Dissimilar regulation of cell differentiation in mesencephalic (cranial) and sacral (trunk) neural crest cells in vitro

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Summary

During development neural crest cells give rise to a wide variety of specialized cell types in response to cytokines from surrounding tissues. Depending on the cranial-caudal level of their origin, different populations of neural crest cells exhibit differential competence to respond to these signals as exemplified by the unique ability of cranial neural crest to form skeletal cell types. We show that in addition to differences in whether they respond to particular signals, cranial neural crest cells differ dramatically from the trunk neural crest cells in how they respond to specific extracellular signals, such that under identical conditions the same signal induces dissimilar cell fate decisions in the two populations in vitro. Conversely, the same differentiated cell types are induced by different signals in the two populations. These in vitro differences in neural crest response are consistent with in vivo manipulations. We also provide evidence that these differences in responsiveness are modulated, at least in part, by differential expression of Hox genes within the neural crest.

Key words: Cranial neural crest, Trunk neural crest, Signaling, Differentiation, Chick

Introduction

Neural crest cells contribute to a wide variety of tissues. For example, neural crest cells are known to produce a diverse set of sensory and autonomic neurons and glial cells of the peripheral nervous system, pericytes and smooth muscle cells of the vascular system, including the major vessels of the heart, chromaffin cells (endocrine cells of the adrenal gland) and most pigment cells (Fig. 1) (Sieber-Blum, 1982; Ito and Sieber-Blum, 1991; Ito et al., 1993; Le Douarin et al., 1993; Anderson, 1997; Etchevers et al., 2001). In addition, neural crest cells originating from the developing head give rise to connective tissue of the cranial muscles and chondrocytes, osteoblasts and odontoblasts, and components of the craniofacial skeleton (Noden, 1983; Couly et al., 1993; Olsson et al., 2001).

Individual premigratory neural crest cells are remarkably pluripotent in the sense that their progeny can give rise to more than one differentiated cell type (Sieber-Blum and Cohen, 1980). Marking individual trunk neural crest cells with fluorescent vital dyes has shown that progeny of a single neural crest cell can include cells as diverse as adrenomedullary cells, Schwann cells, neurons and pigment cells (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989; Selleck et al., 1993). The fate of individual progeny of the initially labeled cells depends to a large degree on the environment in which they come to reside after the completion of their migration. For example, the secreted extracellular signal BMP2 produced by the developing lung, heart and dorsal aorta directs neural crest in their vicinity to differentiate into parasympathetic neurons and smooth muscle cells (Shah et al., 1996). Differences in the fate of neural crest produced at different rostrocaudal levels are, to a certain extent, also based on the different microenvironments they encounter during their migration. Hence, while cholinergic parasympathetic ganglia are produced by the neural crest of the neck and the adrenergic sympathetic ganglia are produced by neural crest of the thorax, these specificities are reversed in reciprocal experiments where one population of neural crest is exposed to the microenvironment normally seen by the other (Le Douarin et al., 1975).

These and other experiments have established a picture of the trunk neural crest as a pluripotent population of stem-like progenitors, the fate of which is determined by signals in the local environment. The molecular nature of the signals regulating differentiation of the neural crest cells has been explored mostly in the trunk, where experiments have provided a wealth of information on how signaling molecules and transcription factors control cell differentiation (Shah, 1996; Anderson, 1997). For example, it has been demonstrated in vivo that the production of pigment cells depends on the WNT pathway, whereas members of the TGFβ1 superfamily have been implicated in formation of smooth muscle cells and sensory and adrenergic neurons (Anderson, 1997; Zhang et al., 1997; Shah et al., 1996; Dunn et al., 2000). Importantly, these in vitro results correlate well with in vivo expression patterns, such that the in vitro defined signals are indeed produced in the regions where the expected neural crest derivatives differentiate.

Similarly, cranial neural crest cells have been shown to receive instructional information from surrounding tissues, such as neuroepithelium, the head surface ectoderm, the

endoderm of the foregut and the paraxial head mesoderm (Francis-West et al., 1998; Golding et al., 2002; Couly et al., 2002; Trainor et al., 2002). A number of candidate signaling molecules have been identified that are expressed in these tissues during head development (Wall and Hogan, 1995; Ferguson et al., 2000) (reviewed by Francis-West et al., 1998). Several members of the fibroblast growth factor family (FGF1, FGF2, FGF4, FGF5 and FGF8) are expressed in the developing facial primordia. FGF2, FGF4 and FGF8 are expressed in the epithelium covering particular regions of the developing face (Crossley and Martin, 1995; Wall and Hogan, 1995; Barlow and Francis-West, 1997; Helms et al., 1997; Richman et al., 1997). Cre-mediated inactivation of *Fgf8* gene showed that FGF8 product is required for cell survival in the first branchial arch (Trumpp et al., 1999). These newborn mutant mice lack all of the structures derived from the first branchial arch except the most distal regions. Moreover, FGF8 signaling contributes to generation of rostrocaudal polarity in the first branchial arch as it provides positional information to the rostral (*Lhx7*-expressing) and caudal (*Gsc*-expressing) domains of the crest-derived ectomesenchyme from the rostral epithelium where FGF8 is expressed (Tucker et al., 1999).

Members of the BMP family are also expressed in the developing head (Roelen et al., 1997; Francis-West et al., 1998). Craniofacial roles of a few members of the BMP family have been studied in some detail (Bennet et al., 1995; Wall and Hogan, 1995; Barlow and Francis-West, 1997). For example, BMP2 and BMP4 are expressed in particular domains of surface epithelium that are associated with mesenchyme expressing Msx1 and Msx2 and ectopic application of beads soaked with either BMP2 or BMP4 can activate the expression of Msx1 and Msx2 and can result in the bifurcation of certain cranial skeletal structures (Barlow and Francis-West, 1997). In addition, Bmp2 signaling increases cell proliferation in the mandibular primordia whereas haplo-insufficiency of BMP4 in C57B1/6 mice results in shorter frontal and nasal bones (Barlow and Francis-West, 1997).

On the cellular level, there has been a number of studies of the specific roles of the above-mentioned signaling molecules in directing the differentiation and proliferation of the trunk neural crest (Nakamura and Ayer-le Lievre, 1982; Shah et al., 1996; Anderson, 1997). However, there is far less information concerning the cellular effects of these molecules on cranial neural crest. Moreover, those in vitro studies that have been carried out with cranial neural crest are often difficult to compare with trunk neural crest as they often used dissimilar media or incubation conditions (Baroffio et al., 1988; Shah et al., 1996; Anderson, 1997; Sarkar et al., 2001). The objectives of the current study were, thus, to directly compare cell differentiation from mesencephalic (cranial) and sacral (trunk) neural crest in response to various factors when grown under similar conditions. We focused on the effects of FGF2/8, BMP2/4 and TGFβ1. We find that cranial and trunk crest cells differ considerably in their responsiveness to these factors, such that the same array of differentiated cell types is produced at different axial levels via interactions with a distinct set of differentiation cues.

As cranial and trunk crest differ qualitatively in their responsiveness to various signaling proteins, one would like to know the upstream cellular factors responsible for these differences. Hox genes, a family of homeodomain transcription factors, are known to play important roles in specifying rostral-caudal differences in many tissues and are hence attractive candidates. Mis-expression of Hox genes in cranial crest cells indeed suggests that these genes are involved in setting up the crucial differences in differentiation response between cranial and trunk crest cells. Among the differences between trunk and cranial neural crest cells, the most well-known has been the unique ability of cranial neural crest to produce chondrocytes. However, it has recently been reported that trunk neural crest cells can produce chondrocytes in long-term in vitro cultures (McGonnell and Graham, 2002). We confirm this observation but find that this long-term change in differentiation potential is correlated with a downregulation of Hox genes in a subset of the neural crest cells and can, moreover, be completely blocked by misexpression of trunk Hox genes in vitro.

Materials and methods

Cranial and trunk neural crest cultures

Fertilized eggs were obtained from SPAFAS (Norwich, CT), incubated at 100F, and the embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). To establish cultures, small explants (0.1-0.2 mm in length) of cranial and trunk dorsal neural folds were collected from stage 8/9 embryos prior to the closure of neural tube (midbrain level neural folds for cranial crest cells) or stage 17-18 (somite 32-33 level dorsal neural folds for trunk crest cells) using tungsten needles and placed in culture (Fig. 1). Neural crest cells that migrated out of the explants were used to seed experimental cultures. The cultures were washed to remove the explants on the next day and the culture media was replaced on alternate days. Cells were grown in 24-well plates (Corning) treated with poly-D-lysine and fibronectin in alpha-modified Eagle's minimal essential medium (alpha MEM with ribosides and deoxyribonucleosides, Gibco BRL, UK) supplemented with 10% fetal calf serum (FCS), 25 units/ml penicillin, 25 μg/ml streptomycin sulfate (Gibco BRL, UK) as previously described (Sarkar et al., 2001). For the cranial crest, in accordance with previously published results, we found no difference in chondrogenesis from explants derived from stage 7/8 or 8/9 embryos (Sarkar et al., 2001). For experiments on anterior trunk neural crest cells explants were collected from the somite level 10 at HH12.

Concentration of all the protein cytokines was 10 ng/ml as it was found to be optimal for chondrogenesis in previously published studies (Sarkar et al., 2001; Petiot et al., 2002). At least seven 24-well plates of cultured cells were used for each of the conditions described unless otherwise indicated. Neural crest cells were cultured for one week except where noted otherwise. To visualize apoptotic cells, we used 'In Situ Cell Death Detection, POD' kit (Roche Diagnostics, GmbH) according to the manufacturer's instructions.

Viral infections of early embryos

The RCAS(A)::CA-β-catenin construct has been described before (Kengaku et al., 1998). To obtain cranial neural crest cell cultures expressing these transgene, we infected future neural plate cells of the early stage 6+ embryos. The following day neural crest tissue was placed in culture. Each of the RCAS-infected cultures was tested with 3C2 antibody with subsequent FITC-conjugated secondary antibody and DAPI stained to ensure that all cells were infected. Only the 3C2-positive (infected) cultures were counted and examined with cell-specific antibodies.

RT-PCR analysis of trunk neural crest cultures

Cultured cells were lysed and processed for RNA analysis using the RNeasy kit (QIAGEN). The method for the RT-PCR analysis was previously described (Munsterberg et al., 1995). The primers used are:

GAPDH (5′-AGTCATCCCTGAGCTGAATG and 5′-ACCATCAAGTCCACAACACG); Noelin-1 (5′-CGTGGAGAAGATGGAAAACC and 5′-GTGCCTGACCACGGGTGAGG); and Id2 (5′-GTCAGCCTCCACCACCAGCG and 5′-GGGTCCTTCTGGTACTCACG). PCR reactions were typically performed at 56°C for 30 cycles with 5% formamide, except for GAPDH, 60°C, 24 cycles, no-formamide. More details are available upon request.

Immunohistochemical procedures and in situ hybridization

The presence of the migrating crest cells in the culture was determined by using HNK-1 or rabbit anti-p75 nerve growth factor (NGF) receptor antibody (Chemicon International, Temecula) (Bannerman and Pleasure, 1993; Rao and Anderson, 1997). p75 is believed to be an excellent marker for undifferentiated neural crest cells (Rao and Anderson, 1997; Young et al., 1998). In addition, we occasionally used mouse antibody 20B4 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City) to detect neural crest cells; monoclonal anti-ColII (Chemicon International, Temecula), monoclonal anti-ColII (Sigma) and rabbit anti-ColII (Collagen II) (Chemicon International, Temecula) to detect chondrocytes; Cy3-conjugated anti-SMA (smooth muscle actin) antibody (Sigma) to detect smooth muscle cells; monoclonal anti-S100 (Sigma), rabbit anti-GFAP (glial acidic fibrillary protein) antibody (Sigma), monoclonal Cy-3 conjugated anti-GFAP antibody (Sigma) and mouse antibody 1E8 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City) to detect glial cells; and rabbit anti-neurofilament M (145 kDa) (Chemicon International, Temecula), mouse anti-NF200 and monoclonal anti-β-3 tubulin, clone 2G10 (Upstate Technology, Lake Placid) to detect neuronal cells. GFAP and SMA proteins are believed to be good markers for glial and smooth muscle cells in chicks, respectively (Kalman et al., 1998; Hoya et al., 2001). We used biotinylated, Texas Red-, FITC-, Cy3- or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove and Vector Laboratories, Burlingame). All purified cytokines were purchased from R&D Systems or were laboratory stocks (obtained from Genetics Institute). Immunochemistry on cell cultures was performed as described before (Bachler and Neubuser, 2001).

In situ hybridization was performed DIG RNA probes, which were detected with antibodies conjugated with alkaline phosphatase (AP). The fluorescent double in situ hybridization on long-term TNC cultures were performed using tyramide signal amplification on HRP-conjugated anti-DIG and anti-FITC antibodies: the red Cy3 signal obtained with the TSA-Plus Fluorescence Palette System (PerkinElmer Life Sciences, Boston) and green Oregon Green 488 signal obtained with the TSA kit #9 (Molecular Probes, Eugene).

Results

The specific effects of various inductive differentiation factors has mostly been defined for trunk neural crest cells. Although cranial crest cells have a distinct capacity to differentiate into chondrocytes that is not shared by the trunk crest cells in vivo, one might predict that in other respect their differentiation might be controlled by the same factors as in the trunk region. To test this model directly, we employed in vitro techniques and conditions previously applied to trunk neural crest cells. Cultures of the premigratory cranial neural crest were derived from the midbrain level of the neural tube in stage 9 chick embryos and premigratory trunk neural crest from the level of somites 32-33 (Fig. 1). The isolated cultures consisted solely of crest cells as determined by immunochemistry with anti-p75 and HNK-1 antibodies (Fig. 2A-C; not shown).

Distinct survival and differentiation requirements of trunk and cranial neural crest cells

Significantly, although cultures of cranial and trunk neural crest initially appeared morphologically similar, over time cranial crest cultures showed high mortality in the absence of additional factors. For the purposes of this study, a culture was scored as dead if less than 5% of the cells of the primary culture remained on the plate after 1 week in culture. By contrast, the trunk crest cultures displayed over 80% survival after a week in culture (Fig. 2D). Trunk neural crest also showed a much higher level of survival than did cranial neural crest when cultured in the presence of BMP2 or TGFβ1 (86% and 61%, respectively) (Fig. 2D). Similar results were obtained when BMP4 was used instead of BMP2 or when anterior trunk neural crest cells were used (somite 10-12) (not shown). However, in the presence of the BMP inhibitor Noggin, the reciprocal result was obtained such that cranial neural crest had a much higher level of survival than did trunk neural crest (56% versus 23%, respectively). This suggests that the medium containing 10% FCS carries a biologically significant amount of BMP activity, which acts to favor trunk neural crest survival in the absence of additional factors. Both trunk and cranial neural crest cultures survived well in the presence of FGF2 added to this media. Similar results were obtained when FGF8 was used instead of FGF2. These results provided a strong indication that important differences in signal responsiveness exist between trunk and cranial neural crest cells. The observed reduction in survival of trunk neural crest upon addition of FGF2 or TGFβ1 (from 83% to 56% and 61%, respectively) may reflect role in regulation of apoptosis that is worthy of further study.

In the presence of both FGF2 and BMP2, the cranial neural crest cells survival (92%)

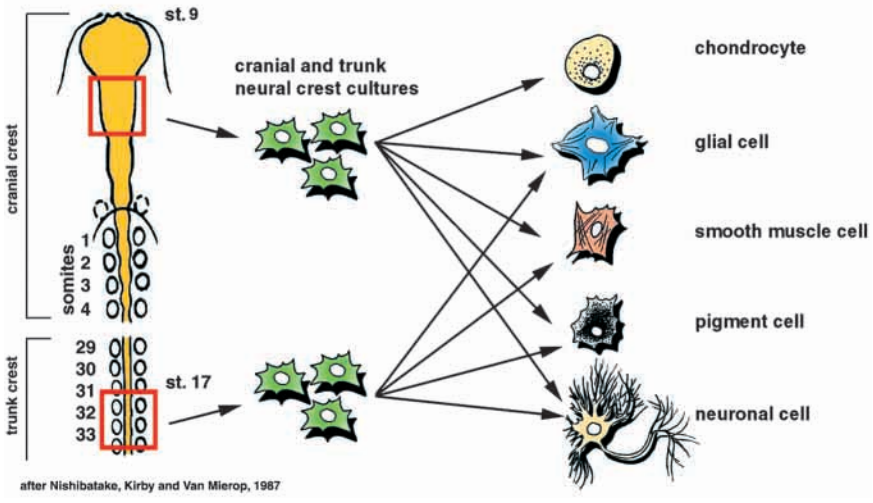

Fig. 1. Explants of dorsal neural tube containing premigratory neural crest cells were used to generate primary cultures of cranial and trunk neural crest cells. After 24 hours of incubation, explants were removed and the media were replaced. Five different cell types were detected using morphology and specific antibodies: chondrocytes, glial cells, smooth muscle cells, pigment cells and neurons.

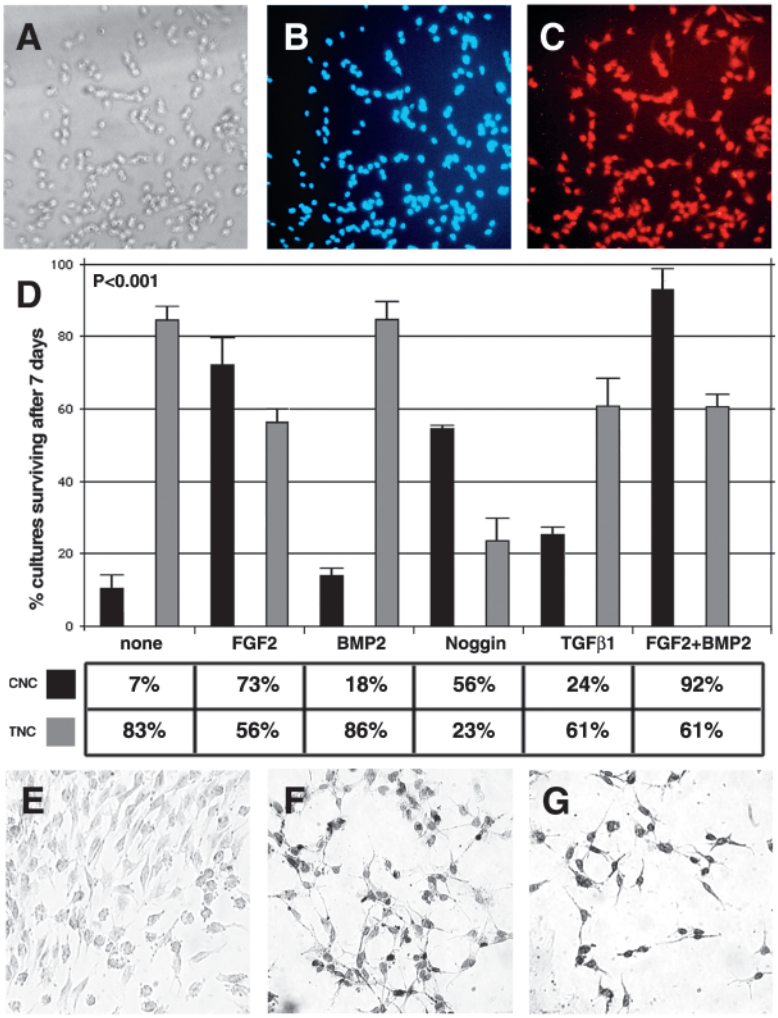

Fig. 2. Survival of cranial and trunk neural crest cell cultures is differentially affected by secreted factors. Bright-field photograph of cranial neural crest culture treated with recombinant FGF2 protein for 36 hours reveals migrating cells (A). The same culture stained with DAPI to reveal nuclei (B) and with anti-p75 nerve growth factor (NGF) receptor antibody that recognizes neural crest cells (C). A histogram of survival rates for cranial and trunk neural crest cells cultures shows that the cranial and trunk neural crest cell cultures had different survival rates under similar culture conditions (*t*-test: $P<0.001$, except for TGFβ1 for which $P<0.03$) (D). % survival=(number of cultures after 7 days in culture/number of explants displaying cell outgrowth on 1st day)×100.
(E-G) Apoptosis is not detected in 48 hour cranial neural crest cultures treated with FGF2 (E), whereas many cranial neural crest cells treated with BMP2 for 18 hours (F) and 36 hours (G) undergo cell death as detected with TUNEL and HRP-conjugated antibody.

was similar to that in the presence of FGF2 alone (73%) and was much higher than in the medium containing BMP2 alone (18%). A different situation was observed for the trunk neural crest cells, which survived more poorly in the FGF2+BMP2 culture (61%) when compared with cultures supplemented with BMP2.

Differentiation of cranial and trunk neural crest cells under similar culture conditions

To examine whether the differential responses result in different cell fate choices in trunk and cranial neural crest cultures, we used cell-type specific antibodies and cell morphology to determine which neural crest-derived cell types were formed. Five distinct types of differentiated cells were assayed: pigment cells (pigment granules), chondrocytes (various anti-ColII antibodies), smooth muscle cells (anti-smooth muscle actin (SMA), glial cells [anti-glial acidic fibrillary protein (GAFP) and anti S100 (calcium-binding protein)] and neuronal cells [anti-β-3-tubulin, anti-neurofilament 145 (NF145) and anti-neurofilament 200 (NF200)] (Fig. 3). In some cases, we used in situ hybridization with cell-specific RNA probes to confirm the antibody results. As used by others in previous studies (Sarkar et al., 2001), we used the percent of treated cultures containing a given cell type to measure whether that cell fate is promoted by the culture conditions. Cultures were considered to display a particular cell fate if it contained a chondrogenic nodule or at least 10% of the cells exhibited a particular marker as determined by antibody staining or in situ hybridization. To calculate the statistical significance, we performed a *t*-test for pair-wise comparison or ANOVA for comparison of multiple groups of samples.

The distinct responses of trunk neural crest to BMPs, TGFβ1 and WNT signaling have been previously described (Graham et al., 1994; Shah et al., 1996; Anderson, 1997; Dunn et al., 2000; Smith and Graham, 2001). We obtained very similar results with each factor in our culture conditions (Fig. 3). We wished to compare these data with the effects of the same factors on cranial neural crest. However, as noted above, cranial neural crest cultures require the presence of FGF2/8 protein for survival. Hence, to make the conditions comparable with the cranial neural crest, we also needed to add FGF2 to the trunk neural crest cultures together with additional factors. We, therefore, first tested whether having FGF2 itself in the culture medium affects the established differentiation pathways of the trunk neural crest cells.

Although, as noted above (Fig. 2), addition of FGF2 decreases the survival of trunk neural crest by about 30%, the same differentiated cell types were found in trunk neural crest cultures with or without FGF2, including smooth muscle cells, neuronal cells and pigment cells with few or no chondrocytes or glial cells (Figs 3, 5). The only significant difference in these cultures was a relative increase in percentage of pigment cells, consistent with previous reports that FGF2 is mildly mitogenic for melanocytes (Sieber-Blum and Zhang, 1997). Similarly, the presence or absence of FGF2 (or FGF8) protein in the culture did not alter the differentiation of trunk neural crest in response to BMP2, TGFβ1 or canonical Wnt signaling (Figs 3, 5; not shown).

Based on these results, we set up parallel cultures of trunk neural crest and cranial neural crest, each containing FGF2, either alone or along with additional factors. As previously reported (Shah et al., 1996; Anderson, 1997), trunk neural crest cells respond to BMP signaling by differentiating preferentially

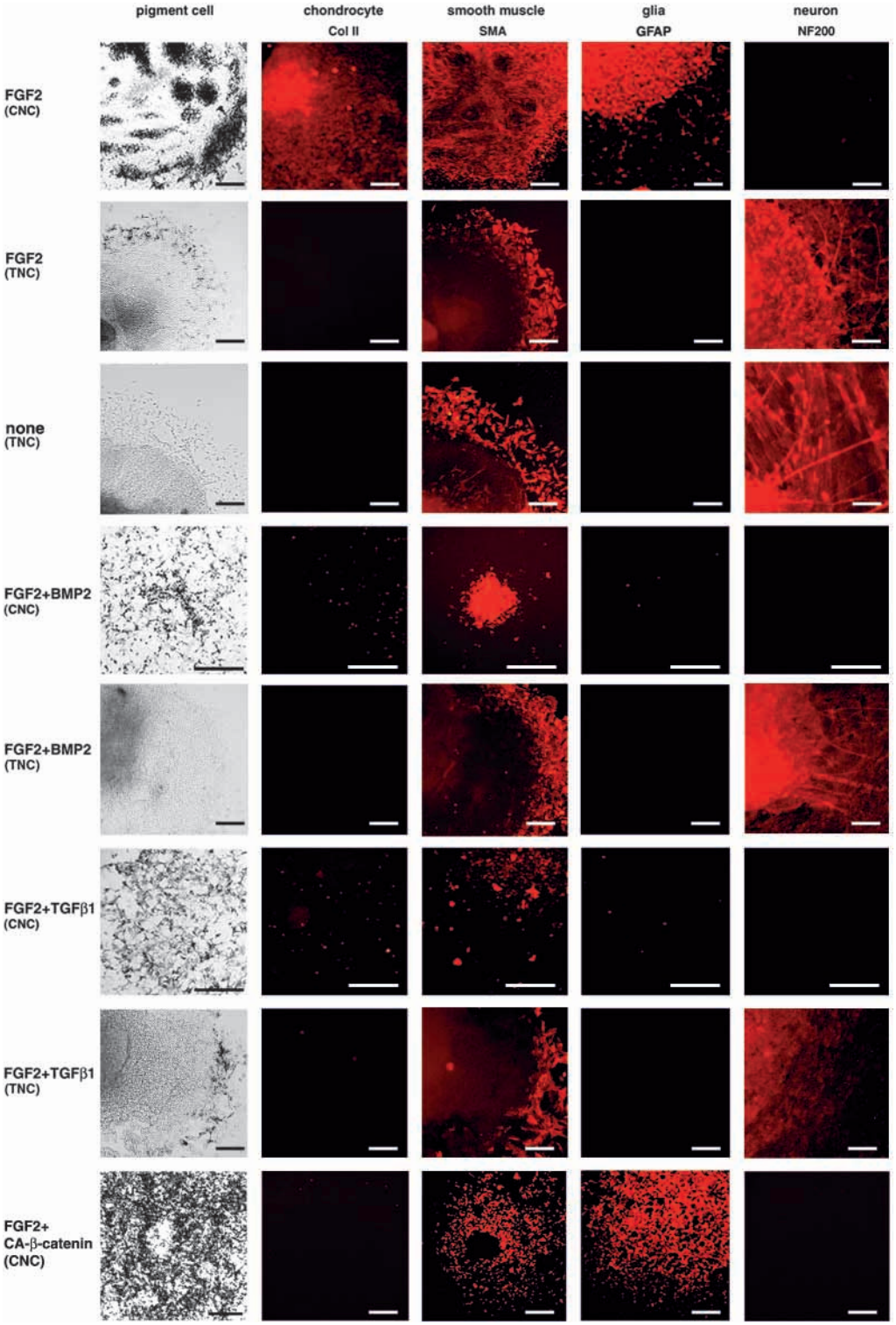

Fig. 3. Immunohistochemical analysis of the cranial and trunk neural crest cells cultured in the presence of FGF2 and in combination with BMP2 and TGFβ1. The bottom row shows cranial crest cultures infected with the virus containing the constitutively active (stabilized) β-catenin. Panels show some of the most representative cultures for each culture condition. Specialized cell types are arranged in columns, whereas rows depict different growth conditions. Note that chondrogenesis is induced in the presence of exogenous FGF2 but is suppressed when BMP2 or TGFβ1 are also added. Scale bars: 100 μm.

into neuronal cells and smooth muscle cells, while we found that the number of cultures undergoing gliagenesis and melanogenesis was dramatically reduced (Figs 3, 5). By contrast, we saw no promotion of neuronal cell fate when 10 ng/ml BMP2 was added to the cranial neural crest cultures. However, BMP2 protein dramatically decreased the number of cultures undergoing chondrogenesis (from 64% to 4% of total; Figs 3, 5). The number of cultures with glial cells decreased substantially as well (from 82% to 17%). Inhibition of melanogenesis was also observed in the presence of BMP2, whereas the number of cultures containing smooth muscle cells was unchanged (Fig. 5). Although on average, smooth muscle cells appeared to form smaller clusters in FGF2+BMP2-treated cranial neural crest cultures.

Dramatic differences in the response of neural crest cells derived from different rostrocaudal levels, were seen with TGFβ1. Trunk neural crest responds to TGFβ1 or FGF2+TGFβ1 signaling by differentiating to become smooth muscle cells (Figs 3, 5) (Shah et al., 1996; Anderson, 1997) (data not shown). In addition, the number of cultures undergoing gliagenesis was sharply reduced. However, when added to the cranial neural crest culture, TGFβ1 actually strongly suppressed the formation of smooth muscle cells (from 76% to 18%, Figs 3, 5). Chondrocyte cell fate was likewise suppressed by TGFβ1, and gliagenesis was also significantly reduced. By contrast, TGFβ1 promoted relatively normal rate of melanogenesis (92% of cultures contained melanocytes) when compared with FGF2-conditioned media alone, although very few melanocyte clusters were observed (Fig. 3). Additionally, in FGF2+TGFβ1 cultures, the pigment cells were dispersed and rarely formed clusters.

WNT1 signaling through the β-catenin pathway induces

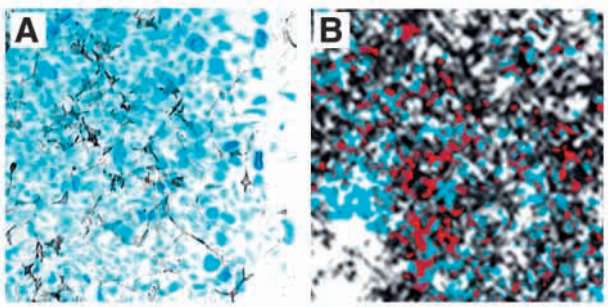

Fig. 4. Mis-expression of a stabilized version of β-catenin using RCAS virus in cranial neural crest cells. The 'exclusion' function of Adobe PhotoShop was used to demonstrate the overlap (red) between pigments cells (black) and smooth muscle cells (blue) in non-infected crest cell cultures (A) or cultures infected (B) with RCAS::β-catenin virus, both treated with FGF2. Note that the relative ratios of smooth muscle cells and pigmented cells are dramatically different.

trunk neural crest cells to differentiate into pigment cells (Dunn et al., 2000). To compare the effect of β-catenin signaling on trunk and cranial neural crest, we used a replication-competent retroviral construct (RCAS) encoding a constitutively active form of β-catenin (CA-β-catenin) (Funayama et al., 1995; Kengaku et al., 1998). Nearly 100% of both trunk crest and cranial neural crest cultures infected with CA-β catenin appeared as dense lawns of heavily pigmented cells (Fig. 3, bottom row). These cultures did not contain chondrocytes or neurons; however, antibodies to SMA (recognizing smooth muscle cells) and GFAP labeled glial cells in a relatively large proportion of the cultures (39% and 37%, respectively). About a third of the cells positive for smooth muscle or glial markers were also pigmented (Fig. 4). These cases might represent either cells with mixed identities or differentiated glial and smooth muscle cells in the process of being transformed into pigment cells [similar process is described elsewhere (Sherman et al., 1993)].

Cranial neural crest cell differentiation in individual cultures

In comparing neural crest differentiation under different culture conditions we adopted the mode of analysis used by previous investigators (e.g. Sarkar et al., 2001; Petiot et al., 2002; Kim et al., 2003) assaying the percentages of cultures in which each cell type was observed. However, we wanted to verify that the changes we observed when assayed in this manner correlated with change in cell number and specific cell fates within individual cultures. To examine this, we set up 10 cultures under each of the following conditions: FGF2 alone, FGF2 and BMP2, and FGF2 and TGFβ1. Each individual colony was analyzed with a double antibody staining against COL2 and SMA to detect chondrocytes and smooth muscle cells, respectively, and pigment cells were directly observed by the presence of the pigment granules (Fig. 6). The average deviation within each sample of 10 colonies is shown as an error bar.

Assaying individual cultures treated with FGF2 alone we found an average of 34% of the cells per culture were positive for COL2 and this ratio ranged from ~20-60% (using the 10% cut-off for counting cultures this would translate to 100% chondrogenic cultures) correlating with the high percentage of cultures showing chondrogenesis in Fig. 5.

By contrast, when cultured in the presence of BMP2 or TGFβ1 in addition to FGF2, there was a dramatic decrease, such that practically no cultures were scored as positive for chondrocytes (Fig. 5). A similar inhibition of chondrogenesis is seen when cells within individual cultures were counted under those culture conditions (Fig. 6). The other significant decrease in cell types under these conditions assayed by percent of cultures is in cultures populated by smooth muscle cells when treated with FGF2 and TGFβ1. This culture condition also gave the lowest percentage of smooth muscle cells when counted within individual cultures (Fig. 6),

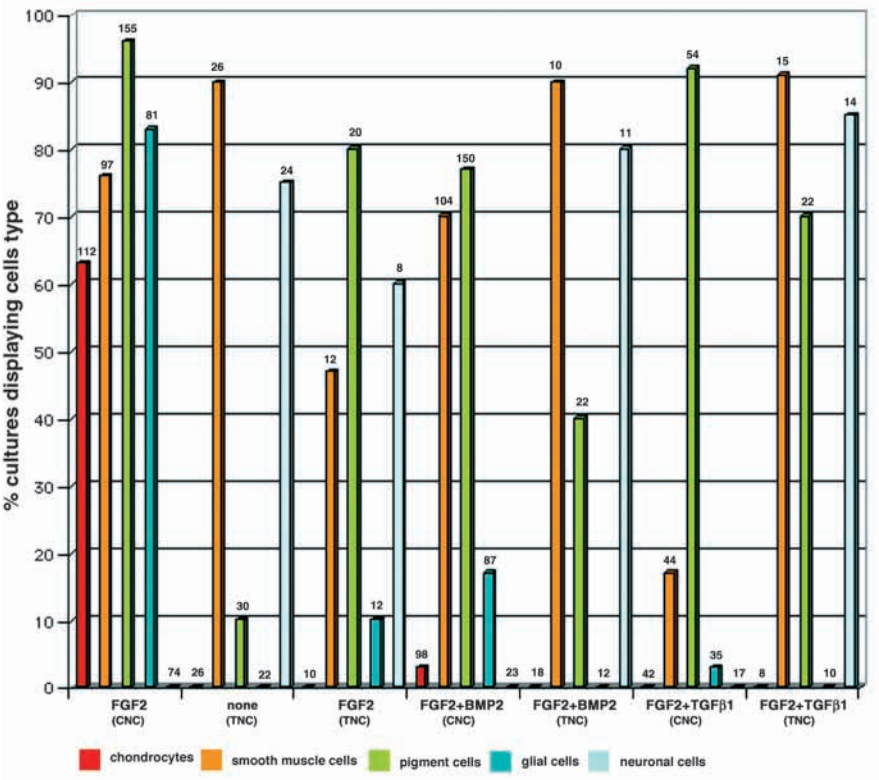

Fig. 5. The effect of FGF2 and other cytokines on cell differentiation of the cranial and trunk neural crest cells in vitro. This histogram of the results described in Fig. 3 compares effects of the cytokines on generation of pigment, glial, smooth muscle, neuronal cells and chondrocytes. As most of the cultures were stained with only two or three different cell-specific antibodies, the histogram represents percentage of independent cultures displaying a particular differentiated cell type from the total number of cultures stained for that marker. Chondrogenesis is inhibited by both BMP2 and TGFβ1 proteins even in the presence of FGF2/8. The total number of cultures tested for each of the cell types is indicated above the representative bars. Note that all cultures were scored for melanogenesis.

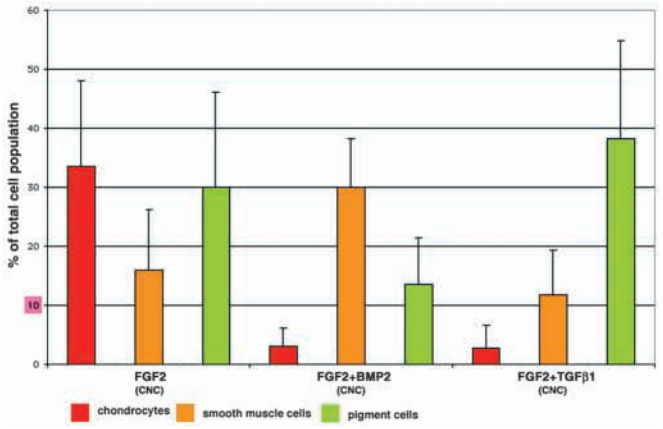

Fig. 6. Individual variation of cell differentiation in the cranial neural crest cultures treated with FGF2, FGF2+BMP2 and FGF2+TGFβ1 based on double immunostaining with antibodies against COL II and SMA. Each color bar represents an average fraction of a particular cell type in ten individual primary cultures. Error bars represent average deviation for the sample for each of the cell types. Color bars indicate different types of cells: red, chondrocytes; orange, smooth muscle cells; green, pigment cells.

although owing to the high variability between cultures, the difference from cultures treated with FGF2 alone is not statistically significant. Using the 10% cut-off for counting individual cultures (pink in Fig. 6) it is apparent that this data set is similar to that assayed in Fig. 5.

Hox genes and differentiation of cranial neural crest cells

Most of the cellular responses by cranial neural crest described above differ dramatically from those of the trunk neural crest cells when exposed to the same signaling factors. One significant difference along the rostrocaudal axis is the expression of Hox genes. Indeed, regional differences between the head and the trunk depend, in part, on the function of Hox genes (Rijli et al., 1993; Grammatopoulos et al., 2000). To examine potential roles of Hox genes in defining the regional differences in responsiveness to various factors, we expressed two different Hox genes in mesencephalic cranial neural crest cultures using RCAS viruses (Figs 7, 8). *Hoxa2* is normally expressed in the posterior head, a region which unlike the trunk produces neural crest-derived cartilage although not as much as in more anterior head regions (Sarkar et al., 2001). It has been shown to be an important regulator of cranial neural crest patterning and morphology of branchial arch skeletal elements in mouse, chick and frog (Rijli et al., 1993; Grammatopoulos et al., 2000; Pasqualetti et al., 2000). *Hoxa2* is normally expressed in cranial neural crest cells migrating into the second branchial arch and loss of its function leads to the homeotic transformation of second branchial elements, into those of the first arch (Rijli et al., 1993). Conversely, misexpression of *Hoxa2* in the anterior head transforms the first branchial arch into a more posterior identity (Grammatopoulos et al., 2000). *Hoxd10* expression is normally observed in the posterior sacral part of the trunk with an anterior boundary at the level of somites 31-32. Neural crest from this region does not yield any skeletal derivatives.

As described above, we found that cranial neural crest cells, but not trunk neural crest cells, require FGF2/8 for survival in culture (Fig. 2D). To test if this FGF dependence relates to Hox gene expression, we infected mesencephalic cranial neural crest cells with RCAS::*HoxA2* and RCAS::*HoxD10*. Survival of *Hoxa2*-expressing cell cultures in the absence of FGF8 (24%) was significantly increased from that of uninfected cranial neural crest cells (7%), whereas survival of *Hoxd10*-expressing cells increased sevenfold (53%) (Fig. 9). Significantly, the overall survival of the RCAS::*Hoxd10* infected cranial neural crest cell cultures in the absence of any exogenous factors was now intermediate between non-infected cranial neural crest cultures and trunk neural crest cultures (7%, 53% and 83%, respectively; Fig. 2D, Fig. 9). Thus, in terms of survival, the *HoxD10*-expressing cranial neural crest cultures (whose survival increased from 7% to over 50%) were similar to trunk neural crest in their lack of FGF dependence (Fig. 9).

We also noted a difference in cell

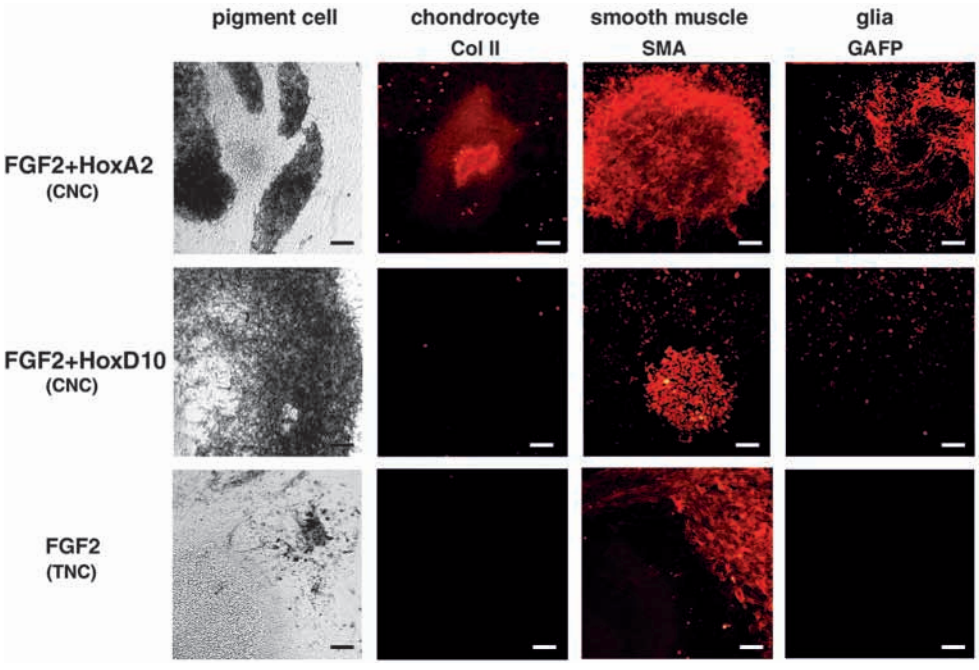

Fig. 7. Immunohistochemical analysis of the cranial neural crest cultures infected with the RCAS::*Hoxa2* and RCAS::*Hoxd10* viruses and comparison with trunk neural crest culture in medium containing the exogenous purified FGF2 or BMP2 protein. All antibodies used are the same as shown in Fig. 3. In RCAS::*Hoxa2*-infected cells, the clusters of melanocytes, glial and smooth muscle cells form similarly to the FGF2 cultures; however, the ColII-positive cells form significantly smaller clusters and in fewer cultures. No chondrocytes are detected in *Hoxd10*-infected cultures, a condition that mimics the trunk neural crest cultures. Scale bars: 100 μm.

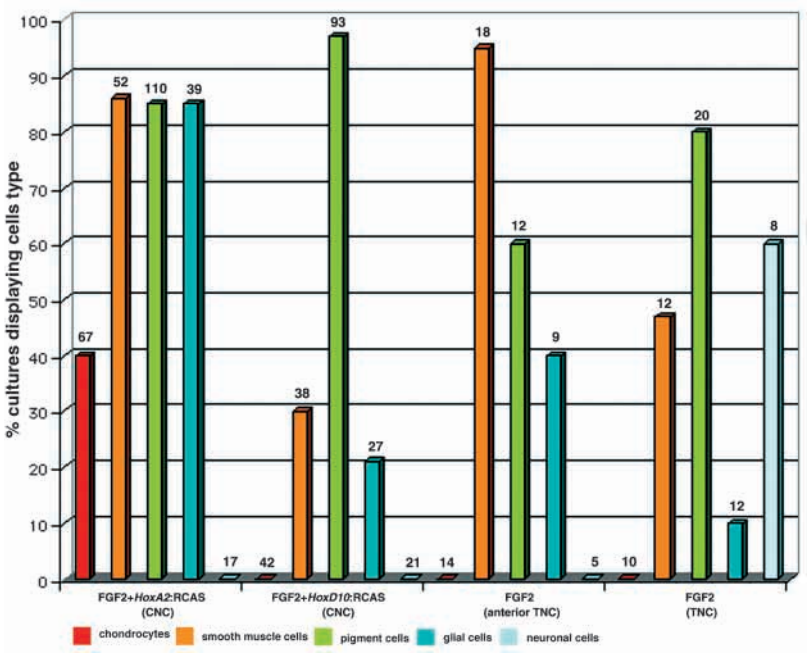

Fig. 8. The effect of FGF2, other cytokines and Hox genes on cell differentiation of the cranial neural crest in vitro. A histogram summarizing the results of the experiments from Fig. 4 showing how Hox genes control cell differentiation of the cranial neural crest cells. For comparison, we included data on differentiation of anterior (somite level 10-12) and posterior (somite level 32-33) trunk neural crest cells. Both Hoxa2 and Hoxd10 can suppress chondrogenesis in cranial crest cells although to a different extent. Hoxa2 limits the rate of chondrogenesis by about 30%, whereas Hoxd10 completely suppresses it. Other cell types are also affected differently: Hoxa2 does not alter the overall rate production of smooth muscle or glial cells but Hoxd10 strongly limits myogenesis and gliagenesis relative to uninfected condition. The total number of cultures tested for each of the cell types is indicated above the representative bars. Note that all cultures were scored for melanogenesis.

survival between trunk and cranial neural crest cells cultured in the presence of BMP2. Trunk neural crest cells survived quite well in the presence of this factor, while cranial neural crest cells undergo apoptosis in response to BMP2 signaling (Fig. 2D-G, Fig. 9). Although, *Hoxa2* did not significantly change survival in the presence of BMP2, RCAS::*Hoxd10*-infected cranial neural crest cells again showed an increase in cell numbers in the presence of BMP2 than uninfected cells (Fig. 2D, Fig. 9). Although a decreased rate of apoptosis in the *Hoxd10*-expressing cranial neural crest cultures would be most consistent with a posterior transformation of these cells, the results could also be explained by an increased proliferation in the infected cultures. A decrease in survival of RCAS::*Hoxd10*-infected cultures in the presence of BMP2 relative to uninfected cultures seems to suggest that although much of the FGF2 dependence was eliminated, some of the sensitivity towards BMP2 remained.

We next examined the ability of Hox genes to modulate differentiation response in neural crest cell cultures. Both trunk and cranial neural crest cells give rise to a mixture of cell types when cultured in the presence of FGF2/8. However, trunk neural crest cells fail to differentiate into chondrocytes and show a more limited range of gliagenesis than CNC cells within our culture period. When mesencephalic cranial neural crest cells were infected with RCAS::*Hoxa2* and treated with FGF2, the number of cultures undergoing chondrogenesis decreased from about 60% to about 40% relative to uninfected cultures (compare Figs 5, 7 and 8). None of the cranial neural crest cell cultures expressing trunk Hox gene *Hoxd10* and treated with FGF2 contained chondrocytes.

This leads to a model where chondrogenesis is blocked by co-expression of anterior (e.g. *Hoxa2*) and posterior (e.g. *Hoxd10*) Hox genes, a pattern normally found in the trunk; low level of chondrogenesis occurs in the presence of only anterior Hox gene expression (e.g. *Hoxa2*), a pattern normally seen in metencephalic (hindbrain) crest, and high level of chondrogenesis takes place in the absence of neural crest Hox gene expression, a condition normally seen in the mesencephalon. To test this hypothesis further, we also mis-expressed *Hoxd10* in primary cultures of metencephalic cranial neural crest from rhombomere 3-4 levels that normally express endogenous *Hoxa2* and produce some cartilage structures. As with mesencephalic crest, we found that mis-expression of *Hoxd10* inhibited chondrogenesis in metencephalic crest (19/20 cultures infected cultures; data not shown).

We also examined the differentiation of neural crest cells into other cell types in our culture conditions under the influence of Hox genes. In the presence of FGF2, mesencephalic cranial neural crest cells infected with RCAS::*Hoxa2* formed large dense cultures containing pigment cells (about 80% of cultures), smooth muscle cells and glial cells, similar to uninfected cultures treated with FGF2 (Figs 5, 7, 8). By contrast, mesencephalic neural crest cultures expressing the trunk Hox gene *Hoxd10* and treated with FGF2 showed a reduced number of smooth muscle cells and glial cells, although melanogenesis was not affected. This pattern of

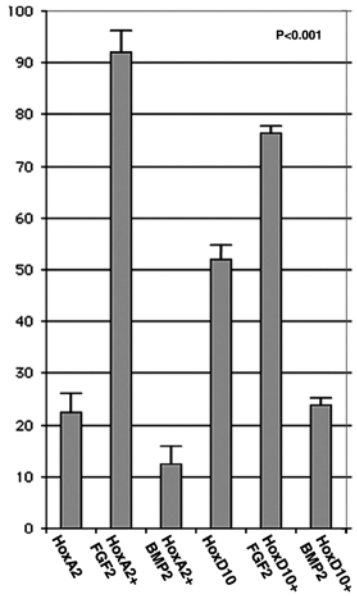

Fig. 9. Comparison of survival in cultures infected with RCAS::*Hoxa2* and RCAS::*Hoxd10* and treated with FGF2 or BMP2. Survival of *Hoxa2*-expressing cells is increased relative to uninfected cranial neural crest cells whereas survival of *Hoxd10*-expressing cells increased almost fivefold and became markedly more similar to that of trunk neural crest (*t*-test: $P<0.001$ for all pair-wise comparisons with a control). All are significantly different except Hoxa2+FGF2.

differentiation was compared with trunk neural crest cultures derived from the anterior trunk (somite 10-12 level), which expressed other Hox genes but not *Hoxd10*, and posterior trunk (somite 32-33 level), which expressed *Hoxd10* and other Hox genes. We found that the two trunk neural crest populations differed significantly in their ability to produce smooth muscle cells, glial cells and neurons (Fig. 8). The RCAS::*Hoxd10*-infected cranial neural crest cells were more similar to the posterior trunk than to the anterior trunk neural crest cells, although they produced very few neurons (Fig. 8). Thus, in several key respects the differentiation pattern of cranial neural crest cells is closer to that of trunk neural crest when cells ectopically express the posteriorly expressed Hox gene *Hoxd10*. Thus, in several key respects the differentiation of pattern of cranial neural crest cells is closer to that of trunk neural crest when cells ectopically express the posterior Hox gene *Hoxd10*.

Chondrogenesis in longer-term trunk neural crest cultures

Transplantation experiments have indicated that trunk neural crest lacks the capacity to undergo chondrogenic differentiation in vivo. Moreover, in our in vitro culture conditions trunk neural crest cells do not produce any chondrocytes. Nonetheless, it has recently been reported that trunk neural crest cells, in long-term culture (2-4 weeks) can undergo chondrogenesis (McGonnell and Graham, 2002). This could be interpreted as reflecting an underlying potential for chondrogenic differentiation in trunk neural crest, which is revealed only under culture conditions that arise within the dish after several weeks. Alternatively, the trunk neural crest cells themselves might be altered by the long-term culture conditions such that at least a subset of these cells gain differentiation potential that is not present in normal trunk neural crest cells. To investigate this issue we allowed our culture to continue growing for 2 weeks. As previously reported, these cultures of trunk neural crest cells ultimately undergo chondrogenesis, although chondrocytes are not readily observed until 12-14 days in culture, while chondrocytes are detected in cranial crest culture as early as 4-5 days of culture (Figs 3-8; data not shown).

One explanation for this could be that the neural crest loses its trunk specificity and converts to a more cranial crest-like cell type during long term culture. To examine this possibility, we used RT-PCR to follow the expression of several cranial neural crest markers, which are preferentially expressed in cranial crest in vivo. We found that Id2 (Martinsen and Bronner-Fraser, 1998) and noelin 1 (Barembaum et al., 2000) were strongly upregulated in trunk neural crest cells after 2 weeks in culture to levels similar to those detected in cranial neural crest cultures (Fig. 10A). Thus, at least in some important respects trunk neural crest resembles cranial crest after long term culture. One possible explanation for this could be that Hox genes, which establish differences in neural crest along the rostrocaudal axis, are dysregulated in long-term culture.

We examined some of the Hox genes normally expressed in vivo at somite 10-12 level from which we established cultures of anterior trunk crest. This anterior trunk only expresses a few Hox genes, including *Hoxa4*, *HoxB4* and *HoxD4*. Of these, only *Hoxb4* is expressed at easily detectable levels in the trunk neural crest cells at the time of explant collection (data not shown). Accordingly, we examined the expression of this gene by in situ hybridization after short and long term culture. *Hoxb4* is downregulated after 2 weeks in a subset the cells in the trunk neural crest cultures, in regions which roughly correlate with chondrogenesis and very few chondrocytes (COLII-positive cells) express *Hoxb4* (Fig. 10C-J). Moreover, overexpression of *Hoxd10* completely blocks chondrogenesis in long-term trunk neural crest cultures (Fig. 10B,K-W).

Discussion

Dramatic differences exist in differentiation of cranial and trunk crest cells

The neural crest is a continuous population of cells that spans both head and trunk. It has long been assumed that with the exception of chondrogenic lineages exclusive to the head, the head and trunk crest follow similar developmental pathways. In particular, both head and trunk tissues produce differentiated cells such as neurons and smooth muscle cells that are identical on morphological, molecular and functional levels. Thus, it was expected that the processes of cell fate acquisition would be similar if not identical in the two parts of the body. Nevertheless, some important regional differences are known to exist, most notably the unique ability of the cranial neural crest cells to form bone and cartilage as well as connective tissues, thus functionally replacing mesoderm. Grafting experiments of the cranial neural crest and trunk neural crest have shown that these regional differences cannot be explained solely by differences in the signaling environment, rather there is an inherent restriction on the potential of trunk neural crest, which prevents it from becoming skeletal tissue. In this study, we have further examined the intrinsic differences between trunk and cranial neural crest by assessing the responsiveness of these cells to secreted factors that are known to be expressed in the developing head.

Fig. 11 summarizes the effect of several signaling pathways on cell differentiation in the cranial and trunk neural crest. Note that FGF2 (and FGF8) seem to play an important role in promoting survival, proliferation and differentiation of cranial neural crest but not trunk neural crest cells, and that freedom from this requirement depends, at least in part, on the expression of trunk Hox genes. Interestingly, some factors that are inductive for a particular cell fate in trunk neural crest cells (Anderson, 1997; Shah et al., 1996; Francis-West et al., 1998) have the opposite functions in the cranial neural crest, e.g. BMP2/4 in neurogenesis and TGFβ1 in smooth muscle formation. On the other hand, the WNT pathway is equally potent in inducing melanogenesis in both head and trunk crest. FGF2 and FGF8 also seem to positively regulate the level of melanogenesis in both systems. In addition, chondrogenesis, which is unique to cranial neural crest cells, is induced by the FGF2/8 but is suppressed or inhibited by BMP2, TGFβ1 and WNT pathways. Taken together, the distinct modes of cell differentiation in cranial and trunk neural crest suggest that the cranial and trunk cells possess significantly different developmental capabilities. Interestingly, different regulatory interactions were also recently found for the head and trunk mesoderm during the process of myogenesis (Mootoosamy and Dietrich, 2002) (E.T., H. Kempf, R. C. Mootoosamy, A. C. Poon, A.A., C.J.T., S. Dietrich and A.B.L., unpublished).

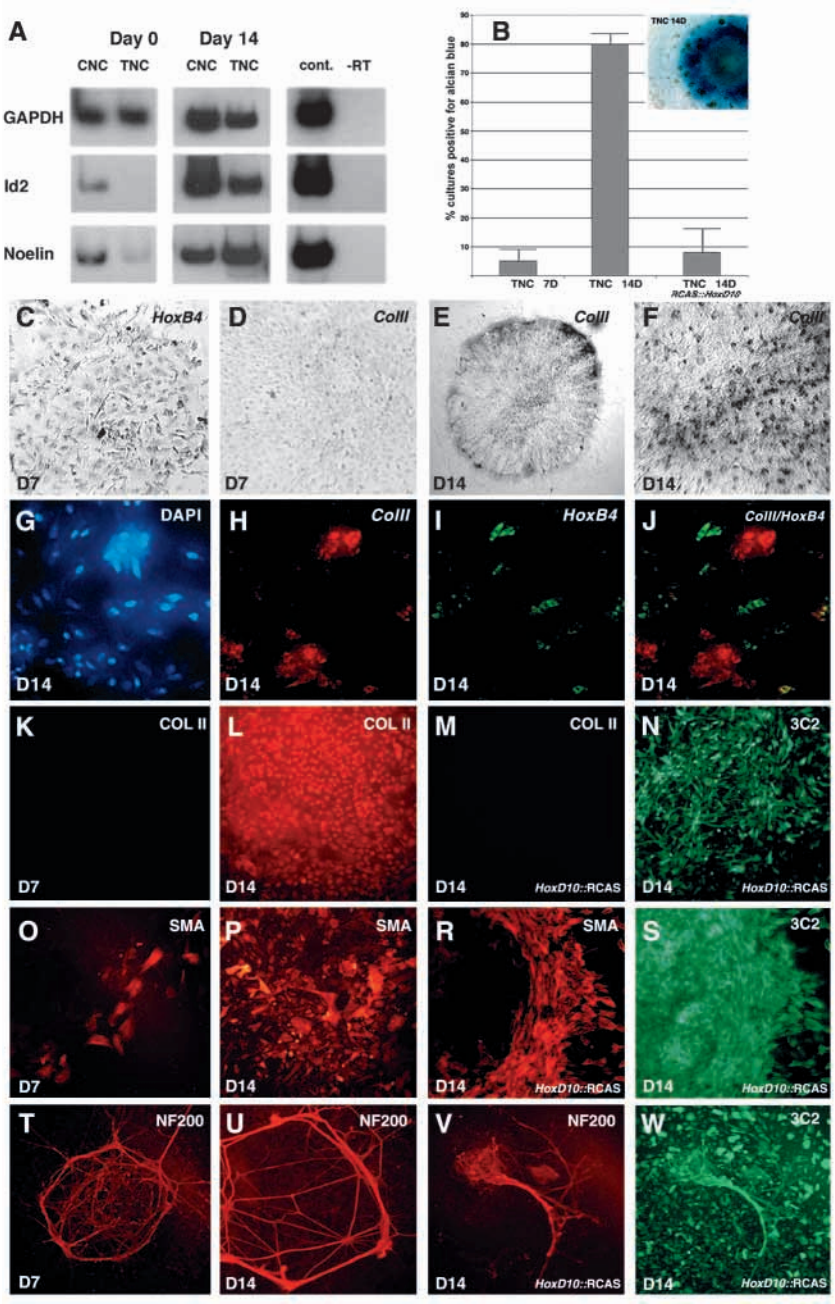

Fig. 10. Analysis of the trunk neural crest cells in long-term culture. RT-PCR amplification of cranial crest-specific markers from cranial and trunk neural crest cultures. Id2 and Noelin are highly expressed in both cranial explants and 14-day-old cranial crest but not in the new trunk crest cultures (A). A histogram showing the number of cultures positive for Alcian Blue staining, i.e. undergoing chondrogenesis (B). Note that on the second week of incubation (D14), about 80% trunk neural crest cultures undergo chondrogenesis and RCAS::*Hoxd10* infection can suppress chondrogenesis in these cultures. (C-J) In situ hybridization on trunk neural crest cultures incubated for 7 or 14 days. *Hoxb4* (C) but not *ColII* (D) signal is detectable in most cells of the week-old trunk neural crest cultures. *ColII* expression (E,F) is detectable in chondrocytes present throughout the 2-week-old cultures. Double in situ hybridization (G-J) revealed that very none or few *Hoxb4*-expressing cells are also positive for *ColII* transcript. (K-W) Immunochemistry with antibodies revealing chondrocytes (αCOLII), smooth muscle cells (αSMA), neuronal cells (αNF200) and 3C2 antibody (RCAS-infected cells) demonstrates the effect of the ectopic trunk Hox expression on chondrogenesis in long-term trunk crest cultures. (K,O,T) Trunk neural crest culture grown for 7 days, analyzed for presence of chondrocytes (K), smooth muscle cells (O) and neurons (T). Note that no COL2-positive cells are detected in these cultures. (L,P,U) Similar cultures grown for 2 weeks have many chondrocytes that appear throughout the culture, whereas smooth muscle cells and neurons are still present. (M,R,V) Trunk neural crest cultures infected with RCAS::*Hoxd10* virus and grown for 2 weeks display a much lower level of chondrogenesis with no effect on myogenesis and neurogenesis. (N,S,W) To ensure that the cultures were indeed expressing the RCAS construct, 3C2 antibody was used to reveal the extent of the retroviral infection.

The inhibitory role for BMP2 is interesting, if somewhat unexpected, as the related BMP4 protein is expressed in the epithelium of the frontonasal and mandibular primordia and BMPs are required for production of chondrogenic cranial neural crest and sufficient for development at least in some of the craniofacial structures in vivo (Wall and Hogan, 1995; Barlow and Francis-West, 1997; Francis-West et al., 1998; Kanzler et al., 2000; Shigetani et al., 2000). However, it was recently demonstrated that the induction of chondrogenesis by BMP4 is positionally dependent (Semba et al., 2000). More specifically, to induce chondrogenesis BMP4 requires a high level of Sox9 expression relative to the level of Msx2, a condition that normally exists in the distal part first branchial arch. It is also possible that the observed induction of chondrogenesis in some regions by BMP4 may require the additional influence of other signaling factors that are expressed in the developing face, but which we have not tested here, such as Shh (with which it broadly overlaps in the face). It is also apparent that BMPs play multiple roles at distinct stages of skeletal development. For example, in the limb bud BMPs have been implicated in regulating cell death (Zou and Niswander, 1996), in early chondrogenesis (Pizette and Niswander, 2000) as well as modulating the growth and differentiation of skeletal elements once they form (Minina et al., 2001; Minina et al., 2002). It is likely, that BMPs act at multiple stages in skeletal development in the cranial region as well. In fact, BMPs are already known to play distinct functions in promoting neural crest formation (Garcia-Castro et al., 2002) and, later, in regulating apoptosis and migration of neural crest cells in vivo (Sela-Donenfeld and Kalcheim, 1999; Smith and Graham, 2001). Interestingly, we find that BMP2/4 are expressed in the mesenchyme surrounding the developing skeletal structures later in development and are capable of positively upregulating their growth both in vitro and in vivo (A.A., unpublished).

We did not aim in this analysis to understand the molecular

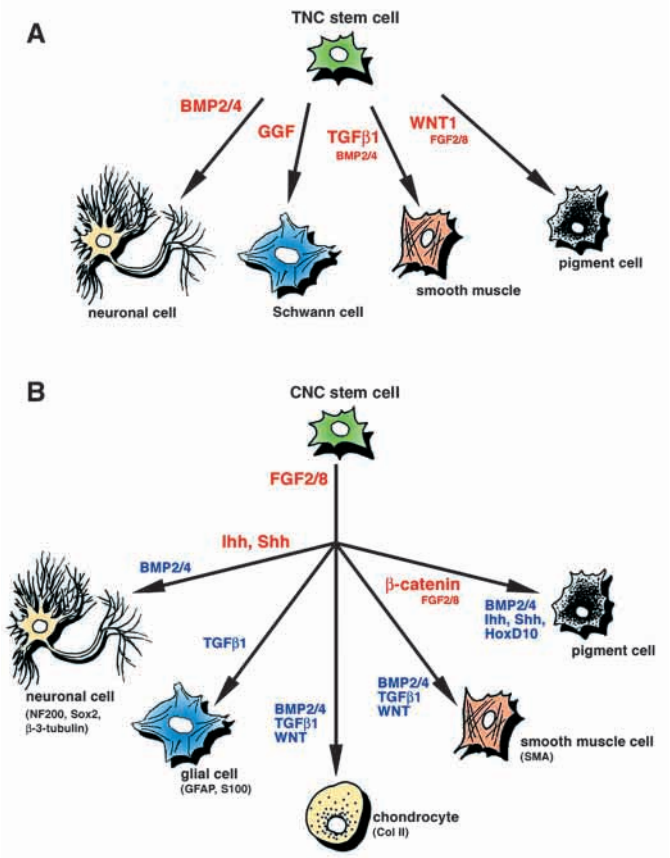

Fig. 11. Distinct modes of regulatory mechanisms of differentiation of the cranial and trunk neural crest cells. BMP2/4, GGF, TGFβ1 and WNT1 act to induce neurogenesis, gliagenesis, myogenesis and melanogenesis, respectively, from the multipotent trunk neural crest stem cells (A) (Shah et al., 1996; Anderson, 1997; Sieber-Blum and Zhang, 1997; Zhang et al., 1997; Francis-West et al., 1998; Dunn et al., 2000). Inductive and repressive roles of the FGF2/8, BMP2/4, SHH, TGFβ1 and WNT pathways on the cranial neural crest differentiation (B). The positive regulators are shown in red and the negative ones are in blue. FGF2/8 appears to be generally required for normal proliferation and survival of the cranial but not trunk neural crest. FGF2/8 also seem to be a positive regulator/survival factor for the melanogenic cells in both cranial and trunk crest cultures. Some of the markers used to identify the different cell types are shown in brackets. Some cell types, particularly smooth muscle cells and pigment cells, differentiate in the surviving cultures grown with media containing no purified exogenous proteins. It is not clear whether these cells rely on the residual factors present in the serum, such as a clearly detectable BMP-like activity, or represent a default state of the cranial neural crest differentiation.

mechanisms for the differences we observed. The differential survival and cell fate diversification could be due to direct regulatory control of differentiation of particular cell types, their differential induction, proliferation or death. In addition, some conditions might be supportive of survival of certain cell type progenitors but not others. All of these issues will need to be addressed in the future studies.

It is important to note that our use of combinations of different factors revealed some interesting synergisms and antagonisms in regards to both survival and differentiation. For example, BMP2 by itself cannot support survival of the cranial neural crest cells in vitro, whereas FGF2 allows the cells to survive and differentiate into chondrocytes. When FGF2 and BMP2 are added together, the survival and proliferation are maintained but chondrogenesis is very strongly inhibited (Fig. 5). In a related study, we found that a combination of FGF2/8 with sonic hedgehog has a very strong synergistic effect on chondrogenesis both in vitro and in vivo (A.A. and C.J.T., unpublished). These and other observations from our study are strongly reminiscent of the previously reported differential effects of combinations of growth factors on proliferation and differentiation of the trunk neural crest cells. For example, the presence of stem cell factor (SCF) is required for early trunk neural crest survival but not for the survival of melanogenic cells. However, a combination of SCF with a neurotrophin, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) or neurotrophin 3 (NT3) can neutralize the activity of SCF. However, a combination of SCF and NT3 can support survival of pigment cell precursors (Zhang et al., 1997; Sieber-Blum, 1998). Moreover, this SCF+NT3 combination can be antagonized by TGFβ1 signaling, which strongly induces differentiation of the trunk neural crest cells into sympathetic and primary sensory neurons, thus emphasizing the importance of the concerted effects of different signaling molecules.

Our in vitro data also correlate well with the expression patterns of the signaling molecules discussed herein. For example, the requirement for FGF2/8 to promote survival and differentiation of cranial neural crest in culture is, moreover, consistent with the broad and persistent expression patterns of different FGF family members (FGF1, FGF2, FGF3, FGF4, FGF5, FGF7 and FGF8) and their receptors in the developing head (Schneider et al., 2001; Bachler and Neubuser, 2001). The in vivo functional significance of these expressions patterns has been partially confirmed in studies where FGF8 activity was conditionally removed from the ectoderm covering the first branchial arch using Cre/loxP technology, resulting in loss of most of the cranial neural crest-derived structures (Trummp et al., 1999).

Neural crest differentiation and Hox genes

Hox genes are important regulators of developmental fates, including specification of structures formed from neural crest. Our data suggest that Hox genes influence neural crest survival, proliferation and differentiation, in part by controlling the differential responsiveness of neural crest cells to various signaling pathways. Cranial neural crest cells infected with retrovirus carrying the trunk Hox gene *Hoxd10* respond to different factors in a manner normally seen with trunk neural crest. For example, *Hoxd10* suppress chondrogenesis from the cranial neural crest cells. Moreover, the overall survival and differentiation abilities of the RCAS*::Hoxd10*-infected cranial crest cells is more similar to those of the posterior trunk neural crest where *Hoxd10* is normally expressed than to either anterior trunk crest cells or RCAS*::Hoxa2*-infected cranial crest cells. However, at least one property of trunk neural crest, the ability to produce high numbers of neurons, was not recapitulated in cultures of cranial neural crest cells infected with *Hoxd10*, suggesting that other important regulatory factors (potentially including other Hox genes) are involved in controlling cell diversification pathways at the trunk level. The effect of *Hoxa2* is more complicated. *Hoxa2* may modulate the

extent of the response of cranial neural crest to chondrogenesis-inducing FGF signals. This might be achieved by locally regulating the balance between proliferative versus differentiative effects of FGF2/8 (Fig. 9). Thus, this interpretation would take into account the fact that *Hoxa2* is required for normal patterning of the 2nd branchial arch and is expressed in cranial neural crest cells destined to become cartilage.

Recently, it has been shown that three anterior trunk Hox genes, *Hoxa2*, *Hoxa3* and *Hoxb4*, when mis-expressed in the head are capable of suppressing chondrogenic structures in the anterior head but differed in their abilities to do so (Creuzet et al., 2002). For example, *Hoxa3* prevents the formation of the first branchial arch but not the nasal septum, whereas *Hoxb4* suppresses formation of nasal skeleton but allows proximal lower jaw development. Together, these results suggest that many Hox genes are capable of regulating differentiation of cranial crest cells, although substantial differences exist in their exact capabilities. It might be important, therefore, to study the effect of Hox genes on differentiation of different crest populations within the trunk and posterior head. It might be predicted, for example, that differentiation of cardiac crest, which originates from a certain anteroposterior level, is also controlled by Hox genes.

It is clear that Hox expression changes the responsiveness of the cranial cultures so that in many respects they resemble cultured trunk crest. Conversely, we have suggested that the ability of long-term cultures of trunk neural crest to adopt chondrogenic fates normally limited to cranial crest may be due to the loss or downregulation of Hox expression in a subset of cells. Although this may not reflect a physiological property of trunk neural crest, it does suggest an underlying plasticity in Hox gene expression. The ability of crest cells to modulate their Hox expression in foreign environments was also seen in experiments where small pieces of dorsal rhombomeric tissue containing crest were transferred from the rhombomere 3, 4 and 5 level to rhombomere 2 level (Trainor and Krumlauf, 2000; Trainor and Krumlauf, 2001).

The ability of Hox genes to alter responsiveness of different regional neural crest populations, while maintaining the extraordinary broad capacity of those cells to differentiate along diverse pathways, generates a flexibility in the way those cells can be directed in development. Thus, a mechanism is generated whereby the proper differentiation of neural crest cells can be coordinated with other tissues by taking advantage of the distinct arrays of secreted signals characterizing different regions of the embryo.

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