and the mesenchyme of the developing teeth. We discuss the implications of these data for the possible functions of *int*-2 in mouse development.

Materials and methods

Northern blot and in situ *hybridization analysis*

Preparation of RNA from microdissected tissues, electrophoresis, blotting and hybridization were all performed as described previously (Wilkinson *et al.* 1987*a*,*b*). *In situ* hybridization was also performed as described (Wilkinson *et al.* 1987*a*,*b*).

Immunocytochemistry

For immunocytochemistry and *in situ* hybridization analysis of serial sections, the brain was dissected from 16·5 day *post coitum* (*p.c.*) embryos and fixed in 4% paraformaldehyde in

PBS at 4°C overnight. Following this, the tissue was dehydrated in an increasing concentration of ethanol, embedded in polyethyleneglycol 400 distearate, 1% cetyl alcohol at 42°C, and then 6 μm sections were cut and mounted on chrome-alum-subbed slides. After overnight drying of these slides, wax was removed with two changes of 100% ethanol for 10 min each, and the sections air dried for at least 20 min. Adjacent sections were used for *in situ* hybridization or for immunocytochemistry with antiserum against chick intestinal calcium-binding protein (Jande *et al.* 1981), which specifically recognizes Purkinje cells in the developing cerebellum. For immunocytochemistry, endogenous peroxidase was blocked with 0·3% hydrogen peroxide in methanol for 30 min, washed in PBS, and nonspecific binding blocked with 1% bovine serum albumin in PBS for 30 min. Incubation with 1/500 diluted antiserum was carried out at 4°C overnight. Washing and visualization of antibody with horseradish peroxidase and diaminobenzidine was performed using the Vectastain kit (Vector Laboratories) as recommended by the manufacturers.

Computer-aided reconstructions

Camera-lucida drawings were made of otic tissue and the sites of *int*-2 expression in serial sections of the inner ear of a 17·5-day mouse embryo. Reconstruction of these data using the SSRCON system (Shepherd *et al.* 1984) was carried out as described previously (Wilkinson *et al.* 1987*b*).

Results

Northern blot analysis of total RNA detects roughly constant levels of *int*-2 transcripts throughout development of the mouse fetus (data not shown). The *in situ* hybridization studies reported here reveal that this reflects complex and dynamic patterns of *int*-2 expression in specific regions of the developing cerebellum, retina, teeth and inner ear. Each of these sites is described in detail below.

int-*2 expression in the cerebellum*

int-2 transcripts were first detected in the developing cerebellum at 14·5 days *p.c.* The cerebellum originates from a population of proliferating neuroepithelial cells, termed the ventricular zone, located at the roof of the hindbrain. Postmitotic cells migrate away from the ventricular zone along routes distinctive for each of the terminal cell types (Altman & Bayer, 1985*a*,*b*) and eventually settle in specific sites to form the stratified structure of the mature cerebellum. These events occur initially in lateral regions and then in progressively more medial parts of the cerebellum. At 14·5 days *p.c.* *int*-2 expression was detected in cells adjacent to the ventricular zone in lateral sections (Fig. 1A,B) but not in more medial sections (data not shown). Two days later, *int*-2 transcripts were detected in cells distal from the ventricular zone in lateral regions (Fig. 1C), but in more medial sections *int*-2 expression occurred in cells adjacent to the ventricular zone (data not shown). This pattern suggests that *int*-2 expression is occurring in Purkinje cells which are known to originate and migrate in this fashion (Altman & Bayer, 1985*a*,*b*). To confirm this, serial sections were used for *in situ* hybridization with *int*-2 probe (Fig. 1C) or for immunocytochemistry with an antibody to a calcium-binding protein that is restricted to Purkinje cells in the cerebellum (Jande *et al.* 1981) (Fig. 1D). At 16·5 days *p.c.*, *int*-2 expression

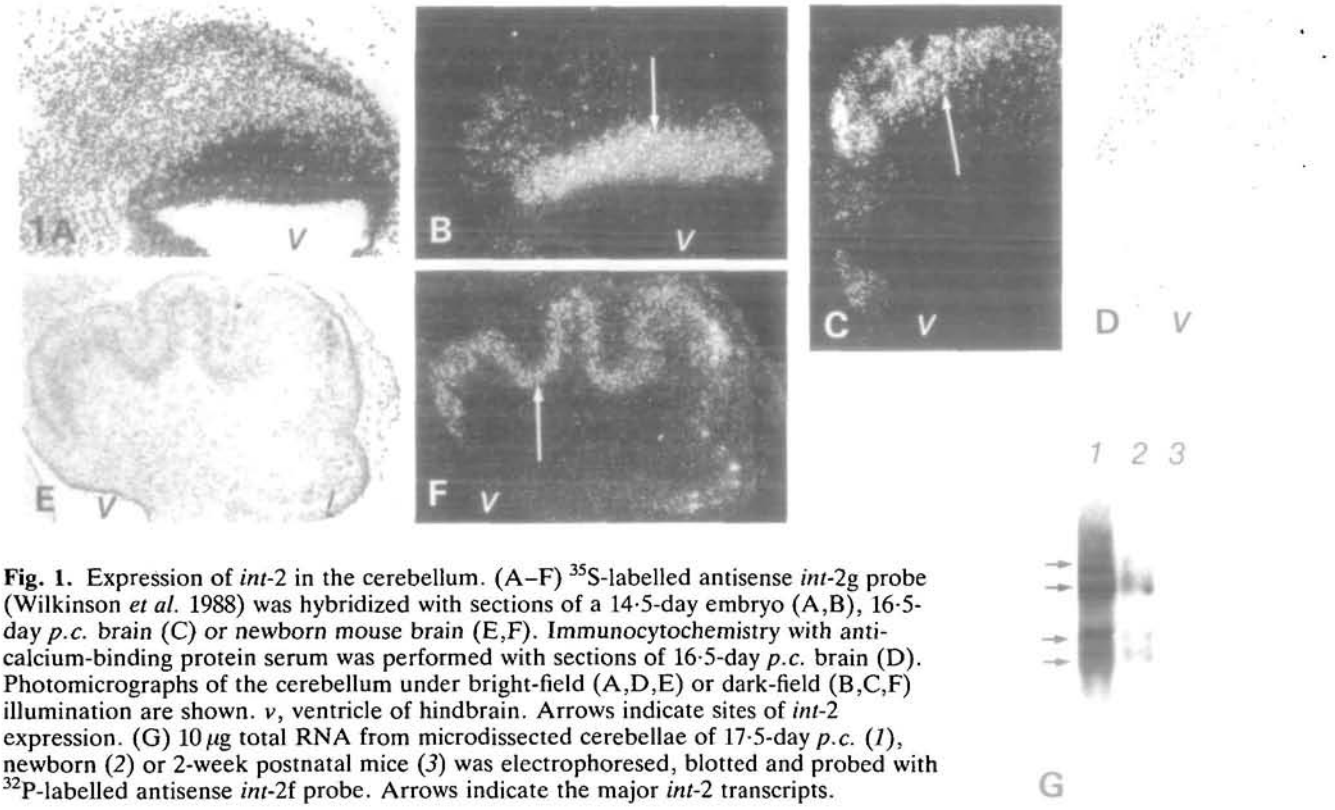


Fig. 1. Expression of *int*-2 in the cerebellum. (A–F) ^{35}S-labelled antisense *int*-2g probe (Wilkinson *et al.* 1988) was hybridized with sections of a 14·5-day embryo (A,B), 16·5-day *p.c.* brain (C) or newborn mouse brain (E,F). Immunocytochemistry with anti-calcium-binding protein serum was performed with sections of 16·5-day *p.c.* brain (D). Photomicrographs of the cerebellum under bright-field (A,D,E) or dark-field (B,C,F) illumination are shown. *v*, ventricle of hindbrain. Arrows indicate sites of *int*-2 expression. (G) 10 μg total RNA from microdissected cerebellae of 17·5-day *p.c.* (*1*), newborn (*2*) or 2-week postnatal mice (*3*) was electrophoresed, blotted and probed with ^{32}P-labelled antisense *int*-2f probe. Arrows indicate the major *int*-2 transcripts.

was confined to regions stained by the Purkinje cell-specific antibody. In the cerebellum of the newborn mouse, *int*-2 RNA was detected in a narrow zone of cells located distal from the hindbrain ventricle, and convoluted in this lateral section (Fig. 1E,F). This is the site of the Purkinje cell layer. Northern blot analysis (Fig. 1G) showed four *int*-2 transcripts (for details of multiple transcripts see Smith *et al.* 1988 and Mansour & Martin, 1988) to be present in the cerebellum at 17·5 days *p.c.* and in the newborn mouse, but barely detectable in the mature cerebellum of a 2-week postnatal mouse. Consistent with this, *int*-2 transcripts were not detected by *in situ* hybridization in the 2-week postnatal cerebellum (data not shown). Thus, high level *int*-2 expression appears to be restricted to early stages of Purkinje cell differentiation.

Expression of int-2 *in the developing retina*

The retina initially consists of a one-cell-thick neuroepithelium which forms a thickened neuroblastic layer following the migration of proliferating cells. Postmitotic differentiating cells first arise in the posterior of the retina, then subsequently in more anterior regions. At 14·5 days *p.c.*, *int*-2 expression was detected in a small group of cells in the posterior region of the retina (Fig. 2A,B). Two days later *int*-2 transcripts are detected throughout the entire neuroblastic layer (Fig. 2C,D). Thus, *int*-2 expression does not correlate with cell proliferation which is occurring throughout the neuroblastic layer during this period, but rather with the onset of appearance of postmitotic cells in early stages of their differentiation (Hinds & Hinds, 1978, 1979). While the neuroblastic layer remains apparently unstructured in the fetal mouse, ganglion cells segregate to form an inner layer. *int*-2 transcripts are not detected in this ganglion cell layer at 16·5 days *p.c.* (Fig. 2E,F) or in the newborn mouse retina (Fig. 2G,H). In the newborn mouse, *int*-2 transcripts are restricted to the anterior periphery of the retina (Fig. 2G,H). This region is the site of mitotic and early-

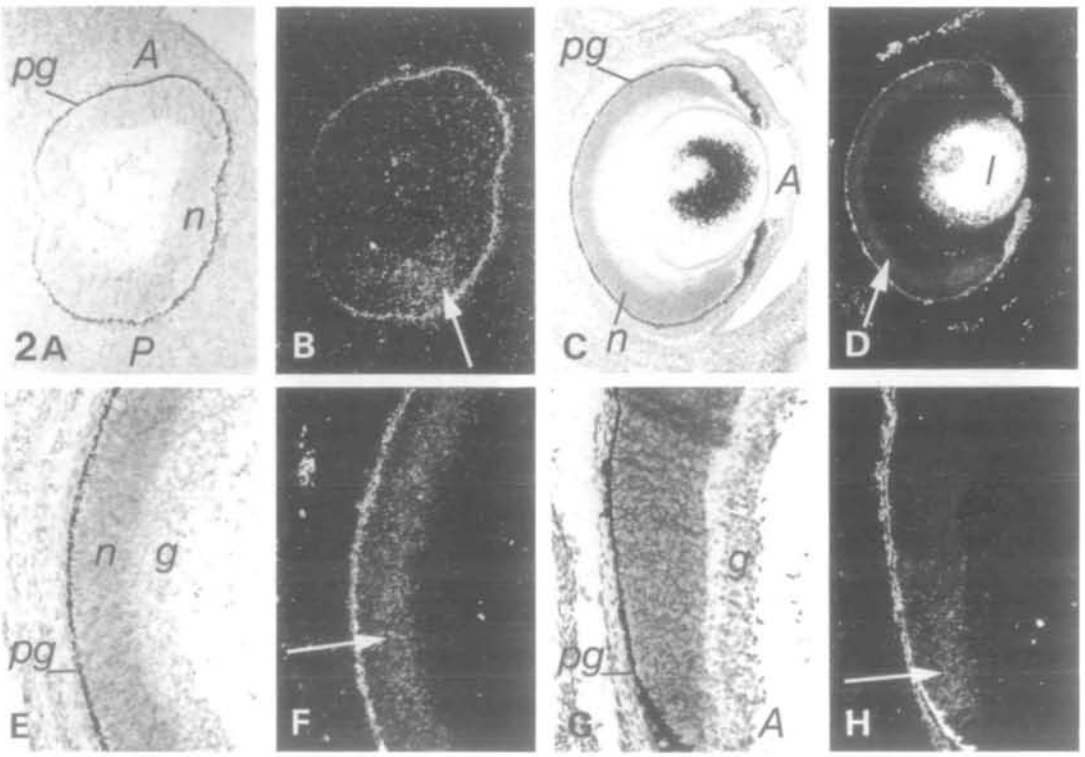


Fig. 2. *int*-2 expression in the developing eye. (A–H) *In situ* hybridization with *int*-2g probe was performed against sections of a 14·5-day embryo (A,B), 16·5-day embryo (C–F) and newborn mouse eye (G,H). Photomicrographs of the whole eye (A–D) or of the retina at high magnification (E–H) were taken under bright-field (A,C,E,G) or dark-field (B,D,F,H) illumination. *A*, anterior; *P*, posterior; *n*, neuroblastic layer; *g*, ganglion cell layer; *l*, lens; *pg*, pigment granules. Arrows indicate sites of *int*-2 expression. Note that the signals due to pigment granules adjacent to the neuroblastic layer, and to refraction by the lens (C,D), do not correspond to silver grains reflecting *int*-2 expression. (I) 10 μg total RNA from eyes of 17·5-day *p.c.* (*1*), newborn (*2*) or adult (*3*) mice were electrophoresed, blotted and hybridized with *int*-2f probe. Arrows indicate the major *int*-2 transcripts.

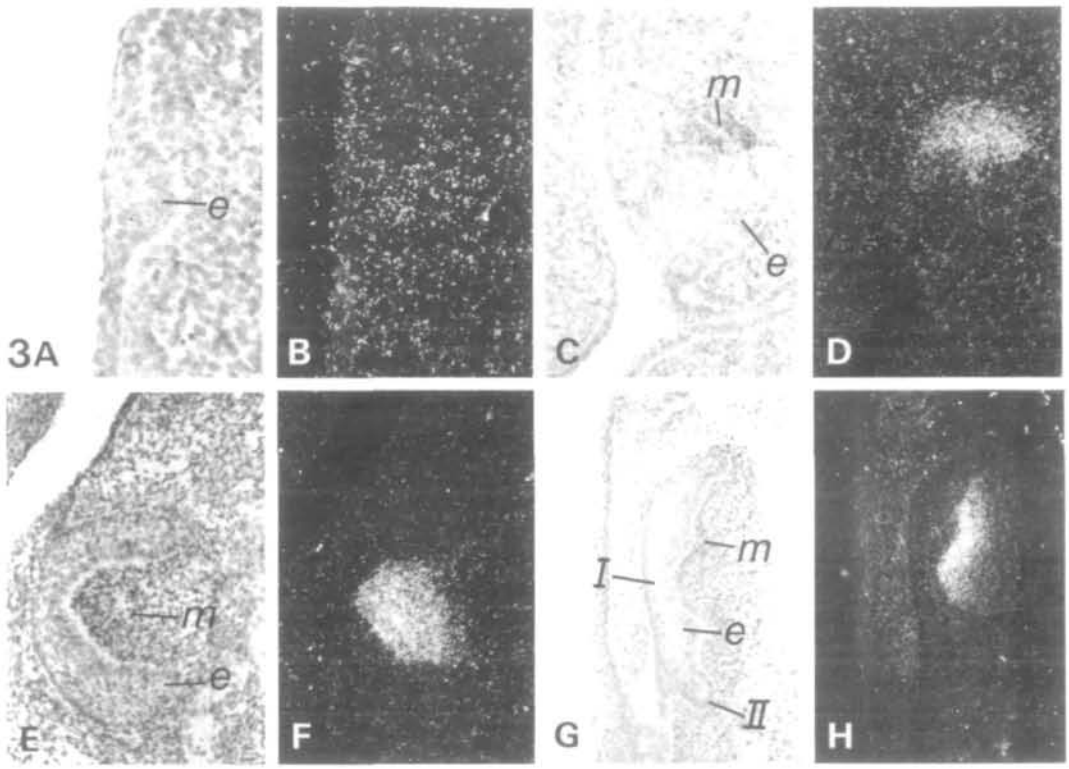


Fig. 3. Expression of *int*-2 in developing teeth. *In situ* hybridization was performed with *int*-2g probe against sections of embryos at 12·5 days (A,B), 14·5 days (C,D) and 17·5 days (E–H). Photomicrographs of tooth buds (A,B), developing incisors (C–F) and molar (G,H) teeth are shown under bright-field (A,C,E,G) and dark-field (B,D,F,H) illumination. *e*, ectoderm; *m*, mesenchyme; *I*, 1st molar tooth; *II*, 2nd molar tooth.

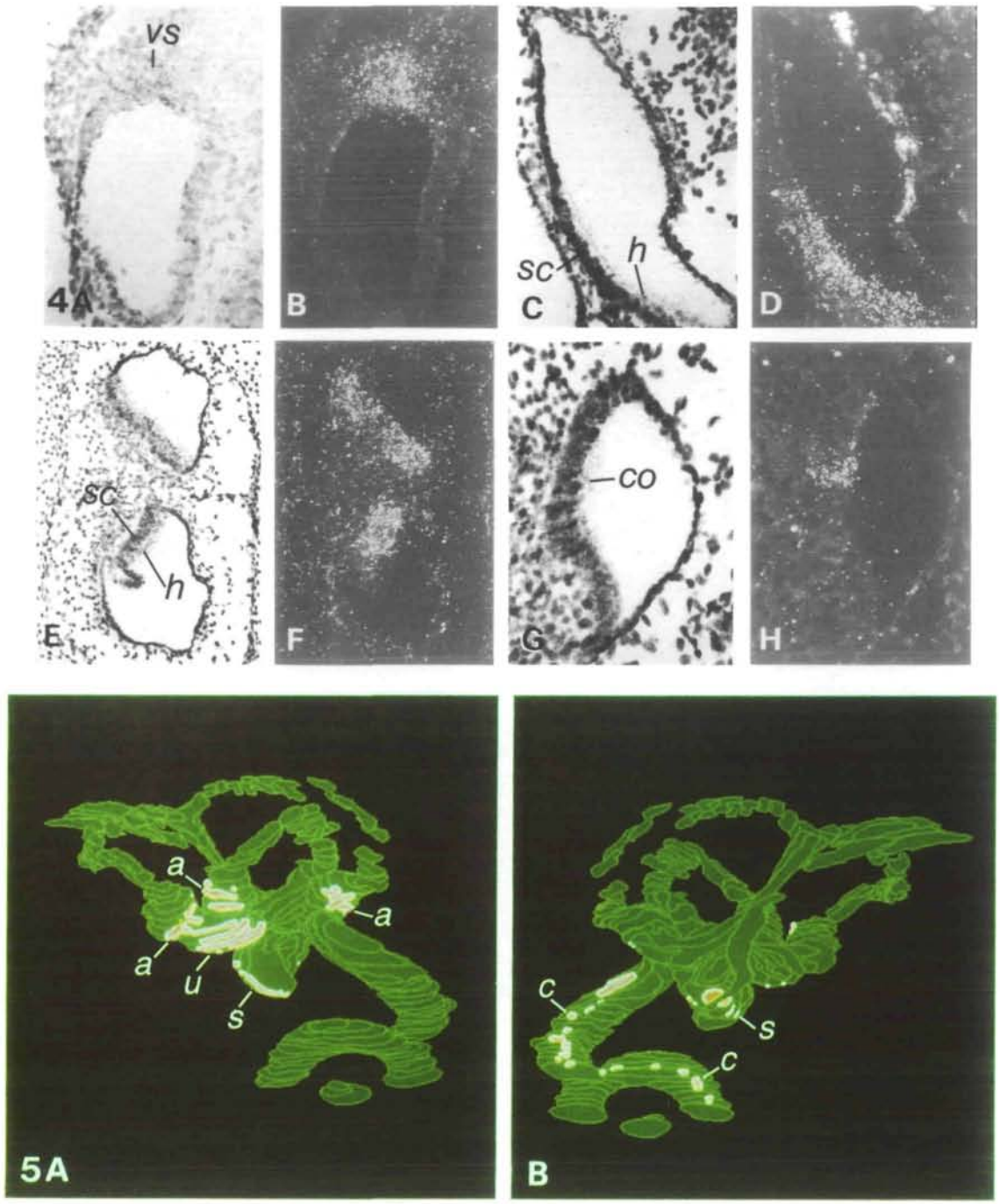
stage postmitotic cells in the retina at this stage, whilst more posterior regions consist only of later-stage differentiating cells. *int*-2 transcripts were not detected by *in situ* hybridization in the mature retina (data not shown) and thus it seems that high-level *int*-2 expression is associated with early stages of retinal cell differentiation. Consistent with this, Northern blot analysis reveals *int*-2 transcripts to be down-regulated between 17·5 days *p.c.* and birth, and barely detectable 2 weeks postnatally (Fig. 2I).

int-*2 expression in the developing teeth*

The major structures of teeth are derived from cells of two distinct origins: ectodermal cells of the jaw epithelium that form the enamel, and neural-crest-derived mesenchymal cells that form the inner dentine and pulp tissue (Lumsden, 1987). Prospective tooth ectoderm is apparent at 12·5 days *p.c.*, but *int*-2 transcripts are not detected at this stage (Fig. 3A,B). By 14·5 days *p.c.*, this thickened ectodermal layer has invaginated and tooth bud mesenchyme has been recruited. *int*-2 expression is detected only in the tooth mesenchyme (Fig. 3C,D). Subsequently, progressive differentiation occurs concurrent with the ectodermal layer forming a morphology characteristic of incisor (Fig. 3E,F) or molar (Fig. 3G,H) teeth. At 17·5 days of development, *int*-2 transcripts are detected in mesenchymal cells destined to form the dentine and pulp of the incisor and first molar teeth until at least 18·5 days *p.c.* (Fig. 3E–H, and data not shown), but not in the second molar which is at an earlier morphological stage (Fig. 3G,H).

Expression of int-*2 in the inner ear*

The inner ear arises from a thickened layer of ectodermal cells that invaginate to form the otic vesicle. At 9·5 days *p.c.* *int*-2 expression is not detected in the otic epithelium, but rather in cells of the hindbrain that are adjacent to this structure (Wilkinson *et al.* 1988). However, at 10·5 days *int*-2 expression is no longer apparent in the hindbrain (data not shown), but is detected in a thickened region of the otic vesicle, distal from the hindbrain, that is destined to form the sensory tissue of the vestibular organ (Carney & Silver, 1983) (Fig. 4A,B). *int*-2 transcripts continue to be detected during the subsequent morphogenesis of the inner ear. By 17·5 days *p.c.*, the major structures of the inner ear have formed, and we have examined the spatial pattern of *int*-2 expression in serial sections of the ear at this stage. Computer-aided reconstructions of these data reveal *int*-2 to be expressed exclusively in the developing sensory regions of the inner ear: in the ampullae of the semicircular canals, the utricle, saccule (Fig. 5A) and the cochlea (Fig. 5B). Detailed examination of the sections shows *int*-2 expression in both the sensory hair cells and the underlying support cells (Fig. 4C–H and data not shown).



Figs 4, 5. Expression of *int*-2 in the inner ear.

Fig. 4. *In situ* hybridization with *int*-2g probe was performed against sections of 10·5-day (A,B) and 17·5-day (C–H) embryos. Photomicrographs of the otocyst (A,B), utricle (C,D), ampullae (E,F) and cochlea (G,H) are shown under bright-field (A,C,E,G) and dark-field (B,D,F,H) illumination. *vs*, prospective vestibular sensory epithelium; *h*, hair cells; *sc*, support cells; *co*, prospective sensory region of the cochlea.

Fig. 5. Computer-aided reconstructions were made of the inner ear tissue (green) and sites of *int*-2 expression (red). A and B represent images rotated through 180° with respect to each other. Only lateral sections through the cochlea were included. Arrows indicate *int*-2 expression in the ampullae of the semi-circular canals (*a*), utricle (*u*), saccule (*s*) and cochlea (*c*).

Discussion

The idea that growth factors and growth-factor-related genes may have multiple roles, in development as well as in tissues of the adult, is an attractive possibility. This view is consistent with the complex pattern of expression of the FGF-related proto-oncogene *int*-2. Based on the cell types expressing *int*-2 from 6·5 to 9·5 days of development, we have suggested that this gene may have roles in cell migration and in the induction of the otocyst (Wilkinson *et al.* 1988). The present report suggests that *int*-2 may have other, unrelated, roles in later fetal development.

Two of the fetal sites of *int*-2 expression are located in the central nervous system. There is already good evidence that basic FGF is expressed in many neural tissues, including the postnatal cerebellum and retina (Hatten *et al.* 1988; Baird *et al.* 1985) and that it may act as a neurotrophic factor promoting the differentiation and/or survival of specific neurones (Morrison *et al.* 1986; Walicke *et al.* 1986; Anderson *et al.* 1988; Hatten *et al.* 1988; for critical review, see Davies, 1988). By analogy, it seems possible that *int*-2 may act as a neurotrophic factor during early phases of Purkinje cell and retinal cell differentiation. The Purkinje cells are migrating during the period of *int*-2 expression, so in view of the apparent association of *int*-2 expression with early mesoderm cell migration (Wilkinson *et al.* 1988), it is also possible that it has a role in Purkinje cell migration.

The detection of *int*-2 transcripts in sensory cells and the underlying support cells in the inner ear is consistent with a role in sensory cell differentiation. These sensory cells form distinct structures in the ampullae, the utricle and saccule and the cochlea, but share the characteristic of being innervated by the vestibulocochlear ganglion. Thus, an alternative idea is that *int*-2 may act as a neurotrophic factor for the vestibular and cochlea nerves. Consistent with this, evidence exists for a trophic influence of the sensory epithelium on the vestibular and cochlea nerves (Sobkowicz *et al.* 1980; Ard *et al.* 1985). Overall, we suggest that *int*-2 may have two distinct roles in the formation of the inner ear; initially as a morphogenetic signal arising from the hindbrain between 8·5 and 9·5 days *p.c.* (Wilkinson *et al.* 1988), and subsequently as a factor involved in sensory cell differentiation and/or innervation. The location of the prospective sensory epithelium distal from the hindbrain at 10·5 days *p.c.* is consistent with this two-step model, rather than with a single role of *int*-2 in inner ear development.

There is at present no experimental evidence for a role of FGF-like proteins in tooth development. In view of the reciprocal inductive tissue interactions between the epithelial and mesenchymal components of the developing tooth (Thesleff & Hurmerinta, 1983; Kollar, 1983), it is possible that *int*-2 acts as a paracrine factor required for differentiation of the ectodermally derived ameloblasts. However, *int*-2 expression does not correlate specifically with the timing of mesenchymal induction of ectodermal differentiation (Lumsden, 1987), and thus it seems more likely that *int*-2 acts as an autocrine factor for the odontoblast pathway.

In summary, we envisage *int*-2 to have multiple roles in mouse development and we have made specific proposals based on correlations of *int*-2 RNA accumulation with developmental events. Rigorous testing of these ideas will require analysis of the biological effects of *int*-2 protein, and of neutralizing antibodies on *in vitro* culture systems, and the identification and characterization of its cellular receptor.

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