Activin and Basic Fibroblast Growth Factor Regulate Neurogenesis of Murine Embryonal Carcinoma Cells[1]

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Abstract

Murine P19 embryonal carcinoma (EC) cells can be differentiated into various germ layer derivatives. The addition of retinoic acid (RA) to P19-EC cell aggregates results in a transient activation of receptor protein tyrosine phosphatase-α (RPTPα). Subsequent replating of these aggregates leads to neuronal differentiation. P19-EC cells expressing constitutively active RPTPα (P19-RPTPα) show extensive neuronal differentiation upon RA treatment in monolayer. P19-RPTPα cells thus provide a suitable *in vitro* model for studying neuronal differentiation. We used P19-RPTPα cells to study the effects of activin and basic fibroblast growth factor (bFGF) on neurogenesis. We show that P19-RPTPα cells express mRNA for types I and II activin receptors. RA addition causes an up-regulation of receptor type IIA expression. Complexes of type I and II receptors were detectable by cross-linking assays both before and after RA treatment. Receptor complexes were functional as determined by transient transfection assays with activin responsive reporter constructs. Undifferentiated as well as differentiated P19-RPTPα cells express also the FGF receptors (FGFRs) FGFR-1 and FGFR-2 but not FGFR-3 and FGFR-4. Their functionality was established by bFGF induced mitogen-activated protein kinase phosphorylation. Activin and bFGF appeared to exert differential actions on RA-induced neuronal differentiation. Although activin irreversibly changes the differentiation fate into nonneuronal directions, bFGF does not affect initial neurogenesis but regulates axonal outgrowth in a concentration-dependent way; low concentrations of bFGF enhance axonal outgrowth, whereas high concentrations inhibit this process. These results strengthen the notion that activin and bFGF are important regulators of neurogenesis in the mammalian embryo.

Introduction

The mechanisms underlying the induction and patterning of the vertebrate nervous system are very complex and only poorly understood, but a number of studies have implicated activins and bFGF[3] as candidate signaling molecules in these processes. Activins are members of the transforming growth factor β superfamily and are dimeric disulfide-linked proteins consisting of two βA subunits (activin A; Ref. 1), two βB subunits (activin B; Ref. 2), or one βA and one βB subunit (activin AB; Ref. 3). A natural antagonist for activin function is the activin-binding protein follistatin (4). This monomeric protein has been shown to bind activin through the activin β-subunit, thereby forming an inactive complex.

For activin, two different types of serine/threonine kinase receptors (types I and II) have been identified, each of which has two subtypes. Activin type II receptors [IIA (5) and IIB (6)] have a high affinity for activin, and these receptors heterodimerize with type I receptors upon activin binding [IA (7) and IB (8)], which cannot bind the ligand on their own. The type II receptor subsequently phosphorylates and activates the type I receptor, which induces additional intracellular signaling (9).

To date, nine different *FGF* genes have been reported (10). Receptors for FGFs are transmembrane tyrosine kinase receptors. Upon binding of the ligand, they dimerize and become autophosphorylated, leading to an intracellular signal transduction cascade (11). Four different *FGFR* genes have been identified thus far: *FGFR-1* (also known as *flg*), *FGFR-2* (also known as *bek*), and *FGFR-3* and *FGFR-4* (reviewed in Ref. 12).

The notion that activin and bFGF play important roles in mesodermal and neuronal induction and patterning in the vertebrate embryo is in particular derived from studies using the experimental features of the *Xenopus laevis* embryo. Inhibition of activin signal transduction by overexpression of a truncated activin type II receptor in *Xenopus* embryos caused inhibition of mesoderm induction (13). Later, it was shown that expression of the same mutant activin receptor caused neuralization and the concomitant expression of the marker protein neural cell adhesion molecule (14). This neu-

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[3] The abbreviations used are: bFGF, basic fibroblast growth factor; FGFR, fibroblast growth factor receptor; EC, embryonal carcinoma; ES, embryonal stem; RA, retinoic acid; RPTP, receptor-like protein tyrosine phosphatase; GAP, growth-associated protein; NF, neurofilament; MAP kinase, mitogen-activated protein kinase.

ralization process takes place in the absence of any detectable mesoderm, suggesting that activin acts as a neural inhibitor. In addition, overexpression of mRNA coding for follistatin caused neuralization of ectodermal explants in the absence of detectable mesodermal structures (15).

Like activin, bFGF is also capable of inducing mesodermal structures in *Xenopus* (16, 17). In addition to this observation, it was demonstrated that the expression of dominant-negative FGFRs caused severe impairments in mesodermal structures (18). As shown by Kengaku and Okamoto (19), low concentrations of bFGF are able to induce gastrula ectoderm and subsequent induction of central nervous system neurons in embryonic cells of *Xenopus*. Recently, Kengaku and Okamoto (20) showed that bFGF directly neuralized *Xenopus* ectodermal explants, without the expression of mesodermal markers. Together, these observations indicate that activin and bFGF are prominent regulators of neurogenesis in *Xenopus* development.

Much less is known about the involvement of these factors in early mammalian development. With respect to activin function, knock-out mice lacking activin βA and βB subunits have been generated (21). These mutants showed a normal early development but died within 24 h after birth. Although this study pointed to a minor role of activin in early development, it could well be that activin has important effects during early mouse development; mutant mice might have been rescued by maternally derived activin or other factors that bind to activin receptors. Besides ligand knock-outs, receptor knock-outs were also generated in which ActRIIA was deleted (22). These mutant mice developed normally and reached adulthood but showed significant suppression of serum follicle-stimulating hormone levels. Like the ligand knock-outs, these mutants also showed no defects in early development. Receptor subtype redundancy could account for a rescue of the mice from severe impairments during early development. Double knock-outs will probably give more insight in receptor ligand interaction.

Targeted mutations in *fgf-3, fgf-4,* and *fgf-5* ligands did not result in defects in early gastrulation (23, 24), probably due to redundant effects by other FGF family members. To investigate the possible effects of FGFs during early murine development at the receptor level, Yamaguchi *et al.* (25) knocked-out the *FGFR-1* gene. It was concluded from this study that although gastrulation and mesoderm induction were normal, mesodermal patterning was aberrant, stressing the importance of *FGFR-1* in mesodermal patterning.

To get additional insight into the possible roles of activin and bFGF during early mammalian embryogenesis, in particular at the cellular level, the use of appropriate *in vitro* model systems will definitively be of help. Murine EC and ES cell lines provide such model systems, because these pluripotent cells resemble inner cell mass cells and can be differentiated *in vitro* to derivatives of the various germ layers, including neuronal cells (26). P19-EC cells, for example, can be differentiated with RA to neuronal cells by replating 3-day-old cell aggregates. Recently, it has been shown that activin as well as bFGF are able to affect the differentiation of P19-EC cells. Activin was found to have an inhibiting effect on the mesodermal and neuronal differentiation in P19-EC cells, when it was present during the aggregation phase (27–29). bFGF was not able to inhibit neuronal differentiation when administered during aggregation but interfered in mesodermal differentiation (29).

P19-EC cells as well as ES cells have to be aggregated for 2–4 days in the presence of RA to become neuronal (30, 31), and this aggregation phase hampers the study of the exact effects and mode of action of other potential signaling molecules, such as activin and bFGF. Recent studies from this laboratory have, however, established that the transmembrane RPTPα is up-regulated during this phase, and that this up-regulation is a key determinant for subsequent neuronal differentiation (32). We could show that P19-EC cells and ES cells (E14-ES, 33) stably transfected with RPTPα are able to undergo enhanced neuronal differentiation in monolayer upon addition of RA, thereby overcoming the need for the aggregation step. P19-RPTPα cells thus provide an advantageous model system for studying the regulation of early neuronal differentiation as compared to the wild-type P19 cells (32).

We have now used P19-RPTPα cells to investigate the effects of activin and bFGF on neuronal differentiation in detail. We demonstrate that the addition of activin leads to the complete absence of a neuronal population in a time- and dose-dependent way. Importantly, only the initial phase of RA-induced neurogenesis is susceptible to this inhibitory action of activin. bFGF shows a quite different and biphasic dose-dependent effect. In contrast to activin, bFGF does not interfere with early neuronal differentiation. However, low concentrations of bFGF (0.1–1.0 ng/ml) enhance subsequent neurite outgrowth, whereas higher concentrations (50–100 ng/ml) result in inhibition of neurite outgrowth. These results indicate that activin and bFGF can serve as differential regulators of neuronal differentiation as well as in early murine development.

Results

Neuronal Differentiation of P19-EC and P19-RPTPα Cells. Previously, we demonstrated that the transmembrane RPTPα is transiently up-regulated in RA-treated P19-EC cell aggregates, which indicates that RPTPα activity is an important determinant in the selection of the neuronal differentiation pathway during cell aggregation. This notion was strengthened by the observation that stably transfected P19 cells expressing RPTPα (P19-RPTPα cells) are selectively induced by RA to differentiate into a neuronal population while cultured in a monolayer (32). Apparently, the constitutive activity of RPTPα overrides the need for a pre-aggregation phase to obtain neuronal differentiation upon RA treatment. P19-RPTPα cells thus constitute a more convenient model system than wild-type P19-EC cells to study processes involved in neuronal differentiation.

In an initial set of experiments, we studied the neuronal differentiation of P19-RPTPα cell cultures by monitoring the expression of two neurospecific proteins, GAP-43 and NF-165 by immunofluorescent staining. GAP-43 is a widely studied determinant in neuronal cells and is up-regulated preceding the process of neurite outgrowth and plasticity (34). NF proteins are specifically localized in the axonal extensions

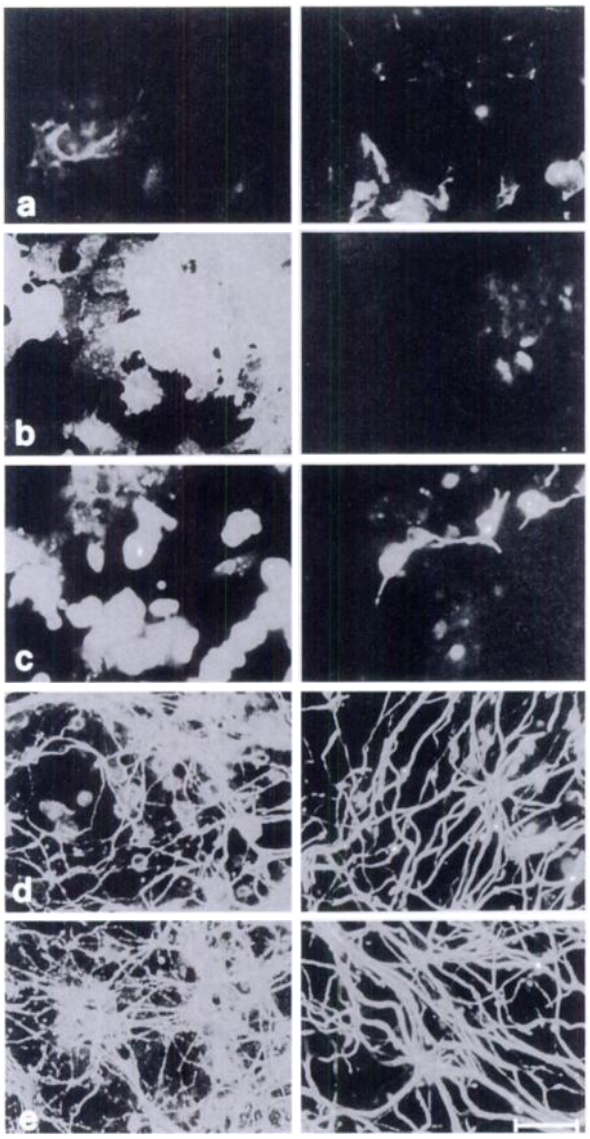

Fig. 1. Neuronal differentiation of P19-RPTPα cells. Immunofluorescent detection of GAP-43 (*left panels*) and NF-165 expression (*right panels*) in P19-RPTPα cell cultures in the presence or absence of 1×10^{-6} M RA. Undifferentiated cells do not express the neuronal marker proteins (*a*). Already 1 day after addition of RA (*b*), the expression of GAP-43 is up-regulated, and after 5 days (*d*), cells with neurites can be detected. GAP-43 staining is localized inside somata and neurites. The expression of NF-165 can be detected from day 3 (*c*) onwards, when the neurite sprouting is initiated. Later on in the differentiation (*e*), dense networks of neurites can be detected. NF-165 expression is localized in neurites. *Bar*, 100 μm.

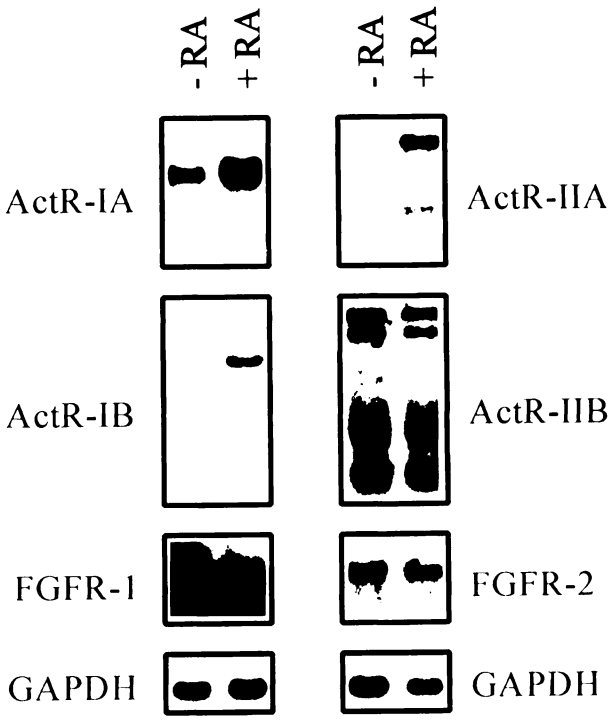

Fig. 2. Activin- and FGFR mRNA expression. Expression of activin receptor and FGFR mRNA in undifferentiated (*−RA*) and differentiated (*+RA*) P19-RPTPα cell cultures. Northern blotting was done as described in "Materials and Methods." *ActR-IA* and *ActR-IB* are activin type I receptor subtypes; ActR-IIA and ActR-IIB are activin type II receptor subtypes; FGFR-1 and FGFR-2 are FGFR subtypes. At the bottom, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is shown as a loading control.

of neuronal cells and exist in different forms, M_r 68,000, 165,000, and 200,000 (35, 36).

Because P19-RPTPα cells show neuronal differentiation upon RA treatment when cultured in monolayer, the initiation of neuronal differentiation is not physically obscured as in P19-EC aggregates. RA induces an up-regulation of GAP-43 and NF-165, but clearly GAP-43 expression precedes that of NF-165 (Fig. 1). Abundant GAP-43 expression is already detectable within 1 day of RA treatment, *i.e.*, before neurite extensions appear, whereas NF-165 expression is restricted to the neurite extensions and can only be detected after 3 days of differentiation, at the time when neurite sprouting is initiated (Fig. 1c). This comparison that various phases of neurogenesis can be readily distinguished in P19-RPTPα cells and additional experiments were thus performed with the use of this *in vitro* model.

P19-RPTPα Cells Have Functional Receptors for Activin and bFGF. Accumulating data in the literature indicate that activin and bFGF are important regulators of mesodermal and neuronal induction and patterning (see "Introduction"). Prior to studying the possible effects of activin and bFGF on neurogenesis in P19-RPTPα cells, we determined the mRNA and protein expression of the respective receptors for these ligands, as well as their functional signaling.

The mRNA expression levels of different activin receptor subtypes (IA, IB, IIA, and IIB) and FGFRs (FGFR-1, FGFR-2, FGFR-3, and FGFR-4) were assessed by Northern blotting experiments on undifferentiated and differentiated P19-RPTPα cells (Fig. 2). All activin receptor subtypes appeared to be expressed in undifferentiated cells. Upon RA-induced neuronal differentiation, a slight up-regulation of both type I receptor mRNAs and a 4-fold increase in activin receptor type IIA expression was observed, whereas the expression of the type IIB receptor is not affected (Fig. 2). Of the various FGFRs, only FGFR-1 and FGFR-2, but not FGFR-3 or

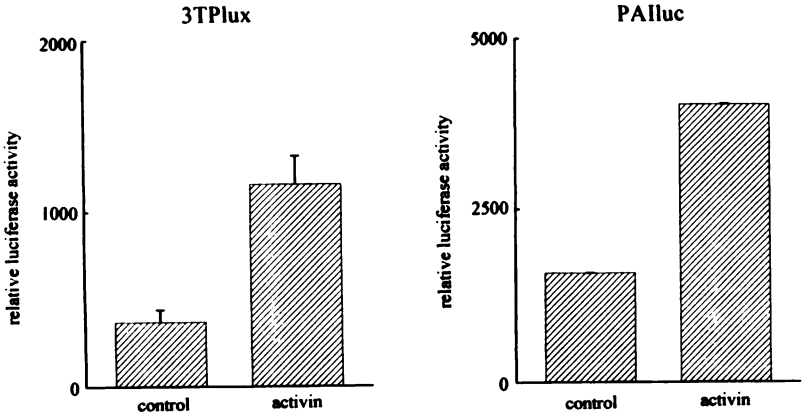

Fig. 3. Induction of activin responsive reporter constructs in undifferentiated P19-RPTPα cells. Undifferentiated P19-RPTPα cells were transiently transfected with the 3TP-lux or the PAI-luc reporter construct in combination with a lac-Z reporter construct. Luciferase activity was measured and corrected for lac-Z activity. Activin was able to induce luciferase activity from both constructs, indicating that functional activin receptor complexes are present. *Bars*, SD.

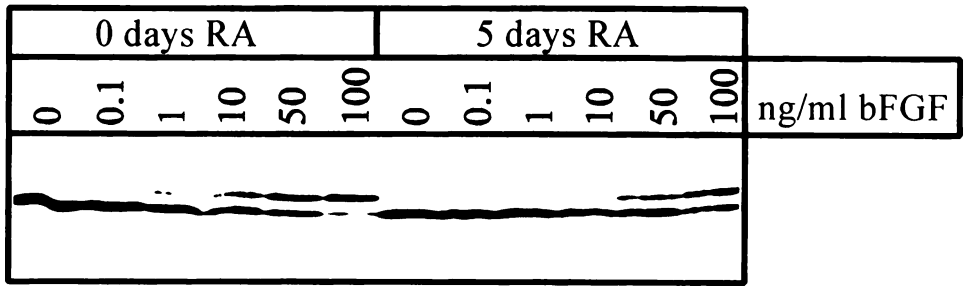

Fig. 4. MAP kinase mobility shift by bFGF. Undifferentiated (*0 days RA*) and differentiated (*5 days RA*) P19-RPTPα cells were treated with increasing concentrations of bFGF for 10 min. Subsequently, cells were lysed, and whole-cell lysates were submitted to SDS-PAGE. MAP kinase protein was detected with a rabbit polyclonal antibody. bFGF caused a MAP kinase mobility shift, characteristic for MAP kinase phosphorylation and activation, in undifferentiated as well as differentiated cells, indicating the presence of functional receptors for bFGF.

FGFR-4 (data not shown), are expressed in P19-RPTPα cells, and their mRNA expression levels remain unchanged during neuronal differentiation (Fig. 2). These mRNA expression patterns are essentially similar to those reported before for P19-EC cells (29, 37).

Activin receptor protein presence in P19-RPTPα cells was determined by cross-linking experiments with iodinated activin A, using the displacement of binding of radiolabeled activin by excess unlabeled ligand as a control. Activin type I and type II receptor proteins are expressed at similar levels in undifferentiated and differentiated P19-RPTPα cells (data not shown). The functioning of the activin receptor complexes was tested in undifferentiated P19-RPTPα cells, transiently transfected with two different luciferase reporter constructs coupled to activin responsive elements, 3TP-lux (38) and PAI-luc (39). Activin induced a significant increase of the luciferase activity (Fig. 3).

FGFRs belong to the tyrosine kinase receptor family, and their signaling involves activation of MAP kinase (11). The functioning of the receptors for bFGF in P19-RPTPα cells was, therefore, assessed by assaying the mobility shift of MAP kinase, which is characteristic for MAP kinase activation and reflects MAP kinase phosphorylation. Increasing concentrations of bFGF induced a mobility shift of MAP kinase in both undifferentiated and differentiated cells, demonstrating that FGFR signaling leads to activation of MAP kinase in these cells (Fig. 4).

From these experiments, we conclude that P19-RPTPα cells express functional receptors for activin and bFGF, which allows the study of the possible effects of these signaling molecules on neurogenesis in these cells.

Activin Acts as an Early Inhibitor of the Neuronal Differentiation. Activin is known for its ability to induce mesoderm in the *Xenopus* animal cap assay (40, 41). Furthermore, it has been shown that inhibition of activin signaling in *Xenopus* leads to direct neuralization of ectodermal cells in the absence of detectable mesoderm (14). Recently, it has been demonstrated that activin inhibits RA-induced neuronal differentiation of P19-EC cell aggregates (27, 29), and that this effect of activin can be prevented by the expression of a truncated, dominant-negative activin receptor (42). These data indicate that activin counteracts neurogenic signaling *in vivo* as well as *in vitro*.

Here we have used RA-induced neuronal differentiation of P19-RPTPα in monolayer cultures to substantiate this potentially important action of activin. The inhibitory action of activin on neurogenesis was confirmed by exposing P19-RPTPα cells to different concentrations of activin during the RA treatment and quantifying neuronal differentiation by immunoblotting of the neurofilament protein NF-165 after 5 days of treatment (Fig. 5). Clearly, activin is capable of inhibiting the neurogenic signaling of RA in P19-RPTPα cells in a concentration-dependent way. Activin concentrations of 5 ng/ml and higher result in a complete inhibition of NF-165

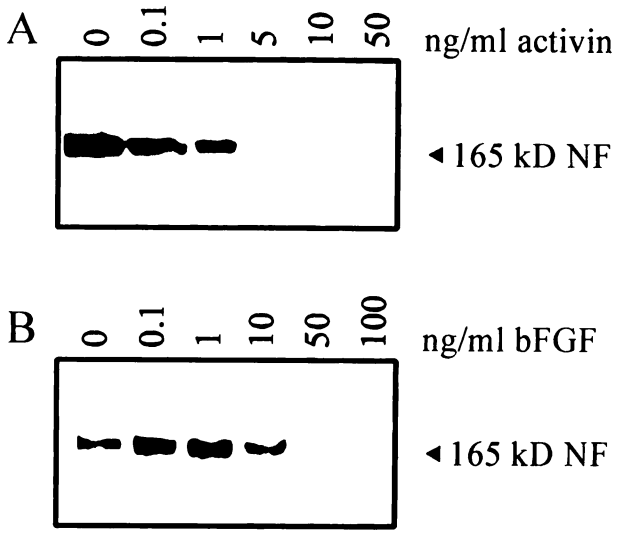

Fig. 5. Effect of activin and bFGF on NF-165 expression. P19-RPTPα cell cultures were treated with 1×10^{-6} M RA and increasing concentrations of activin (*A*) or bFGF (*B*) for 5 days. Cells were lysed, and whole-cell lysates were submitted to SDS-PAGE (equal amounts of protein were loaded). NF-165 expression was detected by immunoblotting as described in "Materials and Methods." Activin inhibits NF-165 expression. Low concentrations (0.1–1 ng/ml) of bFGF were able to increase NF-165 expression, whereas high concentrations (50–100 ng/ml) inhibited the expression.

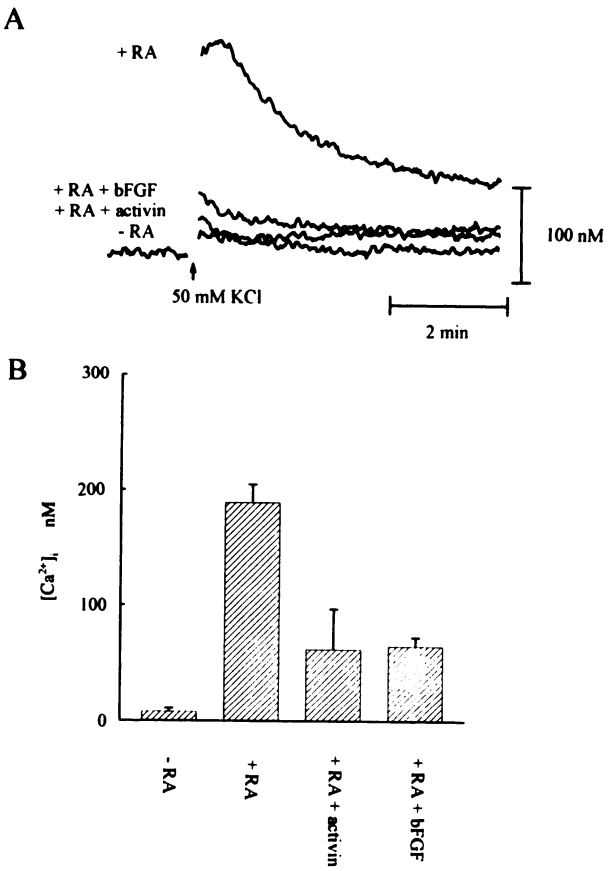

Fig. 6. Effect of activin and bFGF on depolarization-evoked calcium influx. P19-RPTPα cell cultures were tested for electrical excitability by measuring depolarization-induced calcium influx. Cells were cultured in the absence of RA (control), in the presence of RA for 5 days (RA), in the presence of RA and activin for 5 days (*RA + activin*), or in the presence of RA and bFGF for 5 days (*RA + bFGF*). Concentrations: RA, 1×10^{-6} M; activin, 10 ng/ml; bFGF, 100 ng/ml. Depolarization was induced by stimulation with 50 mM KCl. Intracellular free Ca^{2+} concentrations were measured spectrophotometrically, using Indo-1-AM. *A*, on-line registration of individual experiments of KCL-induced $[Ca^{2+}]_i$ influx under the indicated experimental conditions. *B*, averaged results ($n = 4$) of the same experimental conditions; *bars*, SD. Activin and bFGF significantly reduced the depolarization-induced rise in intracellular calcium, indicating that neuronal differentiation was greatly reduced.

expression, whereas lower concentrations of activin (1.0 and 0.1 ng/ml) give rise to a dose-dependent decrease of NF-165 expression.

The expression of NF is indicative for phenotypical neuronal changes but not for the functional state of neuronal cells. We, therefore, studied in parallel the excitability of P19-RPTPα cells before and after RA-induced differentiation. When challenged with a depolarizing stimulus, neuronal cells will respond with a rise in the intracellular calcium concentration $[Ca^{2+}]_i$, which reflects the excitability of the cells. Using this protocol, undifferentiated P19-RPTPα cells show no significant signs of excitability, but cultures exposed to RA for 5 days respond with a clear transient rise in $[Ca^{2+}]_i$, indicating the presence of excitable neuronal cells. When cultures were differentiated for 5 days with RA in the presence of moderate concentrations of activin (10 ng/ml), the appearance of excitable cells was suppressed nearly completely (Fig. 6). These results demonstrate that activin counteracts the neuronal differentiation of P19-RPTPα cells, as induced by RA, both based on phenotypical and on functional criteria.

Finally, we wished to pinpoint the time frame during which activin is capable of exerting its modulatory action. To that end, activin was added for different time intervals during RA exposure, and the extent of neuronal differentiation was visualized by immunofluorescence staining with anti-NF-165 of the cultures after 6 days of RA treatment. Parallel Hoechst staining was used as an indicator for the presence of cell nuclei. As shown in Fig. 7, application of 10 ng/ml activin during the initial 3 days of RA treatment results in a complete inhibition of the expression of NF-165 and of the appearance of neurite extensions, as detected after 6 days (Fig. 7*c*). In marked contrast, neuronal differentiation proceeded completely normally when activin was administered from day 4 onwards (Fig. 7*d*). Using more narrow exposure intervals, it could be shown that the susceptibility of P19-RPTPα cells to the differentiation inhibiting action of activin is limited to a period between days 2 and 3 after the onset of RA treatment (data not shown).

Together, these results demonstrate that activin irreversibly changes the differentiation fate of P19-RPTPα cell cultures into nonneuronal directions, and that its regulatory action is confined to the earliest phases of neurogenesis. Clearly, complete neurogenesis is under multiple, time-dependent control. The initial phase of neurogenic selection involves GAP-43 expression and is activin inhibitable in a dose-dependent way, whereas the subsequent phase of

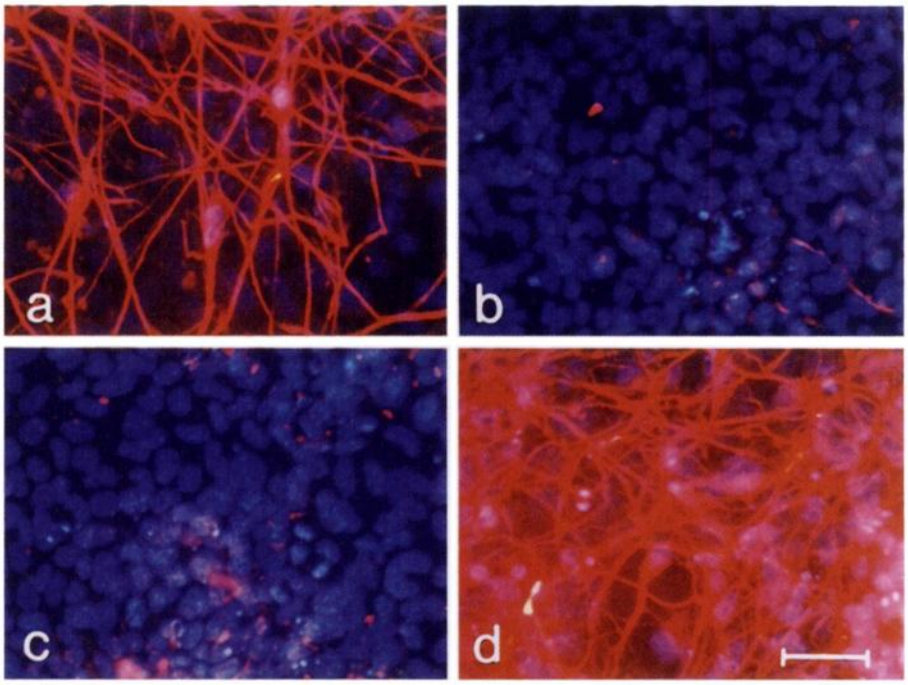

Fig. 7. Time-dependent effects of activin on neuronal differentiation. P19-RPTPα cell cultures were treated for 6 days with 1×10^{-6} M RA (*a*). Activin (10 ng/ml) was present the entire time (*b*), during the first 3 days (*c*), or during the last 3 days (*d*). Cultures were fixed and labeled with an antibody against NF-165 (*red* labeling) and with Hoechst (*blue* labeling). Activin was only able to inhibit neuronal differentiation when it was present during the first 3 days of RA-induced differentiation or when present during all 6 days. *Bar,* 100 μm.

neurite extension, NF-165 expression, and the generation of excitability, once reached, appears to be unaffected by the presence of activin. On first sight, this restricted action of activin does not seem to be related to the activin receptor expression pattern, but additional experiments are necessary to exclude the possibility that subtle, cell type-related differences in expression occur in these P19-RPTPα cultures.

bFGF Affects Neuronal Outgrowth in a Dualistic and Dose-dependent Way. Like activin, bFGF is known for its effects on mesoderm formation in *Xenopus* embryos (13). In addition, Kengaku and Okamoto (19) showed that low concentrations of bFGF are able to induce gastrula ectoderm and, subsequently, to promote the formation of central nervous system neurons in embryonic *Xenopus* cells. In P19-EC cells, bFGF is able to inhibit differentiation toward mesodermal derivatives when administered during the aggregation phase (29). Together, these data indicate a possible positive neurogenic signaling role for bFGF, both *in vivo* and *in vitro*.

As for activin, we have substantiated this possible action of bFGF by studying its effects on RA-induced neuronal differentiation of P19-RPTPα cells. Using a similar strategy as for activin, the possible effects of bFGF on neurogenesis were first determined by exposing these cells to different concentrations of bFGF during RA treatment and determining the degree of neuronal differentiation by immunoblotting of NF-165 protein (Fig. 5*B*). The observed effects were quite opposite to those of activin. At low concentrations (0.1 and 1 ng/ml bFGF), the expression of NF-165 was significantly enhanced as compared to cultures that were only exposed to RA, but at 10 ng/ml, no effect was observed, whereas at higher concentrations (50 and 100 ng/ml), the NF-165 expression was completely inhibited. More detailed analysis by immunofluorescent staining showed that still a small percentage of NF-165 positive cells was detectable under these latter conditions (Fig. 8*b*), but that this limited NF-165 staining is restricted to cell somata, and that no neurite outgrowth takes place when the cells are exposed to high bFGF concentrations. Clearly, bFGF affects neurogenesis in P19-RPTPα cells in a dualistic mode, depending on its concentration; at low concentrations, neurite formation is enhanced, whereas at high concentration, this process is completely inhibited.

The dualistic, concentration-dependent effects of bFGF on the neuronal differentiation of P19-RPTPα cells were further investigated by monitoring the generation of electrical excitability in cultures differentiating in the presence of bFGF. As shown in Fig. 6, inhibition of neurite formation by high concentrations of bFGF (100 ng/ml) is associated with a drastic reduction in the capacity of the cells to respond to a depolarizing stimulus. Subsequently, the effects of different concentrations of bFGF on the excitability of the cultures were measured, and the results confirmed the dualistic mode of action of bFGF on neurogenesis (Fig. 8). Cultures differentiated in the presence of low concentrations of bFGF (0.1 ng/ml) are more excitable than control cultures; at intermediate concentrations (1.0 and 10 ng/ml) no effect is seen, but a pronounced decrease in electrical excitability is observed at a bFGF concentration of 100 ng/ml. These results demonstrate that bFGF has dualistic effects on neuronal differentiation, depending on its dose, which makes it to a candidate signaling molecule for the spatial control of neurogenesis *in vivo*.

Finally, we also determined for bFGF the time frame during which it is effective as an inhibitor of neuronal differentiation in P19-RPTPα cells. To this end, inhibiting concentrations of bFGF were administered to differentiating P19-RPTPα cultures for different intervals, and neuronal differentiation was

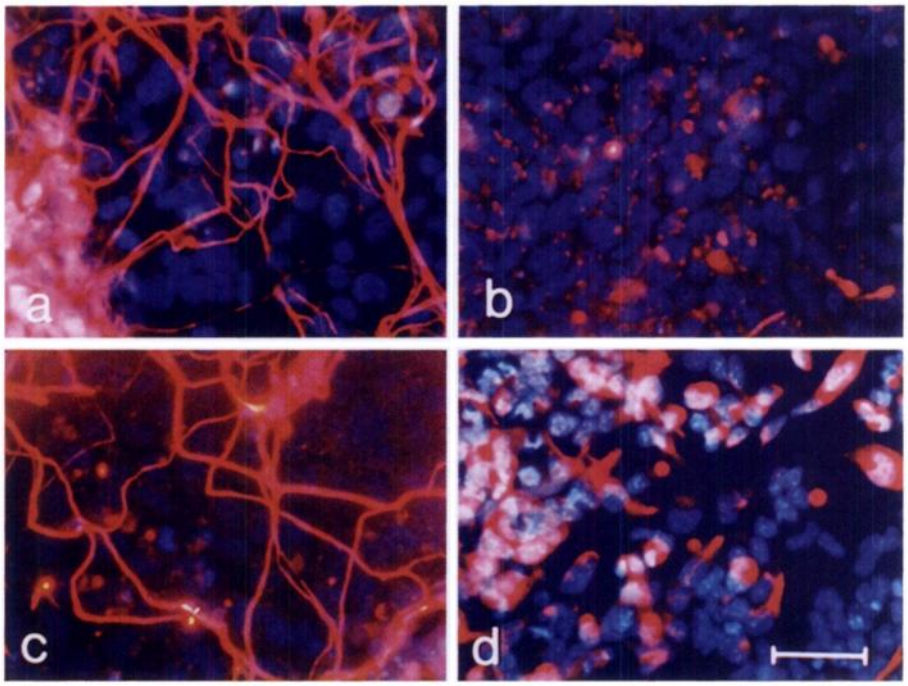

Fig. 8. Time-dependent effects of bFGF on neuronal differentiation. P19-RPTPα cell cultures were treated for 6 days with 1×10^{-6} M RA (*a*). bFGF (100 ng/ml) was present the entire time (*b*), during the first 3 days (*c*), or during the last 3 days (*d*). Cultures were fixed and labeled with an antibody against NF-165 (*red* labeling) and with Hoechst (*blue* labeling). bFGF was able to inhibit neurite outgrowth when present during the entire 6-day differentiating period or when present during the last 3 days. *Bar*, 100 μm.

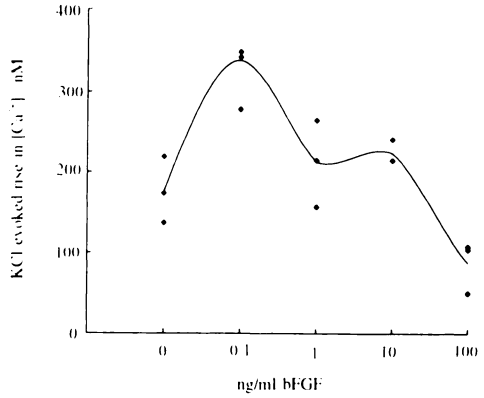

Fig. 9. Effect of increasing concentrations of bFGF on depolarization-evoked calcium influx. P19-RPTPα cells were cultured with 1×10^{-6} M RA in the presence of increasing concentrations of bFGF. Electrical excitability was assessed by measuring depolarization-induced (50 mM KCl) rises in the intracellular calcium concentration. Low concentrations of bFGF were able to increase the excitability, indicating an increased presence of voltage-sensitive channels in the cultures. Higher concentrations of bFGF reduced the excitability.

visualized by NF-165 immunostaining after 6 days of culture. When 100 ng/ml bFGF were present during the first 3 days of RA treatment, a substantial amount of neurons was detectable (Fig. 8*c*). Administration of bFGF during the last 3 days caused a severe inhibition of neurite outgrowth (Fig. 8*d*), comparable to that seen in continuously exposed cultures, but under these conditions, the expression of NF is not completely absent.

Apparently, the inhibitory action of bFGF is confined to the phase of neurite extension and thus does not include early neurogenesis. This contrasts with the action of activin, which appeared to be limited to the earliest phases of neurogenesis. Our results thus indicate that activin may serve as an inhibitor of neural inductive signaling, whereas bFGF may act as dualistic modulator of subsequent neurite maturation.

Discussion

In this study, we have exploited P19-EC cells stably transfected with RPTP α (P19-RPTPα cells) as a convenient *in vitro* model to study regulatory mechanisms underlying neuronal differentiation. Wild-type P19-EC cells are pluripotent stem cells that can differentiate to derivatives of all three germ layers, depending on RA concentration and culture conditions. Neuronal specification occurs in these cells when they are exposed to RA during cell aggregation for 2–4 days, and neuronal outgrowth from these aggregates is detectable upon subsequent replating. RA-treated P19-EC aggregates show a transient activation of RPTPα, which seems to be crucial for their neuronal specification, because monolayer culture of P19-RPTPα cells differentiate directly toward a neuronal phenotype upon addition of RA. Apparently, the constitutive RPTPα activity is an essential trigger for evoking neuronal potency in these cells (35).

Studies on *Xenopus laevis* embryos have implicated activin (13, 14) and bFGF (19) as negative and positive regulators of neurogenesis *in vivo*, respectively. For activin, a similar neurogenic suppressing effect was found in P19-EC cells (27, 28, 29). We have extended these observations by demonstrating that P19-RPTPα cells express functional type I and type II receptors for activin and FGFR-1 and FGFR-2 receptors for bFGF, and that RA-induced neuronal differentiation of these cells is subject to a complex regulation by these factors.

In accordance with the observations in *Xenopus* embryos and P19-EC cells, activin acts as a potent inhibitor of neuronal differentiation in P19-RPTPα cells. Activin suppresses

not only the appearance of morphologically detectable neurite extensions but also suppresses the expression of neuronal marker proteins, such as NF-165, and the generation of electrical excitability. Importantly, this inhibitory action is dose dependent, with a maximally effective dose at 5 ng/ml and above, and confined to the earliest phases of neurogenesis, *i.e.,* between the second and third day of RA treatment, when in control cultures neurite formation has not yet commenced. Extrapolation of these findings to the *in vivo* regulation of neurogenesis would imply that activin could serve as a negative signal for competent neurectodermal cells, which suppresses their neurogenic potency, and thus has been implicated in neuronal patterning. It should be noted that, in addition to its inhibitory action, activin could have other effects on specific subsets of neurogenic cells because it has also been shown that activin is able to induce sets of neural genes in cultured sympathetic neurons (43).

The molecular mechanisms underlying the inhibitory action of activin is as yet obscure. Its restriction to the initiation of neurogenesis cannot be explained on the basis of receptor expression. Undifferentiated P19-RPTPα cells express functional activin receptors, and their expression is not down-regulated upon differentiation. This renders it likely that activin interferes directly with a permissive or inductive signaling pathway, such as RA signaling in the case of P19-RPTPα cells, or that it acts indirectly by interference with as yet unknown intercellular signaling between subpopulations of cells in the culture. To resolve this question, one would like to determine activin receptor expression at the single-cell level, but this has to await the availability of specific antibodies.

There is a contrast between the observed effects of activin in cultured neurocompetent mammalian cells and those reported for mouse embryos thus far. Activin receptor type IIA knock-out mice do not show impairments during early embryogenesis, are born without defects, and develop into adults (22). The main deficiency in these knock-outs is a severe suppression of serum follicle-stimulating hormone levels, resulting in a defective reproductive performance. Early embryonal processes mediated by activin receptors could, however, have been rescued by receptor redundancy, a possibility that can only be excluded by multiple knock-out experiments. Also, mutant mice lacking both activin subunits showed normal early development and were born but died within the first 24 h after birth (21). Interpretation of these results is difficult, because it is possible that maternally derived activin regulates important processes during early development. Whether our *in vitro* results on P19-RPTPα cells can indeed be extrapolated to the mammalian embryo has thus to await validation by future experiments.

Besides activin, we studied the possible effects of bFGF on RA-induced neuronal differentiation of P19-RPTPα cells, and the results are indicative for an important, yet complex, role for bFGF in the regulation of neuronal maturation, which differs from that of activin: (*a*) we demonstrated that bFGF has a clearly dualistic effect on neurite outgrowth, depending on its concentration. Low concentrations of bFGF (0.1 and 1.0 ng/ml) enhance the expression of NF-165 and the level of electrical excitability, which is indicative for a stimulating effect on neurite sprouting, whereas bFGF effectively inhibits neurite formation at concentrations above 10 ng/ml; (*b*) bFGF appeared to exert these actions in a different phase of the RA-induced neuronal differentiation than activin. bFGF has no effects on the early activin-sensitive phase of neurogenesis, during which neuronal selection occurs and early neuronal markers are detectable, but rather serves as a dualistic modulator of subsequent neurite formation and neuronal maturation.

Similar effects of bFGF were not observed in P19-EC cells (29), most likely because bFGF was present only during the aggregation phase in this study. Our finding, that low concentrations of bFGF can stimulate neuronal differentiation, is, however, in accordance with observations on *Xenopus* embryonic cells (19) and strikingly similar to the results obtained with bFGF on neurite outgrowth in rat cerebellar neurons (44).

As for activin, the time dependency of the action of bFGF seems not be related to the expression levels of its receptors. Its relatively late effects make it, however, unlikely that a direct interaction with RA signaling is involved and indicate that the cells have to acquire a neuronal predisposition before bFGF signaling renders any effect. At this moment, one can only speculate about the molecular mechanisms underlying the dose-dependent nature of bFGF on neurite sprouting. An explanation could be that FGFR activation is a prerequisite for neurite formation and neuronal maturation, and that the continuous exposure to high concentrations of bFGF results in receptor down-regulation and thus in inhibition of these processes.

Thus far, evidence obtained from *in vivo* studies on *Xenopus* embryos (18, 45) and mice (25) has stressed the importance of FGFR signaling in mesoderm patterning. Our results suggest that concentration gradients of bFGF could be operative in neuronal patterning *in vivo* as well. Of particular importance for speculation on such a role could be the recent finding of Williams *et al.* (46), that FGF receptors can be activated via certain domains of cellular adhesion molecules, N-cadherin or L1 (46). This raises the possibility that neural cell adhesion molecules interfere with bFGF signaling, or *vice versa,* and that they serve as substrate regulators of neuronal differentiation in a position-dependent manner, both in the early vertebrate embryo as well as in *in vitro* models such as the P19-RPTPα cultures used in this study.

Materials and Methods

Materials. Recombinant bovine activin A was obtained from Innogenetics, S.A. (Ghent, Belgium). bFGF (146 amino acids) was obtained from Boehringer Mannheim. The monoclonal anti-GAP-43 antibody was purchased from Sigma Chemical Co., the monoclonal anti-NF-165 antibody from the Developmental Studies Hybridoma Bank, and the rabbit polyclonal anti-MAP-kinase antibody was a kind gift of Dr. B. Burgering. The transforming growth factor β and activin-responsive 3TP-lux reporter construct, containing three 12-*O*-tetradecanoylphorbol-13-acetate response elements and the −740 to −636 region of the plasminogen activator inhibitor 1 promoter in front of the luciferase gene, was a gift of Dr. J. Massagué. A reporter construct containing the −800 to +75 region of the plasminogen activator inhibitor 1 promoter in front of the luciferase gene (PAI-luc) was obtained from Dr. D. J. Loskutoff.

Cell Culture. P19-EC cells and P19 cells stably transfected with RPTPα (P19-RPTPα) were cultured in DMEM/Ham's-F12 (1:1) medium supplemented with 7.5% FCS on gelatin-coated substrates. For monolayer experiments, cells were seeded at a density of 10×10^3 cells/cm^2. P19-EC cells were first aggregated on a bacteriological dish in the pres-

ence of RA for 3 days, after which they were replated on a gelatin-coated substrate in the absence of RA. All used substrates were coated with 0.1% gelatin. Nunc culture plastics were used, except for immunofluorescence and calcium measurements, in which case glass substrates were used.

Differentiation was induced by the addition of 1×10^{-6} M RA to the culture medium. RA was present during the whole period of differentiation. Culture medium with RA and additional growth factors was replaced after 3 days.

Calcium Measurements. P19-RPTPα cells, differentiated for at least 5 days with 10^{-6} M RA, were incubated in HEPES buffered saline [140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose (pH 7.3) at 33°C] with 10 μM Indo-1-AM (Molecular Probes, Eugene, OR) for 45 min. Coverslips, mounted in a quartz cuvette, were placed in a Perkin-Elmer model 3000 fluorescence spectrometer. The excitation wavelength was 355 nm (slit, 5 nm), and the emission wavelength was 405 nm (slit, 10 nm; Ref. 47).

Calibration of the internal calcium level was determined using 5 μg/ml ionomycin. The maximal and minimal fluorescence was determined by the addition of 4 mM $CaCl_2$ and 6 mM $MnCl_2$, respectively. Absolute values were calculated according to Grynkiewitcz *et al.* (48).

Immunoblotting Experiments. After culturing, dishes with the cells were washed twice with ice-cold PBS, scraped into radioimmunoprecipitation buffer containing 200 μM orthovanadate, 2 mM phenylmethylsulfonyl fluoride, and 3 μl/ml aprotinin and passed several times through a 21-gauge needle. A small amount of the cell lysate was used to determine protein contents; to the rest, appropriate amounts of 5× sample buffer were added, after which the samples were boiled for 5 min.

Equal amounts of protein were loaded on a 8% SDS-polyacrylamide gel. After running, the proteins were blotted to Immobilon polyvinylidene difluoride (Millipore, Bedford, MA). Subsequently, the blot was blocked with Blotto (PBS with 4% milk and 0.05% Tween-20) and incubated with antibodies for 1 h. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies for another hour. Immune complexes were detected by ECL (Amersham International, Buckinghamshire, United Kingdom).

Immunofluorescence. After culturing, the cells were washed with PBS and fixed with 2% paraformaldehyde in PBS for at least 30 min. Antibody incubation was preceded by appropriate washing and blocking. Finally, the coverslips were mounted in Moviol. The detection of immune complexes was performed by photographing the cells using a Zeiss fluorescence microscope.

Luciferase Assay with Activin Reporters. P19-RPTPα cells were cultured in six-well plates (Nunc) and transiently transfected with the 3TP-lux reporter construct or the PAI-luc reporter construct in combination with a lac-Z reporter construct by a standard calcium phosphate precipitation method. After transfection, cells were cultured for 18 h in the presence or absence of 50 ng of activin A or 50 ng of BMP-7 per milliliter of culture medium. Cells were then lysed in 500 μl of lysis buffer (1% Triton, 25 mM glycylglycin, 15 mM $MgSO_4$, 4 mM EGTA, and 1 mM DTT). Luciferase activity was measured in a Lumac 3 M biocounter by the addition of 265 μl of assay buffer (3.8 mM ATP, 34 mM glycylglycin, and 20 mM $MgSO_4$) and 100 μl of 0.2 mM luciferine (Boehringer Mannheim) to 75 μl of lysate. lac-Z activity was determined by measuring the conversion of *o*-nitrophenyl β-D-galactopyranoside (Sigma Chemical Co.) in a Bio-Rad microplate reader at 420 nm. Luciferase activity was corrected for lac-Z activity. The induction of luciferase activity by activin was calculated by comparing luciferase activity in cells cultured in the presence or absence of activin.

RNA Isolation and Northern Blotting. RNA was extracted from undifferentiated and differentiated (5 days of 10^{-6} M RA) P19-RPTPα cells cultured on 150-cm^2 tissue culture dishes (Nunc). The cells were lysed with 4 M guanidine thiocyanate, and total RNA was isolated as described by Chirgwin *et al.* (49). Poly(A)$^+$ RNA was isolated by oligo(dT) chromatography and quantified by measuring the absorbance at 260 nm. Aliquots of 10 μg of poly(A)$^+$ RNA were denatured in 50% formamide containing 2.2 M formaldehyde and MEN buffer (20 mM 3-[*N*-morpholino]propanesulfonic acid, 1 mM EDTA, and 5 mM sodium acetate, pH 7.0) for 15 min at 68°C and electrophoresed in a 0.8% agarose gel containing 2.2 M formaldehyde. After electrophoresis, RNA was stained with ethidium bromide and transferred to nitrocellulose filters in 20× SSC (1× SSC = 150 mM NaCl and 15 mM sodium citrate, pH 7.0). Following transfer, the filters were rinsed in 2× SSC and baked at 80°C for 2 h under vacuum.

Filters were prehybridized at 42°C in 5× SSC, 2× Denhardt's (0.04% BSA, 0.04% Ficoll 400, and 0.04% polyvinylpyrrolidon 360), 50 mM sodium phosphate (pH 6.8), 10 mM EDTA, 0.1% SDS, and 0.1 mg/ml sheared herring sperm DNA containing 50% formamide and hybridized in the same buffer with ^{32}P-labeled probe for 24 h at 42°C. Probes were labeled using the Rediprime random primer labeling kit (Amersham International) and [^{32}P]dCTP (>3000 Ci/mmol; Amersham International). Following hybridization, filters were washed in 2× SSC/0.1% SDS, and labeled products were visualized by autoradiography.

The following probes were used for Northern blot hybridization: mouse activin receptor IA, a 629-bp fragment encoding the extracellular domain, the transmembrane domain, and a small part of the juxtamembrane domain; mouse activin receptor type IB, a 560-bp fragment encoding the extracellular domain, the transmembrane domain, and a small part of the juxtamembrane domain; mouse activin receptor type IIA, a 448-bp fragment encoding the extracellular domain and part of the transmembrane domain; mouse activin receptor type IIB, a 410-bp fragment encoding the extracellular domain; FGFR1, a 1.2-kb fragment encoding the entire extracellular domain; FGFR2, a 1.2-kb fragment encoding the entire extracellular domain; FGFR3, a 2.4-kb fragment encoding the extracellular domain; and FGFR4, a 2.5-kb fragment encoding the extracellular domain.

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References

1. Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D., and Spiess, J. Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. Nature (Lond.), *321:* 776–779, 1986.

2. Nakamura, T., Asashima, M., Eto, Y., Takio, K., Uchiyama, H., Moriya, N., Ariizumi, T., Yashiro, T., Sugino, K., Titani, K., and Sugino, H. Isolation and characterization of native activin-B. J. Biol. Chem., *267:* 16385–16389, 1992.

3. Ling, N., Ying, S-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M., and Guillemin, R. Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. Nature (Lond.), *321:* 779–782, 1986.

4. Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K., and Sugino, H. Activin-binding protein from rat ovary is follistatin. Science (Washington DC), *247:* 836–838, 1990.

5. Mathews, L. S., and Vale, W. Expression cloning of an activin receptor, a predicted transmembrane serine kinase. Cell, *65:* 973–982, 1991.

6. Attisano, L., Wrana, J. L., Cheifetz, S., and Massagué, J. Novel activin receptors: distinct genes and alternative messenger RNA splicing generate a repertoire of serine/threonine kinase receptors. Cell, *68:* 97–108, 1992.

7. Ebner, R., Chen, R-H., Shum, L., Lawler, S., Zioncheck, T. F., Lee, A., Lopez, A. R., and Derynck, R. Cloning of a type-I TGF-β receptor and its effect on TGF-β binding to the type II receptor. Science (Washington DC), *260:* 1344–1347, 1993.

8. Ten Dijke, P., Ichijo, H., Franzén, P., Schulz, P., Saras, J., Toyoshima, H., Heldin, C-H., and Miyazono, K. Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. Oncogene, *8:* 2879–2887, 1993.

9. Attisano, L., Wrana, J. L., Montalvo, E., and Massagué, J. Activation of signalling by the activin receptor complex. Mol. Cell. Biol., *16:* 1066–1073, 1996.

10. Mason, I. J. The ins and outs of fibroblast growth factors. Cell, *78:* 547–552, 1994.

11. Pazin, M. J., and Williams, L. T. Triggering signaling cascades by receptor tyrosine kinases. Trends Biochem. Sci., *17:* 374–378, 1992.

12. Johnson, D. E., and Williams, L. T. Structural and functional diversity in the FGF receptor multigene family. Adv. Cancer Res., *60:* 1–41, 1993.

13. Hemmati-Brivanlou, A., and Melton, D. A. A truncated activin receptor inhibits mesoderm induction and formation of axial structures in *Xenopus* embryos. Nature (Lond.), *359:* 609–614, 1992.

14. Hemmati-Brivanlou, A., and Melton, D. A. Inhibition of activin receptor signaling promotes neuralization in *Xenopus*. Cell, *77:* 273–281, 1994.

15. Hemmati-Brivanlou, A., Kelly, O. G., and Melton, D. A. Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. Cell, *77:* 283–295, 1994.

16. Slack, J. M. W., Darlington, B. G., Heath, J. K., and Godsave, S. F. Mesoderm induction in early *Xenopus* embryos by heparin binding growth factors. Nature (Lond.), *326:* 197–200, 1987.

17. Kimelman, D., Abraham, J. A., Haaparanta, T., Palisi, T. M., and Kirschner, M. W. The presence of fibroblast growth factor in the frog egg: its role as a natural mesoderm inducer. Science (Washington DC), *242:* 1053–1056, 1988.

18. Amaya, E., Stein, P. A., Musci, T. J., and Kirschner, M. W. FGF signalling in the early specification of mesoderm in *Xenopus*. Development (Camb.), *118:* 477–487, 1993.

19. Kengaku, M., and Okamoto, H. Basic fibroblast growth factor induces differentiation of neural tube and neural crest lineages of cultured ectoderm cells from *Xenopus* gastrula. Development (Camb.), *119:* 1067–1078, 1993.

20. Kengaku, M., and Okamoto, H. Basic fibroblast growth factor as a possible morphogen for the anteroposterior axis of the central nervous system in *Xenopus*. Development (Camb.), *121:* 3121-3130, 1995.

21. Matzuk, M. M., Kumar, T. R., Vassalli, A., Bickenbach, J. R., Roop, D. R., Jaenisch, R., and Bradley, A. Functional analysis of activins during mammalian development. Nature (Lond.), *374:* 354–356, 1995.

22. Matzuk, M. M., Kumar, T. R., and Bradley, A. Different phenotypes for mice deficient in either activins or activin receptor type II. Nature (Lond.), *374:* 356–360, 1995.

23. Mansour, S. L., Goddard, J. M., and Capecchi, M. R. Mice homozygous for a targeted disruption of the proto-oncogene *int-2* have developmental defects in the tail and inner ear. Development (Camb.), *117:* 13–28, 1993.

24. Hebert, J. M., Rosenquist, T., Gotz, J., and Martin, G. R. FGF5 as a regulator of the hair growth cycle: evidence from targeted and spontaneous mutations. Cell, *78:* 1017–1025, 1994.

25. Yamaguchi, T. P., Harpal, K., Henkemeyer, M., and Rossant, J. FGFR-1 is required for embryonic growth and mesodermal patterning during mouse gastrulation. Genes Dev., *8:* 3032–3044, 1994.

26. Martin, G. R. Teratocarcinomas and mammalian embryogenesis. Science (Washington DC), *209:* 768–776, 1980.

27. Hashimoto, M., Kondo, S., Sakurai, T., Etoh, Y. Shibai, H., and Maramatsu, M. Activin/EDF as an inhibitor of neuronal differentiation. Biochem. Biophys. Res. Commun. *173:* 193–200, 1990.

28. Van den Eijnden-van Raaij, A. J. M., van Achterberg, T. A. E.. van der Kruijssen, C. M. M., Piersma, A. H., Huylebroeck, D., de Laat, S. W., and Mummery, C. L. Differentiation of aggregated murine P19 embryonal carcinoma cells is induced by a novel visceral endoderm specific FGF-like factor and inhibited by activin A. Mech. Dev., *33:* 157–166, 1991.

29. Van der Kruijssen, C. M. M., van Achterberg, T. A. E., Feijen, A., Hébert, J. M., de Waele, P., and van den Eijnden-van Raaij, A. J. M. Neuronal and mesodermal differentiation of P19 embryonal carcinoma cells is characterized by expression of specific marker genes and modulated by activin and fibroblast growth factors. Dev. Growth Differ., *37:* 559–574, 1995.

30. Jones-Villeneuve, E. M. V., McBurney, M. W., Rogers, K. A., and Kalnins, V. I. Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. J. Cell Biol., *94:* 253–262, 1982.

31. Bain, G., Kitchens, D., Yao, M., Huettner, J. E., and Gottlieb, D. I. Embryonic stem cells express neuronal properties *in vitro*. Dev. Biol., *168:* 342–357, 1995.

32. den Hertog, J., Pals, C. E. G. M., Peppelenbosch, M. P., Tertoolen, L. G. J., de Laat, S. W., and Kruijer, W. Receptor protein tyrosine phosphatase α activates $pp60^{c\text{-}src}$ and is involved in neuronal differentiation. EMBO J., *12:* 3789–3798, 1993.

33. van Inzen, W. G., Peppelenbosch, M. P., van den Brand, M. W. M., Tertoolen, L. G. J., and de Laat, S. W. The role of receptor protein tyrosine phosphatase α in neuronal differentiation of embryonic stem cells. Dev. Brain Res., *91:* 304–307, 1996.

34. Aigner, L., Arber, S., Kapfhammer, J. P., Laux, T., Schneider, C., Botteri, F., Brenner, H. R., and Caroni, P. Overexpression of the neural growth-associated protein gap-43 induces nerve sprouting in the adult nervous system of transgenic mice. Cell, *83:* 269–278, 1995.

35. Nakahira, K., Ikenaka, K., Wada, K., Tamura, T. A., Furuichi, T., and Mikoshiba, K. Structure of the 68-kda neurofilament gene and regulation of its expression. J. Biol. Chem., *265:* 19786–19791, 1990.

36. Bruch, R. C., and Carr, V. M. Rat olfactory neurons express a 200 kDa neurofilament. Brain Res., *550:* 133–136, 1991.

37. Mummery, C. L., van Rooyen, M., Bracke, M., van den Eijnden-van Raaij, J., van Zoelen, E. J., and Alitalo, K. Fibroblast growth factor-mediated growth regulation and receptor expression in embryonal carcinoma and embryonic stem cells and human germ cell tumours. Biochem. Biophys. Res. Commun., *191:* 188–195, 1993.

38. Wrana, J. L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X. F., and Massagué, J. TGF-β signals through a heteromeric protein kinase receptor complex. Cell, *71:* 1003–1014, 1992.

39. Keeton, M. R., Curriden, S. A., van Zonneveld, A. J., and Loskutoff, D. J. Identification of regulatory sequences in the type-I plasminogen activator inhibitor gene responsive to transforming growth factor β. J. Biol. Chem., *266:* 23048–23052, 1991.

40. Van den Eijnden van Raaij, A. J. M., van Zoelen, E. J. J., van Nimmen, K., Koster, C. H., Snoek, G. T., Durston, A. J., and Huylebroeck, D. Activin-like factor from a *Xenopus*-laevis cell line responsible for mesoderm induction. Nature (Lond.), *345:* 732–734, 1990.

41. Smith, J. C., Price, B. M., van Nimmen, K., and Huylebroeck, D. Identification of a potent *Xenopus* mesoderm-inducing factor as a homolog of activin A. Nature (Lond.), *345:* 729–731, 1990.

42. de Winter, J. P., de Vries, C. J. M., van Achterberg, T. A. E., Ameerun, R. F., Feijen, A., Sugino, H., de Waele, P., Huylebroeck, D., Verschueren, K., and van den Eijnden-van Raaij, A. J. M. Truncated activin type II receptors inhibit activin bioactivity by the formation of heteromeric complexes with activin type I receptors. Exp. Cell Res., *224:* 323–334, 1996.

43. Fann, M-J., and Patterson, P. H. Neuropoietic cytokines and activin A differentially regulate the phenotype of cultured sympathetic neurons. Proc. Natl. Acad. Sci. USA, *91:* 43–47, 1994.

44. Williams, E. J., Furness, J., Walsh, F. S., and Doherty, P. Characterization of the second messenger system underlying neurite outgrowth stimulated by FGF. Development (Camb.), *120:* 1685–1693, 1994.

45. Cornell, R. A., Musci, T. J., and Kimelman, D. FGF is a prospective competence factor for early activin-type signals in *Xenopus* mesoderm induction. Development (Camb.), *121:* 2429–2437, 1995.

46. Williams, E. J., Furness, J., Walsh, F. S., and Doherty, P. Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-cam, and N-cadherin. Neuron, *13:* 583–594, 1994.

47. Moolenaar, W. H., Aerts, R. J., Tertoolen, L. G. J., and de Laat, S. W. The epidermal growth factor induced calcium signal in A431 cells. J. Biol. Chem., *261:* 279–285, 1986.

48. Grynkiewitcz, G. M., Poenie, M., and Tsien, R. A new generation of calcium indicators with greatly improved fluorescent properties. J. Biol. Chem., *260:* 5236–5239, 1985.

49. Chirgwin, J. M., Pryzbala, A. E., MacDonald, R. Y., and Rutter, W. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry, *18:* 5294–5299, 1977.