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Growth Factor Regulation of Cell Growth and Proliferation in the Nervous System

A New Intracrine Nuclear Mechanism

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Abstract

This article discusses a novel intracrine mechanism of growth-factor action in the nervous system whereby fibroblast growth factor-2 (FGF-2) and its receptor accumulate in the cell nucleus and act as mediators in the control of cell growth and proliferation. In human and rat brain the levels and subcellular localization of FGF-2 differ between quiescent and reactive astrocytes. Quiescent cells express a low level of FGF-2, which is located predominantly within the cytoplasm. In reactive astrocytes, the expression of FGF-2 increases and the proteins are found in both the cytoplasm and nucleus. In glioma tumors, FGF-2 is overexpressed in the nuclei of neoplastic cells. Similar changes in FGF-2 expression and localization are found in vitro. The nuclear accumulation of FGF-2 reflects a transient activation of the FGF-2 gene by potentially novel transactivating factors interacting with an upstream regulatory promoter region. In parallel with FGF-2, the nuclei of astrocytes contain the high-affinity FGF-2 receptor, FGFR1. Nuclear FGFR1 is full length, retains kinase activity, and is localized within the nuclear interior in association with the nuclear matrix. Transfection of either FGF-2 or FGFR1 into cells that do not normally express these proteins results in their nuclear accumulation and concomitant increases in cell proliferation. A similar regulation of nuclear FGF-2 and FGFR1 is observed in neural crest-derived adrenal medullary cells and of FGF-2 in the nuclei of cerebellar neurons. Thus, the regulation of the nuclear content of FGF-2 and FGFR1 could serve as a novel mechanism controlling growth and proliferation of glial and neuronal cells.

Index Entries: FGF-2; FGF receptor; nuclear localization; cell plasticity; astrocytes; glioma cells.

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Introduction

Cellular growth, proliferation, and differentiation are fundamental processes underlying both development and the regeneration or remodeling of tissues in the mature organism. When perturbed, these processes may lead to the uncontrolled growth that underlies the neoplastic state. The execution of genetic programs for cell growth, proliferation and differentiation is controlled by cell-surface molecules that signal direct contact with other cells and with extracellular matrix. Receptors that interact with soluble growth factors and cytokines are also involved (for reviews, *see* Pardee, 1989; Bishop, 1991; Yamamori, 1992; Hartwell and Kastan, 1994). Expression of these signaling molecules at specific windows of time is thought to control cellular development during ontogeny and reactivation of cellular growth or proliferation in the adult organism (Bogler et al., 1990; Vescovi et al., 1993; Ross 1996). Their expression and functions must be precisely controlled to allow the required degree of plasticity and yet to prevent the uncontrolled proliferation observed in transformed cells. Neoplastic cells proliferate continuously; their response to external cues such as cell-contact inhibition is diminished and their requirement for exogenous growth factors is reduced (Rosenblum et al., 1978). The abnormal expression and/or function of cell-surface signaling molecules could mediate this neoplastic phenotype.

Fibroblast growth factor-2 (FGF-2, bFGF), a member of the family of heparin-binding growth/neurotrophic factors, is one such key signaling protein (Walicke, 1988; Wagner, 1991). FGF-2 is expressed in developing nervous tissue (Ernfors et al., 1990; Grothe and Unsicker, 1990; Grothe et al., 1991). It stimulates the proliferation and differentiation of neuroblasts (Gensburger et al., 1987; Mayer et al., 1993; Stemple et al., 1988; Vescovi et al., 1993), affects their ontogenic death (Dreyer et al., 1989), and promotes neurite outgrowth (Hatten et al., 1988; Gurney et al., 1992) and synapse formation (Peng et al., 1991). FGF-2 also affects the proliferation and differentiation of glial precursor cells (Engele and Bohn, 1992; Vescovi et al., 1993) and stimulates the proliferation of vascular endothelial and smooth muscle cells (Hayek et al., 1987). In some regions of the nervous system, FGF-2 is expressed during adulthood (Woodward et al., 1992; Grothe et al., 1991).

The functions of FGF-2 in mature nervous tissue are still contested. FGF-2 may participate in the response of nervous tissue to injury (Kniss and Burry, 1988) and can enhance the regeneration of damaged neuronal pathways (Otto and Unsicker, 1990). FGF-2 may also act as a neuroprotective agent, increasing the survival of neurons during trans-synaptic stimulation or hypoxia (Nozaki et al., 1993; Grothe and Meisinger, 1997). Afferent stimulation of nervous tissue leads to a structural reorganization similar to that which occurs during development (i.e., proliferation of glial and neuronal cells, expansion of neuritic arbors, formation of new synapses and angiogenesis [Bailey and Kandel, 1993]). These changes could reflect a mobilization of intrinsic growth factors like FGF-2.

In glial cells, episodes of growth and differentiation occur during both development and adulthood. During pre- and early postnatal life, astrocytic precursors actively divide, migrate and differentiate into stationary, quiescent cells (McConnell, 1988). In response to cell atrophy or degeneration, astrocytes in the mature brain become hypertrophic and proliferate until the vacated space is filled (Eng et al., 1992; Liu and Chen, 1994; McMillian et al., 1994). Astrocytic activation also occurs in response to physiological simulation and supports the adaptive plasticity of neural tissue and learning (Anderson et al., 1994; Eng et al., 1992). FGF-2 and its receptors may participate in both astrocytic development and in the plasticity of mature astrocytes. In vitro, FGF-2 stimulates the proliferation of neonatal rat astrocytes (Kniss and Burry, 1988; Arajuo and Cotman, 1992; Engele and Bohn, 1992). It also induces neurotrophic factors and glial fibrillary acidic protein (GFAP), characteristics of reactive astrocytes (Morrison et al., 1985).

During gliosis, the number of astrocytes in the mature rat brain that expresses FGF-2 increases markedly along with intracellular levels of FGF-2 protein and mRNA and correlates with their proliferation (Finkelstein et al., 1988; Frautschy et al., 1991; Liu and Chen, 1994; Gomez-Pinilla et al., 1992; Chadi et al., 1994).

FGF-2 also appears to play a role in the neoplastic transformation of astrocytic cells. FGF-2 is overexpressed in certain gliomas, and its overexpression correlates with the grade of the tumor and the extent of anaplasia (Zagazag et al., 1990). Glioma cells bear FGF receptors and their attachment-independent proliferation is inhibited by FGF-2-specific antisense oligonucleotides (Liberman et al., 1987; Takahashi et al., 1990; Morrison, 1991; Morrison et al., 1994a). The differential splicing of FGF receptors in transformed glia and its contribution to the neoplastic phenotype were reviewed recently (Morrison et al., 1994c) and are not discussed here.

Even though the effects of FGF-2 are well established, the mechanisms by which FGFs and other neurotrophic growth factors produce their biological effects are not completely understood (Mason, 1994). An autocrine loop involving extracellular FGF-2 and membrane FGF receptors has been proposed to explain how FGF-2 stimulates glioma cell proliferation (Liberman et al., 1987). This model is based on the classical theory of signal transduction according to which growth factors are released into the extracellular space and affect cell growth/proliferation by interacting with receptors located in the plasma membrane (Fantl et al., 1993). The only role of membrane-associated receptors is to transmit signals across the cell membrane. Other cytoplasmic proteins and kinases serve as second messengers and propagate the signal downstream to the nucleus.

However, not all the biological effects of growth factors may be produced in this manner. A number of laboratories have reported that peptide growth factors are internalized along with their membrane receptors, suggesting that their actions may continue inside cells (Gorden et al., 1980; Mason, 1994; Jans, 1994). The stimulation of astrocytic proliferation by exogenous FGF-2 could occur through this mechanism (Joy et al., 1997). In addition, reports of an association of growth factors (EGF, PGDF, FGFs, NGF) and hormones or neurotransmitters (growth hormone, insulin, angiotensin II, proenkephalin, or VIP) with the cell nucleus (Burwen and Jones, 1987; Jans, 1994) raise the intriguing question of whether these polypeptides may signal directly from the extracellular environment to the genetic machinery of the cell. The observation that exogenous FGF-2 accumulates in the nuclei of endothelial cells in a cell cycle-dependent manner strongly supports such a mechanism (Bouche et al., 1987; Baldin et al., 1990).

Traditionally, the functions of FGF-1 or FGF-2 have been investigated by adding FGFs to the culture medium. However, neither FGF-1 nor any of the four amino-terminal extended isoforms of FGF-2 contain a secretory signal sequence (Abraham et al., 1986a,b; Florkiewicz and Sommer, 1989; Jaye et al., 1986). Accordingly, only low levels of these types of growth factors are detected outside most cells, whereas large amounts are detected in the cytoplasm and nucleus (Schweigerer et al., 1987; Vlodawski et al., 1987; Moscatelli, 1988; Brigstock et al., 1991; Florkiewicz et al., 1991; Gualandris et al., 1993; Stachowiak et al., 1994). Indeed, the externalization of these growth factors may be a rare event associated with cell injury or death (Schechter, 1992). With the observation that FGF-2 is primarily cell-associated, the regulated nuclear targeting of cytoplasmic FGF-2 to the nucleus by heterologous stimuli found in our laboratory suggests that such growth factors may act as intracrine-signaling molecules (Puchacz et al., 1993; Stachowiak et al., 1994; Moffett et al., 1996; Joy et al., 1997). The direct transfer of FGF-1 and FGF-2 from the cytoplasm to the nucleus is consistent with the presence of a nuclear localization signal in the protein (Courdec et al., 1991; Imamura et al., 1990). Supporting the idea that FGFs can act intracellularly are studies that show that FGF-2 does not need to be secreted to stimulate proliferation of fibroblasts (Ray et al., 1995; Bikfalvi et al.,

1995) or differentiation of avian Schwann cells (Sherman et al., 1993). Furthermore, FGF-2 added to isolated nuclei stimulates rRNA synthesis (Bouche et al., 1987) and affects in vitro gene transcription in nuclear extracts (Nakanishi et al., 1992). The mitogenic action of exogenous FGF-1 on fibroblasts also requires the nuclear translocation of the growth factor and can be separated from its ability to stimulate cell-membrane receptors (Imamura et al., 1990; Wiedlocha et al., 1994; 1996).

Until recently, little was known about the mechanisms that control the nuclear accumulation of growth factors or their nuclear function. We have approached these questions using as a model cells of glial and neuronal lineages in which FGFs stimulate cell growth and proliferation. This article summarizes our investigations on the function of FGF-2 in the reactive and neoplastic transformation of human astrocytes. We describe a novel signaling mechanism by which FGFs and other growth factors may control cell growth and proliferation. This new signaling mechanism operates in a regulated manner in both glial and neuronal cells, but it appears to be deregulated in neoplastic glia (Puchacz et al., 1993; Stachowiak et al., 1994; 1996a,b; 1997; Moffett et al., 1996; Joy et al., 1997). Further elucidation of this process will foster a better understanding of the epigenetic control of development, regeneration, learning, and long-term adaptation in the human nervous system. It may also help to explain the uncontrolled growth of human brain neoplasms and help to develop novel therapeutic strategies for their treatment.

Proliferation and Growth of Human Astrocytes and Glioma Cells Associated with the Nuclear Accumulation of FGF-2

Immunohistochemical examination of normal and gliotic brain tissues and glioblastoma multiforme (GBM) tumors revealed that the content of FGF-2 is increased in reactive astrocytes of the mature human brain, and has confirmed earlier reports that human glioma tumors overexpress FGF-2 (Zagazag et al., 1990; Paulus et al., 1990; Takahashi et al., 1990). The most intriguing finding, however, was that subcellular localization of FGF-2 in quiescent astrocytes differs from that in reactive or neoplastic astrocytes (Joy et al., 1997). In normal tissue from the lateral temporal lobe, stellate astrocytes demonstrate cytoplasmic FGF-2 immunoreactivity (FGF-2-IR). Large neurons also stain diffusely for FGF-2 in the perinuclear cytoplasm. In contrast, reactive astrocytes in the hippocampal region of patients with sclerosis associated with epilepsy display prominent nuclear FGF-2 immunostaining. The intensity of nuclear staining is greatest in areas of severe cell loss. The increased size, enlarged nuclei, and nonstellate morphology of the human astrocytes expressing nuclear FGF-2 resemble reactive proliferating astrocytes in the gliotic tissue of the rat brain (Liu and Chen, 1994). In GBMs, most neoplastic cells display enhanced FGF-2 staining (Joy et al., 1997). As in reactive astrocytes, FGF-2-IR is most intense in the nuclei of glioma cells. Similar to Paulus et al. (1990), we detected no FGF-2-IR in the extracellular space. These findings are consistent with the proposed role of FGF-2 in astrocytic activation (Morrison et al., 1985) and in the malignant progression of gliomas (Liberman et al., 1987; Morrison et al., 1985; 1994b; Zagazag et al., 1990) but suggest an intracrine-nuclear mechanism of growth factor action.

To identify what triggers the nuclear accumulation of FGF-2 in gliotic tissue and in GBM as well as the function of nuclear-growth factor, we developed cultures of astrocytes from adult human brain and used established glioma cell lines. Waymouth 87/3 medium (6) supplemented with 10% serum supported the proliferation of the adult human astrocytes in culture while suppressing the growth of oligodendroglial and endothelial cells. We obtained pure human astrocytic cultures as evidenced by the expression of GFAP by all cells and the lack of expression of galactocerebroside, an

oligodendroglial marker (Moffett et al., 1996a; Joy et al., 1997). These cultures provide a new in vitro model for studying the cycle of growth, proliferation, and differentiation of astrocytes from mature human brain.

Astrocytes maintained in high-density cultures formed a confluent monolayer of quiescent cells with defined processes similar to astrocytes in control brain tissue. After the cell density was reduced, events paralleled the in vivo transition from quiescent to reactive astrocytes. Cells appeared enlarged and reentered a proliferative phase, as detected by incorporation of bromouridine deoxyribose (Joy et al., 1997). Cell hypertrophy and proliferation lasted only until a new confluent state was reached. Similar to what was found in vivo, the induction of nuclear FGF-2 was observed. Before reaching a confluent state, proliferating astrocytes exhibited intense FGF-2-IR within nuclei and nucleoli (Fig. 1A [I]; Joy et al., 1997). As the astrocytes began to extend processes and contact one another, the intensity of the nuclear FGF-2 staining began to decrease (Fig. 1A [II]). The depletion of nuclear FGF-2-IR was reversed by dislodging some cells from the confluent monolayer. In the empty areas, astrocytes were again found with increased expression of nuclear and nucleolar FGF-2 and renewed proliferation (Fig. 1A [III]). Astrocytes that attained a confluent state in the higher-density cultures had a partially depleted nuclear FGF-2 content by 3 wk after plating and were completely depleted at 4 wk. The reduction in the nuclear content of FGF-2 was delayed in astrocytes plated at lower density. The depletion of nuclear FGF-2 was accompanied by a less marked reduction in the cytoplasmic content of FGF-2. Thus, FGF-2 depletion correlated with cell density but was independent of time in culture. The depletion of nuclear FGF-2 in confluent astrocytes was confirmed by confocal microscopy and by Western-blot analysis of biochemically fractionated astrocytes (Joy et al., 1997). The Western analysis showed that the nuclear and cytoplasmic fractions contained both the low and high molecular weight FGF-2 isoforms known to be generated from alternate utilization of CUG and AUG translation initiation codons. This finding is consistent with observations made with other cell types (Florkiewicz and Sommer, 1989; Powell and Klagsbrun, 1991).

A cell density-dependent gradient of FGF-2 expression was observed within the same culture dish, suggesting that the loss of nuclear FGF-2-IR is established by cell-cell contact rather than induced by diffusable agents (Fig. 1B; Joy et al., 1997). Furthermore, when the medium from subconfluent astrocytic cultures was added to confluent cells, it did not affect the pattern of FGF-2 expression. The addition of serum or growth factors also failed to restore FGF-2 expression in the confluent astrocytic monolayer, although these treatments increased the expression of FGF-2 in subconfluent astrocytes. However, the addition of nonastrocytic cells inhibited FGF-2 gene expression in subconfluent astrocytes (Moffett et al., 1996a). These results, along with the correlation between the induction of nuclear FGF-2 in astrocytes in vivo and the extent of degeneration of surrounding cells in human brain tissue, indicate that reduced cell contact may underlie FGF-2 induction in reactive astrocytes (Moffett et al., 1996a; Joy et al., 1997).

The changes in FGF-2 content in confluent astrocytes are not simply a consequence of the cessation of cell division. In subconfluent astrocytes arrested in serum-free medium, expression of FGF-2 or its gene promoter activity was only partially reduced. This depletion was reversed by serum, growth factors, or phorbol ester PMA (Moffett et al., 1996b). In contrast, the cell density-dependent depletion of FGF-2 and the inhibition of FGF-2 gene activity (*see* Subheading entitled Mechanisms Controlling the Expression of FGF-2 and FGFR1 in Glial Cells) was greater than 95% and could not be reversed by any of these agents. Further studies have shown that the molecular mechanisms underlying the loss of FGF-2 expression in confluent astrocytes are different from those underlying its partial depletion in serum-deprived subconfluent astrocytes (Moffett et al., 1996b). The studies indicated that the

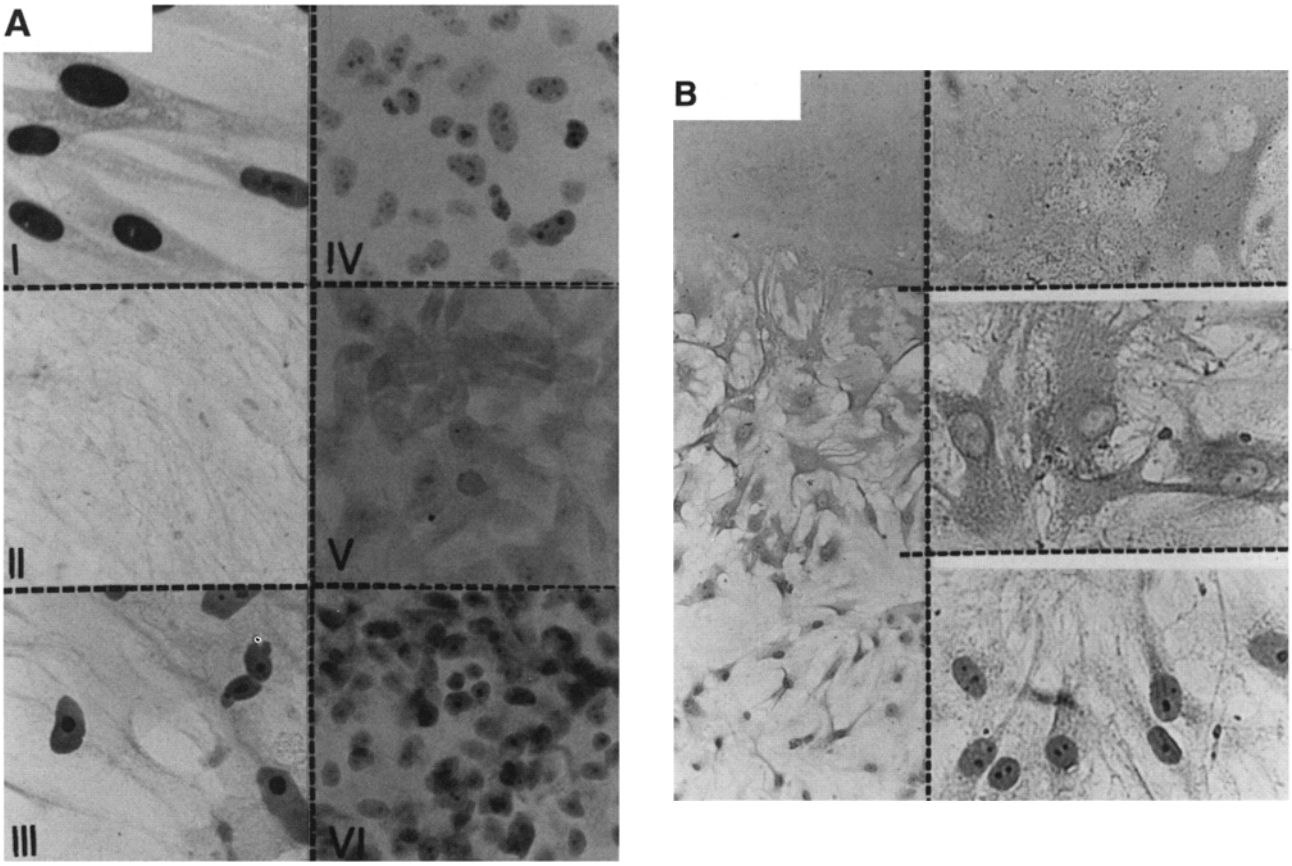


Fig. 1. Cell density-dependent inhibition of FGF-2 expression in human astrocytes and its impairment in glioma cells. Cells were stained with monoclonal antibodies against FGF-2. **(A)** Expression of FGF-2 in astrocytes (IG strain) and U251MG glioma cells. (I) subconfluent astrocytes 1 wk after plating; (II) confluent astrocytes 3 wk in culture; (III) subconfluent astrocytes 5 wk after plating. Confluent astrocytes detached leaving large empty surfaces. Remaining astrocytes show increased expression of nuclear and nucleolar FGF-2. Some show karyokinesis indicating renewed proliferation. (IV, V and VI) glioma U251MG cells 1, 3, and 5 wk after plating, respectively. At 3 wk cells show a small reduction in FGF-2-IR; at 5 wk an additional cell layer formed with intense nuclear FGF-2-IR. **(B)** Gradient of FGF-2 expression in astrocytes (IG) as a function of cell density in a single culture dish (Original magnification 400×). Reproduced from Joy et al., (1997) *Oncogene* **14**, 171–183, with kind permission of Stockton Press, Division of Macmillan Press Ltd., U.K.

FGF-2 gene is in distinct functional states in contact-inhibited and in subconfluent astrocytes arrested in serum-free medium.

Deregulation of FGF-2 Expression in the Nuclei of Glioma Cells

In contrast to the transient and controlled proliferation of astrocytes, neoplastic glioma cells proliferate continuously, showing a diminished response to cell-contact inhibition. After confluent monolayers form in cultures, glioma cells continue to produce additional layers. Cells that attain confluence show only a small reduction in nuclear and cytoplasmic FGF-2-IR (Fig. 1A [IV,V]). With additional time in culture, new cell layers that show intense nuclear and nucleolar FGF-2-IR form (Fig. 1A[VI]). Similarly, Western-blot analysis of cellular fractions detected no reduction in nuclear FGF-2 as the U251MG glioma culture progressed from a subconfluent to a confluent state (Joy et al., 1997).

Discovery of Nuclear FGF-2 Receptors

To determine whether the nucleus may contain functional FGF receptors (FGFR) capable of interacting with FGF-2, we examined the binding of ^{125}I-FGF-2 to subcellular fractions obtained from U251MG glioma cells. The cell surface has both high- and low-affinity binding sites for ^{125}I-FGF-2 (Kd = 6 pM and 17 nM, respectively). Low- and high-affinity binding sites were also observed within the cytoplasmic fraction, whereas only high-affinity binding sites were found in the nuclear fraction (Stachowiak et al., 1996b; 1997).

The high-affinity FGFRs are encoded by four genes that share a high degree of homology (for reviews *see* Johnson and Williams, 1993; Wilkie et al., 1995). Each FGFR gene encodes an extracellular region comprised of two or three immunoglobulin (Ig)-like domains that contain the growth-factor binding site, a transmembrane domain, an intracellular tyrosine kinase domain, and a C-terminal domain.

To determine the types of high-affinity FGFR expressed in astrocytes and in U251MG glioma cells, FGFRs were immunoprecipitated from total-cell extracts with antibodies specific to the C-terminal domains of FGFR1-4. The immunoprecipitates were then subjected to Western analysis with a monoclonal FGFR antibody (McAb6; Hanneken, et al., 1995) that recognizes a common sequence in the extracellular domains of FGFR1–4 (Maher, 1995; Stachowiak et al., 1996a; 1997). The results showed that FGFR1 is the predominant FGFR in both the glioma and astrocytic cells. Three isoforms of FGFR1 (103, 118, and 145 kDa) were observed and all contained both the C-terminal and N-terminal domains (Stachowiak et al., 1995a; 1996; 1997). The nuclei of astrocytes (Fig. 2A) or glioma cells (Fig. 2B) also contain the 103-, 118-, and 145-kDa isoforms of FGFR1. The size of the 103-kDa FGFR1 is similar to the largest FGFR1 isoform predicted by the cDNA (approx 96 kDa; Dionne et al., 1991; Hou et al., 1991), but it is smaller than fully glycosylated receptor (approx 150 kDa, Xu et al., 1992). Treatment of nuclear extracts with N-glycanase converted the 103-, 118-, and 145-kDa forms into a single 100-kDa protein (Fig. 2C), which is consistent with the size of the three Ig-like loop form of FGFR1 (Xu et al., 1992). Furthermore, when the blots were probed with a C-term FGFR1 Ab that interacts preferentially with hypoglycosylated receptor (Maher, 1996), an intense 103-kDa band and weak 118- and 145-kDa bands were seen. Treatment with N-glycanase only lightly affected the migration of the 103-kDa band. Thus, the 103-kDa protein appears to be non- or hypoglycosylated FGFR1 isoform, whereas the 118- and 145-kDa proteins may represent hyperglycosylated isoforms of FGFR1.

In astrocytes, the majority of FGFR1 was associated with the nuclear fraction. Only trace amounts of FGFR1 were detected in the cell membrane or cytoplasmic fractions (Fig. 2A). Thus, in cultured human astrocytes, the nucleus is the main organelle containing FGFR1. Similar results were obtained with U251MG glioma cells (Fig. 2B; Stachowiak et al., 1997). Nuclear FGFR1 binds ^{125}I-FGF-2 and retains tyrosine kinase activity. Incubation of immunoprecipitated nuclear FGFR1 with gamma ^{32}P-ATP resulted in three phosphorylated bands similar in size to the FGFR1 bands detected by immunoblotting. Immunohistochemistry, in combination with confocal microscopy, revealed weak cytoplasmic FGFR1 immunoreactivity (IR) in astrocytes and U251MG glioma cells (Fig. 3). In some cells, the FGFR1 was associated with the plasma membrane. Both astrocytes and U251MG glioma cells also showed a distinct nuclear presence of FGFR1-IR. The pattern of immunofluorescence observed in a series of consecutive confocal sections through cells showed that FGFR1 is present within the interior of the nucleus (Stachowiak et al., 1995a,b; 1996a; 1997). The intranuclear localization of FGFR1 was also demonstrated by immunoelectron microscopy that suggested the translocation of FGFR1 through the nuclear membrane and demonstrated its accumulation inside the nucleus (Stachowiak et al., 1996b). FGF-2-IR and FGFR1-IR were colocalized in the nuclei of

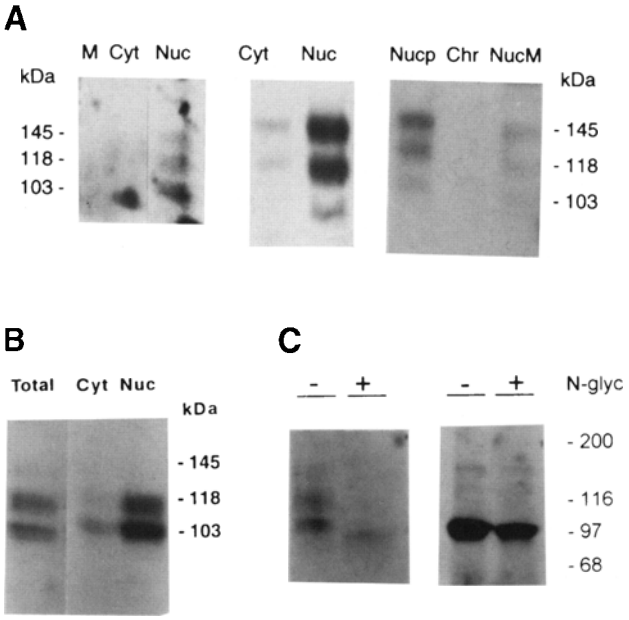


Fig. 2. Subcellular distribution of FGFR1—Western analysis: **(A)** FGFR1 proteins in cell membrane (M), cytoplasmic (Cyt), and nuclear (Nuc) fractions of human astrocytes from QG patient (left panel) and in cytoplasmic and nuclear (middle panel) and subnuclear fractions, (right panel: nucleoplasm—Nucp, chromatin—Chr, nuclear matrix—NucM) of TM patient (panels show independent experiments). 50 µg protein from each fraction were subjected to Western-blot analysis with McAb6 and ^{125}I-protein A (reproduced from *Mol. Brain Res.* **38**, 161–165, 1996, with kind permission from Elsevier Science-NL, Sara Burgerharstraat 25, 10055, KV Amsterdam, the Netherlands). **(B)** (Reproduced from Stachowiak et al., *Oncogene*, **14**, 2201–2211, 1997 with kind permission of Stockton Press.)—FGFR1 in total cell lysates and in cytoplasmic and nuclear fractions from U251MG glioma cells. Each lane contains 50 µg of protein. **(C)** Identification of glycosylated isoforms of nuclear FGFR1. Nuclear extracts from U251MG cells were incubated overnight at 37°C with (+) or without (–) N-glycanase (Genzyme) according to the manufacturer's instructions and were subjected to Western-blot analysis with McAb6 (left panel), which recognizes all glycosylated FGFR1 isoforms, or with the C-term FGFR1 Ab (right panel), which detects predominantly hypoglycosylated FGFR1.

the same cells, indicating that FGFR1 may act as an effector for nuclear FGF-2.

How FGFR1 enters the nucleus is unknown. However, labeling the surface of U251MG cells with an impermeable biotin analog followed by extended incubation at 37°C did not result in any biotin-labeled FGFR1 in the nucleus (P. A. Maher and M. K. Stachowiak, in preparation). Thus, FGFR1 can move to the nucleus without prior insertion into the plasma membrane. Immunoelectron microscopy suggests that FGFR1 is transferred into the nucleus at discrete sites along the nuclear membrane, consistent with transport through nuclear pores. Since FGFR1 lacks typical nuclear localization signal (NLS)-like clusters of basic amino acids, the movement of FGFR1 into the nucleus may be mediated by a chaperone protein, such as FGF-2, that contains a NLS. FGFR1 could also be transferred via a NLS-independent transport system such as the one responsible for the nuclear importation of glycosylated proteins in a sugar-dependent manner (Duverger et al., 1995).

The Nuclear Presence of FGFR1 in Astrocytes Is Coregulated with FGF-2 and Is Constitutive in Glioma Cells

Initially, the reduction in the nuclear content of FGFR1 concomitant with the increase in cell

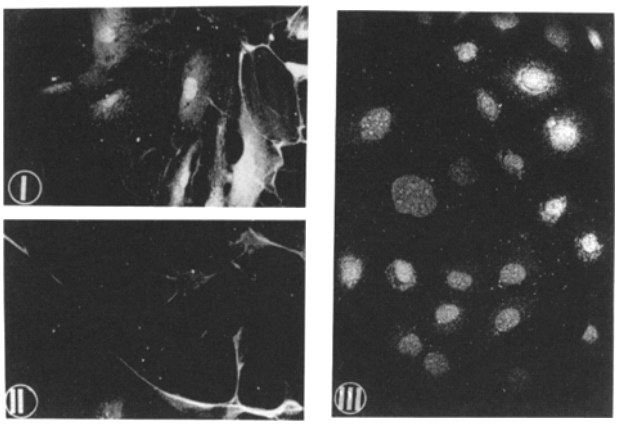


Fig. 3. Immunocytochemical localization of FGFR1 in human astrocytes (I,II) and U251MG glioma cells (III). Cells were incubated with the C-term FGFR1 Ab and the immune complexes stained with CY^3-conjugated anti-rabbit IgG. Preincubation of the C-term FGFR1 Ab (2 μg/mL) for 2 h at 4°C with the FGFR1 C-terminal peptide (20 μg/mL) abolished nuclear FGFR1 immunofluorescence (II). Photographs represent stacked confocal laser sections taken 1 micron apart. Controls for the specificity of the nuclear FGFR-1 staining were documented in (Stachowiak et al.,1996a,b; 1997)

density and the reduction in cell proliferation was detected by immunostaining. Proliferating, subconfluent astrocytes exhibited intense nuclear FGFR1 immunoreactivity (FGFR1-IR) (Fig. 4A [I]). When a confluent monolayer covered the dish, nuclear FGFR1-IR could no longer be detected in the astrocytes (Fig. 4A [II]). As with FGF-2, the depletion of nuclear FGFR1 was reversible. In the vacant surfaces left by detached, confluent astrocytes, we observed nuclear FGFR1-IR in astrocytes invading those areas. Nuclear FGFR1 content increased between 6 and 18 h after cells were replated at a subconfluent density (Fig. 4B). The nuclear accumulation of FGFR1 was accompanied by an increase in FGFR1-associated kinase activity (Stachowiak et al., 1997).

The time course of FGFR1 accumulation was similar to that of FGF-2 although the ligand appeared to begin to accumulate in the nucleus somewhat earlier than the receptor. The expression of nuclear FGF-2 and FGFR1 in astrocytes is regulated in a manner reminescent of cyclin D1. Thus, these three proteins are depleted in confluent astrocytes arrested in G_0 phase, and their levels are restored transiently in G_1 phase. The maximal increases occur 6–18 h after the release of cell-contact inhibition but before the cell division (M. K. Stachowiak and E. K. Stachowiak, in preparation).

In contrast to astrocytes, U251MG glioma cells showed only a small reduction in nuclear FGFR1-IR in the confluent state when compared to subconfluent cells (Fig. 4A [III,IV]). The impaired cell density-dependent depletion of nuclear FGFR1 in glioma cells was confirmed by Western-blot analysis (Stachowiak et al., 1997).

Expression of Nuclear FGF-2 and FGFR1 Stimulated by Growth Factors and cAMP/PKC-Signaling Pathways

In addition to a decrease in direct cell-contact, growth factors and cytokines secreted by glia, neurons, blood-borne macrophages, and monocytes facilitate reactive transformation of astrocytes. EGF and interleukin-1 increase the levels of FGF-2-like-IR in rat astrocytic cultures (Arajuo and Cotman, 1992). We found that treatment of subconfluent serum-free human astrocytic cultures with serum or specific growth factors, such as PDGF, angiotensin II, 18-kDa FGF-2 (Fig. 5A) or EGF, (not shown) that act in the brain as local autocrine/paracrine mitogenic agents increases

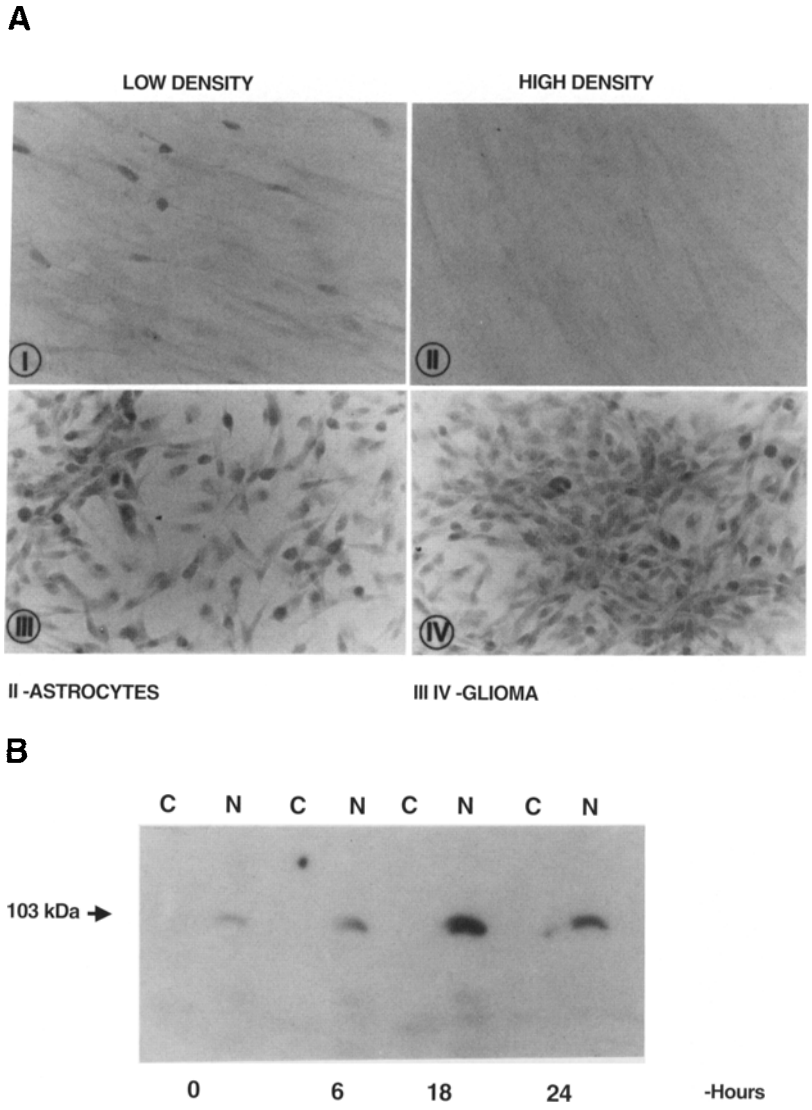


Fig. 4. Inhibition of nuclear FGFR1 expression in confluent astrocytes and its attenuation in glioma cells. **(A)** Immunocytochemical analysis of FGFR1. (I) subconfluent astrocytes (1 wk in culture) show nuclear FGFR1-IR; (II) in confluent astrocytes (3 wk) no nuclear FGFR1 staining is detected. (III) subconfluent (1 wk in culture) and (IV) confluent (3 wk in culture) glioma cells show similar levels of FGFR1 staining. (B) Time-dependent nuclear accumulation of FGFR1 after the reduction of cell density. Astrocytes were maintained in a confluent state for 48 h. Some cultures were replated at a lower density (approx 30% of confluence). Equal amounts of nuclear (N) and cytoplasmic (C) extracts were analyzed for FGFR1 by Western blotting with the C-term FGFR1Ab that recognizes hypoglycosylated FGFR1. Reproduced from Stachowiak et al. (1997) *Oncogene*, **14**, 2201–2211, 1997, with kind permission of Stockton Press.

the intracellular content of all translational isoforms of FGF-2 (Moffett et al., 1996b). Astrocytes are affected by a number of hormonal and neurotransmitter receptors linked to common cAMP- and protein kinase C (PKC)-dependent signaling pathways. Treatment of astrocytes with angiotensin II, with forskolin to directly activate adenyl cyclase, or with the phorbol ester, PMA, to directly activate PKC increased the content of all FGF-2 isoforms (Moffett et al., 1996b).

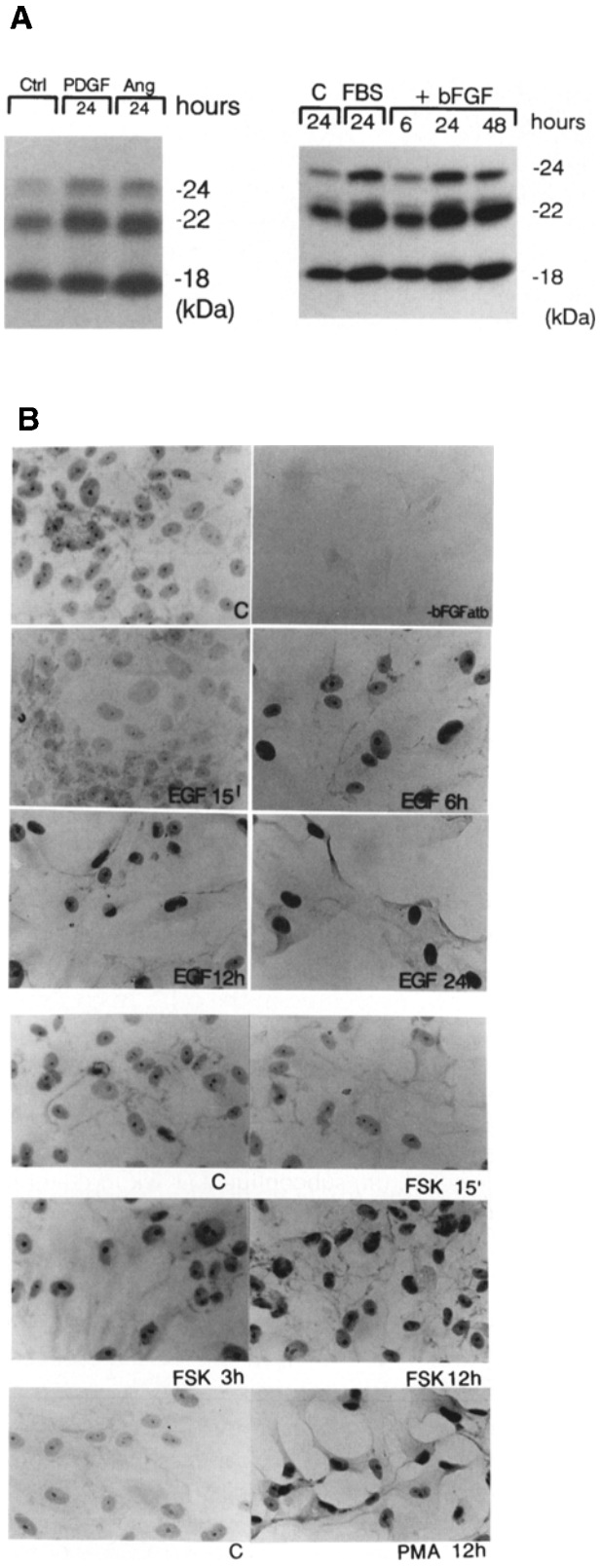


Fig. 5. **(A)** Serum and growth factors increase the intracellular content of FGF-2 in human astrocytes. Addition of 10% fetal bovine serum (FBS), 0.5 n*M* 18-kDa FGF-2 (bFGF), 0.1 n*M* PDGF, or 0.2 µ*M* angiotensin II (Ang) to subconfluent astrocytic cultures increases the cellular content of all translational isoforms of FGF-2 when compared to serum-free control (C or Ctrl) cultures (Western analysis, Moffett et al., 1996b). Scanning of the autoradiograms from 2 to 3 independent experiments showed a 2.5- to 4.5-fold increase in FGF-2 content (M. K. Stachowiak and R. Z. Florkiewicz, unpublished observations). **(B)** Increase in nuclear FGF-2-IR in astrocytes treated with (1) EGF, (2) forskolin (5 µ*M*) or (3) PMA (0.1 µ*M*). **(C)** Treatment with forskolin or PMA increases nuclear FGFR1-IR in human astrocytes. The specificity of the FGF-2 and FGFR1 immunostaining was documented in (Stachowiak et al., 1994; 1996a,b; 1997; Joy et al., 1997).

Western-blot analysis did not detect FGF-2 in the cell culture medium. Immunocytochemistry (Fig. 5B) showed that FGF-2-IR increased predominantly in the nuclei, further supporting our hypothesis that FGF-2 acts as an intracrine nuclear factor in astrocytes (Moffett et al., 1996b; Stachowiak et al., 1996a; Joy et al., 1997). Treatment of astrocytic cultures with forskolin, PMA (Fig. 5C), or serum (not shown) also increases nuclear FGFR1-IR, further demonstrating that the nuclear content of FGF-2 and FGFR1 is regulated in a coordinated manner.

Intracellular FGF-2 and FGFR1 Stimulate Proliferation of Glial Cells

To determine whether nuclear FGF-2 controls cell proliferation, we identified two glioma cell lines, U251MG and SF-767, that are not normally stimulated by exogenous FGF-2 (Joy et al., 1997). Both cell lines were transfected with plasmids that express all FGF-2 isoforms (CMV-bFGF); 18-kDa FGF-2 (CMV-18); only high molecular weight (22-, 23-, and 24-kDa) FGF-2 (CMV-HMW). The latter isoforms do not associate with the plasma membrane and are not found outside of cells (Florkiewicz et al., 1991; Bikfalvi et al., 1995). After reaching a high density, the proliferation of U251MG cells transfected with control CMV-Neo plasmid slowed (Fig. 6A). This inhibition of proliferation was attenuated in cells transfected with any of the three FGF-2-expressing plasmids. This finding suggests that the depletion of nuclear FGF-2 could account for the slowing of proliferation in cells attaining confluence. The rate of proliferation of the SF-767 glioma cell line when transfected with CMV-bFGF, CMV-18, or CMV-HMW also increased compared with CMV-Neo-transfected cells (Joy et al., 1997). Proliferation increased despite the lack of detectable FGF-2 in the medium. Proliferation of SF-767 cells expressing high-molecular-weight isoforms of FGF-2 was resistant to the cell-impermeable FGF-2-binding antagonist, inositol hexakis phosphate (IP6; Morrison et al., 1994a) (Joy et al., 1997). Immunocytochemistry revealed that FGF-2 accumulates predominantly in the nuclei of FGF-2-transfected U251MG or SF-767 cells. In contrast, in another glioma cell line, SF-763, transfected FGF-2 accumulated only in the cytoplasm (Joy et al., 1997) and cell proliferation did not increase. Together, these findings strongly suggest that intracellular/nuclear FGF-2 stimulates the proliferation of glial cells.

To address the role of nuclear FGFR1 in cell proliferation, we transfected SF-763 glioma cells with a plasmid containing the full coding region of FGFR1 under the control of the CMV promoter and examined the proliferation rate in the absence or presence of IP6. Unlike the U251MG cells, the SF-763 glioma cells express FGFR2 and FGFR3 but not FGFR1, consistent with previously reported differences in FGFR expression in human glioma tumors (Morrison et al., 1994b). As determined by both Western blotting (Fig. 6B) and immunohistochemistry, transfected-recombinant FGFR1 accumulated in the nuclear fraction, whereas cells transfected with vector alone showed no specific staining for FGFR1 (Stachowiak et al., 1997). This outcome suggests that the full-length receptor can translocate to the nucleus. In contrast, Johnston et al. (1995), who found FGFR3 in the nuclear fraction of breast cancer cell lines, suggested that this association might be caused by a deletion of its transmembrane domain. The expression of FGFR1 in the nucleus of transfected SF-763 glioma cells was accompanied by an increase in the rate of cell proliferation in the absence of exogenous FGF-2 (Fig. 6B). IP6 inhibited the proliferation of the vector-transfected cells (IC_{50} = 0.23 m*M*), suggesting that they proliferate in response to an extracellular growth factor that acts through cell-surface FGFR (Stachowiak et al., 1997). Since we could not detect FGF-2 in the medium conditioned by SF-763 cells, other members of the FGF family may be responsible for this activity and may act through FGFR2 and/or FGFR3 expressed in the cells. In contrast to the vector-transfected cells, the spontaneous proliferation of the FGFR1-transfected cells was resistant to IP6 (IC_{50} = 3.2 m*M*), providing strong evidence that the cell proliferation increases independently of cell-surface FGFR and may depend on nuclear FGFR (Stachowiak et al., 1997). This accelerated proliferation is likely to represent an increase in the mitotic activity of individual cells. Flow cytometry has shown that in cells transfected with FGFR1, the number of cells residing in S phase increased 30% with a concomitant decrease in the proportion of cells in the G_0 / G_1 phases relative to cells transfected with the vector alone (Stachowiak et al., 1997). Similar changes in the cell-cycle distribution of cells were observed in SF-767 or U251MG cells transfected with FGF-2 (Joy et al.,

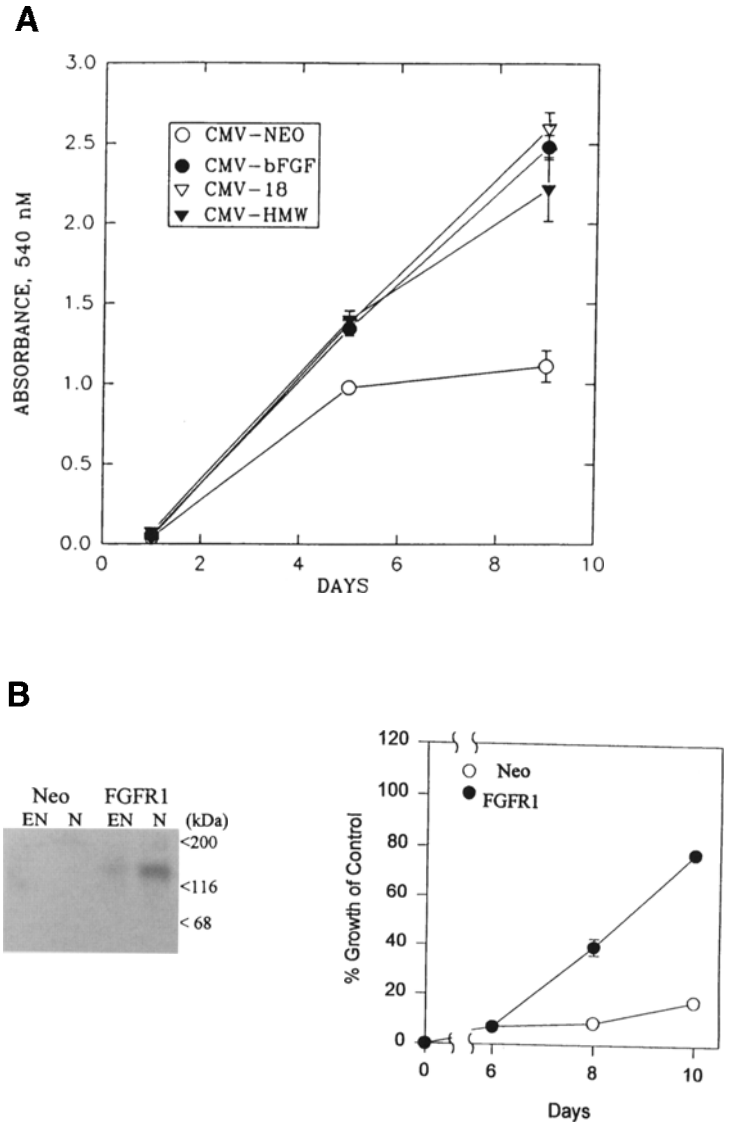


Fig. 6. Proliferation of glioma cells stably transfected with plasmids expressing FGF-2 or FGFR1. **(A)** U251MG glioma cells were transfected with CMV-18 expressing 18-kDa FGF-2, CMV-HMW expressing high-molecular-weight isoforms of FGF-2, CMVbFGF expressing all FGF-2 isoforms or control CMV-NEO without FGF-2 cDNA. Cells were maintained in 5% serum and 250 μg/L of G-418. Each point represents the mean ± SEM of 5–6 samples. **(B)** (Reproduced from Stachowiak et al. (1997), *Oncogene*, **14**, 2201–2211, 1997, with kind permission of Stockton Press.) FGFR1 accumulates in the cell nucleus and stimulates proliferation of transfected SF-763 glioma cells. **(Left)** Western analysis of FGFR1 in the nuclear (N) and extranuclear (EN, cytoplasmic + plasma membrane) fractions of cells stably transfected with plasmids expressing full length FGFR1 or control plasmid. **(Right)** Proliferation of SF-763 glioma cells transfected with the control plasmid or the FGFR1 plasmid. Symbols represent the mean of quadruplicate samples ±SEM.

1997). These results indicate that intracellular/nuclear FGF-2 and FGFR1 may stimulate the transition of cells into the S phase of the cell cycle and are consistent with the nuclear accumulation of endogenous FGFR1 and FGF-2 during the G_1 phase in astrocytes.

Although we detected no extracellular FGF-2 in glial cultures, FGF-2 or FGF-1 could reach the extracellular space in the event of cell lysis, injury, or hyperthermic shock (Schechter, 1992; Shi et al., 1997). Astrocytes treated with 18-kDa FGF-2 became hypertrophic. Their proliferation rate increased as did their rate of incorporating BudR. They also achieved a higher saturation density than control cultures (Joy et al., 1997). Thus, extracellular FGF-2 could signal cell damage via cell surface receptors and activate quiescent astrocytes from the mature human brain to enter the S phase of the cell cycle and to proliferate. However, these mitogenic effects of exogenous FGF-2 were accompanied by the accumulation of endogenous 21-, 23-, and 24-kDa FGF-2 proteins in the nuclei of cells (Moffett et al., 1996b). Thus, even exogenous FGF-2 could stimulate cell proliferation through an intracrine FGF-2 pathway.

Our findings suggest a novel mechanism for growth factor action in which the growth factor and its receptor translocate from the cytoplasm into the nucleus and stimulate entry into the cell cycle. Cells could regulate their responsiveness to FGFs by controlling both the expression and the subcellular localization of FGFR. The presence of functional FGFR1 within the nucleus may explain how intracellular FGFs exert the mitogenic effects observed in this (Joy et al., 1997) and other laboratories (Imamura et al., 1990; Wiedlocha et al., 1994, 1996; Bikfalvi et al., 1995).

Subnuclear Localization of FGF-2 and FGFR1

To begin to identify the mechanisms underlying the action of nuclear FGF-2 and FGFR1, we examined their localization within the nucleus. FGFR1 is associated with the nucleoplasmic fraction and with the nuclear matrix-lamina complex (Fig. 2A). FGF-2 is also present in these two fractions (Joy et al., submitted). Electron microscopy-immunocytochemistry of adrenal medullary cells has shown numerous clusters of FGFR1 and FGF-2 decorating the interior of the nuclear matrix (Stachowiak et al; 1995; 1996b). This patchy distribution suggests that FGFR1 and FGF-2 may bind to specific matrix components and play a role within specialized regions of the nuclear matrix. DNA replication (Berezney and Coffey, 1975; Mcready et al., 1980), transcription (Jackson and Cook, 1985), and RNA processing (Zeitlin et al., 1987) are organized architecturally on the matrix. Topoisomerase, transcriptional factors (Eisenman et al., 1985; Chatterjee and Flint, 1986; Waitz and Loidl, 1991), and retinoblastoma and other proteins that regulate the cell cycle (Chatterjee and Flint, 1986; Deppert and Non Der Weth, 1990; Greenfield et al., 1991; Mancini et al., 1994) associate with the nuclear matrix. Therefore, our findings raise the intriguing possibility that FGF-2 and FGFR1 may exert their influence over a number of cellular processes by acting within the environment of the nuclear matrix. Interestingly, the subnuclear localization of FGF-2 overlaps with, but is not identical to, that of FGFR1. FGF-2 is also present in the nucleoli and in the chromatin, two nuclear fractions that lack FGFR1. Therefore, the nuclear action of FGF-2 could be, at least partially, independent of FGFR1.

Mechanisms Controlling the Expression of FGF-2 and FGFR1 in Glial Cells

Studies with adrenal chromaffin cells and astrocytes revealed a two-stage mechanism for the nuclear translocation of cytoplasmic FGF-2 (Stachowiak et al., 1994, unpublished observations). In the first stage, an increase in the amount of nuclear FGF-2 was accompanied by a depletion in cytoplasmic FGF-2, indicating that nuclear FGF-2 is first derived from cytoplasmic stores. In the second stage, nuclear accumulation of FGF-2 was accompanied by the restoration of the cytoplasmic pool and was prevented by antisense FGF-2 oligonucleotide, indicating that this increase depends upon new FGF-2 synthesis (Stachowiak et al., 1994, un-

published observations). The induction of nuclear FGF-2 in chromaffin cells (Stachowiak et al., 1994) and FGF-2 and FGFR1 in astrocytes (Fig. 7) is paralleled by changes in their levels of mRNA and may reflect, at least in part, regulation of the FGF-2 and FGFR1 genes.

To determine whether the density of cells in culture can affect the transcription of the FGF-2 gene, cells were transfected with chimeric FGF-2 promoter-luciferase constructs or with a control RSVLuc plasmid and plated at different densities (Moffett et al., 1996). The expression of the FGF-2 promoter construct decreased as the cell density increased, reaching 95% inhibition in confluent cells (Fig. 8A). The activity of the FGF-2 promoter was 23.8-fold greater in subconfluent astrocytes than it was in confluent astrocytes (Fig. 8B). The activation of the FGF-2 promoter in subconfluent astrocytes was partially reduced after deletion of the –650 to –512 bp promoter region, indicating that this region is involved in the cell-density regulation of FGF-2 gene promoter activity (Moffett et al., 1996a). Electrophoretic-mobility shift assays (Fig. 9) showed that nuclear proteins from subconfluent cells bind to a fragment of the FGF-2 promoter containing this –650/–512 bp region whereas nuclear proteins from confluent astrocytes do not (Moffett et al., 1996a,b). Hence, the induction of the FGF-2 gene in astrocytes during the transition from the confluent to the subconfluent state may partially reflect the interaction of positive trans-acting factors with the –650/–512 bp promoter region. These factors are either absent from the nuclei of cell-contact-inhibited astrocytes or are unable to interact with the FGF-2 promoter DNA (Moffett et al., 1996a).

The stimulation of FGF-2 expression in astrocytes by growth factors, cAMP, or PKC is also transcriptionally mediated. Northern-blot analysis revealed that forskolin or PMA increases the levels of FGF-2 mRNA (Stachowiak et al., 1994). Similar increases in the level of FGF-2 mRNA in astrocytes treated with serum, growth factors, forskolin, or PMA were detected by a reverse transcriptase-polymerase chain reaction (RT-PCR)-Southern-blot assay (Moffett et al., 1996b). In subconfluent astrocytes transiently transfected with a FGF-2 promoter construct, treatment with serum, EGF, PMA, or forskolin increased promoter activity (Fig. 10) in a manner similar to that seen for the increases in FGF-2 mRNA (Moffett et al., 1996b). Promoter deletion studies have shown that the –650/–512 bp region, which mediates the cell density-dependent regulation of FGF-2 expression, is also essential for the regulation of FGF-2 expression by growth factors, PMA, or forskolin (Moffett et al., 1996b).

Two-Stage Activation of the FGF-2 Gene in Astrocytes

The multiple functions of the –650/–512 bp FGF-2 promoter region suggest that the expression of the FGF-2 gene may be regulated by different stimuli in an interactive manner. Indeed, the activation of the FGF-2 gene by soluble growth factors or by direct stimulation of protein kinases in astrocytes is affected by cell density. In confluent astrocytes, the FGF-2 gene is in an inactive state and cannot be activated by soluble growth factors, serum, or PMA (Moffett et al., 1996b). Thus, in nontransformed human astrocytes, cell-contact inhibition provides the primary control of FGF-2 expression. In brain tissue, the initial stimulus for FGF-2 gene induction in astrocytes could be a reduction in cell contact that shifts an inactive gene to a low-activity state. The expression of the FGF-2 gene could be elevated to a high-activity state by soluble growth factors and/or by agents (i.e., neurotransmitters or hormones) acting via cAMP/PKC signaling pathways.

The binding of nuclear proteins to the –650/–512 bp promoter region shows a similar two-stage regulation (Moffett et al., 1996a,b). In subconfluent, nonstimulated astrocytes, proteins bind at a low level to the –650/–512 bp promoter region. This is in contrast to confluent astrocytes, in which proteins do not bind to the promoter region, even when the cells are treated with serum. In subconfluent cells this low-level binding can be further enhanced by treatment of

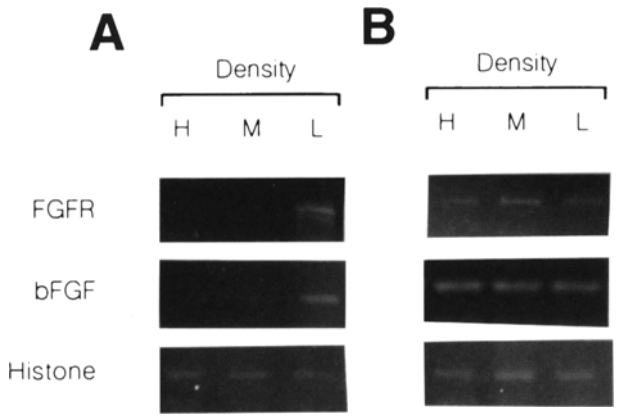
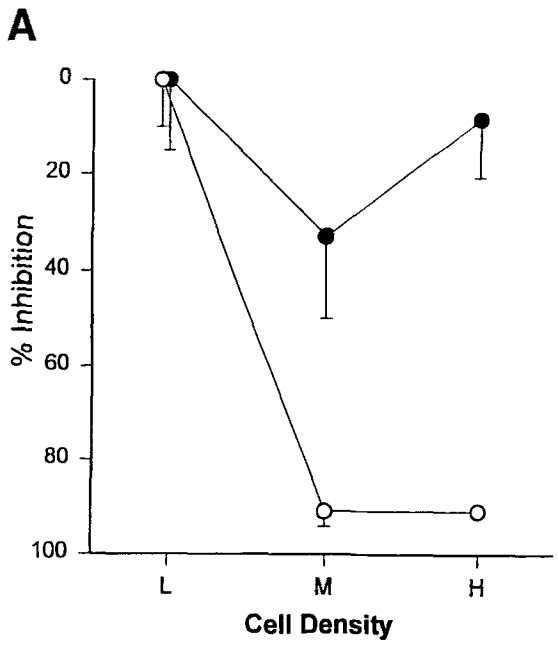


Fig. 7. Effects of cell density on the levels of FGF-2 and FGFR-1 mRNA in astrocytes **(A)** and U251MG glioma cells **(B)** RT-PCR analysis. Total RNA was reverse transcribed and FGF-2, FGFR1, and control histone 3.3 cDNAs were amplified by Moffett and Morrison (Moffett et al., 1996; Morrison et al., 1994b). The linear relationships between the amount of RNA and number of cycles and the amount of cDNA product were established for each reaction (Moffett et al., 1996a). High (H), medium (M), and low densities (L) correspond to 100, 70, and 35% confluence. "H" astrocytic cultures show more than a 95% depletion of FGF-2 and FGFR1 mRNA as compared with "L." cultures. In contrast, histone 3.3 mRNA levels were unaffected by cell density. FGF-2 and FGFR-1 mRNA levels were unaffected by cell density in glioma cells (partially reproduced from Moffett et al., 1996a, *Proc. Natl. Acad. Sci. USA* **93**, 2470–2475, copyright 1996, National Academy of Science, U.S.A.).

astrocytes with serum, growth factors, PMA, or forskolin (Moffett et al., 1996b). The –650/ –512 bp FGF-2 promoter sequence shows no homology to known cAMP, PMA-, or growth factor-responsive elements. Furthermore binding of nuclear proteins to the FGF-2 promoter region is not reduced by competition from oligonucleotides containing such elements (Moffett et al., 1996b). Thus, the –650/–512 bp region in the FGF-2 promoter may contain novel cAMP-, PKC-, and growth factor-responsive sequences that interact with transcriptional mediators.

Altered Transregulation of the FGF-2 Gene Promoter May Underlie Deregulated Nuclear FGF-2 Expression in Human Glioma Cells

Unlike astrocytes, changing the cell density of U251MG glioma cells had no effect on either FGF-2 or FGFR1 mRNA levels (Fig. 7). The expression of the FGF-2-promoter construct was only slightly reduced in confluent glioma cells, and there was no significant difference between the inhibition of the FGF-2 promoter and the inhibition of the control promoter (Fig. 8B). Thus, altered transregulation of the FGF-2 gene promoter, rather than its cis mutation, appears to be responsible for the constitutive expression of the FGF-2 gene in glioma cells. Indeed, unlike in astrocytes, nuclear proteins from glioma cells form complexes with the FGF-2 promoter region independently of cell density (Fig. 9). Also, in glioma cell lines that express high levels of FGF-2, protein binding to the –650/–512 bp promoter region and the activity of FGF-2 promoter are unaffected by treatment of the cells with growth factors, forskolin or PMA (Moffett et al., 1996b). These results suggest that the lack of regulation of FGF-2 gene expression in glioma cells may reflect the constitutive binding of positive trans-acting factors to –650/–512 bp region of the FGF-2 gene promoter. Another mechanism that could contribute to the constitutive overexpression of FGF-2 in some glioma cell lines is the activation of the core FGF-2 promoter by the mutant p53 expressed in those cells (Ueba et al., 1994).



B

	Relative Promoter Activity			
	Low*	High +	Fold '	(n)
-1800 +314 Luciferase	1.00±0.01	0.042±0.01	23.8	(10)
-650 Luciferase	1.59±0.33	0.05±0.01	31.8	(6)
-512 Luciferase	0.04±0.01	0.004±0.001	10.0	(3)
-274 Luciferase	0.07±0.02	0.010±0.002	7.0	(6)
RSV Luciferase	3.0±0.13	1.63±0.34	1.8	(4)

Fig. 8. **(A)** Cell-density regulation of the FGF-2 gene promoter activity in human astrocytes and glioma cells. **(B)** Deletion analysis of the FGF-2 promoter in astrocytes. The numbers given for DNA constructs indicate the included region of the FGF-2 gene. Luciferase activity/pg of transfected DNA is shown relative to the levels of (–1800/+314)FGF-2 Luc activity under low-density conditions. The terms "low" and "high" refer to the density of cultures at the time of harvest for the luciferase assays. Fold; indicates the ratio of specific luciferase activity in subconfluent/confluent astrocytes for each individual plasmid. RSV-Luc; control plasmid expressing luciferase from the RSV promoter (reproduced from Moffett et al., 1996a *Proc. Natl. Acad. Sci. USA* **93**, 2470–2475, copyright 1996, National Academy of Science, U.S.A.).

In summary, these results suggest that the induction of the FGF-2 gene and the nuclear accumulation of FGF-2 in astrocytes released from cell-contact inhibition are stimulated by the binding of activator proteins to the FGF-2 gene promoter. In glioma cells, the binding of trans-acting factors to the FGF-2 gene promoter is unaffected by changes in cell density, leading to the constitutive expression of the FGF-2 gene. The loss of the cell density-dependent regulation of these trans-activating factors may be one of the steps in the progression from normal to neoplastic astrocytes.

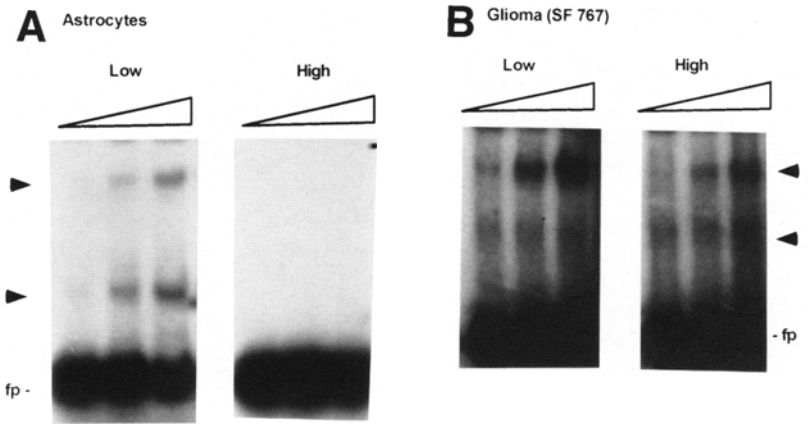


Fig. 9. Effect of cell density on the in vitro binding of astrocytic **(A)** and glioma **(B)** nuclear proteins to the FGF-2 promoter. The target DNA contained nucleotides corresponding to FGF-2 promoter sequences –650 to –512 bp and additional downstream sequences (–511 to –453 bp) that do not bind nuclear proteins from subconfluent or confluent astrocytes. Nuclear proteins (1, 2.5, or 5 μg) from low or high density cultures were incubated with 5 fmol of ^{32}P-labeled FGF-2 promoter fragment and 2 μg dI.dC. Protein binding was displaced by an excess of unlabeled FGF-2 promoter DNA but not by unrelated DNA sequences (Moffett et al., 1996a).

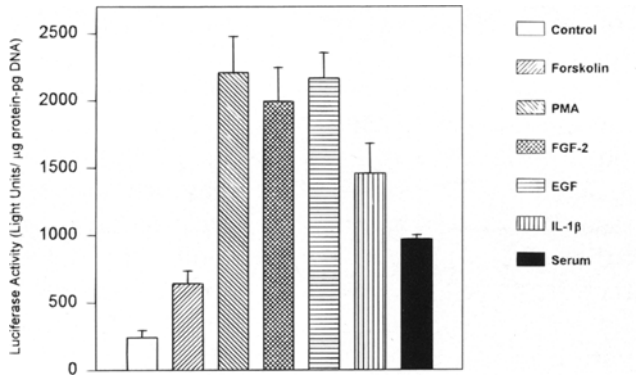


Fig. 10. Stimulation of (–1800/+314)FGF-2-luciferase gene activity by serum, growth factors, or PMA. Specific luciferase activity is shown relative to control (nonstimulated) subconfluent astrocytes. Luciferase activity obtained from cells transfected with the promoterless $pGL2_{basic}$ plasmid was essentially the same as in the reagent blank and showed no increase in cells treated with growth factors, forskolin or PMA (data not shown; from Moffett et al., 1996b).

Mechanisms Controlling the Expression of FGF-2 and Its Intracrine-Nuclear Action Are Similar in Cells of Glial and Neuronal Lineages

FGF-2 localizes in the nucleus of cultured rat brain astrocytes (Gomez-Pinilla et al., 1992) and oligodendrocytes (Vijayan et al., 1993) and in the rat brain (Gomez-Pinilla et al., 1992). Chemically induced seizures increase FGF-2 mRNA levels as well as the nuclear levels of FGF-2 protein in astrocytes and rat forebrain neurons (Humpel et al., 1993). FGF-2 also accumulates in the nucleus of cerebellar pyramidal neurons during neurite outgrowth and synapse formation (Matsuda et al., 1994) as well in dorsal root ganglia neurons after axotomy (Ji et al., 1995). The regulation of FGF-2 expression by cell density in

cells of glial lineages (Westerman and Unsicker, 1990; Murphy et al., 1988) and in endothelial cells (Yu et al., 1993) is now well established.

In recent studies of the rat brain, the presence of FGFR1-like IR was detected in the nuclei of substantia nigra neurons (Gonzales et al., 1995). FGFR1 accumulates in the nucleus of Swiss 3T3 fibroblasts in response to treatment with FGF-2 (Maher, 1996) and in the nucleus of FGF-1-treated NIH 3T3 fibroblasts (Prudovsky et al, 1996). FGFR3 has been found in the nuclear fraction from breast cancer cells (Johnston et al., 1996).

FGF-2 can stimulate proliferation and hypertrophy (Stempele et al., 1990; Tischler et al., 1993; Frodin and Gameltoft, 1994) and the expression of catecholamine and enkephalin biosynthetic genes in neural crest-derived adrenal chromaffin cells (Stachowiak and Goc, 1992; Puchacz et al., 1993). In chromaffin cells, the activation of acetylcholine nicotinic or angiotensin II receptors or the direct stimulation of adenyl cyclase increased the expression of both FGF-2 and FGFR1 as well as their accumulation in the nucleus (Fig. 11; Stachowiak et al., 1994; 1995a; 1996b). Similar to astrocytes, in chromaffin cells, the activation of the FGF-2 gene involves an increased interaction of transactivating factors with the –650/–512 bp FGF-2 promoter region (Stachowiak et al., 1994; J. Moffett, E. Kratz, M. K. Stachowiak, submitted). Hence, the activation of the FGF-2 and FGFR1 genes by heterologous extracellular signals and the subsequent nuclear accumulation of FGF-2 and FGFR1 proteins may serve as a common, novel mechanism mediating the plasticity of a variety of cells in the nervous system.

Receptors for EGF (Rakowicz-Szulczynska 1989; Jiang et al., 1990), insulin (Podlecki et al., 1987), interleukin-1 (Curtis et al., 1990; Solari et al., 1994), angiotensin (Tang et al., 1992; Jimenez et al., 1994), growth hormone (Lobie et al., 1994), vasoactive intestinal peptide (Omary and Kagnoff, 1987), and opioid peptides (Balcheva et al., 1993; Bootger and Spruce, 1995) translocate to the nucleus. With these findings about FGFR, the nuclear accumulation of peptide growth factors and their receptors emerges as a novel and powerful mechanism for control of the cell cycle and gene expression. Identification of molecular targets and the mechanisms controlling the nuclear accumulation and action of growth factor receptors should promote a greater understanding of how the normal and neoplastic growth of cells is regulated within and outside the nervous system.

Summary (Fig. 12)

Regeneration, homeostatic adaptations, or the formation of long-term memory requires renewed growth of astrocytes and specialized regions of neuronal cells and, in some instances, the generation of new neurons and astrocytes. In mature nervous tissue, cell growth and proliferation often coincide with degeneration or involution of other cells as a consequence of pathological degeneration or physiological changes in cell activity. Therefore, decreased cell-cell contact could be an essential, initial stimulus for the remodeling of mature nervous tissue. How changes in cellular interactions may activate cell growth or entry into the cell cycle is unknown. The regulation of the nuclear content of FGFR1 and FGF-2 could serve as a novel mechanism controlling cell proliferation whereby FGF-2 and FGFR1 are intrinsic parts of the cell-cycle machinery. We have shown that the transient induction of the FGF-2 and FGFR1 genes and the subsequent accumulation of FGF-2 and FGFR1 protein in the cell nucleus are operative events in the reversible mitotic activation and hypertrophy of human astrocytes. The expression of the FGF-2 gene in astrocytes is regulated in two stages: transition from an inactive state to a state of low activity induced by reduced cell-cell contact and transition from the low to a high activity state by stimulation with growth factors and hormones. The mechanisms controlling the expression and the nuclear accumulation of FGF-2 and FGFR1 in the cells of glial and neuronal lineages are similar (Puchacz et al., 1993; Stachowiak et al., 1994; 1996a,b; 1997; Joy et al., 1997). In contrast, these pro-

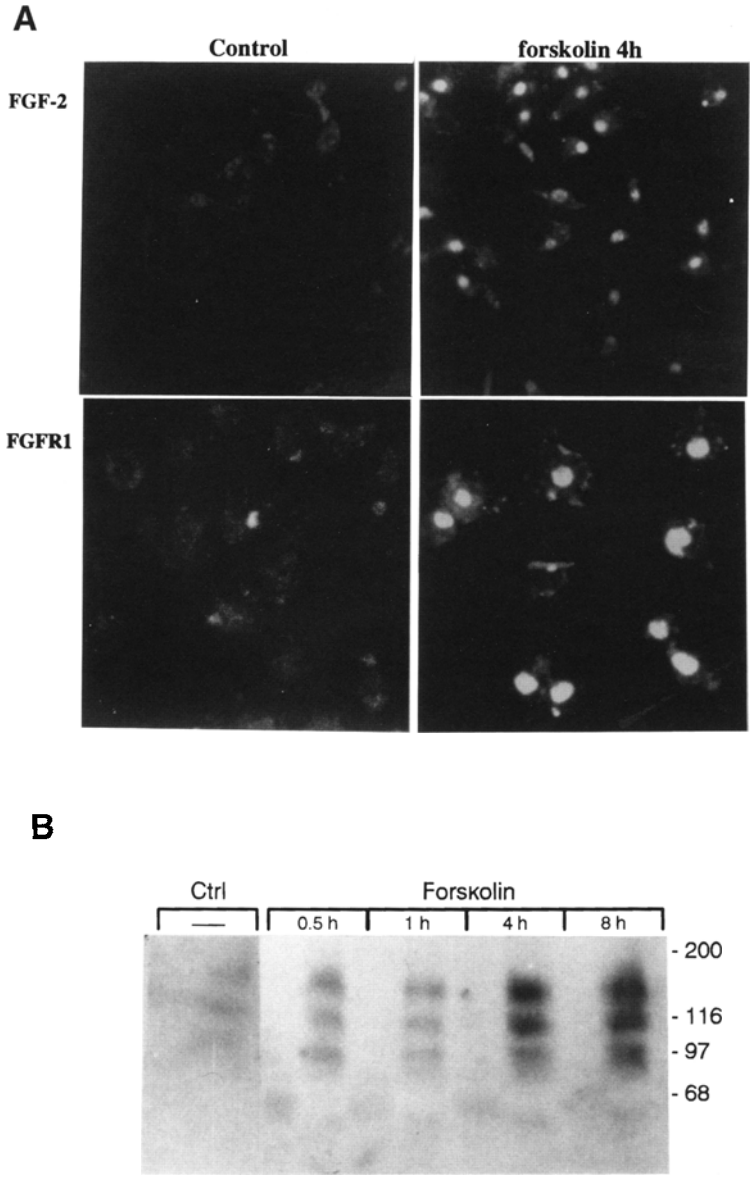


Fig. 11. Forskolin increases the amount of nuclear-associated FGFR1 in cultured adrenal medullary cells. **(A)** immunofluorescent confocal analysis FGF-2 and FGFR1. Cells were cultured in serum-free medium and treated with 5 μ*M* forskolin. Photographs show two-dimensional projections of stacked optical confocal sections taken 1 micron apart. **(B)** Western analysis of cytoplasmic and nuclear FGFR1. Purity of subcellular fractions was shown in Stachowiak et al. (1996b). Forskolin increased nuclear content of all glycosylated isoforms of FGFR1. Migration of molecular-weight protein standards is indicated to the right of the autoradiograms (reproduced from Stachowiak et al. (1996) *Molecular Biology of the Cell* **7**, 1299–1317, with permission of the American Society for Cell Biology).

cesses are deregulated in neoplastic glia resulting in the constitutive nuclear presence of FGF-2 and FGFR1. This promotes proliferation that is insensitive to cell-contact inhibition. The characterization of the mechanisms regulating the expression of the FGF-2 and FGFR1 genes, the nuclear accumulation and action of their proteins, and the malfunction of these path-

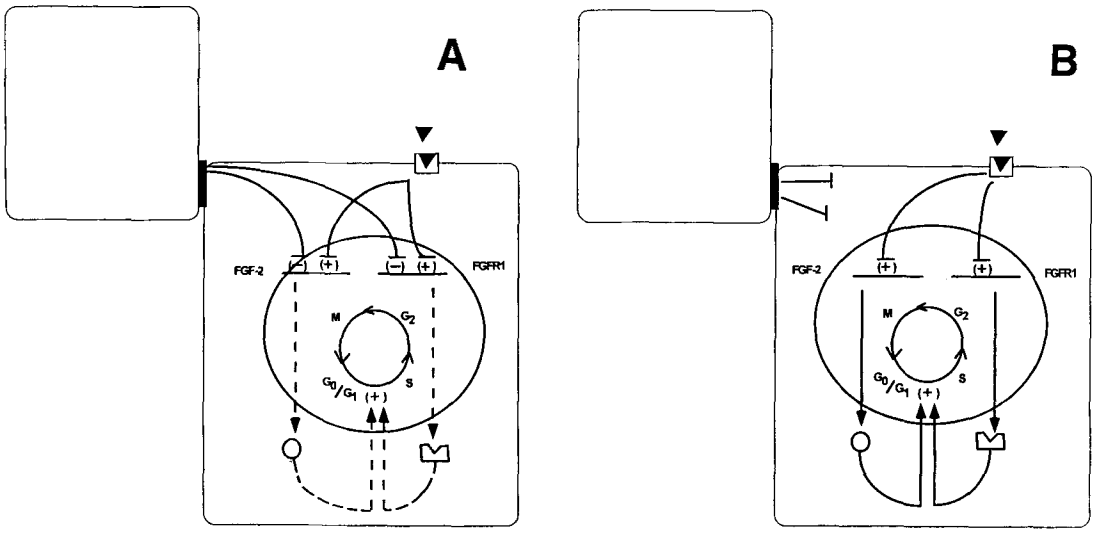


Fig. 12 Intracrine regulation of cell cycle progression by nuclear FGF-2 and FGFR-1 in normal and neoplastic glia. FGF-2 and its receptor, FGFR1, are translocated directly from the cytoplasm to the nucleus where they stimulate (+) the transition from G_0 / G_1 to the S phase of the cell cycle. Whether they translocate and act together in the nucleus remains to be determined. **(A)** In nontransformed astrocytes, the intracrine FGF-2/FGFR1 cycle is subjected to negative control (–) by cell-cell contact stimulated (+) by soluble growth factors and hormones. This control involves changes in FGF-2 and FGFR1 mRNA levels. Changes in FGF-2 mRNA were shown to reflect the activity of the FGF-2 gene promoter. **(B)** In neoplastic glia, FGF-2 and FGFR1 genes are constitutively expressed, independently of the degree of cell contact. The constitutive nuclear presence of FGF-2 and FGFR1 may promote proliferation that is insensitive to cell-contact inhibition. In some glioma cell lines, the FGF-2 gene can be activated by soluble growth factors as in astrocytes, whereas in the others it is maintained at a maximal activated state independent of external simulation (Moffett et al., 1996b).

ways in cancer cells would further contribute to understanding the molecular mechanisms underlying development, regeneration, long-term adaptations, and transformation in the human nervous system.

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