NEURAL STEM AND PROGENITOR CELLS IN CANCER AND DEVELOPMENT

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ABSTRACT

Stem cells are unique cells that possess the capacities to both self-renew and give rise to multiple differentiated progeny. There exist two major types of stem cells that help to create the nervous system: CNS stem cells which produce the neurons and glia of the central nervous system and neural crest cells which produce not only the neurons and glia of the peripheral nervous system, but also structures such as the craniofacial skeleton, cardiac outflow tracts, skin pigment cells, and sympathoadrenal cells. The mechanisms of self-renewal, migration, and differentiation of these two stem cell types have been studied in great detail. Yet despite such insight, much remains to be known about key aspects of neural stem cell development. First, it has long been thought that there might be a lineal relationship between CNS stem cells in human embryos or adults and primary brain tumors, particularly those malignancies occurring in children. To earn better insight into this possibility, I examined fresh pediatric brain tumors and found that they contained a subpopulation of cells with characteristics of neural stem cells that, at a clonal level, could recapitulate properties of the parental tumor. These tumor-derived progenitors shared genetic similarities with normal neural stem cells and could migrate and proliferate in vivo. Second, I have studied whether the late-migrating wave of neural crest cells and their derivatives originates from stem or progenitor cells resident in the embryonic spinal cord by culturing quail neural tube cells as neurospheres. I have found that these cells have the potential to generate melanocytes and possibly other neural crest derivatives both in vivo and in vitro after weeks in culture, suggesting that neural crest or melanocytic progenitor cells in the neural tubes of older embryos might contribute to the late-migrating neural crest populations. Taken together, my results in both model systems suggest that neural stem or progenitor cells that persist in the animal beyond early embryonic development play significant roles at later points in development and life, particularly in the continued development of the peripheral nervous system and the development of malignancies of the central nervous system.

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Chapter 1:

Neural Progenitor Cells in Development and Cancer

[modified from Houman D. Hemmati, Tanya Moreno and Marianne Bronner-Fraser.

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1.1 INTRODUCTION

The peripheral nervous system (PNS) is comprised of groups of neurons and support cells whose cell bodies lie outside the spinal cord and brain. These peripheral ganglia relay sensory input back to the central nervous system (CNS), where the information is processed and physical responses are generated. The PNS is primarily derived from a population of precursor cells called neural crest cells that arise within the developing CNS but subsequently migrate to the periphery and are highly versatile with respect to the types of derivatives that they form.

The neural crest is one of the defining features of vertebrates. Neural crest cells originate in the ectoderm of the early embryo and develop as a ridge of cells flanking the rostrocaudal length of the open neural tube (Fig. 1), resembling a "crest." Initially, these cells appear to be multipotent and subsequently give rise to both neuronal and nonneuronal derivatives, including neurons and support cells of the PNS, pigment cells, smooth muscle cells, and cartilage and bone of the face and skull (Hall, 1998; Le Douarin, 1982). Different populations of neural crest cells arise at different rostrocaudal levels of the neural axis. For example, at cranial levels, neural crest cells contribute to cranial sensory ganglia as well as skeletal elements of the face. In contrast, trunk neural crest cells never contribute to the bone or cartilage, but are the exclusive source of the peripheral ganglia and also contribute to the adrenal medulla (Fig. 2). Thus, neural crest cells at all axial levels appear to have multiple developmental potentials, but they differ from each other according to their level of origin. Recently, it has been shown that some neural crest cells are stem cells that self-renew in vivo and can contribute to at least some

of the derivatives generated by the neural crest (Morrison et al., 1999).

Interest in the mechanisms of induction, migration, and differentiation of neural crest cells has occupied developmental biologists for more than 130 years (Harrison, 1938; His, 1868; Hörstadius, 1950; Landacre, 1921; Stone, 1922), reprinted in (Hall and Hörstadius, 1988). Much is known about the later steps of neural crest development such as migration pathways and cell fate decisions (Bronner-Fraser, 1993; Dickinson et al., 1995; Erickson and Perris, 1993; Le Douarin, 1982; Stemple and Anderson, 1993). However, molecular aspects of these processes have only begun to be uncovered within the last two decades. This review summarizes recent findings regarding neural crest induction and the isolation and characterization of neural crest stem cells and discusses the involvement of neural and neural crest stem cells in the development of tumors of the central and peripheral nervous systems.

1.2 ORIGIN AND INDUCTION OF THE NEURAL CREST

1.2.1 Neural Crest Origin

The ectoderm is the source of the tissues that eventually form the epidermis, CNS, and PNS of all vertebrates. It is initially patterned into neural and nonneural ectoderm by signals emanating from a mesodermal organizing center during gastrulation, i.e., the dorsal lip of the blastopore (Spemann's organizer) in amphibians, Hensen's node in avians, the node in the mouse, and the embryonic shield in zebrafish. This process is called neural induction (Chang and Hemmati-Brivanlou, 1998; Sasai and De Robertis,

1997; Weinstein and Hemmati-Brivanlou, 1997; Wilson and Hemmati-Brivanlou, 1997). Later, the underlying mesoderm also plays a role in supplying rostrocaudal positional information to the neural ectoderm. At the start of neural induction, a broad domain of ectoderm adjacent to the midline thickens to form a columnar epithelium called the neural plate. The ectoderm outside of the neural plate will give rise to the epidermis and, in the head region, placodes. Placodes are regional thickenings of the ectoderm that will contribute to the cranial sensory ganglia and the sense organs of the head such as the eyes, ears, and nose (Barbu et al., 1986; Webb and Noden, 1993). They form the remainder of the PNS that is not generated by the neural crest.

Induction of the neural crest occurs at the border region between the future epidermis and the neural plate (reviewed in refs. (Baker and Bronner-Frase, 1997) and (LaBonne and Bronner-Fraser, 1999)). As development proceeds, the neural plate begins to roll into a tube, causing its lateral edges to form folds that eventually approximate at the dorsal midline of the embryo. The neural folds typically contain the premigratory neural crest cells, although there are some exceptions. For example, in the frog, *Xenopus*, the cranial neural crest is not incorporated into the neural tube, but remains as a separate condensed mass of cells in the border region. Thus, neural crest cells delaminate from the neuroepithelium and begin to migrate before neural tube closure in some species (e.g., mouse, *Xenopus*) (Bartelmez, 1922; Hall and Hörstadius, 1988; Holmdahl, 1928; Nichols, 1981; Olsson and Hanken, 1996; Verwoerd and van Oostrom, 1979), whereas in other species (e.g., chicken), they migrate only after apposition of the neural folds (Bronner-Fraser, 1986). Thus, the CNS is formed from the rolled-up neural plate, and the PNS is formed from the ectodermal placodes and the neural crest cells residing in and

around the dorsal neural tube, which delaminate from the neural epithelium and migrate throughout the embryo (Fig. 1).

It was originally thought that the neural crest was a segregated population of cells, largely based on the fact that these cells appear morphologically distinct from neural tube cells in some species (e.g., axolotl and zebrafish). In other species, however, presumptive neural crest cells are not readily distinguishable from dorsal neural tube cells. Moreover, single-cell lineage analyses of the dorsal neural tube have shown that individual precursors in the neural tube can form both neural crest and neural tube derivatives in chick (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989), frog (Collazo et al., 1993), and mouse (Serbedzija et al., 1992; Serbedzija et al., 1994). Even more strikingly, prior to neural tube closure, the neural folds can give rise to all three ectodermal derviatives: epidermis, neural tube, and neural crest (Dickinson et al., 1995). Recently, genetic screens in zebrafish have identified a mutation called narrowminded, which supports a shared lineage between CNS and PNS cells. This mutant lacks both early neural crest cells (PNS) and sensory neurons in the neural tube (CNS) (Artinger et al., 1999). Further evidence for a common neural progenitor comes from isolation of stem cells from the spinal cord neuroepithelium (NEP) cells that can form both CNS and PNS derivatives (Kalyani et al., 1998).

Not only has it been shown the neural tube/neural crest lineage is shared, but it has also been demonstrated that these cells are not irreversibly committed to either fate until relatively late in development. The ability of the neural tube to produce neural crest cells may persist for long periods. Sharma et al. (Sharma et al., 1995a) identified a late-emigrating population of neural tube cells that form neural crest-like derivatives. When

transplanted into neural crest migratory pathways of younger embryos, these cells can migrate and differentiate into neural crest derivatives (Korade and Frank, 1996). Conversely, it has been shown that early-migrating neural crest cells can reincorporate into the ventral neural tube and express markers characteristic of floor plate cells when challenged by transplantation (Ruffins et al., 1998). In addition to neural crest cells which arise from the dorsal portion of the neural tube, there is evidence that mulitpotent precursors can arise from the ventral neural tube. These "VENT" cells appear to have the ability to form neural crest-like derivatives as well as other cell types (Sohal et al., 1996).

1.2.2 Neural Crest Induction

Cell–Cell Interaction at the Neural Plate Border

Several theories of neural crest induction exist (reviewed in Baker and Bronner-Fraser, 1997). Both the mesoderm and the epidermal ectoderm have been shown to have the ability to induce neural crest. (This section discusses the evidence for ectodermal interactions; see below for a discussion of mesoderm.) The best supported model for neural crest induction is one in which cell-cell interaction at the border between neural and nonneural ectoderm is responsible for inducing the neural crest. In vivo grafting experiments suggest that interactions between presumptive epidermis and neural plate can form neural crest cells. In amphibians, epidermis grafted into the neural plate generates neural crest cells (Moury and Jacobson, 1990; Rollhäuser-ter Horst, 1980). In avians and frogs, neural plate tissue grafted into the epidermal ectoderm results in the production of migratory cells expressing neural crest cell markers (Dickinson et al., 1995; Mancilla and Mayor, 1996). In vitro co-culture experiments have similarly provided

evidence for the sufficiency of the neural plate/epidermal ectoderm interaction to generate neural crest cells (Dickinson et al., 1995; Mancilla and Mayor, 1996). Interestingly, both the epidermis and the neural plate cells contributed to the neural crest cell population (Dickinson et al., 1995; Moury and Jacobson, 1990).

The potential for more ventral neural tube cells to generate neural crest was examined in ablation experiments in which the dorsal region of the neural folds containing the presumptive neural crest cells was removed, thus bringing more ventral regions of the tube into contact with epidermal ectoderm. In this situation, neural crest cells were regenerated at the zone of contact (Couly et al., 1996; Hunt et al., 1995; Scherson et al., 1993; Sechrist et al., 1995; Suzuki et al., 1997), for a limited period. These data show that a very important mechanism of neural crest induction is mediated through cell-cell interactions at the border between the epidermal ectoderm and the neural plate.

The Role of BMPs in Neural Crest Induction: Setting up the Neural Plate Border Region

There is growing evidence, particularly from the *Xenopus* system, that members of the tumor growth factor-ß (TGF-ß) superfamily of signaling molecules play an integral role in setting up the border between neural and nonneural ectoderm. Given that neural crest cells arise at this border, it is likely that these cells are an important target of this signaling process.

Several lines of evidence support the idea that bone morphogenic protein (BMP) molecules play a role in neural induction (for review, see refs. 15 and 16). *Xenopus* BMP-4 is expressed throughout the ectoderm prior to neural induction and then is lost from

regions fated to become the neural plate (Cox and Hemmati-Brivanlou, 1995; Dale et al., 1992; Fainsod et al., 1994). The secreted BMP antagonists noggin (Lamb and Harland, 1995; Lamb et al., 1993), chordin (Piccolo et al., 1996; Sasai et al., 1994), and follistatin (Fainsod et al., 1997; Hemmati-Brivanlou et al., 1994) all are expressed in Spemann's organizer, the tissue responsible for patterning the ectoderm. Thus, the neural plate forms adjacent to the organizer, the source of BMP inhibition, whereas the nonneural ectoderm lies distal to the organizer (Fig. 3).

One possibility is that inhibition of BMP signaling is sufficient to generate both the neural plate and the neural crest, with high levels of inhibition yielding neural tissue and intermediate levels yielding neural crest. The idea that a diffusible morphogen could act to instruct the ectoderm to assume the various available fates was first proposed by Raven and Kloos (Raven and Kloos, 1945), who hypothesized that an "evocator" present in a graded fashion could generate neural crest at low levels and neural plate and neural crest at high levels (reviewed in ref. 19). In *Xenopus* ectodermal explants (animal caps), varying the level of BMP activity leads to varying fates of ectoderm (Knecht et al., 1995; Wilson and Hemmati-Brivanlou, 1997). Overexpression of a dominant-negative BMP receptor (Marchant et al., 1998) or the BMP antagonist chordin in *Xenopus* ectodermal explants causes neural crest marker expression and in whole embryos enhances the neural crest domain in a dose-dependent fashion (LaBonne and Bronner-Fraser, 1998). In contrast, the reciprocal experiment of overexpressing BMP-4 itself in intact embryos does not influence the size of the neural crest domain. Instead, the size of the neural plate decreases in a dose-dependent fashion, thus moving the location, but not the extent, of the presumptive neural crest (LaBonne and Bronner-Fraser, 1998). Furthermore, chordin by

itself cannot induce robust expression of neural crest markers in *Xenopus* animal caps (LaBonne and Bronner-Fraser, 1998). Taken together, these results indicate that inhibition of BMP signaling alone is not sufficient to induce neural crest formation.

Genetic Evidence for the Involvement of TGF-ß Family Members in Neural Crest Induction

Genetic evidence in the zebrafish supports a role for TGF-ß family molecules influencing the fate of the ectoderm. Nguyen et al. (Nguyen et al., 1998) have investigated swirl (Hammerschmidt et al., 1996; Kishimoto et al., 1997), a mutation in the zebrafish BMP-2 gene. Swirl mutants display a loss of neural crest progenitors, whereas mutations in genes downstream of BMP-2b such as somitabun (mutation in Smad5, a BMP signal transducer) expand the neural crest domain (Nguyen et al., 1998). The zebrafish mutant radar, which affects a dpp-Vg1-related molecule distinct from the BMP-2/4 and BMP-5/8 subgroups ((Delot et al., 1999); and see ref. (Hogan, 1996) for a review of TGF-ß relationships), results in the loss of the neural crest marker msxC and -selected neural crest derivatives. Conversely, overexpression of the radar gene causes upregulation of msxC expression, but only in areas contiguous with the endogenous msxC domain (Delot et al., 1999). In these mutants, however, the mesoderm underlying the neural crest is also affected, allowing for the possibility that the strength of the phenotype is not solely due to changes in BMP signaling in the ectoderm. This suggests that the activity of TGF-ß family members contributes to the patterning of the ectoderm. However, only certain regions are competent to respond to these molecules, suggesting that other gene activities may be required for the establishment of the neural crest.

Transgenic mice bearing null mutations in BMP-4 (Winnier et al., 1995), follistatin (Matzuk et al., 1995), or noggin (McMahon et al., 1998) do not display the neural defects that would be expected by extrapolation from the experiments in *Xenopus* described above. It has been suggested that redundancy between different BMP family members, the antagonizing molecules, or other developmental defects may obscure the phenotype (reviewed in ref. (Bjornson et al., 1999)). Alternatively, there may be interesting species differences in the process of neural induction and neural crest formation. Indeed, many studies in the chick embryo have added to the interspecies discrepancies that are found upon investigation of the role of TGF-ß signaling as a mechanism for neural induction and neural crest formation.

BMPs Can Induce Neural Crest in Culture

In the chick embryo, BMP-4 and BMP-7 are expressed in the epidermal ectoderm that contacts the neural tube (Liem et al., 1995; Schultheiss et al., 1997). As development proceeds, however, expression is lost in the epidermal ectoderm but BMP-4 is expressed in the neural folds and dorsal neural tube (Watanabe et al., 1996), along with another TGF-ß family member, dorsalin-1, which is upregulated after neural tube closure (Liem et al., 1995; Streit et al., 1998). When added to isolated intermediate neural plates in tissue culture, both BMP-4 and -7 have been shown to induce neural crest markers and migratory cells (Liem et al., 1995). This seemingly contrasts with the results in *Xenopus*, where inhibition of BMP signaling yields neural fates. However, the paradigm for neural induction by BMP repression in the neural plate does not appear to function in the chick embryo at the time of neural crest/neural plate border formation. Chordin, which inhibits

BMP activity, is expressed in the avian organizer (Hensen's node) but alone cannot neuralize ectoderm (Streit et al., 1998). Additionally, neither BMP-4 nor -7 is sufficient to repress neural induction in the neural plate when ectopically expressed (Streit et al., 1998).

Furthermore, by implanting noggin-producing cells into the neural tube or under the neural fold regions, it has been shown that BMP signaling is required in the chick neural tube for expression of neural crest markers, but not at the stage at which BMP is expressed in the ectoderm (Selleck et al., 1998). Pera et al. (Pera et al., 1998) found that ectopic expression of BMP-2 or -4 under the neural/nonneural border region distorts the neural plate and causes epidermal ectoderm marker expression in areas that would normally give rise to neural plate. Taken together, these results seem to indicate that BMP signaling plays several important roles in neural crest development, beginning with the positioning of the neural plate border and continuing with the maintenance of neural crest induction. Importantly, it is likely that other molecules are involved in the initiation of neural crest induction. Later, BMPs in the dorsal neural tube induce roof plate cells and sensory neurons (Liem et al., 1997). Still later, BMPs are involved in the differentiation of sympathoadrenal precursors from neural crest cells (Anderson, 1993; Schneider et al., 1999; Varley et al., 1998).

There is no direct evidence that either BMP-4 or -7 is the molecule that diffuses from the epidermal ectoderm to induce crest cells (Liem et al., 1995). Indeed, it was shown that BMP-4 induces epidermis at the expense of neural tissue (Wilson and Hemmati-Brivanlou, 1995). The ability of BMP-4 and -7 to induce neural crest from neural plate cultures (Liem et al., 1995; Liem et al., 1997) may be a reflection of the

molecule having first induced epidermis, which in turn interacted with the neural plate to induce neural crest. Another possibility is that exogenous BMP bypasses an epidermal signaling event and mimics a later action of endogenous BMP signaling in the dorsal neural tube that is sufficient to generate neural crest cells. This possibility is supported by the later neural tube requirement for BMP signaling to produce neural crest cells, as demonstrated by Selleck et al. (Selleck et al., 1998). Thus, the action of BMPs may be required within the responding tissues to maintain crest production, rather than being a property of the initial induction (reviewed in ref. 21).

It is important to bear in mind that although many experimental differences between species are reported in the literature, these are most likely to be a result of the rather striking differences among the organisms that are used for study. Differences in morphology and timing of development must require differences in gene expression to achieve the overall goal of properly forming the animal. For example, the frog embryo begins as a hollow ball of cells, whereas the chick embryo begins as a flat sheet of cells. In the frog embryo, development relies for a period on maternal stores of messenger RNAs, which contrasts with the chick embryo. Moreover, the distances between signaling centers and their responding tissues may require different mechanisms in order to effect induction of neural tissue and other developmental events. Although there are many apparent species differences, these may reflect variations in the finer details that accommodate spatial and temporal variations among organisms; the general mechanisms are likely to be common for all vertebrates (LaBonne and Bronner-Fraser, 1999).

Other Sources of Neural Crest-Inducing Signals:

The Mesoderm

It would be overly simplistic to assume that a single signaling event within the ectoderm is sufficient to account for induction of the neural crest. Many lines of evidence suggest that the nonaxial mesoderm is also involved in inducing the neural crest. Although conjugating epidermis and neural plate in vitro is sufficient to induce neural crest markers in the absence of mesoderm (Dickinson et al., 1995; Mancilla and Mayor, 1996; Moury and Jacobson, 1990), mesoderm could represent an important modifier. Mesoderm/neural plate conjugates do not induce early neural crest markers (Dickinson et al., 1995; Mitani and Okamoto, 1991). However, it was demonstrated that paraxial mesoderm conjugated with neural plate could induce the formation of melanocytes, a neural crest derivative (Dickinson et al., 1995). Similarly, non-axial mesoderm from both chick and frog can induce neural crest markers in neural plate co-culture experiments (Bang et al., 1997; Bonstein et al., 1998; Marchant et al., 1998), and removal of the nonaxial mesoderm before neural induction is complete results in a failure of the ectoderm to express neural crest markers (Bonstein et al., 1998; Marchant et al., 1998). The evidence that mesoderm can influence neural crest formation suggests that there may be other molecules involved in the early steps of neural crest induction.

Wnt Family Members

As discussed above, it seems likely that inhibition of BMP alone cannot account for neural crest induction, making it probable that other signaling systems are involved. Possible candidates for involvement in this process are secreted molecules expressed in

both mesoderm and ectoderm, that have been implicated in patterning the neural tube. These include members of the wingless/int family known in vertebrates as Wnts (Wodarz and Nusse, 1998) and the fibroblast growth factor (FGF) family (Sieber-Blum, 1998; Vaccarino et al., 1999). In *Xenopus* ectodermal explants (animal caps), Wnt1 and Wnt3a (Saint-Jeannet et al., 1997), Wnt7b (Chang and Hemmati-Brivanlou, 1998), and Wnt8 (LaBonne and Bronner-Fraser, 1998), in conjunction with inhibition of BMP signaling (i.e., neural induction) can induce the expression of neural crest markers. Furthermore, overexpression of ß-catenin (a downstream component of the Wnt signaling pathway) expands the neural crest domain; expression of a dominant-negative Wnt ligand eliminates the neural crest domain in *Xenopus* embryos (LaBonne and Bronner-Fraser, 1998).

One of the earliest neural crest markers in *Xenopus* is the zinc finger transcription factor, Slug (Mayor et al., 1995) (Fig. 4). When animal caps overexpressing Slug are juxtaposed to Wnt8-expressing explants, neural crest markers are induced, thus bypassing the requirement for inhibition of BMP signaling (LaBonne and Bronner-Fraser, 1998). In contrast, Slug alone cannot induce neural crest (LaBonne and Bronner-Fraser, 1998). Slug, in turn, can expand its own expression domain when overexpressed in the whole embryo (LaBonne and Bronner-Fraser, 1998). These results suggest that a two-signal model may account for the events underlying neural crest formation, such that Wnt signaling together with inhibition of BMP signaling induces the neural crest marker, Slug, with Slug expression abrogating further need for BMP inhibition (LaBonne and Bronner-Fraser, 1998). A recent study has shown that in *Xenopus*, Wnt signals induce the expression of the proto-oncogene c-Myc in neural crest cells prior to the expression of

Slug (Bellmeyer et al., 2003). Knock-downs of c-Myc activity result in a loss of neural crest cells and their derivatives in vivo (Bellmeyer et al., 2003).

Many Wnt molecules are expressed in spatiotemporal patterns appropriate for involvement in various aspects of neural crest development. *Xenopus* Wnt8 is expressed in the ventrolateral mesoderm (Christian et al., 1991), a tissue that has been shown to be a neural crest inducer when conjugated with neural plate in vitro (Bang et al., 1997; Bonstein et al., 1998; Marchant et al., 1998), and avian Wnt-8C is similarly expressed in the nonaxial mesoderm (Hume and Dodd, 1993). In *Xenopus*, an early Wnt8 signal induces expression of Pax3 and Msx-1 in the lateral neural plate (Bang et al., 1999). This establishes a posteriolateral domain from which neural crest cells will eventually arise (Bang et al., 1999). *Xenopus* Wnt7b is expressed throughout the ectoderm at gastrulation (Chang and Hemmati-Brivanlou, 1998), and other Wnts may well be expressed in the ectoderm.

In chick (Dickinson et al., 1995; Hollyday et al., 1995), frog (McGrew et al., 1997; Wolda et al., 1993), and mouse embryos (Parr et al., 1993; Roelink and Nusse, 1991), Wnt1 and Wnt3a are expressed in the dorsal neural tube well after the initial - expression of neural crest markers, although *Xenopus* Wnt3a is also expressed before neural tube closure at the edges of the neural plate (McGrew et al., 1997). Furthermore, avian neural crest can be induced in conjugates of epidermis and neural plate without the concomitant expression of either Wnt1 or Wnt3a (Dickinson et al., 1995). This suggests that Wnt-1 and -3a are not involved in the initial induction of neural crest. However, Wnt1/3a double knockout mice have a reduction in neurogenic and gliogenic neural crest derivatives, suggesting that fewer neural crest cells emerge in embryos lacking both

genes (Ikeya et al., 1997). Not all neural crest derivatives are affected, with ventralmost derivatives such as sympathetic ganglia demonstrating normal morphology, whereas dorsal root ganglia are markedly reduced. This is consistent with the possibility that these Wnts play a later maintenance role in neural crest production by the neural tube. Wnts may be involved in the expansion of neural crest progenitors, most likely by regulating the proliferation of the cells after induction has occurred but prior to commencement of emigration (Ikeya et al., 1997). Garcia-Castro et. al. (Garcia-Castro et al., 2002) have recently shown that a Wnt family member, Wnt6, is likely the early epidermal neural crest inducer in the chick. Wnt6 is expressed in the ectoderm adjacent to the neural folds, but not in the neural folds and neural plate, at a time when neural crest cells are being induced. Drosophila Wingless protein, which activates Wnt singalling in vertebrates, can induce neural crest from naïve neural plate in vitro in the absence of additives, whereas Wnt inhibition blocks neural crest formation in vivo. Taken together, these results demonstrateing that Wnt signaling is both necessary and sufficient to induce neural crest in avian embryos (Garcia-Castro et al., 2002).

Wnt family members may also be able to control some aspects of neural crest cell fate. In zebrafish experiments, single neural crest cells overexpressing molecules of the Wnt signaling pathway form pigment cells at the expense of neurons or glia. Conversely, overexpressing inhibitors of the pathway biases the neural crest cells to form neurons at the expense of pigment cells (Dorsky et al., 1998).

Recent work by Baker et al. (Baker et al., 1999) has put forward a novel model for Wnt function. They demonstrate that expression of *Xenopus* Wnt8, mouse Wnt8, and downstream Wnt targets in frog ectodermal explants can induce expression of the early

panneural marker neural cell adhesion molecule (NCAM), without neural induction by BMP antagonists. Additionally, they demonstrate that Wnt signaling components suppress BMP-4 expression in ectoderm explants as assayed by in situ hybridization. In fact, Wnt8, and not the BMP antagonist noggin, seems to be capable of blocking BMP-4 expression in the neural plate throughout gastrula stages, suggesting that an early Wnt signal and not a direct BMP antagonist is responsible for the early inhibition of BMP-4 expression in the neural plate. Finally, the authors suggest that there may be parallel pathways for the effects of Wnt signaling in neural induction since inhibition of Wnt8-mediated activation of the neural inducers Xnr3 and siamois did not abrogate Wnt8's ability to itself promote neural induction. These results suggest that Wnt signaling may be involved in multiple inductive events in early development. The ramifications of these data for the role of Wnt signaling in neural crest induction are unclear, as these investigators did not explore the effects of the perturbations on neural crest markers. Previous results showing that Wnts could not induce neural crest without a co-expressed neural inducer (Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Saint-Jeannet et al., 1997) taken together with the results of Baker et al. (Baker et al., 1999) may indicate that the precise levels of Wnt signaling are critical. Further investigation will be required to determine exactly what role Wnt plays during neural crest development.

Fibroblast Growth Factors

Other molecules expressed in the mesoderm have been shown to have neural crest inducing activities. FGF signaling can induce neural crest markers in frog ectodermal

explants when in the presence of BMP antagonists (Kengaku and Okamoto, 1993; LaBonne and Bronner-Fraser, 1998; Mayor et al., 1997). Overexpression of a dominant-negative FGF receptor can inhibit expression of the early neural crest marker XSlug in whole embryos (Mayor et al., 1997). Other investigators have demonstrated that FGF signaling has a posteriorizing effect on neural tissue (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1993; Lamb and Harland, 1995; Launay et al., 1996; Xu et al., 1997). Indeed, members of the FGF family are spatiotemporally expressed in a way that is consistent with their playing roles in the process of neural and/or neural crest induction (Bueno et al., 1996; Isaacs et al., 1992; Mahmood et al., 1995; Riese et al., 1995; Storey et al., 1998; Streit et al., 2000). Recent work by Streit et al. has shown that in the chick, an FGF signal from Hensen's node initiates neural induction prior to the onset of gastrulation, inducing the expression of early neural genes such as ERNI, Sox2, and Sox3 (Streit et al., 2000). The results indicate that FGFs may be able to generate both posterior and lateral (i.e., neural crest) fates in the CNS and PNS. The role of FGFs becomes complicated in light of evidence from transgenic frog experiments, however, in which frog embryos expressing a dominant-negative FGF receptor have normally developing posterior neural tissue and border regions including the neural crest, although the investigators did not test a full range of neural crest markers (Kroll and Amaya, 1996). Moreover, FGF-treated neural plate explants do not form neural crest tissue (Mayor et al., 1997). Another recent study suggests that fibroblast growth factor FGF-8 may mediate the inductive effects of paraxial mesoderm on frog animal caps and may be sufficient to induce expression of several neural crest markers (Monsoro-Burq et al., 2003). However, other assays suggest that, neural crest induction by FGF may be a secondary result of its

ability to induce a member of the Wnt family (LaBonne and Bronner-Fraser, 1998).

Notch and Noelin

Recent work has implicated both Notch signaling and Noelin-1 in the generation of neural crest cells. Activation of the Notch receptor results in cleavage of its intracellular domain, which translocates to the nucleus and activates transcription (Kopan, 2002). Notch1 is expressed throughout the neural plate, with higher levels in the neural crest, whereas its ligand Delta1 is expressed in the epidermal ectoderm (Almqvist et al., 2002; Bierkamp and Campos-Ortega, 1993; Coffman et al., 1993; Williams et al., 1995). Notch signaling has been shown to be required for neural crest formation in avian, zebrafish, and *Xenopus* embryos (Almqvist et al., 2002; Cornell and Eisen, 2002) (Glavic et al., 2004), perhaps by repressing Neurogenin-1 function (Cornell and Eisen, 2002).. In the chick and *Xenopus*, Notch promotes neural crest formation by modulating levels of BMP4 expression: in the chick, Notch maintains BMP4 expression (Almqvist et al., 2002), whereas in *Xenopus* Notch represses BMP4 transcription (Glavic et al., 2004). In zebrafish, Notch promotes formation of neural crest by repressing Neurogenin-1 function(Cornell and Eisen, 2002).

Barembaum, et al have recently demonstrated that the secreted glycoprotein Noelin-1 plays a role in the competence of neural tissue to form neural crest formation (Barembaum et al., 2000). Noelin-1 mRNA is restricted to the dorsal neural folds and migrating neural crest cells in avian embryos. Retroviral-mediated overexpression of Noelin-1 in the neural tube increases both the number of neural crest cells generated and

the length of time neural crest cells continue to emigrate from the neural tube. These findings suggest that Noelin-1 may play a role in rendering the neural tube cells competent to form neural crest (Barembaum et al., 2000).

1.2.3 Neural Crest Stem Cells

In the past decade, work by several investigators has led to the prospective identification and purification of neural crest stem cells—cells with the potential to self-renew and also to give rise to the diverse population of derivatives that are generated by the neural crest. The first neural crest stem cells were isolated in vitro by clonal analysis of cells that were fractionated from rat neural crest cultures by cell sorting based on expression of a cell surface epitope (Stemple and Anderson, 1992). These cells can be replated to form new stem cells and also can give rise to "blast" cells that are partially restricted to form neurons or glia. These include the sympathoadrenal sublineage, which includes precursors to sympathetic neurons and adrenomedullary cells (Doupe et al., 1985), that, in the embryo, appear specified by the time that neural crest-derived cells reach their sites of localization around the dorsal aorta.

Specific molecules can instruct neural crest stem cells to adopt specific fates; for example, glial growth factor (neuregulin) causes the development of glia (Schwann cells) BMP-2 biases clones to develop into neurons (and a small number of smooth muscle cells), and TGF-ß1 promotes development of smooth muscle cells (Morrison et al., 1997; Shah et al., 1996; Shah et al., 1994). It has recently been shown that transient Notch activation promotes glial production by neural crest stem cells at the expense of neurogenesis, even in the presence of BMP-2 (Morrison et al., 2000). Thus, it is

interesting to note that members of the TGF-ß superfamily are not only involved in induction of the neural crest but are also implicated in subsequent cell fate decisions.

Although the neural crest stem cells are very useful in testing the ability of factors to promote certain cell fate decisions, there are possible caveats; for example, the stem cell qualities of the purified cells may have been acquired in vitro and may not reflect an actual state that is present in the embryo. The findings of Frank and colleagues (Korade and Frank, 1996; Sharma et al., 1995a) that neural tubes can give rise to neural crest-like cells that emigrate long after the normal period of neural crest formation suggest that neural crest stem cells may persist within the spinal cord and other sites for long periods. Consistent with this possibility, Morrison et al. (Morrison et al., 1999) have recently isolated neural crest stem cells from embryonic rat peripheral nerve at E14.5. The cells were isolated by fluorescence-activated cell sorting using cell surface epitopes p75 and P0. Under proper culture conditions, these cells self-renew and can differentiate into neurons, glia, and smooth muscle cells within single colonies. The cells are also instructively promoted to form neurons or glia by exposure to either BMP-2 or glial growth factor, respectively, in clonal cultures. An important test of the qualities of these neural crest stem cells is to determine whether newly isolated cells are multipotent when transplanted into an embryo. Indeed, freshly isolated cells that were p75 + /P0 - have stem cell properties and can be back-transplanted into chick embryos, giving rise to both neurons and glia as assayed by differential marker expression (Morrison et al., 1999). However, neural crest stem cells sorted from E14.5 rat spinal cords have been shown to have cell-intrinsic differences in developmental potential in vivo from their counterparts cultured E10.5 neural tube explants (White et al., 2001). The older, sorted neural crest

cells produce fewer neurons and appear unable to give rise to noradrenergic neurons, due to reduced sensitivity to the neurogenic signal BMP-2 (White et al., 2001). This phenomenon suggests that neural crest stem cells can change as a function of time and perhaps in response to local environmental factors in the periphery. By labeling actively dividing cells in embryos with the thymidine analog bromodeoxyuridine, it was shown that endogenous neural crest stem cells persist in the embryo by self-renewing (Morrison et al., 1999).

Self-renewing neural crest stem cells have also recently been isolated by flow cytometry from both embryonic (Bixby et al., 2002) and adult (Bixby et al., 2002) gut. Gut neural crest stem cells can be sorted based on expression of both p75 and α4 integrin (Bixby et al., 2002). The prospective isolation of gut neural crest stem cells has proven particularly important toward the understanding of Hirschsprung disease, a common gut motility defect caused by the absence of enteric nervous system ganglia in the hindgut (Iwashita et al., 2003). Gut neural crest stem cells from normal mice were found to express high levels of the glial cell line-derived neurotrophic factor (GDNF) receptor receptor Ret, and GDNF promoted migration of neural crest stem cells (Iwashita et al., 2003). Ret -/-null mice were found to have far fewer neural crest stem cells in the gut as compared to wild type mice (Iwashita et al., 2003). Yet there were no differences in proliferation, differentiation, or survival between neural crest stem cells from normal and mutant mice, suggesting that the absence of enteric ganglia in Ret null mice is caused by a failure of Ret null neural crest stem cells to migrate into the distal gut (Iwashita et al., 2003).

1.3 LINEAGE AND CELL FATE DECISIONS IN THE NEURAL CREST

The existence of neural crest stem cells in the embryo supports the idea that the fate of neural crest cells in vivo is primarily determined by their environment (Barbu et al., 1986). Neural crest cell fate decisions and their relationships to cell lineage have been debated for many years. Although it has been accepted that at least some, if not most, neural crest cells are multipotent, some evidence indicates that other neural crest cells have restricted fates in vivo (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989; Frank and Sanes, 1991). However, in these experiments, the potential of the cells has not actually been tested by challenging the cells with all possible factors that might influence cell fate choice. It is obviously difficult to quantify and compare the environment of one cell with another, beginning from their origins in the neural tube and following their migration trajectories through the periphery. In these lineage experiments, single dye-labeled or retrovirally tagged cells often gave rise to clones of progeny with multiple derivatives but sometimes gave rise to clones of only one cell type, suggesting an earlier specification for that progenitor cell. Thus, alternate methods of marking and challenging neural crest cells will be necessary to define the state of multipotency at the single cell level. This is an area in which the neural crest stem cells and their blast cells promise to provide new and important information.

Recent work has shown that the HMG-group transcriptional regulator Sox10 maintains the multipotency of neural crest stem cells (Kim et al., 2003). In rodents, Sox10 is expressed in neural crest cells at the time of their emigration from the dorsal neural tube. Loss-of-function mutations in Sox10 result in defects in multiple neural crest derivatives (Southard-Smith et al., 1998). Interestingly, over-expression of Sox10 in

neural crest stem cells maintains their neurogenic and gliogenic potentials, despite challenges by opposing differentiation factors such as BMP2 or TGF-ß (Kim et al., 2003).

1.3.1 Mechanisms of Neural Crest Diversification

If neural crest cells are truly multipotent and only receive instructions for differentiation when migrating to or reaching their final destinations, then it is interesting to consider how cells are instructed to take on different fates. For example, neural crest cells in the dorsal root ganglia differentiate into both sensory neurons and glia. An asymmetric cell division could produce a blast cell of each type, which could in turn replicate. Alternatively, the progenitor may replicate itself and produce a more restricted daughter cell, which then goes on to form the final derivatives. The latter seems more likely given the ability of neural crest stem cells to self-renew.

1.3.2 Environmental Cues versus Timing of Emigration

Both the environment and the timing of emigration from the neural tube have been proposed to affect the cell fate decisions of the neural crest. A restriction in available cell fate accompanies the time of emigration from the neural tube: the latest migrating cells only populate the dorsal root ganglia as neurons and form melanocytes in the skin and feathers (Serbedzija et al., 1989; Sharma et al., 1995a). However, when transplanted into earlier embryos, neural crest-like cells derived from much older spinal cords were able to migrate more ventrally and make sympathetic and peripheral neurons

(Korade and Frank, 1996; Weston and Butler, 1966). Similarly, in the head, late-migrating cells only formed dorsal derivatives because of the presence, ventrally, of earlier migrating cells; however, they are not restricted in potential (Baker and Bronner-Frase, 1997). Furthermore, the latest migrating cells of the main wave of crest emigration make melanocytes in the skin, but skin culture experiments show that they have the potential to form neurons (Richardson and Sieber-Blum, 1993). This suggests that the restriction in available fates in these cases is made by the environment that the cells occupy rather than the time that they emerge from the neural tube (Fig. 1).

Additional evidence for the influence of environment on neural crest cell fate comes from neural crest stem cells, in which single progenitor cells can generate smooth muscle cells when exposed to TGF-ß molecules. However, a community effect takes place when denser cultures are exposed to TGF-ß molecules, such that either neurons form or cell death occurs, rather than differentiation of smooth muscle cells (Hagedorn et al., 1999). These data suggest that cell fate in the embryo could also be determined by community effects in which cells respond differently to the same factors depending on the density of neighboring cells (Hagedorn et al., 1999). Other interesting studies on neural crest stem cells reveal that they can integrate multiple instructive cues and are biased to certain levels of responsiveness based on the growth factors to which they are exposed. If cultures of neural crest stem cells are exposed to saturating levels of both BMP-2 and glial growth factor (neuregulin), BMP-2 appears dominant and neurons differentiate. However, BMP-2 and TGF-ß1 seem to be co-dominant (Morrison et al., 1997).

There is evidence, however, that some neural crest cell populations may undergo

early fate restrictions. By culturing “early-migrating” and “late-migrating” trunk neural crest cells, Artinger and Bronner-Fraser (Artinger and Bronner-Fraser, 1992) found that the latter are more restricted in their developmental potential than the former; although they can form pigment cells and sensory-like neurons, they fail to form sympathetic neurons. Additionally, late-migrating cells transplanted into an earlier environment can colonize the sympathetic ganglia but failed to form adrenergic cells (Artinger and Bronner-Fraser, 1992). Thus, the time that a precursor leaves the neural tube may contribute to its potency. Perez et al. (Perez et al., 1999) have provided evidence for early specification of sensory neurons by the basic helix-loop-helix transcription factors neurogenins 1 and 2. These molecules are expressed early in a subset of neural crest cells, and ectopic expression of the molecules biases migrating neural crest cells to localize in the sensory ganglia and express sensory neuron markers.

The mechanism for formation of sensory neurons from neural crest cells has remained a mystery until recently. In an elegant set of experiments, Lee et al have demonstrated that activation of the canonical Wnt signaling pathway instructs neural crest cells to adopt a sensory neuronal fate (Lee et al., 2004). Transgenic mice in which a constitutively active form of ß-catenin, a key mediator in the canonical Wnt signaling pathway, was selectively expressed in neural crest cells produced only sensory neurons at the expense of other neural crest derivatives (Lee et al., 2004). Moreover, exogenous Wnt added to clonal cultures of neural crest cells in vitro similarly biased the cells toward a sensory neuronal phenotype without altering their proliferation (Lee et al., 2004).

Recent work implicates FGF and Wnt signaling in influencing the fate decisions of neural crest cells(Lee et al., 2004; Trainor et al., 2002). Hoxa2 expression in hindbrain

neural crest appears to confer second brancial arch identity at, causing them to form second arch skeletal elements in the head (Grammatopoulos et al., 2000; Pasqualetti et al., 2000). Trainor et al have shown that FGF8 signals alone or from the isthmic organizer can inhibit Hoxa2, allowing second-arch cells to adopt a first-arch fate and duplicate first-arch skeletal structures (Trainor et al., 2002).

The study of neural crest stem cells has yielded new insight into the effects of environment and timing on the function of neural crest cells (Bixby et al., 2002; Morrison et al., 1999). Bixby et al have compared the properties of neural crest stem cells purified from the sciatic nerve and gut of rat embryos at E14, a time at when neurogenesis is predominant in the gut while gliogenesis is most prevalent in nerves (Bixby et al., 2002). In both cell culture and transplantation experiments, gut neural crest stem cells produced primarily neurons, a function of their increased sensitivity to BMP4, whereas sciatic nerve neural crest stem cells formed mostly glia, due to their enhanced sensitivity to neuregulin and the Notch ligand Delta (Bixby et al., 2002). These stem cells maintain their difference even after many days of culture and subcloning, suggesting that the differences are intrinsic to the cells (Bixby et al., 2002). In contrast, neural crest stem cells purified from postnatal rat gut formed primarily glia after they were transplanted into the peripheral nerves of chick embryos, demonstrating that gut neural crest stem cells undergo temporal changes that help determine their cell fates in vivo (Bixby et al., 2002). Some important questions arise from this work: What signals cause the spatial and temporal differences observed in different neural crest stem cells? What are the normal functions of neural crest stem cells that persist into adulthood?

Another way to account for the process of promoting two different cell fates from

one precursor population within a single tissue is the proposal that temporal changes in the target environment bias the cell fate decision (Frank and Sanes, 1991). This is supported by the fact that first neurons and then glia are born in the dorsal root ganglia (e.g., ref. (Carr and Simpson, 1978)). The target environment could be influenced to change by early differentiating neural crest cells themselves; for example, some neurons produce glial-promoting factors (Lemke, 1996; Marchionni et al., 1993; Meyer and Birchmeier, 1995; Meyer et al., 1997; Morrison et al., 2000; Orr-Urtreger et al., 1993). The strongest evidence of this phenomenon comes from the effects of Notch activation on cell fate of neural crest stem cells (Morrison et al., 2000). Notch activation by ligands such as Delta, which are expressed on differentiating neuroblasts, induces glial differentiation at the expense of neurogenesis, even in the presence of BMP2 (Morrison et al., 2000). These findings suggest that differentiating neuroblasts might activate Notch in neighboring neural crest cells as a feedback signal to promote gliogenesis. Also, the loss of certain inhibitory glycoconjugates from the extracellular matrix in the dorsolateral migration pathway has been linked to the migration of late-emigrating neural crest cells along this pathway (Oakley et al., 1994), where they are exposed to melanogenic factors and hence adopt a melanocyte fate (Perris, 1997). Thus, there is evidence for the influence of both the timing of emigration and environmental cues in determining neural crest fates.

1.3.3 Progressive Lineage Restriction

It has been proposed that neural crest cells adopt specific fates by progressive lineage restrictions (Anderson, 1993; Anderson, 1999; Stemple and Anderson, 1993).

One way to explain the intermingling of clonally related neurons and glia is that the choice is made stochastically, such that each cell has the capacity to adopt either fate, and environmental factors act by influencing the probability of a fate choice rather than imposing strict commitments (Frank and Sanes, 1991). Trentin et al have recently taken individual avian neural crest cells through multiple rounds of serial recloning to demonstrate that both cranial and trunk neural crest cells give rise to progeny cells in a hierarchical manner, with progressively restricted developmental potentials, akin to the lineage hierarchy formed by hematopoietic stem cells (Trentin et al., 2004). Strikingly, they found that only two types of intermediate bipotent precursors, glial-melanocytic and glial-myofibroblast precursors had the ability to self-renew (Trentin et al., 2004). Support for the idea of progressive fate restriction comes also from the NEP, which can give rise to both CNS- and PNS-type stem cells. PNS stem cells (indistinguishable from neural crest stem cells, as described in ref. 11) are formed on addition of BMP-2/-4 to the NEP cell cultures (Kalyani et al., 1998). BMP-2, a molecule that is known to instruct neural crest stem cells toward an adrenergic neuronal fate, is expressed in the dorsal aorta, near where sympathetic ganglia form (Bitgood and McMahon, 1995; Lyons et al., 1995; Morrison et al., 1997). Thus, there is evidence that environmental cues may be able to promote progressive restriction of neural crest cell fates. Many factors act selectively by affecting the proliferation or survival of neural crest derivatives; others act instructively on multipotent progenitors to promote one fate over another. Further work will be required to answer the complex question of how individual cells within the same environment can adopt different fates. The evidence in support of both multipotentiality and lineage restriction may imply that neural crest cells take cues from both the timing of

emigration from the neural tube and the environments to which they are exposed in cell lineage decisions. For more discussion on the topic of neural crest diversification, the reader is referred to several recent reviews (Anderson, 1993; Groves and Bronner-Fraser, 1999; LaBonne and Bronner-Fraser, 1998).

1.4 Cancer Stem Cells in the Central and Peripheral Nervous System

A great deal of recent interest has focused on the striking similarities between stem cells and cancer cells in the nervous and hematopoietic systems as well as in various epithelial cell types (Iwashita et al., 2003; Reya et al., 2001). In particular, stem cells and some cancer cells share the fundamental properties of self-renewal and the ability to differentiate into multiple distinct cell types (Bonnet and Dick, 1997; Sell and Pierce, 1994; Wechsler-Reya and Scott, 2001).. The mechanisms underlying self-renewal of normal stem cells are known to be tightly regulated (Lessard and Sauvageau, 2003; Molofsky et al., 2003; Reya et al., 2003). In contrast, self-renewal of cancer cells is, by definition, aberrant.

The multipotency and self-renewal of some cancer cells raises the possibility that cancer might arise from the transformation of normal somatic stem or progenitor cells. In many cancers, it is known that only a small subset of cancer cells (called tumor or cancer stem cells) is able to drive the growth of the tumor and give rise to secondary tumors in vivo. However, the origins and phenotypes of those cells were unknown (Hamburger and

Salmon, 1977). One interesting possibility is that these cancer stem cells may be lineally related to transformed stem or progenitor cells.

Recent work has identified and characterized cancer stem or stem-like cells in leukemia, breast cancer, and brain tumors, and shown that these cells can self-renew, are multipotent, and can recapitulate characteristics of the original tumor in vitro and/or in vivo (Al-Hajj et al., 2003; Bonnet and Dick, 1997; Hemmati et al., 2003; Ignatova et al., 2002; Kondo et al., 2004; Singh et al., 2003). The existence of cancer stem cells was first proven in acute myeloid leukemia (Bonnet and Dick, 1997). Bonnet and Dick showed that a rare subset of cells from this cancer was able to proliferate extensively; in contrast, to the majority of leukemic cells have limited proliferative ability (Bonnet and Dick, 1997). Moreover, these leukemia stem cells, but not non-stem cells from the same patients' population of leukemic cells, could transfer the disease after transplantation into immunodeficient mice (Bonnet and Dick, 1997). This hyperproliferative subset of cells from these tumors was found to be phenotypically similar to normal hematopoietic stem cells based on expression of cell-surface antigens, suggesting that they might be related to the normal stem cells (Bonnet and Dick, 1997). However, the possibility exists the leukemic stem cells arise from the transformed committed progenitor cells that have reacquired stem cell characteristics (Cozzio et al., 2003). To address this question, Cozzio et al. recently transduced both hematopoietic stem cells and myeloid progenitor cells with a leukemogenic fusion protein, and found that transformation of both cell types produces an identical type of tumor in vivo (Cozzio et al., 2003). Therefore, it is possible that cancer stem cells arise from cells other than multipotent stem cells (Cozzio et al., 2003).

Similar experiments in breast cancer have demonstrated the existence of breast

cancer stem cells (Al-Hajj et al., 2003). Al-Hajj et al. used cell surface markers on uncultured breast cancer cells to fractionate them into different subsets, and transplanted the cells into immunodeficient mice (Al-Hajj et al., 2003). Only cells that phenotypically resembled normally mammary epithelial progenitor cells were able to give rise to tumors in the mice (Al-Hajj et al., 2003). Moreover, the tumors that formed in mice histologically resembled the tumors from which their stem cells were derived and themselves contained progenitor cells that formed tumors in other mice, suggesting that breast cancer stem cells can self-renew in vivo (Al-Hajj et al., 2003).

The search for cancer stem-like cells in central nervous system malignancies has recently borne fruit (Hemmati et al., 2003; Ignatova et al., 2002; Kondo et al., 2004; Singh et al., 2003). It has been proposed for some time that brain tumors arise from the transformation of self-renewing neural stem or progenitor cells (Brustle and McKay, 1995; Holland, 2000). Aside from the fact that some brain tumors contain both neurons and glia, many brain tumors (particularly those occurring in children) arise from the ventricular zone, where neural stem cells reside postnatally (Choe et al., 2003; Koos and Horaczek, 1985; Sutton et al., 1992) (Sanai et al., 2004). Like neural stem cells, some brain tumors express the intermediate filament nestin, as well as other genes that regulate the proliferation of normal neural progenitor cells, such as EGF-R (Almqvist et al., 2002; Gilbertson et al., 2001; Rorke et al., 1997; Taipale and Beachy, 2001; Tohyama et al., 1992; Wechsler-Reya and Scott, 2001). Moreover, expression of oncogenes in neural stem or progenitor cells in mice results in the formation of tumors that resemble primary human brain tumors (Holland, 2000).

Ignatova et al. first showed that primary human cortical anaplastic astrocytoma

and glioblastoma multiforme contain cells that, in clonal cultures, produce cells expressing neuronal and/or glial markers (Ignatova et al., 2002). Two groups recently demonstrated that some cells from pediatric brain tumors (primarily medulloblastomas and astrocytomas), like normal neural stem cells, could be directly cultured in serum-free medium as floating aggregates known as neurospheres, which were multipotent and could self-renew for long periods of time to give rise to secondary neurospheres (Fig. 5) (Hemmati et al., 2003; Singh et al., 2003). The neurosphere-forming subset was shown to reside in the fraction of cells expressing the cell-surface antigen CD133, a marker of human neural stem cells (Singh et al., 2003; Uchida et al., 2000). Hemmati et al. showed that tumor-derived progenitors from each patient had a stereotypic pattern of differentiation in vitro, yet those patterns were highly variable from one patient to another (Hemmati et al., 2003). Strikingly, however, the proportions of neurons, glia, and nestin-expressing cells produced from differentiated brain tumor-derived progenitor cells in vitro strongly correlated with their proportions in the primary tumor of origin (Hemmati et al., 2003). Unlike neural stem cells, the tumor-derived progenitors proliferated aberrantly and differentiated into unusual cells that co-expressed both neuronal and glial markers (Fig. 5G) (Hemmati et al., 2003). These results are consistent with the possibility that brain tumor-derived progenitors might be cancer stem cells (Hemmati et al., 2003). Short-term studies have demonstrated that brain tumor-derived progenitors can migrate, proliferate, and differentiate after transplantation into rat brains, but tests of their ability to form tumors in immunodeficient mice are ongoing (Hemmati et al., 2003).

In light of these data, might neural crest stem cells or their committed progenitor cells be the origins of peripheral nervous system tumors? This seems like a tractable

possibility since neural crest stem cells persist into adulthood and are therefore susceptible to acquiring transforming throughout life (Bixby et al., 2002). It has been posited that neuroblastomas, the most common solid extracranial tumor in children, arise from the transformation of neural crest stem cells or neural crest-derived sympathetic precursor cells (Brodeur, 2003; Nakagawara and Ohira, 2004). The recent finding by Trentin et. al. that multipotent neural crest cells form a hierarchy that contains lineage-restricted precursor cells that retain self-renewal ability suggests that those progenitor cells might be targets for transformation in the development of certain neural crest-derived malignancies (Trentin et al., 2004). Specifically, transformation of glial-melanocyte progenitor cells, which are able to self-renew in vivo, might lead to human tumors characterized by the overproduction of glia and melanocytes, such as neurofibromas and melanotic Schwannomas (Ferner and O'Doherty, 2002; Riccardi, 1991). Likewise, glial-myofibroblast progenitor cells might give rise to tumors that involve both neural and mesectodermal cells, like Ewing's sarcomas (Cavazzana et al., 1987; Thiele, 1991). To address these possibilities, it will be necessary to prospectively isolate cancer stem cells from the aforementioned tumors using similar methods to those used for CNS tumors.

What molecular mechanisms might drive normal stem or progenitor cells to become malignant? One possibility is that failure of stem cells to regulate their endogenous self-renewal pathways causes their neoplastic transformation. One candidate molecule that has received much attention in this regard is bmi-1, a polycomb family transcriptional repressor that promotes proliferation partly by repressing the expression of two cyclin-dependent kinase inhibitors p16-ink4a and p19-Arf (Jacobs et al., 1999). Bmi-

1 was recently shown to be required for the self-renewal of both normal and leukemic hematopoietic stem cells (Lessard and Sauvageau, 2003; Molofsky et al., 2003). In the nervous system, bmi-1 is required for the self-renewal of both neurosphere-forming central nervous system stem cells and neural crest stem cells (Molofsky et al., 2003). Expression of bmi-1 has recently been shown to be elevated in cerebellar precursor cells, whole human medulloblastomas, and in tumor-derived progenitor cells from medulloblastomas and other pediatric brain tumors, even after differentiation (Hemmati et al., 2003; Leung et al., 2004). Therefore it is possible that bmi-1 and other regulators of proliferation play a role in the pathogenesis of tumors of the central and peripheral nervous system.

1.5 CONCLUSIONS

The demonstration that multiple molecules from different gene families have the capacity to induce neural crest implies that the mechanism of neural crest induction involves complex and perhaps parallel pathways. It is further interesting to note that the same molecules can have multiple inductive capabilities at different times in development. Although great strides have been made toward understanding the induction and cell fate decisions of the neural crest, many mysteries remain. The field of neural crest research is rich in unanswered questions whose solutions will not only offer deeper understanding of the mechanisms of neural crest development but will also give more general insight into phenomena such as cell migration and differentiation, as well as the development of cancers of the peripheral nervous system.

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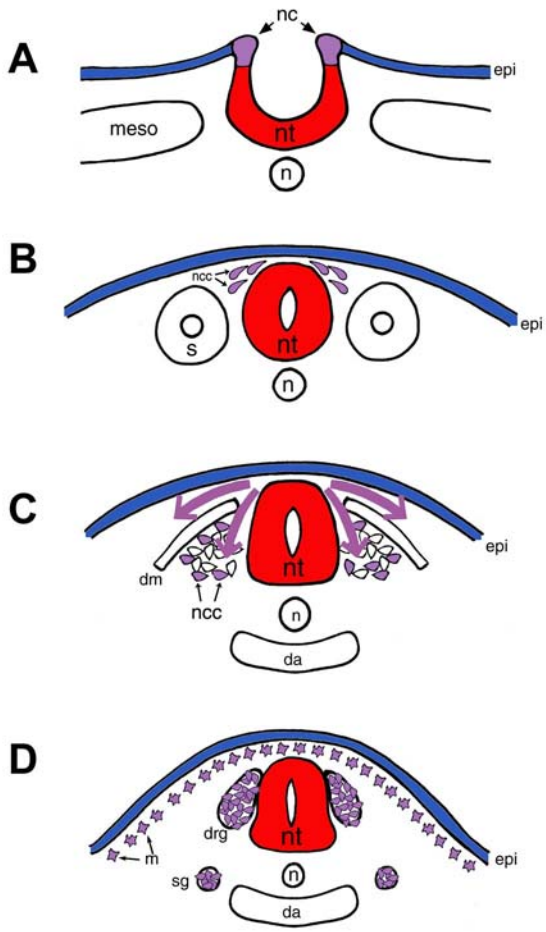

Figure 1: Neural crest-forming regions and migration pathways in avians: cross-sectional view

(A) E1.5-2. Thickened epithelium at the midline begins to fold into a tube. The border of the neural and non-neural ectoderm is the site of neural crest formation.

(B) E2-2.5. Neural crest cells delaminate from the the dorsal neural tube and begin to migrate.

(C) E3. Two migration pathways are shown: the dorsolateral pathway passes between dermomyotome and epidermis, and the ventral pathway passes through the sclerotome of the somites.

(D) E4: Neural crest cells populate the dorsal root ganglia, sympathetic ganglia, and form melanocytes in the skin.

da, dorsal aorta; dm, dermomyotome; drg, dorsal root ganglion; epi, epidermis; m, melanocyte; meso, non-axial mesoderm; nc, neural crest; nt, neural tube; s, somite; sg, sympathetic ganglion.

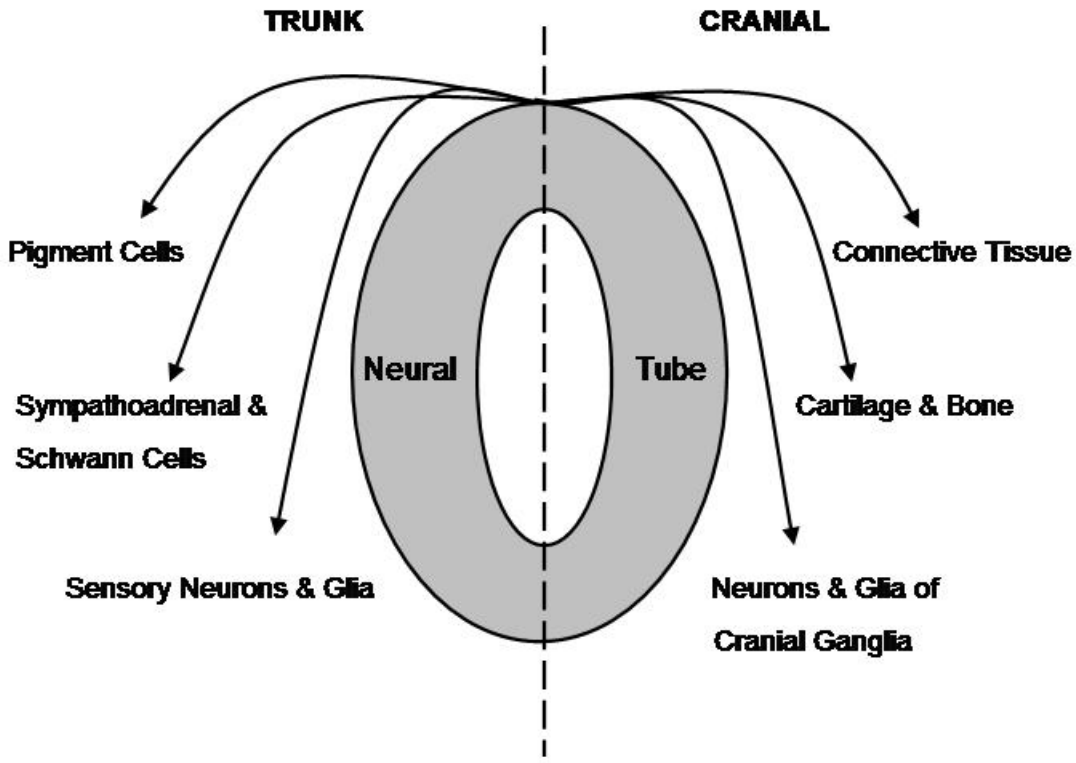

Figure 2: Diagram illustrating the derivatives arising from trunk and cranial neural crest cells.

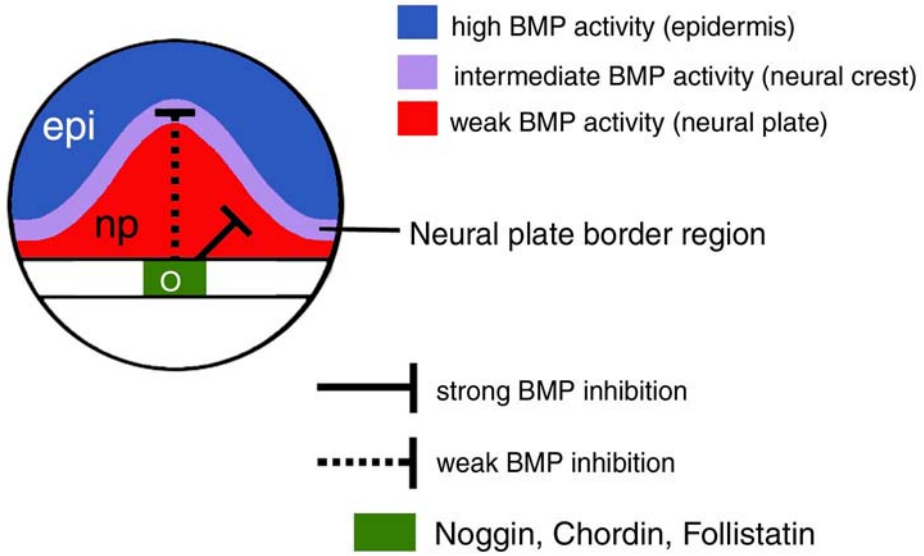

Figure 3: Schematic diagram of the *Xenopus* model of neural induction

The BMP antagonists noggin, chordin and follistatin are secreted from Spemann's Organizer (black box) to modulate BMP activity in the ectoderm. The activity of BMP molecules establishes three fates of ectoderm: lowest activity = neural plate; intermediate activity = neural crest; highest activity = epidermis. This simplistic model does not include the evidence for the involvement of other molecules in neural and neural crest induction, but is intended as a simplified model of neural induction. Modified from LaBonne & Bronner-Fraser.

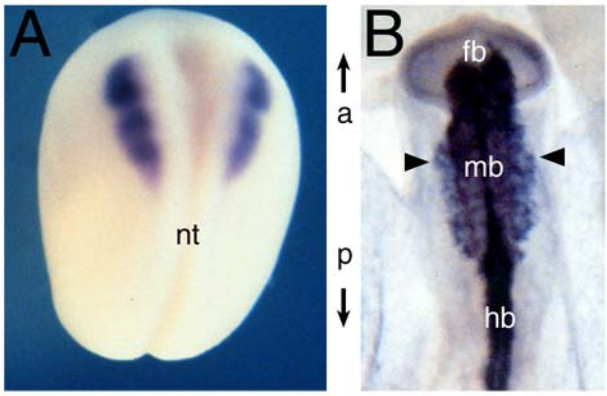

Figure 4: *Slug* expression pattern in *Xenopus* and chick

The zinc-finger transcription factor Slug is an early marker for the neural crest. (**A**): a late-neurula *Xenopus* embryo with *Slug* mRNA expression in the cranial neural crest on both sides of the closing neural tube. The groove down the central portion of the embryo is the forming neural tube. (**B**): an E1.5 (10-somite stage) chick embryo with *Slug* mRNA marking the early-migrating neural crest in the head (arrowheads) and pre-migratory neural crest at more posterior levels of the neural tube.

a, anterior; p, posterior; fb, forebrain; mb, midbrain; hb, hindbrain; nt, neural tube.

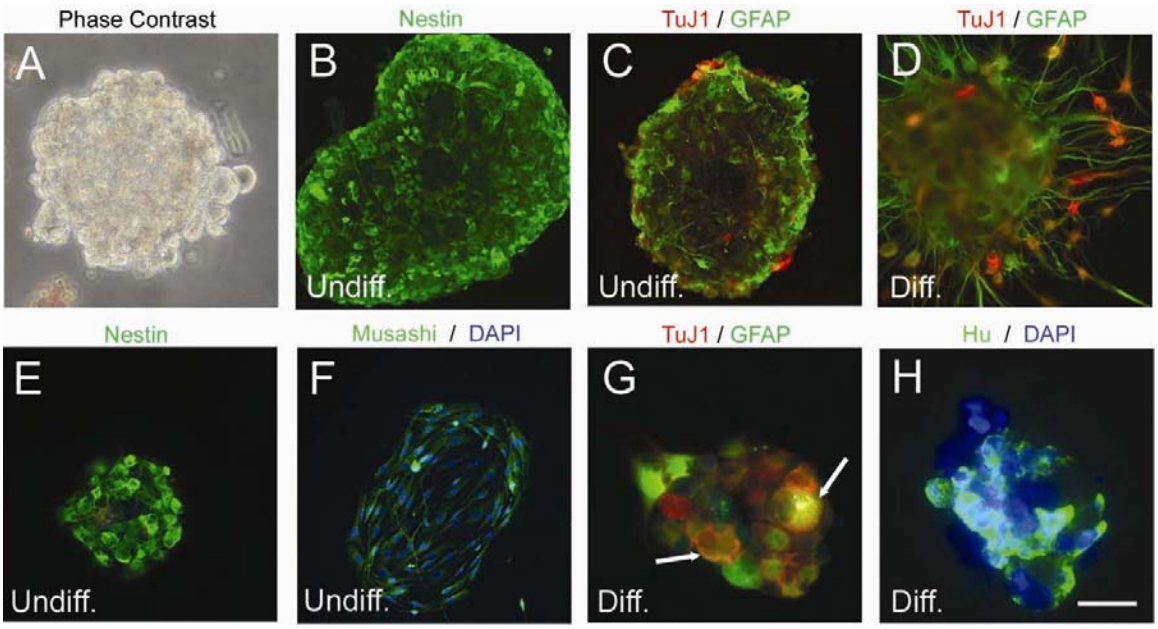

Figure 5: Tumor-derived progenitors form neurospheres in culture that give rise to both neuronal and glial cells. Neurospheres from one tumor, BT1, were cultured at medium (**A-D**) and clonal (**E-H**) densities. (**A**) A typical primary neurosphere is round in morphology and contains numerous birefringent cells when viewed under phase contrast optics. (**B** and **C**) Undifferentiated primary neurospheres expressed high levels of nestin protein (**B**, green) and low levels of (**C**) βIII-tubulin (red) and GFAP (green). (**D**) After 7 days of exposure to differentiation conditions, primary neurospheres significantly increased numbers of cells expressing β–III-tubulin and GFAP, and produced cells extending processes onto the substrate. (**E**) Undifferentiated clonal neurosphere cells have high levels of nestin expression. (**F**) Undifferentiated clonal neurospheres expressed the neural stem cell marker musashi-1 (green) in nearly every cell. (**G**) A neurosphere derived from a single cell that, under differentiation conditions, formed cells expressing β–III-tubulin (red) which is characteristic of neurons and GFAP (green), which is characteristic of astrocytes. Some cells expressed both markers. (**H**) Differentiated clonal neurospheres produced cells expressing the neuronal marker Hu (green) in similar proportions to β–III-tubulin. Some nuclei were counterstained with DAPI (**F** and **H**, blue). Scale bar in **H** = 30 μm in **A**, **G**, and **H**, 60 μm in **B**-**F**. *Reproduced from PNAS with permission.*

Chapter 2:

Cancerous Stem Cells Can Arise from Pediatric Brain Tumors

[modified from Houman D. Hemmati, Ichiro Nakano, Jorge A. Lazareff, Michael Masterman-Smith, Daniel H. Geschwind, Marianne Bronner-Fraser and Harley I. Kornblum

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Abstract

Pediatric brain tumors are significant causes of morbidity and mortality and are of unknown cellular origin. An intriguing possibility is that they derive from self-renewing, multipotent neural stem cells. Here, we tested whether different pediatric brain tumors, including medulloblastomas and gliomas, contain cells with properties similar to neural stem cells. We find that tumor-derived progenitors form neurospheres that can be passaged at clonal density and are able to self-renew. Under differentiative conditions, individual cells are multipotent, giving rise to both neurons and glia, in proportions that reflect the tumor of origin. Unlike normal neural stem cells, however, tumor derived progenitors have an unusual capacity to proliferate and sometimes differentiate into abnormal cells with multiple differentiation markers. Gene expression analysis reveals that both whole tumors and tumor-derived neurospheres express many genes characteristic of neural and other stem cells, including CD133, Sox2, musashi-1, bmi-1, maternal embryonic leucine zipper kinase, and phosphoserine phosphatase, with variation from tumor to tumor. After grafting to neonatal rat brains, tumor-derived neurosphere cells migrate, produce neurons and glia, and continue to proliferate for more than four weeks. The results show that pediatric brain tumors contain neural stem-like cells with altered characteristics that may contribute to tumorigenesis. This may have important implications for treatment via specific targeting of stem-like cells within brain tumors.

Introduction

Primary malignant central nervous system tumors are the most frequent form of solid malignancy in children (Smith et al., 1998). While they are a heterogeneous group of tumors, some of the most common histological types are astrocytomas (52%), primitive neuroectodermal tumors (PNETs), including medulloblastoma (21%), and high grade gliomas (19%) (Sklar, 2002). Despite several advances in therapy, morbidity and mortality remain high (MacDonald et al., 2003).

The cellular origin of pediatric brain tumors remains a mystery whose solution is critical for the effective diagnosis, treatment, and basic understanding of brain tumor biology. One possibility is that pediatric brain tumors arise by transformation of proliferating neural stem cells (Brustle and McKay, 1995; Holland et al., 2000). Neural stem cells are defined by their fundamental ability to self-renew and differentiate into neurons and glia of the central nervous system (Morrison et al., 1997). There are several lines of indirect evidence in support of the possibility that brain tumors arise from stem-like cells. First, pediatric brain tumors often contain multiple cell types, suggestive of an origin from a cell with multilineage potential (Valtz et al., 1991). Second, many pediatric brain tumors, particularly PNETs and medulloblastomas, appear to arise from the ventricular zone, the region of the brain that houses proliferating progenitors, including neural stem cells (Alvarez-Buylla and Garcia-Verdugo, 2002; Koos and Horaczek, 1985; Sanai et al., 2004; Sutton et al., 1992). Third, both pediatric brain tumors and neural stem cells express nestin, an intermediate filament characteristic of progenitor cells for multiple lineages, including astrocytes, neurons, oligodendroglia, ependymal cells, melanocytes, and smooth muscle (Almqvist et al., 2002; Rorke et al., 1997; Tohyama et

al., 1992). Fourth, pediatric brain tumors often express genes that regulate proliferation and self-renewal of normal neural stem cells, such as growth factor receptors and components of the Wnt and Sonic hedgehog signaling pathways (Gilbertson et al., 2001; Taipale and Beachy, 2001; Wechsler-Reya and Scott, 2001). Mutations in genes that normally regulate neural stem cell proliferation are frequently found in pediatric brain tumors, suggesting that aberrant proliferation of neural stem cells may underlie tumorigenesis. For example, the Pten tumor suppressor gene is often mutated in glioblastomas, medulloblastomas and PNETs (Mischel et al., 2003); similarly, the epidermal growth factor receptor is amplified or mutated in high grade gliomas (Watanabe et al., 1996). Finally, forced expression of oncogenes in neural stem and progenitors cells in mice produces tumors that are histologically and genetically similar to primary human tumors, demonstrating the capability of transformed neural stem cells to produce tumors (Holland et al., 2000).

If pediatric brain tumors contain cells with stem cell properties, an important question is whether these cells behave like "normal" stem cells or whether individual stem cells have abnormal properties that are responsible for the aberrant and persistent growth of the tumor (Reya et al., 2001). In models of breast cancer and acute myelogenous leukemia, "cancer stem cells" have been isolated and re-passaged into experimental animals to form new tumors, providing strong evidence that these cells are the root cause of the tumor (Al-Hajj et al., 2003; Bonnet and Dick, 1997). Such individual cells are capable of self-renewal, proliferation and differentiation to create the complex, heterogeneous tumor. It is unknown whether pediatric brain tumors contain such cancer stem cells, and, if so, whether such cells are derived from neural stem cells.

In the current study, we explored the fundamental properties of pediatric brain tumors and asked whether they contained progenitor cells with characteristics similar to those of neural stem cells. We have isolated and characterized multipotent, self-renewing cells from tumor samples, here referred to as "tumor-derived progenitors" which have both similarities to and differences from normal neural stem cells. Cells derived from pediatric brain tumors were able to produce proliferating neurospheres that could be passaged at clonal density and differentiated into cells with defining antigenic characteristics of neurons and glia (Reynolds and Weiss, 1992). These neurospheres expressed many genes characteristic of neural stem cell-derived spheres. Furthermore, like normal neurospheres, tumor derived spheres migrated and continued to proliferate when transplanted into neonatal rat brain. Unlike neural stem cells, however, these tumor-derived progenitors were more long-lived and often gave rise to abnormal dual-phenotype cells. Our data suggest that pediatric brain tumors arise from cells with many of the characteristics of neural stem cells, but with abnormal ability to propagate and differentiate. These studies also raise the possibility that some tumor-derived cells may be cancer stem cells with the ability to generate pediatric brain tumors.

Materials and Methods

Tissue Collection and Grading

Pediatric brain tumors and non-tumor human brain specimens from patients undergoing neocortical resections for intractable epilepsy were obtained within 30 minutes of surgical resection in accordance with protocols approved by institutional review boards at UCLA Medical Center and the California Institute of Technology. The epilepsy surgery tissue was taken from the lateral ventricular surface and immediately adjacent tissues. Tumors were graded at UCLA Medical Center by the attending neuropathologist in accordance with World Health Organization (WHO) established guidelines (Kleihues et al., 2002).

Neurosphere Culture

Tumors and neurosphere cultures were performed as according to the protocol for human neurospheres described by Svendsen with some modifications (Svendsen et al., 1998). 100-500 mm^3 pieces of tumor or "normal" brain ventricular tissue were washed in chilled sterile phosphate buffered saline (PBS, pH 7.4) with 0.6% glucose. The tissues were minced with scissors into small pieces. Pieces were incubated in 0.1% trypsin (Gibco-Invitrogen), 0.04% DNAase (Sigma type II) in Hank's balanced salt solution (HBSS) for 45 minutes at 37°C. The digested tissues were washed three times in 0.04% DNAase in HBSS and then triturated successively in the same solution using first a 5 ml pipette (Falcon), a1000 μm pipette tip, and then a fire-polished Pasteur pipette. Dissociated cells were passed through a 100 μm cell strainer (Falcon), followed by passage through a 70 μm cell strainer. Cells were seeded at a concentration of 100,000 per ml into tissue

culture flasks and grown in proliferation medium containing DMEM-F12 (Gibco Invitrogen), penicillin G, streptomycin sulfate, amphotericin B (1:100; Gibco-Invitrogen), B27 (1:50; Gibco-Invitrogen), recombinant human fibroblast growth factor (FGF-2, 20 ng/ml; Peprotech), recombinant human epidermal growth factor (EGF, 20 ng/ml; R&D Systems), LIF (20 ng/ml; Chemicon) and heparin (5 μg/ml; Sigma). Fresh FGF-2 and EGF were added at the same concentrations twice each week. Spheres were passaged by gentle trituration through a fire-polished Pasteur pipette, followed by re-seeding into fresh proliferative medium. To create clonal density spheres, moderate density spheres (described above) were triturated through a fire-polished Pasteur pipette, and the resulting cell suspension was passed through a 40 μm cell strainer to create a single-cell suspension (Geschwind et al., 2001). Cell counts were performed, and cells were reseeded in 2:3 filtered mouse-neurosphere conditioned medium diluted in proliferation medium at a density of 1000 cells per ml.

Immunocytochemistry of Neurospheres

Immunocytochemistry of neurosphere cultures was performed as previously described (Kornblum et al., 1997). Differentiation was induced by plating the neurospheres onto coverslips pre-coated with poly-L-lysine (Sigma) in Neurobasal medium (Gibco-Invitrogen) supplemented with B27 and in the absence of added bFGF or EGF. After seven days, the plated neurospheres were fixed in 4% paraformaldehyde and immunostained with rabbit anti-nestin (1:200; Chemicon) or rabbit anti-musashi (1:200; Chemicon) for neural stem and progenitor cells, mouse anti-TuJ1 (1:500; Berkeley Antibodies) or anti-Hu (1:300; Molecular Probes) for neurons, rabbit anti-GFAP (1:500;

DAKO) for astrocytes, and anti-O4 (1:40; Chemicon) for oligodendrocytes, followed by Alexa flurophore-conjugated secondary antibodies (1:2000 Molecular Probes). In some cases, nuclei were counterstained with DAPI. Visualization was performed with fluorescence microscopy using a Zeiss Axiophot and images captured with a Zeiss Axiocam.

Statistical Analysis

Total cell counts of clonal neurospheres were performed by counting the number of nuclei within the neurospheres incorporating DAPI, as observed under fluorescence microscopy. Cell counts are reported as a percentage of total cells expressing nestin, TuJ1, and/or GFAP among several clonal neurospheres plated across two to three coverslips. Data were analyzed using the paired *t*-test and presented as the mean ± standard error of measurement. Controls consisted of the identical cultures incubated in the absence of primary antibodies.

Immunohistochemistry of Whole Tumor Sections

Formalin-fixed, paraffin-embedded tissue specimens from BT1-3 and BT5 were sectioned at 8μm. Sections were mounted on Superfrost Plus slides (Fisher), deparaffinized in Histosol (National Diagnostics), and processed for antigen retrieval by microwave heating as previously described (Imam et al., 1995). Briefly, the slides were placed in a glass jar containing 0.01M sodium citrate buffer, pH 6.0, and heated twice for 5 minutes each in a microwave oven at the highest power setting. The slides were cooled in the jar until they returned to room temperature. The slides were washed in distilled,

deionized H_2O for 5 minutes, then in PBS, and stained with the appropriate primary and secondary antibodies, as described above.

BrdU-Labeling of Neurospheres

Neurospheres were cultured in proliferative medium containing 2 μM 5-Bromo-2'-Deoxyuridine (BrdU) for 14 hours. The cells were fixed in 4% phosphate-buffered paraformaldehyde solution. Staining with anti-BrdU antibody (Becton-Dickinson) was performed according to manufacturer's suggestions and $BrdU^+$ nuclei were observed and counted with fluorescence microscopy.

Semi-Quantitative RT-PCR

Total RNA was isolated from normal ventricular brain and tumor samples, neurosphere cultures, and neurospheres differentiated for 7days by TRIzol (GIBCO BRL), and 1μg of RNA from each sample was converted to cDNA by reverse transcriptase, following the manufacturer's protocol (Superscript II, Life Technologies). The amount of cDNA was examined by rt-PCR using primers for a glyceraldehyde-3-phosphate-dehydrogenase-gene (GAPDH) as an internal control from 25 to 33 cycles. After a confirmation of the equal amount of cDNA for each sample, the resultant cDNA was subjected to PCR analysis using primers specific to genes, listed below. The protocol for the thermal cycler was denaturation at 94ºC for 5 min, followed by 36 cycles of 94ºC (30 sec), 60ºC (1 min), and 72ºC (1 min), with the reaction terminated by a final 10 min incubation at 72ºC. Control experiments were run either without reverse transcriptase or without template cDNA to ensure that the results were not due to amplifications of genomic or

contaminating DNA. Each reaction mixture was visualized after 2% agarose gel electrophoresis for 30 min, and the expression levels of each gene were compared between the cDNA samples on a same gel. The intensities of signal were scored as -, ±, +, ++, +++ by an independent observer.

Msi1 S	5'gagactgacgcgccccagcc3'
Msi1 AS	5'cgcctggtccatgaaagtgacg3'
CD133 A	5'ctggggctgctgtttattattctg3'
CD133 AS	5'acgccttgtccttggtagtgttg3'
Sox2 S	5'accggcggcaaccagaagaacag3'
Sox2 AS	5'gcgccgcggccggtatttat3'
Melk S	5'cttggatcagaggcagatgtttggag3'
Melk AS	5'gttgtaatcttgcatgatccagg3'
PSP S	5'ggcggggcagtgcctttcaaa3'
PSP AS	5'tgttggctgcgtctcatcaaaacc3'
Bmi-1 S	5'ggagaccagcaagtattgtccttttg3'
Bmi-1 AS	5'cattgctgctgggcatcgtaag3'

Transplantation of Neurosphere Cells into Neonatal Rats

Intracranial transplantation of neurosphere cells into neonatal rat brain was performed according to the methods of Uchida et. al. with some modifications (Uchida et al., 2000). Animals were treated in accordance with protocols approved by the UCLA Institutional Review Board. Neurosphere cells were dissociated with gentle trituration through a fire-polished Pasteur pipette and the number of live cells determined by Trypan blue exclusion. Neonatal (postnatal day 1) rats were anesthetized by placing them on ice for 5-10 minutes. Once cryoanesthetized, the pups were placed on a stereotactic device and injected with 1 µl of cells containing 50,000 cells into the neostriatum. The injected rats were kept for 4 weeks before they were examined for human cells.

Immunohistochemical Analysis of Transplanted Rat Brain

Four weeks after transplantation, the injected rats were perfused with 4% paraformaldehyde. Brains were removed, post-fixed for 2 hours in 4% paraformaldehyde, cryoprotected in 20% sucrose overnight, and serially sectioned at 20 μm on a cryostat. Sections were incubated in primary and secondary antibodies as described above. To detect human cells in the transplanted brains, sections were stained with a mouse monoclonal antibody against human nuclei (1:100; Chemicon).

Results

Primary brain tumors from 22 patients were used in the current studies. The tumors included: 10 gliomas including 2 glioblastomas, 6 medulloblastomas, 5 primitive neuroectodermal tumors (PNET) and 1 ependymoma. For detailed analysis of cell fate and gene expression, we studied a subgroup of 6 tumors from 5 pediatric patients, aged 5 months to 6 years, [designated tumors 1-5 (BT1-BT5); Table 1] that were obtained within 30 minutes of surgical resection for this study. These tumors included a midline anaplastic astrocytoma, as defined by the World Health Organization (WHO grade III astrocytic tumor; BT1), two cerebellar medulloblastomas (BT2 and BT3), one glioblastoma multiforme (WHO grade IV astrocytic tumor; BT4), and one desmoplastic medulloblastoma (BT5), distinguished from medulloblastoma by marked neuronal differentiation (Kleihues et al., 2002).

Brain tumor cells have the ability to form neurospheres

As a first step to test for the presence of stem-like cells in pediatric brain tumors, tumors were dissociated into single cells and assayed for their ability to form neurospheres using the same methods as described previously for human neural stem cells (Svendsen et al., 1998; Uchida et al., 2000). From all 22 primary brain tumors studied, this resulted in the production of proliferating neurospheres. Small spheres of approximately 4-10 cells in diameter were observed in culture flasks between 3 days and 1 week after seeding the cells at moderate density (100,000 cells/ml; Fig. 1A). The frequency with which dissociated cells formed viable microspheres was very high, as each flask contained a far greater number of spheres than did flasks with cells propagated

from noncancerous ventricular zone seeded at the same density. Within 2 weeks, most spheres had increased their diameters approximately 5 to 10-fold. Cells from a local recurrence of one tumor (BT4, glioblastoma multiforme) also gave rise to spheres in culture. Neurospheres could be passaged multiple times by mechanical dissociation of large spheres and reseeding in fresh proliferative medium every 2-3 weeks. Under these circumstances, neurospheres could be maintained for 16 weeks or more.

We next asked whether tumor-derived progenitors maintained the differentiative capacity of neural stem cells. To test this, we examined the types of molecular markers expressed by neurospheres grown under both proliferative and differentiating conditions. Early-passage spheres maintained in culture for 4 weeks or less were used for further characterization. For most tumors, immunocytochemical analysis of undifferentiated tumor spheres in proliferation medium revealed many cells expressing the neural progenitor marker nestin (Lendahl et al., 1990) (Fig. 1B; except BT3) and relatively few cells expressing the neuronal marker ßIII tubulin and/or the astrocytic marker GFAP (Fig. 1C). Tumor-derived neurospheres passaged in culture between 10 and 16 weeks maintained these high levels of nestin expression and low levels of ßIII-tubulin and GFAP expression, similar to typical neurospheres derived from embryonic mammalian germinal zones.

In order to assess differentiation potential, neurospheres were transferred to differentiating medium (lacking bFGF and EGF) and were grown for 7 days on an adherent substrate. Under these conditions, cell division in the spheres slowed dramatically and attached cells appeared to migrate on the substrate. Immunocytochemical analysis revealed an increase in the proportion of cells expressing

ßIII tubulin and/or GFAP. Differentiated neurospheres produced numerous cells resembling neurons and astrocytes, with many cells extending processes on the substrate (Fig. 1D). These results show that pediatric brain tumors contain cells with properties similar to those of neural stem cells. These cells have the ability to form neurospheres and can differentiate into cells with neuronal and glial characteristics spontaneously at low incidence under proliferative conditions and at high incidence when grown under differentiative conditions.

Tumor-derived progenitors can be serially recloned

The above results show that primary tumor cells have the ability to form neurospheres. Self-renewal and multipotency are critical features of any stem cells, including neural stem cells (Morrison et al., 1997). Accordingly, we next tested whether individual cells derived from these neurospheres had the ability to form new neurospheres that subsequently differentiated into multiple cell types. To this end, primary neurospheres were tested for their ability to form secondary spheres when dissociated into single-cell suspensions and reseeded at clonal density (1000 cells/ml) in proliferative medium. Conditions were similar to those used previously to establish clonality for primary neurospheres (Groszer et al., 2001). When tumor samples are seeded at this density and examined the following day, only individual cells and no clusters could be observed, verifying their clonal origin. In all cases, clonally-derived neurospheres were visible within 2-4 weeks after reseeding. This result suggests that neurospheres contain individual stem-like cells with the ability to self-renew and form neurosphere colonies.

Immunocytochemistry was used to compare the molecular markers expressed by clonal neurospheres grown under proliferative and differentiation conditions. We first examined the expression of two proteins known to be present in a majority of neural stem and progenitor cells. Undifferentiated clonal spheres from 4 out of 5 tumors expressed nestin in a large percentage of their cells (> 45%) with little variability between clones (Figs. 1E and 2A, B, D, E). However, nestin protein expression was undetectable in BT3, a medulloblastoma (Fig. 2C). In addition, tumor-derived spheres expressed significant immunostaining for musashi-1, a marker of neural stem and progenitor cells (Fig. 1F) (Sakakibara et al., 2002). After transfer to differentiating conditions for 7 days, the proportion of nestin expressing-cells declined in clonal spheres derived from 3 out of the 4 tumors (Fig. 2A, B, D) remaining unchanged only in BT5, a desmoplastic medulloblastoma (Fig. 2E).

To understand the differentiative capacity of clonal tumor-derived neurospheres, we quantitatively compared the cell types produced by spheres prior to and after differentiation using cell type-specific markers. Clonal neurospheres gave rise to cells with neuronal and glial properties. Under differentiation conditions, the majority of clones from each tumor gave rise to cells resembling both neurons and glia, with many cells per sphere expressing ßIII-tubulin and/or GFAP (Figs. 1G). The average percentage of ßIII-tubulin^{+} cells in clonal spheres increased significantly after transfer to differentiation conditions in all tumors except BT5, a desmoplastic medulloblastoma (Fig. 2, left panel). The average percentage of GFAP^{+} cells in clonal spheres increased significantly after differentiation in all tumors except BT4, a glioblastoma multiforme (Fig. 2, left panel). Differentiated clonal spheres expressed the neuronal HuC/D antigen

in similar proportions to their expression of ßIII-tubulin (Fig. 1H). In contrast, the oligodendrocyte marker O4 could not be detected by immunocytochemistry in clonal or high density neurospheres. These results show that tumors contain progenitor cells that are multipotent, giving rise to cells with neuronal and glial characteristics.

Although all tumors gave rise to both neuronal and glial-like cells, the percentage varied considerably from one tumor to another (Fig. 2, right panel). BT5 produced neurospheres which differentiated into both neurons and glia. The remaining tumors gave rise to a mixture of clones, some of which formed multiple cell types and others that formed only neuron-like cells. Curiously, BT1 produced some clones that formed neither neurons nor astrocytes. It is interesting to note that the lowest proportion of neurospheres differentiating into multiple cell types was observed in BT3, the only tumor studied that had very low levels of nestin protein (Fig. 2C).

Properties of clonally derived spheres recapitulate characteristics of the parental tumors

The results described above demonstrate that tumor-derived progenitors are self-renewing and multipotent. In order to determine whether these findings recapitulate the properties of the parental tumors or are simply an artifact of culture, we examined the antigenic characteristics of 4 tumors for which we had both neurosphere counts and sufficient tumor tissue for further analysis (Fig. 3). Tumors were stained for nestin, GFAP and TuJ1. Interestingly, the staining patterns demonstrated broad similarities to those observed in tumor-derived spheres. For example, BT1 expressed high levels of nestin within the tumor (Fig. 3A) as well as in neurospheres. In addition, many cells in

this same tumor expressed either TuJ1or GFAP (Fig. 3E). BT2 (Fig. 3 B, F) also express all three markers but at lower levels than BT1, both in the parent tumor and in the neurospheres. BT3 had little nestin staining in the tumor (although nestin is obvious in the adjacent normal brain; Fig. 3C); this low level of nestin parallels that observed in the neurospheres (compare Figs. 2C and 3C). With respect to TuJ1 and GFAP (Fig. 3G), BT3 had strong reactivity for both, as did the neurospheres (Fig. 2C). BT5 showed good correlation between the numbers of TuJ1 and GFAP in both the tumors (Fig. 3H) and neurospheres (Fig. 2E); in contrast, nestin expression did correlate well between tumor and neurosphere in this one particular case (Fig. 3D and 2E). Taken together, these data indicate that the tumor derived spheres, even after months in culture, differentiate similarly to the original tumors from which they were derived in the majority of cases.

Tumor-derived neurospheres express neural stem cell-related genes

We used semi-quantitative RT-PCR analysis to determine whether neurospheres cultured from pediatric brain tumors expressed multiple genes enriched in neural and other stem cells, and to compare expression amongst proliferating and differentiating tumor progenitors as well as non-propagated tumor. Primers were designed for the following human genes: CD133, a cell surface protein expressed on all fetal human neural stem cells (Uchida et al., 2000); musashi-1, an RNA binding protein robustly expressed by mammalian neural stem and progenitor cells (Kaneko et al., 2000); Sox2, an early transcription factor expressed in neural stem cells and the developing neural tube (Cai et al., 2002; Zappone et al., 2000); bmi-1, a Polycomb Group gene required for self-renewal and proliferation of normal and leukemic hematopoietic stem cells (Lessard and

Sauvageau, 2003; Park et al., 2003). We also examined the expression of two genes that we have identified from mouse neural progenitors by a subtractive microarray screening approach: melk, maternal embryonic leucine zipper kinase and PSP, phosphoserine phosphatase (Easterday et al., 2003; Geschwind et al., 2001; Terskikh et al., 2001). Both of these genes are highly and selectively expressed in murine central nervous system germinal zones and are present in both neural progenitors and hematopoietic stem cells.

All tumors as well as cells derived from the ventricular zone of normal brain had detectable gene expression of CD133, Sox2, melk, PSP, and bmi-1 (Table 2). Those genes expressed in both tumor-derived and normal neurospheres were also expressed in their parental tissues, suggesting that gene expression observed in neurospheres is not an artifact of cell culture. We consistently detected expression of Sox2, melk, and bmi-1 across all whole tumor, normal brain, and undifferentiated neurosphere samples. Interestingly, expression of some genes, most noticeably PSP, was reduced or lost in spheres, as compared to whole tumor or ventricular zone-containing tissue. This suggests that either uncultured tissues expressed these genes at higher levels than their neurosphere-initiating cells and their progeny, or that these genes were down-regulated as a consequence of cell culture. Neurospheres from BT1 and BT4, both glial tumors of different pathological grades, expressed nearly all stem cell-related genes, but BT3 and BT5, both medulloblastomas, had significantly lower or absent detectable expression of those genes, indicating that cells in glial tumors might have a closer molecular relation to neural stem cells. In 11 instances, expression of specific genes in the tumor-derived spheres was reduced after growth factor withdrawal, consistent with our prediction that tumor-derived progenitors would produce differentiated cells at the expense of

multipotent progenitor cells. For example, expression of melk was reduced after differentiation in all neurospheres, except those from BT1. In only two instances did expression of specific genes increase after differentiation; for example, PSP in BT2 and CD133 in BT3.

The expression of Sox2 by brain tumor-derived progenitors is consistent with their being multipotent cells related to neural stem cells. Expression of this transcription factor is highly restricted being generally limited to embryonic stem cells (Easterday et al., 2003) and neural progenitors (D'Amour and Gage, 2003; Graham et al., 2003) and likely regulates multipotentiality of ES cells (Nichols et al., 1998; Niwa et al., 2000) and inhibits differentiation of neural progenitors (Graham et al., 2003).

Interestingly, all whole tumors and normal brain samples tested expressed high levels of bmi-1, a protein thought to be involved in stem cell self-renewal (Lessard and Sauvageau, 2003; Park et al., 2003). All tumor-derived neurospheres maintained high bmi-1 expression both under proliferative and after differentiative conditions. In contrast, normal neurospheres had significantly less bmi-1 expression, which further declined after differentiation.

Tumor-derived neurospheres incorporate into rat brain

It has previously been shown that cells derived from normal human neurospheres migrate, proliferate and differentiate within multiple sites after transplantation into developing rodent brains (Brustle et al., 1998; Uchida et al., 2000). To test whether tumor-derived progenitors possess the same abilities, 50,000 dissociated cells from neurospheres derived from BT4 at passage 4 were injected into the neostriata of neonatal

rats. Using an anti-human nuclear antibody, human cells were found seeding the subventricular zone of the lateral ventricle (Fig. 4A), in the corpus callosum (Fig. 4B), and at the injection site (Fig. 4C) by four weeks post-injection. Those human cells localized to the corpus callosum had elongated nuclei, consistent with the possibility that transplanted cells were migrating (Fig. 4B) (Yang et al., 2000). Some human cells in all locations expressed the neuronal marker Hu (Fig. 4 A and B) whereas others expressed the astrocytic marker GFAP (data not shown). To test whether transplanted tumor-derived progenitor cells and their progeny continued to proliferate in vivo, they were examined for co-expression of the Ki-67 antigen, a nuclear marker of proliferation expressed by cells in late $G_1/S/G_2/M$ phases of the cell cycle (Gerdes et al., 1983). Approximately 50% of transplanted cells co-expressed Ki-67, indicating that at least half of the tumor-derived progenitors and their derivatives continued to proliferate 4 weeks after transplantation into rat brains (Fig. 4C).

Tumor-derived neurospheres have similarities to and differences from normal neurospheres

We compared the properties of tumor-derived neurospheres to those of normal neurospheres cultured from the brain tissue of pediatric epilepsy surgery patients with age ranges similar to those of the tumor patients studied. For tumor-derived spheres, neurospheres could be maintained at moderate to high density for at least 4 months with no obvious change in their proliferative properties. In contrast, normal neurospheres grown under identical conditions typically persisted no longer than 1 month in culture. This suggests that tumor-derived neurospheres maintain proliferation for much longer

time periods than normal neurospheres. Consistent with this possibility, tumor-derived spheres demonstrated unusual proliferation even in apparently differentiated cells. We observed cells that expressed both ßIII-tubulin and Ki-67, a proliferation-specific nuclear antigen, suggesting that cells with the appearance of differentiated neurons were dividing (Fig. 5A). Moreover, approximately 50% of ßIII-tubulin$^+$ cells incorporated BrdU after a 14 hour pulse, indicating that a significant proportion of tumor-derived neurons divided over a brief time (Fig. 5B).

A second distinction was noted in the differentiative capacity of normal versus tumor-derived neuropheres. Neurospheres from all tumors tested gave rise not only to neurons and glia, but also to unusual individual cells that expressed both the neuronal marker ßIII-tubulin and the astrocytic marker GFAP (Fig. 2). Such dual-fate cells were common and represented a significant fraction of the population. ßIII-tubulin$^+$GFAP$^+$ cells often appeared larger than other cells in the same sphere (Fig. 1G, arrows). In two tumors, BT1 and BT5, the percentage of ßIII-tubulin$^+$ GFAP$^+$ cells exceeded the percentage of cells expressing ßIII-tubulin or GFAP alone (Fig. 2). These abnormal cells were observed in pediatric brain tumor-derived neurospheres at both moderate and clonal density. Previous studies have reported similar abnormal cells derived from adult brain tumors (Ignatova et al., 2002).

These results demonstrate that tumor-derived neurospheres, while sharing many features with neurospheres derived from non-neoplastic tissue, also have important differences. Both populations contain individual progenitor cells that are multipotent, having the ability to form neurospheres comprised of both neurons and glia. However, the cancer-derived stem-like cells appear to proliferate at a higher rate and can be

maintained in culture for significantly longer than neural stem cells isolated under similar conditions. In addition, they frequently undergo abnormal differentiation into dual phenotype neuronal/glial cells not seen in normal neuropheres.

Discussion

Recently, it has been recognized that some tumors contain cancer stem cells, a rare subpopulation of tumor cells that phenotypically resemble stem cells (Al-Hajj et al., 2003; Bonnet and Dick, 1997). An intriguing possibility is that these may represent the exclusive tumorigenic portion of the cancer. The discovery of such a population may have profound implications for the study and treatment of cancer (Dick, 2003; Reya et al., 2001).

In the present study, we asked whether pediatric brain tumors contain neural stem-like cells that may be responsible for the formation of pediatric brain tumors. Our results show that tumor-derived cells indeed have the ability to form neurospheres and can be propagated for prolonged times in culture. These results with pediatric brain tumors mirror those previously found for adult gliomas, demonstrating that both share the ability to produce neurospheres (Ignatova et al., 2002). We further showed that this property is not unique to a single type of tumor, but rather is a general property of all of the 22 tumors examined in this study. After transplantation into neonatal rat brains, neurosphere cells from one representative tumor seeded the subventricular zone and corpus callosum, gave rise to cells with antigenic properties of neurons and glia, and continued to proliferate in the brain one month after transplantation.

To test whether these had properties of stem cells, we demonstrated that, at clonal density, cells derived from these neurospheres are capable of self-renewal and are multipotent, differentiating into cells resembling neurons and astrocytes. Our results show for the first time that the properties of self-renewal are common to all of the pediatric brain tumors tested. Interestingly, the types of progeny arising from

neurospheres vary from tumor to tumor and, to some extent, recapitulate the properties of their tumor of origin. Further demonstrating the relationship between tumor-derived progenitors and neural stem cells, both expressed many of the same genes and proteins.

Among the characteristics in common between tumor-derived spheres and normal neural stem cells is the expression of specific genes, including CD-133, musashi-1, sox-2, melk, psp, bmi-1 and nestin. The latter is of particular interest, as it has been clearly demonstrated to be expressed by most, if not all, mammalian neural stem and progenitor cells. Only one of the tumors we studied, a medulloblastoma (BT3), failed to express nestin in most cells within undifferentiated clonal neurospheres. Interestingly, BT3 was the least stem cell-like of all the tumors we tested. It gave rise to the lowest percentage of multipotent neurospheres, did not express nestin protein, and failed to express most of the stem cell genes in its spheres. It is possible that the cells that formed spheres from BT3 were not the stem-like cells within the tumor, or that our cell culture conditions changed the phenotype of the cells, since the original tumor expressed many of the genes characteristic of stem cells. Another key gene is Sox2. This transcription factor is exclusively expressed in neural progenitors during mouse development and plays a critical role in the maintenance of the neural progenitor state (Graham et al., 2003). Interestingly, all tumors and neurospheres tested expressed this transcription factor. In addition, tumor derived spheres express other markers that are specifically enriched in neural stem cells, such as MELK and PSP (Easterday et al., 2003; Terskikh et al., 2001).

Although tumor-derived progenitors have many similarities to neural stem cells and to each other, it is important to note that differences exist between them and neural stem cells as well as between tumor-derived progenitors from different tumors. For

example, unlike normal neurospheres, cancer-derived neurospheres undergo aberrant proliferation and differentiation. Clonally-derived spheres from an individual pediatric brain tumor generally gave rise to similar percentages of neuron- and glial-like cells, suggesting that each tumor contained a fundamental type of stem cell. However, the degree of differentiation and types of cells produced differed from tumor to tumor. This may reflect a difference in the original stem cell, or simply stochastic differences in how the cell responds to environmental influences.

One surprising finding of our study is that the tumor-derived neurospheres tend to differentiate into an array of progeny with the same general profile as the parental tumor. Immunohistochemical analysis of the original tumor revealed striking similarities with the corresponding neurosphere. For example, BT3 had a high predominance of TuJ1-positive cells in both the tumor and the tumor-derived neural stem cells, while BT1 and BT2 contained a greater mix of neurons and glia. Importantly, our data demonstrate that the presence of diverse cell types in the tumors is not simply a result of the presence of "host" cells within the tumor mass, but rather is a property of the tumor itself.

Our finding that pediatric brain tumors contain neural stem-like cells is consistent with the hypotheses that at least some pediatric brain tumors are derived from transformed neural stem cells in the central nervous system. It has previously been hypothesized that medulloblastomas and PNETs are derived from multipotent progenitor cells (Valtz et al., 1991). While supporting that notion, our data also suggest that pediatric astrocytomas and high-grade gliomas are derived from either neural stem cells or progenitor cells, given that clonal spheres derived from such tumors are multipotent. Previous studies have demonstrated that high grade gliomas can form multiple

differentiated cell types (Mischel et al., 2003), and that some cells derived from adult gliomas in a manner similar to that described here, can form neurospheres with multiple differentiated cell types (Ignatova et al., 2002). One possibility is that gliomas are derived from a fully committed glial cell that then "de-differentiates" to become multipotent. In this case, while the source of the tumor-forming cell would not be a stem cell, per se, the net result would still be that the tumor contained self-renewing, multipotent cells. Alternatively, gliomas may be derived from a neural stem cell that is fundamentally the same as that which gives rise to PNETs or medulloblastomas. In the mouse, there is strong evidence to indicate that there is a lineage of cells that progresses from primitive cells within the neural tube to radial glia to GFAP-expressing "astrocytes" that all retain the fundamental properties of neural stem cells: self-renewal and multipotency (Doetsch et al., 1999) (Imura et al., 2003). We hypothesize that phenotypically different tumors derive from oncogenic transformation of cells at different points along this pathway, but retain broad developmental potential and the capacity to self-renew. However, as stated above, it is possible that the same result would be obtained if more committed cells re-attained a stem cell-like state during oncogenesis.

The mechanism whereby normal neural stem cells become malignant is not clear. Our data demonstrate that normal and tumor-derived spheres express the oncogene bmi-1, with tumor-derived spheres expressing higher levels. This gene has been demonstrated to be important for self-renewal of both leukemic and normal hematopoietic stem cells (Lessard and Sauvageau, 2003) (Park et al., 2003). It is possible that the high level of bmi-1 expression in tumor cells indicates a greater capacity for self-renewing divisions. Consistent with this possibility, bmi-1 levels drop in normal neurospheres derived from

postnatal brain grown under differentiating conditions. However, bmi-1 levels do not fall for tumor-derived progenitors grown under differentiating conditions. Thus, maintenance of bmi-1 correlates with prolonged proliferative ability of tumor-derived progenitors.

Cancer stem cells have been prospectively isolated from acute myelogenous leukemia and, more recently, from breast cancer (Bonnet and Dick, 1997) (Al-Hajj et al., 2003). These cells can be sorted using surface markers expressed by stem cells in normal bone marrow or mammary epithelium. The existence of these cells is consistent with the possibility that cancer stem cells derive from transformed somatic stem cells. Moreover, cancer stem cells share with their non-tumorigenic counterparts the properties of self-renewal and multipotency. Individual cancer stem cells transplanted into immunodeficient NOD-SCID mice have the ability to generate more cancer stem cells as well as all of the cell types that are found in the tumor, thereby recapitulating the human tumor (Reya et al., 2001).

While we have shown that tumor-derived progenitors, like normal neural stem cells, are able to migrate, proliferate and differentiate when transplanted into developing rat brains, it is not yet known if the tumor-derived cells capable of forming neurospheres also have the ability to form tumors in an animal model. Given the ability of rats to mount an immune response against engrafted human cells, it is not surprising that we did not observe tumors in transplanted animals. Future assays for tumor formation will be carried out in long-term studies using immunodeficient mice, as previously described (Al-Hajj et al., 2003).

Although we hypothesize that tumor-derived progenitor cells are, in fact, clonally derived from tumor cells, it is formally possible that normal neural stem cells or

progenitors may "contaminate" the tumor mass and thus account for the formation of neurospheres. There are two reasons for this concern: first, normal neural stem cells residing in the ventricular zone might have been included in the specimens obtained from surgical resection of the tumors, many of which were located at or near the midline. Second, others have reported that neural stem cells transplanted into rodent brains carrying human brain tumor cells have an affinity for tumors and can "track" infiltrating tumor cells and disseminate within the tumor bed (Aboody et al., 2000). To address these concerns, we compared the proliferation and differentiation of tumor-derived progenitors to those of cells derived from age-matched human brain specimens obtained from epilepsy surgeries. Quite strikingly, tumor-derived specimens produced far more spheres, which proliferated faster, and persisted in culture months longer than those not derived from tumors. Only tumor-derived spheres gave rise to individual cells expressing both neuronal and glial markers. Moreover, only tumor-derived spheres maintained high expression levels of bmi-1 before and after differentiation.

Knowledge of the developmental origin of pediatric brain tumors has important implications for therapy. We have explored the intriguing possibility that these tumors may be derived from progenitor cells with stem-cell like properties. Indeed the results show that cells derived from pediatric brain tumors form neurospheres that can be serially recloned and contain self-renewing stem cells. These stem cells are multipotent, giving rise to both neurons and glia in similar proportions to their tumors of origin. Although they resemble normal neural stem cells, they also exhibit important differences including an increased proliferative rate, persistently elevated bmi-1 expression, and the tendency to form abnormal dual phenotype cells. This approach can be used to elucidate

tumorigenic pathways, as well as to evaluate candidate anti-tumor therapies. These have the potential to identify improved diagnostic markers and therapeutic targets for pediatric brain tumors. Demonstration that tumor-derived progenitors are cancer stem cells for pediatric brain tumors suggests that therapies for treating pediatric brain tumors should include methods for targeting and elimination of the stem cell population.

Acknowledgements

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Table 1
Tumor and patient characteristics

Tumor	Patient Age	Diagnosis	Site of Tumor
BT1	5y	Anaplastic astrocytoma	Midline hypothalamus
BT2	5mo	Medulloblastoma	Cerebellum
BT3	6y	Medulloblastoma	Cerebellum
BT4	6y	Glioblastoma multiforme	Temporal lobe
BT5	15 mo	Desmoplastic medulloblastoma	Cerebellum

Table 2
Gene expression analysis

Whole Tumors	BT1	BT2	BT3	BT4	BT5	Normal
CD133	ND	±	++	+	++	+
Msi1	ND	++	-	+	++	++
Sox2	ND	ND	++	++	+	+
Melk	ND	+++	+	+++	++	+++
PSP	ND	+++	+++	++	++	++
Bmi-1	ND	ND	ND	+++	+++	+++
Spheres Undiff.						
CD133	+	±	-	+	-	-
Msi1	++	-	-	++	±	++
Sox2	++	++	ND	++	±	+
Melk	+++	++	+	+++	+	+++
PSP	+++	+	-	++	-	-
Bmi-1	+++	+++	ND	+++	ND	+
Spheres Diff.						
CD133	+	-	++	-	-	-
Msi1	++	-	ND	-	±	++
Sox2	++	++	±	-	-	±
Melk	+++	+	-	++	±	+
PSP	-	+++	-	-	-	-
Bmi-1	+++	+++	ND	+++	+++	±

Table 2: Semi-quantitative RT-PCR of pediatric brain tumors (BT1-5) and normal human brain analyzed for expression of stem cell markers. ND = not determined; - = no signal; ± = minor signal; + = weak signal; ++ = moderate signal; +++ = strong signal.

Figure 1

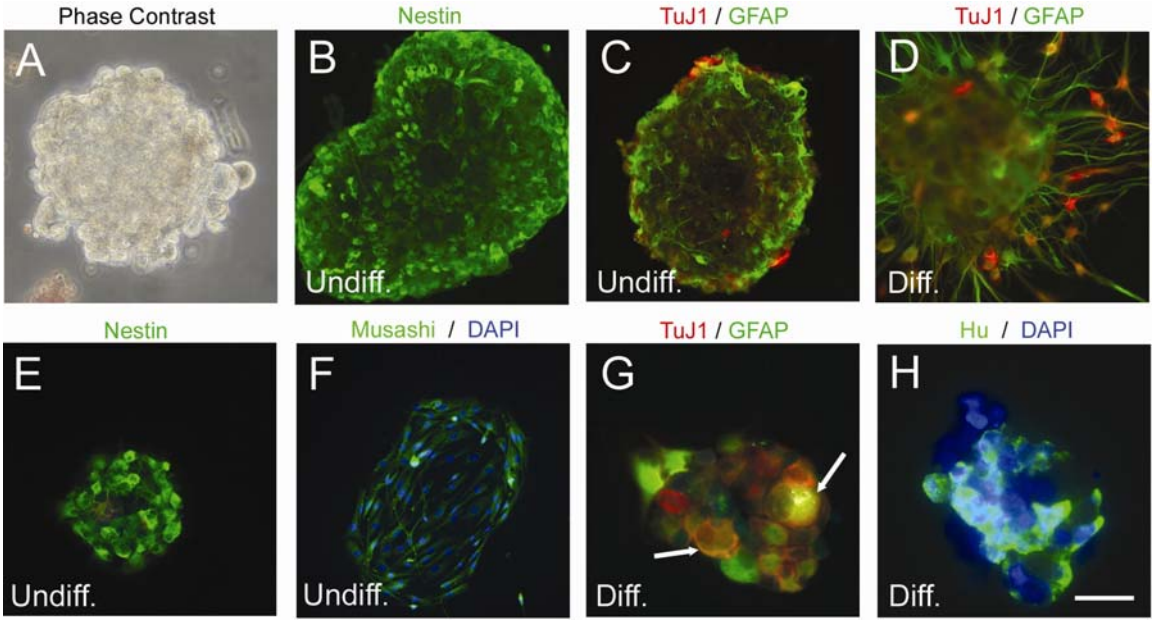

Fig. 1: Tumor-derived progenitors form neurospheres in culture that give rise to both neuronal and glial cells. Neurospheres from one tumor, BT1, were cultured at medium (**A-D**) and clonal (**E-H**) densities. (**A**) A typical primary neurosphere is round in morphology and contains numerous birefringent cells when viewed under phase contrast optics. (**B** and **C**) Undifferentiated primary neurospheres expressed high levels of nestin protein (**B**, green) and low levels of (**C**) βIII-tubulin (red) and GFAP (green). (**D**) After 7 days of exposure to differentiation conditions, primary neurospheres significantly increased numbers of cells expressing β–III-tubulin and GFAP, and produced cells extending processes onto the substrate. (**E**) Undifferentiated clonal neurosphere cells have high levels of nestin expression. (**F**) Undifferentiated clonal neurospheres expressed the neural stem cell marker musashi-1 (green) in nearly every cell. (**G**) A neurosphere derived from a single cell that, under differentiation conditions, formed cells expressing β–III-tubulin (red) which is characteristic of neurons and GFAP (green), which is characteristic of astrocytes. Some cells expressed both markers. (**H**) Differentiated clonal neurospheres produced cells expressing the neuronal marker Hu (green) in similar proportions to β–III-tubulin. Some nuclei were counterstained with DAPI (**F** and **H**, blue). Scale bar in **H** = 30 μm in **A**, **G**, and **H**, 60 μm in **B-F**.

Figure 2

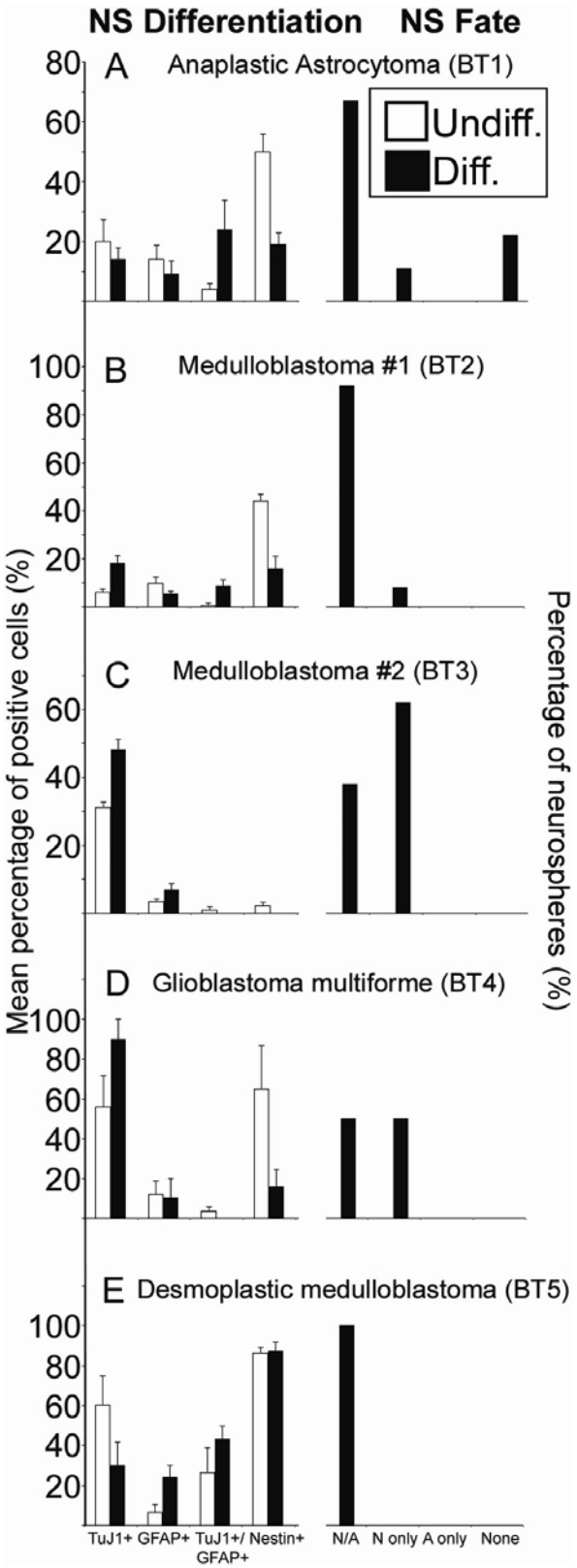
NS Differentiation
NS Fate
A
Anaplastic Astrocytoma (BT1)
Undiff.
Diff.
B
Medulloblastoma #1 (BT2)
C
Medulloblastoma #2 (BT3)
D
Glioblastoma multiforme (BT4)
E
Desmoplastic medulloblastoma (BT5)
Mean percentage of positive cells (%)
Percentage of neurospheres (%)
TuJ1+
GFAP+
TuJ1+/ GFAP+
Nestin+
N/A
N only
A only
None

Fig. 2: Neurospheres derived from multiple types of tumors give rise to cells expressing neuronal and glial markers in various proportions. **Left panel:** average count of cells expressing nestin, TuJ1 alone, GFAP alone, or both markers in clonal neurospheres (NS) from BT1-5 (**A-E**) prior to (white) and after (black) differentiation. **Right panel:** Fates of clonal neurospheres (NS) after differentiation. Markers used are TuJ1 for neurons (N) and GFAP for astrocytes (A).

Figure 3

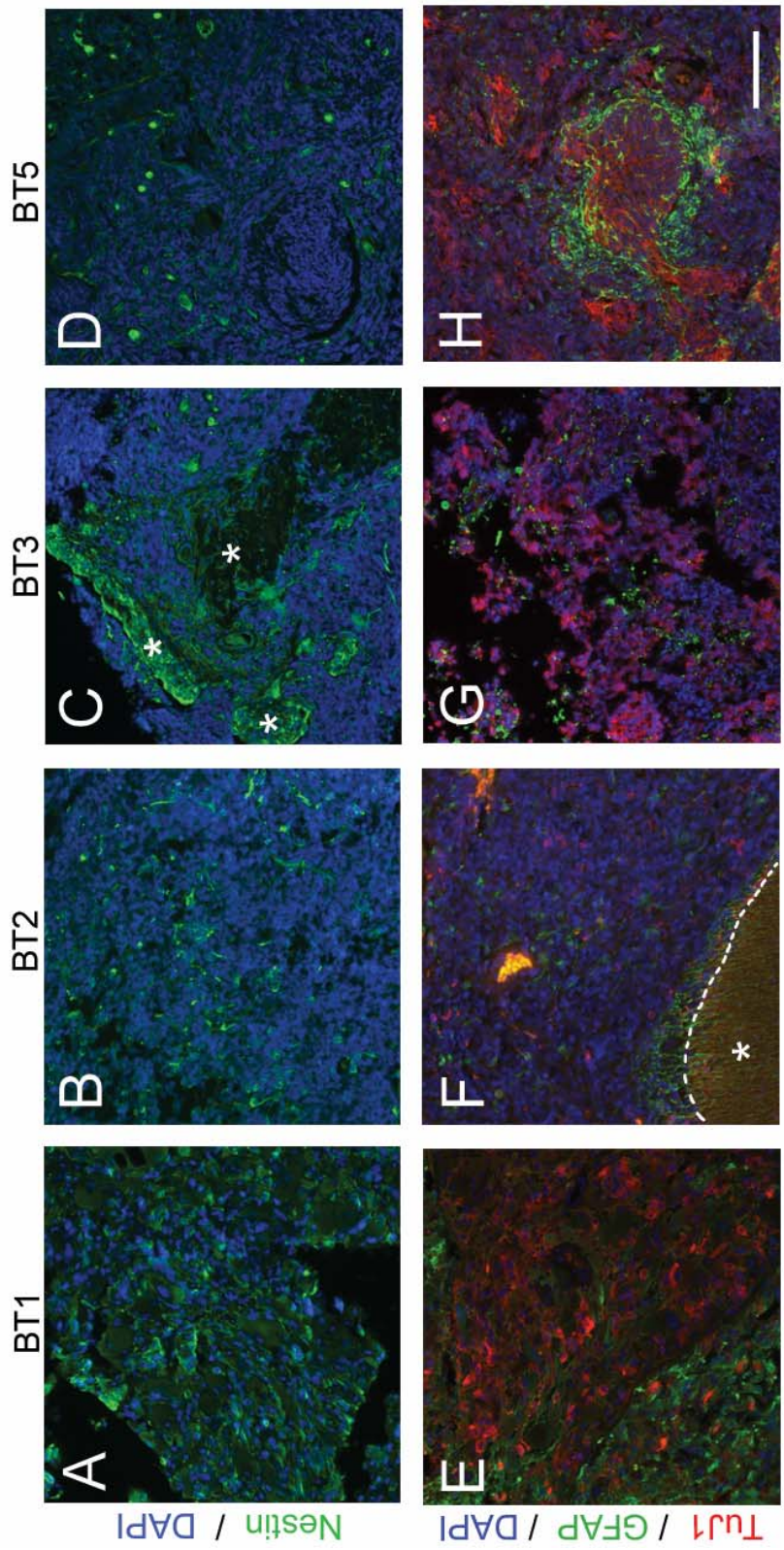

Fig. 3: Immunohistochemical characteristics of original tumor samples. Paraffin-embedded sections were labeled with antibodies to nestin (green; **A-D**), TuJ1 (red; **E-H**) to recognize neurons or GFAP (green; **E-H**) to recognize glia. Area denoted by asterisk in **C** and **F** delineates normal brain tissue adjacent to the tumor. Scale bar in **H** = 60 μm in **A-H**.

Figure 4

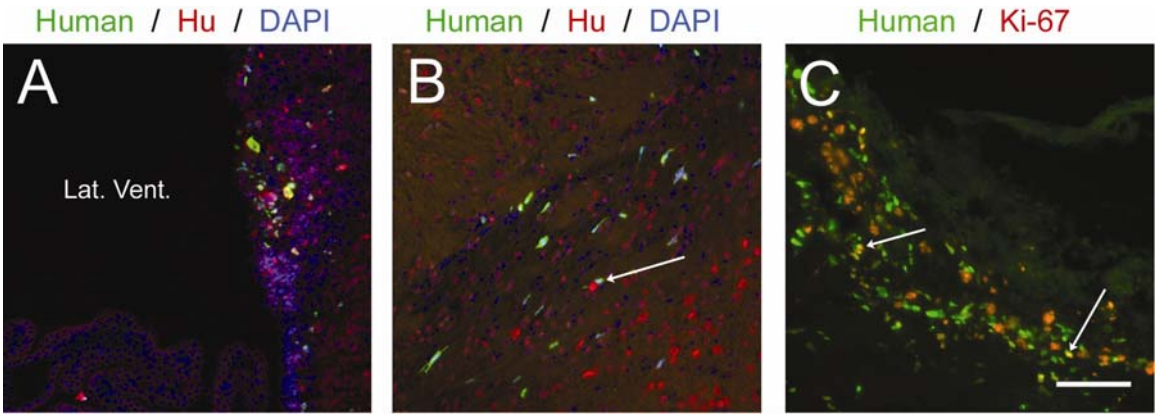

Fig. 4: Neurospheres derived from BT4 tumor become incorporated into the ventricular zone and corpus callosum after grafting to the brain of neonatal rats. **(A)** A coronal section showing the grafted cells stained for a human nuclear antigen (green) are in the ventricular zone. Some express the neuronal marker Hu (red). (**B**) A coronal section through the corpus callosum shows elongated human cells (green) with the morphology of migratory cells. Some of these also express Hu (red; arrow). (**C**) A coronal section through the injection site in the ventral portion of the brain shows that many (~50%) of the injected cells (green) also express the proliferation marker Ki-67 (red). Arrows indicate some of the double-labeled cells. Sections were counterstained with DAPI to identify all nuclei in **A** and **B**. Scale bar in **C** = 90 µm in **A** and **B**, 45 µm in **C**.

Figure 5

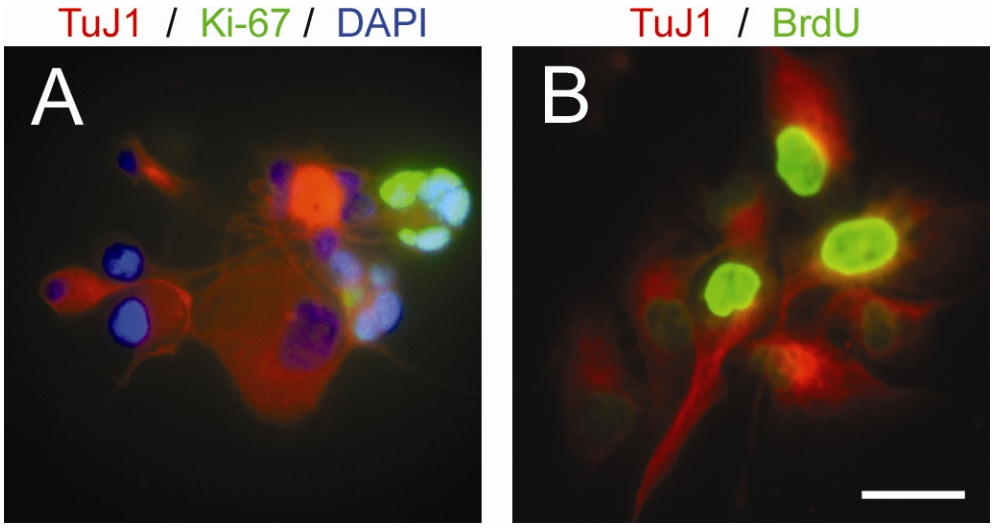

Fig. 5: Tumor-derived neurospheres give rise to neurons that proliferate aberrantly. (**A**) In this clonal neurosphere derived from the BT4 tumor, some cells co-expressed β–III-tubulin (red) as well as the proliferation marker Ki-67 (green). Nuclei were counterstained with DAPI (blue). (**B**) 50% of cells that expressed β–III-tubulin incorporated BrdU (green) after a 14 hour pulse, indicating that they have undergone cell division. Scale bar in **B** = 15 μm in **A** and **B**.

Chapter 3:

Neural Crest Progenitor cells in the Embryonic Avian Spinal Cord

Houman D. Hemmati and Marianne Bronner-Fraser

Introduction

In vertebrate embryos, neural crest cells arise at the border between the prospective epidermis and prospective neural plate, and migrate away from the neural tube to give rise to a wide variety of derivates including craniofacial bone and cartilage, melanocytes, neurons and glia of the peripheral nervous system, and smooth muscle (LaBonne and Bronner-Fraser, 1999).

In chicken embryos, neural crest cell migration occurs between E2 and E3, from stages 13-22 (Bronner-Fraser, 1986). In response to the observation that the number of sensory neurons and Schwann cells in dorsal root ganglia increases long after neural crest migration has ended (Carr and Simpson, 1978), Eric Frank's group injected the vital dye DiI into the central canal of stage 25-26 (E4.5 to E5) chick embryo neural tubes to determine if the neural tube is a source of those cells (Sharma et al., 1995a). They observed a wave of late-migrating neural crest cells originating in the dorsal root entry zone, exiting the neural tube through the dorsal roots, and populating the dorsal root ganglia. This finding was confirmed by transplanting isochronically and isotopically quail neural tubes into stage 25-26 chick embryo hosts and following the fates of the quail cells by staining chimeric embryos with the quail-specific nuclear antibody QCPN (Sharma et al., 1995a).

In order to locate the source of the late-migrating neural crest cells from the late-stage avian spinal cords, the same group injected large numbers of dissociated, uncultured, dorsal and ventral spinal cord cells from stage 18 (E2.5) and stage 27 (E5.5) quail embryos into the neural crest migratory pathway (cranial half of forelimb-level somites) of stage 16-20 (E2.5) chick embryo hosts (Korade and Frank, 1996). After 1-10

days, host embryos were harvested, and sections were stained for QCPN, neuron-specific TuJ1, and quail neuron-specific QN to detect migration and differentiation of the injected quail spinal cord cells. Korade and colleagues found that while both young and old dorsal spinal cord cells could produce neural crest derivatives such as sensory and sympathetic neurons, Schwann cells, and melanocytes, only young (E2.5) ventral spinal cord cells could produce all of those derivatives; old (E5.5) ventral cells were restricted to several crest derivates with the specific exceptions of sensory and sympathetic neurons (Korade and Frank, 1996). Moreover, old ventral spinal cord cells migrated significantly less than their dorsal or younger ventral counterparts (Korade and Frank, 1996).

The aforementioned findings prompt the question of what the source is for these late-persisting cells that have the potential to generate neural crest derivatives as a population. Are these cells existing as or derived from multipotent stem or progenitor cells in the neural tube that persist well after neural crest emigration has traditionally ceased? The results of several studies support this possibility. In mouse (Serbedzija et al., 1992; Serbedzija et al., 1994), frog (Collazo et al., 1993), and chick (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989) embryos, single-cell lineage analyses of dorsal neural tube have shown that individual precursor cells in the neural tube can form both neural tube and neural crest derivatives. Further evidence for the existence of neural tube progenitors with neural crest potential comes from the isolation in monolayer cell culture of neuroepithelial (NEP) stem cells from rodent neural tubes that, on a clonal level, can generate derivatives both of the central nervous system and the neural crest (Mujtaba et al., 1998). Moreover, Morrison et al. have prospectively isolated neural crest stem cells from embryonic rat peripheral nerve at E14.5 using flow cytometry (Morrison

et al., 1999). These cells can generate neural crest-specific neurons and glia in clonal culture and after back-transplantation into chick embryos (Morrison et al., 1999). Similarly, self-renewing neural crest stem cells have been recently isolated from both embryonic (Bixby et al., 2002) and adult (Bixby et al., 2002) gut from rodents. Taken together, these findings suggest that multipotent, self-renewing cells that possess the ability to generate neural crest derivatives can persist in both older embryos and adults, and in some cases can be cultured as undifferentiated cells.

In this study, we explored the question of whether the potential for cells from spinal cords of older avian embryos to generate neural crest derivatives originates in multipotent stem or progenitor cells that persist in the neural tube after the cessation of neural crest emigration. By culturing spinal cord cells from avian embryos at different stages in development as neurospheres, using culture methods similar to those used for neural stem and progenitor cells from the embryonic and adult mammalian nervous system, we asked how late into development such progenitors persist, how their developmental potentials change as a function of embryonic age, whether they can be cultured long-term in an undifferentiated state, and whether they can generate multiple neural crest derivatives at both bulk and clonal densities both *in vivo* and *in vitro*. We show that dissociated cells from E3, E5, E7, and E10 quail neural tubes can be cultured as neurospheres at both bulk and clonal densities. When cultured in the presence of fibroblast growth factor (FGF) and epidermal growth factor (EGF), these cells cultured as neurospheres fail to generate melanocytes, a neural crest derivative, after one month in culture, but cells derived from E5 embryos produce melanocytes *in vivo* and *in vitro* after two or more months in culture. Our data suggest that the late-emigrating wave of neural

crest derivatives from avian embryos arise from progenitor cells that persist in the neural tube.

Materials and Methods

Isolation of Avian Spinal Cord Cells

Fertile quail or hen's eggs obtained from local farms were incubated for 3 to 10 days at 38°C to obtain embryos at appropriate stages for these experiments (Hamburger and Hamilton, 1992). Spinal cords were dissected and dissociated according to methods previously reported, with some modifications (Korade and Frank, 1996). Briefly, quail embryos of the appropriate stages were dissected, removing the head, tail below the hindlimb, and all tissue extraneous to the neural tube. Trunk segments were incubated in dispase solution first for 15 minutes on ice, then at 37°C in a humidified incubator for 15 minutes. Dispase was removed and trunk segments were recovered for 15 minutes on ice in Hank's Balanced Salt Solution (HBSS; Invitrogen) containing 0.2% Bovine Serum Albumin (BSA; Sigma). Trunk segments were triturated gently with a Pasteur pipet to separate neural tubes from non-neural tissue. Neural tubes were then incubated in 0.1 mg/mL Ca/Mg-free trypsin solution (Invitrogen) diluted in HBSS for 10 minutes at room temperature. Trypsin was removed and neural tubes were recovered in HBSS containing 0.2% BSA. Neural tubes were triturated with a flame-polished Pasteur pipet to dissociate the cells. The single-cell suspensions were centrifuged for 5 minutes at 3500 RPM and cell pellets were resuspended in neurosphere culture medium.

Neurosphere Culture

Neurosphere cultures were performed as previously reported, with some modifications (Hemmati et al., 2003). Cell pellets resulting from a single-cell suspension of dissociated quail neural tubes were resuspended in Neurobasal medium (Invitrogen)

supplemented with basic fibroblast growth factor (20 ng/ml), epidermal growth factor (20 ng/ml), and B27 supplement (Invitrogen; 1x) at a density of 100,000 cells/ml. Clonal cultures were plated at a density of 1,000 cells/ml in quail neurosphere conditioned medium.

Transplantation of Neurospheres into Chick Embryos

Both whole and dissociated quail neurosphere cells were concentrated in neurosphere culture medium by brief centrifugation in a tabletop microcentrifuge at low speed. Concentrated cells were backfilled into fine glass needles and injected into for forelimb-level somites of E2.5 (stage 18) white leghorn chick embryos.

Immunohistochemical Analysis of Transplanted Chick Embryos

Between 1 and 72 hours after transplantation, embryos were harvested from the eggs and all membranes dissected from them. Brachial segments were dissected from the embryos and retained for further analysis. For immunohistochemistry, brachial segments were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 1 h and embedded in gelatin for cryosectioning. Immunostaining was performed as described previously (Hemmati et al., 2003) using anti-rabbit, -mouse or -sheep secondary antibodies coupled to Alexa fluor 488 (green) and 594 (red; Molecular Probes). Nuclei were visualized with DAPI (Molecular Probes).

Results

Embryonic Quail Spinal Cord Cells Generate Neurons, Glia and HNK-1$^+$ Cells When Transplanted into Chick Embryos

To determine whether dissociated cells from embryonic quail spinal cord could engraft chick embryos after transplantation into the neural crest migratory pathway of chick, cells from E3.5 quail embryos were transplanted into the forelimb-level somites of stage 18 chick embryos as previously performed (Korade and Frank, 1996). Transplanted embryos were harvested and fixed at 1 and 48 hours after injection of cells and incubation of eggs. They were sectioned through the area of transplantation and stained for QCPN (a marker of quail nuclei), HNK-1 (a marker for neural crest and other embryonic cell types), and Hu (a protein expressed by early neurons). In embryos fixed immediately after transplantation, quail cells were confined to the area of transplantation, adjacent to the neural tube (Fig. 1A). No donor cells could be seen ventral to the area of injection. Moreover, immediately after transplantation, very few (< 1%) of donor cells expressed Hu or HNK-1, suggesting that donor cells at those stages had not yet differentiated to express those differentiation markers (Fig. 1A). In embryos fixed 48 hours after transplantation, far larger numbers of cells were localized ventral to the site of injection (Fig. 1B) as well as around the transplantation site (Fig. 1C). While a relatively higher proportion of ventrally-localized quail cells expressed HNK-1 but not Hu (Fig. 1B), donor cells localized adjacent to the neural tube expressed either HNK-1 or Hu in significant proportions (Fig. 1C). Some quail cells in both dorsal and ventral aspects of the older transplanted embryos had elongated nuclei, consistent with Schwann cells (data not shown). These data confirm the migratory capacity of neural tube-derived cells

previously reported (Korade and Frank, 1996) and suggest that dorsally-localized cells preferentially produce neurons.

Embryonic Quail Spinal Cord Cells Can Be Cultured as Neurospheres

To examine the stem or progenitor cell properties of cells from embryonic spinal cords, dissociated cells from quail neural tubes at varying embryonic stages were assayed for their ability to form neurospheres using the same methods as previously described for human neural stem cells from both fetal and tumor tissue (Hemmati et al., 2003; Uchida et al., 2000). Large numbers of small spheres (~5-10 cells in diameter) were observed within 24 or 48 hours in flasks seeded with 100,000 cells/ml of dissociated cells from quail embryos harvested at E3 (Fig. 2A), E5 (Fig. 2B), E7 (Fig. 2C), and E10 (Fig. 2D). Within 1 week, most spheres had increased their diameters ~5- to 10-fold. Neurospheres could be passaged multiple times by mechanical dissociation of large spheres and reseeding in fresh proliferative medium every 1-2 weeks and could be maintained in culture for 10 weeks or more. These neurospheres could also be dissociated into single-cell suspensions and reseeded at clonal density (1,000 cells/ml; data not shown). Furthermore, the growth properties of these neurospheres were independent of their embryonic age of origin.

This high rate of neurosphere growth was seen only when culturing the cells in Neurobasal medium under serum-free conditions, supplemented with recombinant FGF and EGF. Fewer spheres and lower overall growth rate were appreciated when culturing cells in DMEM-F12 under otherwise identical conditions (data not shown). Dissociated neural tube cells from embryos at any stage failed to produce neurospheres when cultured

in the presence of fetal bovine serum, chick embryo extract, or both (data not shown). These data suggest that neural tube cells from quail embryos respond similarly to those from mammalian sources, raising the possibility that avian cells likewise contain multipotent neural stem and/or progenitor cells.

Quail Neurosphere Cells Can Generate Melanocytes *In Vitro* and *In Vivo*

To assess the capacity of stem or progenitor cells in embryonic quail spinal cord to generate neural crest derivatives, we examined the production of melanocytes from spinal cord-derived neurospheres over time both in culture and after transplantation into the neural crest migratory pathway of chick embryos. 3 weeks after seeding into proliferative medium, pigmented cells, presumed to be melanocytes, were rare in neurospheres cultured from E3, E5, and E7 embryos (Fig. 2A-C) and altogether absent from E10 embryos (Fig. 2D), suggesting that neurosphere culture conditions allow spinal cord progenitors to remain undifferentiated and that between E7 and E10, melanocyte-producing cells change in a way that hinders their ability to produce melanocytes at this stage. After 8 weeks under the same culture conditions, nearly all neurospheres derived from E7 embryos at bulk (Fig. 3A) density and many spheres grown at clonal density (data not shown) were composed of a significant proportion of melanocytes, estimated to be range from 10 to 40% of the total number of cells within each sphere. Neurospheres from E3, E7 and E10 embryos were not examined at this time point in culture.

To determine whether neural tube-derived neurosphere cells can migrate and differentiate within embryos similar to neural crest cells, small numbers of neurospheres from E5 quail embryos were injected into the forelimb level somites of stage 18 chick

embryos. Embryos were collected 48 hours after transplantation, sectioned, and examined for the presence of melanocytes. In some embryos, pigmented cells derived from quail donor cells were found localized to regions distant to the site of transplantation, such as the gut (Fig. 3B and 3C), suggesting that melanocytes or their precursor cells within neurospheres have the ability to migrate *in vivo.*

Discussion

In the present study, we asked whether spinal cords from avian embryos at stages older than the time of cessation of traditional neural crest emigration contain stem or progenitor cells that might contribute some or all of the neural crest derivatives shown previously to arise from the late-emigrating population of neural crest cells (Korade and Frank, 1996; Sharma et al., 1995a). Our results show that quail spinal cords from E3 through E10 embryos contain cells capable of being cultured as neurospheres, which can be propagated in culture for at least 8 weeks at both bulk and clonal densities. We show that during early passages of these neurospheres, they fail to produce significant numbers of melanocytes, a neural crest derivative, but that spheres cultured from at least one embryonic stage (E5) are capable of generating many melanocytes after 8 week of culture under identical conditions. This temporal change suggests that melanocytes precursor cells within the neurospheres might be maintained in an undifferentiated state for some time, but that this inhibition of differentiation decreases with time. Moreover, we demonstrate that neurosphere cells from E5 quail neural tubes can become localized to the host gut and produce melanocytes 48 hours after transplantation into the neural crest migratory pathway of chick embryos.

The ability of cells within avian embryonic neural tubes to respond similar to mammalian neural stem and progenitor cells when cultured in classical neurosphere culture medium suggests that avian neural tubes might contain neural precursor cells with similar properties of self-renewal and multilineage differentiation. To begin to assess whether such progenitor cells exist, we showed that after long-term (8+ week) culture, neurosphere cells from at least one embryonic stage in culture cold generate substantial

numbers of melanocytes, a neural crest derivative. We also demonstrated that avian neurosphere cells can migrate and exist as melanocytes after transplantation into chick embryo hosts. It remains to be seen whether, at a bulk or clonal level, these same neurosphere cells can generate other neural crest or central nervous system cell types as well, including neurons, glia, and/or smooth muscle.

Might the neurosphere-generating cells we have isolated via cell culture from embryonic avian neural tubes be stem or progenitor cells that ultimately produce the late-emigrating wave of neural crest cells and their derivatives (Korade and Frank, 1996; Sharma et al., 1995a) ? Recently, another group has reported an intriguing finding in mouse embryos that neural crest-derived boundary cap cells, that form clusters at surface of the neural tube, at entry and exit points of peripheral nerve roots, are responsible for generating neurons and glia that until now were attributed to a late-emigrating wave of neural crest cells from the neural tube (Mora and Gerald, 2004). While doing so, they do not show that boundary cap cells contribute to any melanocytes lineage cells, which others have attributed to cells with neural crest potential residing in the dorsal neural tube (Mosher and Morrison, 2004; Sharma et al., 1995b). This suggests the interesting possibility that two different cell types might play critical but unique roles in generating the repertoire of cells traditionally classified as “late-migrating” neural crest derivatives: boundary cap cells might contribute the neuronal and glial components, while progenitors in the neural tube might exclusively contribute the melanocytes component (Mosher and Morrison, 2004).

Several avenues of study must be pursued in order to determine whether the neural tube of older-stage embryos contains multipotent neural crest progenitor cells or

only melanocytes-restricted precursors that can be cultured as neurospheres. First, clonal neurospheres must be differentiated *in vitro,* stained for various markers of neurons and glia, and examined for the presence of melanocytes. Failure to produce neurons and glia, while maintaining melanocytic potential, would support the latter model. Clonal spheres should also be transplanted into chick embryos to explore their cells' potentials within the embryonic microenvironment. Similar experiments should be conducted using neurospheres cultured from E3, E7, and E10 quail embryos, in addition to the E5 stage described in the present study. Finally, these neurosphere cells must be serially recloned to establish whether they have self-renewal capacity. These and other experiments are presently underway and promise to shed important light on the elusive cell of origin for late-migrating melanocytes. Future studies should also examine the possibility that transformation of melanocyte precursor cells leads to the development of malignant melanoma or other skin cancers (Nakano et al., 2004).

Figure 1

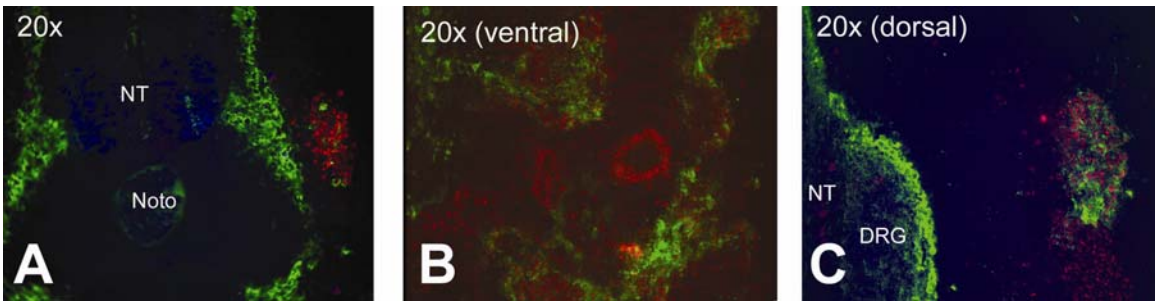

Fig. 1: Dissociated E3.5 quail neural tube cells migrate, proliferate, and produce neurons and glia when transplanted into the neural crest migratory pathway of Stage 18 chick embryos. Large numbers of uncultured, dissociated cells from E3.5 quail embryos were injected into forelimb level somites of Stage 18 chick embryos. **(A)** Small cluster of quail cells expressing QCPN (red) but which contains few HNK-1^{+} (green) cells and no neurons (Hu; blue) confined to the injection site lateral to the neural tube (NT) in host embryos fixed immediately after transplantation. **(B and C)** Relatively large numbers of quail cells, some expressing HNK-1 or Hu, localized to the ventral **(B)** and dorsal **(C)** aspects of transplanted chick embryos fixed 48 hours after transplantation.

Figure 2

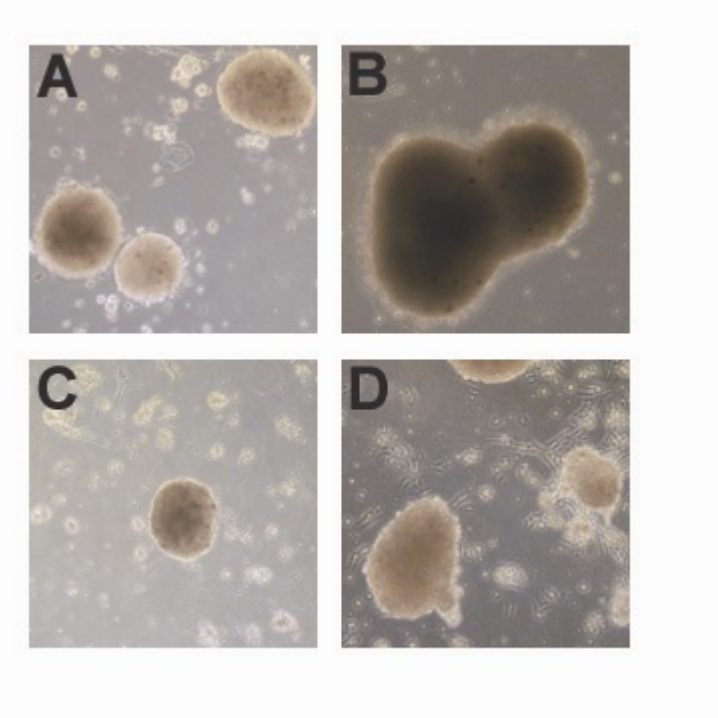

Fig. 2: Dissociated quail neural tube cells from varying embryonic stages can be cultured for weeks as neurospheres with minimal differentiation. Cells from E3 **(A)**, E5 **(B)**, E7 **(C)**, and E10 **(D)** quail embryos form neurospheres that, after 3 weeks in culture, contain relatively few pigmented cells, presumably melanocytes. Pigmented cells are confined to E3-E7 embryo-derived neurospheres **(A-C)**, but are not seen in E10-derived spheres **(D)** 3 weeks after onset of culture.

Figure 3

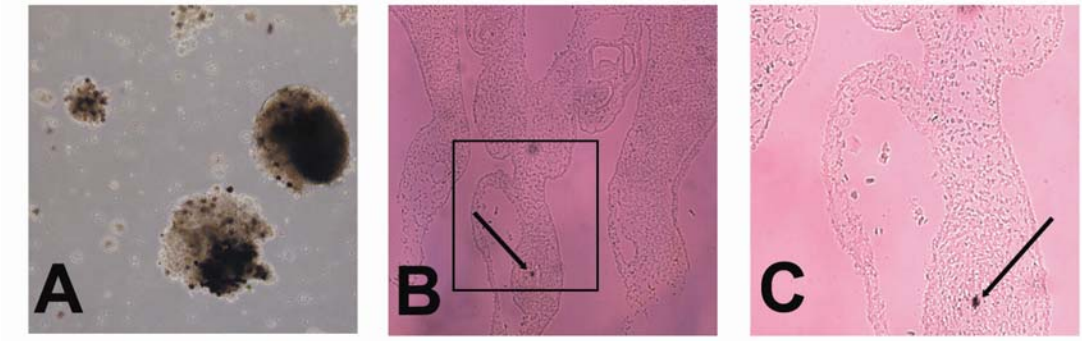

Fig. 3: After 2 months in culture, neurosphere cells from E5 quail neural tubes generate melanocytes *in vitro* and after transplantation into chick embryo hosts. (A) Neurospheres derived from E5 quail neural tubes generate melanocytes in large proportions in nearly every sphere in culture at 8 weeks in culture. **(B & C)** 2 days after transplantation of 8 week-old neurospheres from E5 quail embryos into the forelimb-level somites of stage 18 chick embryos, melanocytes can be seen in the foregut of transplanted embryos (arrows; magnified inset from **B** in **C**).

Chapter 4:

Conclusions

STEM AND PROGENITOR CELLS IN DEVELOPMENT

Stem cells are, at a minimum, defined as cells that possess the capacities to both self-renew and give rise to multiple differentiated progeny (Morrison et al., 1997). The term "progenitor" is used as a general term to describe any dividing cell that can generate differentiated progeny, irrespective of its ability to self-renew. Given these properties, stem and progenitor cells have long intrigued scientists, for they play important developmental roles and have the potential to replace cells lost or damaged through disease processes (Lagasse et al., 2001).

Stem cells have been isolated and characterized from different tissues from a variety of organisms, in both embryos and adults, including blood-forming hematopoietic stem cells from fetal liver (Morrison et al., 1995) or adult bone marrow (Spangrude et al., 1988), and the neural crest (Morrison et al., 1999). Thus far, only hematopoietic stem cells are routinely used therapeutically in humans, though many promising stem cell therapies are on the horizon (Lagasse et al., 2001; Meyer et al., 2006; Moore and Lemischka, 2006). Moreover, the recent explosion is stem and progenitor cell research has led to the identification of genes and molecular pathways critical for the regulation of self-renew and differentiation of stem and progenitor cells from a variety of normal and malignant tissues (Moore and Lemischka, 2006). The scientific and clinical significance of some of these genes and pathways in the nervous system is discussed later in this chapter.

IDENTITY AND PROPERTIES OF NEURAL STEM AND PROGENITOR CELLS

Though it was originally believed for some time that the central and peripheral nervous system were unable to generate new neurons during adulthood, evidence of continued neurogenesis in the adult pointed to a long-lived neural progenitor cell (D'Amour and Gage, 2003). A series of studies over the last several year demonstrated that neurogenesis does indeed occur in the adult mammalian brains, especially at the midline and hippocampal areas, from a pool of progenitor cells (Alvarez-Buylla and Garcia-Verdugo, 2002). Isolation of stem-like cells from the embryonic central nervous system, including the basal forebrain (Reynolds et al., 1992; Temple, 1989), hippocampus (Johe et al., 1996), cerebral cortex (Davis and Temple, 1994), spinal cord (Kalyani et al., 1997; Kalyani et al., 1998), and the peripheral nervous system (Stemple and Anderson, 1992) as well as evidence for multipotent, stem-like progenitors in vivo (Leber and Sanes, 1991; Sanes, 1989; Walsh and Cepko, 1993) suggested that stem and progenitor cells are important components of the developing nervous system. More recent evidence for continued presence of stem cells in the adult in areas not previously considered to be neurogenic, such as the spinal cord (Weiss et al., 1996) and cortex (Gould et al., 1999; Marmur et al., 1998), suggests that stem and progenitor cells are also an important feature of the adult nervous system.

Reynolds and Weiss demonstrated that neurogenesis in the central nervous system is owed to a population of neural stem cells, that can self-renew, generate neurons, astrocytes, and oligodendrocytes, and be cultured as floating spherical aggregates known as neurospheres (Reynolds and Weiss, 1992). Very elegant studies in the mouse demonstrated that neural stem cells are exclusively GFAP-expressing astrocytes located

in the subventricular zone (SVZ), lining the walls of the ventricles in the central nervous system (Doetsch et al., 1999; Imura et al., 2003; Suslov et al., 2000). More recently, it has been shown that in adult human brain, some SVZ astrocytes can also behave like neural stem cells, yet active neurogenesis from those progenitors was not proven (Sanai et al., 2004). In this study, we report that we have cultured neural crest progenitor cells at clonal density from quail embryos at various stages of development (E3-E10). We have found in preliminary studies that the capacity of these cells to generate melanocytes diminishes with embryonic age, and disappears altogether by E10.

Gene expression studies have recently shed light on the characteristics of neural stem and progenitor cells from various sources. Several groups, for example, have reported that neural stem cells share common expression of a subset of genes with other stem cells, including hematopoietic stem cells and embryonic stem cells (Burns and Zon, 2002; Easterday et al., 2003; Geschwind et al., 2001; Ivanova et al., 2002; Ramalho-Santos et al., 2002). Genes found to be critically important in regulating the self-renewal and/or differentiation of neural stem or progenitor cells include PTEN (Groszer et al., 2006; Groszer et al., 2001), MELK (Nakano et al., 2005), bmi-1 (Molofsky et al., 2003), PBK/TOPK (Dougherty et al., 2005), epidermal growth factor receptor (EGFR) (Kornblum et al., 1998; Reynolds et al., 1992), and Wnt pathway members (Chenn and Walsh, 2002). These genetic similarities among stem and progenitor cells must be examined in further detail, as they undoubtedly contain valuable clues regarding the molecular mechanisms responsible for the fundamental properties of stem cells.

RELATIONSHIP BETWEEN CANCER AND STEM CELLS IN THE NERVOUS SYSTEM

In recent years, many groups have been interested in investigating the similarities between cancer cells and stem cells (Iwashita et al., 2003; Reya et al., 2001), particularly in leukemia (Bonnet and Dick, 1997), breast cancer (Al-Hajj et al., 2003), and brain tumors (Galli et al., 2004; Hemmati et al., 2003; Ignatova et al., 2002; Kondo et al., 2004; Singh et al., 2003; Singh et al., 2004). The fact that both cancer cells as a population and stem cells share the abilities to self-renew and differentiate into multiple phenotypes is likely not purely coincidental; rather, they likely share common molecular mechanisms that are responsible for their fundamental properties. Further, it is possible that stem or progenitor cells (or, more specifically, their critical genetic regulators) are targets for malignant transformation, since they are already equipped with the cellular machinery required for self-renewal and proliferation (and, in some cases, migration and invasion) that most somatic cells do not ordinarily possess (Hemmati et al., 2003; Sanai et al., 2005; Singh et al., 2004).

In this study, we report that midline human pediatric brain tumors each contain a unique population of multipotent, self-renewing progenitor cells that, when differentiated in culture at a clonal level, consistently recapitulate the cellular characteristics of the primary tumor from which they are derived (Chapter 2 and Hemmati et al., 2003). While brain tumor-derived progenitor cells vary in their developmental potentials from one patient to another, there is very low interclonal variability in clonal progenitors derived from each tumor, suggesting a common origin for those progenitor cells. Tumor-derived

progenitor cells proliferate and differentiate aberrantly in vitro, producing neurons that continue to incorporate BrdU and express the proliferation antigen Ki-67, as well as cells that coexpress neuronal and glial markers. We report that tumor-derived progenitor cells can migrate, proliferate, and differentiate for at least one month after transplantation into brains of immunocompromised rodents.

Brain tumor-derived progenitor cells express many genes traditionally associated with neural stem and progenitor cells, including *nestin*, *musashi-1, bmi-1, melk, Sox2, CD133,* and *bmi-1.* Our study found that strikingly, even after differentiation, tumor-derived progenitors maintain high levels of expression of *bmi-1*, which helps to positively regulate self-renewal of multiple types of stem and progenitor cells (Lessard and Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003), whereas normal human neural progenitor cells express *bmi-1* at significantly lower or undetectable levels. This finding raises the possibility that *bmi-1* overexpression is involved in maintaining the extensive self-renewal capacity of tumor-derived progenitors and, possibly, that of the tumor as a whole. This scenario has recently been addressed by another group for medulloblastoma, a common pediatric brain tumor (Leung et al., 2004). Moreover, *bmi-1* has been shown to play an important role in the transformation of hematopoietic progenitor cells into leukemic progenitor cells (Lessard and Sauvageau, 2003).

Other groups have recently published similar findings from primary adult glial tumors (Galli et al., 2004; Ignatova et al., 2002), cultured glial tumor cell lines (Kondo et al., 2004), and pediatric brain tumors (Singh et al., 2003). Dirks and colleagues have positively sorted for the human neural stem cell surface marker CD133 (Uchida et al., 2000) to prospectively identify similar neurosphere-forming cells from pediatric brain

tumors (Singh et al., 2003). Later, this same group tested the tumor-initiating capacity of these CD133-expressing cells by transplanting $CD133^+$ and $CD133^-$ cells into the brains of immunodeficient nonobese diabetic/severe combined immunodeficiency mice (Singh et al., 2004). In their study, only the $CD133^+$ brain tumor fraction contained cells that were capable of tumor initiation in xenografts. Injection of as few as 100 $CD133^+$ cells produced a tumor that was serially transplantable and was a phenocopy of the patient's original tumor, whereas injection of 10^5 $CD133^-$ cells produced engraftment but did not cause a tumor (Singh et al., 2004). This set of experiments gave strong support to the cancer stem cell hypothesis as the basis for brain tumor development (Dirks, 2005).

Another research group has recently shown that large numbers of cultured neurosphere cells from adult glioblastoma multiforme can form glioblastoma-like tumors with high efficiency after transplantation into immunodeficient mice, and that those tumors can yield tumor-forming neurospheres after serial propagation *in vivo* (Galli et al., 2004). Taken together, these studies, along with ours, suggest that brain tumor growth is initiated and driven by a rare subpopulation of cancer stem or progenitor cells (Nakano and Kornblum, 2006).

There is also mounting evidence that some peripheral nervous system tumors or melanomas might be derived from transformation of neural crest stem cells or their committed progenitor cells (Fang et al., 2005; Mora and Gerald, 2004). Fang and colleagues have recently shown that CD20-expressing cells cultured from human melanoma samples as floating spheres self-renew, give rise to multiple neural crest derivatives in culture, and forum tumors in vivo (Fang et al., 2005). Similar experimental

approaches should be applied to prospectively identify, purify, and evaluate candidate cell types from other tumors as cancer stem cells for neural crest lineage tumors.

FUTURE EXPERIMENTS

Our studies, as well as those of other groups, have generated as many questions as they have answered. Several critical questions remain to be answered, and we propose the following lines of investigation in guiding future experiments in this field:

What are some other markers of brain tumor stem cells?

Further research into and therapeutic exploitation of cancer stem cells for brain tumors requires the prospective identification of a cell that, on a clonal level, can self-renew and regenerate its parental tumor in vivo. As discussed above, to answer this question will require purification of candidate cells using flow cytometry and transplantation into NOD-SCID or SCID-beige mice, which do not possess the residual non-specific immune responses that have traditionally biased and plagued tumor xenograft studies that have used SCID or nude mice as transplant recipients. Though those mouse strains are technically immunodeficient, the residual activity of natural killer cells and macrophages in those mice might be sufficient to cause rejection of small numbers of transplanted human cells, thereby confounding negative results from these experiments.

Most groups, including ours, have identified stem-like cells from cancer using *in vitro* cell culture selection, without cell purification using cell surface markers (Galli et

al., 2004; Hemmati et al., 2003; Ignatova et al., 2002). Until now, only one group has reported the ability to prospectively enrich for cancer stem cells from primary brain tumors in an *in vivo* xenografts assay in immunodeficient mice (Singh et al., 2004). Unfortunately, positive selection for CD133 enriches, but does not purify for cancer stem cells in human brain tumors. The fact that 100 or more $CD133^+$ had to be injected by Singh and colleagues into mice for tumors to form suggests that the tumor initiating fraction comprises a small percentage of CD133-expressing cells, at best. For this reason, additional cell-surface markers of brain tumor stem cells must be identified, for purposes of positive or negative selection, which would enable further enrichment or purification of cancer stem cells from brain tumors. Others in our group are actively engaged in this effort.

What are the genetic and epigenetic similarities and differences between tumor-derived progenitors or brain cancer stem cells and normal neural stem and progenitor cells and whole tumors?

A more thorough understanding of gene expression similarities between tumor-derived cells and normal neural progenitor cells will yield insights into the potential lineal relationship between cancer cells and stem cells, whereas gene expression differences will highlight potential molecular mechanisms involved in or responsible for malignant transformation. This study should be carried out as a cDNA microarray or gene chip-based comparison of cDNAs from uncultured progenitor cells freshly purified from primary brain tumor or normal brain specimens. A recently published microarray-based gene expression analysis of many medulloblastoma samples reported that gene

expression patterns predict clinical outcome (Pomeroy et al., 2002). Another recent study reported that proteome-based clustering in human gliomas significantly correlated with patient survival (Iwadate et al., 2004). We have already begun preliminary experiments to this effect, including microarray-based studies of microRNA (oncomir) expression in brain tumor-derived progenitors and normal neural progenitor cells (Esquela-Kerscher and Slack, 2006).

What are the functions of candidate oncogenes in tumorigenic potential or transformation of brain tumor-derived progenitors or cancer stem cells?

This question elaborates on the previous question by elucidating the roles of genes such as *melk* and *bmi-1* in the transformation of neural progenitor cells and maintenance of self-renewal and multipotency of tumor-derived progenitor cells. Both overexpression and gene knockdown studies should be performed, using both uncultured and cultured progenitor cells and cell lines derived from primary brain tumor or normal brain specimens. In vitro neurosphere-forming, self-renewal and differentiation assays should be combined with *in vivo* tumor-formation assays in immunodeficient mice in order to understand the function of these genes in the tumor formation process at the progenitor level.

We have already commenced some of these studies using *melk*, PTEN, and EGFR-VIII. The expression of the latter two genes in human gliomas has recently been shown to have strong clinical significance, as it can guide treatment decisions using

EGFR kinase inhibitors erlotinib or gefitinib (Friedman and Bigner, 2005; Mellinghoff et al., 2005).

THERAPEUTIC IMPLICATIONS

Exploitation of these findings for the development of improved brain tumor-specific therapies is no longer a fantasy, and will require careful and deliberate investigation. Several new classes of tumor-specific drugs have recently become approved for treatment of non-CNS tumors, most of which block the functions of protein kinases, but through varying mechanisms of action (Noble et al., 2004). Each of these new drugs targets a specific molecular pathway that has previously been shown to be important in either the original transformation event or the maintenance of tumor proliferation. We and others hope to exploit some of the aforementioned molecular pathways active in brain tumor-derived progenitor cells to develop new and customized therapeutics for brain tumor patients.

Gleevec (imatinib), a small molecule drug that inhibits the product of the Bcr-Abl translocation that is responsible for the transformation of hematopoietic progenitor cells into leukemic stem cells for chronic mylogenous leukemia, is a notable example of a new-generation targeted cancer therapy (Noble et al., 2004). Evidence has shown much of the anti-cancer activity of Gleevec in chronic mylogenous leukemia can be attributed to its activity against leukemic cancer stem cells (Galmozzi et al., 2006). Gleevec also inhibits the c-kit receptor tyrosine kinase, as well as the PDGF receptor tyrosine kinase, which is expressed on the surface of normal human neural stem cells and brain tumor

stem cells (Reardon et al., 2005). A recently-completed Phase II clinical trial of Gleevec in patients with recurrent glioblastoma multiforme discovered that the treatment is well-tolerated and associated with durable antitumor activity in those patients (Reardon et al., 2005). It remains to be seen whether Gleevec's activity against malignant gliomas parallels its action against leukemia by acting through the cancer stem cell.

Iressa (gefitinib) and Tarceva (erlotinib) are small-molecule inhibitors that bind the intracellular tyrosine kinsase domain of the epidermal growth factor receptor (EGFR) and were originally designed to treat (and are FDA approved for) metastatic non-small cell lung cancer and pancreatic cancer (Noble et al., 2004). These drugs are highly effective against the tumors that they target, and do so with minimal side effects due to their molecular specificity. EGFR, which is expressed by both normal and tumor-derived neural stem cells, is known to be frequently amplified, overexpressed, or mutated in glioblastomas (Dancey and Freidlin, 2003; Smith et al., 2001), but only 10 to 20 percent of patients exhibited a response to EGFR kinase inhibitors in recent clinical trials (Prados et al., 2006; Rich et al., 2004). Glioblastomas often express EGFRvIII, a constitutively active genomic deletion variant of EGFR (Frederick et al., 2000; Wong et al., 1992) that strongly activates the PI3 kinase signaling pathway, leading to tumor cell survival, motility, and proliferation (Choe et al., 2003). This persistent PI3 kinase signaling is thought to cause "pathway addition;" addicted tumor cells die after disruption of the pathway (Weinstein, 2002). Bianco and colleagues hypothesized that EGFRvIII would sensitize tumors to EGFR kinase inhibitors, whereas PTEN loss would impair the response to such inhibitors (Bianco et al., 2003). In a recent clinical study, Mischel and colleagues found a strong association between the coexpression of EGFRvIII and PTEN

in glioblastoma cells and responsiveness to EGFR kinase inhibitors (Mellinghoff et al., 2005). This finding of glioblastomas with specific genetic and phenotypic profiles that respond to EGFR kinase inhibitors is an important first step rational, tumor-specific therapy for malignant brain tumors (Friedman and Bigner, 2005). Recent evidence from our group suggests that the PTEN and EGFRvIII mutations in brain tumors function at the level of cancer stem cells (data not shown).

The aforementioned clinical studies support the concept that targeting specific mutations and molecular pathways active in tumor stem and progenitor cells is effective in treating cancer. Since similar genetic mechanisms regulate the survival and development of normal stem and progenitor cells (Bonnet and Dick, 1997; Hemmati et al., 2003; Pardal et al., 2003), it is necessary to examine whether drugs that target cancer stem cell pathways also inhibit the growth or survival of normal stem cells. A recent study by Morrison and colleagues identified different self-renewal mechanisms for maintaining pools of leukemic stem cells and normal hematopoietic stem cells (Yilmaz et al., 2006). Conditional deletion of the PTEN tumor suppressor gene in adult hematopoietic stem cells led to transplantable leukemias within weeks. PTEN deletion also promoted proliferation of hematopoietic stem cells, eventually depleting the hematopoietic stem cells, and preventing the hematopoietic stem cells from stably reconstituting lethally irradiated mice. This illustrated a contrast between normal and leukemic stem cells, in that normal hematopoietic stem cells, but not leukemic stem cells, required PTEN for self-renewal. These effects were mediated in large part by mTOR, as they were inhibited by rapamycin, which targets the mTOR protein. This work is the first clear illustration that mechanistic differences between normal stem cells and cancer stem

cells can be targeted to deplete cancer stem cells without damaging normal stem cells (Rossi and Weissman, 2006).

Several steps must be taken in order to develop effective brain tumor-specific therapies. First, appropriate drug targets must be discovered and evaluated. As discussed above, cDNA microarray screens, coupled with in vitro and in vivo functional studies of candidate gene function, will likely yield interesting targets for drug action. Some candidate drug targets that might arise from our work with brain tumor-derived progenitors include the PTEN tumor suppressor gene, *melk, bmi-1,PSP,* and *PBK/TOPK*.

Second, candidate drugs need to be evaluated for efficacy in preclinical trials using purified tumor-derived progenitors or brain cancer stem cells, as well as NOD-SCID mouse xenograft models. Current methods of screening candidate drugs in vitro rely on the responsiveness of tumor cell lines, some of which have been in serum-containing culture for many years or decades, to determine whether those drugs will graduate to a more complex level of preclinical testing. It is possible that some agents that would otherwise be effective against primary tumor-derived progenitor cells fall through the cracks during this screening process because they are tested against tumor cell lines that no longer resemble tumors found in patients and might not contain progenitor cells that are responsible for growth of the tumor in vivo. The recent study by Vescovi and colleagues highlights the fact that a popular glioblastoma cell line (U87-MG) fails to generate glioblastoma-like tumors after transplantation *in vivo* (Galli et al., 2004). The pharmaceutical industry has many compounds in its warehouses that can be rescreened using primary progenitor cultures.

The characterization of neural progenitor cells from both normal and malignant human brain has yielded interesting insights into both normal development of the nervous system and the molecular and cellular mechanisms that might be involved in tumorigenesis. In the coming years, further investigation will hopefully answer even more questions about the origins of brain tumors and create a knowledge base the might eventually lead to the development of improved methods to prevent, diagnose, and treat cancers of the nervous system.

Appendix:

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