Neuronal Differentiation in Response to Epidermal Growth Factor of Transfected Murine P19 Embryonal Carcinoma Cells Expressing Human Epidermal Growth Factor Receptors[1]

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Abstract

The human epidermal growth factor receptor (hEGF-R) was introduced into murine P19 embryonal carcinoma (EC) cells, which do not express endogenous EGF-R. Undifferentiated stable P19 EC transfectants containing multiple copies of the hEGF-R complementary DNA were isolated. These cells express functional EGF-R, exhibiting characteristic biphasic EGF binding and intrinsic tyrosine protein kinase activity. Whereas normally EGF induces the expression of multiple nuclear protooncogenes, only *jun*B expression is induced by EGF in the HER-transfected cells. This indicates that undifferentiated P19 EC cells contain at least part of a signal transduction machinery capable of coupling to the ectopically expressed hEGF-R. Interestingly, neuronal differentiation is induced in these cells in response to EGF under culture conditions resembling those during early preimplantation embryogenesis. These results indicate that neuronal differentiation of pluripotent P19 EC cells can be induced via activation of a tyrosine protein kinase signaling pathway.

Introduction

The generation of different cell types from a single pluripotent stem cell, as occurs during early embryogenesis, is a highly regulated process involving selective proliferation and differentiation of cells according to a specific program. Polypeptide growth and differentiation factors have been shown to be crucial components of this process in early vertebrate development (1–4), implying that the stage- and cell type-specific expression of growth factor receptors coordinates the development of multicellular systems.

The EGF-R[3] has been detected in preimplantation mouse embryos as early as the eight-cell stage (5) and after gestation from day 10 onward (6, 7). Additionally, one of the ligands of the EGF-R, transforming growth factor α, is found to be expressed in the 32–64-cell stage of early murine preimplantation embryos (8).

EC cells resemble the pluripotent stem cells of the inner cell mass of preimplantation embryos and have been widely used as a model system to study early murine development (9, 10). The pluripotent murine P19 EC cell line (11) has the capacity to differentiate *in vitro* in response to agents such as RA and DMSO (12, 13). Differentiated P19 derivatives, resembling all three germ layers, have been obtained (14). Differentiation of P19 EC cells is accompanied by characteristic changes in growth factor and growth factor receptor gene expression. The EGF-R gene is not expressed in undifferentiated P19 EC cells, in accordance with the absence of EGF-binding activity in the inner cell mass of mouse blastocysts (5). The EGF-R is expressed in P19 cells following treatment with RA for 3–5 days (15).

The human EGF-R is a glycosylated transmembrane protein of M_r 170,000, consisting of an extracellular ligand-binding domain, a single hydrophobic transmembrane region, and an intracellular domain with tyrosine protein kinase activity (for a review, see Ref. 16). Ligand binding to the EGF-R occurs through binding to high and low affinity receptors (17–19) and leads to receptor internalization and degradation (16). The intrinsic tyrosine protein kinase of the EGF-R is activated upon ligand binding and leads to tyrosine autophosphorylation of the receptor as well as tyrosine phosphorylation of a number of cellular substrates (16, 20). Among these is phospholipase Cγ, which is probably activated by tyrosine phosphorylation, resulting in increased phosphoinositide turnover and thus production of the second messengers diacylglycerol and inositol-1,4,5-trisphosphate (21, 22). These, in turn, give rise to a number of cellular responses, including activation of the Na^+-H^+ exchanger and Ca^{2+} (influx and) release (23). In addition, activation of the EGF-R leads to the induction of a number of immediate early genes, including c-*fos*, c-*myc*, c-*jun*, and *jun*B (24–28). Eventually, activation of the EGF-R and subsequent signal transduction lead to stimulation of cell proliferation (16). Analysis of mutant EGF-R has demonstrated that activation of the tyrosine kinase is essential for the im-

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[3] The abbreviations used are: EGF(-R), epidermal growth factor (receptor); h-, human; HER, human epidermal growth factor receptor; EC, embryonal carcinoma; RA, retinoic acid; DMSO, dimethyl sulfoxide; IL-3, interleukin 3; kb, kilobase(s); bp, base pair(s); cDNA, complementary DNA; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.

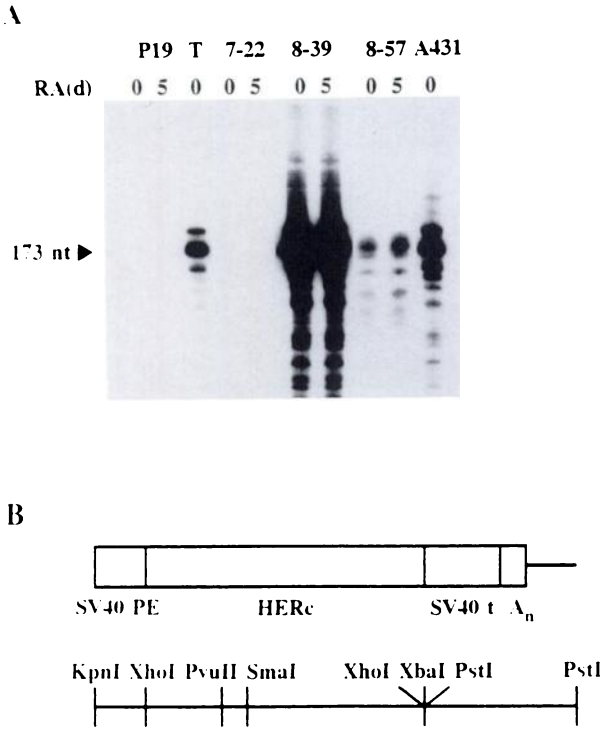

Fig. 1. *A*, HER mRNA expression of P19 EC transfectants. P19 EC is a wild-type murine embryonal carcinoma cell line, and A431 is a human squamous carcinoma cell line expressing high levels of hEGF-R. Stable transfectants of P19 EC cells were obtained by cotransfection of pSV2neo with an empty expression vector (P19 7-22) or with pSV2HERc (P19 8-39 and P19 8-57) and subsequent G418 selection. *Lane T*, P19 EC cells transiently transfected with pSV2HERc. Total RNA of undifferentiated cells (0 days RA) and of cells treated with RA (10^{-6} M) for 5 days (5 days RA) was isolated and analyzed. RNase protection was carried out using 50 μg of total RNA (2 μg for A431) and a synthetic antisense mRNA probe derived from a *PvuII/SmaI* fragment of the hEGF-R cDNA. *nt*, nucleotide. *B*, structure of the hEGF-R expression vector pSV2HERc, derived as described in "Materials and Methods." The 5.5-kb transcription unit of pSV2HERc together with a restriction endonuclease map, displaying relevant sites, is depicted. The pUC18-based construct contains the hEGF-R cDNA (extending from 51 bp upstream of the translation initiation codon to 151 bp downstream of the termination codon) under transcriptional control of the SV40 early promoter (*SV40 PE*). The SV40 small t intron (*SV40 t*) and polyadenylation signal (A_n) are located 3′ of the hEGF-R cDNA.

mediate cellular responses as well as for stimulation of cell proliferation (29–32).

Ectopically expressed hEGF-R can couple to preexisting signal transduction pathways in a variety of cell lines. EGF is able to transduce its mitogenic signal via exogenous EGF-R on CHO cells (33), NIH 3T3 cells (29, 30, 34), and IL-3-dependent hemopoietic stem cells (35–37). In addition, Wang *et al.* (37) have shown that in the presence of IL-3 and EGF, hEGF-R-expressing, IL-3-dependent pre-mast cells differentiate into more mature mast cells than in the presence of IL-3 alone.

To investigate the biological function of tyrosine protein kinase-containing receptors during early murine development, we introduced functional hEGF-R into EGF-R-less, undifferentiated murine P19 EC cells. We show that the exogenous hEGF-R is capable of transducing a signal upon ligand binding, resulting in early response gene expression. In addition, we show that in response to EGF, ectopically expressed hEGF-R alters the direction of P19 EC cell differentiation, leading to neuronal differentiation.

Results

Introduction and Expression of the HER Gene in Murine P19 Embryonal Carcinoma Cells. To introduce the gene encoding the hEGF-R in undifferentiated P19 EC cells, an expression vector was constructed as described in "Materials and Methods." This construct, pSV2HERc, contains the hEGF-R cDNA under transcriptional control of the early SV40 promoter (Fig. 1). pSV2HERc was transfected via calcium phosphate precipitation-mediated DNA transfer in P19 EC cells together with pSV2neo, containing the selectable *neo* gene (38). After selection for 14 days, about 80 G418-resistant colonies had formed, of which 40 were cloned and established as stable cell lines. Genomic DNA of these cell lines was isolated and analyzed by Southern blotting. A fragment of 4.1 kb, indicative for intact copies of the SV40/HER chimeric transcription unit, could be detected after hybridization with a hEGF-R cDNA probe. Two P19 EC clones, P19 8-39 and P19 8-57, each containing multiple genomic copies of the HER gene, were selected for further study (Table 1). A control cell line, P19 7-22, was isolated after cotransfection of pSV2neo together with an empty expression vector (*i.e.*, pSV2HERc lacking the hEGF-R cDNA) and subsequent G418 selection and cloning.

Expression of HER mRNA in the selected cell lines was investigated by RNase protection using an antisense RNA probe, synthesized from a *PvuII/SmaI* fragment, located in a region encoding the extracellular domain of the hEGF-R (Fig. 1*B*). With this probe, a 173-nucleotide protected fragment was detected following hybridization with RNA isolated from P19 EC cells, transiently transfected with pSV2HERc (Fig. 1*A*). Additionally, protection of this 173-nucleotide fragment was detected after hybridization of the probe to RNA isolated from A431 cells, a human squamous carcinoma cell line expressing the hEGF-R at a high level (39). No protection of this probe was observed with RNA isolated from both undifferentiated P19 EC cells and their RA-differentiated derivatives, which do express murine EGF-R mRNA (15), indicating that this probe permits specific detection of HER mRNA. P19 7-22 does not express HER mRNA, whereas P19 8-39 and, to a lesser extent, P19 8-57 express HER mRNA (Fig. 1*A*; Table 1). For P19 8-39, no significant difference in the level of HER mRNA expression between undifferentiated and RA-differentiated transfectants was observed (Fig. 1*A*), although SV40 early promoter activity is (slightly) enhanced in differentiated derivatives of P19 EC cells in transient transfection assays (40).[4] Possibly, the chromosomal locations of the exogenous HER gene copies account for the fact that HER mRNA expression is not up-regulated in P19 8-39 cells after RA-induced differentiation.

All selected transfected P19 cell lines had retained SSEA-1 (41) and ECMA-7 (42) surface antigen expression, as determined by indirect immunofluorescence (Fig. 2;

[4] J. den Hertog and W. Kruijer, unpublished observations.

Table 1 Properties of HER-transfected P19 cells

Cell line	HER copies/cell	Expression[a]			Soft agar growth (%)	No. of EGF-Rs/cell (× 10^{-4})			
						0 days RA		5 days RA	
		HER mRNA	SSEA-1	ECMA-7		Human	Murine	Human	Murine
P19 EC	–	–	+++	++	26.4	–	<0.2	–	18.3
P19 7-22	–[b]	–	+++	++	24.8	–	<0.2	–	19.4
P19 8-39	~1500	+++	+++	++	18.4	28.1	1.5	52.9	13.3
P19 8-57	~40	++	+++	++	19.3	ND[c]	ND	ND	ND

[a] –, ++, and +++ indicate relative levels of expression in column 2 and relative degrees of immunofluorescence intensity in columns 3 and 4.
[b] P19 7-22 contains approximately 40 copies of the empty expression vector/cell.
[c] ND, not determined.

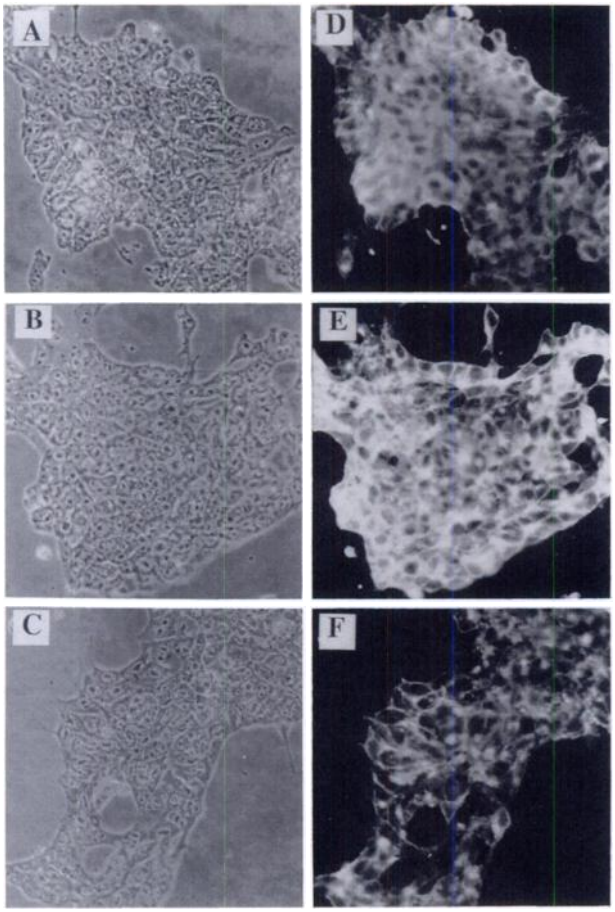

Fig. 2. SSEA-1 expression of the HER-transfected P19 EC cells. After fixation with methanol-glacial acetic acid (95/5, v/v) the cells were labeled with the undifferentiated stem cell-specific antibody anti-SSEA-1 and subsequently with a FITC-conjugated second antibody. Photographic magnification was 200×. *A–C*, phase micrographs; *D–F*, immunofluorescence. *A* and *D*, P19 EC; *B* and *E*, P19 7-22; *C* and *F*, P19 8-39.

Table 1). The observed immunofluorescence is specific, since no immunofluorescence could be detected after incubation of the cells with nonimmune serum instead of anti-SSEA-1 and ECMA-7 antibodies. Additionally, indirect immunofluorescence showed that after differentiation with RA for 5 days, the SSEA-1 and ECMA-7 antigens had disappeared from the cell surface of the wild-type and transfected P19 cells (data not shown). Furthermore, all undifferentiated transfected cell lines had retained the ability to grow in soft agar (Table 1). Both assays showed that the isolated cell lines resemble the parental undifferentiated P19 EC cells, indicating that transfection and subsequent G418 selection had not resulted in differentiation of the isolated HER-transfected cell lines.

Expression of the hEGF-R in the HER-transfected Cell Lines. The capacity of the HER-containing, stably transfected cell lines to bind ^{125}I-EGF was measured to confirm the presence of cell surface-localized hEGF-R. Undifferentiated P19 EC cells lack detectable levels of ^{125}I-EGF binding (15). Scatchard plots of ^{125}I-EGF binding experiments to undifferentiated P19 8-39 show characteristic biphasic EGF binding, caused by the presence of two classes of receptors of high (K_d ~ 0.2 nM) and low (K_d ~ 3 nM) affinity (17, 18) (Fig. 3*B*; Table 1). To discriminate between expression of human and endogenous murine receptors, we preincubated the cells prior to ^{125}I-EGF binding with the anti-EGF-R antibody Ab 528, specifically blocking EGF binding to human EGF-R (43, 44). Preincubation of differentiated P19 cells with Ab 528 did not result in lower levels of ^{125}I-EGF binding (Fig. 3, *A* and *C*), indicating that Ab 528 does not block EGF binding to murine EGF-R. In contrast, ^{125}I-EGF binding to A431 cells, expressing high levels of hEGF-R, was almost completely lost after preincubation with Ab 528 (Fig. 3*C*). Five to 10% of the EGF-Rs were not blocked by Ab 528, due to the competitive interaction of Ab 528 and EGF with the EGF-R. Undifferentiated P19 8-39 and P19 8-57 showed ^{125}I-EGF binding, which could be blocked by preincubation with Ab 528. P19 7-22, on the other hand, did not show enhanced EGF binding compared to wild-type P19 EC cells (Fig. 3*C*). For P19 8-39, the number of hEGF-Rs per cell was calculated by subtracting the number of non-Ab 528-blocked murine EGF-Rs from the total number of EGF-Rs per cell, as estimated by Scatchard plot analysis. Undifferentiated P19 8-39 contain a total of 30,000 EGF-Rs, of which about 1,500 remain after preincubation with Ab 528 (Fig. 3; Table 1). The remaining sites are most likely human EGF-R, as the blocking capacity of Ab 528 is incomplete. Only in 5-day RA-differentiated P19 8-39 and P19 8-57 cells were murine EGF-Rs expressed (Fig. 3; Table 1), in line with the RA-induced expression of EGF-R mRNA (15).

The presence of hEGF-R on the HER-transfected P19 cells was further investigated by immunoblotting analysis with anti-EGF-R antibodies. The polyclonal antiserum 281-7, which was used, specifically recognizes an epitope present in the intracellular domain of both murine

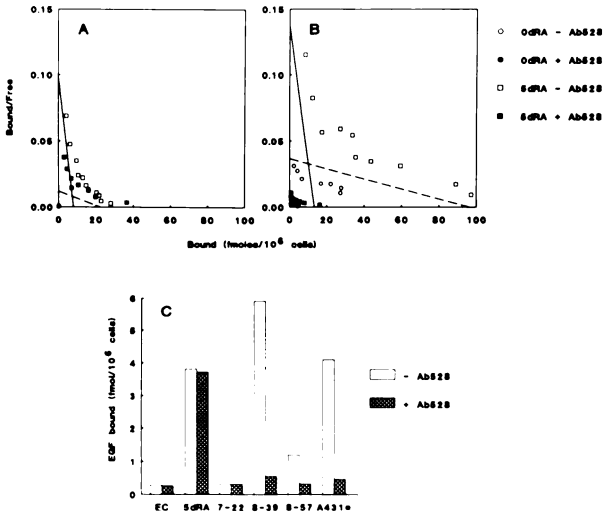

Fig. 3. EGF binding to HER-transfected P19 cells. Scatchard plots showing receptors with high and low affinity for EGF are depicted for wild-type P19 (*A*) and P19 8-39 (*B*). The cells were preincubated with anti-EGF-R antibody Ab 528 (1 μg/ml) to block EGF binding to human EGF-Rs. The *straight lines* (*solid line*, high affinity; *dashed line*, low affinity) from which the number of receptors and EGF affinities were estimated are depicted for the RA-treated cells. *C*, summary of the Ab 528 competition. EGF binding is given in fmol/10^6 cells, except for A431 cells (*A431**). EGF binding to A431 cells is given in fmol/5 × 10^4 cells. All EGF binding was corrected for nonspecific binding of ^{125}I-EGF by incubation with a 1000-fold excess of unlabeled EGF.

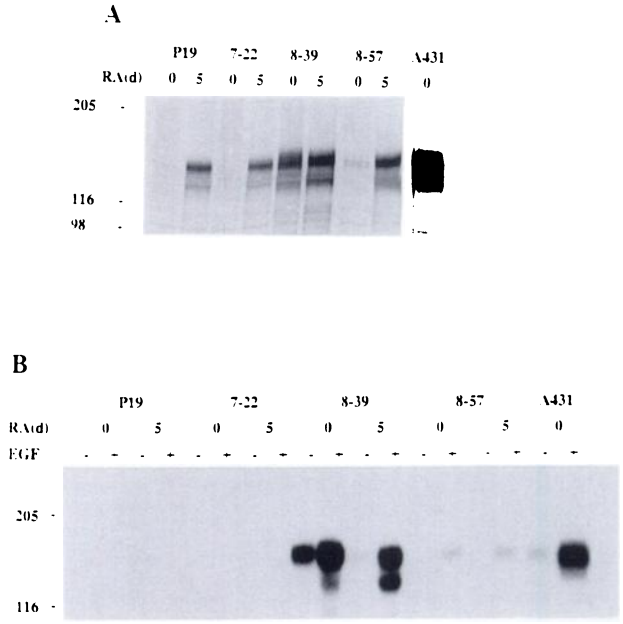

Fig. 4. hEGF-R expression and autophosphorylation. *A*, total cell lysates (200 μg; A431, 20 μg) were fractionated on a linear 5–15% SDS-polyacrylamide gel and blotted. The filter was incubated with the affinity-purified polyclonal antiserum 281-7, which is specific for the intracellular domain of the EGF-R. The EGF-R was made visible after incubation with an alkaline phosphatase-coupled second antibody and subsequent color development of the filter. *B*, total cell lysates (P19, P19 7-22, P19 8-57, 10 mg protein; P19 8-39, 1.0 mg; A431, 0.1 mg) were treated with EGF (1.0 μg/ml) or buffer for 15 min at 20°C. Phosphorylation was allowed to proceed for 10 min at 0°C, after which period the hEGF-R was immunoprecipitated with 1.0 μg of anti-hEGF-R antibody (Ab 528). Immunoprecipitates and a high molecular weight marker were electrophoresed on a linear 5–15% SDS-polyacrylamide gel.

and human EGF-Rs (19). Total cell lysates of the HER-transfected P19 cells were isolated, fractionated on a denaturing SDS-polyacrylamide gel electrophoresis gel, and subsequently blotted. A band corresponding to the size of the EGF-R (M_r 170,000) was detected in A431 cells, as well as in RA-differentiated, but not in undifferentiated, P19 cells (Fig. 4*A*). P19 7-22 showed the same EGF-R expression pattern as wild-type P19 (Fig. 4*A*). On the other hand, EGF-R was detected in undifferentiated P19 8-39, in accordance with the Scatchard analysis (Figs. 3*B* and 4*A*). Only a slight increase in immunodetectable EGF-R was found upon differentiation of P19 8-39 (Fig. 4*A*), although the number of hEGF-Rs per cell is increased 2-fold (Fig. 3; Table 1). This is probably due to the 2–3-fold larger cell surface area of the differentiated cells. The M_r 170,000 EGF-R is hardly immunodetectable in undifferentiated P19 8-57 cells (Fig. 4*A*), although undifferentiated P19 8-57 does contain EGF-binding activity (Fig. 3*C*). After differentiation of P19 8-57, EGF-R expression is more pronounced than that of differentiated wild-type P19 cells.

The molecular weight of the exogenous hEGF-R on the HER-transfected cells was similar to the molecular weight of the endogenous hEGF-R of A431 cells (Fig. 4*A*). The unglycosylated hEGF-R protein core has a molecular weight of 130,000 (45), suggesting that the extent of glycosylation of the hEGF-R in the murine cells is similar to that in human cells.

Activation of the hEGF-R in the Transfected P19 EC Cells. Activation of the hEGF-R was characterized by EGF-induced autophosphorylation *in vitro*. Aliquots of the total cell lysates of the HER transfectants (amount of protein: P19, P19 7-22, P19 8-57: 10.0 mg; P19 8-39: 1.0 mg; A431: 0.1 mg) were treated with EGF (1.0 μg/ml) or buffer, incubated with a phosphorylation assay mix, and subsequently immunoprecipitated with Ab 528, as described in "Materials and Methods." Undifferentiated P19 8-39 and P19 8-57 showed EGF-dependent autophosphorylation of the M_r 170,000 hEGF-R (Fig. 4*B*). We note that EGF-independent basal autophosphorylation levels decrease in P19 8-39 and P19 8-57 following induction of differentiation with RA. Due to detection limits, this effect is less clear in P19 8-57. However, this observation was consistent, suggesting either that the intrinsic tyrosine protein kinase is activated in undifferentiated cells by (an) endogenous signal molecule(s) or that, due to differentially expressed tyrosine protein phosphatases, the EGF-R is dephosphorylated to a higher extent in differentiated cells. Taken together, Scatchard analysis, Western blotting, and immunoprecipitation indicate that undifferentiated P19 EC cells are competent to express typical cell surface hEGF-R, containing tyrosine protein kinase activity.

EGF-dependent Gene Expression in the Transfected Cell Lines. The nuclear response to activation of the EGF-R by EGF is the rapid induction of a group of genes, including c-*fos*, c-*myc*, c-*jun*, and *jun*B (24–28). To investigate the induction of these genes by EGF in the hEGF-R-expressing P19 cell lines, RNA was isolated at different times after EGF treatment and analyzed by Northern blotting. In undifferentiated P19 EC and P19 7-22 cells, EGF fails to induce expression of c-*fos*, c-*myc*, c-*jun*, and

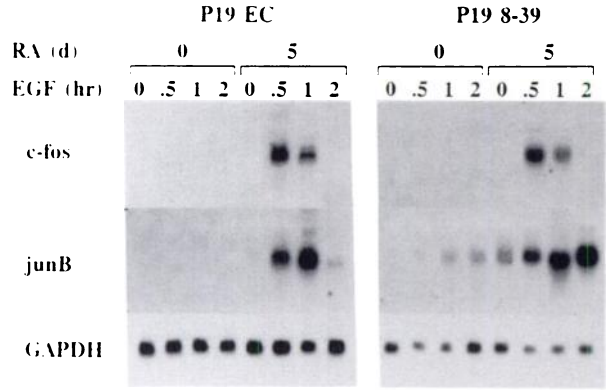

Fig. 5. Northern blot analysis of EGF-induced *c-fos* and *junB* expression in P19 and P19 8-39 cells. Total RNA of wild-type and hEGF-R-expressing undifferentiated (0 days RA) and differentiated (5 days RA) P19 cells, treated with EGF (50 ng/ml) for increasing times, was isolated and electrophoresed on formaldehyde-agarose gels (15 μg total RNA/lane). After transfer, filters were sequentially hybridized to ^{32}P-labeled probes specific for *junB*, *c-fos*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) to correct for the amount of RNA loaded per lane. Autoradiography was for 4 days (*c-fos* and *junB*) or overnight (GAPDH), at −70°C using intensifying screens.

Table 2 Rapid induction of early response genes by EGF in HER-transfected P19 cells

	Induction[a] after RA (days)							
	c-fos		*c-myc*		*c-jun*		*junB*	
	0	5	0	5	0	5	0	5
P19	−	+++	−	++	−	+	−	+++
P19 7-22	−	+++	−	++	−	+	−	+++
P19 8-39	−	+++	−	++	−	+	++	+++
P19 8-57	−	ND[b]	−	ND	−	ND	++	ND

[a] −, +, ++, and +++ indicate relative induction of the gene under observation after treatment with EGF.
[b] ND, not determined.

junB, whereas they are transiently induced in their 5-day RA-differentiated derivatives (Fig. 5; Table 2), in line with the differentiation-dependent expression of murine EGF-R by these cells. In the undifferentiated hEGF-R-expressing cell lines, P19 8-39 and P19 8-57, EGF fails to induce *c-fos*, *c-myc*, and *c-jun* expression. Only *junB* is transiently induced by EGF in undifferentiated HER-containing P19 cells (Fig. 5; Table 2). Scanning of the autoradiogram showed that peak expression of *junB* (2 h EGF) is 50-fold higher in differentiated than in undifferentiated cells. However, *junB* induction is only 5-fold higher in differentiated cells, due to the high basal level of *junB* expression in the differentiated cells. These results show that the hEGF-R activates signal transduction upon binding of EGF, leading to the induction of at least one of the early response genes.

Neuronal Differentiation Is Induced by EGF in the HER-transfected P19 Cells. EGF can have biological consequences other than stimulation or inhibition of cell growth via ectopically expressed EGF-R. Wang *et al.* (37) have shown that EGF, in the presence of IL-3, induces differentiation of an IL-3-dependent pre-mast cell line via ectopically expressed EGF-R.

P19 EC cells, growing in monolayer, can be induced to differentiate *in vitro* by adding RA (10^{-6} M) to the medium. This results in the formation of a mixed population of endoderm- and mesoderm-like cells. Furthermore, culturing of P19 EC cells in hanging droplets of medium leads to aggregation of the cells. The thus-formed embryoid bodies (aggregates) can be replated by transfer to tissue culture dishes. The replated aggregate consists of a bulk of cells, which can be regarded as the remnants of the aggregate, and a small number of monolayer-like cells, growing from the aggregate outward. The commitment of the P19 cells in the aggregate to differentiate in certain directions can be steered by the addition of chemical agents. In the absence of chemical agents, endoderm-like as well as undifferentiated EC cells are found in the outgrowth of the aggregate. When RA is present, aggregation will induce neuronal differentiation, whereas in the presence of DMSO, mesodermal differentiation prevails (12). However, the outgrowth of the aggregates is a heterogeneous population of cells. For instance, RA-induced neuronal differentiation yields approximately 30% neuronal cells, the rest of the outgrowth being predominantly endoderm-like cells.

To investigate EGF-dependent modulation of differentiation in the HER-transfected cell lines, a cellular characterization was carried out, following induction of differentiation under different conditions as outlined above. The differentiated cells were tested against a panel of immunofluorescence markers, including SSEA-1 [expressed by undifferentiated stem cells (41)], TROMA-1 [a marker for endoderm (46)], brushin [present in primitive and visceral endoderm (47)], laminin (expressed by parietal endoderm cells), NF-70 (an *M*, 70,000 neurofilament component), N-CAM [expressed in neural ectoderm and neural derivatives (48)], and MF-20 [expressed by cardiac and skeletal muscle (49)].

The undifferentiated hEGF-R-expressing cells growing in monolayer in the presence of EGF show positive staining in indirect immunofluorescence with the anti-SSEA-1 antibody. Furthermore, P19 8-39 and P19 8-57 retain the ability to grow in soft agar when EGF is present (data not shown). The additional presence of EGF during differentiation of P19 7-22, P19 8-39, and P19 8-57 in monolayer with RA as inducing agent did not alter the commitment to form endoderm- and mesoderm-like cells. Thus, when grown in monolayer, EGF has no differentiation-inducing or -modulating potential, although *junB* expression is affected (Fig. 5). Furthermore, cell proliferation of the HER-transfected P19 cells growing in monolayer is slightly inhibited by EGF (data not shown).

The immunofluorescence profile of the differentiated cells, obtained by aggregation and subsequent replating of P19 and P19 7-22 cells, was not altered by the presence of EGF (Table 3). However, when P19 8-39 and P19 8-57 were aggregated and subsequently cultured in the presence of EGF, neuronal differentiation was induced, as indicated by the high percentage of cells with neurite extensions. This was further confirmed by indirect immunofluorescence staining with the antibodies anti-NF-70 and anti-N-CAM (Fig. 6; Table 3). The amount of cells staining positive with these nerve cell-specific antibodies was estimated at 10% of the total outgrowth of the aggregates. Neuronal differentiation of P19 8-39 and P19 8-57 was dependent on the presence of EGF, since the outgrowth of aggregates made and replated in the absence of EGF showed a 10-fold lower amount of cells

Table 3. Immunofluorescence profile of hEGF-R-expressing P19 EC cells after differentiation by aggregation in presence of chemical agents (RA, DMSO) and EGF

	Immunofluorescence staining[a] in presence or absence of EGF													
	SSEA-1		TROMA-1		Brushin		Laminin		NF-70		N-CAM		MF-20	
	−	+	−	+	−	+	−	+	−	+	−	+	−	+
EGF														
P19/P19 7-22	+	+	±	±	±	±	+	+	−	−	−	−	−	−
P19 8-39/8-57	+	+	+	+	±	±	+	+	−	+	−	+	−	−
RA														
P19/P19 7-22	−	−	++	++	−	−	+	+	+	+	+	+	−	−
P19 8-39	−	−	++	++	−	−	+	+	++	++	++	++	−	−
DMSO														
P19/P19 7-22	±	±	++	++	−	−	±	±	±	±	±	±	++	++
P19 8-39	±	±	+	+	−	−	±	±	±	+	±	++	+	±

[a] ++, +, ±, and − indicate relative levels of immunofluorescence staining. Averages of 3–5 independent experiments in duplicate are depicted.

staining positive with anti-NF-70 and anti-N-CAM than aggregates made and replated in the presence of EGF (Fig. 6; Table 3). On the other hand, no cells could be detected in the outgrowth of wild-type P19 and P19 7-22 aggregates made in the absence or presence of EGF that stained positive with anti-NF-70 or anti-N-CAM (Fig. 6; Table 3). RA-induced neuronal differentiation of P19 8-39 aggregates was more pronounced than that of wild-type P19 cells. The additional presence of EGF during aggregation and replating of P19 8-39 cells in the presence of RA did not further increase the expression of NF-70 or N-CAM, nor of any of the other markers tested (Table 3). On the other hand, P19 8-39 aggregated in the presence of DMSO showed less staining with the mesoderm-specific antibody anti-MF-20 than wild-type P19 aggregates. MF-20 expression was even further lowered by the additional presence of EGF, whereas under these conditions, staining with the antibodies anti-NF-70 and anti-N-CAM was enhanced (Table 3). These results show that activation of the exogenous EGF-R can alter the commitment of P19 aggregates to differentiate in different directions in favor of neuronal differentiation.

Discussion

Undifferentiated EC cells do not express EGF-R, whereas upon RA-induced differentiation, functional EGF-R can be detected within 3–5 days by EGF binding and tyrosine kinase assays (15, 50). To study the role of tyrosine protein kinase-containing receptors in early mouse de-

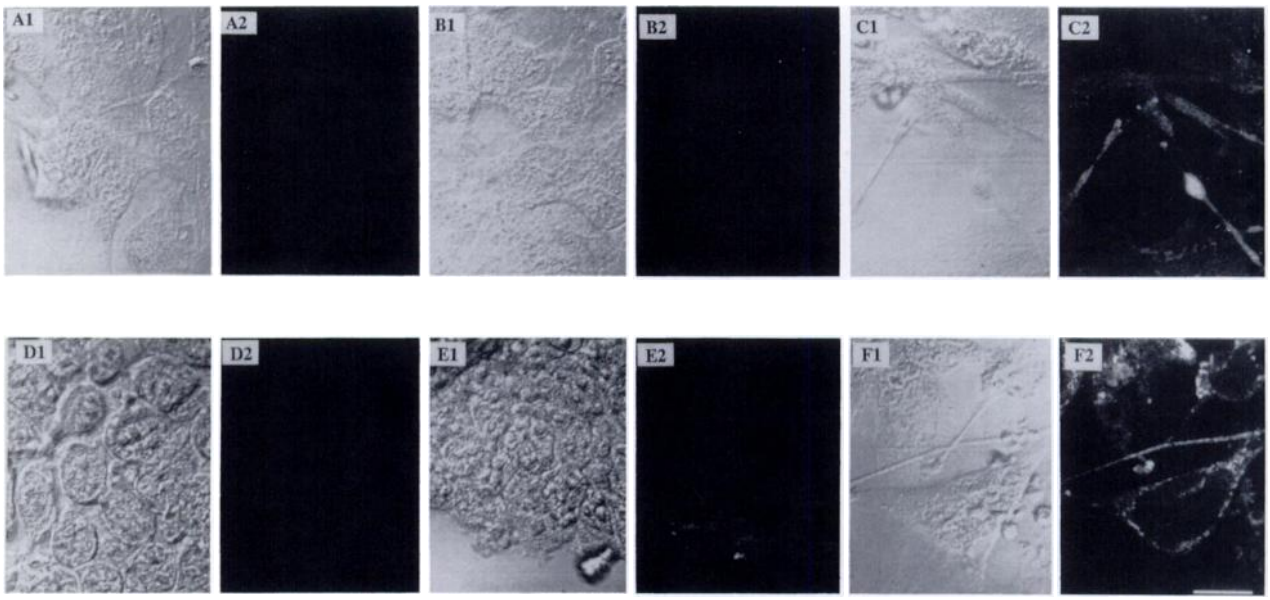

Fig. 6. Differentiation by aggregation of hEGF-R-expressing P19 EC cells. Cells were allowed to aggregate in absence or presence of EGF (50 ng/ml) for 5 days. Subsequently, the aggregates were replated on glass coverslips. After 14 days, the cells were fixed with methanol-glacial acetic acid (95/5, v/v) and labeled with the neuron-specific antibodies anti-NF-70 or anti-N-CAM. Subsequently, the cells were incubated with second (FITC-conjugated) antibody. *Bar* in *F2*, 25 μm. The phase micrographs (*1*) are represented by the corresponding immunofluorescence pictures (*2*). *A–C*, staining with anti-NF-70; *D–F*, staining with anti-N-CAM. *A* and *D*, P19 EC + EGF; *B* and *E*, P19 8-39 − EGF; *C* and *F*, P19 8-39 + EGF.

velopment, a HER-containing expression vector was transfected in undifferentiated P19 EC cells. Two P19 EC clones containing multiple copies of the intact HER gene and expressing HER mRNA were selected. The differentiation state of the HER-containing clones was not altered by the transfection procedure and subsequent cloning.

Analogous to EGF-R found on epithelial cells and on HER-transfected CHO, NIH 3T3, and IL-3-dependent hemopoietic stem cells, the exogenous EGF-Rs on P19 EC cells exhibit characteristic biphasic EGF binding of both high and low affinity (18, 29, 30, 33–37). Additionally, EGF-dependent intrinsic tyrosine protein kinase activity, which is essential for signal transduction via the EGF-R (29–32), can be detected in the HER-transfected P19 cells.

One of the early responses of EGF-R-bearing cells to ligand binding and subsequent activation of the EGF-R signal transduction pathway is the rapid induction of a number of nuclear protooncogenes, including c-*fos*, c-*myc*, c-*jun*, and *jun*B (24–28). Of these early response genes, only *jun*B is induced by EGF in both undifferentiated HER-transfected cell lines, whereas all four genes are induced in differentiated derivatives of P19 EC cells. This finding implies that a functional signal transduction machinery, capable of coupling to the hEGF-R, is present in undifferentiated P19 EC cells. Until now, this could not be proved, due to the lack of expression of known ligand-inducible receptors with tyrosine protein kinase activity by these cells. The inability of the hEGF-R-expressing P19 EC cells to induce c-*fos* is not completely understood. Although c-*fos* and *jun*B are co-induced by a number of external stimuli in differentiated cells, both genes are not inducible by 12-*O*-tetradecanoylphorbol-13-acetate in undifferentiated P19 cells (51). Apparently, the induction of c-*fos* and *jun*B by EGF is uncoupled in the undifferentiated HER-transfected P19 cells. This is probably due to the fact that the c-*fos* and *jun*B enhancers are structurally dissimilar and bind different *trans*-activating factors (52, 53).

EGF can have cellular effects other than stimulation or inhibition of growth via ectopically expressed EGF-R. Wang *et al.* (37) have shown that hEGF-R-expressing, IL-3-dependent pre-mast cells differentiate into more mature mast cells in the presence of EGF and IL-3. EGF in itself has no differentiation-inducing capacity on the HER-transfected P19 cells growing in monolayer. However, a slight inhibition of cell proliferation was observed in response to EGF. The commitment of HER-transfected P19 cells to differentiate into a mixed population of endoderm- and mesoderm-like cells during RA-induced differentiation is not altered by the presence of EGF. By contrast, neuronal differentiation of the hEGF-R-expressing P19 cells is induced when these cells are aggregated and replated in the presence of EGF. In this respect, it is worthwhile to mention that pluripotent P19 cells resemble neuroectoderm of early preimplantation mouse embryos. The sequence of events that take place during early embryogenesis is that mesoderm is formed between ectoderm and endoderm. During development, the mesoderm delivers signal(s) capable of inducing differentiation of neuroectoderm in neural tissues. This process is mimicked during P19 EC cell aggregation. Neuronal differentiation of P19 aggregates is dependent on RA, but the mechanism by which this process is steered is unknown. In this study, we show that the presence and activation of the hEGF-R in P19 EC cells trigger neuronal differentiation. Possibly, the exogenous EGF-R substitutes for a tyrosine protein kinase receptor that is expressed on undifferentiated P19 EC cells and that normally fulfills this role upon activation by signaling molecules derived from mesoderm. It is noteworthy that neuronal cells express high levels of an activated form of the tyrosine protein kinase $pp60^{c\text{-}src}$ (54). In addition, nerve growth factor, capable of enhancing nerve cell proliferation and neuronal differentiation, induces tyrosine protein phosphorylation in target cells (55). Hence, tyrosine protein kinases probably play an important role in neuronal differentiation and maintenance of the neuronal state. The mechanism of the EGF-induced neuronal differentiation of HER-transfected P19 cells could be analogous to the neural induction by 12-*O*-tetradecanoylphorbol-13-acetate of *Xenopus laevis* embryos, which is mediated by activation of protein kinase C (56, 57). Since activation of the EGF-R by one of its ligands also leads to activation of protein kinase C, similar events might take place during neural induction of *X. laevis* embryos and neuronal differentiation of the hEGF-R-expressing P19 cell lines. Our results indicate that the activation of a tyrosine kinase receptor signaling pathway might be an important event in neuronal differentiation and should lead to a further characterization of the role of tyrosine protein kinase receptors and their ligands in neuronal differentiation during murine embryogenesis.

Materials and Methods

Cells and Cell Culture. P19 EC cells (11) were cultured as described previously (58) in bicarbonate-buffered DF medium, containing 7.5% fetal calf serum at 37°C and 7.5% CO_2. A431 human squamous carcinoma cells, which express an amplified EGF-R gene, were cultured as described previously (39). All cell lines were routinely passaged three times weekly.

Plasmids and Construction of pSV2HERc. pSV2neo (38), an expression vector containing the selectable *neo* gene under transcriptional control of the SV40 early promoter, was used for cotransfections. As probes for hybridization studies, a 1.0-kb *Pst*I mouse genomic fragment homologous to v-*jun*,[5] a 1.5-kb *Eco*RI cDNA fragment of *jun*B (28), a 0.8-kb *Pst*I fragment of v-*fos* (59), a 3.0-kb genomic *Bam*HI/*Hind*III fragment of mouse c-*myc* (60), and a 1.4-kb fragment of rat glyceraldehyde-3-phosphate dehydrogenase (61) were used.

A 4.1-kb *Kpn*I/*Xba*I fragment from the hEGF-R expression vector pLSX (33), containing the SV40 early promoter and the hEGF-R cDNA (extending from 15 bp upstream of the initiation codon to 151 bp downstream of the termination codon), was ligated to *Kpn*I/*Xba*I-opened pUC18. pSV2HERc was constructed by insertion of the SV40 small t intron and polyadenylation site from pSV2neo (38) in the *Pst*I site 3′ of the HER gene. An empty expression vector, which was used for the isolation of a negative control cell line, was derived from pSV2HERc by deletion of a 3.8-kb *Xho*I fragment containing the hEGF-R cDNA.

Transfections. Transfections were performed using a slightly modified calcium phosphate precipitation

[5] W. Kruijer, unpublished observations.

method (62). Twenty-four h prior to transfection, cells were plated in 60-mm dishes (4×10^5 cells/dish). DNA precipitates were made by adding plasmid DNA (20 μg pSV2HERc + 2 μg pSV2neo) to 250 μl 2× HBSP (42 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-275 mM NaCl-10 mM KCl-1.4 mM Na_2HPO_4-10 mM glucose, pH 7.05) and subsequently, while vortexing, 250 μl $CaCl_2$ (250 mM). After 25 min at 20°C, the precipitate was added directly to the medium (5 ml) on the cells. Following incubation with the DNA precipitate for 20 h at 37°C, the cells received fresh medium, and 24 h later, selection with G418 (200 μg/ml) was started. After selection for 14 days, the colonies were cloned and established as stable cell lines. The frequency of stable integration of pSV2neo in the genome was approximately 2×10^{-5} (*i.e.*, about two G418-resistant colonies formed/10^5 initially plated P19 EC cells).

Analysis of Genomic DNA and mRNA. Genomic DNA was isolated as described previously (63). Five μg of genomic DNA were double digested with *Kpn*I and *Xba*I. Tris-borate-EDTA-agarose gel electrophoresis and transfer to nylon filters (Hybond; Amersham) was done according to standard protocols (64).

RNA, used for Northern blotting analysis, was isolated using a phenol extraction method (65). Fifteen μg of total RNA were fractionated on a 0.8% formaldehyde-agarose gel and subsequently transferred to nylon filters as described previously (64).

The Southern and Northern blots were hybridized to ^{32}P-labeled probes. Hybridization of the filters was performed in 50% formamide-5× SSC-50 mM NaH_2PO_4, pH 6.8-10 mM EDTA-0.1% SDS-0.1 mg/ml sonicated salmon sperm DNA-2× Denhardt solution at 42°C overnight. After hybridization, the filters were washed three times with 2× SSC-0.1% SDS and once with 0.1× SSC-0.1% SDS for 20 min at room temperature and once with preheated (55°C) 0.1× SSC-0.1% SDS for 10 min at room temperature while shaking. The ^{32}P-labeled probes were obtained using a multiprime labeling kit (Amersham), routinely with 50 ng DNA fragment and 50 μCi [α-^{32}P] dCTP (NEN Research, DuPont).

RNase protection was performed according to Melton *et al.* (66), using total RNA, isolated by the isothiocyanate-cesium chloride method (67). Fifty μg of total RNA were hybridized at 45°C for 18 h to the synthetic antisense mRNA probe, labeled with [α-^{32}P]UTP (Amersham). RNase A (0.1 mg/ml; Boehringer) digestion was performed at 37°C for 3 h. The protected mRNA fragments were run on a 5% polyacrylamide gel.

Autoradiography was performed with Kodak XAR-5 films at −70°C, using intensifying screens.

EGF Binding Analysis. Cells grown to near confluency in 6-well plates (Nunc) were washed twice with ice-cold PBS and incubated in duplicate in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 mM)-buffered Dulbecco's modified Eagle's medium containing varying amounts of unlabeled EGF (receptor grade; 0–500 ng/ml; Collaborative Research) and 0.50 ng/ml ^{125}I-EGF (1.0–6.0 × 10^6 cpm/ng) in a final volume of 0.5 ml for 2 h at 4°C on a rocking plateau. The cells were washed four times with ice-cold PBS, and subsequently 1 ml 1 M NaOH was added to each well. After 5 min at room temperature, the solubilized cells were resuspended, and the incorporated radioactivity was determined in a gamma counter. The cell number was determined in wells, passaged, and grown simultaneously with the wells under observation.

For the competition assay, cells were incubated with Ab 528 (1 μg/ml; Oncogene Science) (43) for 1 h at 4°C, after which period both unlabeled EGF (0–500 ng/ml) and ^{125}I-EGF (0.5 ng/ml) were added to a final volume of 0.5 ml. After gentle rocking for 2 h at 4°C, cell-associated radioactivity was determined as described above. ^{125}I-EGF was a kind gift of Johannes Boonstra.

Western Blotting and EGF-R Autophosphorylation-Immunoprecipitation. Total cell lysates were prepared by scraping nearly confluent cells (100–600 cm^2) in PBS and subsequent incubation for 20 min on ice in 500 μl cell lysis buffer (10 mM Tris-HCl, pH 7.4-150 mM NaCl-1% Triton X-100-10% glycerol-1 mM phenylmethylsulfonyl fluoride). The protein concentrations of these preparations were determined using the Bio-Rad protein assay mix.

For Western blotting, 200 μg total cell lysate (20 μg of A431 lysate) were fractionated on a linear 5–15% polyacrylamide gel and blotted onto nitrocellulose filters (Schleicher and Schuell). The filters were incubated with diluted (1:200), affinity-purified polyclonal anti-EGF-R antiserum 281-7 (19). Subsequently, they were incubated with an alkaline phosphatase-coupled second antibody, using the protoblot Western blot alkaline phosphatase system, exactly as described by the manufacturer (Promega).

The phosphorylation reactions were performed exactly as described by Defize *et al.* (19). Total cell lysates (0.1–10 mg protein) were incubated with buffer or with EGF (0.5 μg/ml) for 15 min at 20°C. The phosphorylation reactions, containing 25 μCi [γ-^{32}P]ATP/sample, were allowed to proceed for 10 min on ice. Subsequently, the hEGF-R was precipitated with 1.0 μg Ab 528 and 40 μl of a 1:1 slurry of Protein A-Sepharose beads (Pharmacia). After extensive washing, the beads were resuspended in 50 μl Laemmli sample buffer containing SDS and boiled for 5 min. The supernatants were electrophoresed on a linear 5–15% SDS-polyacrylamide gel.

Differentiation and Immunofluorescence. Cells were differentiated either in monolayer by incubation in medium containing RA (10^{-6} M) or by aggregation of the cells in hanging droplets. Aggregation was performed by incubation of 20 μl cell suspension (40×10^3 cells/ml), hanging from the top of a bacterial dish over PBS. The aggregates were generated in standard media without any additions or with RA (10^{-7} M) or DMSO (1%) and in the absence or presence of EGF (50 ng/ml). After aggregation for 5 days, the aggregates were replated on gelatinized glass coverslips in the corresponding media. Every 2 days, the cells received fresh medium. After 14 days, the cells were fixed with methanol-acetic acid 95:5 (v/v). The fixed cells were preincubated for 1 h with 0.1% bovine serum albumin in PBS, incubated with antibody for 1 h at 37°C, washed with PBS three times, and then incubated with the appropriate FITC-labeled conjugate for 1 h at 37°C. After washing (PBS, five times), the coverslips were mounted in Moviol. The antibodies were diluted prior to labeling according to the recommendations of the suppliers. Anti-SSEA-1 was kindly supplied by Dr. D. Solter, TROMA-1 by Dr. R. Kemler, anti-brushin by Dr. T. Muramatsu, anti-N-CAM by Dr. J. P. Thiery, anti-MF-20 by Dr. D. Fischman, and anti-NF-70 by Dr. S. Warnaar. Anti-laminin was a commercial preparation

from BRL. All FITC-conjugated antibodies were from Nordic. Immunofluorescence and phase contrast pictures were obtained using a Confocal laser scanning microscope (Bio-Rad).

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