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Review

Effects of growth factors on the differentiation of neural crest cells and neural crest cell-derivatives

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Introduction

Growth factors are proteins or glycoproteins as small as 6045 daltons that regulate cellular functions such as proliferation and cytodifferentiation by acting at the cell surface to trigger second messenger systems that lead to the activation of DNA-binding proteins. Growth factors were unknown until the early 1950s when the first, NGF, was discovered (Levi-Montalcini and Hamburger, 1951; Levi-Montalcini, 1952, 1976, 1987). Since then, whole families of growth factors have been identified (Table 1). This review examines the effects of growth factors on neural crest cells and neural crest cell derivatives with special attention to their possible role in specification of cell fate, and to their differing roles in specification of mesenchymal, endocrine and neuronal neural crest-derived cells.

Growth factor families

Certain growth factors show such a remarkable similarity in amino acid sequence, function, and competitive binding with the same receptor, that they can be grouped into growth factor families (Table 1, and see Mercola and Stiles,1988 for a review). For example, the EGF supergene family is composed of EGF, TGF-α and VGF (Marquardt *et al.*, 1984; Brown *et al.*, 1985). EGF-like activity reported in fetal tissues is due to TGF-α, a finding that led Nexo *et al.* (1980) to suggest that TGF-α is the fetal form of EGF. TGF-ßs are a multifunctional supergene family of growth factors consisting of TGF-ßs 1-5 along with decapentaplegic protein from *Drosophila* and Vg1 from *Xenopus* (Table 1). TGF-ß1 and TGF-ß2 are homologous to cartilage-inducing factors A and B (CIF-A and -B) isolated from demineralized bovine bone (Seyedin *et al.*, 1985, 1986, 1987). Other growth factor families are summarized in Table 1.

Sources of growth factors

No specific glands secrete growth factors; they are produced in minute amounts by many cell types. Nor do growth factors necessarily depend on the circulatory system to reach target cells; they reach their target by simple diffusion, *i.e.*, growth factors are *paracrine factors*. **For** example, media conditioned by glial cells, glioma cells and brain astrocytes support the growth and survival of neurons because of growth factors that are released (Ebendal and Jacobson, 1975, Barde *et al.*, 1978, and Lindsay, 1979). This feature is of immense importance for early embryonic development when there is no circulatory system to transport growth factors from the place of production to the target cell. However, later in development, IGF, PDGF and TGF-ßs are stored in platelets and circulate in the blood bound to specific binding proteins (Rinderknecht and Humbel, 1976; Witte *et al.*, 1978, Assoian *et al.*, 1983). Once secreted, growth factors can act upon the cell that secreted them, a phenomenon of *autocrine regulation*. The effect of TGF-ß on calvarial cells is a good example (Centrella *et al.*, 1986; see below, and see Sporn and Roberts, 1990 for a recent insightful overview). Growth factors are also found in association with extracellular matrixes (see last two sections in this review), a binding that localizes them at their site of action.

Mechanism of growth factor action

The presence of a growth factor is not sufficient for its action. The target cell has to be able to respond, a responsiveness that is determined by receptors at the cell surface. Binding of a growth factor or growth factor-matrix molecule complex to a receptor is the first step in growth factor action. Knowledge of growth factor receptors, especially the NGF receptors which are tyrosine kinases (known stimulators of cell proliferation) is advancing rapidly; see Bothwell (1991) for a recent review. Once bound to the receptor, a second message is transduced across the cytoplasm and into the nucleus. Signal transduction is a complex process that varies from cell type to cell type and growth factor to growth factor (see Gospodarowicz, 1983; Rozengurt and Collins, 1983; Pratt, 1987, and Sporn and Roberts, 1988 for reviews). Once a signal transferred from a growth factor reaches the nucleus, it affects nuclear DNA; growth factor-induced DNA replication or transcription leads to such cellular responses as proliferation and cytodifferentiation.

Interaction of growth factors with hormones and other growth factors

The action of a growth factor varies depending on other growth factors, hormones and other molecules or ions in the microenvironment. For example, TGF-α stimulates *in vitro* proliferation of fibroblasts in the presence of PDGF, but inhibits proliferation in the presence of EGF (Roberts *et al.*, 1985). The mitogenic effect of EGF on palatal epithelial cells is enhanced by retinoic acid and by glucocorticoids (Yoneda and Pratt, 1981a, b; Abbott *et al.*, 1988 and see below). Interactions of growth factors with components of mesenchymal and epithelial extracellular matrices have already been noted and are discussed in the last two sections. Given such interactions we should exercise caution in interpreting *in vitro* studies in which soluble growth factors are presented to cells.

Differences between growth factors and polypeptide hormones

Despite the fact that hormones are produced by specialized glands and transported by the circulatory system to target sites,

Abbreviations used in this paper: BDNF, brain-derived neurotrophic factor; BMP, bone morphogenetic protein; CIF-A and -B, cartilage inducing factors A and B; CRGP, calcitonin gene-related peptide; DPP, decapentaplegic protein of *Drosophila;* DRG, dorsal root ganglion; ECGF, endothelial cell growth factor; ECM, extracellular matrix; EGF, epidermal growth factor; Egr-1, early growth response gene-1; FGF, fibroblast growth factor; HBGF, heparin-binding growth factor; HCGF, hemopoietic cell growth factor; IGF, insulin-like growth factor; IL, interleuken; MIS, Müllerian inhibiting substance; m RNA, messenger ribonucleic acid; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PTH, parathyroid hormone; RNA, ribonucleic acid; TGF, transforming growth factor; Vg, vegetal pole mRNA of *Xenopus;* VGF, vaccinia growth factor; Vgr, mouse homologue of Vg.

TABLE 1

GROWTH FACTOR FAMILIES

Family	Members	Key references
EGF	EGF, TGF-a, VGF	Cohen (1962); De Larco and Todaro (1978, 1980)
NGF	NGF	Levi-Montalcini and Hamburger (1951), Levi-Montalcini (1952)
IGF	IGF-I[a], IGF-II[b]	Rindertnecht and Humbel (1976)
TGF-ß	TGF-ß1-ß5[c]	Roberts *et al.*, (1981)
Inhibin	Inhibin A and B, Activin A and B	
DPP/Vg1[d]	DPP, Vg-1, Vgr-1. BMP 2-7[e]	Urist *et al.*, (1973,1984); Sampath *et al.*, (1987); Padgett *et al.*, (1987)
FGF	a-FGF, b-FGF, k-FGF (oncogene product), int-2, FGF-5, FGF-6	Gospodarowicz (1974)
PDGF	PDGF-A, PDGF-B, PDGF-AB	Ross *et al.*, (1974); Kohler and Lipton (1974)
MIS	MIS	Blanchard and Josse (1974)
HCGF	IL 1, 3-6	
BDNF	Brain Derived Neurotrophic Factor	Barde *et al.*, (1982)

BDNF, brain-derived neurotrophic factor; BMP, bone morphogenetic protein; DPP, decapentaplegic protein of *Drosophila;* ECGF, endothelial cell growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; HBGF, heparin-binding growth factor; HCGF, hemopoietic cell growth factor; IGF, insulin-like growth factor; IL, interleuken; MIS, Müllerian inhibiting substance; NGF, nerve growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; Vg, vegetal pole mRNA of *Xenopus;* VGF, vaccinia growth factor; Vgr, mouse homologue of Vg.

a. IGF-I is also known as somatomedin A and somatomedin C (Rechler and Nissley, 1977).
b. IGF-II is also known as MSA (Multiplication Stimulating Activity).
c. TGF-B1 is also known as cartilage-inducing factor A. TGF-B2 is also known as cartilage-inducing factor B.
d. Together the TGF-ßs, DPP and Vg1s constitute the TGF-ß superfamily.
e. BMP-1 which can be isolated from bone does not belong to the TGF-ß superfamily. BMP-2 was previously known as BMP-2BA; BMP-4 as BMP-2A

their mechanism of action upon cells is similar to that of growth factors; polypeptide hormones also act through cell surface receptors, second messengers and ultimate action on nuclear DNA. Moreover, some growth factors were initially identified as hormones, *e.g.*, the gastric antisecretory hormone, urogastrone, which competitively binds with EGF receptors, and is, in fact, EGF (Gregory, 1975; Hollenberg and Gregory, 1976). Because of these similarities in structure and mechanism of action, the traditional boundary between hormones and growth factors is gradually disappearing. Both can act as paracrine regulators of proliferation and cytodifferentiation. The effects of relevant peptide hormones are discussed below only insofar as they pertain to the differentiation of neural crest cells or their derivatives. The effect of steroid hormones and substances such as vitamin A (retinol) will be mentioned when they affect growth factor action on neural crest cells or their derivatives.

The neural crest

The neural crest, an ectodermal derivative, gives rise to a plethora of cell types including most of the autonomic and peripheral sensory nervous systems, the craniofacial skeletal and connective tissues, thyroid and adrenal endocrine tissues, chemoreceptive cells of the carotid body and aortic arches, and, at least as demonstrated for birds, certain parts of the walls of the heart, aortic arches and semilunar valves (Weston, 1963; Johnston, 1966; Le Douarin, 1973, 1982; Le Liévre and Le Douarin, 1975; Noden, 1975, 1978a, b; Kirby *et al.*, 1983, 1985; Kirby and Bockman, 1984; Hall and Hörstadius, 1988; Kirby, 1989; Sumida *et al.*, 1989; Takamura *et al.*, 1990).

Neural crest cells migrate from the crests of the folding neural ectoderm during neurulation, the specific timing varying from region to region along the neural axis and from Class to Class (Fig. 1). Such premigratory neural crest cells are not committed to only one particular cell fate (Weston and Butler, 1966; Noden, 1975; Le Douarin, 1986, 1988a, b; Weston, 1986; Hall and Hörstadius, 1988). Bi- or pluripotentiality becomes established as neural crest cells migrate along definite pathways or after they reach their destination, in part, due to interactions with components encountered in the microenvironments (Schweizer *et al.*, 1983; Kalcheim and Le Douarin, 1986; Hall and Hörstadius, 1988).

The importance of extracellular components for neural crest cell migration has been recognized for some time, *e.g.*, the pioneering studies of Pratt *et al.* (1975, 1976) and Weston *et al.* (1978) on hyaluronic acid and chondroitin sulfate. Components of the extracellular matrixes that neural crest cells migrate on or through are also widely studied putative initiators of neural crest cell differentiation. For example, building on earlier studies, Perris *et al.* (1991) demonstrated a spatially- and temporally-complex segmented pattern of distribution of chondroitin and keratin proteoglycans with variable degrees of sulfation, both in regions occupied by migrating neural crest cells and in regions from which neural crest cells were absent, the presumption being that cells were excluded from certain regions because of the particular proteoglycans present there.

The differentiation of chromatophores (melanophores, xanthophores) is promoted by extracellular matrix materials located in the migration pathway, evidenced by implanting Millipore filter barriers into the pathway and using the filters coated with matricial components as substrata for the culture of neural crest cells (Perris and Löfberg, 1986). This technique has been used to demonstrate regional specificities in matricial components that correlate with cytodifferentiation; sub-epidermal extracellular matrices promote pigment cell differentiation; matrices from the sites of dorsal root ganglia promote neuronal differentiation (Epperlein, 1988; Perris *et al.*, 1988). Mutants may exert their effects by modifying these matrixes; pigment cells fail to differentiate in embryos of the white axolotl mutant because the matrix in the migration pathway is abnormal (Löfberg *et al.*, 1989).

Not all cell types that arise from the neural crest have been given equal attention with respect to effects of growth factors. Neurons and related cell types which have been studied more extensively than have mesenchymal neural crest-cell derivatives are discussed first.

Neural crest cell contribution to the peripheral sensory and autonomic nervous systems

Neural crest cells give rise to sympathetic and parasympathetic ganglia, as well as to dorsal root and some cranial sensory ganglia. Pre-migratory precursors of sensory and autonomic neurons are not regionalized along the neural axis. That sensory and autonomic ganglia can arise from multiple regions of the neural crest (Le Douarin *et al.*, 1975) indicates the pluripotency of these premigratory

cells. Even after migration, cells may remain pluripotent. For example, when early developing dorsal root (sensory) or ciliary (parasympathetic) ganglia are back-transplanted into the neural crest, cells from the graft make their way to different destinations including sympathetic ganglia and adrenal medulla and differentiate according to the environment encountered there (Le Douarin *et al.*, 1979; Le Lièvre *et al.*, 1980; Schweizer *et al.*, 1983). However, during normal development, segregation of autonomic and sensory cell lines occurs early in migration. Fig. 2 illustrates the migratory routes of those neural crest cells which give rise to sensory and sympathetic ganglia (see Le Douarin (1982) and Gilbert (1991) for descriptions of these migratory routes). Different environmental factors, including growth factors, acting upon these neuronal precursor cells at different destinations direct them along particular differentiative pathways (Le Douarin, 1986). Sieber-Blum (1990) has emphasized that both positive and negative signals are involved in specification of neural crest cell fate.

Sensory and autonomic neuronal precursors segregate from one another early in development, in some instances while neural crest cells are still in the neural tube (Ciment and Weston, 1985; Le Douarin, 1986). Subsequent segregation within these lineages is controlled by factors in the the microenvironment (Loring *et al.*, 1982; Sieber-Blum and Sieber, 1985; Kalcheim and Le Douarin, 1986; Perris and Löfberg, 1986). Pluripotential precursors also exist in pre- and post-migratory populations of neural crest cells as beautifully demonstrated by Bronner-Fraser and Fraser (1988, 1989) and Fraser and Bronner-Fraser (1991) using *in vivo* injection and labeling of individual trunk neural crest cells. The injected label was subsequently found in sensory and sympathetic neurons, Schwann cells, pigment cells and in neural and neural crest-derived neurons. Pluripotentiality has also been demonstrated in back-transplanted peripheral ganglia which produce pigment cells, Schwann cells, glia and autonomic and sensory neurons (Le Douarin, 1982) and using clonal culture of migrating cranial neural crest cells (Baroffio *et al.*, 1988, 1991).

Effects of growth factors on neuronal differentiation

There are three lines of evidence supporting growth factor involvement in sensory and sympathetic ganglion development.

1) Injection of antiserum, either to NGF or to a source rich in NGF, such as snake venom or mouse submaxillary gland, into newborn mice or pregnant females results in the complete disappearance of sympathetic ganglia (Levi-Montalcini and Brooker, 1960). A similar response from dorsal root ganglia has been observed in embryonic mice treated with anti-NGF (Levi-Montalcini and Angeletti, 1968).

2) Neuron-enriched cultures prepared from developing sympathetic and sensory ganglia do not survive *in vitro* unless small amounts of NGF are added to the culture medium, in which case they produce neurite outgrowths (Levi-Montalcini and Angeletti, 1963; Chun and Patterson, 1977; Green, 1977).

3) Injection of exogenous NGF prevents the programmed death which normally occurs during development of certain neuronal precursors in sympathetic ganglia (Hendry and Campbell, 1976). Prevention of neuronal cell death by NGF was, of course, the classic discovery by Levi-Montalcini (1952).

All neural crest-derived cells in sympathetic and sensory ganglia are responsive to NGF (Davis and Lindsay, 1985; Lindsay and Rohrer, 1985; Lindsay *et al.*, 1985b). When these embryonic ganglia are treated with NGF in organ culture, induced neurite outgrowth can be seen as a fibrillary halo (Levi-Montalcini, 1952, 1976, 1987; Davis and Lindsay, 1985). Similarly, individual cells in neuron-enriched cultures prepared from these ganglia produce remarkable neurite outgrowth in the presence of NGF (Lindsay *et al.*, 1985a). However, the responsiveness of neural crest-derived parasympathetic ganglia to NGF remains controversial. Although some studies (Coughlin and Rathbone, 1977; Helfand *et al.*, 1978; Rohrer and Sommer, 1982) indicated an EGF effect, others did not (Collins and Dawson, 1983). Part of these differences no doubt lies in the fact that the responsiveness of peripheral sensory neurons to NGF varies with the developmental stage of the embryo from which the neurons are derived.

In contrast to the neural crest-derived sympathetic and sensory neurons, and in apparent commonality with neural crest-derived parasympathetic neurons, placode-derived ganglionic cells are unresponsive to NGF; they were thought to lack NGF receptors (Davis and Lindsay, 1985; Lindsay and Rohrer, 1985; Lindsay *et al.*, 1985b). Again this conclusion is controversial; neurons in nodose ganglia at early stages in their development can respond to NGF; indeed NGF receptor mRNA is present in many tissues (neuronal and non-neuronal, neural crest and placodal) of the early chick embryo (Hallböök *et al.*, 1990).

Timing of NGF action

Although NGF has a dramatic effect on neurite outgrowth of neural crest cells in ganglionic primordia, premigratory and early migratory neural crest cells are unresponsive to NGF; they do not possess NGF receptors (Bernd, 1985). Receptor expression occurs as a later differentiative event, possibly in response to microenvironmental signals. Even after aggregation into ganglionic primordia, further time is required before these neurons become responsive to NGF.

Effects of NGF are evident in both cranial and spinal sensory ganglia obtained from 6-12 day-old chick embryos, with a maximal effect at embryonic days 8-11 (Barde *et al.*, 1980; Davis and Lindsay, 1985; Frenkel *et al.*, 1990). Aggregation of neural crest cells into the trigeminal ganglion occurs as early as 3 days of incubation (Johnston, 1966; Noden, 1975). After embryonic day 12, the effect of NGF on ganglionic cells declines, a time course that correlates with cessation of cell proliferation and subsequent onset of differentiation of spinal sensory ganglionic cells as reported from ^{3}H-thymidine autoradiography (Carr and Simpson, 1978) and RNA measurements (Yates, 1961). NGF therefore affects these neuronal precursor cells during their initial differentiation.

Sympathetic ganglia require NGF at somewhat later stages than do sensory ganglia in the same organism (Levi-Montalcini and Angeletti, 1968). This is probably due to their later onset; the longer distance over which future autonomic cells have to migrate means later aggregation into sympathetic primordia, in comparison to the shorter migration route and earlier aggregation of sensory neuronal cells (Fig. 2).

Despite the evidence implicating NGF in the differentiation of sensory and sympathetic neurons, NGF does not *initiate* neuronal differentiation, but *enhances* an already initiated differentiation process. According to Ernsberger and Rhorer (1988) a substrate coated with laminin (or less effectively, fibronectin) is required for initiation of differentiation of sensory neurons, determined by the presence of neuron-specific Q211 antigen and short neurite outgrowth. After this initiation phase, which lasts only about 9 hours *in vitro*, cells become dependent on NGF for survival and progression of neurite outgrowth (Ernsberger and Rhorer, 1988). These findings

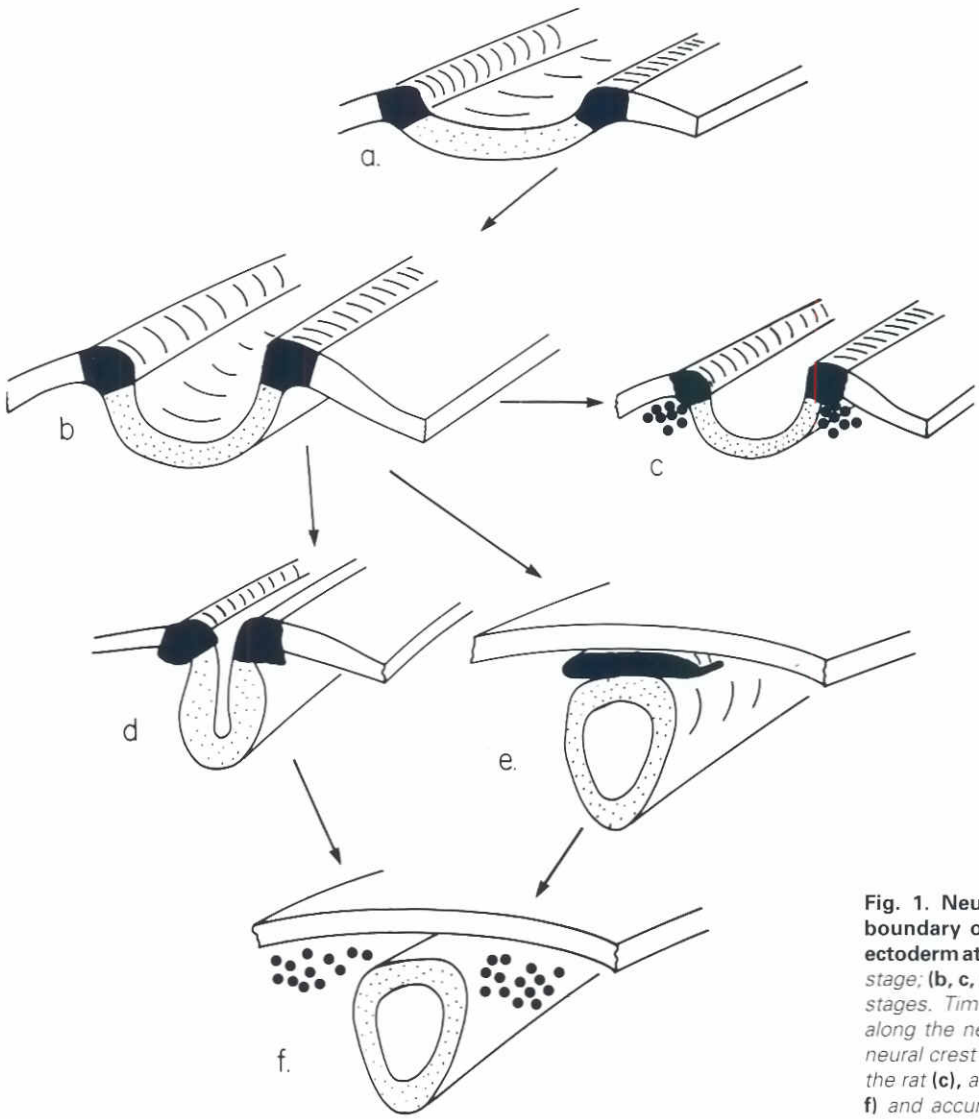

Fig. 1. Neural crest cells (black) are shown at the boundary of neural (stippled) and epidermal (white) ectoderm at various stages of neurulation; (a) *neural plate stage;* **(b, c, d)** *neural fold stages;* **(e, f)** *closed neural tube stages. Timing of migration of neural crest cells varies along the neural axis and between species, e.g., cranial neural crest cells migrate at the open neural fold stage in the rat* **(c),** *at the closed neural tube stage in the chick* **(d, f)** *and accumulate above the closed neural tube before migrating in amphibians* **(e, f).**

are further supported by Perris *et al.* (1988) who demonstrated that matrix material from presumptive sites of dorsal root ganglia induces neuronal expression in premigratory neural crest cells. A similar finding has been reported for sympathetic neurons; fibronectin and heparan sulfate proteoglycan induce neuronal expression before sympathetic precursor cells become responsive to NGF (Sieber-Blum *et al.*, 1981; Lander *et al.*, 1982). It has also been suggested that in addition to sustaining the differentiation process, NGF directs growing axons of both sympathetic and sensory neurons along an NGF concentration gradient towards their targets (Levi-Montalcini, 1976; Gundersen and Barrett, 1979, 1980; Campenot, 1982a, b).

Not all cells in ganglionic primordia develop into neurons; some differentiate into satellite cells such as Schwann cells and glia. All the non-neuronal cells in neural crest-derived ganglia are derived from the neural crest. Indeed, non-neuronal cells in all cranial ganglia, irrespective of the placodal origin of some neuronal cells, are derived from the neural crest (Noden, 1978b; D'Amico-Martel and Noden, 1983). These cells express certain neuronal properties such as catecholamine uptake (Rohrer, 1985) and NGF receptors (Zimmerman and Sutter, 1983; Rohrer, 1985; Sonnenfeld *et al.*, 1986) and respond to NGF *in vitro* by neurite outgrowth (Davis and Lindsay, 1985; Lindsay and Rohrer, 1985). Neuronal potentiality in non-neuronal (glial) cells of sensory ganglia was first demonstrated when glial cells were back-transplanted into the migration pathway and shown to transform into neuronal cells (Ayer-Le Lièvre and Le Douarin, 1982). Such findings may indicate that postmigratory neural crest cells in ganglionic primordia have the potential to become neuronal cells but that spatially and/or temporally limited amounts of NGF (and possibly other growth factors) only allow some cells to differentiate into neurons. Precursor cells which are not reached by NGF may become satellite cells. A similar binary control for the generation of cell type has been proposed by Yamada (1986, 1989) and indeed is a general feature of vertebrate and many

invertebrate developing systems (Maclean and Hall, 1987; Hall, 1991a).

Effects of other growth factors

The discovery during the last decade of several other factors that enhance the *in vitro* differentiation and survival of sensory and sympathetic neurons has revealed the complex and multistep nature of neuronal differentiation (Le Douarin, 1988a). Among these BDNF has been purified (Barde *et al.*, 1982). Other factors reported in chicken heart cell-conditioned medium (Edgar *et al.*, 1981), glioma cell-conditioned medium (Barde *et al.*, 1978) including a partially purified glial factor and factors in neural tube extracts (Kalcheim and Le Douarin, 1986) await identification.

The factor derived from the neural tube and present in the migratory environment may also be involved in initiating the differentiation of sensory neurons and may be BDNF. Both neural tube ablation (Teillet and Le Douarin, 1983) and placement of a silastic membrane barrier between the neural tube and the ganglionic primordia causes ganglionic degeneration (Kalcheim and Le Douarin, 1986). However, impregnating the barrier with neural tube extract enhances neural crest cell survival and induces neurite outgrowth from some cells (Kalcheim and Le Douarin, 1986). The neural tube extract can be replaced by BDNF and ganglia which have been separated from the neural tube can be rescued from the cell death that otherwise accompanies separation (Kalcheim *et al.*, 1987; Kalcheim and Gendreau, 1988).

In vitro studies suggest that BDNF, like NGF, can induce neurite outgrowth of dorsal root ganglionic neurons at early differentiative stages and even as late as day 16 of chick embryonic life (Lindsay *et al.*, 1985a). During early differentiative stages, the combined *in vitro* effect of NGF and BDNF on sensory ganglionic cells is greater than the effect of either alone (Barde *et al.*, 1980; Lindsay *et al.*, 1985a), indicating that sensory ganglia contain subpopulations of cells with different growth factor requirements as proposed by Edgar *et al.* (1981) and that NGF and BDNF act via separate cellular mechanisms, although both bind to the same cell surface receptor. However, BDNF is not an initiator of neuronal differentiation, but like NGF, is required for progression of ongoing differentiation (Ernsberger and Rhorer, 1988).

Unlike NGF, BDNF is ineffective on neural crest-derived sympathetic and parasympathetic neurons (Lindsay *et al.*, 1985a). BDNF injected into 3-7 day-old chick embryos increases survival of both placode- and neural crest-derived sensory neurons by preventing the programmed cell death that normally occurs. Sympathetic neurons are unaffected (Hofer and Barde, 1988). Therefore, BDNF does not selectively act on placode-derived or neural crest-derived neurons but affects sensory neurons of either origin. In contrast, NGF acts on neural crest-derived neurons regardless of their sympathetic or sensory function. Clonal cultures of sympathetic neurons have been shown to modulate their differentiation in response to NGF, heart-conditioned medium and glucocorticoids; see Patterson (1990) for a review.

Although neural crest cells differentiating into sympathetic neurons do not require BDNF for differentiation, requirements for other growth factors have been reported. Insulin and IGFs, especially somatomedin-C, induce neurite outgrowth from sympathetic precursor cells in culture (Xue *et al.*, 1988; see Table 2 for the responsiveness of various categories of neuronal precursor cells to growth factors). Insulin and IGF could act as cofactors for sympathetic neuronal differentiation (Le Douarin, 1988b). The presence of insulin-like immunoreactivity and insulin gene activity at the site of sympathetic cell migration and differentiation *in vivo* (Alpert *et al.*, 1988; Monier *et al.*, 1988) is consistent with *in vitro* effects of insulin and IGF.

bFGF is expressed in both central and peripheral neurons such as those of the dorsal root ganglia (Kalcheim and Neufeld, 1990). Pigmentation is induced in some twenty percent of neurons from dorsal root ganglia cultured in the presence of bFGF. PDGF, TGF-α, TGF-ß or NGF cannot elicit this transformation, although TGF-ß1 inhibits the bFGF-induced transformation in cell phenotype (Stocker *et al.*, 1991).

Effects of other molecules

If NGF and BDNF are secondary factors involved in the propagation of ongoing differentiation, what might the factor(s) that initiate differentiation of sensory and sympathetic neurons be? A definitive answer cannot yet be given but two possible candidates are preferred.

Laminin

The following evidence suggest that laminin may play a role as an initiating factor for neuronal differentiation.

a) Post-migratory neural crest cells from dorsal root ganglia express neuron-specific antigens and short neurite outgrowth in the absence of growth factors but in the presence of laminin (Ernsberger and Rhorer, 1988), more specifically in the presence of fragment 8 of laminin (Abbott, 1988).

b) Initial neurite outgrowth can be prevented by reducing the active laminin receptors (Cohen *et al.*, 1987, Edgar and Nurcombe, 1988).

c) The production of extracellular matrix containing laminin by glial cells surrounding neurons and the presence of laminin in the migratory pathway of neuronal precursor cells (Le Douarin, 1982) indicates that laminin is available at appropriate times *in vivo* to play an initiating role.

However, laminin may also act to propagate already induced neuronal differentiation, acting one step ahead of BDNF and NGF in a multistep differentiative cascade. As laminin is abundant in basement membranes and other extracellular matrixes it may 'merely' provide a suitable substrate either for cell attachment, enabling cells to react to growth factors in the environment, or for localization of growth factors themselves; see last section.

Vitamin A

Vitamin A (retinol) is another candidate for regulation of the differentiation of neural crest-derived neuronal cells. A cDNA probe to a chick retinoid receptor of unknown ligand (cRXR) has been localized to neural crest cells in the neural tube and during migration of trunk cells through the somites in stage-16 (2.25 day) chick embryos and in dorsal root, cranial and enteric ganglia and peripheral nerve tracts at stages 24-27 (4. 5-5. 5 days; Rowe *et al.*, 1991). This finding, when coupled with several other lines of evidence, implies that retinol and/or retinoic acid plays a role in differentiation and/or morphogenesis of both neuronal and mesenchymal neural crest cell-derivatives (for the latter see below). Cellular retinoic acid-binding protein is localized in dorsal root, sympathetic and enteric ganglia and in sensory nerves, craniofacial and visceral arch mesenchyme (Denker *et al.*, 1990; Dollé *et al.*, 1990; Maden *et al.*, 1990; Vaessen *et al.*, 1990). Synthetic retinoic acid (isotretinoin) decreases adhesion to substrata of cranial and trunk neural crest

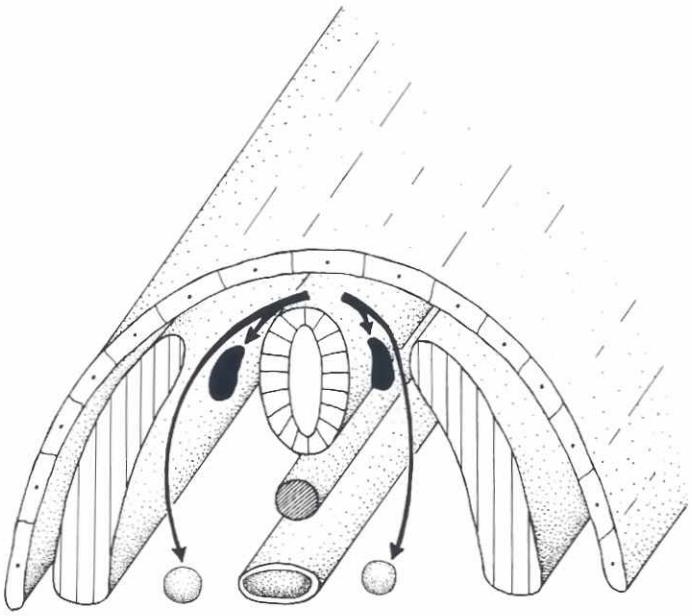

Fig. 2. A representation of the migratory routes (arrows) of neural crest cells that give rise to dorsal root sensory ganglia (black) and sympathetic chain ganglia (stippled). *Some neural crest cells migrate for a short distance over the neural tube ((horizontal shading, short arrow) and give rise to dorsal root ganglia. Others migrate over longer distances (long arrow) first over the neural tube and then beneath the dermamyotome (vertical shading) as well as through intersomite (sclerotomal) spaces (not shown) to the level of the dorsal aorta to give rise to sympathetic ganglia, shown as circles on either side of the dorsal aorta and below the notochord (close hatching). Migration under the ectoderm at the cervical level to the site of enteric ganglia is not shown.*

cells but not non-neural crest-derived fibroblasts (Smith-Thomas *et al.*, 1987). Local application of retinoic acid elicits malformations in neural crest-derived facial structures in both embryonic chicks and mice (Morriss and Thorogood, 1978; Lammer *et al.*, 1985; Wedden, 1987; Hart *et al.*, 1990; Osumi-Yamashita and Eto, 1990).

Effects of growth factors on neural crest-derived endocrine cells

Adrenal medullary (chromaffin) cells

Adrenal medullary cells are of neural crest origin (Weston, 1970; Le Douarin and Teillet, 1971; Teillet and Le Douarin, 1974). They share a common precursor with sympathetic neurons, arise from the same level of the neural crest and retain noradrenergic properties during early stages of adrenal development (Rothman *et al.*, 1978; Bohn *et al.*, 1981; Landis and Patterson, 1981).

Differentiation of medullary precursor cells into chromaffin endocrine cells is influenced by glucocorticoid hormones. That a noradrenergic clonal cell line of rat adrenal pheochromocytoma cells (PC12) produces neurite outgrowth in response to NGF gave the first indication of a possible transformation of functional endocrine cells into a neuronal phenotype under the influence of NGF (Green and Tischler, 1976; Dichter and Tischler, 1977).

It is now well documented that NGF can induce neurite outgrowth from adrenal chromaffin cells *in vitro* (Unsicker *et al.*, 1978; Landis and Patterson, 1981; Naujoks *et al.*, 1982; End *et al.*, 1983) and *in vivo* (Aloe and Levi-Montalcini, 1979; Anderson and Axel, 1985). The NGF-induced neuronal phenotype is similar to the sympathetic counterpart in morphology, neurotransmitters produced (Unsicker *et al.*, 1978; Naujoks *et al.*, 1982) and in bearing functional synapses (Ogawa *et al.*, 1984). NGF switches adrenal medullary cells into sympathetic neurons.

NGF can alter the phenotypic expression of chromaffin cells even after they exhibit endocrine properties. Such NGF-induced neuronal expression of chromaffin cells can be abolished by glucocorticoids, both *in vitro* and *in vivo* (Unsicker *et al.*, 1978; Aloe and Levi-Montalcini, 1979; Bohn *et al.*, 1981). The same studies indicate that glucocorticoids also induce these cells to regain the endocrine phenotype. There is a remarkable similarity between the endocrine secretory product of chromaffin cells (adrenaline) and one of the major neurotransmitters of sympathetic neurons (noradrenaline). Noradrenaline is converted to adrenaline by addition of a methyl group under the control of methyl transferase; both products could be processed in two different ways under the influence of different growth factors.

It appears that during normal development, cells in the sympathoadrenal branch of the neural crest lineage have the potential to differentiate into sympathetic neurons. Cells that stop migrating at the level of the dorsal aorta (Fig. 2) face an NGF rich-microenvironment and continue differentiating into sympathetic neurons. Cells that continue migrating to the level of the adrenal gland differentiate into endocrine cells. Once in the adrenal medulla, which is rich in glucocorticoids and probably other undescribed factors, the endocrine pathway of differentiation is expressed, but not terminally; neuron-specific genes that were expressed during ontogeny can still be transcribed, if the cells are provided with an appropriate stimulus such as NGF.

Calcitonin-producing cells

A similar situation can be seen in the other neural crest- derived endocrine tissue, the calcitonin-producing cells of the ultimobranchial body (parafollicular cells of the thyroid in mammals). These calcitonin-producing cells and enteric neurons of the gut arise from the vagal region (level of somites 1-7) of the neural crest (Le Douarin, 1982; Rothman *et al.*, 1986a, b). Even in the adult, parafollicular endocrine cells share some neuron-specific characters with serotonergic enteric neurons. These characters, such as the presence of 5-hydroxytryptamine-binding protein and the ability to store 5-hydroxytryptamine, indicate their common lineage, (Bernd *et al.*, 1979, 1981; Gershon *et al.*, 1983; Barasch *et al.*, 1987a). They normally produce calcitonin whereas serotonergic neurons produce calcitonin gene related peptide (CRGP). However, even after having functioned as endocrine cells, these cells can express the ontogenically related neuronal phenotype by producing neurite outgrowth and CGRP in response to NGF (Barasch *et al.*, 1987b). Clearly, growth factors can modulate these cells between endocrine and neural phenotypes. As discussed below, mesenchymal cell derivatives are not so modulated by growth factors.

Mesenchymal neural crest derivatives

The neural crest gives rise to a variety of mesenchymal derivatives, including bone, cartilage, dentine, other connective tissues and smooth muscles. These are mainly derivatives of the cephalic

neural crest, forming craniofacial structures and certain parts of the heart and walls of the main arteries (see Le Douarin, 1982; Noden, 1984; Hall and Hörstadius, 1988, and Smith and Hall, 1990 for reviews). Mesenchymal derivatives of the neural crest include all of the cartilage, bone and connective tissues of the facial skeletal (mandible, maxilla, hyoid, palate) much of the bone and cartilage of the cranial skeleton (skull and cranial base), dentine of the teeth, connective tissues of thyroid, parathyroid, thymus, pituitary and lachrymal glands, and portions of the wall and semilunar valves of the heart and aortic arches, and the connective tissue of dorsal and ventral fins in amphibians and fishes (Le Douarin, 1982; Hall and Hörstadius, 1988). Mesenchymal neural crest-derivatives segregate early from neural crest-derived neuronal and pigment precursors (Le Douarin, 1982; Hall and Hörstadius, 1988).

Unlike sympatho-adrenal derivatives of the neural crest, induced transformation of one mesenchymal derivative into another by a growth factor(s) has not been reported. In fact, most studies indicate that extracellular matrix-mediated interactions with adjacent epithelia are the major mechanism leading these mesenchymal cells into a particular differentiative pathway (see Wessells, 1977; Sawyer and Fallon, 1983; Hall, 1986, 1987a, b, 1988, 1991b, c for reviews). Growth factors are associated with stromal-epithelial interactions in the adult (Anderson *et al.*, 1990; Hall, 1991f, and see below). How far growth factors are involved in embryonic epithelial-mesenchymal interactions is the subject of the remainder of this review.

In the following sections the localization of growth factors and the effects of exogenous growth factors are discussed for the following mesenchymal neural crest-derivatives; calvarial osteoblasts, palatal mesenchyme, dentine-forming odontoblasts of the teeth, mandibular mesenchyme, Meckel's cartilage and mandibular membrane bones. Additionally, possible roles for growth factors in epithelial-mesenchymal interactions involving palatal, dental and mandibular mesenchyme are discussed.

Distribution of growth factors in neural crest mesenchymal derivatives

Various growth factors have been localized in mesenchymal derivatives of the neural crest as discussed below. Growth factor distribution in dental and palatal mesenchyme is discussed separately and in more detail below (also see Ferguson, 1988; Sharpe and Ferguson, 1988; Nilsen-Hamilton, 1990 and Foreman *et al.*, 1991 for reviews).

TGF-ß1 mRNA has been localized in osteocytes of murine mandibular membrane bone at 14.5 days gestation; mRNA but not the protein has also been localized in cranial epithelia at 14.5 days (Lehnert and Akhurst, 1988), suggesting a role for growth factors in epithelial-mesenchymal interactions. TGF-ß2 mRNA is found in murine mesenchyme, osteoblasts and chondroblasts (Pelton *et al.*, 1989, 1990b) in patterns that also suggest a role in epithelial-mesenchymal interactions. Similar distributions of TGF-ß1 and ß2 mRNA have been reported from human embryos (Gatherer *et al.*, 1990).

TGF-ß1-ß3 mRNAs are found in palatal mesenchyme and epithelia in patterns related to palatal shelf elevation, *i.e.*, at active sites of epithelial-mesenchymal interactions (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990a; Millan *et al.*, 1991; and see below). In a different pattern, palatal mesenchyme has been reported to have very high levels of mRNA for TGF-ß1 but not of TGF-ß2 or ß3 (Schmid *et al.*, 1991).

IGF-I and/or IGF-II mRNAs have been localized in both neural crest- and mesodermally-derived mesenchyme, in endoderm but not in ectoderm or ectodermally-derived epithelia. IGF-1 and -II mRNA is present in high amounts in cartilage, but decreases when cartilage undergoes hypertrophy or is replaced by bone (Beck *et al.*, 1987, 1988; Han *et al.*, 1987; Stylianopoulou *et al.*, 1988; Ayer-Le Lièvre *et al.*, 1991). Receptors for IGF-I and IGF-II can be isolated from fetal osteoblasts and IGF-I and IGF-II have been reported as products of bone cells stored in bone matrix (Centrella *et al.*, 1990).

NGF has been identified in cartilage and bone from femorae of embryonic chicks both by visualization using a polyclonal antibody and by stimulation of neurite outgrowth from dorsal root ganglia cultured in homogenates of bone, a stimulation that is abolished by anti-NGF (Frenkel *et al.*, 1990).

Effects of growth factors on the development of calvaria

We now turn to an examination of the effects of growth factors on one neural crest mesenchymal derivative, the osteoblasts of the calvaria that form the embryonic skull. In particular, we examine the effects of TGF-ßs, EGF and the BMPs, for it is these factors that appear to play a role in differentiation of osteogenic mesenchyme and/or in the epithelial-mesenchymal interactions that regulate that differentiation. For recent overviews of the role of growth factors in the differentiation of cartilage and bone see Goldring and Goldring (1990), Hauschka (1990), Nilsen-Hamilton (1990) and Stein *et al.* (1990).

TGF-ß is known to induce cartilage formation even from mesenchymal cells normally destined to form muscle (Seyedin *et al.*, 1986). In addition, TGF-ß induces proliferation of certain cell types (De Larco and Todaro, 1978), promotes wound healing (Sporn *et al.*, 1983) and bone resorption (Tashjian *et al.*, 1985). Cells from fetal rat calvaria are affected *in vitro* by TGF-ß in a number of different ways as described below.

Mitogenic effects of TGF-ß on calvarial cells

Centrella *et al.* (1986) reported that TGF-ß enhanced DNA synthesis by calvarial cells in organ culture, a finding confirmed by Katagiri *et al.* (1990) for murine MC3T3-E1 cells derived from calvarial osteoblasts, by Pfeilschifter *et al.* (1990b) for osteoblast-like cells, and by Hock *et al.* (1990) in intact calvaria in which synthesis of bone matrix was also enhanced. The mitogenic response required a higher TGF-ß concentration (30ng/ml) than did the effect on matrix synthesis (1-30ng/ml). Differential responses to TGF-ß at different concentrations may also explain how TGF-ß can act as a stimulator of both matrix synthesis and of matrix resorption (Tashjian *et al.*, 1985; Hock*et al.*, 1990)

TGF-ß does not exert a direct proliferative effect on calvarial cells *in vitro*, but rather potentiates the proliferative effect of a- and bFGF (Globus *et al.*, 1988; Nicholas *et al.*, 1990). bFGF and to a lesser extent a FGF decrease alkaline phosphatase and increase collagen synthesis in calvarial cell cultures (McCarthy *et al.*, 1989). Continuous exposure to EGF reduces osteoblastic differentiation, as evidenced by decrease in bone nodule formation, while pulsed exposure to EGF increases bone nodule formation (Antosz *et al.*, 1989). Thus, mode of application of growth factors affects their actions. PDGF increases both cell replication and collagen synthesis (Canalis *et al.*, 1989; Centrella *et al.*, 1989b). When considering that bone contains a number of growth factors including FGFs (Hauschka *et al.*, 1986; Hauschka, 1990), enhanced proliferation

TABLE 2

RESPONSIVENESS OF VARIOUS CATEGORIES OF NEURONAL PRECURSOR CELLS TO GROWTH FACTORS

	sympathetic neural crest	**sensory** neural crest	**sensory** placodal	**parasympathetic** neural crest
NGF[a]	+	+	-	?
BDNFb	-	+	+	-
IGFc	+	-	?	?

+ and - indicate responsiveness or unresponsiveness of cells respectively.
? indicate uncertainty of the response.
a. Lindsay and Rohrer (1985) and Levi-Montalcini (1987)
b. Lindsay *et al.*, (1985b)
c. Xue *et al.*, (1988)

following addition of TGF-ß to calvaria in organ culture (Centrella *et al.*, 1986) may be an indirect effect of TGF-ß.

There is also a recent report of the isolation of three low molecular weight (770, 1050 and 1600 daltons) peptides from murine calvariae that stimulate mitotic activity of osteoblasts but not fibroblasts. These peptides are much smaller than the smallest growth factors known from bone, and growth factors such as TGF-ß and EGF stimulate mitosis in both osteoblasts and fibroblasts (Birnbaum and Andress, 1990). The relationship, if any, of these peptides to growth factor action has to be determined.

Differentiative effects of TGF-ß on calvarial cells

There are now numerous reports of TGF-ß modulating phenotypic expression of osteoblasts derived from rat calvariae, assessed by decrease in alkaline phosphatase (Centrella *et al.*, 1988, 1989a; Rosen *et al.*, 1988) or decrease in the number of bone nodules produced (Antosz *et al.*, 1989). Effects of hormones such as PTH on calvarial cells are also in part modulated by TGF-ß (Centrella *et al.*, 1989c).

Calvarial cells are not homogeneous and heterogeneity for response to TGF-ß has been reported (Guenther *et al.*, 1989). Bone contains cell populations that differ from one another in growth and hormone response (Aubin *et al.*, 1982). Distinct sub-populations ranging from predominantly fibroblastic to osteoblastic have been isolated from calvariae (Wrana *et al.*, 1988). Differentiative effects of TGF-ß vary with calvarial cell type and culture conditions. By examining these cell populations for their responsiveness to TGF-ß, it has been shown that calvarial fibroblast- cultures, but not osteoblast-enriched cultures, produce osteonectin or related proteins together with other matrix components such as collagen and fibronectin in response to TGF-ß (Grigoriadis *et al.*, 1988, 1989, 1990; Wrana *et al.*, 1988). In fact, in osteoblast-enriched cultures, osteoblastic features including alkaline phosphatase activity are suppressed by TGF-ß (Rosen *et al.*, 1988; Wrana *et al.*, 1988).

Inhibition of the phenotypic expression of already differentiated skeletal cells by TGF-ß has been reported from other sources as well. For example, MC 3T3-E1 cells (Noda and Rodan, 1986, 1989) and chondroblasts from rat long bone (Rosen *et al.*, 1988) lose their differentiated status in the presence of TGF-ß. Moreover, TGF-ß has been shown to stimulate prostaglandin synthesis and bone resorption in cultured mouse calvariae (Tashjian *et al.*, 1985). TGF-ß1 and ß2 have also been shown to initiate osteogenesis *in vivo* following injection over the frontal and parietal bones in mice (Mackie and Trechsel, 1990).

How can these multifunctional roles of TGF-ß be explained? At least two variables determine the final outcome of TGF-ß action. First, interactions with other growth factors, hormones and other effective molecules present in bone modulate the effect of TGF-ß. Second, different bone cell populations (or bone cells at different developmental stages) respond differently to the same growth factors present at the same concentrations. Four lines of evidence can be provided.

(a) Bone is a tissue rich in growth factors, some such as TGF-ß and IGF-II secreted by osteoblasts themselves, others such as FGF and PDGF exogenously derived but trapped in the bone matrix (Hauschka *et al.*, 1986; Hauschka, 1990) and able to modulate other growth factor effects.

(b) The action of TGF-ß on calvariae can be modulated by other factors present in bone such as parathormone and vitamin D_3 (Petkovich *et al.*, 1984; Pfeilschifter and Mundy, 1987).

(c) TGF-ß can stimulate the production of factors such as prostaglandin (Tashjian *et al.*, 1985).

(d) Diverse cell populations are present in the calvarium, each with its own responsiveness to growth factors (Grigoriadis *et al.*, 1988, 1989, 1990; Wrana *et al.*, 1988).

Effects of other growth factors on calvarial cells

Seven bone morphogenetic proteins (BMPs) have now been isolated and cloned (Wozney *et al.*, 1988; Celeste *et al.*, 1990). BMP 2 - BMP-7, members of the TGF-ß supergene family (Table 1) are important in skeletogenesis. BMP-2 is capable of initiating chondrogenesis when used alone (Wozney *et al.*, 1988). However, the BMPs do not initiate osteogenesis directly; cartilage is first induced, and *in vivo*, chondrogenesis is followed by a phase of cartilage erosion and endochondral ossification. Takuwa *et al.* (1991) reported that BMPs have variable effects upon MC3T3-E1 cells; BMP-2 increases differentiative activity, as evidenced by increase in alkaline phosphatase activity; BMP-2 and BMP-3 slightly enhance collagen synthesis, and none of BMP 1-3 affected proliferation.

a and bFGF have been reported to stimulate bone cell proliferation and to inhibit collagen synthesis and alkaline phosphatase activity in fetal rat calvariae or calvarial cell cultures (Canalis *et al.*, 1988 Globus *et al.*, 1988; Nicolas *et al.*, 1990). FGF is both a less potent mitogen and a more effective inhibitor of alkaline phosphatase than EGF (Nicolas *et al.*, 1990).

EGF exerts a biphasic effect on fetal rat calvarial cells. Exposure to EGF for a short period of time (4 h-48 h) enhances bone nodule formation *in vitro*. Exposure for longer than 4 days decreases bone nodule formation (Antosz *et al.*, 1987). IGF-I, PDGF and TGF-ß1 act synergistically to enhance bone formation in cultured fetal rat calvariae. When used alone TGF-ß1 is the more potent stimulator of bone formation (Pfeilschifter *et al.*, 1990a). IGF-I also stimulates the formation of bone matrix in fetal rat calvaria (Hock *et al.*, 1988). Studies on TGF-ß and these other growth factors indicate their involvement in calvarial development, especially during late embryonic stages. However, how far growth factors are involved in *directing* neural crest-derived mesenchymal cells towards a particular skeletogenic pathway is unclear.

Studies on growth factor involvement in calvarial development have treated the calvarium as a single unit, without special

emphasis on the neural crest-derived portion of the calvarium. In fact, the calvarium is composed of both neural crest and mesodermally-derived membrane bones (Noden, 1978a, 1984). Most of the calvarium is occupied by the large, flat parietal and frontal bones. Part of the frontal is derived from neural crest while the remainder of the frontal and the parietal bones are mesodermal derivatives (Le Lièvre and Le Douarin, 1975; Le Lièvre, 1978; Noden, 1978a, 1984; see Le Douarin, 1982 for an excellent review). Therefore, it is difficult to tell whether both neural crest-derived and mesodermally-derived cells are present in prepared calvarial cell cultures and respond equally to growth factors or whether cells from different origins respond to growth factors differently. Studying the neural crest-derived portion of the calvarium or cell lines derived from it separately from the mesodermal portion would allow recognition of any effects of growth factors on calvarial cells of different origins.

Palatogenesis

The mammalian secondary palate is a fusion product of two palatal processes; see Pratt (1984) and Ferguson (1988) for reviews. Palatal shelf fusion is also the developmental event that separates the nasal passage from the oral cavity. Interruption to the fusion of these processes can lead to cleft palate as seen in 1 in every 600 births in North America (Pisano and Greene, 1986).

Palatal mesenchyme is derived from the cephalic neural crest (Le Liévre and Le Douarin, 1975; Le Liévre, 1978; Noden, 1978a). Development of the palate involves a hierarchy of complex mechanisms, including but not limited to, mesenchymal cell proliferation, palatal shelf elevation; death of the medial epithelial cells on each palatal process (and/or the transformation of epithelial cells into mesenchyme), subsequent fusion of the palatal shelves into a continuous palatal shelf, and epithelial-mesenchymal interactions governing osteogenesis within the palatal mesenchyme (see Pratt, 1984; Ferguson, 1988, and Sharpe and Ferguson, 1988 for overviews). Mesenchyme and epithelium of the embryonic palate require growth factors and hormones for the normal progression of these phases (see Pisano and Greene, 1986 and Pratt, 1987 for reviews). Interruption to the action of growth factors and hormones during palatal fusion can lead to cleft-palate (Pratt, 1987). The most widely studied factors in this regard are EGF and steroids such as glucocorticoids and retinoic acid; see below.

Localization and effects of growth factors on palatogenesis

TGF-ß1-ß3 have been visualized in the murine palate between 11.5 and 15.5 days of gestation. TGF-ß3 appears first, initially in the epithelium of the vertical palatal shelves; TGF-ß2 appears in the epithelium of horizontal shelves, followed by TGF-ß2 in mesenchyme under the median epithelium of the fusing shelves (Sharpe and Ferguson, 1988; Fitzpatrick *et al.*, 1990; Foreman *et al.*, 1991; Gehris *et al.*, 1991). TGF-ß mRNAs are present at these active sites of epithelial-mesenchymal interactions, indicative of local synthesis (Pelton *et al.*, 1990a; Millan *et al.*, 1991).

Exogenous TGF-ß increases synthesis of extracellular matrix proteins such as fibronectin and collagen types III, IV and V but inhibits synthesis of collagen type I. EGF and TGF-α stimulate collagen and glycosaminoglycan synthesis and proliferation of palatal mesenchyme. PDGF also stimulates proliferation, while both a- and bFGF decrease extracellular matrix synthesis (Sharpe and Ferguson, 1988). Linask *et al.* (1991) report differences between murine and human palatal mesenchymal cells, both in response to TGF-ß1, which increases proliferation in human but decreases proliferation in murine cells, and in TGF-ß receptors; mouse palatal mesenchyme has receptor types I-III, human has only I and III. Whether these differences reflect alterations in a long-established human cell line or real species differences that obtain *in vivo* is unclear.

Murine palatal mesenchymal cells bear high levels of EGF receptors during mid-gestation (Yoneda and Pratt, 1981a, b; Sharpe and Ferguson, 1988). Removal of EGF from a chemically defined medium results in markedly reduced growth of palatal mesenchymal cells (Yoneda and Pratt, 1981a, b). However, exposure of mice to high doses of EGF *in vivo* results in cleft palate, mainly due to prevention of programmed cell death in the palatal epithelium (Tyler and Pratt, 1980). Effects of high doses of EGF can be seen in mesenchyme as well. Increased proliferation and enhanced production of ECM components such as hyaluronic acid and fibronectin leaves large extracellular spaces among mesenchymal cells (Silver *et al.*, 1981,1984; Turley *et al.*, 1985). However, TGF-α has been found in higher levels than EGF during embryonic development (Nexo *et al.*, 1980). Nexo *et al.* (1980) suggested that TGF-α, which is functionally and structurally related to EGF (Gregory, 1975; Marquardt *et al.*, 1984), and which binds to EGF receptors with high affinity, may be the embryonic form of EGF.

Although the importance of EGF involvement during normal palatogenesis is still obscure, the dramatic EGF-induced effects seen *in vitro* may indicate the importance of EGF in modulating synthesis of ECM by mesenchymal cells that normally occurs during the fusion process *in vivo*.

EGF is not responsible for initiation of the differentiation of palatal mesenchymal cells. Rather, studies regarding osteogenesis indicate the importance of extracellular matrix-mediated epithelial-mesenchymal interactions in the differentiation of osteogenic cells in palatal mesenchyme (Tyler and McCobb, 1981). It remains to be determined whether growth factors are involved in such tissue interactions but their localization at active sites of epithelial-mesenchymal interaction is highly suggestive of a functional role.

Glucocorticoids and palatogenesis

Glucocorticoids are also involved in normal palatal mesenchymal development (Salomon and Pratt, 1976, 1978, 1979; Goldman *et al.*, 1978; Yoneda and Pratt, 1981a, b). Physiological doses of glucocorticoids promote proliferation and growth of mesenchymal cells *in vitro* (Salomon and Pratt, 1978, 1979). High doses inhibit mesenchymal cell proliferation and decrease ECM synthesis (Saxén, 1977; Salomon and Pratt, 1979), an effect that is opposite to the action of high doses of EGF. Interactions between EGF and glucocorticoids occur in the growth of mesenchymal cells *in vitro* (Yoneda and Pratt, 1981a, b). Dexamethasone, a synthetic glucocorticoid, enhances the binding of EGF while EGF decreases the number of glucocorticoid binding sites per cell. This *in vitro* evidence may indicate regulatory mechanisms of growth factor action on the normal growth and differentiation of palatal mesenchymal cells *in vivo*. However, that different strains of mice possess remarkably different degrees of susceptibility to high doses of glucocorticoids (Biddle and Fraser, 1976) and that there are 2-3 fold lower levels of glucocorticoid receptors per cell in less susceptible strains (Salomon and Pratt, 1976; Goldman *et al.*, 1977) indicates that normal palatogenesis occurs under different glucocorticoid (and therefore different growth factor?) levels in different strains.

TABLE 3

GROWTH FACTORS IN DEVELOPING MAMMALIAN TEETH TO ILLUSTRATE THE STAGE-DEPENDENT DISTRIBUTION IN EPITHELIAL AND MESENCHYMAL COMPONENTS

	NGFr	EGFr	TGF-ß1	BMP-4
dental lamina	+			
enamel organ		+		
dental papilla	+			
bud stage				
epithelium		+++	-	+
mesenchyme		-	+	-
cap stage				
epithelium		-	+	-
mesenchyme		+++	-	+
bell stage				
epithelium		+		
mesenchyme		+		

See text for details and literature

Vitamin A and palatogenesis

A number of studies have indicated the possible effect of another steroid, vitamin A, on palatogenesis. Inclusion of vitamin A in media in which early chick embryos are cultured inhibits migration of neural crest cells into the first branchial arch from which palatal mesenchyme is derived, resulting in embryos with facial malformations (Hassell *et al.*, 1977). Such exogenously applied vitamin A probably acts through retinoic acid. Cleft palate induced by retinoic acid has been reported to be caused mainly by action on the medial epithelium (Kochhar and Johnson, 1965; Abbott and Pratt, 1987). However, palatal mesenchyme in organ culture has also been reported to be affected by retinoic acid (Abbott and Pratt, 1987). Although the mechanism has not been fully described, there is evidence that retinoic acid mimics effects of EGF (Abbott and Pratt, 1987) and enhances EGF binding and synthesis of EGF receptors in several cell culture systems (Rees *et al.*, 1979; Jetten, 1980, 1982), including palatal mesenchymal cells (Kukita *et al.*, 1987).

Tooth formation

Until very recently, evidence for the neural crest-origin of the odontoblasts and dentine of vertebrate teeth rested on the elegant studies on the urodele *Pleurodeles waltl*, evidence that was extrapolated to other toothed vertebrates such as fishes and mammals (Chibon, 1966, 1967; see reviews in Le Douarin, 1982; Hall, 1987a; Hall and Hörstadius, 1988). However, recently Lumsden (1987) has provided experimental evidence for tooth production from tissue recombinations of cranial neural crest and mandibular epithelium obtained from mouse embryos and Smith and Hall (1990) have marshalled evidence for the neural crest origin of odontoblasts in fishes.

Mammalian teeth develop by a series of epithelial-mesenchymal interactions between oral epithelium and mesenchyme in a series of typological stages from dental lamina through enamel organ, dental papilla, bud, cap and bell stages, such that odontogenesis is exquisitely controlled both at the cellular and at the molecular levels (Berkovitz and Moxham, 1981; Lesot, 1986; Lumsden, 1987; Ruch *et al.*, 1987; Slavkin *et al.*, 1988; Ruch, 1990; Slavkin, 1990a, b; Andujar *et al.*, 1991). Growth factors are emerging as important elements in that control.

Localization of growth factors

A feature of the distribution of growth factors and their receptors that is emerging from studies on tooth development in murine embryos is stage-dependent shifts in localization between the epithelial and mesenchymal components (Table 3). Such shifts are precisely the behavior expected of a molecule(s) involved in mediating epithelial-mesenchymal interactions as will now be discussed.

Distinctive patterns of distribution of EGF and TGF-ß in developing rodent teeth have been described in both the oral epithelium and the enamel-forming ameloblasts that arise from it and in the neural crest-derived mesenchyme and the dentine-forming odontoblasts that arise from it (Cam *et al.*, 1990; D'Souza *et al.*, 1990a, b; Nilsen-Hamilton, 1990; Partanen, 1990; Slavkin, 1990a, b; Slavkin *et al.*, 1990). The level of EGF receptors is high between bud and cap stages; in the oral epithelium at the bud stage and in the mesenchyme at the cap stage. Receptor levels are very low in both tissues at the bell stage (Partanen, 1990). Studies using ^{125}I-EGF also indicate the presence of EGF binding sites in dental mesenchymal cells at the cap stage (Partanen and Thesleff, 1987). Receptors for EGF are found in enamel organs and in apical mesenchyme of rodent incisors after birth (Cam *et al.*, 1990).

TGF-ß1 is found in dental mesenchyme but not epithelium at the bud stage (12.5 days in the mouse) and in dental epithelium but not mesenchyme at the cap stage (14.5 days in the mouse; Lehnert and Akhurst, 1988). D'Souza *et al.* (1990a, b) reported TGF-ß1 localization in both epithelial and dental papilla at the bell stage, the different patterns with two antibodies reflecting intra- and extracellular localization. BMP-4 (BMP-2A) is found in epithelial cells at the base of the tooth bud at 12.5 days and in dental mesenchyme and odontoblasts at 14.5 days, a pattern that holds in newborn mice (Lyons *et al.*, 1990).

Receptors for NGF are found in both the epithelial dental lamina and in dental mesenchyme of injured rodent teeth (Byers *et al.*, 1990). The growth factor-inducible genes, early growth-response-1 (*Egr*-1) and the proto-oncogene *c-fos* are induced in dental mesenchyme at the bud stage (McMahon *et al.*, 1990) while the FGF-like proto-oncogene *int*-2 is expressed in dental mesenchyme at cap and bell stages (Thesleff *et al.*, 1990).

Effects of EGF on tooth formation

Dental papilla and mandibular mesenchyme have been reported to be affected by EGF and some other growth factors. When exposed to EGF in organ culture, morphogenesis and differentiation of odontoblasts and ameloblasts is inhibited in tooth germs taken at the cap stage but not in bell and older stages (Partanen *et al.*, 1985; Partanen, 1990; Thesleff *et al.*, 1990). As evident from (^{3}H) thymidine incorporation, inhibition results from inhibition of the proliferation of mesenchymal cells (Partanen *et al.*, 1985).

EGF also acts on cells of the dental follicle, a population of neural crest- derived mesenchymal cells that produces the periodontal ligament surrounding and anchoring the tooth into the bone. EGF is mitogenic for these cells (Partanen and Thesleff, 1987) inducing premature eruption of mouse incisors (Rihtniemi and Thesleff, 1987; Topham *et al.*, 1987). EGF also inhibits differentiation at later stages of tooth development, inhibiting type I collagen synthesis by odontoblasts in parallel with inhibition of enamel protein

synthesis by ameloblasts in cultured tooth germs from 17-19-day-old embryonic mice (Hata *et al.*, 1990).

In the palate, EGF maintains epithelial proliferation via the mesenchyme (Tyler and Pratt, 1980). As it is the dental epithelium that responds to EGF by enhanced proliferation, while receptors are high in the mesenchyme, odontogenic mesenchymal cells are also the target for EGF, enhancing epithelial proliferation via an epithelial-mesenchymal interaction. Epithelial cells in turn regulate proliferation of mesenchymal cells (Partanen, 1990). A similar situation is seen in the response of mammary glands to testosterone; receptors are localized on mesenchymal cells but the testosterone-specific response is effected by mammary epithelial cells (Kratochwil and Schwartz, 1976).

In addition to EGF, certain serum factors are also required by developing tooth germs in organ culture. EGF increases the proliferation of disaggregated dental papilia mesenchymal cells cultured in monolayers (Partanen *et al.*, 1985), indicating that the effect of EGF on dental papilla mesenchymal cells in intact tooth germ is regulated by other factors locally present. Serum requirements for morphogenesis extend from the bud to the cap stage in mouse tooth germs (Partanen, 1990). Transferrin is one serum factor that is necessary for tooth morphogenesis and subsequent differentiation of odontoblasts and ameloblasts (Partanen *et al.*, 1984).

Inhibition of ameloblast and odontoblast differentiation as a result of the mitogenic effects of EGF on dental epithelial and dental papilla mesenchymal cells is not surprising; the final differentiation of ameloblasts and odontoblasts results from a series of mutual interactions between dental epithelium and mesenchyme (see Lumsden, 1987; Ruch, 1990; Slavkin, 1990a, b for reviews). Interruption of this series of mutual interactions could inhibit the appearance of final differentiative epithelial (enamel) and/or mesenchymal (dentine) products. In fact, in two recent reports, Kronmiller *et al.* (1991a, b) have reported the isolation of EGF mRNA from both epithelial and mesenchymal tooth components of 9 and 10 day mouse embryos (when the interactions are initiated) and that exposure of mandibular tissues from 9-day-old embryos to an EGF antisense oligodeoxynucleotide inhibits odontogenesis.

Facial mesenchyme and mandibular skeletogenesis

As space does not permit discussion of all the mesenchymal neural crest derivatives, development of facial mesenchyme and skeletogenesis in mandibular mesenchyme have been selected to illustrate the current status of knowledge of growth factor involvement in proliferation and differentiation of neural crest-derived mesenchyme and mesenchymal derivatives. Mandibular mesenchyme, derived from the neural crest, is the source of Meckel's cartilage and the membrane bone(s) of the mandibular skeleton. The following growth factors have been localized in craniofacial cartilage and bone.

D'Souza *et al.* (1990a) reported TGF-ß1 localization within both extracellular matrix and osteoblasts of mandibular membranous bone surrounding tooth germs and in the extracellular matrix of proliferating Meckelian chondrocytes in the rat. Hypertrophic chondrocytes are labelled with a second antibody for intracellular TGF-ß (*ibid*, Jakowlew *et al.*, 1991). IGF-I is weak or absent in prechondrogenic mesenchymal condensations but appears in chondrocytes, especially hypertrophic chondrocytes. It is also weak in recently-deposited bone but stronger in later bone, *i.e.*, IGF-I is associated with differentiation rather than proliferation, although IGFs have a general stimulatory effect on proliferation in general (Ralphs *et al.*, 1990). IGF-II mRNA is also high in facial mesenchyme, in cartilage and in perichondria (Beck *et al.*, 1987, 1988; Stylianopoulo *et al.*, 1988; Ayer-Le Lièvre *et al.*, 1991). IGF-I and -II bind to the same receptor. bFGF acts differentially on different neural crest-derived mesenchymal populations, affecting growth and chondrogenesis of avian frontonasal but not maxillary or mandibular mesenchyme maintained in micromass culture (Richman and Crosby, 1990).

Meckelian chondrocytes differentiate after an interaction between mesencephalic cranial neural crest-derived mesenchyme and cranial epithelium prior to mesenchymal migration from the neural crest, an epithelial-mesenchymal interaction that is typical of all craniofacial cartilages and bone (Bee and Thorogood,1980; Hall, 1987a). Epithelial-mesenchymal interactions promote cytodifferentiation, in part by maintaining proliferative activity within subpopulations of mesenchymal cells (Hall, 1982, 1991d; Fyfe and Hall, 1983; Hall and Coffin-Collins, 1990). EGF has been shown to be mitogenic for mandibular mesenchyme, to delay differentiation of chondrocytes that give rise to Meckel's cartilage but not to alter their differentiative fate (Coffin-Collins and Hall, 1989; Hall and Coffin-Collins, 1990). The latter is in contrast to the role of EGF in neuronal and endocrine neural crest derivatives.

Minkoff and his colleagues have documented regional differences in mitotic activity in facial processes (chiefly the maxillary) within the embryonic chick, cell proliferation being higher at boundaries or tips of processes and in sub-epithelial mesenchyme (Minkoff, 1980, 1984; Bailey *et al.*, 1988). Cells that cycle more slowly are chondrogenic (Minkoff and Martin, 1984). Facial mesenchyme, in common with chondrogenic and osteogenic mandibular mesenchyme, requires an epithelial influence to maintain viability and normal rates of cell proliferation (Saber *et al.*, 1989). Therefore, epithelia of facial processes regulate mesenchymal proliferation and, at least *in vitro*, EGF can substitute for that epithelial signal (Hall and Coffin-Collins, 1990). The promotion of mesenchymal cell proliferation by EGF may, in part, explain the delay in chondrocyte differentiation elicited by EGF.

BMP-4 is found in facial mesenchyme of 9-day and older murine embryos, especially associated with condensing mesenchyme of whisker primordia (Jones *et al.*, 1991) where NGF and NGF receptor mRNA is also localized. BMP is also found in mandibular membrane bone (Silbermann *et al.*, 1990). Hall (1988) described BMP at the epithelial-mesenchymal interface in mandibular processes of embryonic chicks at the stage (HH 22; 3 1/2-4 days of incubation) when the epithelial-mesenchymal interaction initiating osteogenesis is occurring. Epithelial cells and bone may both act as skeletogenic inducers because they share a common mechanism such as the production of BMP (Anderson, 1990). This is an attractive idea and it is known that the synthesis of mesenchymal products that are deposited into epithelial basement membranes (laminin, fibronectin) is facilitated by epithelial-mesenchymal interaction (Xu *et al.*, 1990a, b). However, it has so far not been possible to initiate osteogenesis in isolated embryonic mandibular mesenchyme exposed to BMP (Hall, unpublished. observation).

Although BMP appears ineffective, recently, EGF in combination with sodium fluoride has been shown to provide a sufficient stimulus for osteogenesis to be initiated in mandibular mesenchyme from the embryonic chick maintained *in vitro* in the absence of serum (Hall, 1991e). Osteogenesis does not occur with EGF or NaF alone unless serum is included in the culture medium *(ibid.)*. The impli-

cation is that the correct mix of growth factors *in vitro* (and their correct organization on the basement membrane *in vivo;* see below) can initiate osteogenesis in neural crest-derived mesenchyme.

Taken together, actions on dental and mandibular mesenchyme show that growth factors are involved in the normal development of both systems; see Mina *et al.* (1990) for a recent discussion. However, there is no evidence that growth factors, other than EGF, either alone (dental mesenchyme; Kronmiller *et al.*, 1991b) or combined with NaF (mandibular mesenchyme; Hall, 1991e) are involved in generation of specific cell types from neural crest-derived mesenchymal cells as occurs in neuronal or endocrine neural crest cell derivatives.

Growth factor sequences in extracellular matrix products

Studies in which growth factors such as BMP are localized at sites of epithelial-mesenchymal interactions, or in which growth factors are shown to substitute for a signal normally provided to mesenchymal cells by an epithelium (*e.g.*, EGF providing the proliferative signal normally provided to mandibular mesenchyme by mandibular epithelium) are of especial interest in the light of the dual findings that normal constituents of epithelial basement membranes contain growth factor sequences (this section) and that growth factors bind to extracellular matrix products (next section).

The B1 chain of laminin, the 800 kDa triple chain glycoprotein of basement membranes, has seven domains with cysteine-rich regions homologous to EGF (Sasaki *et al.*, 1987). That fragments of laminin containing these EGF-like repeats stimulate division in cells possessing EGF receptors but not in cells that lack such receptors indicates that laminin can function as does EGF and over the same dose response range (Panayatou *et al.*, 1989).

Entactin, a 150kDA sulfated glycoprotein which covalently complexes with laminin has six EGF-type domains acting as a cell attachment region (Durkin *et al.*, 1988).

Tenascin, a hexameric, multidomain protein of 190-240 kDa subunits has thirteen consecutive EGF-like regions (Adams *et al.*, 1988). Interestingly, the synthesis of tenascin can be induced by TGF-ß.

Other molecules, such as some of the blood clotting factors (thrombospondin) and cartilage matrix components (cartilage matrix protein) also contain EGF-like repeats, with the former acting in calcium binding. There may be a diversity of roles of such growth factor sequences in matrix and plasma molecules; see Engel (1989, 1991) for reviews. Such findings raise the possibility that growth factor sequences in basement membrane components could act as signaling systems for neural crest-derived mesenchymal cells.

Binding of growth factors to matrix components

The importance of the binding of growth factors to components of extracellular matrixes is only now being realized as it becomes apparent that growth factors form an integral part of the 'extracellular matrix code' required for the regulation of cell differentiation (Hall, 1988) and see Nathan and Sporn (1991) for a recent overview.

Specificity of binding of growth factors to components of both mesenchymal and epithelial extracellular matrixes has recently been described; selective binding of a- and bFGF to syndecan and other heparan sulfate proteoglycans (Jeanny *et al.*, 1987; Folkman *et al.*, 1988; Vigny *et al.*, 1988; Bernfield and Sanderson, 1990), and of IL-3 to heparin and heparan sulfate (Ruoslahti and Yamaguchi, 1991). TGF-ß binds to decorin, a proteoglycan associated with type I collagen, to biglycan, to the core protein of proteoglycans and to fibronectin (Fava and McClure, 1987; Moordian *et al.*, 1990; Okuda *et al.*, 1990; Ruoslahti and Yamaguchi, 1991). TGF-ß1 and osteogenin (BMP-3; Sampath *et al.*, 1990), bind to type IV collagen (Paralkar *et al.*, 1990, 1991; Vukicevic *et al.*, 1990). That the binding of TGF-ß to the proteoglycans can inhibit TGF-ß activity (Yamagouchi *et al.*, 1990) highlights the functional significance of growth factor-matrix molecule interactions.

Therefore, basement membrane molecules such as laminin, entactin and tenascin, contain EGF- sequences; growth factors bind to and are functionally modified by components of extracellular matrixes; growth factors are localized at boundaries where epithelial-mesenchymal interactions that control initiation of differentiation occur; and basement membrane components such as laminin can potentiate the effects of growth factors on neuronal neural crest-derivatives. Given these observations, growth factors and growth factor-matrix molecule complexes are likely regulators of mesenchymal proliferation and modulators of epithelial-mesenchymal interactions.

Summary

During neurulation, neural crest cells migrate to many regions of the body to give rise to a wide variety of cell types. Many premigratory neural crest cells are pluripotent, their potency for differentiation being gradually restricted as they migrate along definite pathways and interact with factors present in the microenvironment. Effects of growth factors on these cells have been discussed in the present review. Mediation of growth factors in differentiation varies with the cell type. Growth factors exert a direct influence on the differentiation of neural and other related neural crest-derived tissues such as endocrinal tissues but evidence for such influences on neural crest-derived mesenchymal tissues is limited. For example, NGF, BDNF, and other factors present in neural tube extracts and glioma cell conditioned medium are essential for the differentiation of sensory neurons. Similarly, NGF, insulin, IGFs and possibly other undescribed factors are necessary for the differentiation of sympathetic neurons. IGFs also enhance the proliferation of mesenchymal derivatives of both neural crest and mesodermal origin. Glucocorticoid-mediated differentiation of neural crest-derived chromaffin endocrine cells that are ontogenetically closely related to sympathetic neurons can be inhibited by NGF, and chromaffin cells can be induced to express the neuronal phenotype by NGF. Some growth factors, such as NGF, act on neural crest- and not on placodally-derived neurons, whether the former are sensory or sympathetic. Placodal sensory neurons possess NGF receptors, but only display a limited response to NGF, perhaps because of low affinity of the receptors. Other growth factors, such as BDNF, selectively act upon sensory neurons, whether neural crest- or placodally-derived.

Although extracellular matrix products play a role in initiating the differentiative process, signals from growth factors are necessary for the establishment of the functionally competent phenotype of neural crest-derived neurons, a situation that does not apply for neural crest-derived mesenchymal cells. It is interactions with ECM components deposited by epithelia that govern the differentiation of mesenchymal derivatives. Growth factors do effect proliferation of mesenchymal derivatives and inhibit mesenchymal differentiation. Although direct involvement of *single* growth factors in trans-

formation of one mesenchymal phenotype to another has not been reported so far, their localization at sites of epithelial-mesenchymal interactions in palate, teeth and mandible, and the ability of excess growth factors to interrupt normal development is suggestive of their possible involvement during normal development. One group of growth factors, BMPs, can influence differentiation of cartilage, including those of neural crest origin. The combination of EGF and NaF can provide a sufficient signal to initiate mandibular osteogenesis. EGF is required for tooth formation.

Presence of growth factor sequences in components of epithelial basement membranes, binding of growth factors to such extracellular matrixes, and modulation of growth factor function by such binding, makes plausible a relationship between growth factors and epithelial-mesenchymal interactions, and in fact, indicates that growth factor-matrix complex interactions are important but as yet relatively unexplored components of epithelial-mesenchymal interactions.

KEY WORDS: *neural crest, growth factors, differentiation, mesenchymal neural crest derivatives, neuronal neural crest derivatives, endocrine neural crest-derivatives, extracellular matrix, epithelial-mesenchymal interactions, proliferation.*

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