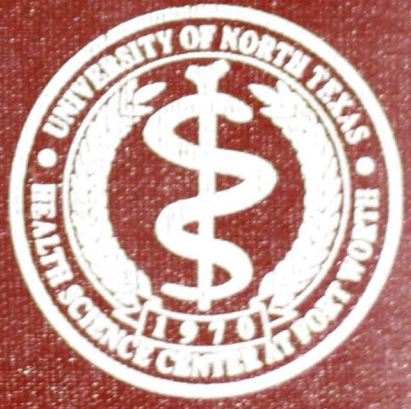
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

ABSTRACT

These studies evaluated the possibility of inducing the cells of human neonatal neurospheres to commit to the neuronal phenotype. Neurospheres are hollow, multicellular aggregates held together by combinations of adherens junctions and gap junctions. Their walls were seen to be 6-8 cell layers thick, with proliferating cells randomly distributed throughout these layers. The cells of the neurosphere wall were found to be organized into an outer "glial basket" and an inner layer of putative neuroblasts, and this arrangement facilitated orchestrated cellular outgrowth on immobilized extracellular matrix proteins, with $GFAP^{+}$/$nestin^{-}$ cells forming radial tracks upon which $GFAP^{-}$/$nestin^{+}$ cells migrated.

Using a novel technique, it was demonstrated that FGF2 downregulated nestin and vimentin, induced transient upregulations of α-internexin, and induced sustained upregulations of neurofilament M (NF-M). β-tubulin was most strongly upregulated by long-term (9 days) exposure to basal medium without growth factors. Dose response studies indicated that 5ng/ml FGF2 was optimal for promoting upregulations of the neuronal intermediate filament proteins, but that 0-1ng/ml FGF2 was optimal for β-tubulin upregulation. Commitment-promoting FGF2 treatments were shown to have little effect on the proliferation of neurosphere cells with the exception of treatment with growth factor-free basal medium, which strongly reduced proliferation.

The α, βI, βII, δ, ε, η, and θ isoforms of PKC were detected in neurospheres, and these expression profiles were quantitatively but not qualitatively altered by treatments with various growth factors. Blockade of PKC activity by administration of the general PKC inhibitor GF109203X ablated FGF2-induced upregulations of α-internexin and NF-M, although FGF2 and GF109203X upregulated the expression of β-tubulin.

We propose a model in which high FGF2 coupled with EGF drives cellular proliferation, the removal of EGF and decreased FGF2 stimulates upregulation of neuronal intermediate filaments, and a further lowering of FGF2 (down to 0ng/ml) stimulates the upregulation of β-tubulin and axonal extension. During the first two stages, cellular proliferation is not altered, and it is not until the final stage that cells begin to exit the cell cycle. It is presumed that PKC drives the first two stages, while the final stage is inhibited by PKC.

INDUCTION OF NEURONAL COMMITMENT IN PLURIPOTENT NEUROSPHERES

Michael L. Moeller, M.S.

APPROVED:

Major Professor

Committee Member

Committee Member

Committee Member

Committee Member

Committee Member

University Member

Chair, Department of Molecular Biology and Immunology

Dean, Graduate School of Biomedical Sciences

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Michael L. Moeller, M.S.

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CHAPTER I

Introduction

Model Systems Used in Neuronal Research

Investigations in neuroscience have relied traditionally on the use of *in Vitro* models designed to simulate the functions of the cells and tissues of the nervous system. Mature neurons are postmitotic and incapable of significant proliferation under *in Vitro* conditions. Therefore, establishment of cell populations large enough to permit detailed investigation has been a major hurdle in the development and selection of desirable models. In 1976, the rat pheochromocytoma cell line (PC12) was established from rat adrenal tumor tissue. These cells could be rapidly expanded in the presence of serum and induced to exit the cell cycle and develop neurite extensions in response to serum withdrawal and exposure to nerve growth factor (NGF). Withdrawal of NGF allowed the cells to resume proliferation with atrophy of the extended neurites, and neurite-bearing cells were found to spontaneously synthesize the norepinephrine and dopamine. These cells could also synthesize epinephrine in response to dexamethasone, indicating a catecholinergic phenotype (Greene and Tischler, 1976). Since that time, the PC12 line has been used in investigations spanning the entire spectrum of neuroscience research, including apoptosis (Anantharam et al., 2002; Viswanath et al., 2001; Mota et al., 2001; and Troy et al., 2000), survival (Foehr et al., 2000), differentiation (Zhang et al., 2000), and

neurite extension (Foehr et al., 2000). These examples represent only a fraction of the more than 6,600 publications dealing with PC12 cells. The main benefit of this line lies in the ease with which the cell populations can be expanded and differentiated. It has also been possible to stably transfect PC12 cells, thus establishing stable subclones that may be used in a variety of studies (Gottlieb, 2002). However, it has been found that some subclones lose their neuronal and catecholaminergic properties (Blum et al., 2000). PC12 cells also lack a complete genome. Normal rat cells contain a complement of 40 autosomes and a pair of X/Y chromosomes, while PC12 cells contain only 38 autosomes and an XY pair (Greene and Tischler, 1976). Thus, despite convenience, it is questionable how realistic the PC12 line is as a model.

The NTera 2 (NT-2) line is also derived from tumor tissue, in this case embryonic human teratocarcinoma. Initial characterization of this line demonstrated that these cells express a number of phenotypic marker proteins, including nestin, vimentin, intermediate filament (IF) proteins, and microtubule-associated protein (MAP) 1b. Weaker expression of the neuronal cytoskeletal proteins neurofilament M (NF-M), neurofilament L (NF-L), MAP2c, and α-internexin were also found to be present. Like the PC12 cell line, these cells proliferate and exhibit neuroepithelial characteristics in the presence of serum. Serum withdrawal accompanied by 4 weeks of exposure to retinoic acid (RA) induces differentiation, as evidenced by increases in the expressions of the neuronal markers MAP2b, MAP2c, polysialylated neural cell adhesion molecule (PSA-NCAM), NF-L, NF-M and α-internexin and concommitant decreases in the immature markers keratin 8, keratin 18, and nestin. Gross morphological changes are also apparent during NT-2 differentiation. Collectively, these data indicate that NTera 2 cells undergo differentiation to a neuronal phenotype. A substantial increase in the level of neurofilament phosphorylation following RA treatment further supports this claim (Pleasure and

Lee, 1993). Since that time, the NT-2 line has been used to investigate a number of problems in neurobiology, including apoptosis (Zigova et al., 2001; and Gibson, 1999) and neuronal differentiation (Leypold, Flajolet, and Methner, 2002; Leypold, Lewrenz, and Methner, 2001; and Przyborski et al., 2000).

A major drawback to tumor-derived cell lines lies in a lack of information concerning the event or events that have allowed the cells to escape the intrinsic barriers to continued proliferation. It is for these reasons that stem cell lines transformed with specific oncogenes were developed. In these lines, the genetic changes enabling the cells to sustain proliferation are known, so that a particular cell line becomes less of a "black box." The HiB5 cell line was produced by the transfection of rat embryonic hippocampal cells with a temperature-sensitive variant of the SV40 large T antigen (Gottlieb, 2002). More recently, the C17 cell line has been developed by transfection of neonatal mouse cerebellar cells with the v-myc oncogene. Numerous studies have shown that these cells are capable of reintegrating into the mouse central nervous system, where they differentiate into various mature phenotypes (Renfranz, Cunningham, and McKay, 1991; Snyder et al., 1992; and Rosario et al., 1997).

Although oncogene-transformed cell lines are better defined than tumor-derived cell lines, some major drawbacks still remain. Insertion of transforming oncogenes is an inherently random process, and transgene expression is influenced by the site of insertion. It is possible that heterogeneity exists within cell line populations due to different insertion sites (Gottleib, 2002). In addition, transgene insertion may disrupt the expressions of surrounding genes, including phenotype-specific ones. This may lead to translation of mutant proteins or reductions in overall protein synthesis. Thus, transformed cell lines also possess drawbacks that make them less than ideal.

Much interest has recently been focused on the use of wild type stem and progenitor cells obtained from the mammalian forebrain germinal zones. These cells proliferate through a large number of population doublings when expanded in serum-free medium supplemented with growth factors, generally epidermal growth factor (EGF) and/or basic fibroblast growth factor (FGF2). In the first study using this approach, the striata of adult mice were enzymatically dissociated and grown as monolayers in the presence of EGF. Spherical cell aggregates termed "neurospheres" were observed to bud from the monolayers, and these could be expanded by mechanical disruption, followed by regrowth in suspension culture of the resulting fragments in the presence of EGF and/or FGF2 (Reynolds and Weiss, 1996; and Caldwell et al., 2001). The addition of leukemia inhibitory factor (LIF) to medium containing both EGF and FGF2 has also proven beneficial in the expansion of neurospheres (Carpenter et al., 1999; and Fricker et al., 1999).

The cells found in both rodent and human neurospheres have been shown to be multipotent, meaning that they are capable of differentiating into a number of mature phenotypes, including neurons, astrocytes, and oligodendrocytes (Reynolds and Weiss, 1996; Kukekov et al., 1999; and Suslov et al., 2000). It has been suggested that the fate decisions of neurosphere cells is dependent upon the precise anatomical location from which they were harvested (Ostenfeld et al., 2002), although this may represent a species-specific artifact (Kukekov et al., 1999). However, it should be pointed out that expansion of forebrain germinal zone cells may be accomplished without resorting to neurosphere culture, however. For example, hippocampal neurons can be harvested and cultured as a monolayer in the presence of high concentrations (10-20ng/ml) of FGF2 for more than 5 months in culture (Ray et al., 1993). Regardless of whether they are grown as monolayers or as neurospheres, these cells represent excellent potential model systems

as they are neither aberrant, as are tumor-derived cell lines, nor genetically modified. The use of unaltered wild type cells in neuroscience research will allow results to be collected that are more appropriate to the *in Vivo* situation.

CNS Germinal Zones: A Source of Neuronal Stem/Progenitor Cell Populations

It has recently been realized that germinal zones exist in multiple locations throughout the central nervous system. The germinal zones first identified were the ventricular and subventricular zones (VZ and SVZ, respectively) of the mammalian forebrain (**Fig. 1A**). As their names suggest, these regions are located around the lateral ventricles of the brain, and both produce large populations of multipotent progenitors in the fetus. However, following maturation, only the SVZ retains this ability. The organization of the SVZ has been extensively investigated and reviewed, as well as the migrations of cell clusters from the SVZ to the olfactory bulb during replacement of olfactory neurons, a process that occurs throughout life. It is now known that the SVZ is comprised of chains and "nests" of proliferative, migratory neuroblasts (termed "Type A cells") surrounded by sheaths of astrocytes that express glial fibrillary acidic protein (GFAP). The astrocytes are termed "Type B cells", and Type B1 and Type B2 cells have been identified in the sheaths based on location. It is speculated that the ensheathing glia may serve to protect migrating neuroblasts from surrounding adverse chemical and electrical interactions during migration, and that they may also provide trophic support for neuroblasts. Type C cells bearing the morphological and biochemical characteristics of immature cells are found also in association with the neuroblast chains, but may be discerned from neuroblasts based on morphology and the absence of neuronal or glial phenotypic markers (**Fig. 1B, C**). It is thought that Type C cells represent uncommitted precursors which have not

yet made a phenotypic fate decision, a speculation supported by the presence of the intermediate filament protein nestin, a marker of uncommitted progenitors (Doetsch, Garcia-Verdugo, and Alvarex-Buylla, 1997; and Garcia-Verdugo et al., 1998). It is also suspected that Type B and/or Type C cells give rise to new neuroblasts, while Type A cells do not self-renew (Lim and Alvarez-Buylla, 1999). It has been demonstrated through ^{3}[H]thymidine labeling that the Type C cells are the most actively proliferating cells in the SVZ (Doetsch, Garcia-Verdugo, and Alvarex-Buylla, 1997). It has also been shown that, following ablation of Type A and Type C cells, Type B cells divide to form Type C cells, which, in turn, become Type A cells. Retrovirally-labeled Type B cells have also been shown to become neurons within the olfactory bulb (Doetsch et al., 1999). Furthermore, SVZ astrocytes have been shown to be capable of generating pluripotent neurospheres responsive to both EGF and FGF2. While it has been proposed that the ciliated ependymal cells directly lining the ventricles might be neural progenitors, it has been shown that these cells are actually unipotent and destined to become glia (Laywell et al., 2000).

Following establishment of the chains described above, the collections of progenitors migrate to their targets, which are some distance away from the SVZ. In the adult mouse, such migrations may span distances of up to 5mm (Lois, Garcia-Verdugo, and Alvarez-Buylla, 1996). Migrations have also been suggested, but not proven, in the larger brains of adult primates, as well (Gould et al., 1999). That such long distances could be traversed in such a precise fashion is most impressive, and a number of mechanisms appear to exist to ensure properly-directed migrations. The cells migrating within the rostral migratory stream (RMS) leading from the SVZ to the olfactory bulb are guided by binding of the Deleted-in-Colorectal-Carcinoma (DCC) receptor to netrin-1 on the surfaces of olfactory mitral cells combined with the differential

expressions of integrins (Murase and Horwitz, 2002). The interaction between DCC and netrin-1 would seem to suggest that surface molecules expressed on target tissue cells are important in the guidance of progenitor chain migrations. However, evidence has accumulated that progenitor chain migrations can persist even in the absence of the olfactory bulb (Kirschenbaum et al., 1999), suggesting the existence of mechanisms other than target-specific cues. One such mechanism is the astrocyte-derived migration-inducing activity (MIA), a powerful chemoattractant identified in rats (Mason, Ito, and Corfas, 2001). It appears that the combination of MIA and the repellent protein Slit is largely responsible for the directed and targeted migrations of progenitor chains.

Since the discovery and characterization of the SVZ, additional CNS germinal zones have been identified in the olfactory bulb, itself (Gritti et al., 2002), dentate gyrus of the adult hippocampus (Gage et al., 1998), the cerebral cortex (Arsenijevic et al., 2001), and the adult spinal cord (Weiss et al., 1996), raising the possibility that germinal zones may be dispersed throughout the whole of the CNS. If this is indeed the case, then the mature central nervous system may be capable of more extensive repair and regeneration than was previously suspected.

Factors Affecting Neuronal Commitment

Much work has been done to elucidate how the cells of the mature nervous system are derived from the initial pool of stem and progenitor cells. The general scheme proposed involves asymmetric mitoses of pluripotent stem cells. These stem cells, by definition, are self-renewing and phenotypically undefined. Each asymmetric mitotic event creates an identical daughter stem cell and a more phenotypically restricted cell referred to as a progenitor cell. Progenitor cells are highly proliferative and ultimately give rise to even more restricted progenitor phenotypes

(referred to as precursors) which will eventually differentiate to form neurons, astrocytes, or oligodendrocytes. With each step of this progression, fate decisions become more restricted and proliferative potential decreases (Gage, 2002) (**Fig. 2**). This scheme is complicated by the speculation that progenitor populations generated early in development are generally destined to become neurons, while those produced later in development are destined to become glia (Qian et al., 1998; and Qian et al., 2000).

Soluble Agents. Using isolated, individual neural stem cells as starting material, it has been shown that cells committed to the neuroblast phenotype are produced largely through asymmetric divisions in the early stages of clonal expansion, while glioblasts were produced largely through symmetric divisions later in clonal expansion (Qian et al., 1998). Furthermore, this shift from neuroblasts to glioblasts is contingent upon an increase in responsiveness to EGF (Qian et al., 2000) through upregulation of EGF receptors (EGFRs) (Liilen and Raphael, 2000; and Burrows et al., 1997). This finding parallels earlier studies which suggested that EGF directly promotes gliogenesis (Kuhn et al., 1997), while early neural progenitors tend to be preferentially responsive to FGF2 (Tropepe et al., 1999; Martens, Tropepe, and van der Kooy, 2000; Ciccolini, 2001; and Ciccolini and Svendsen, 1998). Upregulation of EGF receptors appears to be driven, as least in part, by FGF2 (Burrows et al., 1997), although FGF2 has also been proposed as a promoter of neuronal commitment (Kuhn et al., 1997). This seeming disparity is explained by dose-responsiveness, as low concentrations of FGF2 appear to be able to promote neuronal commitment, while higher doses encourage increasing EGF responsiveness (Qian et al., 1997). Curiously, although adherent cultures of neural progenitors exhibit this temporal pattern of phenotypic commitment, neurospheres formed from these cultures are

apparently unrestricted and can differentiate into neurons, astrocytes, and oligodendrocytes at all stages of expansion (Qian et al., 2000).

NGF has long been known to promote neurogenesis in PC12 cells (Greene and Tischler, 1976). In addition, it has been identified as being capable of supporting the survival and growth of sensory and sympathetic neurons derived from the spinal cord (Levi-Montalcini, 1987). A number of studies have elucidated the signaling pathway associated with NGF, and the overall picture that has emerged has been recently reviewed (Vaudry et al., 2002). NGF dimers associate with two known receptors, TrkA and $p75^{NTR}$ (Bothwell, 1995). Subunits of TrkA are single pass transmembrane proteins which act as receptor tyrosine kinases (RTKs) (Sofoniew, Howe, and Mobley, 2001). Signaling through TrkA produces the more characteristic effects attributed to NGF. TrkA is known to stimulate neurite extension primarily through the same signaling pathways used by most growth factor receptors (**Fig. 3**). The MAPK (Levi-Montalcini, 1987) and phospholipase C-γ/protein kinase C-δ (PLCγ/PKCδ) pathways are thought to be responsible for proliferation and neurite extension, while neuronal survival is reinforced through the phosphatidylinositol-3 kinase/protein kinase B (PI3K/PKB) pathway (Huang and Reichardt, 2001). The p75 NGFR subunit is a neurotrophin-binding transmembrane glycoprotein (Sofoniew, Howe, and Mobley, 2001) which activates the NF-κB pathway at the same time that apoptosis is activated through the production of ceramide (Huang and Reichardt, 2001). A number of studies have indicated that the balance between TrkA and p75 receptor subunits determines the fate of cell exposed to NGF. Both TrkA homodimers and TrkA/p75 heterodimers have been shown to promote cell survival, while p75 homodimers cause apoptosis (Niederhauser et al., 2000; and Yoon et al., 1998). Transfection of p75-expressing cells with TrkA has been shown to rescue them from p75-mediated apoptosis in response to NGF (Yoon et al., 1998; and

Eggert et al., 2000). Recently, a novel signaling pathway has been identified in which TrkA/NGF activates the MAPK pathway in the distal axonal segment, resulting in axonal extension, followed by internalization of the TrkA/NGF complexes. Following receptor/ligand internalization, retrograde transport of the "signaling endosomes" carries them to the cell body, where activation of the PI3K/Akt pathway promotes neuronal survival (Ginty and Segal, 2002). Signaling endosomes have been shown to be clathrin-coated vesicles that incorporate not only TrkA/NGF complexes, but also components of the MAPK, PI3K, and phospholipase C (PLC)-γ pathways. Thus, not only do signaling endosomes carry TrkA/NGF complexes to the cell body, but they also serve as vehicles for intracellular signaling (Howe et al., 2001). NGF signaling in PC12 cells has also been shown to involve translocation of PKCζ from the cytoplasm to the nucleus, where activation of differentiation is affected at the genetic level (Wooten et al., 1998; Wooten et al., 1997; and Zhou, Seibenhener, and Wooten, 1997). PKCζ-mediated differentiation appears to be opposed by the phosphorylation and activation of PKCδ (Wooten et al., 1998). These findings are discussed below.

Platelet-derived growth factor (PDGF) has recently been identified as another growth factor capable of inducing neurogenesis in stem/progenitor cells. This is apparently not through stimulation of neuronal commitment, but rather through support of the growth of neuronal progenitors at the expense of other progenitor types (Erlandsson, Enarsson, and Forsberg-Nilsson, 2001). However, PDGF has been more commonly associated with the development of oligodendrocyte precursors as opposed to neuronal ones. Tripotent glial-restricted progenitors (GRPs) have been shown to preferentially become bipotential oligodendrocyte precursors in response to PDGF and thyroid hormone (TH) (Gregori et al., 2002). Injections of PDGF and FGF2 into the cerebrospinal fluid have also been shown to promote oligodendrogenesis in mouse

SVZ (Lachapelle et al., 2002). In this case, it is impossible to discern whether PDGF or FGF2 is actually promoting oligodendrogenesis, however. The signaling pathways activated by PDGFRs are essentially the same as those described for NGF (**Fig. 3**), although the use of signaling endosomes has not been reported with regards to this growth factor's signaling.

In Vivo infusion of insulin-like growth factor 1 (IGF-1) into the cerebrospinal fluid has been shown to stimulate proliferation of distinctly neuronal progenitors in the adult rat hippocampus (Aberg et al., 2000), a result that has also been seen *in Vitro* (Arsenijevic et al., 2001). Intraventricular administration of EGF has been shown to result in considerable expansion of the rat SVZ and VZ zones, although it is difficult to discern which *in Vivo* factors might modify the EGF signal (Craig et al., 1996). The signaling pathways activated by IGFRs are essentially the same as those described for NGF (**Fig. 3**).

As stated earlier, it has been suggested that EGF promotes gliogenesis, while FGF2 promotes neurogenesis (Kuhn et al., 1997). A link between neurogenesis and FGF2 has been shown in a number of studies. It has been suggested that FGF2 is capable of activating latent "neuronal programming" in cell derived from multiple brain regions (Palmer et al., 1999), and neuronal progenitors cultured in the presence of FGF2 have been shown to develop into spontaneously active neuronal networks (Mistry et al., 2002). Differentiation of H19-7 cells may also be affected by FGF2 by sustained phosphorylation of (CRE)-binding protein (CREB) in the nucleus (Sung et al., 2001). It has been documented that the default state of cortical stem cells is neuronal and that FGF2, at a low concentration ($\leq$ 0.1ng/ml), allows cells to retain this state in culture. Higher concentrations of FGF2 (1-10ng/ml) are associated with the development of glia from these same stem cell populations (Qian et al., 1997), which may at least partly explain why injections of both FGF2 and PDGF promote oligodendrogenesis (Lachapelle et al., 2002). It has

been pointed out that, during development, early neurons are known to produce comparatively high levels of FGF2, which may stimulate surrounding stem/progenitor cells to adopt glial phenotypes (Qian et al., 1997).

Retinoid acid (RA), a derivative of vitamin A, has also been known for some time to promote neuronal differentiation. Its mechanism of action is shown in **Figure 3**. RA diffuses across cell membranes and is bound to cytoplasmic RA receptors (RAR, RXR) which translocate to the nucleus, where activation of neuron-specific genes takes place. The NTera-2 cell line undergoes differentiation in response to RA (Lee and Andrews, 1986), and it has been shown that RA-treated cultures yield greater than 95% pure cultures of postmitotic neurons (Pleasure, Page, and Lee, 1992). RA has also been used to generate populations of functional neurons from P19 murine embryonal carcinoma cells as well as murine pluripotent stem cells (Finley, Kulkarni, and Huettner, 1996; and Wichterle et al., 2002). This treatment is particularly relevant physiologically as it has been suggested that RA functions as a local paracrine factor to induce localized neuronal commitment and differentiation (Maden, 2001; and McMaffrey and Drager, 2000). It has even been suggested that the radial glia upon which neuronal progenitors are known to migrate during development may induce neuronal differentiation through the localized release of RA (Chanas-Sacre et al., 2000).

Growth Substrata. It is well known that contacts between cells and the surrounding extracellular matrix (ECM) regulate a variety of cellular processes, including differentiation. Such adhesions are generated by the binding of particular integrins on the cell surfaces to specific extracellular matrix molecules. Integrins are heterodimeric trasmembrane glycoproteins consisting of α and β subunits that, in combination, bind with specific ECM molecules.

Currently, at least 16 α and 8 β subunits have been identified. Intracellular signaling mediated by integrin binding takes place through the association of focal adhesion kinase (FAK) with the cytoplasmic tails of β integrin subunits (**Fig. 4**). These signaling pathways may be characterized as being "inside-out" or as "outside-in". In the case of inside-out signaling, integrin/matrix interactions are modified by gene activation stimulated, in many cases, by signaling resulting from prior ECM/integrin interactions. In the case of outside-in signaling, signals are transduced from ECM/integrin associations through FAK and the internal signaling pathways. Inside-out and outside-in signaling create a reciprocal relationship between cells and their environment, patricularly with ECM (Boudreau and Jones, 1999). Signaling pathways known to be associated with FAK include the PI3K, Rho, Rac, and MAPK pathways, and many more including the Jun N-terminal kinase (JNK) and PKB pathways are suspected. As integrins are known to play roles in cell survival, migration, proliferation, and differentiation, the associations of integrins with so many signal transduction pathways is not surprising (Schwartz, 2001).

Specific ECM/integrin interactions have been known for some time to promote neuronal commitment. One well-characterized promoter of neuronal commitment and growth is laminin, a large, heterotrimeric glycoprotein found in the basement membranes of adult mammals. Early work determined that laminin supported the growth and axonal extension of primary neurons, and the MAPK and PKC pathways were found to be responsible for transducing laminin-specific signals (Luckenbill-Edds, 1997). β1 and β3 integrin subunits have been shown to be associated with the growth, axonal extension, and ultimate differentiation of embryonic neurons (Bates and Meyer, 1997), and α3β1 and α6β1 integrin dimers have been implicated in the laminin-dependent growth and differentiation of PC12 cells (Ivins, Yurchenco, and Lander, 2000).

The integrin-mediated attachment of cells to the ECM protein fibronectin has also been studied. Fibronectin is a large, multidomain glycoprotein which dimerizes via disulfide bonds, and these dimers associate to form long fibrils. It has been found that the fibronectin-associated associated signaling cascade is the same as that for laminin (Li, Daniels, and Pant, 2001). Thus, it is not surprising that neurite extension can be mediated by attachment to fibronectin (Stallcup, 2000). Neurite outgrowth of rat hippocampal neurons also takes place on tenascin-C (Rigato et al., 2002) and collagen IV (Ali, Pappas, and Parnavelas, 1998). In the latter case, growth in the absence of FGF2 promoted a drive toward neuronal differentiation with concomitant loss of proliferative capacity and glial differentiation, while the addition of FGF2 enabled the differentiation of both glia and neurons.

Signaling Pathways Associated With Neuronal Commitment and Differentiation. NGF-mediated differentiation of PC12 cells is known to be associated primarily with activation of the MAPK (Levi-Montalcini, 1987) and PLCγ/PKC pathways (Huang and Reichardt, 2001). Although the MAPK pathway was traditionally considered to be the major signaling pathway involved in PC12 differentiation, its function is not absolutely required for differentiation. It has been shown that differentiation induced by bone morphogenetic protein-2 (BMP-2) is capable of stimulating PC12 differentiation without activation of MAP kinases or MEK (Iwasaki et al., 1996). Therefore, other pathways must be involved in PC12 differentiation.

The role of PKC isoforms in NGF-induced differentiation has been studied extensively. NGF-induced differentiation in PC12 cells is known to be accompanied by an increase in *de novo* diacylglycerol (DAG) biosynthesis (Neri et al., 1999), which is capable of activating PKC. All known isoforms of PKC have been identified in PC12 cells, and all of these have been shown

to be activated by treatment with NGF (Wooten et al., 1998; Wooten et al., 1997; Zhou, Seibenhener, and Wooten, 1997; and Wooten et al., 1992). Furthermore, all PKC isoforms appear to play distinct roles in NGF-mediated differentiation. Initial studies showed six-fold induction of PKCβII, indicating a potential role for this isoform in differentiation (Wooten et al., 1992). Later work demonstrated that this was not the only isoform involved, however. PKCζ has been shown to undergo nuclear translocation during the differentiation of PC12 cells (Wooten et al., 1997; Zhou, Seibenhener, and Wooten, 1997; and Neri et al., 1999) and associate directly with the nuclear scaffolding (Zhou, Seibenhener, and Wooten, 1997). This action is opposed by activation of PKCδ (Wooten et al., 1998).

RA-induced differentiation of cells in multiple model systems is dependent upon RA binding to RA receptors (RARs) and retinoid X receptors (RXRs). In neuroblastoma cell lines, it has been found that RARβ and RARγ form heterodimers with RXRβ, which directly activate genes associated with differentiation (Rana et al., 2002). Thus, multiple pathways have been implicated in neuronal differentiation, but these pathways have been largely studied in cell lines, and it is difficult to ascertain how relevant they are to the *in Vivo* situation.

Points Addressed By The Studies Presented in This Dissertation

Thus far, neuronal commitment and differentiation has been studied primarily in cell lines that may or may not accurately represent the *in Vivo* situation. Elucidation of the mechanisms involved in neuronal commitment and differentiation in neurospheres derived from primary tissue, therefore, represents a significant breakthrough. Neurospheres were selected as a preferred model system because neurogenic potential is apparently maintained indefinitely, as opposed to monolayer cultures, which are only able to generate neurons early in their

development (Qian et al., 2000). Based upon the literature concerning FGF2 and stem cell commitment, it was felt that manipulation of the FGF2 concentrations to which the neurospheres were exposed might be exploited to induce neuronal commitment. It was further speculated that the optimal concentration for such commitment would be lower than that used to stimulate proliferation (10ng/ml), and that exposure of neurospheres to optimal doses of FGF2 would downregulate immature phenotypic markers associated with the neuroepithelial state and upregulate neuronal markers. Based on studies conducted exclusively in monolayers, this phenotypic shift was expected to be driven by a PKC isoform or isoforms.

Based upon this hypothesis, these studies were divided into five specific aims:

1. Definition of the cellular nature and tissue architecture of neurospheres;
2. Development and validation of a method to study the changes in expression of phenotypic marker proteins in whole, intact neurospheres;
3. Induction of neuronal commitment in whole, intact neurospheres by exposure to optimal FGF2 doses. Phenotypic shifts would be assessed by comparing expression levels of key phenotypic marker proteins between treated (commitment-induced) cultures and control (proliferative) cultures using the method developed in the previous stage;
4. Determination of PKC isoforms found in neurospheres, determination of changes in expression levels of various isoforms under a variety of different growth factor treatments;
5. Determination of a role for PKC in FGF2-mediated commitment to the neuronal phenotype.

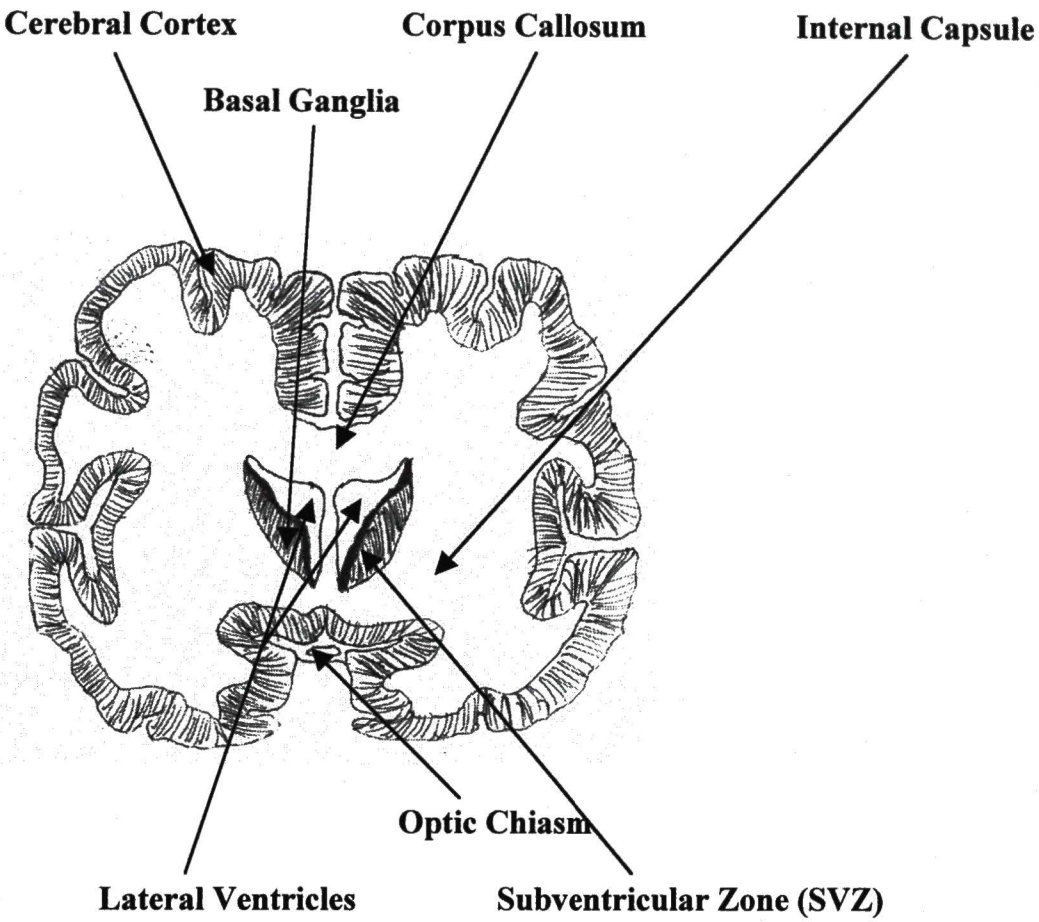
It is hoped that the completion of these studies will significantly advance neurobiology by defining critical events in the differentiation of neurons from initially multipotent stem and progenitor cells. These studies are further significant in that the potential for neurogenesis has never before been adequately assessed in human neurospheres harvested directly from primary tissue. While the mechanisms underlying such events have been well mapped, it remains to be seen whether or not the same mechanisms apply to neuronal commitment and differentiation in intact neurospheres. As neurospheres are complex structures consisting of multiple cell layers, it is suspected that the stimuli encouraging neuronal commitment and differentiation will differ from those described for monolayer cultures.

It is also proposed that experiments conducted with intact neurospheres could aid in the development of a conditioning regimen that will "program" the cells within neurospheres to the neuronal phenotype prior to attachment to specific extracellular matrix substrates. For example, conditioning of neurospheres followed by outgrowth on a neuron-promoting substrate such as laminin might allow cell cultures greatly enriched in neurons to be obtained. If this is indeed the case, then it may also be possible to create cell cultures enriched in astrocytes or oligodendrocytes, as well. In addition to providing more useful *in Vitro* models, the creation of such models might also facilitate the development of artificial tissue constructs suitable for transplantation. Another possible application of preconditioned neurospheres lies in the creation of three dimensional model systems. Investigations of neuronal outgrowth, axonal extension, and axonal targeting have been conducted for several years in systems involving the implantation of nervous tissue explants into three-dimensional collagen gels (Varela-Echavarria et al., 1997; Ringstedt et al., 2000; Mouveroux, Lakke, and Marani, 2001; and Hernandez-Montiel et al., 2003). A key benefit of this model system lies in the freedom of the cells of cultured explants to

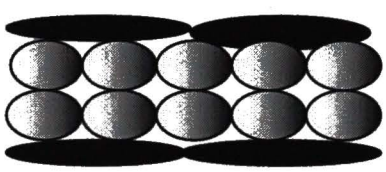
expand or extend cellular processes in three dimensions, thus more accurately simulating *in Vivo* nervous tissue. This model system could be easily modified for use with conditioned neurospheres rather than explants, and the use of neurospheres transfected with specific genes combined with conditioning regimens could greatly enhance the understanding of CNS development, repair and regeneration. With greater understanding of the signals, both soluble and insoluble, controlling specific phenotypic fate decisions, these neuronal models could be designed according to need. With such diversity of attractive possibilities, investigations into methodologies to direct phenotypic commitment events are vital. Most groups working with neurospheres rely on mechanical dissociation of neurospheres followed by monolayer expansion of the resulting cells. Such investigations do not address how far commitment events may proceed within the intact starting material. We felt that it was time to address these issues in intact neurospheres.

Figure 1: A schematic diagram of cellular organizations in migratory cell streams of the rostral migratory stream (RMS, A) and the subventricular zone (SVZ, B). A. A representation of a coronal section of the mature human brain showing the relative positions of the lateral ventricles and the subventricular zones (SVZs) lying immediately beneath them. B. In the migratory cell streams, cores of Type A cells showing the phenotypic characteristics of neuroblasts are surrounded by sheaths of Type B cells showing the phenotypic characteristics of astrocytes. C. The organization of the so-called "cell nests" of the subventricular zone is similar, with the addition of Type C cells showing the phenotypic characteristics of uncommitted progenitors.

A.

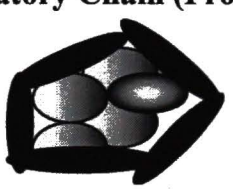


B.



Migratory Chain (From Rostral Migratory Stream, RMS)

C.



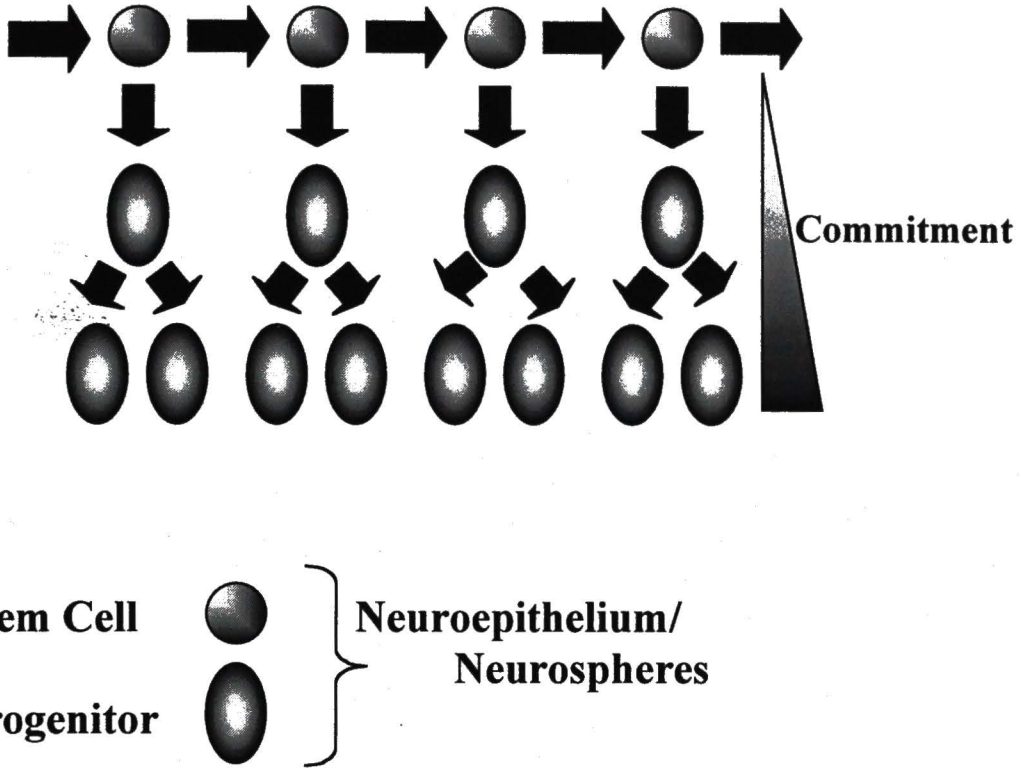
"Cell Nest" (From Subventricular Zone, SVZ)

Type A Cells Neuroblast **Type C Cells Uncommitted Progenitors**

Type B Cells Astrocytes

Figure 2: A diagram showing the development of the various cell types in the central nervous system (CNS). A. Stem cells, defined as self-renewing, phenotypically uncommitted cells, undergo asymmetric mitoses to generate more stem cells as well as partially lineage-committed progenitor cells, which are restricted in phenotypic commitment. B. In response to environmental cues delivered by soluble signals (growth factors, cytokines, etc.) and attachment-dependent signals (extracellular matrix protein activation of integrins), the ultimate phenotypic fates of progenitors are determined.

A.


Commitment
Stem Cell
Progenitor
Neuroepithelium/
Neurospheres

B.

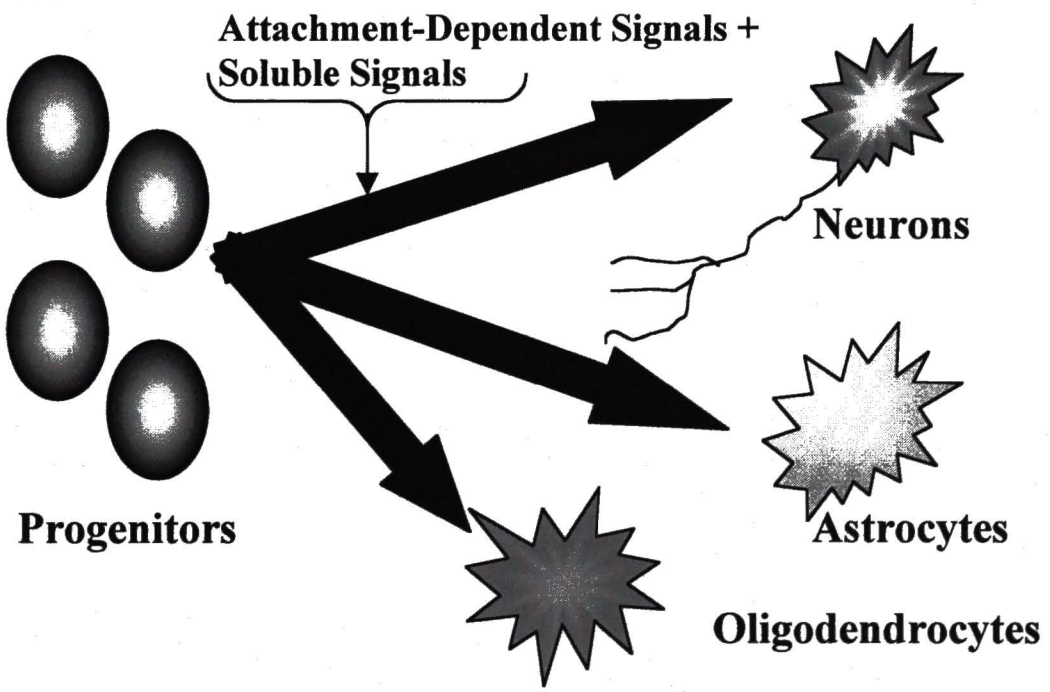

Attachment-Dependent Signals +
Soluble Signals
Neurons
Astrocytes
Oligodendrocytes
Progenitors

Figure 3: A schematic diagram showing some of the major signaling pathways associated with proliferation, differentiation, and survival of neural progenitors. Retinoic acid (RA) diffuses directly across cell membranes, becomes associated with nuclear retinoic acid receptor (RAR)/retinoid receptor (RXR) dimers and activates genes associated with differentiation. Growth factor (GF) signaling is accomplished through the dimerization of receptor tyrosine kinases (RTK) and activation of multiple downstream signaling pathways, including the MAP kinase (MAPK) pathway, the PI3K/Akt pathway, and the protein kinase C (PKC) pathway. These pathways ultimately affect aspects of proliferation, commitment and differentiation, and survival. In addition to signaling through the general GF pathways, NGF may also accomplish neuronal apoptosis through activation of p75/p75 dimers and the concomitant production of ceramide.

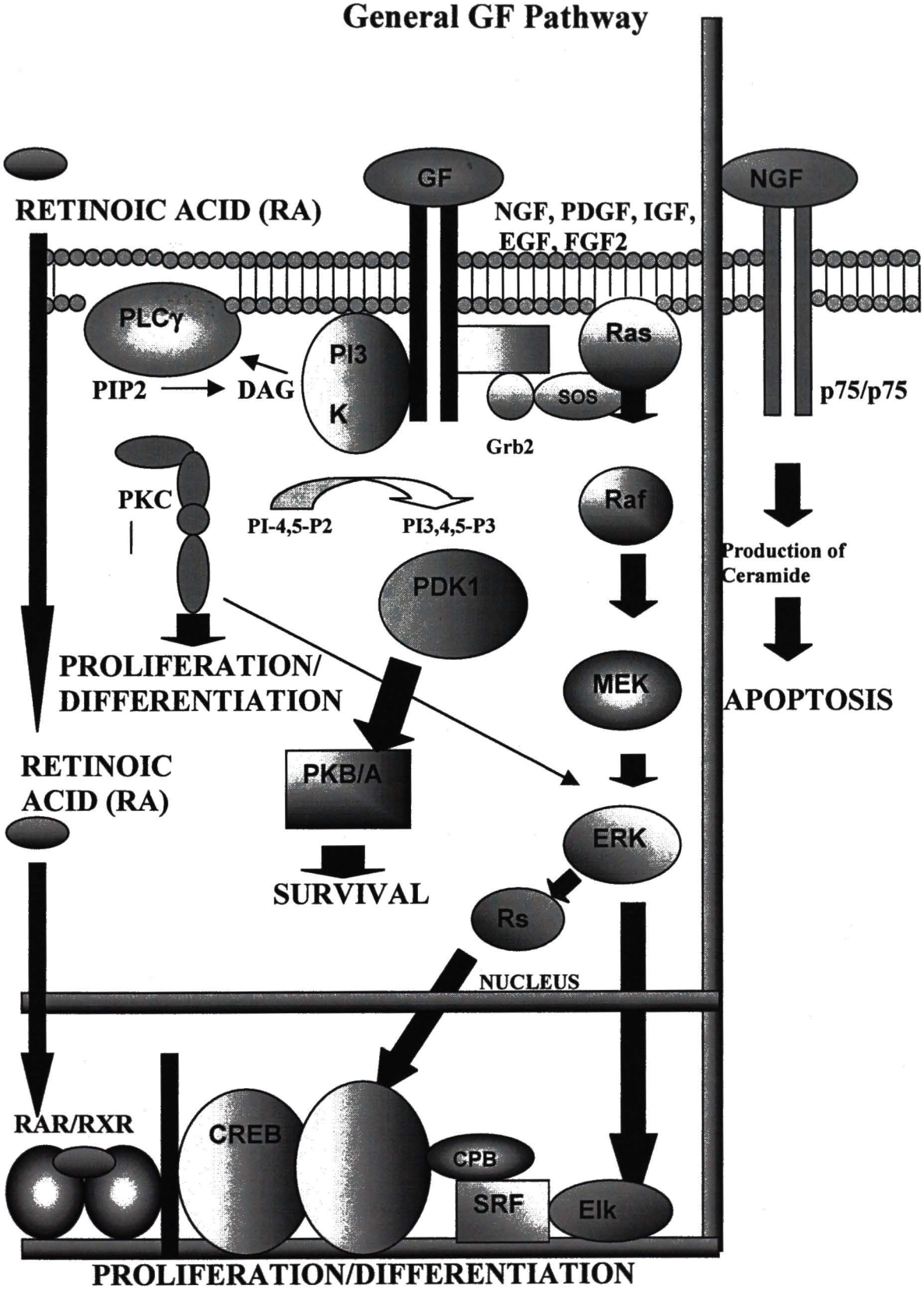
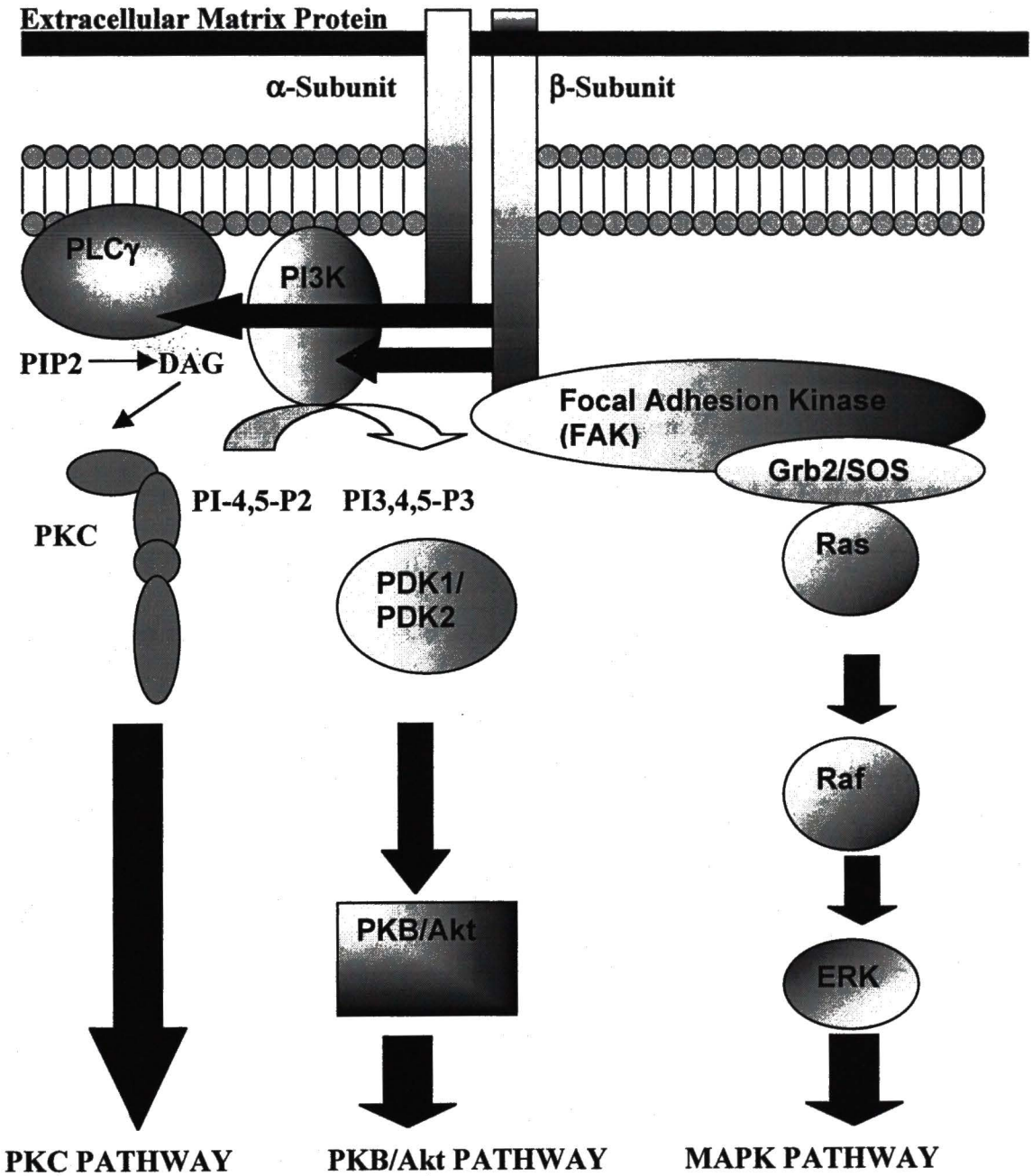

General GF Pathway
RETINOIC ACID (RA)
GF
NGF, PDGF, IGF, EGF, FGF2
NGF
PLCγ
PIP2
DAG
PI3 K
Ras
SOS
Grb2
p75/p75
PKC
PI-4,5-P2
PI3,4,5-P3
Raf
Production of Ceramide
PDK1
PROLIFERATION/ DIFFERENTIATION
MEK
APOPTOSIS
RETINOIC ACID (RA)
PKB/A
ERK
SURVIVAL
Rs
NUCLEUS
RAR/RXR
CREB
CPB
SRF
Elk
PROLIFERATION/DIFFERENTIATION

Figure 4: A schematic diagram of some of the major signaling pathways associated with integrin-mediated cell attachment to the extracellular matrix. A. The integrin subunits (α and β) bind to extrecellular matrix proteins, and focal adhesion plaques (FAPs) form largely around the cytoplasmic tails of the β subunits. A major component of FAPs is focal adhesion kinase (FAK), which, once tyrosine phosphorylated, serves as a downstream activator of the MAP kinase (MAPK) pathway. Additional signaling from the FAP occurs through the PI3K/Akt and PKC pathways. B. A chart of all known combinations of the alpha and beta integrin subunits as well as their extracellular matrix ligands, where known (compiled from multiple sources).


Integrin Dimer
Extracellular Matrix Protein
α-Subunit
β-Subunit
PLCγ
PI3K
PIP2
DAG
Focal Adhesion Kinase
(FAK)
Grb2/SOS
Ras
PKC
PI-4,5-P2
PI3,4,5-P3
PDK1/
PDK2
Raf
PKB/Akt
ERK
PKC PATHWAY
PKB/Akt PATHWAY
MAPK PATHWAY

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CHAPTER II

The following manuscript is in preparation for submission to *Gene Expression Patterns*.

Regional Distribution of Progenitor Cells in Human Neonatal Neurospheres

Michael L. Moeller, Julian Borejdo, and S. Dan Dimitrijevich

Department of Molecular Biology and Immunology

The University of North Texas Health Science Center at Fort Worth

3500 Camp Bowie Blvd., Fort Worth, Texas

***Corresponding author. Present address: Department of Integrative Physiology and Cardiovascular Research Institute, UNT Health Science Center, 3500 Camp Bowie Blvd. Fort Worth, TX 76107. e-mail: ddimitri@hsc.unt.edu**

ABSTRACT

Numerous models have been used in neuroscience research for the last 30 years. Initially, tumor cell lines with neuronal characteristics were used, primarily because of the ease with which they could be proliferated and differentiated. However, uncertainty concerning the initial transformative event(s) has generated reservations regarding these lines. The development of cells transformed with specific oncogenes has allowed studies to be conducted with more defined lines, but problems may exist due to transgene insertion effects. Recently, wild type stem and progenitor cells from mammalian forebrain germinal zones have received attention as potential models. These cells may be obtained from the subventricular zone (SVZ) of the forebrain and expanded in various ways, including as floating cell aggregates termed neurospheres. The cells comprising these structures are multipotent and may give rise to astrocytes, oligodendrocytes, or neurons when differentiated. Until now, the various progenitor cell populations found within neurospheres have been considered to be randomly distributed. We report here the first evidence that this may not be the case. Through double immunofluorescent labeling experiments, we show that human neurospheres are surrounded by $GFAP^+$ astrocytes encasing a core of putative neuroblasts expressing several neuronal markers. This structure resembles that of the "cell nests" of the SVZ and the migratory "cell chains" of the rostral migratory stream (RMS), we propose that neurospheres may represent partial recapitulations of the *in Vivo* situation. Based on the cell interactions during outgrowth, we also propose a mechanism by which cell chains of the RMS may colonize target regions.

INTRODUCTION

Investigations in neuroscience have traditionally relied on the use of experimental models to simulate the *in Vivo* condition. In the 1970s, the PC12 line of rat pheochromocytoma cells was established as one such model. These cells proliferate in the presence of serum, and can be induced to cease proliferation and develop neurite extensions in response to serum withdrawal and exposure to nerve growth factor (NGF) (Greene and Tischler, 1976). The NTera 2 (NT-2) cell line is another cell line derived from tumor tissue, in this case an embryonic human teratocarcinoma. In the presence of fetal bovine serum (FBS), these cells are also proliferative, but can be differentiated in the presence of retinoic acid (RA) (Pleasure and Lee, 1993).

A major drawback to tumor-derived cell lines lies in a lack of knowledge concerning the mechanism by which the line has overcome the intrinsic barriers to continued cell division. In the 1980s, a number of laboratories attempted to address this problem by producing stem cell lines transformed with specific oncogenes. These oncogene-transformed cell lines may be more defined models than tumor-derived cell lines. However, insertion of transforming oncogenes is random, and expression is influenced by the insertion site. This may lead to heterogeneity within cell lines due to different insertion sites. Insertion of transgenes may also disrupt the expressions of surrounding genes, including phenotype-specific ones. Thus, transformed cell lines possess certain drawbacks which make them less than ideal (Gottlieb, 2002).

Much recent interest has been focused on the use of wild type stem and progenitor cells obtained from the mammalian forebrain germinal zones. These cells divide continuously for long periods of time when cultured in serum-free defined medium supplemented with growth factors. In the first study using this approach, adult mouse striata were dissociated and grown as

monolayers in the presence of epidermal growth factor (EGF). The resulting monolayers gave rise to floating spherical cell aggregates ("neurospheres") that could be expanded by mechanical disruption, followed by regrowth of the fragments (Reynolds and Weiss, 1996). Since that time, several other culture conditions have been used to expand neurosphere populations, including growth in the presence of basic fibroblast growth factor (FGF2) or combinations of EGF and FGF2 (Caldwell et al., 2001; Svendsen et al., 1998). In all cases, the neurospheres derived exhibit multipotency (i.e. can differentiate to give rise to all cell types found in the nervous system).

It has been suggested that the cells in neurospheres exhibit no regional organization of phenotypes (Kukekov et al., 1999). We report here that the cells contained within embryonic human forebrain-derived neurospheres are arranged in a manner suggestive of the arrangement of cells in the "cell nests" of the mammalian subventricular zone (SVZ) and the migratory cell chains of the rostral migratory stream (RMS). Based upon this similarity, we propose that neurospheres are a highly realistic model system. Based on the interactions seen between these different cell types during outgrowth on extracellular matrix proteins, we also propose a model by which cell chains might populate target regions.

MATERIALS AND METHODS

Neurospheres. Cell cultures used to create neurosphere populations were purchased from Biowhittaker (Clonetics). Starting cultures consisted of individual cells and small cell clusters obtained the forebrain from 16-week old human fetal forebrain. Within 2-4 weeks of initiation, cultures began to become populated by neurospheres visible to the naked eye.

Culture conditions. Neurosphere cultures were raised in serum-free defined medium (NPMM, Biowhittaker (Clonetics)) containing EGF (20ng/ml), FGF2 (10ng/ml), gentamycin/amphotericin, and neural survival factors (NSF-1). All cultures were maintained under constant humidity at 37°C and 5% CO_2. For early cultures, medium was completely replaced once every 7-10 days by removal of medium, pelletting of cells by centrifugation at 4,000-5,000rpm, decanting of spent medium and resuspension of cells in fresh medium. In later cultures, 1/2-2/3 of the medium volume was replaced every 2-4 days, resulting in the adherence of large numbers of cells and small neurospheres to the flask bottoms and extensive zones of outgrowth as monolayers. When large numbers of neurospheres were desired, medium replacement frequency was decreased, leading to fragmentation of the adherent monolayers and formation of substantial numbers of neurospheres.

Antibodies used. α-internexin was labeled using 1:200 dilutions of monoclonal rabbit anti-α-internexin (Chemicon International Inc., Temecula, CA) in PBS. β-tubulin was labeled with rabbit polyclonal β-tubulin (Santa Cruz Biotechnologies Inc., Santa Cruz, CA) diluted to 1:100 in PBS. Neuron-specific enolase was labeled with mouse monoclonal anti-neuron-specific enolase (NSE) (Chemicon International Inc., Temecula, CA) used at a dilution of 1:200 in PBS. Polyclonal rabbit anti-neurofilament M (Chemicon International Inc., Temecula, CA) was used at a dilution of 1:200 in PBS to label NF-M. Glial fibrillary acidic protein was labeled with monoclonal mouse anti-GFAP (Chemicon International Inc., Temecula, CA) at a dilution of 1:200 in PBS. Monoclonal mouse anti-nestin (Chemicon International Inc., Temecula, CA) was used at a dilution of 1:200 in PBS to label nestin. Vimentin was labeled with mouse monoclonal anti-vimentin (Sigma RBI, St. Louis, MO) at a dilution of 1:200 in PBS. Connexin-43 was labeled with rabbit anti-connexin-43 polyclonal antisera, which was generously provided by Dr.

Mario Wolosin, Department of Ophthalmology, Mount Sinai School of Medicine, New York, New York. Secondary antibodies used were AlexaFluor 488 goat anti-mouse, AlexaFluor 594 goat anti-mouse, AlexaFluor 488 goat-anti-rabbit, and AlexaFluor 594 goat anti-rabbit (Molecular Probes, Inc. Eugene, OR), and were used at dilutions of 1:250 in PBS.

Transmission Electron Microscopy (TEM). Neurospheres were embedded in a proprietary mixture of collagen Type I gel, fixed at 4^{O}C overnight in 2% glutaraldehyde and 1% tannic acid (low MW, Polysciences Inc., PA) buffered in 0.125M PIPES, pH 7.4. Secondary fixation using 1% osmium tetroxide in PIPES was carried out for 45 minutes, followed by staining with aqueous uranyl acetate and dehydration through graded concentrations of ethanol (10%, 30%, 50%, 70%, 95%, and 100%x3). After substitution into propylene oxide, the samples were infiltrated overnight in Polybed 812 (Polysciences Inc., PA) and polymerized at 70^{O}C. Thins sections were cut with diamond knives on a Sorvall MT2-B ultramicrotome, collected on 150 mesh Ni grids, and stained with saturated ethanolic uranyl acetate and Sato's calcined lead citrate. The stained sections were examined on a Hitachi H-600 transmission electron microscope, and micrographs were recorded on Kodak SO-163 electron image film.

Indirect immunofluorescence. Neurospheres were removed from culture flasks, rinsed briefly in PBS, and fixed/permeabilized in ice cold 1:1 (v:v) methanol:acetone for 12 minutes at 4^{O}C. Following fixation/permeabilization, neurospheres were soaked in PBS for 30 minutes and blocked at 4^{O}C overnight in PBS containing 1%BSA. Following blocking, neurospheres were rinsed briefly in PBS and incubated at 4^{O}C overnight in 1^{O} antibody solutions. Following 1^{O} staining, neurospheres were rinsed 3x 10 minutes in PBS containing 0.25% Triton X-100 and incubated in 2^{O} antibody solutions (1:250 in PBS) for 1.5 hours at room temperature, followed by rinsing in 0.25% Triton X-100 for 3x 10 minutes. This procedure was then repeated to label

the second marker. Fully labeled specimens were rinsed 3x 10 minutes in PBS and soaked for 30 minutes in distilled water prior to being mounted on clean glass slides with FluorSave™ mounting medium (Calbiochem, La Jolla, CA).

Assessment of the multipotency of neurosphere cells. To determine whether or not neurosphere cells are multipotent, neurosphere cultures were switched to medium containing 50ng/ml phorbol myristoyl acetate (PMA). Within 72hrs. neurospheres were seen to attach to the flask bottoms and undergo morphological differentiation to give the distinctive morphologies of neurons, astrocytes, and oligodendrocytes.

Identification of phenotypic marker proteins present. Initial experiments were carried out to identify proteins present within neurosphere cells which are considered to be representative of specific phenotypes. The intermediate filament proteins nestin and vimentin were taken to represent phenotypic immaturity, α-enolase was taken to represent the epithelial stem cell phenotype, glial fibrillary acidic protein (GFAP) was taken to be representative of the glial (specifically astrocytic) phenotype, α-internexin, β-tubulin, neuron-specific enolase (NSE), neurofilament M (NF-M), and substance P were taken to represent the neuronal phenotype. For each study, 3-6 neurospheres were labeled.

Double labelings performed. Double labeling was carried out using antibodies against the following pairs of phenotypic marker proteins: GFAP/NF-M, GFAP/β-tubulin, β-tubulin/NF-M, NSE/β-tubulin, α-internexin/β-tubulin, α-internexin/NF-M, vimentin/nestin, α-internexin/nestin, vimentin/GFAP, vimentin/β-tubulin, and NSE/NF-M. For each dual labeling study, 3-6 neurospheres were labeled. Adherent outgrowths grown on coverslips were labeled for GFAP/nestin and GFAP/β-tubulin.

BrdU incorporations. Assessment of cell proliferation in neurospheres was performed by incubating neurospheres in proliferation medium containing 10μM BrdU (Sigma RBI, St. Louis, MO) for 24 hours at 37°C, 5% CO2, and constant humidity. Following BrdU incorporation, neurospheres were fixed/permeabilized in ice cold 1:1 methanol:acetone for 12-15 minutes at 4°C, and rinsed in PBS. Genomic DNA was denatured for 30 minutes at room temperature using the manufacturers solution, and samples were soaked at room temperature for 30 minutes in PBS and blocked at 4°C overnight in the blocking solution provided with the kit. Specimens were incubated over the next night in the biotinylated anti-BrdU 1° antibody provided with the kit. Secondary staining was done by incubating specimens in the presence of 10μg/ml Texas Red-labeled streptavidin (Molecular Probes, Inc. Eugene, OR) for 1 hour at room temperature. Fully-labeled specimens were rinsed 3 x 10 minutes in PBS and soaked for 30 minutes in distilled water prior to mounting on clean glass slides with FluorSave™ mounting medium (Calbiochem, La Jolla, CA). Imaging was performed on a laser scanning confocal microscope as described.

Outgrowth Studies. Coverslips were coated with either collagen IV (BD Biosciences, MA; 1.0mg/ml; 10μg/cm^2) or fibronectin (Collaborative Biomedical Products, MA; 1.0mg/ml; 5μg/cm^2) and allowed to air dry completely under a sterile hood. Individual neurospheres were placed on the coated coverslips in minimal (15-20μl) volumes of medium and allowed to attach overnight under incubation at 37°C. Following attachment, adherent neurosphere cultures were cultured in NPMM medium (Biowhittaker Clonetics) containing both EGF (20μg/ml) and FGF2 (10μg/ml). Medium was changed out every 2-3 days until significant growth was achieved, generally after 7-10 days. Labeling of antigenic markers was performed as described above.

Laser-scanning confocal microscopy. Laser-scanning confocal microscopy was performed using a Zeiss Micro Systems 410 LSM laser scanning microscope package with an Axiovert 135 microscope and Omnichrome 43 laser. Images were captured using the software which came with the package.

RESULTS

Organization of Progenitor Phenotypes in Neurospheres. Surface views of our neurospheres indicated that the cells present within our neurospheres exhibited neuroepithelial morphology, and optical sections obtained via laser scanning confocal microscopy demonstrated the hollow nature of our neurospheres (**Fig. 5A**). In previous work, DAPI staining has demonstrated that the walls of neurospheres were composed of ≈6-8 cell layers (Moeller and Dimitrijevich, 2003, in review). BrdU incorporation studies further demonstrated that proliferating cells were not segregated into discrete compartments, but were apparently randomly distributed throughout the walls (**Fig. 5B**). Labeling of connexin-43, a component protein of gap junctions, demonstrated the presence of extensive numbers of connexin-43^{+} gap junctions surrounding the individual cells (**Fig. 5C**). TEM images of neurospheres embedded in collagen Type I gel and sectioned revealed that numerous structures resembling adherens junctions were also seen connecting neurosphere cells (**Fig. 5D**).

To better characterize the phenotypes present within our neurospheres, two separate approaches were taken. First, neurosphere cultures were exposed to medium containing 50ng/ml PMA, a differentiation-promoting agent. Within 72hrs. of exposure, neurospheres were seen to adhere to the flask bottoms and undergo differentiation to give morphologically-distinct neurons,

astrocytes, and oligodendrocytes (**Fig. 6**). Secondly, proteins considered to be indicative of specific phenotypes were labeled by indirect immunofluorescence. The immature phenotypic markers nestin and vimentin are expressed, as are the more committed markers GFAP, α-internexin, β-tubulin, NSE, and NF-M. α-enolase and substance P were not expressed (**Fig. 7**).

To assess the distributions of positively-identified marker relative to one another, an extensive series of double indirect immunofluorescent labeling studies was performed. These studies revealed the presence of a surrounding shell or basket (**Fig. 8A,B**) of $GFAP^+$/$vimentin^+$ (**Fig. 8C**) and $GFAP^+$/$nestin^+$ (**Fig. 8D**) cells, with an inner core of neuroblasts expressing a variety of neuronal antigenic markers including α-internexin, β-tubulin, and neurofilament M (NF-M). It was found that in some, but not all, neurospheres, various neuronal markers (α-internexin, β-tubulin, and NF-M) were coexpressed with GFAP in some of the cells of the glial basket layer (**Fig. 8E-I**), although interdigitation of separate neuroblasts and $GFAP^+$ cells was more frequently observed. In some cases, neuroblasts were seen to form discrete, thin layers immediately outside the $GFAP^+$ layer (**Fig. 8H**). The cells expressing neuronal markers were arranged in laminate strata, with $NF\text{-}M^+$/$\alpha\text{-internexin}^+$ cells being located closer to the periphery of the neurosphere wall (**Fig. 8J**), and $\alpha\text{-internexin}^+$/$\beta\text{-tubulin}^+$/$NF\text{-}M^-$ cells being located immediately beneath this layer (**Fig. 8K**).

Although nestin and vimentin were observed to be coexpressed with many of the same marker, they did not colocalize with each other in double labelings (**Fig. 8L**). Vimentin was found to colocalize with α-internexin (**Fig. 8M**), NF-M (**Fig. 8N**), and GFAP (**Fig. 8C**). Nestin was also found to colocalize with α-internexin (**Fig. 8O**), NF-M (**Fig. 8P**), and GFAP (**Fig. 8D**).

A Function of The Architecture Is Suggested by Outgrowth Studies. Why the cells contained within neurospheres should exhibit such a clear and reproducible structural organization is difficult to say. One possibility is that this organization of cells facilitates colonization and outgrowth of neurosphere cells at specific target sites. To address this hypothesis, neurospheres were allowed to attach and grow into monolayers on coverslips coated with either collagen Type IV or fibronectin. Within 48hrs. reticulate outgrowths could be seen radiating from the adherent neurospheres (**Fig. 9A**). Double labeling experiments performed against GFAP/nestin and GFAP/β-tubulin demonstrate that the cells located in the radiating network express GFAP and bear long cellular extensions. Some of these cells were $GFAP^+/\beta\text{-tubulin}^+$ (data not shown). $GFAP^-/nestin^+$ cells were seen to be distinctly different morphologically from the $GFAP^+/nestin^-$ cells, being less elongated and more rounded, and were seen to emerge from the bodies of attached neurospheres as distinct clusters or swarms which would occasionally migrate outward along the extended tracks formed by the radiating $GFAP^+$ network (**Fig. 9B**).

DISCUSSION

Many angles have been pursued in the creation of neuronal models suitable for either basic or applied research. Cell lines have long formed the backbone of neuronal cellular research, with rat pheochromocytoma (PC12), neuroblastoma, and glioma cell lines becoming some of the most commonly used models. Transfection of stem cells with transforming oncogenes to create highly proliferative, immortalized cell lines has created many other models which have been used extensively. These models have several inherent problems, including a lack of knowledge concerning the initial transformative event or events in tumor cell lines and

the potential for phenotypic heterogeneity due to the randomness of transgene insertions in the artificially transformed cell lines. The use of unmodified wild type cells obtained from CNS germinative zones represents the most recent line of thinking in model development (Gottlieb, 2002). Cell obtained from these regions may be cultured as monolayers (Ray, et al., 1993) or as neurospheres, hollow clusters of cells grown in suspension culture. Neurospheres have been regarded as lacking phenotypic organization, with different progenitor types arranged randomly relative to one another (Kukekov, et al., 1999). We report here a high degree of spatial organization of progenitor types within human neurospheres derived from human neonatal forebrain. The arrangement of glial and neuroblast cells described in this work closely resembles that of the "cell nests" or cell chains described in the mammalian subventricular zone (Doetsch, Garcia-Verdugo, and Alvarez-Buylla, 1997; Garcia-Verdugo, et al., 1998). In these cell nests, Type B cells, which are $GFAP^+$/$vimentin^+$ and bear the morphologies of astrocytes, encircle cores of Type A cells which are PSA-$NCAM^+$/$TuJ1^+$ and resemble migrating neuroblasts. During migration down the rostral migratory stream (RMS) during olfactory neuron replacement, these cells form migrating chains, also encircled by $GFAP^+$/$vimentin^+$ cells and containing cores of PSA-$NCAM^+$/$TuJ1^+$ cells. Here, we describe the neurosphere architecture as consisting of a "basket" or shell of putative glia surrounding a core of neuroblasts.

The glial basket layer consists primarily of a mixture of $GFAP^+$/$vimentin^+$ and $GFAP^+$/$nestin^+$ cells interspersed with cells which coexpress GFAP with neuronal markers as well as $GFAP^-$ neuroblasts expressing a variety of neuronal markers. $GFAP^+$ /$vimentin^+$ cells might be in an intermediate stage of development, as vimentin is thought to be progressively replaced by GFAP during glial maturation (Menet, et al., 2001). Indeed, vimentin expression has been shown to be indicative of mitotically active (immature) glia (Alonso, 2001). Vimentin has

also been seen to be associated with the radial glial phenotype (Noctor, et al., 2002), and it has been suggested that radial glia are capable of differentiating into neurons (Noctor, et al., 2001). This speculation is supported by the observation that cells coexpressing GFAP and a variety of neuronal markers were seen to be abundant in the glial basket layer. Thus, the radiating network of $GFAP^+$ cells formed during outgrowth might not only promote the outward migrations of neural progenitors, which can then differentiate into neurons, but the glial network, itself, might differentiate to form a fully-formed neural network. Indeed, we have observed coexpression of GFAP and β-tubulin in both some of our outgrowths and neurospheres (data not shown), possibly indicating that the network which grows out from adherent neurospheres is actually composed of radial glia, and that they form directly from the radial glia of the glial basket. This possibility is supported also by the colocalization of GFAP and NF-M in some of the cells of the glial basket layer. The presence of both nestin and NF-M in the outermost layer of cells appears to represent the "first wave" of neural progenitors which migrate out of the neurosphere following attachment. These might correlate with the $nestin^+$ cells observed to migrate onto coated coverslips during outgrowth studies. A proposed arrangement of progenitor phenotypes in neurospheres is shown in **Figure 10**.

All cells of the neurospheres appear to be joined together primarily by tight junctions, with smaller numbers of connexin-43-containing gap junctions. That tight junctions were found to be the major mode of cell/cell attachment explains an observation that we have made regarding the expansion of neurospheres. Neurosphere populations have traditionally been expanded by mechanical disruption of the neurospheres through vigorous pipetting, followed by regrowth of the fragments to form new neurospheres. We have estimated that this method results in relatively high mortalities, possibly as high as 50-60%, as evidenced by the accumulation of

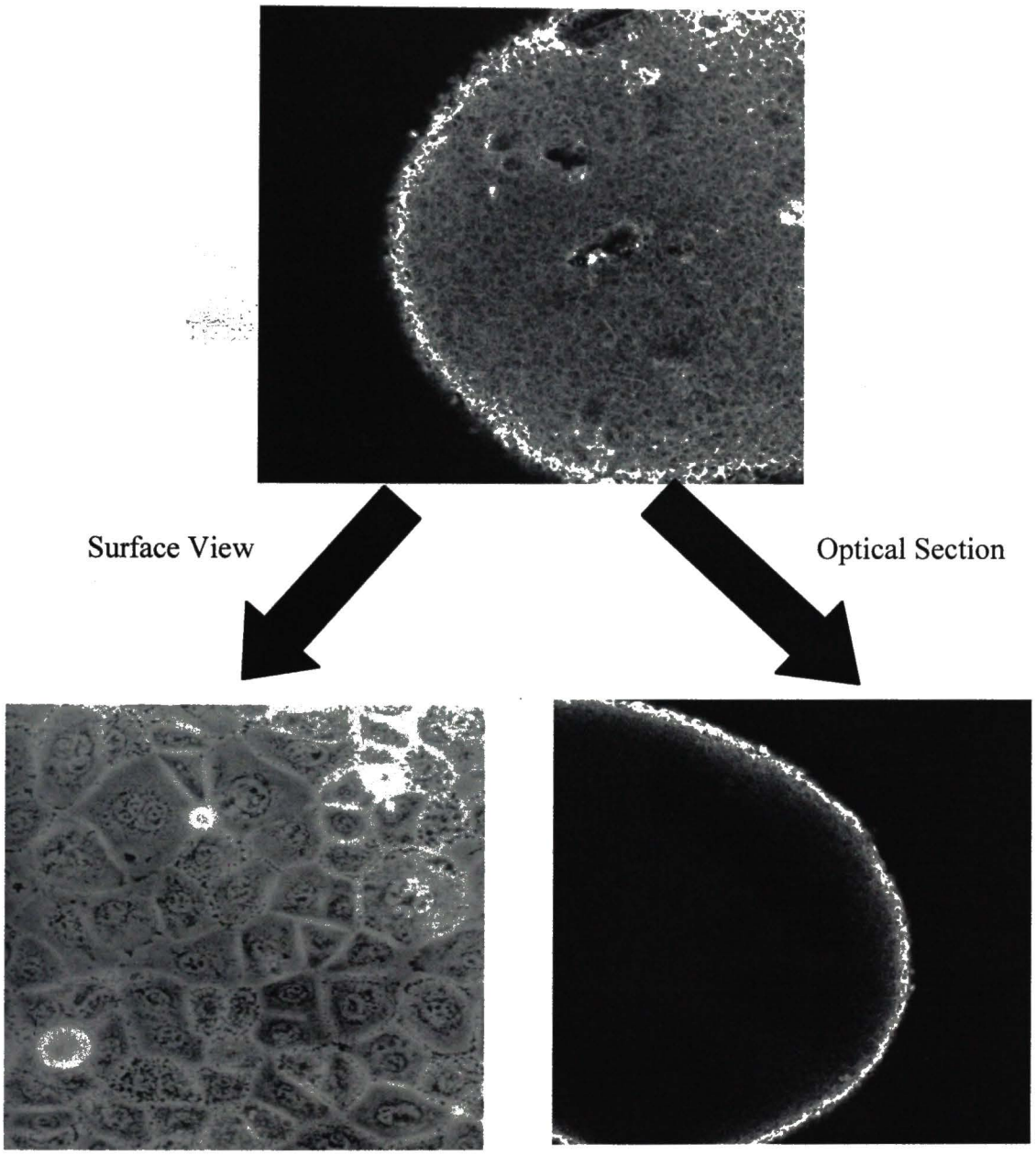
cellular fragments and debris following this procedure. It is logical that mechanical disruption of cellular populations held together by large numbers of tight junctions would result in significant disruption of cell membranes, leading to cell death. One method to circumvent this problem has been the sectioning of neurospheres using automated tissue sectioning equipment (Svendsen, et al., 1998).

The similarity in cellular arrangement between neurospheres, SVZa cell nests, and migratory cell streams suggests that the neural progenitor cell populations which form neurospheres are adopting an organizational pattern that mimics the environment from which they were obtained. In addition, the structural similarities and behavior during outgrowth suggest a potential mechanism whereby these cell aggregates might actively colonize specific target areas. In this model, neurospheres would adhere to specific extracellular matrix locations, form radiating glial scaffolds that grow along the colonized surface, and develop rapidly into tissue through the outward migrations of neural progenitors from the neurosphere cores to colonize the area and eventually differentiate. That attachment is necessary for full commitment to differentiation is shown by the appearance of substance P in poly-D-lysine-adherent outgrowths and neurospheres grown in floating disks composed of collagen Type I (data not shown), even though substance P is not expressed in intact neurospheres floating in suspension. The presence of substance P in these outgrowth also raises the question of the exact fates which the cells in neurospheres can adopt. Substance P has been known for some time to be involved in neurogenic inflammation and nociception in the peripheral nervous system (Harrison and Gepetti, 2001), including newly-elucidated nociception associated with the Meissner corpuscles of skin (Pare, et el., 2001). Both of these points raise the possibility that neurospheres or neurosphere-like dispersal vehicles could conceivably participate in the formation of the peripheral nervous system as well as the CNS.

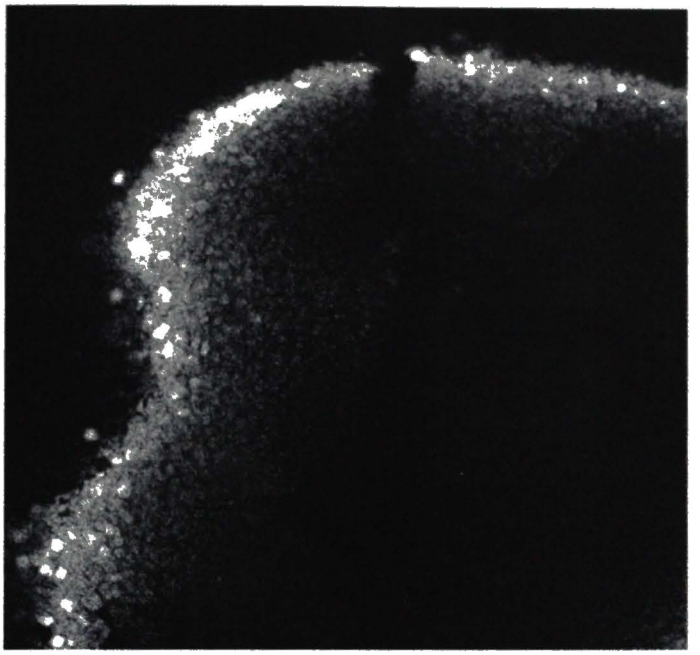
However, substance P has also been shown to have a role in photic resetting of the Circadian pacemaker of the mammalian hypothalamus (Kim, et al., 2001). Thus, the exact fate of these substance P^+ cells is difficult to discern.

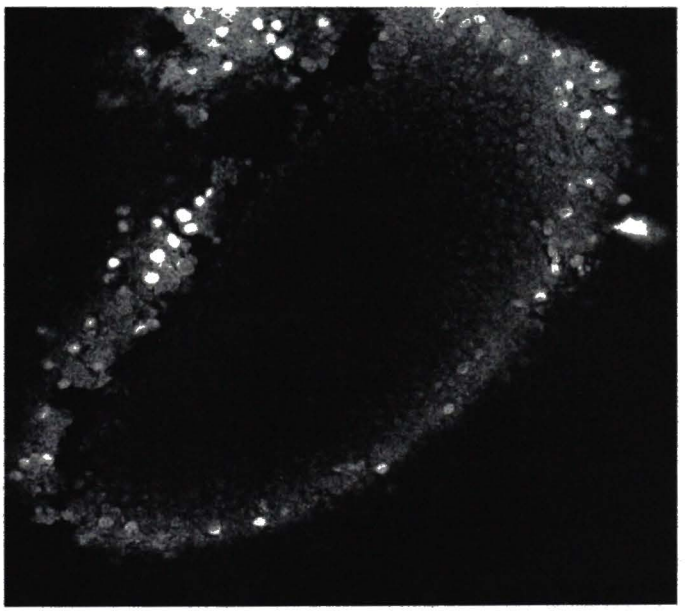
The similarity in arrangement suggests that the cells which give rise to neurospheres form aggregates with a global architecture intrinsic to the cell clusters of the SVZ, and it might mean that neurospheres represent a superior model system for investigations into neuronal development. Moreover, if the organization of cell types in neurospheres is indeed intrinsic to the SVZ, and this organization facilitates colonization of extracellular matrix-encoded targets, neurospheres also show great promise in repopulation and reconstruction of areas of damaged central nervous system.also possible that neurospheres represent an ideal starting material for the construction of innervated artificial tissues. If, as is suspected, neurospheres are organized in such a way that they colonize target locations in the same way as the migratory cell chains described in the RMS, it may be possible to create situations in which neurospheres are induced to innervate either artificial tissue constructs or regions of the nervous system which have been damaged. As the cells contained within neurospheres are in an early stage of development and, therefore, somewhat phenotypically plastic, it is suspected that final phenotypic fate commitments will be driven by the nature of the surrounding milieu into which the neurospheres are implanted.

Figure 5: Examples of the morphology and basic architecture of neurospheres. A. Surface view and optical section of a typical neurosphere. Above, a whole view of a typical neurosphere (250x); below left, the neuroepithelial arrangement of cells characteristic of neurospheres (1000x); and below right, an optical section of a typical neurosphere showing its hollow nature (250x). B. Cross-sections of typical neurospheres, showing BrdU incorporation following a 24hr. pulse. Proliferating cells did not appear to be segregated within the layers of the neurosphere wall, but were randomly distributed throughout the wall layers. C. Connexin-43 distribution in the neurosphere wall. Cells were seen to express high levels of connexin-43 around the peripheries of all cells (1200x). D. Transmission electron microscope (TEM) image of the cell/cell junctions present between neurosphere cells. Numerous structures bearing the morphologies of adherens junctions were seen to comprise the bulk of cell/cell junctions (50,000x).



A.





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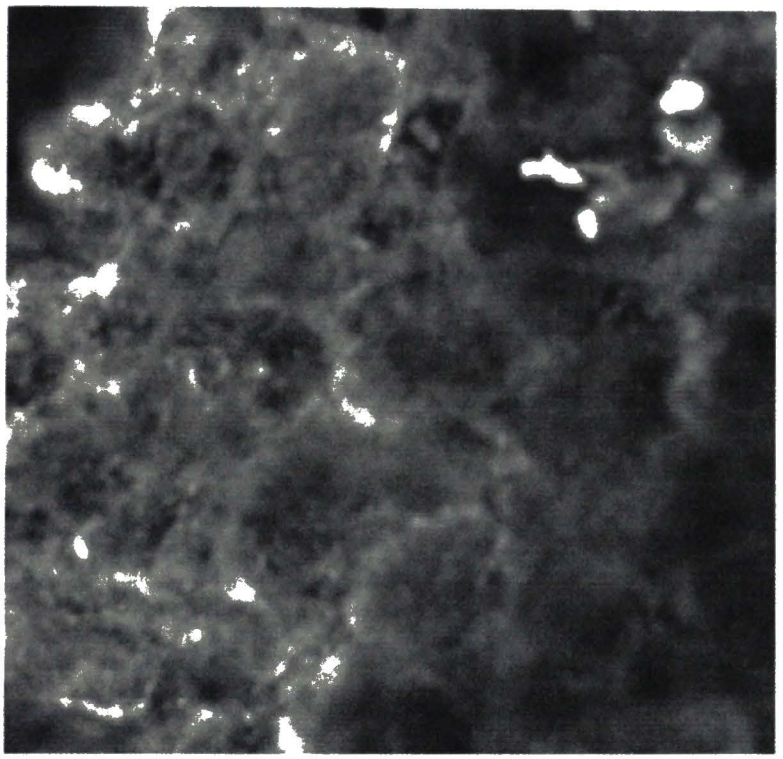

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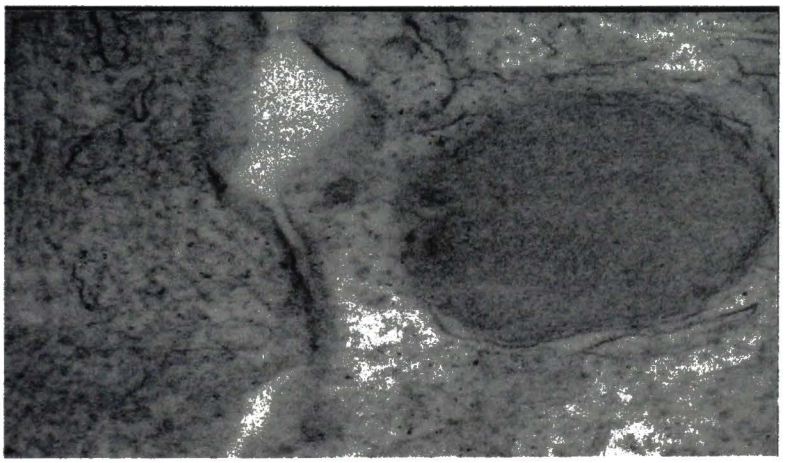


D.

Figure 6: Neurosphere multipotency is seen following differentiation induced by exposure to PMA. Exposure to 50ng/ml PMA for 5-7 days resulted in the differentiation of the neuroepithelial cells (upper left) of neurospheres to cells bearing the morphologies of oligodendrocytes, (lower left) neurons (upper right), and astrocytes (lower right), confirming the multipotency of neurosphere cells (400X).

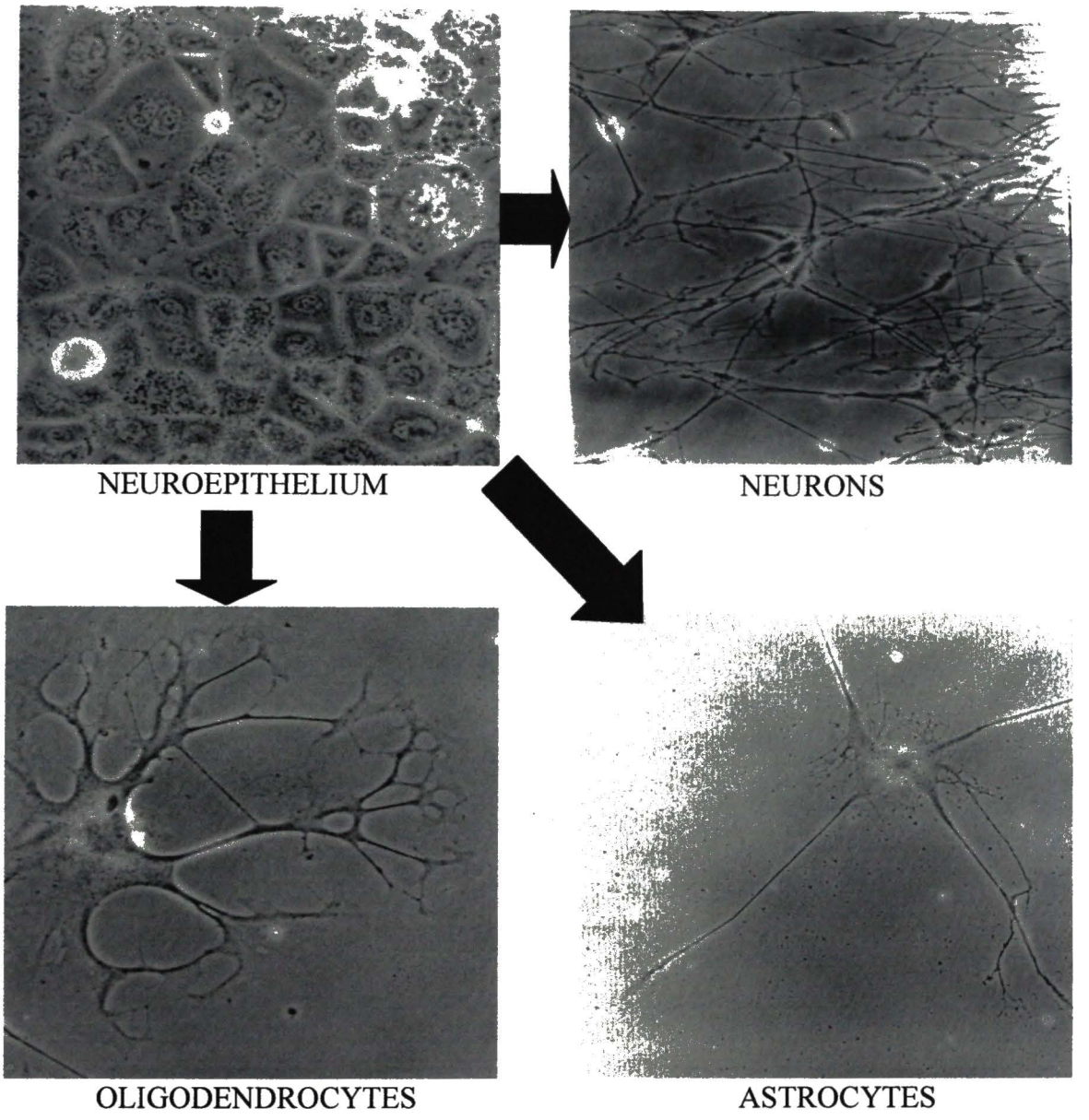
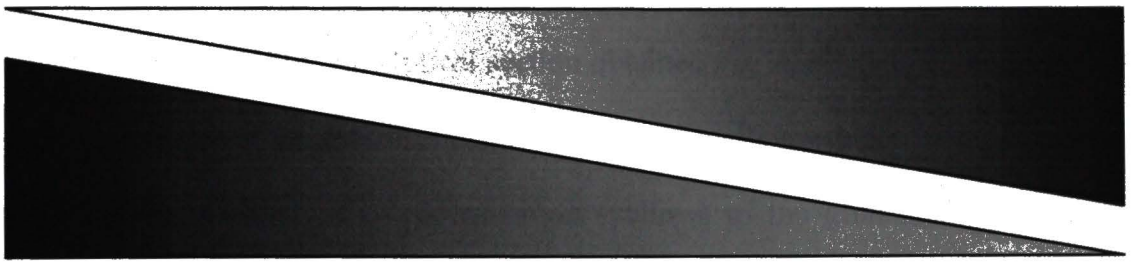

NEUROEPITHELIUM
NEURONS
OLIGODENDROCYTES
ASTROCYTES

Figure 7: Phenotypic marker protein identified in human embryonic forebrain-derived neurospheres. α-enolase is regarded as a marker of the stem cell compartment of epithelia, vimentin as a marker of neuroectoderm and/or general epithelia, nestin as a marker of uncommitted neural progenitors, α-internexin and β-tubulin as markers of neurons in the early stage of development, neurofilament M (NF-M) and neuron-specific enolase (NSE) as markers of more mature neurons, substance P as a marker of sensory neurons, and glial fibrillary acidic protein (GFAP) as a marker of the glial phenotype. All markers with the exceptions of α-enolase and substance P were positively identified in neurospheres. As indicated, the cell in these neurospheres appear to span a broad range of phenotypes between true stem cells and fully-committed precursors.

Maturity



Immaturity

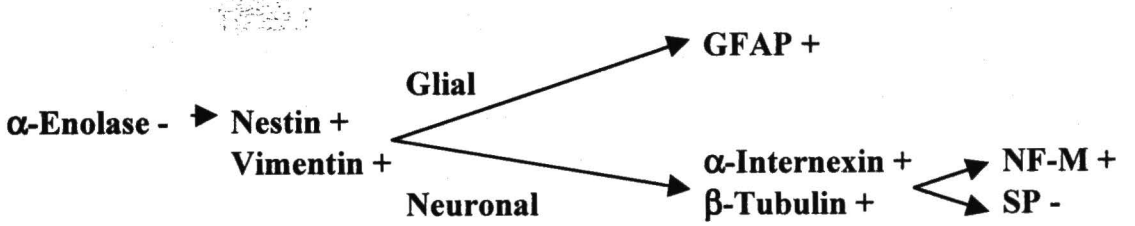
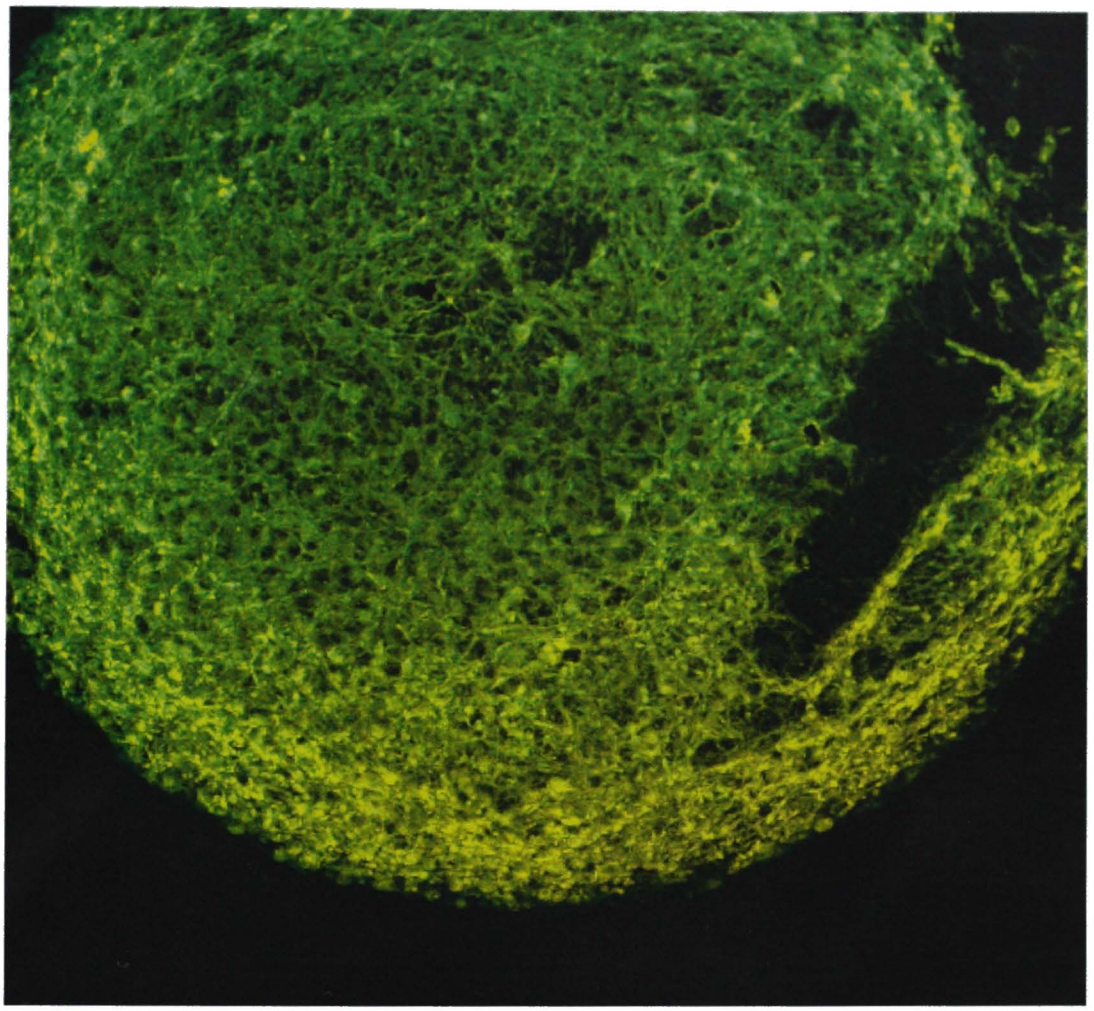


Figure 8: Structural and phenotypic organization of the cells in neurospheres. A. Surface view of the glial basket showing the network of interlacing $GFAP^+$ glia (green) which surround the neurosphere (250x). B. Optical section obtained via laser scanning confocal microscopy of the organization of the neurosphere wall. $GFAP^+$ cells are labeled in green and $NF\text{-}M^+$ are labeled in red (800x). C. Colocalization (yellow) of the glial markers GFAP (green) and vimentin (red) in an optical section of the neurosphere wall (250x). D. Colocalization (yellow) of GFAP (green) and nestin (red) in an optical section of the neurosphere wall (250x). E. Colocalization (yellow) of GFAP (green) and α-internexin (red) in an optical section of the neurosphere wall (800x). F. Colocalization (yellow) of GFAP (green) and β-tubulin (red) in an optical section of the neurosphere wall (250x). G, H. Colocalization (yellow) of GFAP (green) and NF-M (red) in an optical section of the neurosphere wall (800x). I. Colocalization (yellow) of GFAP (green) and NF-M (red) in an optical section of the neurosphere wall (250x). J. Colocalization (yellow) of α-internexin (green) and NF-M (red) in an optical section of the neurosphere wall (250x). K. Colocalization (yellow) of α-internexin (red) and β-tubulin (green) in an optical section of the neurosphere wall (250x). L. Colocalization (yellow) of nestin (red) and vimentin (green) in an optical section of the neurosphere wall (250x). M. Colocalization (yellow) of α-internexin (red) and vimentin (green) in an optical section of the neurosphere wall (250x). N. Colocalization (yellow) of vimentin (green) and NF-M (red) in an optical section of the neurosphere wall (800x). O. Colocalization (yellow) of nestin (red) and α-internexin (green) in an optical section of the neurosphere wall (250x). P. Colocalization (yellow) of nestin (red) and NF-M (green) in an optical section of the neurosphere wall (250x). Q. Colocalization (yellow) of nestin (red) and β-tubulin (green) in an optical section of the neurosphere wall (250x). R. Colocalization (yellow) of nestin (red) and vimentin (green) in an optical section of

the neurosphere wall (250x). S. Colocalization (yellow) of nestin (red) and NF-M (green) in an optical section of the neurosphere wall (800x).



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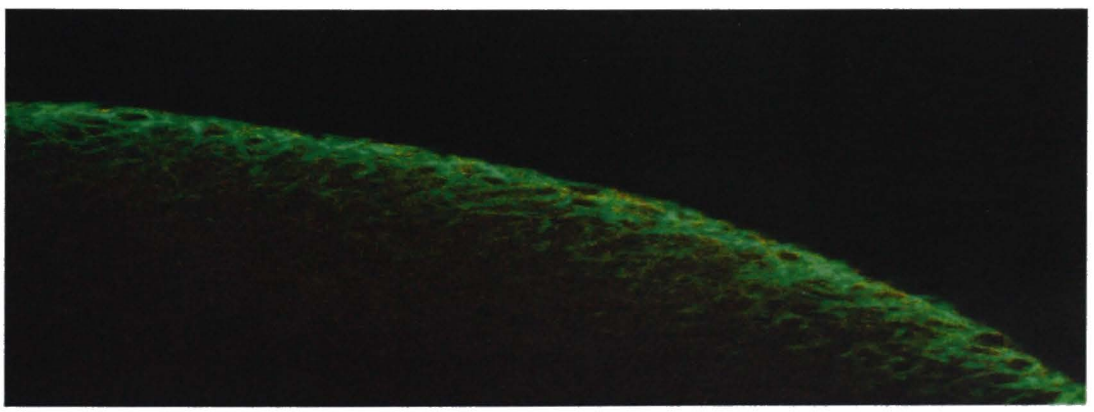

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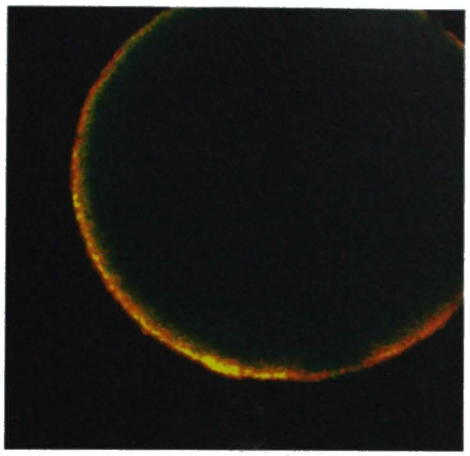

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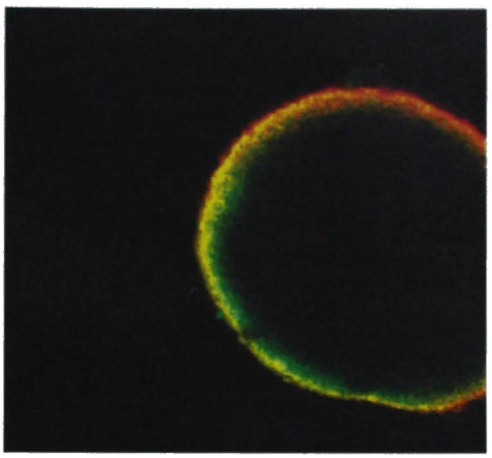

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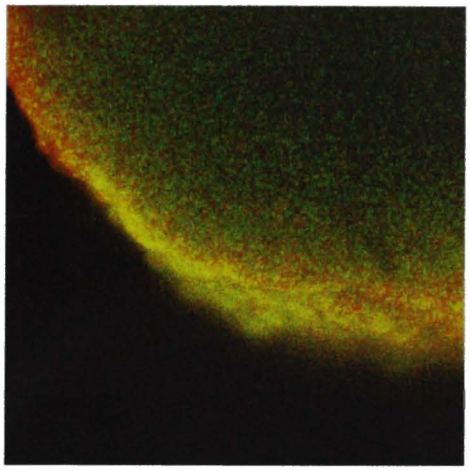

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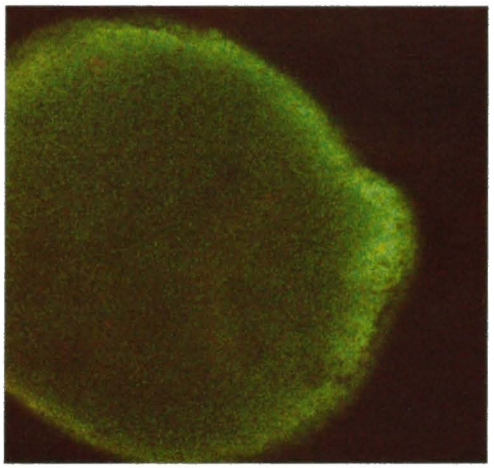

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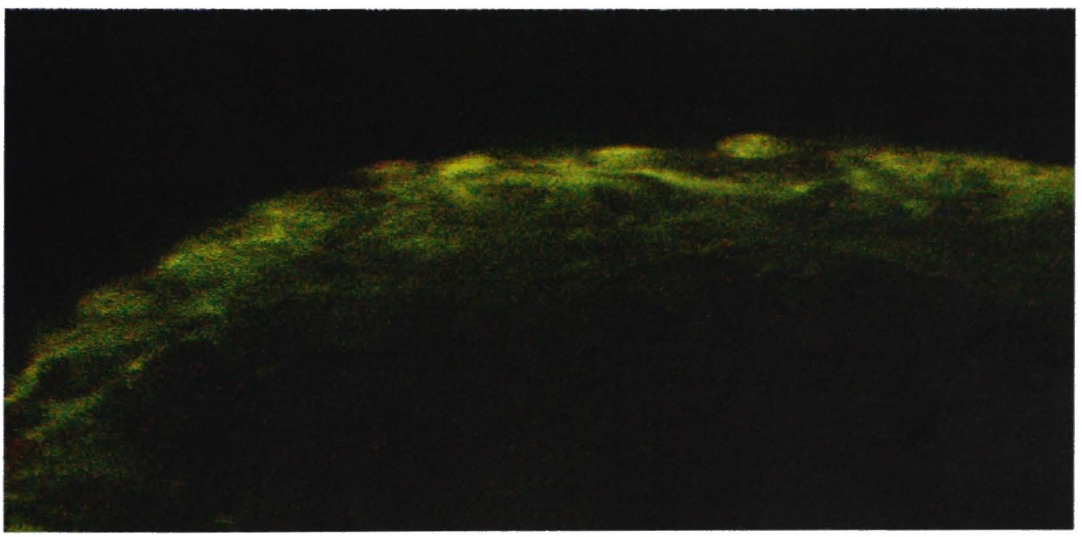

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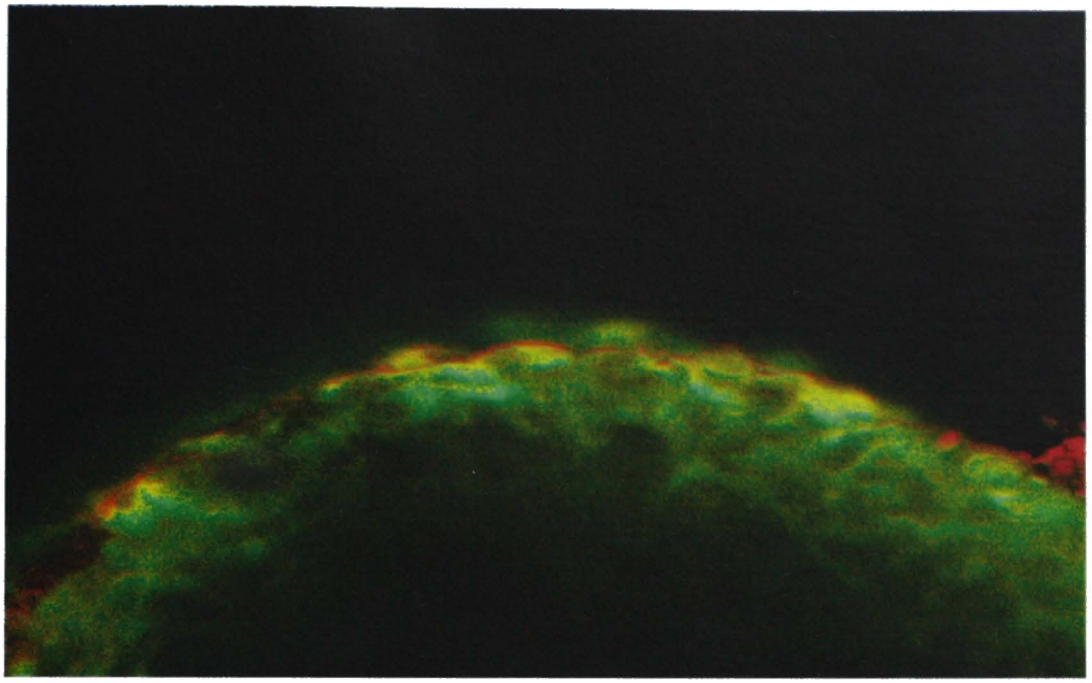

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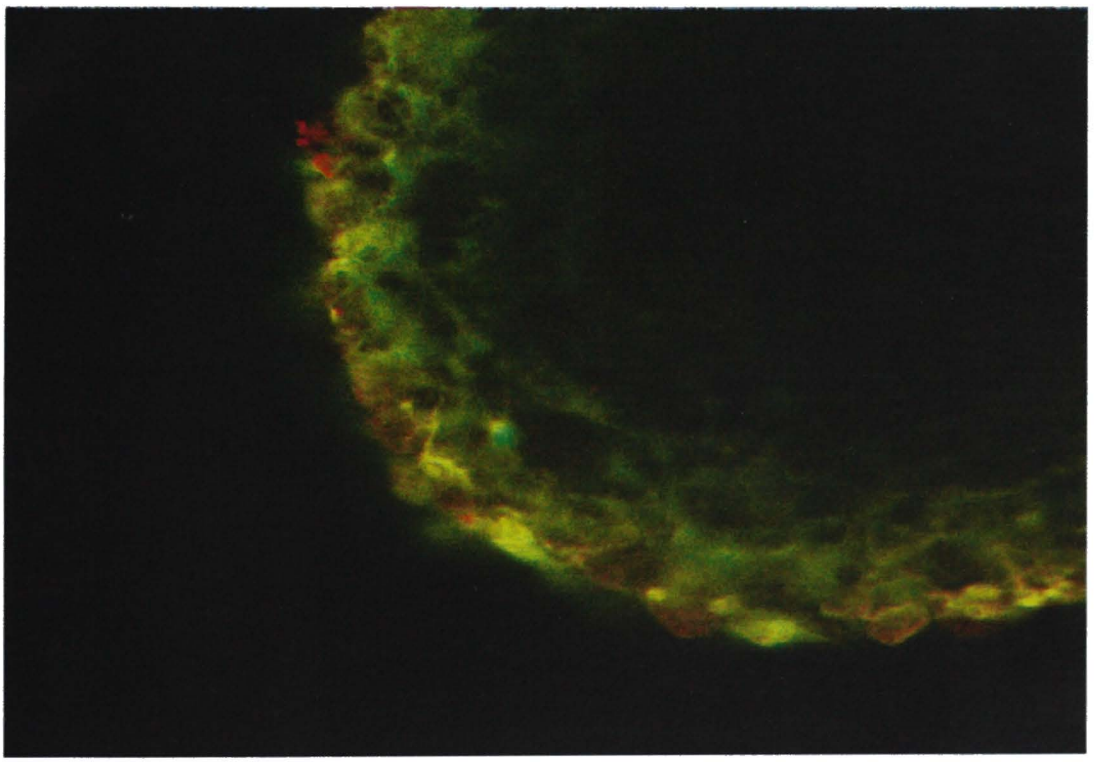

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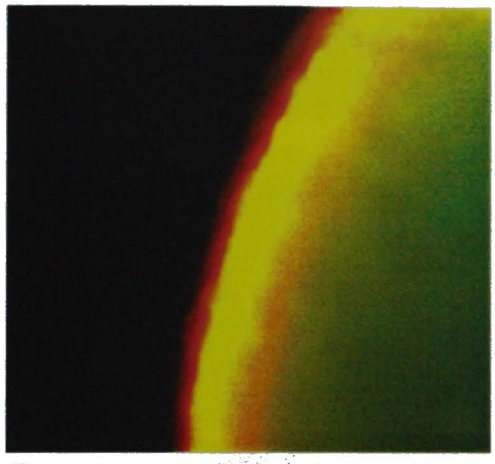

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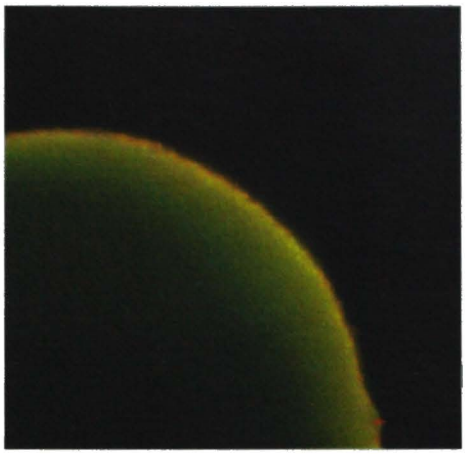

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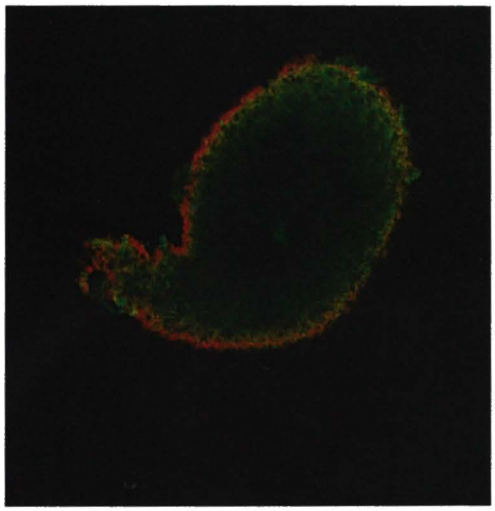

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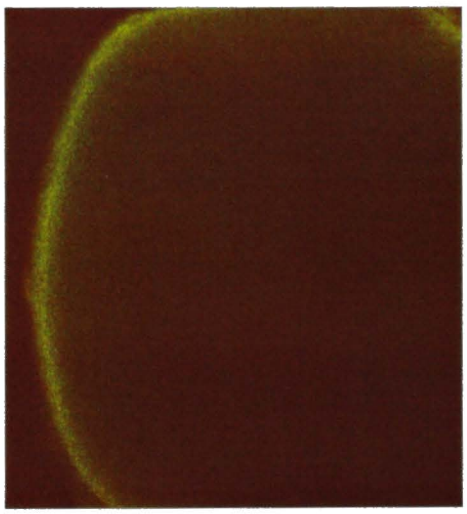

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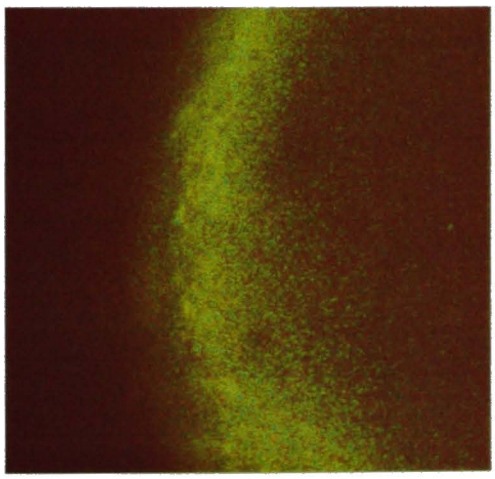

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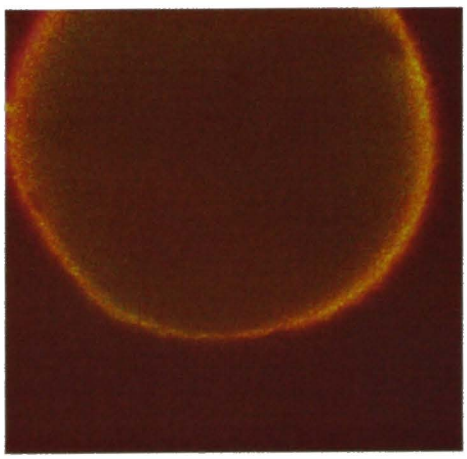

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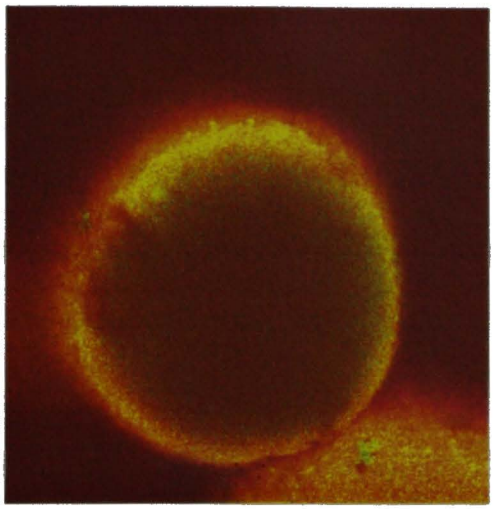

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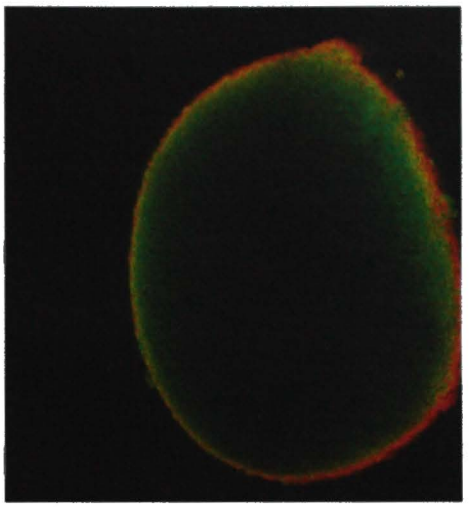

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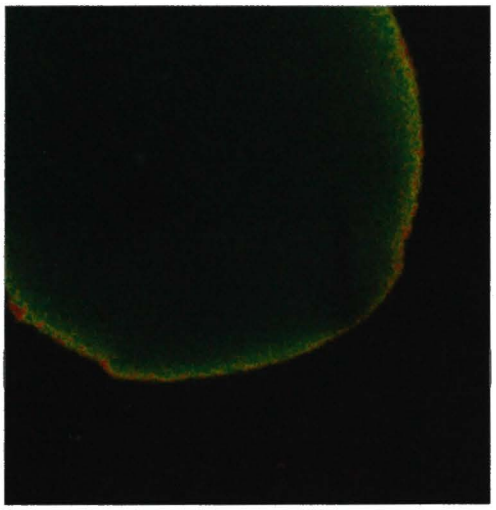

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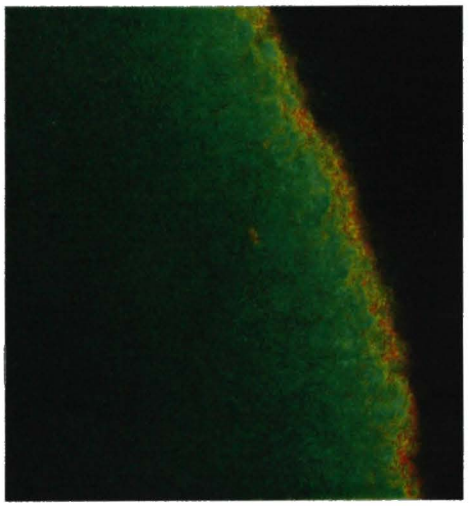
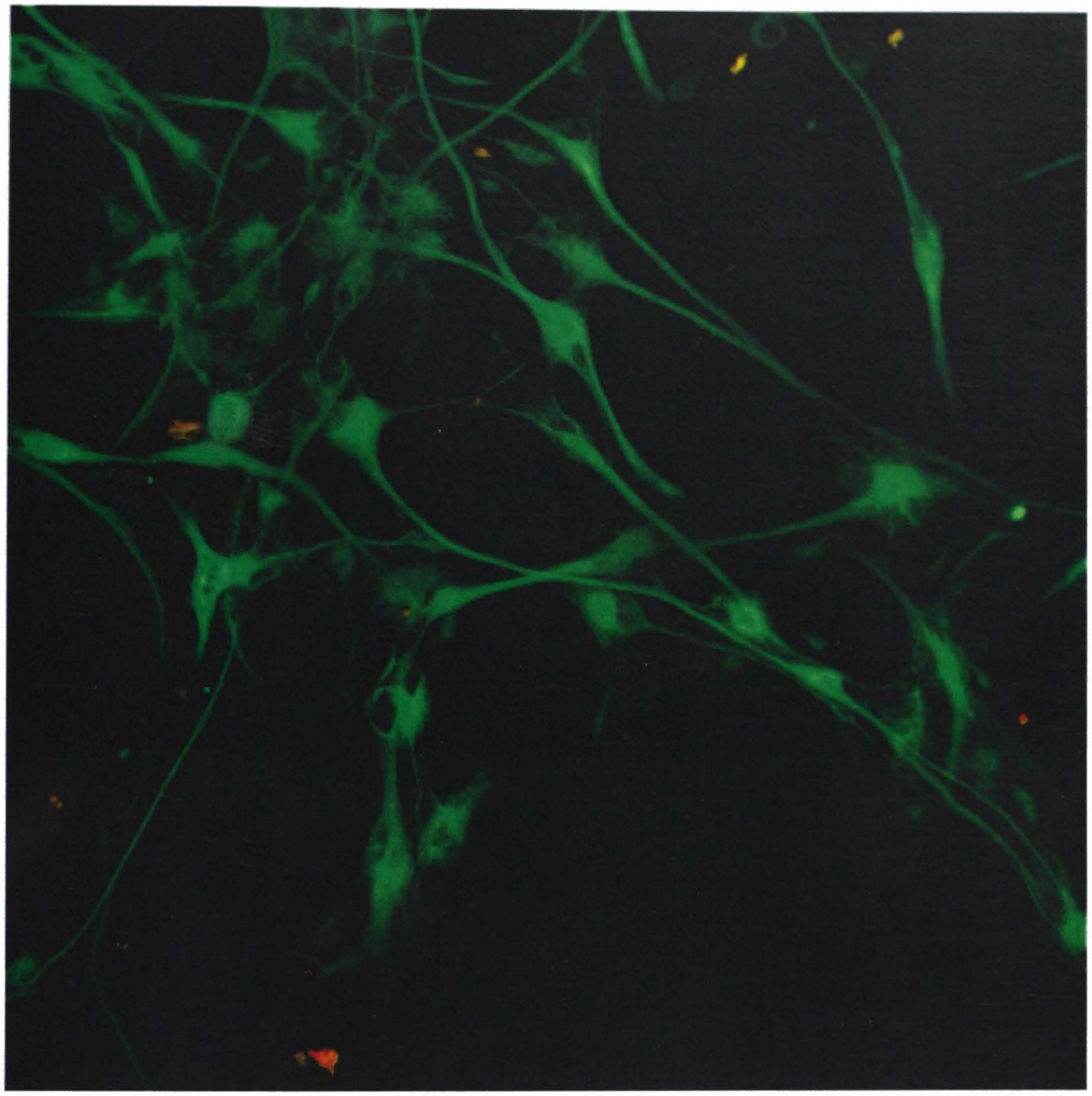


S.

Figure 9: Outgrowth studies on immobilized extracellular matrix proteins reveals a possible function of neurosphere architecture. A. During outgrowth on a variety of extracellular matrix substrates, $GFAP^+/nestin^-$ cells (green) were observed to form radiating, reticulate outgrowths first (800x), and B. $GFAP^-/nestin^+$ (red) cells were observed to then migrate from the neurospheres, occasionally using the $GFAP^+/nestin^-$ cells (green) as "tracks" (250x).



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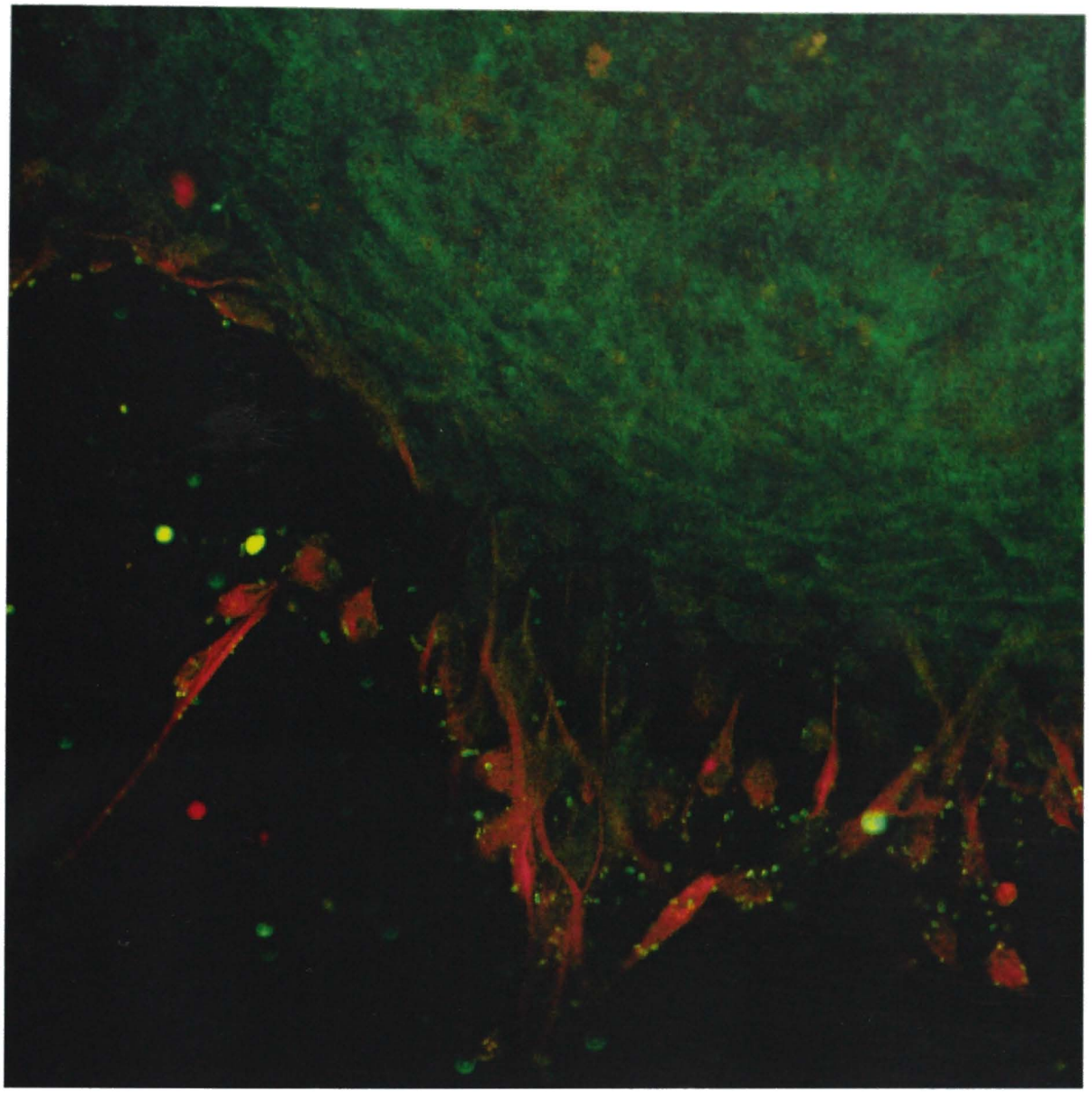
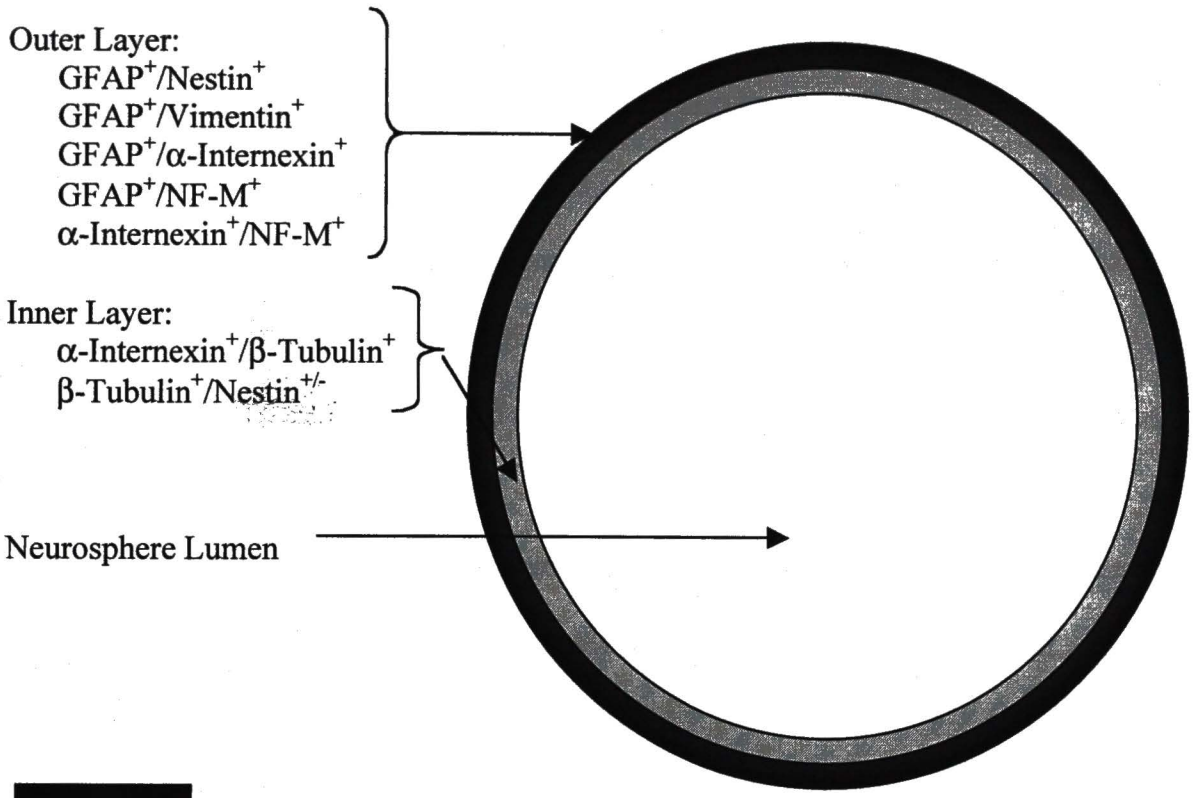


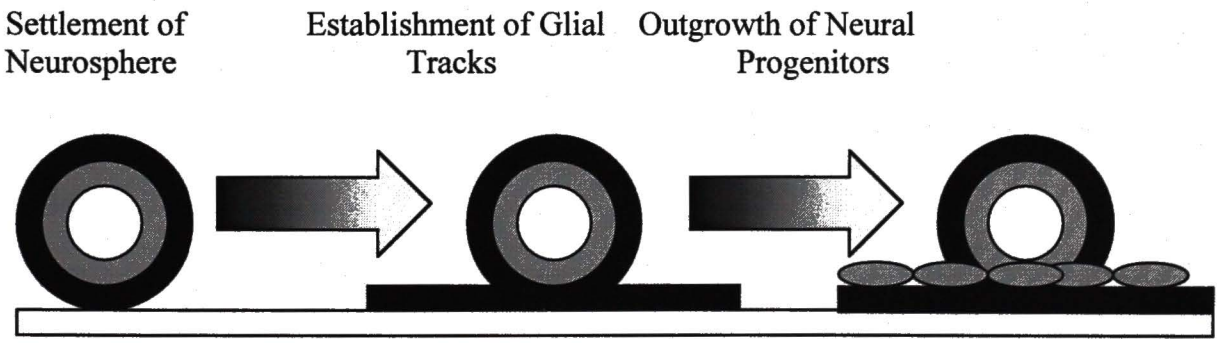
B.

Figure 10: Proposed organization of cellular phenotypes within the neurosphere wall. A. Based upon numerous sequential double immunostains, it is proposed that human embryonic neurospheres possess a glial shell layer ($GFAP^+$/$Vimentin^+$, $GFAP^+$/$nestin^+$, and cells coexpressing glial and neuronal markers) which is periodically interpenetrated and overlain by a layer of $NF\text{-}M^+$/$nestin^+$ neuroblasts. Located inside the glial basket layer is a layer of neuroblasts expressing various neuronal markers. B. These two layers are speculated to participate in the recognition and colonization of specific extracellular matrix-encoded targets (outer layer) followed by the establishment of networks of neurons (inner layer).

A.


Outer Layer:
GFAP+/Nestin+
GFAP+/Vimentin+
GFAP+/α-Internexin+
GFAP+/NF-M+
α-Internexin+/NF-M+
Inner Layer:
α-Internexin+/β-Tubulin+
β-Tubulin+/Nestin+/-
Neurosphere Lumen
Recognition and Colonization of Target
Generation of Neurons

B.


Settlement of Neurosphere
Establishment of Glial Tracks
Outgrowth of Neural Progenitors

1. **Greene, L.A.; and A.S. Tischler.** 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proceedings of The National Academy of Sciences, USA 73(7): 2424-2428.

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4. **Reynolds, B.A.; and S. Weiss.** 1996. Clonal and Population Analyses Demonstrate That an EGF-Responsive Mammalial Embryonic CNS Precursor Is a Stem Cell. Developmental Biology 175: 1-13.

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7. **Kukekov, V.G.; et al.** 1999. Multipotent Stem/Progenitor Cells with Similar Properties Arise from Two Neurogenic Regions of Adult Human Brain. Experimental Neurology 156: 333-344.

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13. **Noctor, S.C.; et al.** 2002. Dividing Precursor Cells of the Embryonic Cortical Ventricular Zone Have Morphological and Molecular Characteristics of Radial Glia. The Journal of Neuroscience 22(8): 3161-3173.

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CHAPTER III

The following manuscript was submitted to *The Journal of Neuroscience Methods*.

A Novel Method For Determining Changes in Protein Expression in Neurospheres

Michael L. Moeller and S. Dan Dimitrijevich*

Department of Molecular Biology and Immunology

The University of North Texas Health Science Center at Fort Worth

3500 Camp Bowie Blvd., Fort Worth, Texas

***Corresponding author. Present address: Department of Integrative Physiology and Cardiovascular Research Institute, UNT Health Science Center, 3500 Camp Bowie Blvd. Fort Worth, TX 76107. e-mail: ddimitri@hsc.unt.edu**

ABSTRACT

Changes in phenotypic marker expression in intact neurospheres are difficult to determine easily and accurately. This particularly so in view of the diversity of cell populations involved and when there is variable expression across the neurosphere wall or circumferentially. We here report a novel direct method for measuring protein expression by densitometric image analysis of immunofluorescently labeled neurospheres optically cross-sectioned by confocal microscopy. We exposed human neurosphere to basic fibroblast growth factor (FGF2), which has been reported to induce neuronal commitment in monolayer cultures of neuroprogenitors cells derived from neurospheres. We determined that this treatment downregulated nestin and vimentin, the two protein markers accepted to be indicative of an immature, uncommitted phenotype. Neuron specific enolase was only marginally affected. We demonstrate that our method allows quantitation of changes in expression of marker proteins that are least comparable to densitometry following Western blot analysis. We further propose that this novel strategy, with or without confocal microscopy, is applicable when tubular or cylindrical structures or approximately spherical cell aggregates, have to be analyzed for changes in expression of proteins caused by various changes in external environment.

INTRODUCTION

A number of compartments containing self-renewing progenitor cells have been identified in the central nervous systems of embryonic and adult mammals. The first of these to be characterized was the subventricular zone (SVZa), the region located immediately beneath the lateral ventricles. Cells derived from these regions have been cultured in a variety of ways, including as floating multicellular aggregates called neurospheres (Reynolds and Weiss, 1996).

Human, rat, and murine neurospheres have been demonstrated to be multipotent (i.e. capable of generating neurons, astrocytes, and oligodendrocytes; Reynolds and Weiss, 1996; Kukekov et al., 1999; and Suslov, 2000). Considerable effort has been invested in studying conditions that would enrich cell population derived from neurospheres with a specific neuronal phenotype. Determination of phenotypic shifts is typically decided by quantifying the expressions of phenotype-specific markers, and may be measured at the transcriptional (mRNA, Ignatova et al., 2002; Suslov et al., 2002, 2000) or the translational (protein) level. However, measurement of changes in mRNA pools does not provide information about protein levels or post-transcriptional events. Furthermore, the methodology for expansion of neurosphere population to provide sufficient protein for Western blot analysis is labor-intensive, time-consuming and, for human neurospheres, quite expensive. Although attempts have been made to utilize FACS analysis on neurospheres, the label was targeted to intracellular antigens and a single-cell suspension immediately obtained (McLaren et al., 2001). Analyses based on FACS sorting are more appropriate for neurospheres bearing the markers of interest on the cell surface. Some conclusions are also derived from analysis of fluorescently labeled monolayer outgrowths from neurospheres although it seems very likely that in the process of attachment additional signaling cues are involved in fate determination. Thus, there is a need for new methods to determine changes in the expressions of phenotypic marker proteins. As neurospheres are multicellular aggregates, such a method would ideally be able to demonstrate regional changes in protein expressions within neurospheres. We report here a novel method that combines indirect immunofluorescent labeling of phenotype markers with laser scanning confocal microscopy and image analysis of the neurosphere wall of the equatorial optical sections. We show that this method allows quantification of changes in protein expression as a result of changes in growth

factor exposure with accuracy comparable to Western blot analysis. We believe that this method has wide-ranging applicability and may be used to quantitatively and qualitatively define changes in protein expressions in a variety of biological structures bearing hollow circular cross-sections.

MATERIALS AND METHODS

Neurospheres. Neurospheres used to generate our cultures were purchased from Biowhittaker (Clonetics). Starting cultures consisted of a mixture of individual cells, small cell clusters and small neurospheres derived from human fetal forebrain. Within 2-4 weeks of initiation, cultures began to produce neurospheres visible to the naked eye.

Culture conditions. Neurospheres were cultured in defined medium (NPMM, Biowhittaker-Cambrex (Clonetics)) containing 20ng/ml EGF, 10ng/ml FGF2, gentamycin / amphotericin, and neural survival factors (NSF-1). All cultures were maintained at constant humidity at 37^{O}C and 5% CO_2. In early cultures, medium was completely replaced once every 7-10 days by pelletting neurospheres by careful centrifugation, and re-suspending them in fresh medium. In later cultures, 1/2-2/3 of the medium volume was replaced every 2-4 days. Suppliers suggest careful fragmentation of neurospheres into small clusters using a Pasteur pipette with a tip drawn into a capillary to markedly increase neurosphere numbers. This culture process resulted in some adherence of individual cells and small neurospheres to the flask growth surface and outgrowth of cells monolayers. Decreasing medium replacement frequency, lead to formation of substantial numbers of neurospheres including those from adherent monolayer outgrowths.

Antibodies used. Monoclonal mouse anti-nestin (Transduction Laboratories, Lexington, KY; or Chemicon International Inc., Temecula, CA) was used at a dilution of 1:200 in phosphate

buffered saline (0.256g/L NaH_2PO_4 H_2O, 1.19g/L Na_2HPO_4, 8.76g/L NaCl, pH 7.4) (PBS) for indirect immunofluorescence or 1:500 for Western blot analysis. Mouse monoclonal anti-vimentin (Sigma RBI, St. Louis, MO) was used at a dilution of 1:200 in PBS for indirect immunofluorescence or 1:500 for Western blot analysis. Mouse monoclonal anti-neuron-specific enolase (NSE) (Chemicon International Inc., Temecula, CA) was used at a dilution of 1:200 in PBS for indirect immunofluorescence or 1:500 for Western blot analysis. Secondary antibodies used in these experiments were AlexaFluor 488 goat anti-mouse, AlexaFluor 594 goat anti-mouse, AlexaFluor 488 goat-anti-rabbit, and AlexaFluor 594 goat anti-rabbit (Molecular Probes, Inc. Eugene, OR). All were used at dilutions of 1:250 in PBS.

Growth factor treatment of neurospheres. Neurospheres were removed from proliferative culture conditions (20ng/ml EGF, 10ng/ml FGF2), rinsed multiple times in PBS, and cultured for 9 days in the presence of FGF2-only (10ng/ml). Medium was replaced every 2-3 days during this incubation period.

Indirect immunofluorescence. Neurospheres were removed from culture medium, rinsed briefly in PBS, and fixed/permeabilized in ice cold 1:1 (v:v) methanol : acetone for 12 minutes at $4^{O}C$. Neurospheres were then soaked in PBS for 30 minutes to allow specimen re-hydration and blocked at $4^{O}C$ overnight in PBS containing 1%BSA. Following blocking, specimens were rinsed briefly in PBS and incubated at $4^{O}C$ overnight in 1^{O} antibody solutions. Following 1^{O} antibody incubation, samples were rinsed 3x10 minutes in PBS containing 0.25% Triton X-100 , incubated in 2^{O} antibody solutions (1:250 in PBS) for 1.5 hours at room temperature, and rinsed in 0.25% Triton X-100 for 3x 10 minutes. This procedure was then repeated to label a second marker. Fully labeled specimens were rinsed 3x10 minutes in PBS and soaked for 30 minutes in

distilled water prior to mounting on clean glass slides with FluorSave™ mounting medium (Calbiochem, La Jolla, CA).

Positive controls were: WI-38 cell lysate for anti-nestin, and human keratinocytes, fibroblasts, and melanocytes for anti-vimentin. Negative controls for all experiments consisted of specimens labeled with 1^{O}-only or 2^{O}-only, and cellular auto-fluorescence was measured in unlabeled specimens. All positive controls confirmed 1^{O} antibody binding specificity, and all negative controls were relatively dark. Fluorescence detected in negative controls as well as mean background fluorescence intensities were subtracted from fluorescence of experimental neurospheres.

DAPI Nuclear labeling. To determine the cellular makeup and stratification of within the neurosphere wall, DAPI labeling was performed on neurospheres cultured under standard proliferation conditions. Neurospheres were fixed/permeabilized in ice cold 1:1 methanol : acetone for 12 minutes at 4^{O}C, rinsed in PBS, and incubated in 600nM DAPI for 5 minutes at 37^{O}C. Following incubation, neurospheres were rinsed repeatedly in PBS and mounted on clean glass slides with FluorSave™ mounting medium (Calbiochem, La Jolla, CA).

Image Acquisition. Laser-scanning confocal microscopy was performed using a Zeiss Micro Systems LSM 410 connected to an Axiovert 135 laser scanning confocal microscope (Zeiss, West Germany). Illumination was provided by an Ominchrome Series 43 laser. All objectives were provided by Zeiss, as was the image acquisition software. Images of individual neurospheres were obtained at 250x, and the plane of focus was adjusted so that optical sections were taken at the equatorial region (the midline) of the neurosphere. Individual sections of the neurosphere wall (1-2 per neurosphere) were selected for magnification at 750x.

Image Analysis, Densitometry of Neurosphere Wall Sections, and Statistical Analysis of Results. Images of magnified wall sections were recorded digitally and analyzed using ImagePro, Plus™. All treatments involved 3-24 neurospheres, 1-2 magnified wall sections from each neurosphere, and 6-10 uniform-sized sampling boxes of approximately 42–49 cells (42-49 nuclei, verified by independent DAPI staining) within each magnified wall section. Mean intensity values were calculated for individual neurospheres, pooled together for individual treatments, and used to establish ratios with control values

Western blot analysis of neurosphere lysates. Neurospheres were raised to large numbers under the conditions described above, collected, pelleted by centrifugation, and lysed at room temperature in 300µl lysis buffer for 10 minutes. Lysis buffer consisted of 2.5ml 1M Tris buffer (pH = 7.0), 1g SDS, and 2.5g sucrose in 50ml distilled water. Genomic DNA was sheared by 10 passes through a syringe tipped with a 22-gauge needle, and samples were stored at –20^{O}C until needed. It was not possible to define an internal standard whose expression would remain constant while expression of phenotypic markers was changing. Therefore, multiple BCA protein assays (Pierce, Rockford, IL) were performed in combination with staining of gels with Coomassie Blue to accurately determine protein concentrations of lysates and to ensure equal loading of lanes. SDS PAGE was performed with 20µg protein/lane at room temperature on 4-15% Tris-HCl Ready Gels (Bio Rad, Hercules, CA) for 1 hour, 10-15 minutes at 95V in Tris/glycine/SDS running buffer. Electro-blotting was done on ice overnight at 25mA in Tris/glycine buffer with 20% methanol onto nitrocellulose membranes, and protein transfer was evaluated by staining of the membranes with Ponceau Red. After de-staining in distilled water, membranes were blocked for 30 minutes at room temperature and then overnight at 4^{O}C in blocking buffer consisting of 5% milk powdered milk and 1% BSA in PBS. Membranes were

then incubated in 1^{O} antibody solutions (1:500 in PBS + 1%BSA) for 30 minutes at room temperature, incubated at $4^{O}C$ overnight, and for 30 minutes at room temperature the following morning. Membranes were rinsed 3x 10 minutes in PBS containing 0.5% Tween-20 and incubated in 2^{O} antibody solutions (1:2000) for 1 hour at room temperature. After rinsing 3x10 minutes in PBS containing 0.5% Tween-20, the membranes were developed using ECL™ Western Blot analysis system (Amersham Pharmacea Biotech.). Densitometric analysis of bands was carried out using Adobe Photoshop™.

RESULTS

Effect of FGF2 on the expressions of nestin, vimentin, and neuron-specific enolase (NSE) - comparison of two methods. Human neonatal neurospheres were shown by laser scanning confocal microscopy to be hollow aggregates of several hundred-several thousand cells. DAPI labeling of nuclei has shown that the walls of neurospheres are typically 6-8 cells thick (**Fig. 11**). In neurospheres cultured exposed to FGF2-only, the expressions of the immature phenotypic markers, nestin and vimentin, was markedly downregulated, while the expression of neuron-specific enolase (NSE) was marginally upregulated. These changes were detected after indirect immunofluorescent labeling of these markers followed by laser scanning confocal microscopy and quantification of fluorescence intensity of the neurosphere wall along the equatorial optical section (**Fig. 12**). Labeling intensities for FGF2-treated neurospheres were normalized to the average values of control (untreated) specimens, and changes in expression derived were confirmed by quantification of Western blots. A comparison of the values obtained by the two methods is shown in **Figure 13**. Nestin was downregulated by approximately ≈37-41% depending on the method used, and vimentin was downregulated by ≈32-34%. Both methods

showed NSE to be upregulated marginally to ≈14% above base levels by treatment with FGF2. For all phenotypic markers, the agreement between the values obtained by two methods was quite close, varying by ≈2-4%.

DISCUSSION

In this study, we demonstrate the validity of a novel method designed to determine changes in the expressions of phenotypic marker proteins in whole, intact neurospheres during changes in their external environment, in this case growth factor treatment. A major advantage of this method is that it allows experiments to be conducted using relatively small numbers of neurospheres. Initiating and maintaining cultures to provide large enough number of neurospheres for lysates that would give sufficient protein for Western blot analysis is labor-intensive and time consuming, often taking several months. Such expenditure of time and effort could be wasted if a particular treatment produces little or no change in protein expression from untreated controls. In contrast, the method described here allows investigations to be accomplished quickly, efficiently, and inexpensively. Furthermore, rapid screening of complex protocols designed to elicit specific changes in the phenotypes within the neurosphere cell population may be implemented prior to establishing large-scale long-term studies for Western analysis. Thus, time and effort may be invested in analysis of only those treatments that produce definitive changes.

As shown, densitometric analysis of the neurosphere wall taken at the equatorial optical section allows quantification of changes in protein expression at a level comparable to those obtained by Western blot analysis. Changes in the expressions of nestin, vimentin, and NSE appeared to occur homogeneously throughout the layers of the neurosphere wall. However, if

changes in marker expressions were to occur in specific regions of the neurosphere wall (i.e. the inner region near the lumen or the outer periphery), these could also be determined with high resolution of the individual cell layers. Thus, in contrast to Western analysis, our method can pinpoint expression changes anywhere within the intact neurosphere and accurately quantify them. Since neurospheres represent a rather heterogeneous cell population that may respond in differential and non-uniform manner, such resolution is essential, as Western analysis of cell lysates does not allow analysis of changes in protein levels at regional levels.

Although in this study we have applied this method to neurospheres, it could be adapted for analysis of any structure bearing a tubular, cylindrical spherical or near spherical structure which would generate a circular cross section. Thus, changes in protein expressions could be determined, qualitatively or quantitatively, in blood vessels, lymphatic vessels, intestinal sections, or any type of duct in the body. It should also be pointed out that this method could be applied to samples prepared for electron microscopy or immunohistochemistry. One such system that might lend itself to this sort of analysis is the aortic ring organotypic culture system. This is a hollow segment of aorta maintained in culture and has been used for some time to study cardiovascular phenomena ranging from angiogenesis (Masson et al., 2002) to regulation of vasotension (Nishida and Satoh, 2003), but has to date not been evaluated by densitometric analysis of the type we propose.

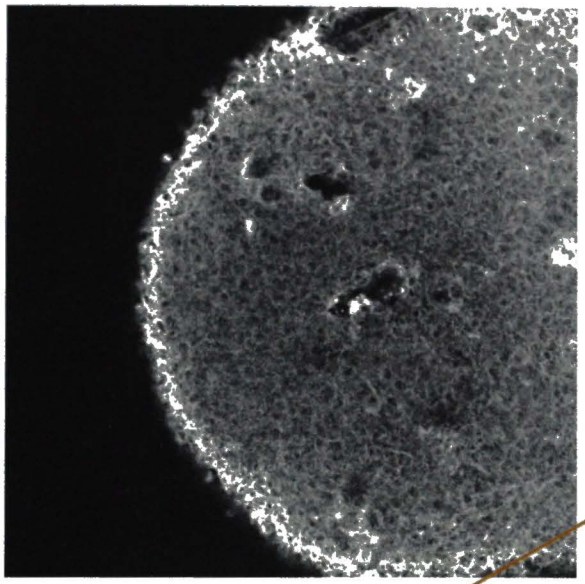
Another model system that would lend itself to this type of analysis is the embryoid body model, derived from inner blastocyst stem cells. These cell clusters that initially develop as solid cell masses eventually undergo cavitation to form hollow masses with distinctive ectodermal, mesodermal, and endodermal layers. The cavitation process involves programmed cell death (PCD) of the core cells of the aggregate induced by the production and deposition of laminin in

the interstitium between endoderm and the upper cell layers (Murray and Edgar, 2000). The embryoid body model has been used to investigate the formation of the proamniotic cavity during embryogenesis and the more wide-ranging phenomenon of cavity formation, a phenomenon that is widespread during development (Murray and Edgar, 2000). Embryoid bodies have also been investigated as a potential source of neurons suitable for neuroimplantation (Sasai, 2002).

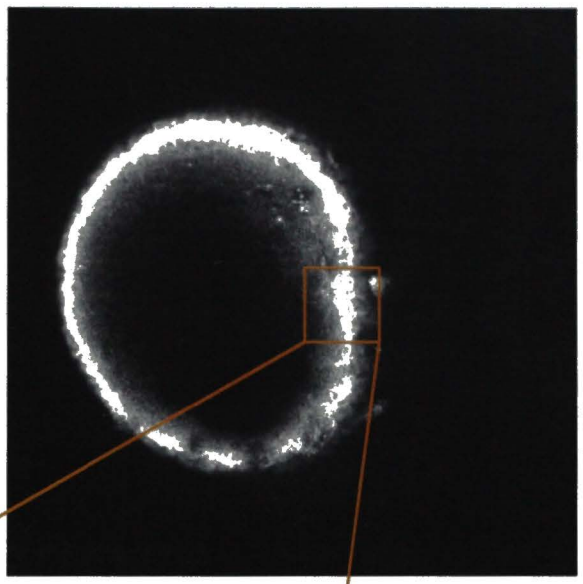
Equally pertinent this discussion is solid cell clusters, spheroids, that may be derived from various types of tumors. The architecture and cellular nature of these structures makes them suitable model for studies of solid tumors (Kelm et al., 2003). A different type of spheroidal entity has also been described in the literature, being composed of cells derived from the ductal epithelium of the pancreas (Peck; et al., 2002). These *in vitro* generated immature islets neither have the same composition as the native Islets of Langerhans, nor definitive localization of a- b- d and *pp* cells.

In both of the above examples, it would be possible to monitor the changes in expression of specific proteins using the wall densitometric analysis method described here. Although in some cases confocal microscopy may not be necessary or even possible the methodology proposed here appears adaptable to suit the application.

Figure 11: A brief survey of neurosphere structure. Human neonatal neurospheres are hollow, multicellular aggregates with walls 6-8 cells in thickness. A. A surface view of a typical human neonatal neurosphere showing the distinctive "cobblestone" morphology of neuroepithelium (250x). B. An equatorial optical section obtained by laser scanning confocal microscopy of a typical neurosphere (250x). C. DAPI labeling of the nuclei present within the neurosphere wall. Neurospheres are fairly uniform in size and structure (800x).



A.



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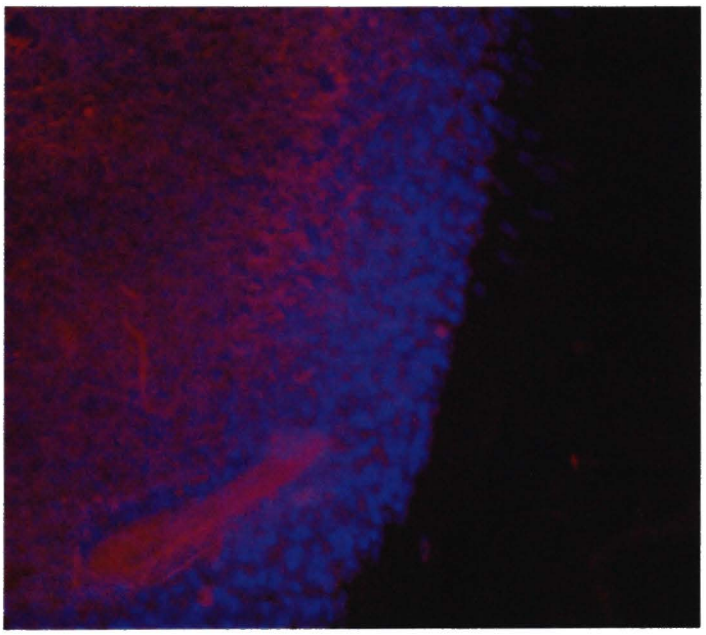
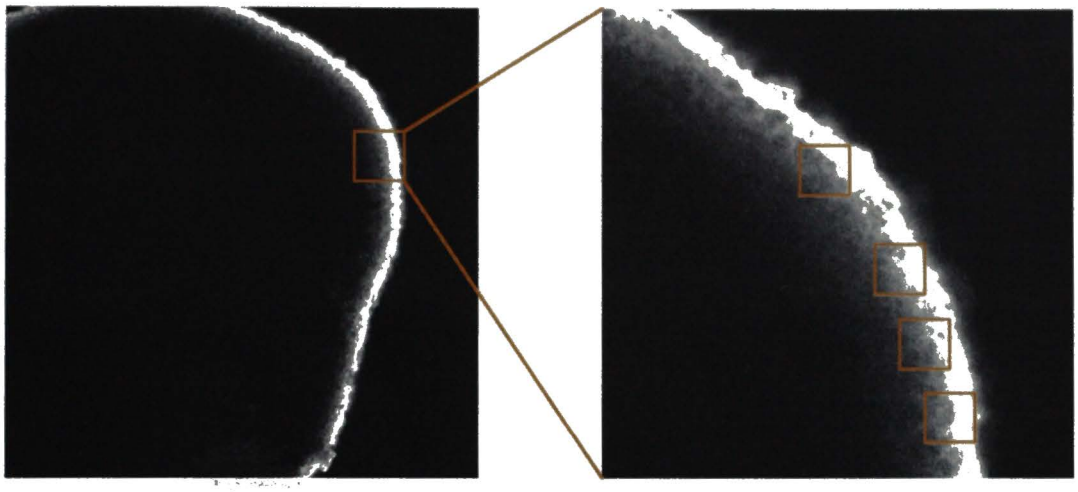


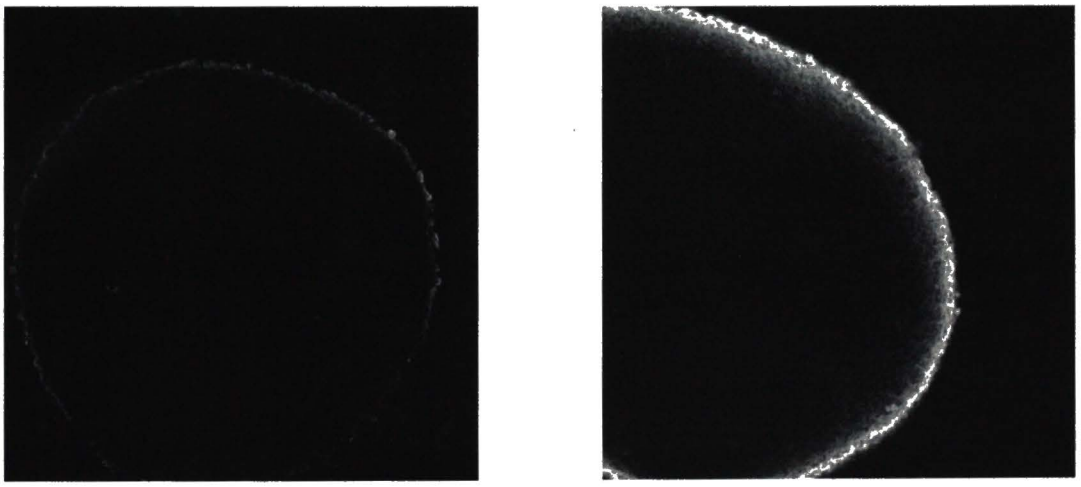
C.

Figure 12: Wall densitometry method for determining changes in protein expressions in intact neurospheres. Neurospheres were fixed/permeabilized in ice-cold 1:1 methanol : acetone, blocked in PBS containing 1%BSA, subjected to indirect immunofluorescent labeling of marker proteins. Images of equatorial optical sections were of the neurospheres were obtained scanning confocal microscopy (A), and densitometric analysis was performed on segments of the neurosphere wall (B). Mean signal intensities were calculated for individual neurospheres and pooled to obtain mean values for individual treatments. In some treatments, densitometric values showed several fold difference between the untreated controls (C) and experimentally-treated (D) neurospheres (data not shown).



A. Optical section at neurosphere equatorial midline (250x)

B. Expanded view of neurosphere wall (800x) with sampling boxes superimposed



C. Untreated control neurosphere

D. FGF2-treated neurosphere

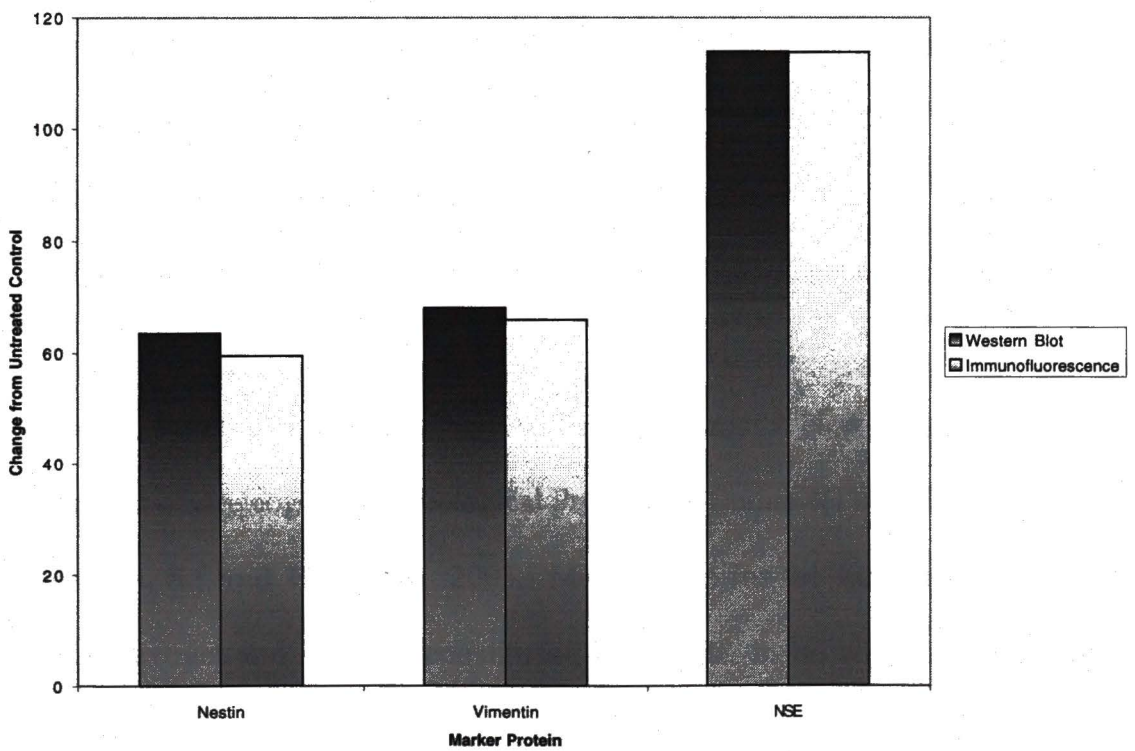
Figure 13: A comparison of the results from wall densitometry and Western blot analysis. Lysates were prepared from neurosphere cultures incubated in the presence of FGF2-only and compared by Western blot analysis with control lysates. Nestin and vimentin, markers of phenotypic immaturity, were shown to be substantially downregulated by exposure to FGF2, while neuron-specific enolase (NSE) was marginally upregulated. Densitometric analysis of the walls of neurospheres labeled by indirect immunofluorescence and optically sectioned by scanning confocal microscopy gave very similar results to those obtained from Western blot analysis.

Nestin	**Control**	**FGF2-Treated**	**Change**
			
Western Blot	100% (1.000)	63.5% (0.635)	36.5%↓
Immunofluorescence	100% (1.000)	59.4% (0.594)	40.6%↓

Vimentin	**Control**	**FGF2-Treated**	**Change**
			
Western Blot	100% (1.000)	68.0% (0.680)	32.0%↓
Immunofluorescence	100% (1.000)	65.9% (0.659)	34.1%↓

Neuron-Specific Enolase (NSE)

	Control	**FGF2-Treated**	**Change**
			
Western Blot	100% (1.000)	113.9% (1.139)	13.9%↑
Immunofluorescence	100% (1.000)	113.8% (1.138)	13.8%↑



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CHAPTER IV

The following manuscript was submitted to *The Journal of Neuroscience*.

Enhancement of Neuronal Commitment in Human Neurospheres

Michael L. Moeller and S. Dan Dimitrijevich*

Department of Molecular Biology and Immunology

The University of North Texas Health Science Center at Fort Worth

3500 Camp Bowie Blvd., Fort Worth, Texas

***Corresponding author. Present address: Department of Integrative Physiology and Cardiovascular Research Institute, UNT Health Science Center, 3500 Camp Bowie Blvd. Fort Worth, TX 76107. e-mail: ddimitri@hsc.unt.edu**

ABSTRACT

Cells isolated from the mammalian ventricular and subventricular germinal zones can form neurospheres, hollow cell aggregates that may be expanded in suspension culture using serum-free defined medium containing epidermal growth factor (EGF) and basic fibroblast growth factor (FGF2). Neurospheres have been shown by several laboratories to consist of progenitors which may give rise to neurons, astrocytes, and oligodendrocytes. Exploiting this multipotency by using growth factors to direct differentiation towards the neuronal phenotype has stimulated new avenues of investigation. Invariably these studies have focused on cellular outgrowth from neurospheres that were attached prior to the induction of differentiation, and were predominantly concerned with murine and rat cells. We hypothesized that phenotypic commitment/differentiation could be initiated in the cells of intact neurospheres without the complication of attachment cues. Utilizing a novel strategy of immunofluorescence and scanning laser confocal microscopy (SLCM), we show that the expressions of nestin and vimentin are downregulated by FGF2. Expression of the neuronal markers α-internexin, β-tubulin, and neurofilament M (NF-M) are also modulated by FGF2 with or without heparin. Since FGF2-mediated regulation of neuronal commitment does not suppress proliferation of the neurosphere cells, their progenitor/precursor status is maintained in spite of the commitment to specialize towards specific functional phenotype. This "preconditioning" of the neuorsphere cells allows progressive studies of differentiation signaling and tracking of changes in other phenotypic characteristics, such as proliferative capacity. This strategy is important when evaluating critical *in vitro* effects on cells destined for therapeutic use.

INTRODUCTION

In adult organism cells of the central nervous system have traditionally been considered to be terminally differentiated and, therefore, incapable of participating in the tissue repair process. Characterization of the subventricular zone (SVZa) of the mammalian forebrain and the isolation and expansion of neural progenitor cells from this region are changing this preconception (Weiss et al., 1996; Doetsch, Garcia-Verdugo et al., 1998; Luskin, 1998). Cells derived from germinal zones in a number of species have been cultured using a variety of strategies. One of these involved incubation of cell aggregates, "neurospheres", in the presence of epidermal growth factor (EGF) and/or basic fibroblast growth factor (FGF2) (Reynolds and Weiss, 1996). Several studies have shown that murine or human neurospheres can give rise to astrocytes, oligodendrocytes, and neurons (Reynolds and Weiss, 1996; Kukekov et al., 1999; and Suslov, 2000). To take advantage of this multipotency, induction of specific phenotypic commitment in the monolayer cultures of neuroprogenitors, and the role of growth factors in proliferation and differentiation are being vigorously studied.

Although EGF has frequently been used to expand the population of undifferentiated neural progenitors (Reynolds and Weiss, 1996; Caldwell et al., 2001), some groups have been unable to expand neurospheres in its presence (Kalyani, Hobson, and Rao, 1997). FGF2 has also been used to stimulate proliferation (Svendsen, 1998), and due to the successful expansion of monolayer cultures, a combination of EGF and FGF2 has also been studied (Caldwell, 2001; Svendsen, 1998). The presence of distinct populations of EGF-responsive and FGF2-responsive cells within such cultures validates the relevance of both the growth factors (Tropepe et al., 1999). The two cell populations differ in their growth kinetics with EGF-responsive cells undergoing primarily symmetric mitoses and FGF2-responsive cells undergoing asymmetric

mitoses (Martens, Tropepe, and van der Kooy, 2000). EGF- and FGF2-responsive neural progenitors have also been localized to different regions of the CNS (Parmar et al., 2002). It has also been proposed that EGF directs progenitor populations toward gliogenesis, while FGF2 encourages neurogenesis (Kuhn et al., 1997; Palmer et al., 1999). FGF2-mediated commitment of murine progenitors to neural phenotype is dose dependent, with low concentrations favoring predominantly the neuronal phenotype, while higher doses generate mixed populations of neurons and glia (Qian et al., 1997).

In the present study of human neonatal neurospheres we show that growth factors induce changes in expression levels of the markers of phenotypic immaturity (nestin and vimentin) as well as the neuronal markers α-internexin, β-tubulin, and neurofilament M (NF-M). Exposure of neurospheres to FGF2 downregulated the expression of nestin and vimentin, while, at optimal doses, the expression of neuronal markers is upregulated. Although there is an overall shift toward the neuronal phenotype, these changes in commitment do not appear to affect the proliferation of neurosphere cells, supporting the premise that they remain a part of the progenitor/precursor pool.

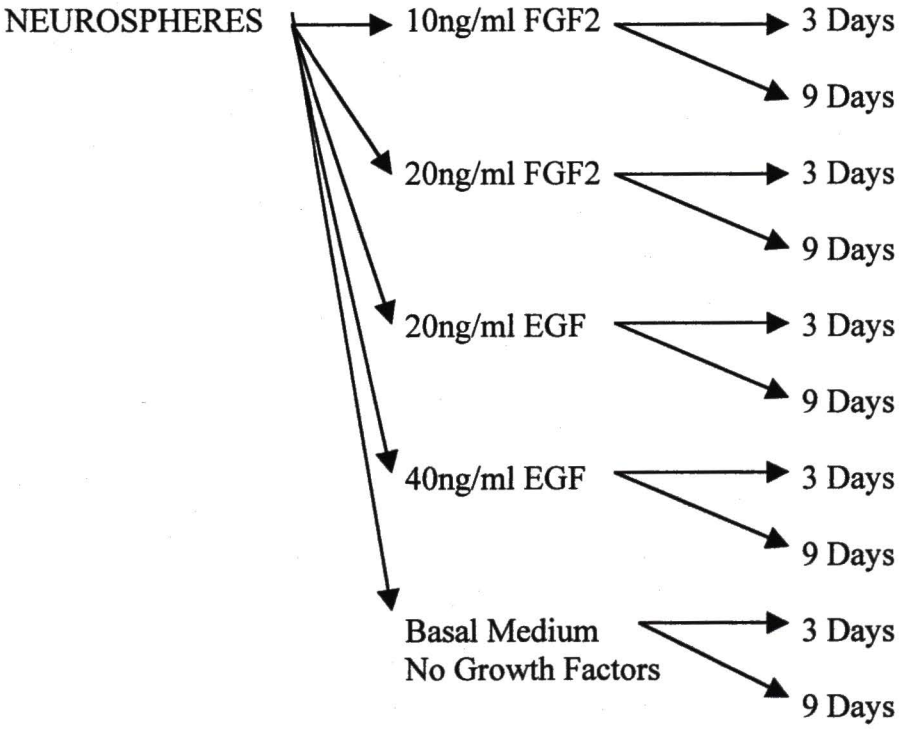
MATERIALS AND METHODS

Neurospheres. Starting cultures, consisting of small cell clusters and individual cells derived from 16-week old human fetal forebrain, were purchased from Biowhittaker-Clonetics, (San Diego, CA). Within 2-4 weeks after initiation, suspension cultures began to yield increasing numbers of neurospheres of the size visible to the naked eye.

Culture conditions. Neurospheres were cultured in defined medium [NPMM, Biowhittaker (Clonetics)] containing EGF (20ng/ml), FGF2 (10ng/ml), gentamycin and amphotericin, and

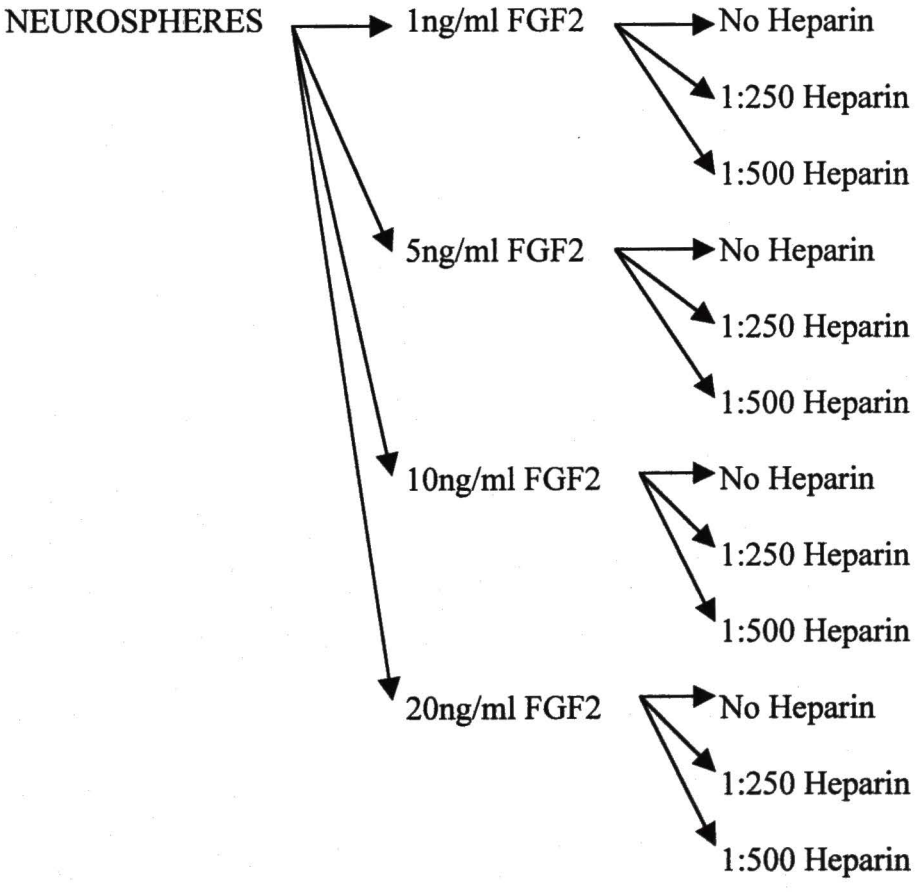
neural cell survival factor (NSF-1). All cultures were maintained at constant humidity at 37°C and 5% CO_2. For early cultures, complete medium replacement was carried out every 7-10 days, by pelletting neurospheres with gentle centrifugation, and re-suspending them in fresh medium. In later cultures, 1/2-2/3 of the medium volume was replaced every 2-4 days. When substantial numbers of neurospheres were required, the neurospheres were fragmented according to the supplier's instructions (pass through Pasteur pipette whose tip is drawn into a capillary) and medium replaced every 2-3 days.

Media Compositions. In initial experiments designed to study the influence of growth factors on the expression of immature phenotypic markers, four media compositions were used: EGF (20ng/ml), FGF2 (10ng/ml), FGF2 (10ng/ml) with heparin (5µg/ml), and EGF (20ng/ml) with FGF2 (10ng/ml) and heparin (5µg/ml). Basal, growth factor free medium [NPBM, Biowhittaker (Clonetics)] was also used as a control environment. In later experiments designed to elucidate the relationship between neuronal markers and growth factors, the scheme below was used:



Above treatments were compared with untreated control populations of neurospheres maintained in proliferation medium (NPMM).

Effects of FGF2 and Heparin on Neuronal Marker Expression. To study the effects of heparin on FGF2-mediated upregulation of neuronal markers, neurospheres were exposed to media according to the flow-chart shown below. FGF2 doses of 1,5,10, and 20ng/ml were used without heparin, with an FGF2:heparin ratio of 1:250, or with an FGF2:heparin ratio of 1:500. Specimens were maintained for 9 days with complete medium replacement every 2-3 days. Following incubation, specimens were processed for indirect immunofluorescent labeling, laser scanning confocal microscopy, and wall cross-section densitometric analysis as described (Moeller and Dimitrijevich, in review).



Indirect immunofluorescence. Neurospheres were removed from culture media, rinsed in PBS, and fixed/permeabilized in ice-cold 1:1 methanol : acetone (v: v) for 12 minutes at $4^{O}C$. Neurospheres were then re-hydrated in phosphate buffered saline (PBS, 0.256g/L NaH_2PO_4 H_2O, 1.19g/L Na_2HPO_4, 8.76g/L NaCl, pH 7.4) for 30 min. and blocked (overnight at 4°C) in PBS containing 1% BSA. Specimens were rinsed with PBS and incubated at $4^{O}C$ overnight with 1^{O} antibody. The neurospheres were then rinsed in PBS (3x10min.) containing Triton X-100 (0.25%), incubated with 2^{O} antibody (1:250 in PBS) for 1.5 hours at room temperature, and rinsed in Triton X-100 (0.25%, 3x10min.). This procedure was repeated for each antibody when multiple labeling experiments were performed. Finally, the specimens were rinsed in PBS (3 x 10 min.), incubated in distilled water (30 min.), and mounted on clean glass slides using FluorSave™ (Calbiochem, La Jolla, CA).

Antibodies. Monoclonal rabbit anti α-internexin antibody (Chemicon International Inc., Temecula, CA) was used at 1:200 dilution in PBS. Rabbit polyclonal anti-β-tubulin antibody (Santa Cruz Biotechnologies Inc., Santa Cruz, CA) was used at 1:100 dilution in PBS. Polyclonal rabbit anti-NF-M antibody (Chemicon) was used at a dilution of 1:200 in PBS. Monoclonal mouse anti-nestin (Signal Transduction Laboratories, Lexington, KY; or Chemicon) was used at a dilution of 1:200 in PBS. Monoclonal mouse anti-vimentin (Sigma RBI, St. Louis, MO) was used at a dilution of 1:200 in PBS. Secondary antibodies were Alexa Fluor 488nm and 594nm goat anti-mouse, Alexa Fluor 488 and 594 goat-anti-rabbit (from Molecular Probes, Inc. Eugene, OR) and were used at dilutions of 1:250 in PBS.

Controls. For positive controls lysates from WI-38 for anti-nestin, lysates from keratinocytes, fibroblasts, and melanocytes for anti-vimentin, and lysates from melanocytes for anti-β-tubulin were used to confirm 1^{O} antibody binding specificity. Binding specificity for all antibodies was

also confirmed using Western blot analysis of neurosphere lysates (Moeller and Dimitrijevich, 2003). Specimens labeled with 1^{O}-antibody only, or 2^{O} antibody only, were used as negative controls in all experiments; these showed virtually no fluorescence. Any fluorescence detected in negative controls as well as mean background fluorescence intensities were subtracted from experimental fluorescence values. Cellular auto-fluorescence was measured in unlabeled specimens.

Image Acquisition. SLCM was performed using a Zeiss Micro Systems LSM 410 connected to an Axiovert 135 laser scanning confocal microscope (Zeiss, West Germany). Illumination source was an Ominchrome Series 43 laser, and all objectives and image acquisition software were from Zeiss. Equatorial optical cross sections of neurospheres labeled for specific target proteins were obtained using SLCM and analyzed densitmetrically as described (Moeller and Dimitrijevich, in review) at a magnification of 250X. Segments of the neurosphere wall, from which "sampling boxes" of uniform size (42-49 nuclei per box) were chosen, were recoded at a magnification of 800X.

Image Analysis: Densitometry of Neurosphere Wall Sections, and Statistical Analysis. This was performed as previously described (Moeller and Dimitrijevich, 2003). Briefly, images of magnified equatorial wall sections were recorded digitally and intensities analyzed using ImagePro, Plus™. The average image intensity values were determined for each neurosphere by averaging image intensities of individual sampling boxes. All values used in graphical representation of treatment results (**Figs. 14 - 18**) were obtained as the ratio of the average signal intensity of treated neurospheres to the average signal intensity of untreated control neurospheres. All treatments and subsequent analyses were carried out on 3-24 neurospheres, 1-2 magnified wall sections from each neurosphere, and 6-10 uniform-sized sampling boxes

(approximately uniform cell numbers; 42-49 nuclei verified by DAPI labeling) within each magnified wall section. Mean intensity values were calculated for individual neurospheres, pooled together for each treatment, and used to establish ratios with control values. All data points are reported as mean values ± SEM and compared using Student's T-tests . Statistical significance was set at $p<0,05$ or lower. The effect of growth factors, growth factor concentrations and exposure times were evaluated by 2X2X2 analysis of variance (ANOVA), and the effect of growth factor and heparin doses on mean expression of neuronal phenotypic markers by 4x3 ANOVA.

Effect of Growth Factors on Proliferation of Neurosphere Cells. To determine the effects of growth factor treatments on the proliferation of component cells, neurospheres were exposed to FGF2 (1, 5, 10, or 20ng/ml), EGF (20 or 40ng/ml), or basal medium (NPBM) for 9 days. Concurrent control experiments were carried out on neurospheres maintained in commercially available proliferation medium (NPMM). In all experiments, the medium was changed every 2-3 days. During the final 24hrs of growth factor exposure, neurospheres cultures were pulse labeled with BrdU (10μM) using a commercially-available kit (Oncogene, Boston, MA). Following the BrdU pulse, samples were fixed/permeabilized in ice cold methanol:acetone – 1:1 for 12 minutes at 4^{O}C. Genomic DNA was denatured for 30 minutes at room temperature (denaturing solution from the kit), and samples were incubated (30 min, room temperature) in PBS and blocked at 4^{O}C overnight (blocking solution from the kit). Specimens were incubated overnight with the biotinylated anti-BrdU 1^{O} antibody provided with the kit, rinsed (3x10min, PBS), and then incubated with Texas Red-l conjugated streptavidin (10μg/ml), for 1 hour at room temperature. Fully-labeled specimens were rinsed in PBS (3 x 10 min.), incubated in distilled water (30 min.) and mounted on clean glass slides with FluorSave™ (Calbiochem, La

Jolla, CA). Imaging was performed by LSCM as described above. Random fields (2-3) were selected within each neurosphere, and BrdU-labeled nuclei were counted and averaged for each neurospheres. The average number of BrdU-labeled nuclei per neurosphere were determined for all treatments (n=3), and compared using Student's T-tests.

RESULTS

Effects of FGF2 on markers of phenotypic immaturity.

In order to determine the effects of growth factors on the expressions of nestin and vimentin, neurospheres were exposed to: EGF (20ng/ml), FGF2 (10ng/ml), FGF2 (10ng/ml) with heparin (5μg/ml), a combination of EGF (20ng/ml), FGF2 (10ng/ml) and heparin (5μg/ml), or basal medium without growth factors, for 8 days. Control neurospheres were maintained in proliferation medium containing EGF (20ng/ml) and FGF2 (10ng/ml). In all experiments the medium was changed every 2-3 days. After exposure to growth factors neurospheres were immunolabeled and digital images of equatorial optical sections (SLCF) analyzed as previously described (Moeller and Dimirijevich, in review).

Nestin expression levels were substantially increased (~1.5-2.5 fold) after all treatments except when FGF2 alone was used, when there was a significant decrease (~40%) (**Fig. 14**). The combination of FGF2 and heparin also caused significant upregulation of nestin expression (~2 fold), as did the treatment with EGF (~2.5 fold). While only the presence of FGF2 downregulated vimentin expression level (~40% of control value), no other treatments produced significant changes (**Fig. 14**).

Dose response of neuronal markers to EGF, FGF2 and FGF2 with heparin.

To determine the effects of growth factors on the expression of the neuronal phenotypic markers (α-internexin, neurofilament M, and β-tubulin) neurospheres were exposed for 3 or 9 days to EGF (20 or 40ng/ml), FGF2 (10 or 20ng/ml), or basal growth factor free medium. Increase in the expression of α-internexin (~ 1.4 fold for 20ng/ml, no change for 40ng/ml) (**Fig. 15A**), and NF-M (~4.8 fold for 20ng/ml, ~3.3 fold for 40ng/ml) (**Fig. 15B**) were observed following 3 days of exposure to both concentrations of EGF tested. This was followed, after 9 days of exposure, by sharp decreases particularly in NF-M expression (**Fig. 15B**). A similar trend was observed for expression of α-internexin in neurospheres treated with both concentration of FGF2. In contrast, 3 days of FGF2 treatment increased NF-M expression levels, which then remained elevated through 9 days treatment (**Fig.15B**). Exposure to basal medium did not significantly change the expression of α-internexin and NF-M when compared with their levels in the controls (see **Fig. 15A, 2B**). Changes expression of β-tubulin followed a different trend (**Fig. 15C**), with highest expression (~2.4 fold) following 9 days in the absence of growth factors (basal medium, NBMP). Exposure to 20ng/ml FGF2 for 9 days also produced significant upregulation of β-tubulin (~2.3 fold) (**Fig. 15C**). ANOVA analysis indicated that exposure time rather than specific growth factor environment regulated the expression of α-internexin and NF-M while growth factor choice and concentration influenced the expression of β-tubulin (**Table 1**).

In order to further delineate the effect of FGF2 on phenotypic commitment of progenitor cells in neurospheres, FGF2 dose responses were investigated. The effect of the combination of FGF2 with heparin was also examined. The majority of FGF2 treatments resulted in significant upregulation of the expression levels of neuronal markers. Particularly striking was the increase of α-internexin (~4.5 and 6.4 fold) and NF-M (~4.2 and 3.8 fold) at 5ng/ml FGF2 and 10ng/ml,

FGF2 with a 1:250 ratio of FGF2: heparin (**Figs. 16A, 16B**). The trend of responses observed for β-tubulin was again different than those observed for α-internexin and NF-M. The most significant increases in expression levels of β-tubulin were observed at 1ng/ml FGF2 with (~3.2 fold) or without (~2.4 fold) heparin and the increases were comparable to the upregulation induced by basal medium (~2.4 fold) (**Fig. 16C**). ANOVA analysis indicated that in the expression of NSIFs, FGF2 concentration was always the primary factor within and between the treatment groups. The expression of α-internexin was also significantly influenced by FGF2 and heparin concentrations while NF-M expression was dependent on the presence of heparin. (**Table 2**).

Effect of EGF and FGF2 on Proliferative Capacity

To determine if the upregulation of neuronal markers occurred at the expense of decline in proliferation, neurospheres were exposed to FGF2 (1, 5, 10, or 20ng/ml), EGF (20 or 40ng/ml), basal medium, and proliferation medium (controls) for 9 days. During the last 24hrs of exposure, all cultures were treated with 10μM BrdU. Exposure of neurospheres to the basal medium (NPBM) resulted in significantly lower numbers of proliferative cells (BrdU labeled nuclei)(5±2, $p<0.01$, $n=3$) than in control neurospheres. A significant reduction in labeled cells ($p<0.01$) was also found in neurospheres treated with 1ng/ml FGF2 when compared with those treated with 10ng/ml FGF2. However, the numbers of proliferative cell in all other treatments were not significantly different from those in controls. (**Fig. 17**)

DISCUSSION

In vivo, proliferation and differentiation of neural progenitors are under regulatory control of signals derived from a wide spectrum of interactions. Attachment cues arising from the interaction of cells with specific components of the extracellular matrix have been documented as one group of the signals. Differentiation of progenitor (precursor) cells isolated from the CNS of several species to the neuronal phenotype followed attachment to laminin (Barami et al., 2001), a mixture of poly-D-lysine and laminin (Mistry et al., 2002; Feldman et al. 1996), fibronectin (Fitzakerley, 2001), and collagen IV (Samina, Pappas, and Parnavelas, 1998). This specialization was characterized on the basis of extension of various cellular processes, and was assumed to be attachment dependent phenomena (Caldwell et al., 2001; Ostenfeld et al., 2002). In a number of studies with attached cells the role for FGF2 and other growth factors in the progression of differentiaion to the neuronal phenotype has been proposed. In contrast to the majority of reports that deal with rat or murine systems, we have undertaken the study of the effect of FGF2 on the specialization /commitment of neuro-progenitors comprising intact unattached human neurospheres.

Initially we were interested to demonstrate growth factor induced phenotypic shift from an immature, unspecialized, phenotype to a more committed specialized one. In several species nestin and vimentin have been proposed as markers of an uncommitted neural progenitors, while their downregulation indicated phenotypic maturation (Shihabuddin, Ray, and Gage, 1997; Palmer, Ray, and Gage, 1995). In our experiments only the 8-day exposure to FGF2 reduced the expressions of these two markers.

Heparin interacts with FGF2 and it from proteolysis and stabilizes its receptor complex. Interestingly, the addition of heparin to FGF2-containing medium increased the expression of

nestin (**Fig. 14**). At 5ng/ml FGF2 the expressions of α-internexin and NF-M were reduced significantly and progressively by increases in heparin concentration, declining to base level at an FGF2:heparin ratio of 1:500 (**Fig. 15A,15B**). A similar trend was observed at 1ng/ml FGF2, but 10ng/ml FGF2 with a 1:250 FGF2:heparin ratio significantly increased the expressions of all markers, notably α-internexin (~6 fold) and NF-M (~4 fold). Increasing the FGF-heparin ratio to 1:500 reduced the expressions of all three markers to near base levels. We therefore propose that the presence of exogenous heparin reduces the availability of FGF2. It has been shown that neural progenitors synthesize and express heparan sulfate proteoglycans (HSPGs) that interact with FGF2 (Chipperfield et al., 2002). Thus increasing the concentration of non-cellular heparin would be expected to limit FGF2 available for interact with cell surface HSPGs .

The trends in expression of the NSIFs under the various conditions were not paralleled by β-tubulin. Upregulation of β-tubulin expression occurred primarily at lower doses of FGF2, or in the absence of growth factors (**Fig. 15C**). This suggests that the cytoskeletal elements of the mature neurons are differentially regulated during development.

Upregulation of NSIFs and β-tubulin might not be expected since that would imply attachment related morphological changes. Such changes may occur in the neurospheres cells in the absence of attachment cues because early neuronal commitment may involve synthesis of neuronal cytoskeletal precursors. Thus although upregulation of cytoskeletal protein expressions may occur their assembly into the mature neuronal cytoskeleton is not required and would not be observed. However, it seems unlikely that 3-6 fold increases in expression level would take place without feedback inhibition occurring at some stage.

We have observed cellular processes extending from the β-tubulin$^+$ cell layer across the surfaces of the cells located beneath them in shallow optical sections taken just below the surface

of neurospheres labeled for neuronal markers (**Fig. 18**). We therefore propose that neuronally-committed cells within neurospheres, undergo morphological changes characteristic of neuronal differentiation also due to the attachment cues generated within the multilayered wall of the neurospheres. Since neurospheres are several cell layers thick (DAPI labeling, Moeller and Dimitrijevich, 2003), they may provides a discrete tissue like cell mass within which neuronal specialization may occur.

EGF has been proposed to promote gliogenesis and to support substantial proliferation of SVZ progenitors (Kuhn et al., 1997). While we did not determine glial commitment, 8-day exposure of neurospheres to EGF (20ng/ml) increased the expression of nestin (~2.5 fold, **Fig. 14**) and maintained at approximately base level the expression of a-internexin after 9 days (**Fig 15A**). Exposure to 40ng/ml EGF (9 days) strongly downregulated the expression of α-internexin, while the expression of β-tubulin and NF-M was near base levels. Thus long-term exposure to EGF does not appear to promote the commitment of progenitors to the neuronal phenotype. Increases in the expressions of α-internexin and NF-M and β-tubulin after short exposure to EGF (3 days), are interpreted as a response to reduction in total mitogen content of the medium. Whatever the cause, this effect is abolished by 9 days of treatment.

Based on numerous studies with attached cells from several species (Lee et al., 2000; Ben-Hur et al., 1998; Shihabuddin, Ray, and Gage, 1997; Okabe et al., 1996; Palmer, Ray, and Gage, 1995), total mitogen withdrawal should be a powerful stimulant of neuronal commitment as mitogens predominantly support a proliferative, (immature) phenotype. Since total mitogen withdrawal increased the expression of nestin (~100% after 3 days) and β-tubulin (~2 fold, after 9 days) while the expressions of NF-M and α-internexin remained at base levels (after 9 days), our studies suggest a tendency toward a neuronal progenitor phenotype rather than more

definitive neuronal commitment. The reason for this response is not clear, but may be associated with the complex network of cell-cell interactions within neurospheres, which *in vivo* might constitute a pool of progenitor or precursor cells that are capable of further differentiation as the need arises. Since it has been proposed that neurospheres can produce FGF2 (Lobo et al., 2003), growth factor withdrawal could activate an autocrine FGF2 loop, so that high levels of endogenously generated mitotic activity may delay commitment to a neuronal phenotype while maintaining the progenitor pool in the proliferative mode.

BrdU labeling studies show that FGF2 stimulated neuronal commitment does not prevent proliferation of neurosphere cells. This may be expected from a population of progenitors, and also suggests that in the absence of additional signals, an intrinsic limit to phenotypic maturation is reached. Exposure to basal medium reduced significantly the number of BrdU-labeled nuclei, indicating that exit from the cell cycle may depend on growth factor withdrawal. EGF did not lead to significantly higher BrdU-incorporation than FGF2. This is in contrast to the results reported for *in vivo* experiments when growth factors are introduced directly into rat ventricles (Kuhn et al., 1997), but might reflect differences in the two experimental models. Injection of growth factors into the cerebrospinal fluid (CSF) would be expected to have inherently unpredictable consequences, since the precise interactions with the variety of endogenous growth factors and extracellular matrix cannot be determined. Moreover, the ventricular injection study cited was conducted in the rat whereas our studies were conducted in a human model system *in vitro*.

It has been suggested that monolayer cultures are temporally restricted from producing differentiated neurons whereas the cells in neurospheres are not (Qian et al., 2000). The results from our studies indicate that neurospheres may be an excellent source of neurons, since the cells

in neurospheres display much greater plasticity. Furthermore, we propose that the exposure of neurospheres to a "conditioning" environment would predispose the component cells to more readily commit to the neuronal phenotype upon attachment. It is now evident that a substantial level of specialization is attainable during expansion of neurospheres in suspension culture.

Our studies also suggest that the commitment to specialize and the differentiation process itself may consist of discrete phases in which the cells within a progenitor/precursor pool participate. Induction of phenotypic maturation occurs under the control of specific growth factors, and is optimal at particular concenration. Our findings are corroborated by observations made in other model systems (Qian et al., 1997).

Figure 14: Downregulation of nestin and vimentin expression after growth factor treatment of neurospheres for 8 days with proliferation medium containing 5μg/ml heparin, 10ng/ml FGF2, 10ng/ml FGF2 with 5μg/ml heparin, 20ng/ml EGF, or NPBM (no growth factors). All densitometric values are normalized to neurospheres maintained in NPMM (20ng/ml EGF, 10ng/ml FGF2) and are reported as mean ± SEM. For each treatment, n=3-5. * Indicates significance of $p<0.05$ relative to controls, and **indicates significance of $p<0.01$ relative to controls.

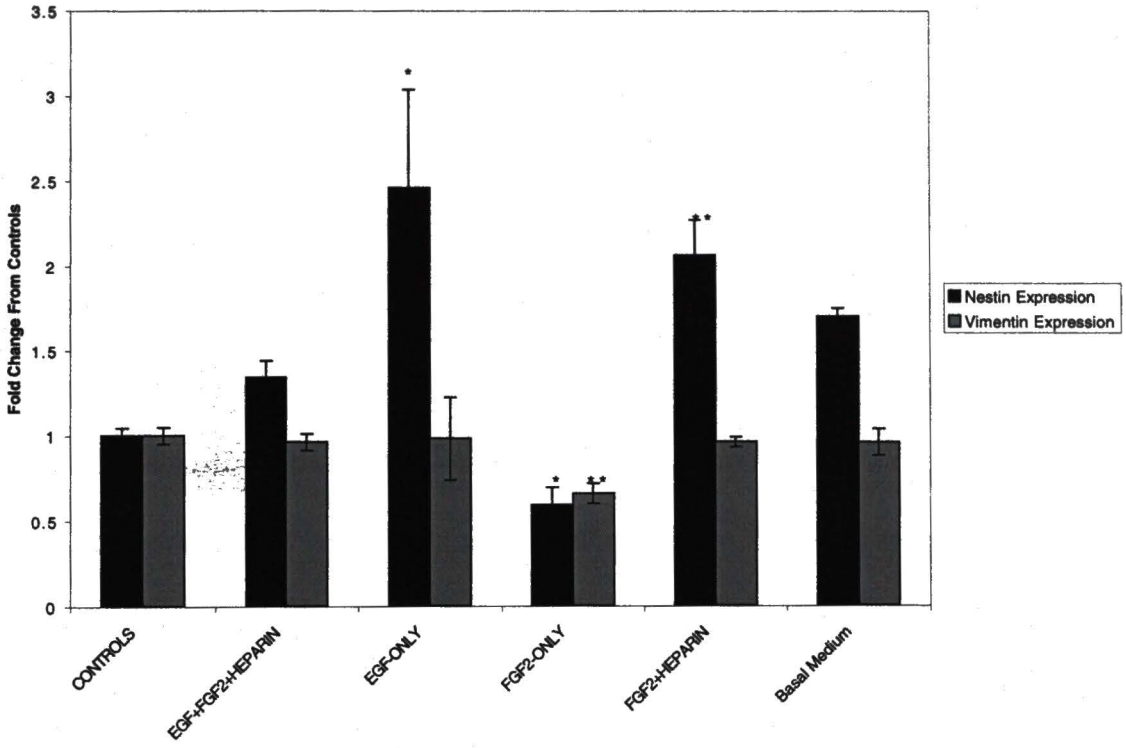
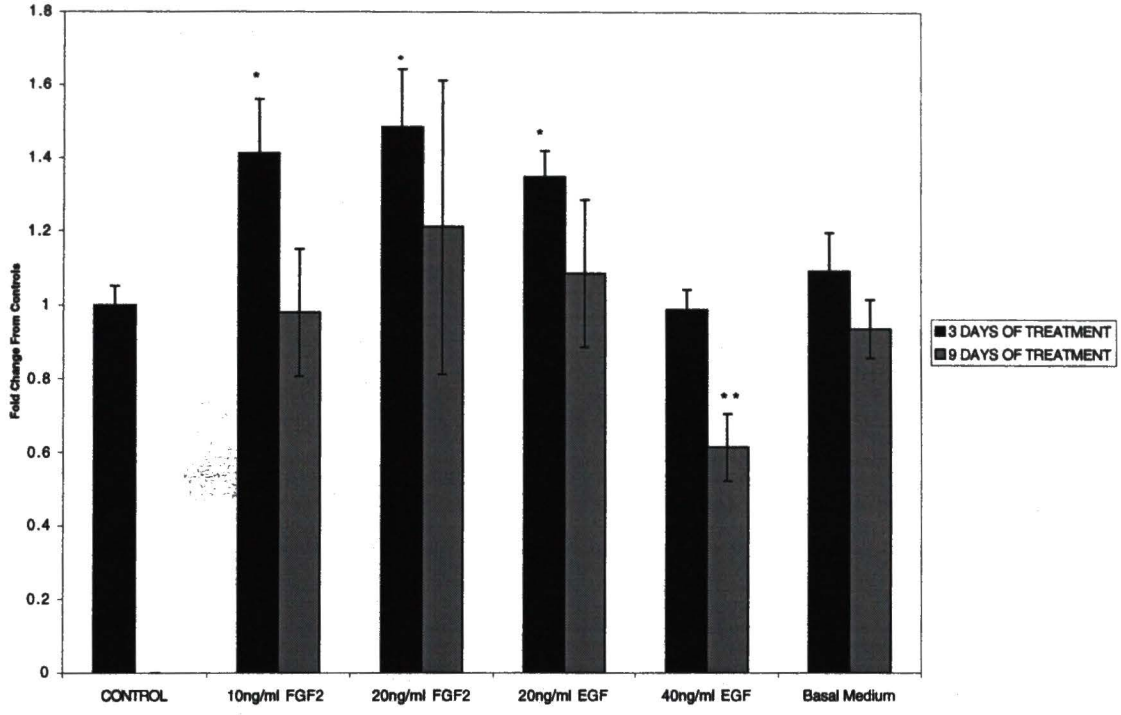

3.5
3
2.5
2
1.5
1
0.5
0
Fold Change From Controls
*
**
*
**
Nestin Expression
Vimentin Expression
CONTROLS
EGF+FGF2+HEPARIN
EGF-ONLY
FGF2-ONLY
FGF2+HEPARIN
Basal Medium

Figure 15: Changes in the expression of α-internexin (**A**), neurofilament M (NF-M) (**B**), and β-tubulin (**C**) after growth factor treatment of neurospheres treated for 3 or 9 days with media containing 10ng/ml FGF2, 20ng/ml FGF2, 20ng/ml EGF, or 40ng/ml EGF or growth factor free basal medium. Densitometric values for all groups were normalized to those of control neurospheres maintained in NPMM (20ng/ml EGF,10ng/ml FGF2) and are reported as mean ± SEM. For each treatment, n= 3-9. *Indicates significance of $p<0.05$ relative to controls, and **indicates significance of $p<0.01$ relative to controls.

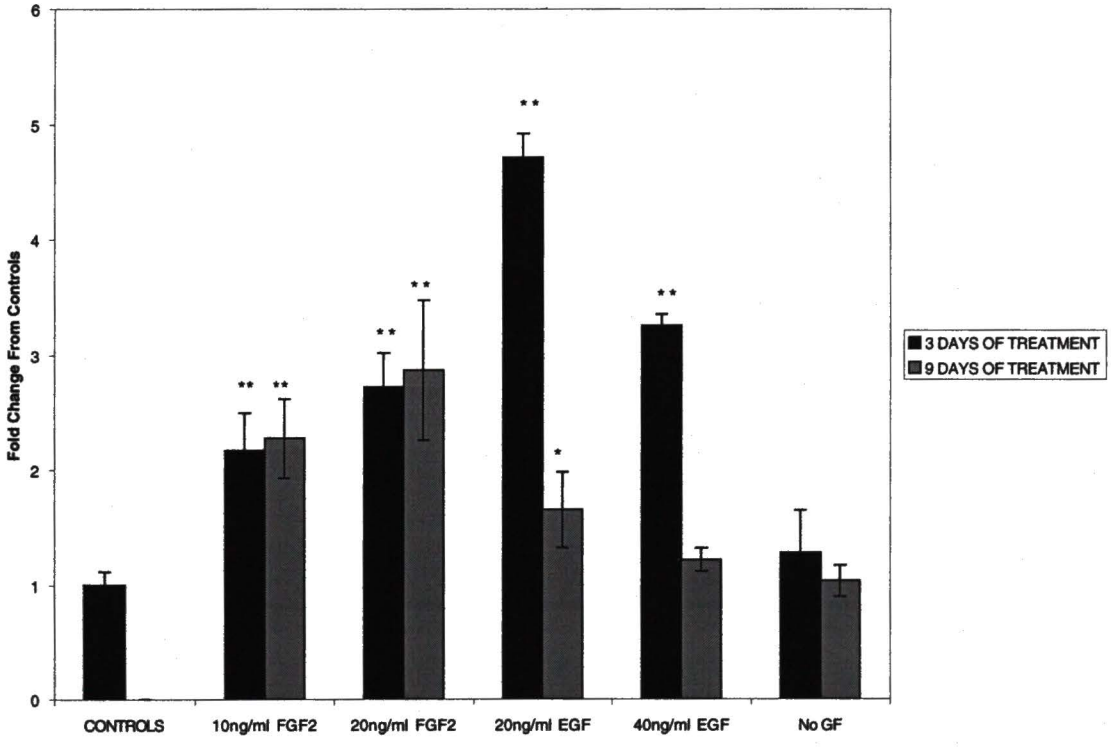
A. The expression of α-internexin was elevated after 3 days of treatment with FGF2 and the lower dose of EGF, but declined to approximately base levels by 9 days. 9 Days of exposure to 40ng/ml EGF resulted in significant reduction of α-internexin expression (relative to 20ng/ml), and exposure to NPBM did not affect the expression.

B. The expression of NF-M was upregulated after 3 days of treatment with FGF2 and remained elevated at 9 days. 3 days of treatment with EGF upregulated NF-M expression, but this effect was mostly ablated by 9 days of exposure. NPBM had no effect on expression of NF-M.

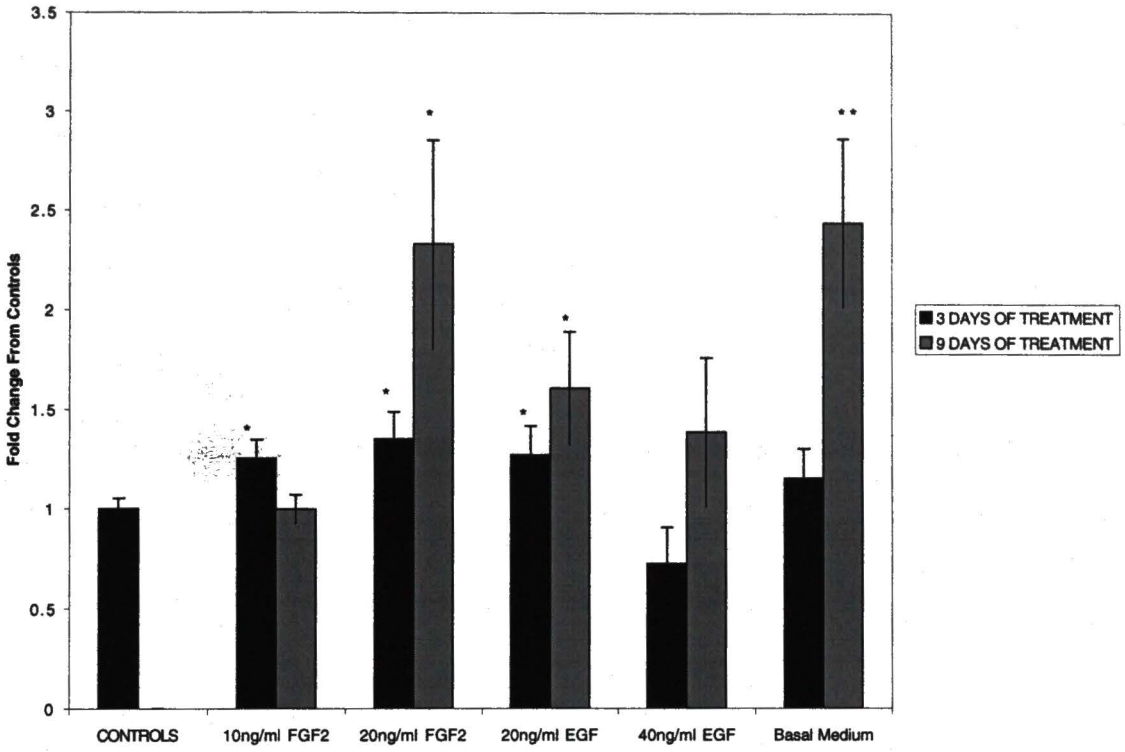
C. The expression of β-tubulin was not strongly affected by most growth factor regimens, but 9 days of exposure to either 20ng/ml FGF2 or NPBM resulted in large upregulation of expression.


Fold Change From Controls
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CONTROL
10ng/ml FGF2
20ng/ml FGF2
20ng/ml EGF
40ng/ml EGF
Basal Medium
3 DAYS OF TREATMENT
9 DAYS OF TREATMENT

A.


Fold Change From Controls
6
5
4
3
2
1
0
CONTROLS
10ng/ml FGF2
20ng/ml FGF2
20ng/ml EGF
40ng/ml EGF
No GF
3 DAYS OF TREATMENT
9 DAYS OF TREATMENT

B.


3.5
3
2.5
2
1.5
1
0.5
0
Fold Change From Controls
CONTROLS
10ng/ml FGF2
20ng/ml FGF2
20ng/ml EGF
40ng/ml EGF
Basal Medium
3 DAYS OF TREATMENT
9 DAYS OF TREATMENT

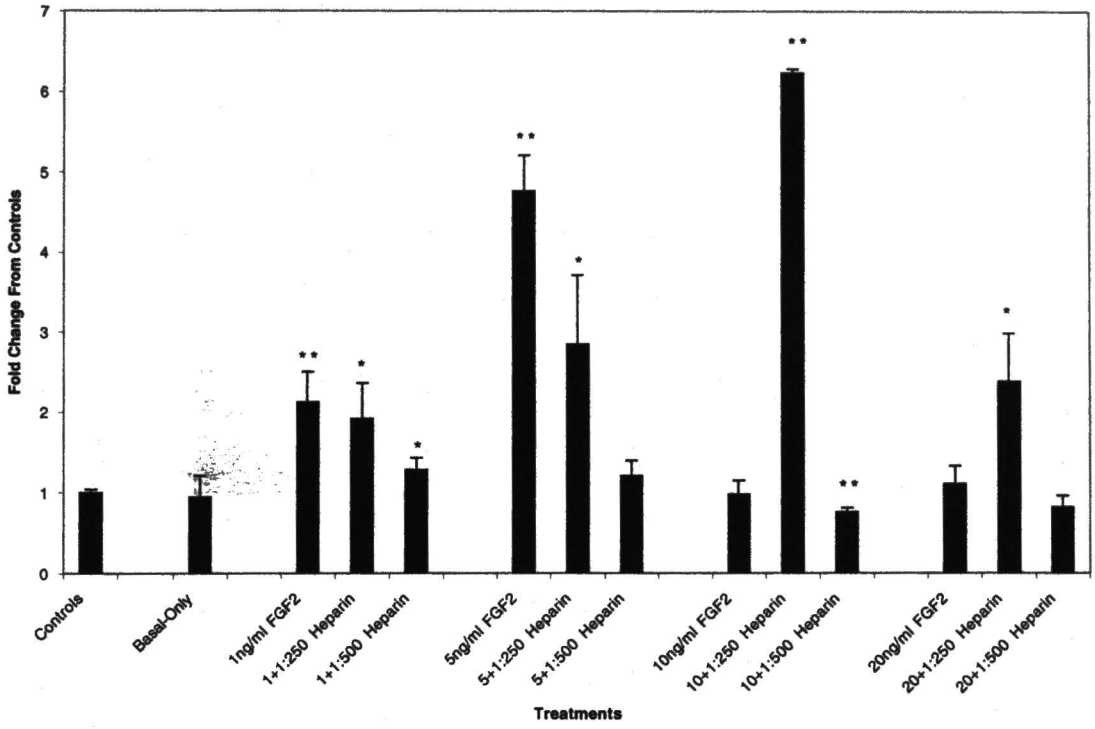
C.

Figure 16: Changes in the expression of α-internexin (**A**), NF-M (**B**), and β-tubulin (**C**) with varying concentrations of FGF2 and heparin. Neurospheres were treated for 9 days with 1,5,10, or 20ng/ml FGF2 without heparin, with an FGF2:heparin ratio of 1:250, or with an FGF2:heparin ratio of 1:500. Densitometric values for all groups were normalized to those of control neurospheres maintained in NPMM (20ng/ml EGF, 10ng/ml FGF2) and are reported as mean ± SEM. For each treatment, n= 3-12.

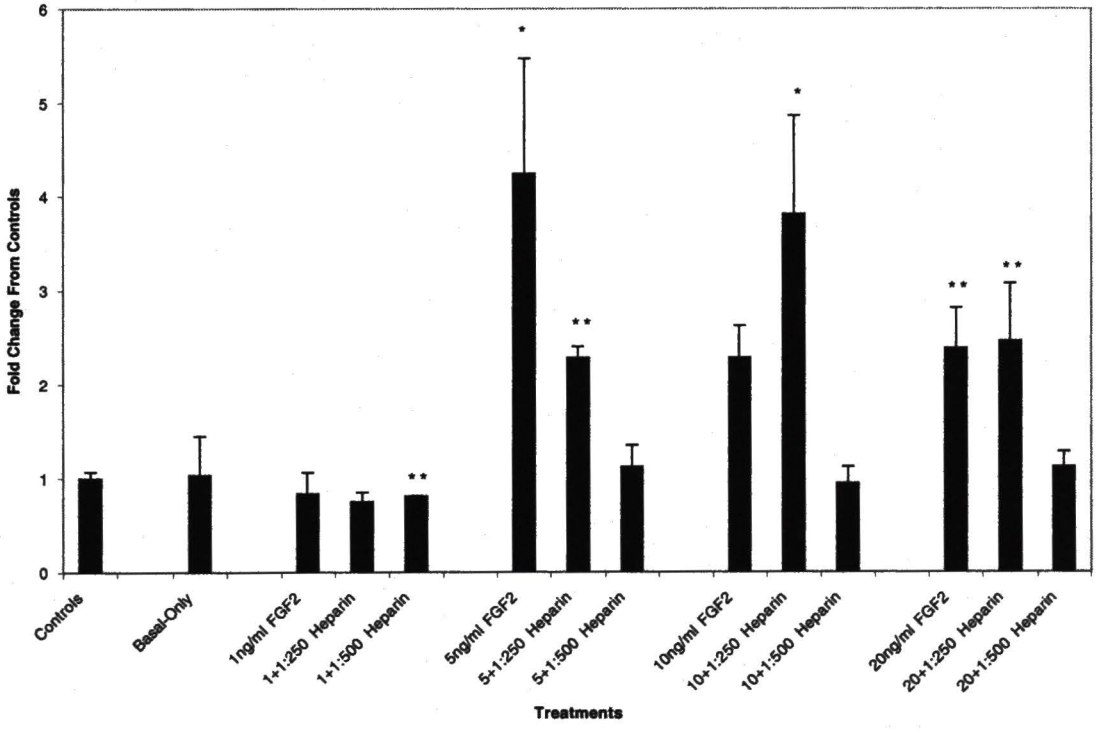
A. At low doses of FGF2 (1-5ng/ml), α-internexin expression is upregulated most strongly in the absence of exogenous heparin and exhibits dose-dependence with increasing heparin doses. At higher doses of FGF2 (10-20ng/ml), the upregulation only occurs in the presence of heparin at a ratio of 1:250 to FGF2.

B. The expression of NF-M is either maintained at base level or downregulated by exposure to 1ng/ml FGF2, but is strongly upregulated by 5ng/ml FGF2. At 5ng/ml FGF2, dose-dependent decreases in NF-M expression are observed with increasing doses of exogenous heparin. At higher doses FGF2 (10-20ng/ml), strong upregulation was observed either in the absence of heparin or at the lower dose (1:250) used.

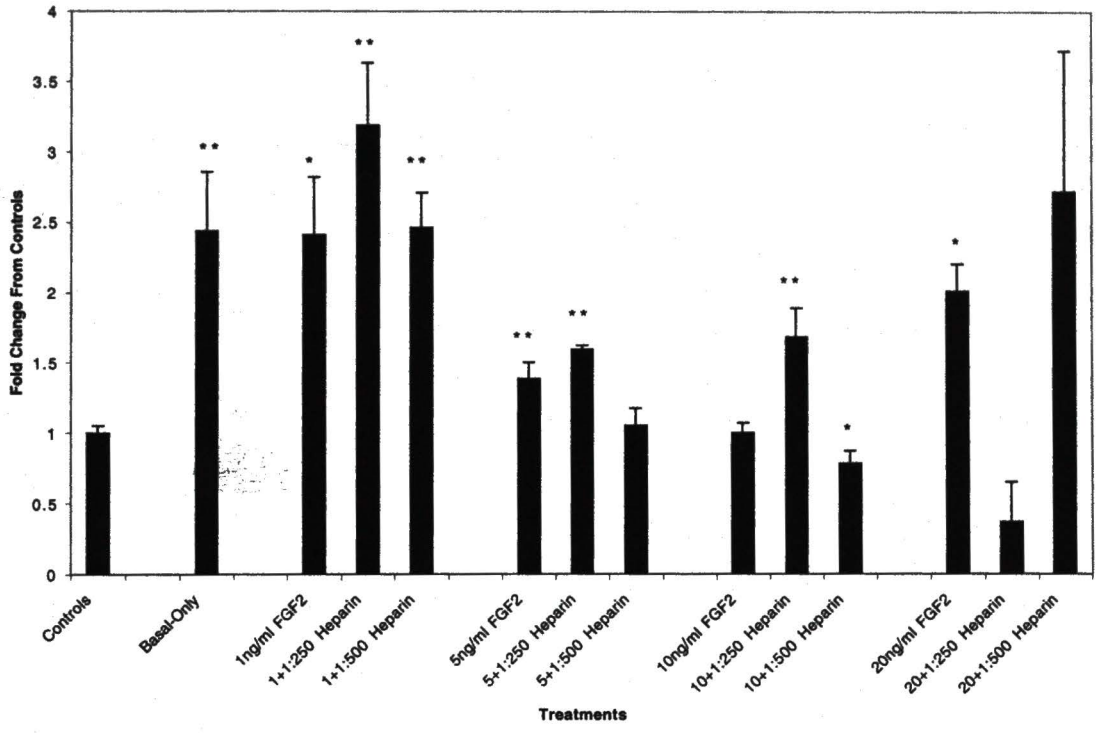
C. Several treatment regimens produced strong upregulation of β-tubulin expression but the most significant resulted from the treatment with 0-1ng/ml FGF2 with or without heparin.


Fold Change From Controls
Controls
Basal-Only
1ng/ml FGF2
1+1:250 Heparin
1+1:500 Heparin
5ng/ml FGF2
5+1:250 Heparin
5+1:500 Heparin
10ng/ml FGF2
10+1:250 Heparin
10+1:500 Heparin
20ng/ml FGF2
20+1:250 Heparin
20+1:500 Heparin
Treatments

A.

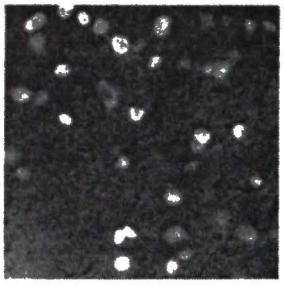

Fold Change From Controls
Controls
Basal-Only
1ng/ml FGF2
1+1:250 Heparin
1+1:500 Heparin
5ng/ml FGF2
5+1:250 Heparin
5+1:500 Heparin
10ng/ml FGF2
10+1:250 Heparin
10+1:500 Heparin
20ng/ml FGF2
20+1:250 Heparin
20+1:500 Heparin
Treatments

B.

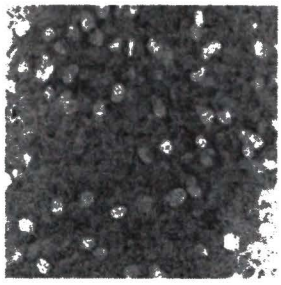

Fold Change From Controls
4
3.5
3
2.5
2
1.5
1
0.5
0
Controls
Basal-Only
1ng/ml FGF2
1+1:250 Heparin
1+1:500 Heparin
5ng/ml FGF2
5+1:250 Heparin
5+1:500 Heparin
10ng/ml FGF2
10+1:250 Heparin
10+1:500 Heparin
20ng/ml FGF2
20+1:250 Heparin
20+1:500 Heparin
Treatments

C.

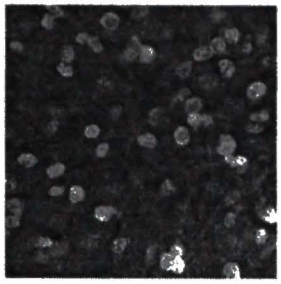
Figure 17: Changes in the proliferative capacity of neurosphere cells following growth factor treatments. Neurospheres were treated with growth factors or NPBM for 9 days, and pulsed with BrdU over the last 24hrs. Multiple sample boxes were selected for individual neurospheres, and the number of BrdU +ve nuclei were determined, averaged and values reported as mean ± SEM (n=3 for all treatment groups). Treatment with NPBM resulted in a statistically lower number of BrdU-labeled nuclei than any of the other treatments, which were all similar. (800x).



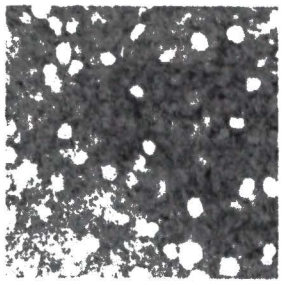
1ng/ml FGF2 (27±2)



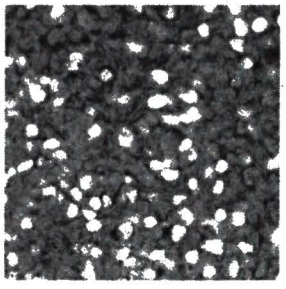
5ng/ml FGF2 (34±6)



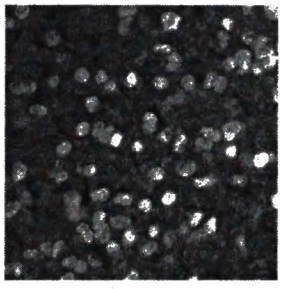
10ng/ml FGF2 (36±2)



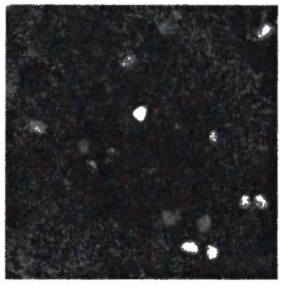
20ng/ml FGF2 (34±3)



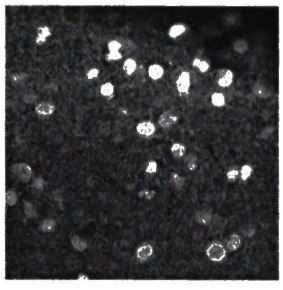
20ng/ml EGF (49±16)



40ng/ml EGF (56±13)



Basal Medium (5±2)



Control (41±13)

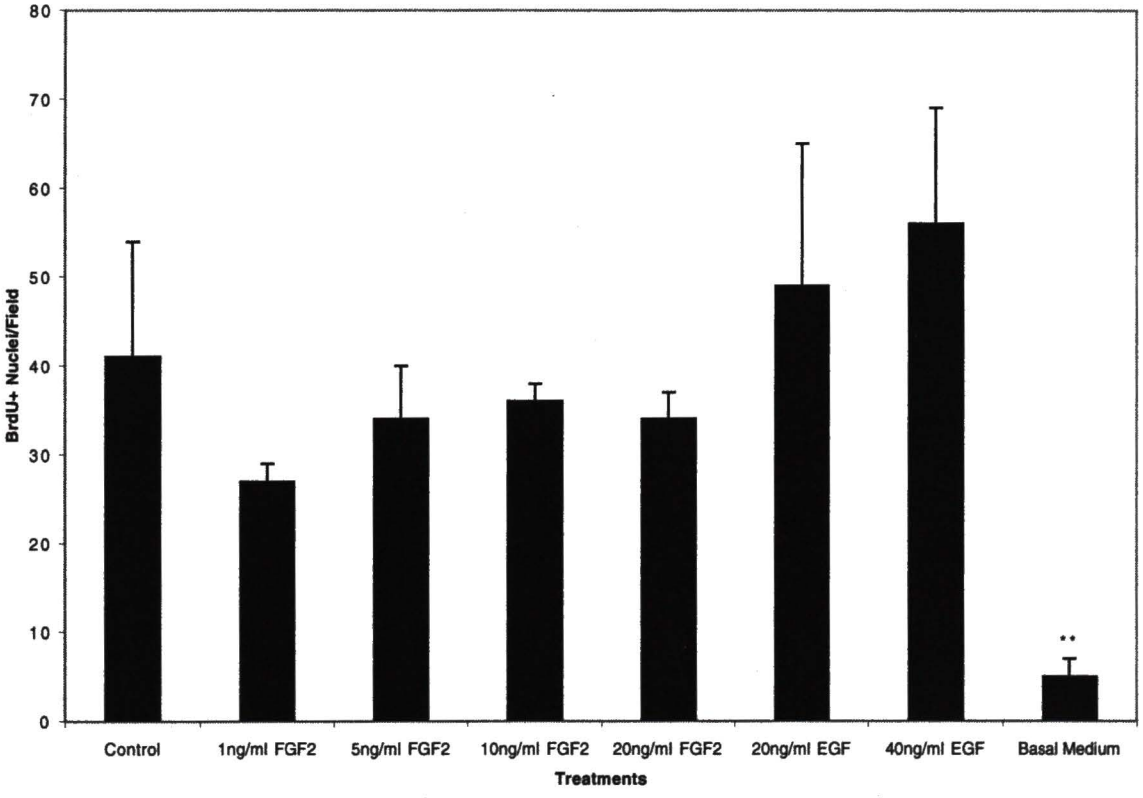


Figure 18: Extension of cellular processes across the neurosphere wall of stratified cells. Although cellular processes resembling axons and/or dendrites were not observed to project from the surfaces of neurospheres, cells labeled +ve for neuronal markers extended processes (indicated by arrowheads) across the surfaces of cell layers beneath them. (800x)

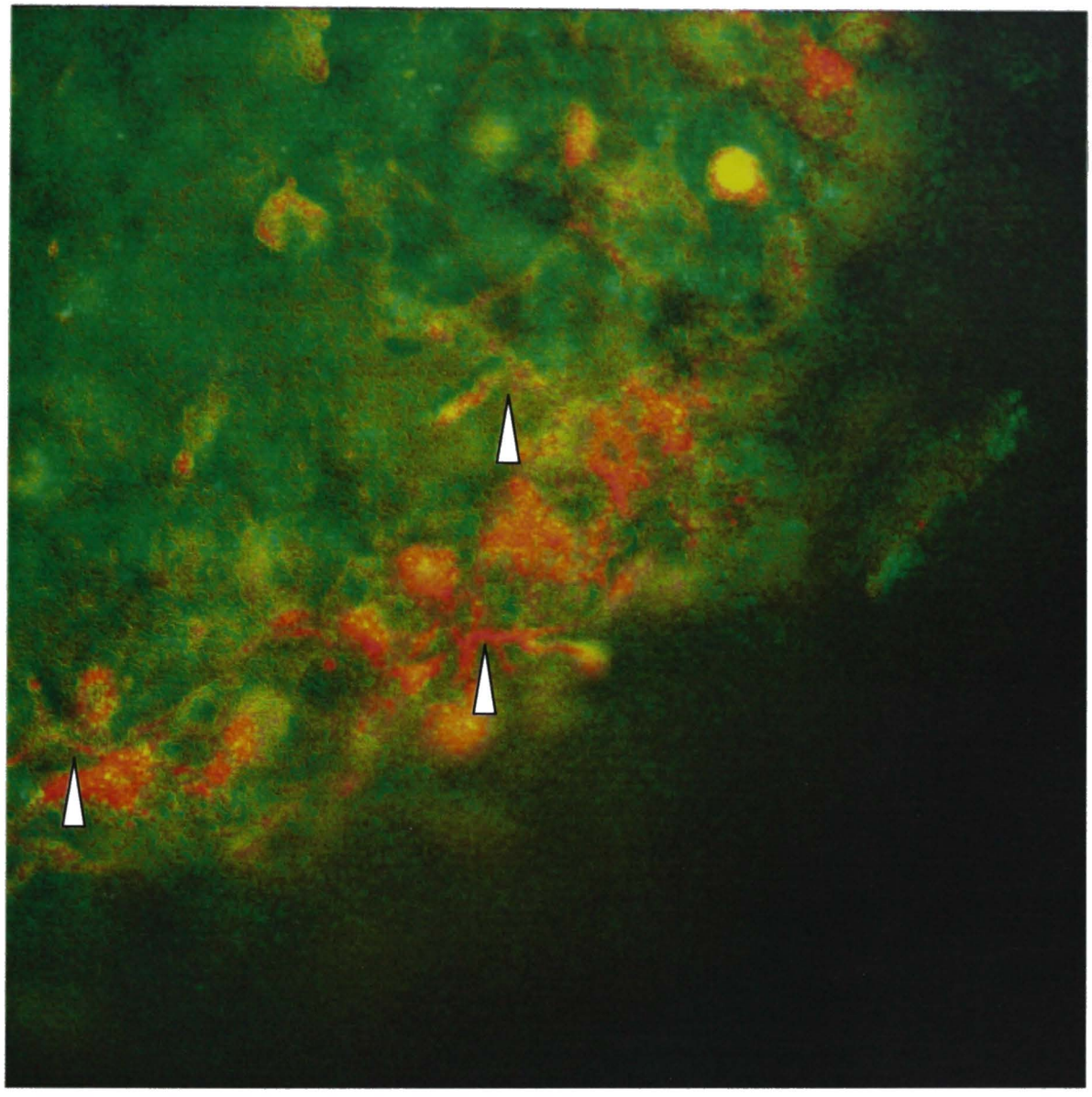


Table 1: Effects of growth factors, growth factor doses, growth factor exposure times, and the combined effects of these variables on the expressions of the neuronal phenotypic marker proteins α-internexin, NF-M, and β-tubulin. Neurospheres were subjected to either 3 or 9 days of exposure to 10 or 20 ng/ml FGF2 or 20 or 40ng/ml EGF. Following treatment, samples were fixed/permeabilized and immunofluorescently labeled for α-internexin, NF-M, or β-tubulin, subjected to wall densitometric analysis as described, and normalized to untreated control samples run in parallel. 2x2x2 analysis of variance (ANOVA) was performed to assess the separate effects of growth factors (EGFor FGF2), doses (10 or 20ng/ml for FGF2, and 20 or 40ng/ml for EGF), exposure times (3 or 9 days), and the combinations of the different effects. * indicates significance at $p<0.05$, and ** indicates significance at $p<0.01$.

MARKER	GROWTH FACTOR EFFECT	DOSE EFFECT	EXPOSURE TIME EFFECT	GF+ DOSE	GF + TIME	DOSE + TIME	GF + DOSE + TIME
α-INTERNEXIN	0.115	0.613	0.020*	0.099	---------	---------	0.337
NF-M	0.808	0.366	0.044*	0.720	0.022*	0.546	0.552
β-TUBULIN	0.779	0.184	0.073	0.006**	0.649	0.062	0.131

Table 2: Assessments of the effects of FGF2 and increasing heparin concentrations on the expressions of the neuronal phenotypic marker proteins α-internexin, NF-M, and β-tubulin. Neurospheres were incubated for 9 days in the presence of 1,5,10, or 20ng/ml FGF2 either without heparin, with a 1:250 ratio of FGF2:heparin (ng:ng), or with a 1:500 ratio of FGF2:heparin. 4x3 analysis of variance (ANOVA) was used to assess the effects of FGF2 dose, heparin dose, and the combination of FGF2 and heparin doses on the expressions of the neuronal markers. * indicates significance at $p<0.05$, and ** indicates significance at $p<0.01$.

MARKER	FGF2 DOSE EFFECT	HEPARIN DOSE EFFECT	FGF2 DOSE AND HEPARIN COMBINED
α-INTERNEXIN	<0.001**	0.060	<0.001**
NF-M	0.007**	0.032*	0.138
β-TUBULIN	0.073	<0.001**	0.001**

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CHAPTER V

The following manuscript is being prepared for submission to *The Journal of Neuroscience.*

The Role of Protein Kinase C in Neuronal Commitment of Neurosphere Cells

Michael L. Moeller and S. Dan Dimitrijevich

Department of Molecular Biology and Immunology

The University of North Texas Health Science Center at Fort Worth

3500 Camp Bowie Blvd., Fort Worth, Texas

***Corresponding author. Present address: Department of Integrative Physiology and Cardiovascular Research Institute, UNT Health Science Center, 3500 Camp Bowie Blvd. Fort Worth, TX 76107. e-mail: ddimitri@hsc.unt.edu**

ABSTRACT

The PKC family of serine/threonine kinases has long been implicated in neuronal differentiation stimulated by nerve growth factor (NGF). Using a novel technique, we demonstrate here that the PKC kinases also contribute to some aspects of basic fibroblast growth factor (FGF2)-mediated neuronal commitment in the cells of intact human neonatal neurospheres. We have shown that the α, βI, βII, δ, ε, η, and θ isoforms of PKC are expressed in human neonatal neurospheres, and that this expression profile is altered quantitatively, but not qualitatively, by the presence of either epidermal growth factor (EGF) or FGF2 or by outgrowth on immobilized extracellular matrix proteins. We have previously shown that optimal concentrations of FGF2 upregulate the expressions of the neuronal marker proteins α-internexin, neurofilament M (NF-M), and β-tubulin in the cells of whole, intact neurospheres. It is shown here that the upregulations of α-internexin and NF-M, both neuronal intermediate filament proteins, may be diminished in the presence of GF109203X, a global PKC inhibitor. However, at an optimal FGF2 concentration, the presence of GF109203X upregulates β-tubulin expression. Since the expression of β-tubulin does not appear to be regulated by growth factors in the same way as the intermediate filament proteins α-internexin or NF-M, and as PKC appears to differentially effect the relative expressions of these two broad classes of cytoskeletal proteins, we propose that intermediate filaments associated with the cell body and microtubular components associated with axonal extensions are regulated in different manners during FGF2-mediated differentiation.

INTRODUCTION

The protein kinase C (PKC) family is a group of 11 related serine/threonine kinases which play pivotal roles in many signaling pathways. They have molecular weights ranging from 77 to 87kDa and are divided into three structural classes. Classical PKC isoforms (cPKCs; α, βI, βII, and γ) possess four conserved domains (C1-C4) separated by five variable regions (V1-V5, **Fig. 19A**). Novel PKC isoforms (nPKCs; δ, ε, η, and θ) are similar in size to cPKCs, but lack the C2 domain (**Fig. 19B**). Atypical PKC isoforms (aPKCs; ζ, and ι/λ) lack both the C2 domain and half of the C1 domain (**Fig. 19C**) (Liu, 1996).

PKC isoforms have been implicated in neuronal differentiation imposed by nerve growth factor (NGF). Work with various neuronal models has shown correlations between differentiation, neurite extension, and increased PKCβ activity (Wooten et al., 1992; Wooten, 1992). PKCζ has also been shown to play a role in the differentiation in certain models (Coleman and Wooten, 1994) through nuclear translocation, chromatin binding, and direction of nuclear events necessary for differentiation (Wooten, 1997). Additional studies have shown that PKCβ activates transcription of genes associated with neuronal differentiation (Troller et al., 2001), that PKCδ may inhibit neuronal differentiation (Gallagher, Odumeru, and Regan, 2000), and that PKCε encourages neurite outgrowth during differentiation (Hundle et al., 1995). These results indicate that members of the PKC family are pivotal in all aspects of neuronal differentiation.

Basic fibroblast growth factor (FGF2) has been shown to influence neuronal differentiation (Kuhn et al., 1997; Palmer et al., 1999; Mistry et al., 2002). We have recently shown that basic fibroblast growth factor (FGF2) induces neuronal commitment in neurospheres derived from neonatal human forebrain (Moeller and Dimitrijevich, in review I). These

neurospheres are clusters of multipotent progenitor cells capable of becoming astrocytes, neurons, and oligodendrocytes (Reynolds and Weiss, 1996; Kukekov et al., 1999; and Suslov et al., 2000). In our studies, FGF2 downregulates nestin and vimentin and upregulates α-internexin, β-tubulin, and neurofilament M (NF-M) (Moeller and Dimitrijevich, in review II).

Based on the literature indicating a connection between NGF-induced differentiation and PKC, we wondered if PKC was involved in FGF2-induced neuronal commitment. We demonstrate here the presence of the α, βI, βII, δ, ε, η, and θ PKC isoforms in neurospheres, show that the isoforms appear to colocalize throughout the stratified layers of the neurosphere wall, and that growth factor treatment and outgrowth on immobilized extracellular matrix proteins affects isoform expressions quantitatively but not qualitatively. We further demonstrate that FGF2-induced upregulations of the neuronal marker proteins α-internexin and neurofilament M (NF-M) can be abrogated by GF109203X, a global PKC inhibitor, indicating that the PKC family of signaling proteins plays a role in the FGF2-directed upregulations of neuronal intermediate filaments. In contrast, the expression of β-tubulin was elevated by exposure to FGF2 and GF109203X, possibly indicating that different classes of neuronal cytoskeletal elements are regulated through different mechanisms.

MATERIALS AND METHODS

Neurospheres. Starting cultures used to generate neurosphere populations were obtained commercially from Biowhittaker (Clonetics). The starting cultures consisted of individual cells and small cell clusters obtained from human neonatal forebrain. Within 2-4 weeks of initiation, cultures began to become populated by neurospheres visible to the naked eye.

Culture conditions. Neurospheres were cultured in commercially-available serum-free defined medium (NPMM, Biowhittaker (Clonetics)) containing 20ng/ml EGF, 10ng/ml FGF2, gentamycin/amphotericin, and neural survival factors (NSF-1). All cultures were incubated under constant humidity at 37^{O}C and 5% CO_2.

Outgrowth of neural progenitors on immobilized extracellular matrix proteins. T-25 culture flasks were coated with FNC, a mixture of fibronectin, collagen I, and bovine serum albumin (BSA) (BRFF, Maryland), by covering the flask bottoms for approximately 1 minute, recovering the excess, and allowing the flasks to air dry for 1 hour under a sterile hood. Flasks were then seeded with 10-20 neurospheres in minimal volumes of media (≈2ml.), and the neurospheres were allowed to adhere for 4hrs. before being given medium. Flasks coated with collagen IV were coated by 3 equal applications of collagen IV solution (BD Biosciences, MA; 1.0mg/ml; 10μg/cm^2). Between applications and before seeding, flasks were allowed to air dry completely under a sterile hood. Flasks were seeded as described above. Cultures were maintained in NPMM (proliferation medium) under the culture conditions described above for approximately two weeks before lysis.

Western blot analysis of neurosphere lysates. Neurospheres were cultured to large numbers under the conditions described, collected, pelleted by centrifugation, and lysed at room temperature in 300μl lysis buffer for 10 minutes. Lysis buffer consisted of 2.5ml 1M Tris buffer (pH = 7.0), 1g SDS, and 2.5g sucrose in 50ml distilled water. Genomic DNA was sheared by several (10-20) passages through a syringe tipped with a 22 gauge needle, and samples were stored at –20^{O}C until needed. Multiple BCA protein assays (Pierce, Rockford, IL) were performed in concert with staining of gels with Coomassie Blue to accurately determine protein concentrations of lysates and to ensure equal loading of lanes. SDS PAGE was performed with

20µg protein/lane at room temperature on 4-15% Tris-HCl Ready Gels (Bio Rad, Hercules, CA) for 1 hour, 10 minutes at 95V in Tris/glycine/SDS running buffer. Electroblotting was carried on ice overnight at 25mA in Tris/glycine buffer with 20% methanol onto nitrocellulose membranes, and protein transfer was confirmed by staining the membranes with Ponceau Red. Following destaining in distilled water, membranes were blocked for 30 minutes at room temperature in blocking buffer consisting of 5% milk powdered milk and 1% BSA in PBS. Following room temperature incubation, membranes were allowed to incubate at 4^{O}C overnight, followed by 30 minutes at room temperature the following morning. Membranes were incubated in 1^{O} antibody solutions for 30 minutes at room temperature, incubated at 4^{O}C overnight, and for 30 minutes at room temperature the following morning. Membranes were rinsed 3x 10 minutes in PBS containing 0.5% Tween-20 and incubated in 2^{O} antibody solutions for 1 hour at room temperature. After rinsing 3x 10 minutes in PBS containing 0.5% Tween-20, membranes were developed via ECL chemiluminescence (Amersham Biosciences, UK). Densitometric analysis of bands was carried out using Adobe Photoshop™.

Indirect immunofluorescence. Neurospheres were removed from cultures, rinsed briefly in PBS, and fixed/permeabilized in ice cold 1:1 (v:v) methanol:acetone for 12 minutes at 4^{O}C. Following fixation/permeabilization, neurospheres were soaked in PBS for 30 minutes and blocked at 4^{O}C overnight in PBS containing 1%BSA. After blocking, neurospheres were rinsed briefly in PBS and incubated at 4^{O}C overnight in 1^{O} antibody solutions, rinsed 3x 10 minutes in PBS containing 0.25% Triton X-100, and incubated in 2^{O} antibody solutions (1:250 in PBS) for 1.5 hours at room temperature, followed by rinsing in 0.25% Triton X-100 for 3x 10 minutes. This procedure was then repeated to label the second marker.

Image acquisition. Laser-scanning confocal microscopy was performed using a Zeiss Micro Systems LSM 410 connected to an Axiovert 135 laser scanning confocal microscope (Zeiss, West Germany). Illumination was provided by an Ominchrome Series 43 laser. All objectives were provided by Zeiss, as was the image acquisition software. Images of individual neurospheres were obtained at 250x, and the plane of focus was adjusted so that equatorial optical sections were recorded for the neurosphere. Individual sections of the neurosphere wall (1-2 per neurosphere) were selected and magnified to 800x.

Image analysis, densitometric measurements of neurosphere wall sections, and statistical analysis. Digitial image analysis, densitometric assessments of expressional changes of protein markers, and statistical analysis of expressional changes were performed as described (Moeller and Dimitrijevich, in review I). All values are expressed as mean values±SEM.

Antibodies used. Rabbit polyclonal anti-PKCα, anti-PKCβI, anti-PKCβII, anti-PKCγ, anti-PKCδ, anti-PKCε, anti-PKCη, anti-PKCθ, and anti-PKCζ (Santa Cruz Biotechnologies Inc., Santa Cruz, CA) were all used at a concentration of 1:500 in PBS with 1% BSA. Peroxidase-conjugated 2^{O} antibodies were used at a concentration of 1:1000. α-internexin was labeled using 1:200 dilutions of monoclonal rabbit anti-α-internexin (Chemicon International Inc., Temecula, CA) in PBS. Rabbit polyclonal anti-β-tubulin (Santa Cruz Biotechnologies Inc., Santa Cruz, CA) was used at a dilution of 1:100 in PBS. Polyclonal rabbit anti-neurofilament M (Chemicon International Inc., Temecula, CA) was used at a dilution of 1:200 in PBS.

Effects of global PKC inhibition on FGF2-mediated upregulation of neuronal markers. To delineate the role of the PKC family of proteins on FGF2-mediated neuronal commitment, neurosphere cultures were exposed to 50ng/ml PMA (global PKC activator), 500nM GF109203X (global PKC inhibitor), 50ng/ml PMA together with 500nM GF109203X, 5ng/ml

FGF2, or 5ng/ml FGF2 with 500nM GF109203X for 9 days. Following incubation, the samples were processed as described and subjected to wall densitometric analysis.

RESULTS

Identification of The PKC Isoforms Present In Neurospheres Cultured Under A Variety of Conditions. Lysates of neurospheres raised under standard proliferative conditions were probed for PKCα, PKCβI, PKCβII, PKCγ, PKCδ, PKCε, PKCη, PKCθ, and PKCζ. The classical PKC isoforms PKCα, PKCβI, and PKCβII as well as the novel isoforms PKCδ, PKCη and PKCθ were positively identified, but the classical isoform PKCγ and the atypical isoform PKCζ were undetectable. To determine whether or not PKC isoform profiles would change with growth factor treatements, profiles were also obtained for cultures raised in the presence of 1, 5, or 20ng/ml FGF2 or 40ng/ml EGF. It was found that different treatment regimens did not qualitatively affect the expression profiles of PKC isoforms, but did quantitatively affect the expression levels of some of the isoforms. Relative to control cultures, expression of PKCα was suppressed by treatment with 1ng/ml and 20ng/ml FGF2, but elevated by treatment with 5ng/ml FGF2. Expression of PKCβI was elevated by all concentrations of FGF2 tried as well as by exposure to a high dose (40ng/ml) of EGF, but expression of PKCβII was suppressed by all of these treatments. Outgrowth of neurosphere cells on either FNC or collagen Type IV suppressed the expressions of all classical isoforms to similar degrees with the notable exception of PKCβII, which was expressed at much higher levels in monolayers grown on collagen Type IV than on FNC (**Fig. 20**). Expressional changes in novel PKC isoforms were fairly mixed with the various treatment groups. PKCδ expression was downregulated at all concentrations of FGF2 tried as well as by outgrowth on either FNC or collagen Type IV and to a lesser degree by treatment with

40ng/ml EGF when compared to control neurosphere populations. PKCε exhibited dose-dependant increases in expression with increasing FGF2 dose but was suppressed to varying degrees by outgrowth on either FNC or collagen Type IV. PKCθ was suppressed to varying degrees by treatment with FGF2 as well as by outgrowth on either FNC or collagen Type IV, but was expressed at approximately base levels in neurospheres treated with 40ng/ml EGF (**Fig. 21**).

Indirect immunofluorescent double labeling revealed high levels of colocalization of PKCα/PKCβI, PKCα/PKCβII, PKCα/PKCε, PKCβI/PKCβII, PKCβI/PKCε, and PKCβII/PKCε throughout the neurosphere walls. Thus, it appears that specific PKC isoforms are not expressed in discrete regions of the neurosphere wall, but are, rather, distributed globally throughout all cell layers (**Fig. 22**).

Effects of global PKC inhibition on FGF2-mediated upregulation of neuronal markers. To assess the role of the PKC family of proteins on FGF2-mediated neuronal commitment, neurosphere cultures were exposed to 50ng/ml PMA (global PKC activator), 500nM GF109203X (global PKC inhibitor), 50ng/ml PMA together with 500nM GF109203X, 5ng/ml FGF2, or 5ng/ml FGF2 with 500nM GF109203X for 9 days. Exposure to PMA strongly upregulated the expressions of both α-internexin (3.091±0.149, n=3, $p<0.001$) and β-tubulin (3.750±0.285, n=3, p=0.001) relative to untreated control populations, and NF-M was also seen to be upregulated although not to a statistically significant degree (1.788±0.355, n=3, p=0.189). Addition of GF109203X ablated PMA-mediated increases in the neuronal intermediate filament proteins α-internexin (1.084±0.182, n=4, p=0.349) and NF-M (0.931±0.015, n=3, p=0.352). β-tubulin expression was reduced from the levels seen following PMA exposure, but were still significantly elevated over base levels (1.714±0.269, n=3, p=0.058). Treatment with

GF109203X alone resulted in significant upregulations in the expressions of α-internexin (1.658±0.218, n=7, p=0.012) and β-tubulin (1.897±0.260, n=4, p=0.025), although this was not seen with NF-M (1.554±0.419, n=3, p=0.274) (**Fig. 23A, Table 3**). Treatment with 5ng/ml FGF2 resulted in significant upregulations of α-internexin (3.100±0.713, n=7, p=0.027) and NF-M (3.351±0.859, n=8, p=0.015), and β-tubulin was also upregulated, but not to a significant degree (1.379±0.125, n=5, p=0.121). The addition of GF109203X completely ablated the FGF2-induced upregulations of α-internexin (0.970±0.146, n=4, p=0.434) and NF-M (1.308±0.272, n=3, p=0.110), although the combination of FGF2 and inhibitor elevated the expression of β-tubulin (2.574±0.600, n=3, p=0.053) (**Fig. 23B, Table 3**).

DISCUSSION

PKC isoforms have been implicated in the differentiation of a variety of neuronal model systems. Many of these studies have focused on differentiation driven by nerve growth factor (NGF), and links between NGF, numerous PKC isoforms, and neuronal differentiation have been established (Wooten et al., 1992; Wooten, 1992; Coleman and Wooten, 1994; and Wooten, 1997). Numerous connections have been established between FGF2 and PKC-mediated signaling in a number of processes occurring in neurons. FGF2 has been shown to promote survival in serum-deprived PC12 cells through a PKCδ-dependent mechanism (Wert and Palfrey, 2000), and the intracellular localization of growth-associated protein 43 (GAP-43), a protein involved in axonal elongation and growth cone guidance, is regulated by FGF2-stimulated PKC phosphorylation of GAP-43 in cultured rat hippocampal neurons (Tejero-Diez et al., 2000). Associations of FGF2 receptors (FGFRs) and PKC have been demonstrated within lipid raft domains (Ridyard and Robbins, 2003), and connections between FGFRs, syndecan-4, PIP_2, and

PKCα have also been described (Horowitz, Tkachenko, and Simons, 2002; and Murakami et al., 2002). In this model, syndecan-4 core proteins are dephosphorylated on their cytoplasmic tails, leading to PIP_2-dependent oligomerization and activation of PKCα (Horowitz, Tkachenko, and Simons, 2002). PKCδ attenuates FGF2-mediated activation of PKCα by phosphorylating the cytoplasmic tails of syndecan-4 monomers and leading to a breakdown of syndecan-4 oligomers (Murakami et al., 2002). It is not known if syndecan-4 is present on the surfaces of human neonatal neurospheres, but the presence of endogenous heparan sulfate proteoglycans has been documented (Chipperfield et al., 2002). It has further been speculated that endogenous heparan sulfate proteoglycans may explain dose-dependent decreases in neuronal marker expressions observed with increasing doses of exogenously administered heparin (Moeller and Dimitrijevich, in review II). Thus, syndecan-4 may well be a component of the extracellular matrix shell that could envelop neurospheres.

In the present study, we show that FGF2-mediated commitment to the neuronal phenotype, as evidenced by the upregulations of neuronal marker proteins, is also regulated at least partially by PKC in the cells of human neonatal neurospheres. We have shown that the α, βI, βII, δ, ε, η, and θ isoforms of PKC are expressed in neurospheres subjected to a variety of treatments and that this profile is altered quantitatively, but not qualitatively, by growth factor treatments as well as by outgrowth on either FNC or collagen IV. It also appears that these PKC isoforms are coexpressed homogeneously throughout the stratified cell layers of the neurosphere wall and are not segregated into discrete domains. We have been unable to detect the γ and ζ isoforms, which is significant as PKCζ has been strongly implicated in the NGF-mediated differentiation of PC12 cells (Coleman and Wooten, 1994; Wooten et al., 1997).

The connection between FGF2, PKC, and neuronal commitment in neurosphere cells is not a simple one, however. It has been shown that upregulations of the neuronal intermediate filament proteins α-internexin and NF-M are optimally stimulated by concentrations of FGF2 higher than those which stimulate optimal upregulations of β-tubulin (Moeller and Dimitrijevich, in review II). This might indicate that a gradually decreasing FGF2 concentration may progressively drive first the upregulation of neuronal intermediate filaments followed by β-tubulin and axonal components. If this is the case, the cytoskeletal architecture of the cell body is altered prior to axonal formation. Such a hierarchical process would be expected to involve fundamental differences in signal transduction pathways. Exposure of neurospheres to 5ng/ml FGF2 upregulates the expressions of α-internexin and NF-M 2.5-4.25 fold, and these upregulations may be completely ablated by treatment with GF109203X, indicating that these upregulations are associated with PKC activity. At this concentration of FGF2, the expression of the α and βI isoforms of PKC were seen to be elevated relative to untreated controls, suggesting that these isoforms may be responsible for these upregulations.

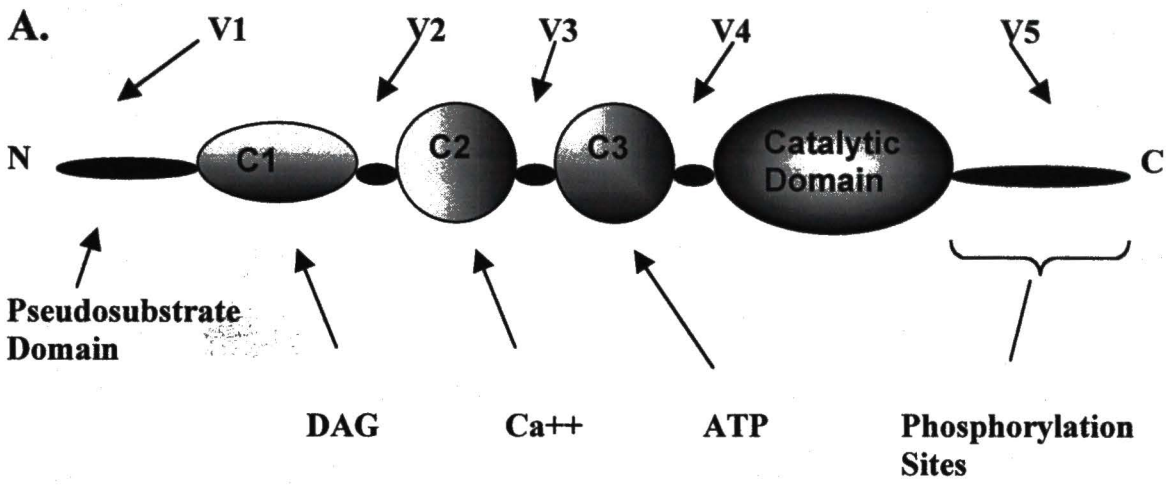
The expression of β-tubulin is also upregulated by exposure to 5ng/ml FGF2, though not to a significant degree relative to controls. The combination of 5ng/ml FGF2 and GF109203X does result in significant upregulation (≈2.5 fold). In previous work, we have demonstrated that β-tubulin upregulations are more profound at concentrations of FGF2 (0-1ng/ml) lower than those which most strongly upregulate the neuronal intermediate filaments (5ng/ml) (Moeller and Dimitrijevich, in review II). When FGF2 is lowered from 5ng/ml to 1ng/ml, the expression of PKCα expression decreases sharply, as do the expressions of the δ, ε, and θ isoforms. Thus, upregulation of β-tubulin may be related to decreases in the expressions of these isoforms. Perhaps the administration of GF109203X to neurospheres treated with 5ng/ml FGF2

sufficiently lowers the activities of these isoforms such that β-tubulin expression is increased. Regardless of whether or not this is the case, it is apparent that the global suppression of PKC results in the ablation of FGF2-mediated increases in the expressions of the neuronal intermediate filament proteins α-internexin and NF-M while that of β-tubulin is increased. Together with other lines of evidence, it appears that neuronal intermediate filaments and microtubular components are differentially regulated during neuronal commitment and differentiation.

Figure 19: Domain structure and activation of the members of the protein kinase C (PKC) family of protein kinases. The classical isoforms of PKC (α, βI, βII, and γ) consist of 4 constant domains (C1-C4) separated and flanked by 5 variable regions (V1-V5). The C1 domain confers responsiveness to both diacylglycerol (DAG) and phorbol esters such as PMA, the C2 domain confers responsiveness to calcium ions, the C3 domain binds and hydrolyses ATP, and the C4 domain serves as the catalytic domain for the protein. The N-terminal variable region (V1) serves as a "pseudosubstrate" domain which disables the catalytic activity of the enzyme by binding to the catalytic domain, folding the enzyme into a closed conformation. When activated, the enzyme is moved into an open conformation (B). The novel isoforms of PKC (δ, ε, η, and θ) lack the C2 domain, thus making them insensitive to Ca+2, and the atypical isoforms of PKC (ζ and λ/ι) lack the C2 domain as well as a portion of the C1 domain.

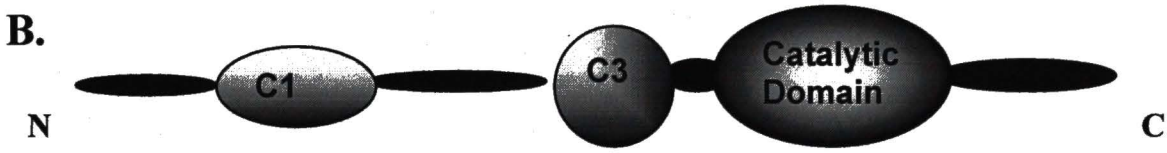
CLASSICAL ISOFORMS OF PROTEIN KINASE C (PKC)

α, βI, βII, γ



NOVEL ISOFORMS OF PROTEIN KINASE C (PKC)

δ, ε, η, θ



ATYPICAL ISOFORMS OF PROTEIN KINASE C (PKC)

ζ, ι/λ

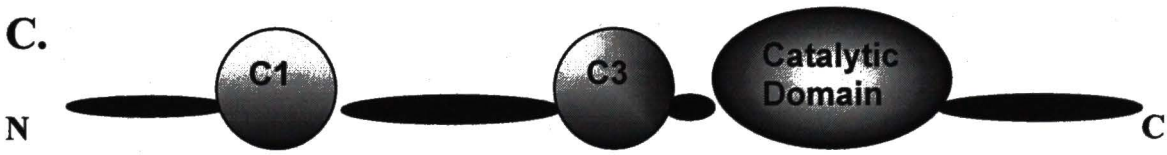


Figure 20: Expression of classical PKC isoforms in neurospheres exposed to different growth factor regimens. Neurospheres were incubated for 9 days in the presence of 1, 5, or 20ng/ml FGF2 or 40ng/ml or grown out into monolayers on either FNC (collagen Type I and fibronectin) or collagen Type IV. A. The classical PKC isoforms α,βI, and βII were identified in lysates of all treatments. Lane 1: Untreated Control; Lane 2: 1ng/ml FGF2, 9 Days; Lane 3: 20ng/ml FGF2, 9 Days; Lane 4: 5ng/ml FGF2, 9 Days; Lane 5: 40ng/ml FGF2, 9 Days; Lane 6: Untreated Neurospheres; Lane 7: FNC-Adherent Monolayer; Lane 8: Collagen IV-Adherent Monolayer. B. Expressions of these isoforms were quantified relative to neurospheres raised under standard proliferative conditions.


α
βI
βII

1 2 3 4 5 6 7 8

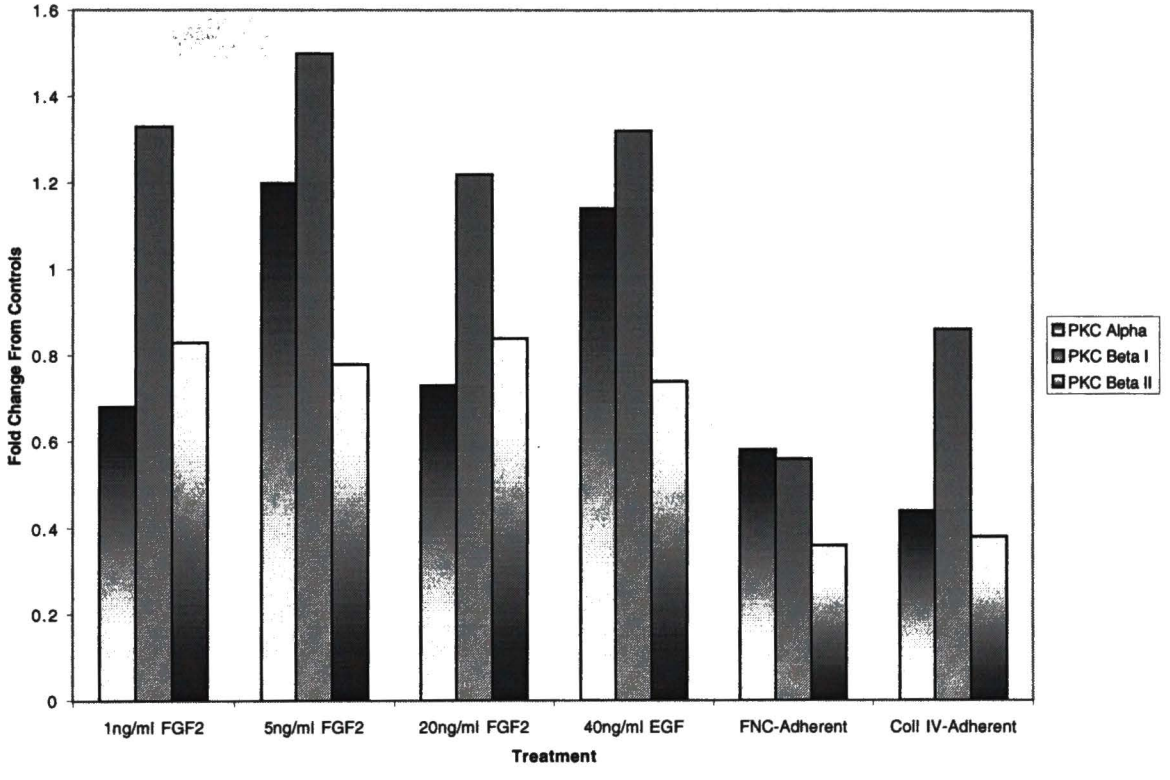
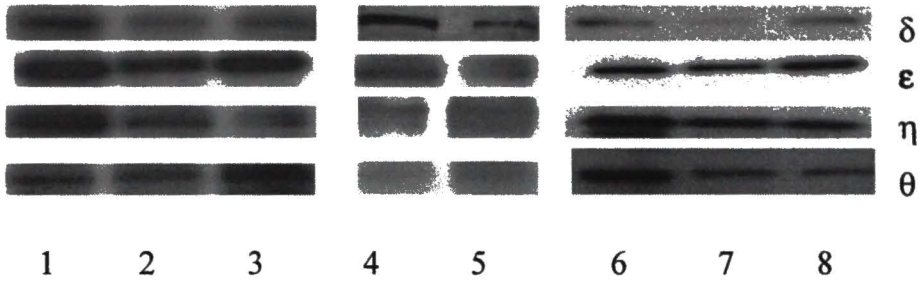

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Fold Change From Controls
1ng/ml FGF2
5ng/ml FGF2
20ng/ml FGF2
40ng/ml EGF
FNC-Adherent
Coll IV-Adherent
Treatment
PKC Alpha
PKC Beta I
PKC Beta II

Figure 21: Expression of novel PKC isoforms in neurospheres exposed to different growth factor regimens. Neurospheres were incubated for 9 days in the presence of 1, 5, or 20ng/ml FGF2 or 40ng/ml or grown out into monolayers on either FNC (collagen Type I and fibronectin) or collagen Type IV. A. The novel PKC isoforms δ, ε, η, and θ were identified in lysates of all treatments. Lane 1: Untreated Control; Lane 2: 1ng/ml FGF2, 9 Days; Lane 3: 20ng/ml FGF2, 9 Days; Lane 4: 5ng/ml FGF2, 9 Days; Lane 5: 40ng/ml FGF2, 9 Days; Lane 6: Untreated Neurospheres; Lane 7: FNC-Adherent Monolayer; Lane 8: Collagen IV-Adherent Monolayer. B. Expressions of these isoforms were quantified relative to neurospheres raised under standard proliferative conditions.


δ
ε
η
θ
1 2 3 4 5 6 7 8

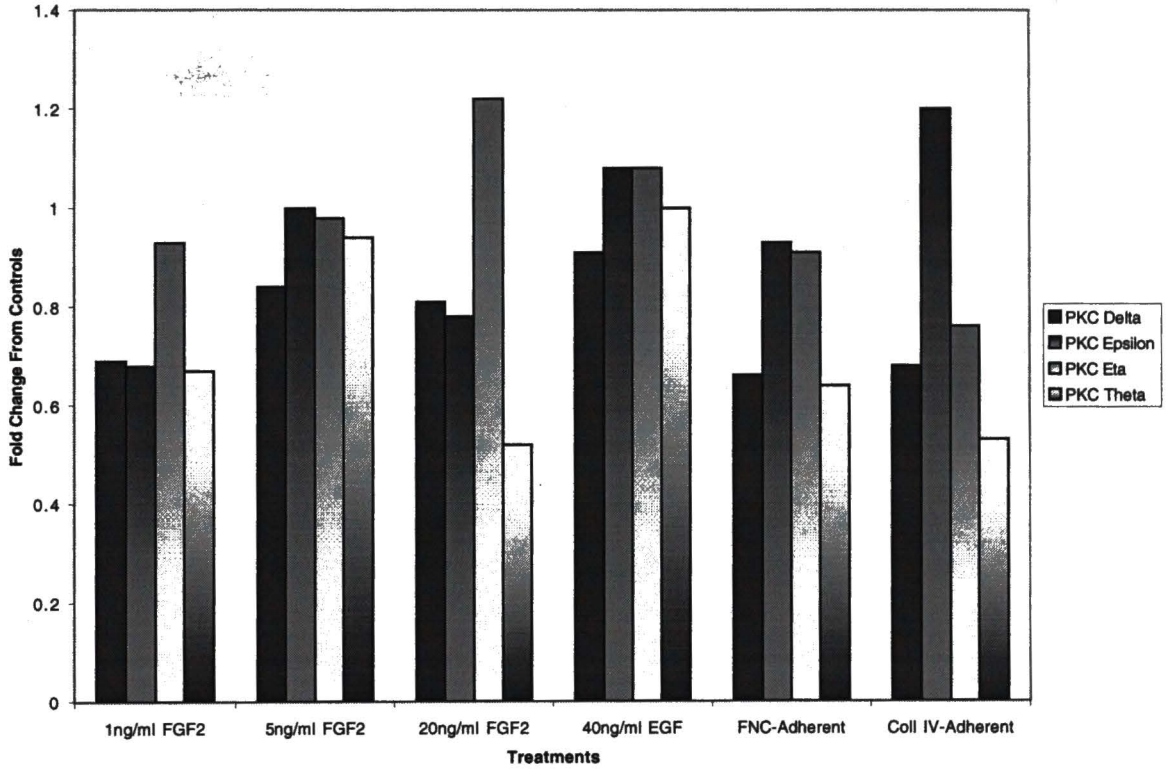
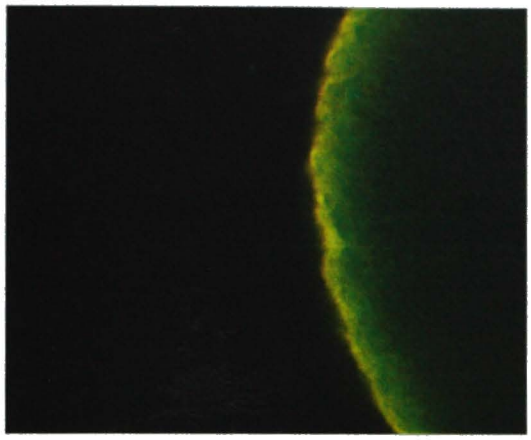

1.4
1.2
1
0.8
0.6
0.4
0.2
0
Fold Change From Controls
1ng/ml FGF2
5ng/ml FGF2
20ng/ml FGF2
40ng/ml EGF
FNC-Adherent
Coll IV-Adherent
Treatments
PKC Delta
PKC Epsilon
PKC Eta
PKC Theta

Figure 22: Colocalization of PKC isoforms in the walls of neurospheres. Neurospheres raised under standard proliferative conditions were fixed/permeabilized and subjected to indirect immunofluorescent staining of pairs of PKC isoforms. A. PKCα and PKCβI colocalize in intact neurospheres. B. PKCα and PKCβII colocalize in intact neurospheres. C. PKCα and PKCε colocalize in intact neurospheres. D. PKCβI and PKCβII colocalize in intact neurospheres. E. PKCβI and PKCε colocalize in intact neurospheres. F. PKCβII and PKCε colocalize in intact neurospheres. All neurosphere wall sections 800x.



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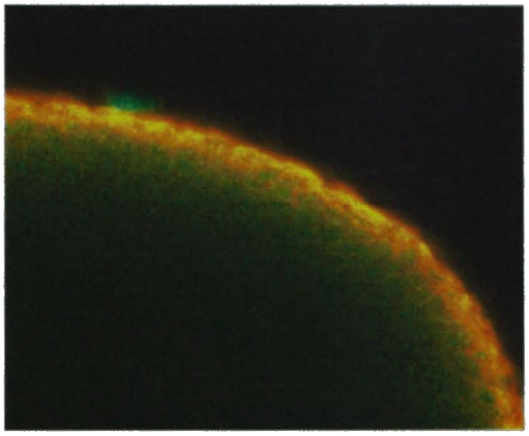

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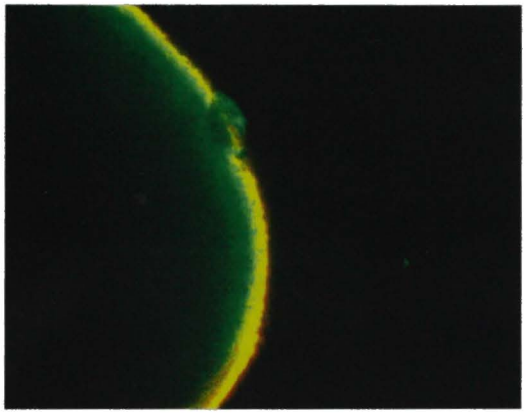

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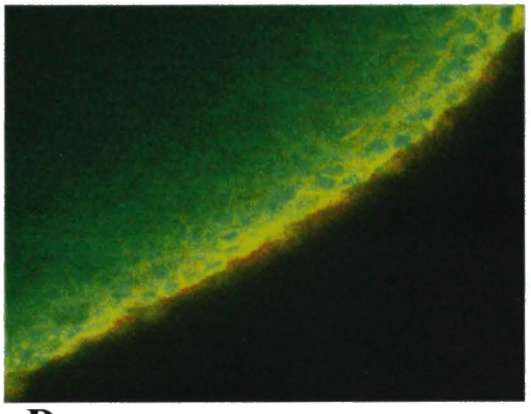
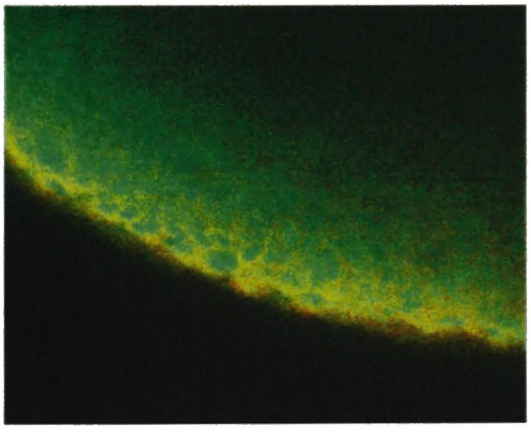
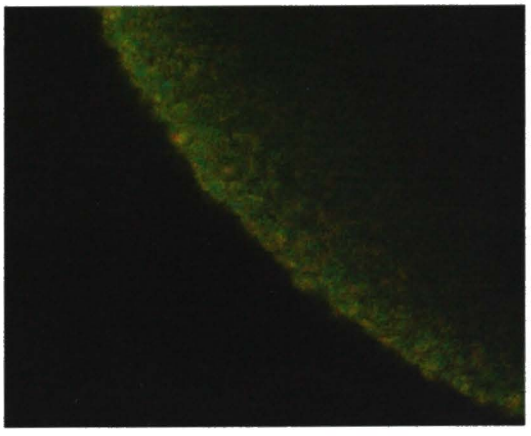


D.

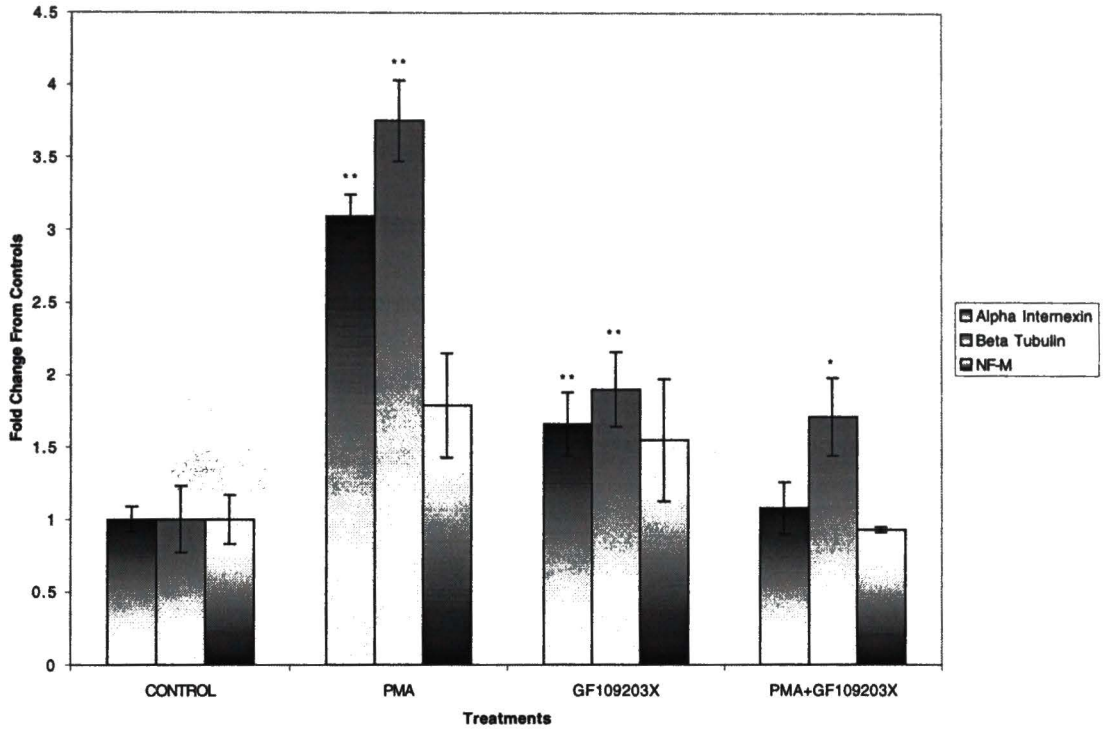


E.

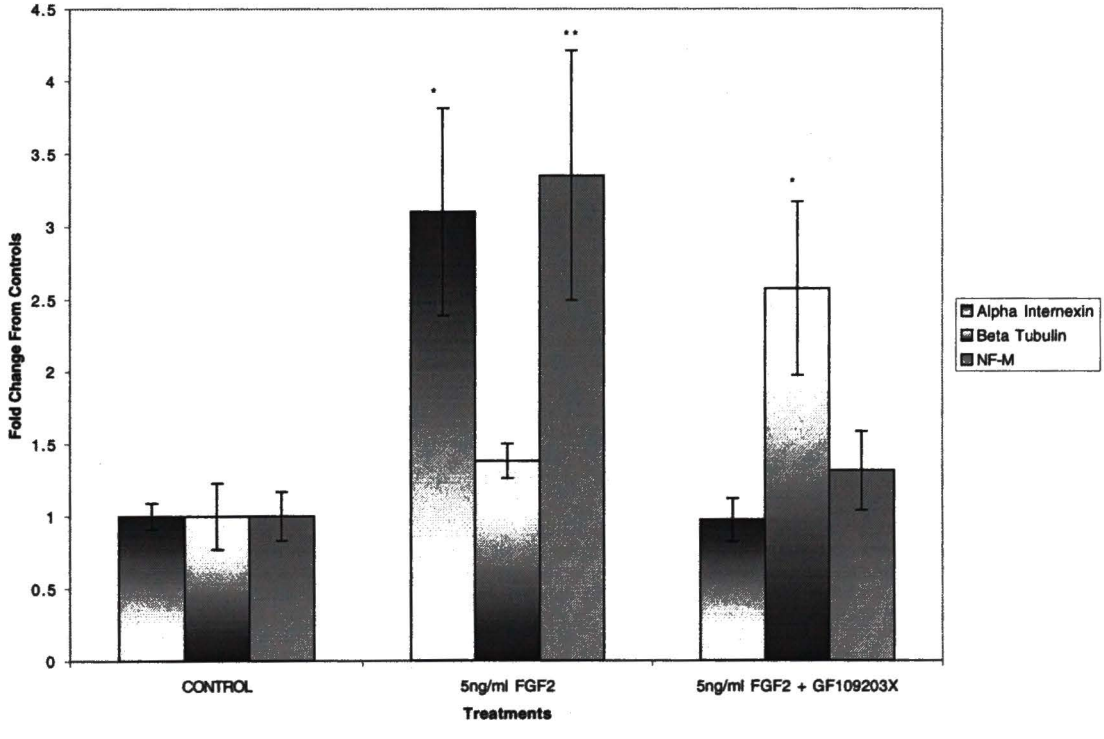


F.

Figure 23: General activation of PKC by exposure to PMA upregulates all markers of neuronal commitment, and general blockade of PKC activity by exposure to GF109203X alters FGF2-mediated changes in the expressions of neuronal markers. A. Neurospheres were exposed to PMA (50ng/ml), GF109203X (500nM), or PMA and GF109203X (50ng/ml and 500nM, respectively) for 9 days, fixed/permeabilized, and immunofluorescently labeled against the neuronal markers α-internexin, β-tubulin, or NF-M. As shown, exposure to PMA stimulated significant upregulations of α-internexin and β-tubulin, effects which were largely or completely ablated by the addition of GF109203X. B. Neurospheres were exposed to 5ng/ml FGF2, or 5ng/ml FGF2 and GF109203X for 9 days. Exposure to 5ng/ml FGF2 for 9 days stimulated significant upregulations of α-internexin and NF-M, although β-tubulin was not significantly upregulated. FGF2-mediated upregulations of the neuronal intermediate filaments α-internexin and NF-M were completely ablated by the addition of GF109203X, although the combination of 5ng/ml FGF2 and GF109203X stimulated the upregulation of β-tubulin.


4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Fold Change From Controls
CONTROL
PMA
GF109203X
PMA+GF109203X
Treatments
Alpha Internexin
Beta Tubulin
NF-M

A.


4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Fold Change From Controls
CONTROL
5ng/ml FGF2
5ng/ml FGF2 + GF109203X
Treatments
Alpha Internexin
Beta Tubulin
NF-M

B.

Table 3: The effects of global PKC inhibition on FGF2-mediated upregulations of neuronal phenotypic markers. Neurospheres were exposed to 50ng/ml PMA, 500nM GF109203X, the combination of PMA and GF109203X, 5ng/ml FGF2, or FGF2 with GF109203X for 9 days. Following immunofluorescent processing, samples were subjected to densitometric analysis of the neurosphere walls. All data values are reported as mean values SEM, and data sets are compared using both 1-way analysis of variance (ANOVA) and Student's T-tests. * indicates significance at $p<0.05$, and ** indicates significance at $p<0.01$.

MARKER	FIRST SET	MEAN±SEM	SECOND SET	MEAN±SEM	ANOVA	T-TEST
α-INT	Control	1.000±0.087	PMA	3.091±0.149	N	<0.001**
α-INT	Control	1.000±0.087	GF109203X	1.658±0.218	N	0.012*
α-INT	Control	1.000±0.087	PMA+GF109203X	1.084±0.182	N	0.349
α-INT	Control	1.000±0.087	5ng/ml FGF2	3.100±0.713	N	0.027*
α-INT	Control	1.000±0.087	FGF2+GF109203X	0.970±0.146	N	0.434
NF-M	Control	1.000±0.165	PMA	1.788±0.355	N	0.189
NF-M	Control	1.000±0.165	GF109203X	1.554±0.419	N	0.274
NF-M	Control	1.000±0.165	PMA+GF109203X	0.931±0.013	N	0.352
NF-M	Control	1.000±0.165	5ng/ml FGF2	3.351±0.859	N	0.015*
NF-M	Control	1.000±0.165	FGF2+GF109203X	1.454±0.258	N	0.110
β-TUB	Control	1.000±0.232	PMA	3.755±0.285	P<0.01**	0.001**
β-TUB	Control	1.000±0.232	GF109203X	1.897±0.260	N	0.025*
β-TUB	Control	1.000±0.232	PMA+GF109203X	1.714±0.269	N	0.058
β-TUB	Control	1.000±0.232	5ng/ml FGF2	1.379±0.125	N	0.121
β-TUB	Control	1.000±0.232	FGF2+GF109203X	2.574±0.600	P<0.05*	0.053

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CHAPTER VI

DISCUSSION

These studies were intended to evaluate human neonatal neurospheres as a potential source of neuron-enriched cell cultures and also to investigate growth factor treatments that could stimulate neuronal commitment and the molecular mechanisms behind these commitment events. Human neonatal neurospheres were shown to be hollow aggregates of multipotent cells with walls 6-8 cell layers thick held together by combinations of connexin-43^{+} gap junctions and adherens junctions. Proliferating cells were randomly distributed throughout the wall strata and not segregated into discrete compartments. Multiple phenotypic marker proteins were identified, and it was discovered that a shell or basket of putative glia surrounds a core of neuroblasts expressing neuronal markers. This arrangement is similar to that seen in the cell nests of the subventricular zone (SVZa) of the mammalian forebrain as well as the cell chains known to migrate down the rostral migratory stream (RMS) during neuronal replacement of the olfactory bulb. During outgrowth on both fibronectin and collagen Type IV, the cells of the glial basket were seen to form extensive radiating networks away from attachment foci, and nestin^{+} neuronal progenitors were seen to emerge later during outgrowth, migrating on radial tracks of glia. This sequence of events may indicate a basic colonization paradigm for migratory cell aggregates such as the cell chains of the RMS.

Later studies focused on the potential of specific growth factors, notably FGF2, to stimulate and direct neuronal commitment. FGF2 downregulated expressions of the immature phenotypic markers nestin and vimentin, suggesting that FGF2 promotes maturation of immature progenitors to more mature phenotypes. Later studies assessed the effects of growth factors on the expressions of neuronal markers, and these generated mixed results. α-internexin was upregulated by short-term exposure (3 days) to either 10 or 20ng/ml FGF2 but relatively unaffected by long-term exposure (9 days) to either concentration. This may be explained by the sustained upregulations of neurofilament M (NF-M) following both short term and long term exposure to both concentrations of FGF2 as NF-M is known to replace α-internexin during neuronal maturation. β-tubulin, the primary component of axonal microtubules, showed a different pattern of expressional changes from the two intermediate filaments, with the strongest upregulations being generated by long term exposure (9 days) to either high doses (20ng/ml) of FGF2 or basal medium devoid of growth factors.

Further studies assessed dose dependency of FGF2-mediated upregulations of neuronal markers. These studies also investigated the effects of increasing heparin doses on the upregulations of neuronal markers. The intermediate filament proteins (α-internexin and NF-M) exhibited similar responses, with 5ng/ml FGF2 or 10ng/ml FGF2 with heparin added to a 1:250 FGF2:heparin ratio showing the highest upregulations. An FGF2:heparin ratio of 1:500 invariably reduced intermediate filament protein expressions to base levels, suggesting a titrating effect of exogenous heparin. As neurospheres have been suggested to produce their own endogenous heparan sulfate proteoglycans, it was speculated that exogenous heparin creates a competitive inhibitory effect by reducing the amount of FGF2 available for binding to endogenous HSPGs and, hence, for presentation to FGF2 receptors. This was supported by the

stepped decreases seen in the expressions of α-internexin and NF-M at 5ng/ml FGF2 with increasing doses of heparin.

β-tubulin showed different responses from either of the two intermediate filament proteins. Although most of the treatments tried produced upregulations of β-tubulin expression, the highest upregulations were seen at low or absent concentrations of FGF2. The upregulation seen at 5ng/ml was not as pronounced as those at 0-1ng/ml, supporting the idea that upregulations of microtubular components such as β-tubulin are regulated differently than are upregulations of intermediate filaments.

Later studies sought to determine the mechanism behind FGF2-stimulated neuronal commitment. Isoform expression profiles were generated for neurospheres raised under standard proliferative conditions as well as neurospheres subjected to various growth factor treatments, and it was shown that growth factors alter the PKC expression profiles quantitatively but not qualitatively. Subsequent experiments sought to connect PKC with FGF2-mediated neuronal commitment using the global PKC inhibitor GF109203X. Treatment with 5ng/ml FGF2 again resulted in the upregulations of the neuronal intermediate filaments α-internexin and NF-M, and β-tubulin was also upregulated, though not by a significant degree. Upregulations of the neuronal intermediate filaments were abolished by GF109203X, but the combination of FGF2 and GF109203X stimulated upregulation of β-tubulin, suggesting that PKC may play different roles in the FGF2-mediated upregulations of the different classes of neuronal cytoskeletal proteins. Our overall model of FGF2-mediated neuronal commitment is presented in **Figure 24**. We believe that initially high concentrations of FGF2 combined with EGF drive the expansions of progenitor populations. This stage is replicated in our model system during proliferative expansion in the presence of EGF and FGF2. The loss of EGF and a lowered concentration of

FGF2 leads to initial commitment events, including the synthesis and accumulation of neuron-specific intermediate filaments. Proliferation is not hindered at this stage, and this stage is represented in our model system by exposure to 5ng/ml FGF2. The PKC family of proteins is speculated to strongly drive this stage, as evidenced by the ablation of FGF2-mediated effects by GF109203X. The further lowering and eventual ablation of the FGF2 concentration is speculated to drive exit from the cell cycle as well as the construction of axons, finally leaving mature postmitotic neurons. This stage is represented in our model system by exposure to 0-1ng/ml FGF2. The PKC family of proteins is speculated to inhibit this stage of development, as evidenced by the upregulation of β-tubulin in the presence of FGF2 and GF109203X. It was initially speculated that PKC may direct these events through downregulation of the gap junctional protein connexin-43, but this was shown not to be the case.

Future Directions

There are obviously many questions left to be answered concerning the suitability of neurospheres for the production of neuron-enriched cell cultures, the ability of growth factors to drive neuronal commitment in neurosphere cells, and the intracellular signaling mechanisms involved in growth factor-mediated neuronal commitment. Assessments of the expressions of EGF and FGF2 receptors (EGFRs and FGFRs, respectively) under the various growth factor treatments tried would be useful in providing information concerning both the potential reciprocal interactions between these two growth factors as well as possible changes in receptor distribution during treatments.

Investigations of the effects of soluble factors controlling neuronal commitment of neurosphere cells should also include other soluble factors besides EGF and FGF2.

Investigations into the effects of nerve growth factor (NGF), platelet-derived growth factor (PDGF), transforming growth factors α and β (TGFα and TGFβ) as well as the various neurotrophins (NTs) could provide valuable information on the control of neurogenesis by soluble factors. Studies exploring the directional control of commitment to the neuronal phenotype should also include studies that combine outgrowth on specific extracellular matrix protein substrates with specific growth factor regimens. Although the visible extension of cellular processes was seen to occur in intact neurospheres treated to commit to the neuronal phenotype, the upregulations of β-tubulin seen under these conditions were not large, possibly indicating that full axonal development does not occur in the absence of additional signals. Investigations incorporating specific growth factor regimens in combination with adherent outgrowth on immobilized extracellular matrix substrates such as collagen Type I, collagen Type IV, fibronectin, or laminin would allow full complements of commitment-promoting signals to be investigated.

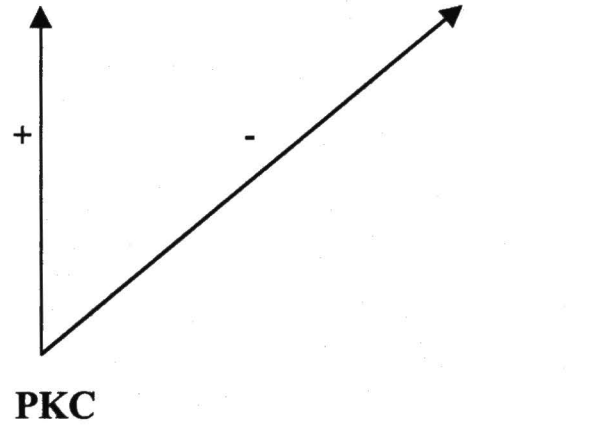
Although downregulation of connexin-43 in response to increased neuronal commitment was not seen, gating of the gap junctions might occur during commitment. We had intended to pursue this hypothesis through cell coupling studies involving microinjection of Lucifer Yellow into individual neurosphere cells, followed by timed tracking of dye transmission to surrounding cells. These studies were ultimately not pursued as microinjection of the unusually small neurosphere cells proved to be too difficult to perform consistently. In addition, the outer surfaces of the neurospheres used for trial experiments appeared to possess a tunic-like structure, which may correspond to either the tenascin-enriched extracellular matrix layer reported by some groups or a layer of adherent heparan sulfate proteoglycans. Penetration of this tunic during microinjection proved to be difficult without damaging the neurospheres, and precise targeting of

individual cell layers became prohibitively difficult. As an indirect way of assessing gap junctional gating, future studies have been designed to address PKC phosphorylation of connexin-43 subunits of gap junctions, which is known to affect gap junctional closure.

It is also worth noting that signaling pathways other than the PKC pathway are no doubt intimately involved in FGF2-mediated neuronal commitment and differentiation. Future investigations should include the cAMP/PKA pathway as well as the MAPK and PI-3K pathways to fully elucidate the cascade of pro-commitment signals generated by FGF2/FGFR interactions. From there, transcriptional control of neuron-specific genes could be investigated, thus leading to a complete picture of FGF2-mediated neuronal commitment and differentiation.

Figure 24: A model of FGF2-regulated control of neuronal commitment in neurosphere cells. The combination of high FGF2 and EGF is speculated to drive the expansion of the progenitor pool. Elimination of EGF and lowering of the FGF2 concentration (to 5ng/ml) results in the initial events of neuronal commitment. Notably, neuron-specific intermediate filaments begin to replace nestin and vimentin, leading to changes in the makeup of the cytoskeleton. At this stage, proliferation is not impaired. Gradually decreasing FGF2 concentrations (down to 0-1ng/ml) lead to the upregulation of β-tubulin, which allows axonal construction to take place. At this point, proliferation begins to decrease leading to exit from the cell cycle and the gradual adoption of the mature, postmitotic neuronal phenotype. It is speculated that PKC stimulates the early commitment event involving neuronal intermediate filament protein upregulation and inhibits the later commitment events involving accumulation of β-tubulin and construction of microtubule-supported axons.

High FGF2 + EGF	Moderate FGF2 (5ng/ml)	Low FGF2 (0-1ng/ml)
Expansion of progenitor pool	Early commitment events	Late commitment events
Nestin/Vimentin predominate as intermediate filaments	Neuronal intermediate filaments begin to accumulate	Axonal proteins upregulate
Immature cell body No neuronal cellular extensions	Changes in cell body	Cellular extensions
Proliferative	Proliferative	Proliferation ↓



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