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in Promoting Lineage Commitment of Mouse Embryonic Stem (ES) Cells

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To examine the role of secreted signaling molecules and neurogenic genes in early development, we have developed a culture system for the controlled differentiation of mouse embryonic stem (ES) cells. In the current investigation, two of the earliest identified BMP antagonists/neural-inducing factors, noggin and chordin, were expressed in pluripotent mouse ES cells. Neurons were present as early as 24 h following transfection of ES cells with a pCS2/noggin expression plasmid, with differentiation peaking at 72 h. With neuronal differentiation, stem cell marker genes were down-regulated and neural determination genes expressed. Coculture experiments and exposure to noggin-conditioned medium produced similar neuronal differentiation of control ES cells, while addition of BMP-4 to noggin expressants strikingly inhibited neuronal differentiation. Transfection of ES cells with a pCS2/chordin expression vector or exposure to chordin-conditioned medium produced a more complex pattern of differentiation; ES cells formed neurons, mesenchymal cells as well as N-CAM-positive, nestin-positive neuroepithelial progenitors. These data suggest that, consistent with their different expression fields, noggin and chordin may play distinct roles in patterning the early mouse embryo.

Key Words: **BMP; differentiation; induction; neuron; nervous system; node; transfection.**

INTRODUCTION

The induction and sequential rounds of neurogenesis and glial cell differentiation that shape the nervous system appear to be controlled by local niches of secreted signaling factors and inhibitors. Recent attention has focused particularly on the role of the bone morphogenetic proteins (BMPs) and their extracellular inhibitors noggin, chordin, cerberus, and follistatin in neural induction and differentiation. It now appears that BMPs may alternatively stimulate (Li *et al.*, 1998) or inhibit (Mabie *et al.*, 1999; Li and LoTurco, 2000; Lim *et al.*, 2000) neuronal differentiation, depending on the stage of development (Grinspan *et al.*, 2000; Gross *et al.*, 1996; Li *et al.*, 1998), distance from the source (Mehler *et al.*, 1997), or the expression by target cells of particular transcription factors (Sun *et al.*, 2001). Although in *Xenopus* embryos, it is clear that inhibition of BMP signaling in the ectoderm by the dorsalizing factors noggin, chordin, Xnr3, cerberus, and follistatin plays an important role in neural induction, gene deletion experiments in mouse embryos have been less conclusive, presumably due to the presence of additional, "compensatory" factors in these embryos.

To examine the role of these secreted signaling molecules and their inhibitors in early development, we have developed a controlled differentiation paradigm using mouse embryonic stem (ES) cells. Because ES cells express and respond to many of the same factors as the pluripotent ectoderm, they provide a simple model to study the signaling cascades involved in early mouse development. Exposure of ES cells to the BMP inhibitor/neural inducer noggin in defined medium produced widespread differentiation of primitive neurons, which was strongly inhibited by BMP-4. Consistent with its broader expression in forming mesen-

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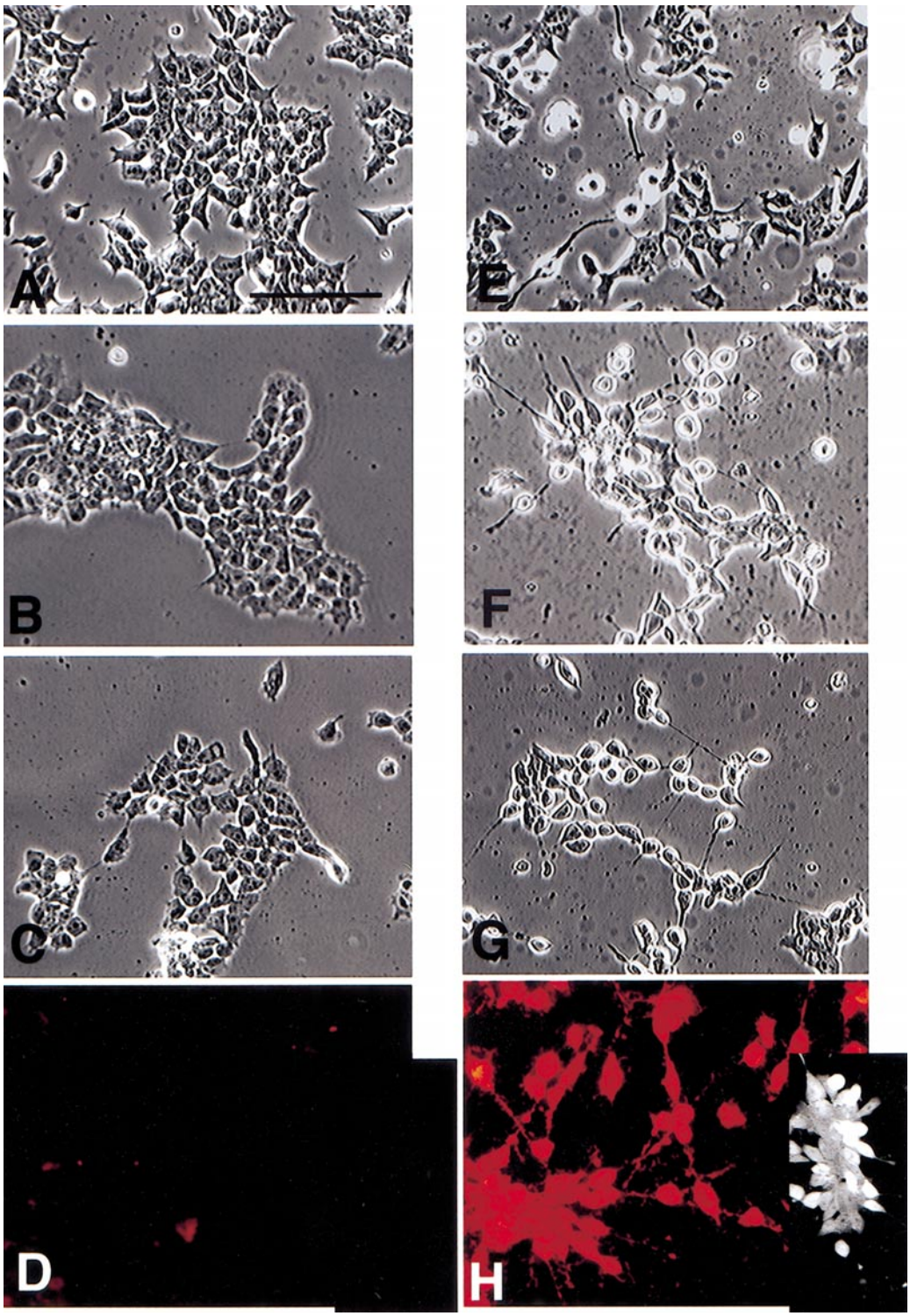

FIG. 1. Differentiation of ES cells transfected with pCS2 vector alone (A–D) or with pCS2/noggin (E–H), after 24 (A, E), 48 (B, F), and 72 h (C, D, G, H) *in vitro*. There is little differentiation in the control cultures based either on morphology (A–C), or as indicated by immunohistochemical localization of antibodies to neurofilament protein (D). When ES cells were transfected with pCS2/noggin, neuronal differentiation was observed as early as 24 h later (E). There was a gradual morphological change from an undifferentiated to a neuronal

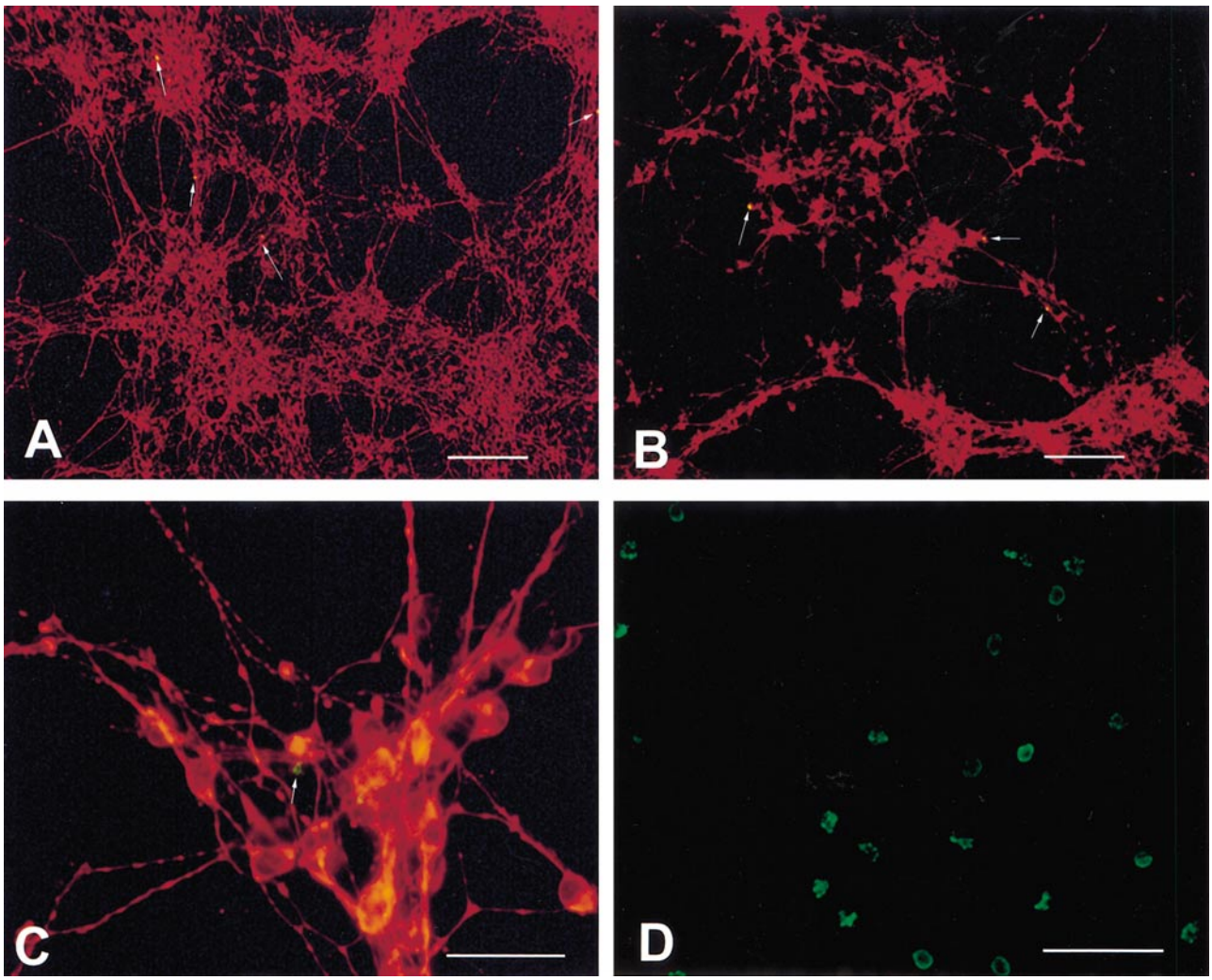

FIG. 2. Localization of anti-phospho histone H3 (FITC secondary) and neuronal tubulin (anti-TUJ1, CY3 secondary) in ES cells transfected with pCS2/noggin (A–C) or pCS2 alone (D). After 96 h, neuronal differentiation is extensive in noggin-exposed cultures, with dividing cells (white arrows in A–C) rarely observed in these cultures. In pCS2 control cultures, there were scattered, dividing cells indicated by FITC labeling of H3, but very little neuronal differentiation (CY3-neuronal tubulin). Scale bars, 200 μm in (A), 100 μm in (B), and 50 μm in (C) and (D).

chymal wings of the embryo (Bachiller *et al.*, 2000) as well as the node, exposure of ES cells to chordin produced a more complex pattern of pan-neural as well as mesenchymal cell differentiation. These results support recent views regarding the molecular organization of the node into discrete signaling, migratory, and stem cell populations (Mathis *et al.*, 2001), and provide an *in vitro* system to begin to dissect the relative roles of the molecules that contribute to these zones.

MATERIALS AND METHODS

Vector construction. Genomic DNA isolated from D3 ES cells was employed to obtain mouse *noggin* and *chordin* cDNAS. Primers for PCR were designed from the mouse *noggin* (GenBank U79163) and *chordin* (AF069501) sequences to encompass the complete coding regions: *Nog* F GGA AGC TTG GAA TTG CGA CCA ACT CGT GCG CGT CTT CTG CG, *Nog* R GGA TCC ATC AAG TGT CTG GGC GCG GGC AG, and *Chrd* F GGG

appearance at 48 h (F), with the majority of the cells adopting a neuronal morphology after 72 h (G, H). Images in (D) and (H) (insets) indicate the lack of expression of neurofilament protein in control cultures (D) compared with the pattern of neuronal differentiation at the edges of cell aggregates in noggin transfectants (H). Scale bar, 100 μm.

GAA TTC CCC AGC TGT CTG CTC CGC GTC, *Chrd* R GGG TAC GTA CTG CTC AGC TCA GGG GTA GC. PCR products of 1 kb (noggin) and 3.2 kb (chordin) were cloned directly downstream of the cmv promoter in the pCS2 mammalian expression vector (Turner and Weintraub, 1994), and verified by sequence analysis.

ES cell culture, transfection, and differentiation paradigms. The D3 ES line is routinely passaged in gelatin-coated flasks in DMEM supplemented with 10% FBS, β-mercaptoethanol, and 1000 units/ml LIF. Prior to transfection, D3 cells were washed free of serum proteins and LIF by overnight growth in defined medium. ES cells were then trypsinized, resuspended in F12 medium, passed through Tetko 50-μm pore mesh to remove cell clumps, and counted by using a Coulter counter. ES cells were electroporated with 20 μg of DNA (pCS2, pCS2/noggin, or pCS2/chordin) by using a Biorad Genepulser (current of 0.3 kv, capacitance = 250 μF). Cells were transferred to six-well plates and grown at 5×10^5 cells/well in 2 ml defined medium (N2 or N2/B27; GIBCO) supplemented with 100 mM sodium pyruvate. Plates were previously coated with poly-omithine (0.1%) followed by laminin-1 (20 μg/ml). These culture conditions permit, but do not alone promote significant neuronal differentiation, which requires additional growth factor manipulation (FGF-2 withdrawal and exposure to additional growth and survival factors; O'Shea, 2002). At 24-h intervals, medium was changed and cells were counted and examined by using a Leitz Fluovert inverted microscope.

"Neurosphere" differentiation. In a second series of experiments, pCS2, pCS2/noggin, and pCS2/chordin-transfected ES cells were plated on low adhesive tissue culture plastic (Primaria, uv inactivated) in N2/B27 medium. Under these conditions, uniform aggregates of cells reminiscent of neurospheres lift from the tissue culture plastic after 24 h *in vitro*. Aggregates were gravity sedimented, plated on PORN-laminin-treated substrates in N2/B27 medium, and after 8 days *in vitro*, cultures were fixed in methanol prior to immunohistochemical localization of neuronal tubulin. The number of aggregates containing neurons (TUJ1-positive cells) was determined from 5 fields of 20 aggregates per field, with 4 replicates.

Conditioned medium and coculture experiments. To demonstrate that noggin and chordin were expressed and active in promoting ES cell differentiation, conditioned medium (CM) was removed from transfected cells at 24-h intervals, centrifuged, and supernatant added to control ES cells. Chordin CM was also concentrated by using Millipore Biomax filter units. In addition, COS cells were transfected with the chordin, noggin, and control expression plasmids, supernatant concentrated 10 and 100×, then added to untransfected ES cells.

For coculture experiments, ES cells were grown on PORN-laminin-coated glass coverslips. After 24 h, a coverslip containing ES cells transfected with either pCS2, pCS2/noggin, or pCS2/chordin and a coverslip containing untransfected ES cells were added to a 35-mm dish containing defined medium, and differentiation of the nontransfected ES cells was monitored.

BMP-4 exposure. BMP-4 was added at plating to cultures of pCS2/noggin transfected cells and to untransfected ES cells at 0, 10, and 100 ng/ml; pCS2/chordin-transfected cells were exposed to 0 and 10 ng/ml BMP-4. The number of neurons per microscopic field (10×) was counted at 24, 48, and 72 h. Five fields from each culture well, with five replicate cultures, were scored at each 24-h period. Mean number of neurons per field was computed and analyzed by using Student's *t* test.

Assessment of differentiation. Differentiation was monitored throughout the culture period by using morphology, immunohistochemical localization of cell-type restricted proteins, and RT-PCR. For neuronal differentiation, the number of birefringent cells with processes at least the length of the cell body, was counted at 10× from at least 5 fields in each culture well. Counts were made on 4 replicates from 10 to 20 individual transfection experiments (number of fields = 200 minimum). Since each transfection was considered to be an independent experiment, data from each transfection (5 fields from 4 wells) were averaged and used to compute a population mean (average of 10–20 electroporations) and are expressed as mean ± sem. To assess proliferation in these cultures, anti-phospho-histone H3-positive and -negative cells were counted (10×) from 5 fields with 4 replicates. Data are expressed as mean number of proliferating cells ± sem compared with mean number of cells/field. Statistical analysis was carried out by using Student's *t* test, $P \leq 0.05$ was considered statistically significant.

Immunohistochemistry. Cells were fixed in methanol or 2% paraformaldehyde, washed, and stored at 4°C in PBS. Nonspecific antibody binding was blocked, then cells exposed to primary antibody: anti-neurofilament antibody (Sigma, 1:100), anti-neuronal tubulin (TUJ1, BABCO, 1:100), anti-N-CAM (Carl Lagenaur, 1:100), anti-nestin (PharMingen, 1:60), anti-alpha actin (Sigma, 1:250), anti-vimentin (Sigma, 1:200), anti-pan-cytokeratin (Sigma, 1:200), anti-glial fibrillary acidic protein (Chemicon, 1:250), anti-SSEA1 (marker of undifferentiated stem cells; MC480 supermatant DSHB, U of Iowa, 1:10), anti-PCNA/Cyclin (Roche, 1:100), anti-phospho-histone H3 (1:500, Upstate Biotech), followed by secondary antibody conjugated to FITC or to CY3.

Cells were photographed by using a Nikon or SPOT camera, images were imported into Photoshop 5.5, and plates were assembled and printed by using a Kodak XLS 8600 color printer.

RT-PCR. Cytoplasmic RNAs were extracted from transfected ES cells at 24, 48, and 72 h posttransfection, DNAsed, and quantified. RNAs from control ES cultures and from blastocysts were used to assess the endogenous expression of each gene. RNAs (1 μg ES cells, 100 ng blastocysts) served as templates in reverse transcriptase reactions with oligo-dT primers. General PCR conditions were 94°C/3min, 94°C/1min, 51–60°C/1min, 72°C/2min for 25–35 cycles. The RT-PCR products were electrophoresed in 2% agarose gels in the presence of ethidium bromide, then photographed on a uv light box. At least five replicates were done at each time point. Primers were: *Rex1* (F, AAAGTGAGATTAGCCCCGAG; R, TCCCATCCCCTTCAATAGCA; 930-bp product, 25 cycles); *Brachyury (T)* (F, AGAAAGAAACGACCACAAAGATG; R, ATTTATTTATTTTTCCCTTGTCC; 746 bp, 30 cycles), *noggin* (F, AAGGATCTGAACGAGACG; R, GCAGGAACACTTACACTCG; 520 bp product, 25 cycles), *chordin* (F, ACCAACGCAGTAGAGACCTCCC; R, GGGGTAGCAGGAATGGTGTG; 905-bp product, 25 cycles), *Sox1* (F, GCACACAGCGTTTTCTCGG; R, ACATCCGACTCCTCTTCCC; 407-bp product, 25 cycles), *Neurod3/ngn1* (F, CATCTCTGATCTCGACTGC; R, CCAGATGTAGTTGTAGGCG; 405-bp product, 35 cycles), *BMP4* (F, CTCCCAAGAATCATGGACTG; R, AAAGCAGAGCTCTCACTGGT; 468-bp product, 30 cycles), and *β-actin* (F, AACCCTAAGGCCAACCGTG; R, CAGGATTCCATACCCAAGAAGG; 494-bp product, 20 cycles).

TABLE 1
Neuronal Differentiation (mean percent + SEM)

Group	Hours *in vitro*		
	24	48	72
Primary Transfections:			
pCS2	0.45 ± 0.2	4.4 ± 0.7	16.6 ± 1.0
pCS2/noggin	21.9 ± 1.0*	71.6 ± 1.3*	91.7 ± 1.3*
pCS2/chordin	3.1 ± 0.3**	21.6 ± 0.5**	54.7 ± 0.1**
Conditioned Medium:			
ES + pCS2 CM	1.3 ± 0.4	4.9 ± 0.6	12.7 ± 1.5
ES + pCS2/noggin CM	46.3 ± 2.4*	77.8 ± 2.1*	88.2 ± 2.4*
ES + pCS2/chordin CM	2.0 ± 0.6	17.2 ± 0.7**	47.9 ± 1.6**
ES + pCS2/chordin CM 10X	2.6 ± 1.1	24.1 ± 2.5**	61.6 ± 1.9**
ES + pCS2/chordin CM 100X	4.5 ± 0.4**	33.9 ± 1.0**	69.4 ± 0.2**
Coculture experiments:			
ES + pCS2 ES	0.8 ± 0.4	3.2 ± 1.1	12.0 ± 1.4
ES + pCS2/noggin ES	21.8 ± 2.3*	68.4 ± 1.9*	89.0 ± 1.7*
ES + pCS2/chordin ES	1.1 ± 0.9	8.4 ± 0.4**	39.7 ± 1.9**

* indicates pCS2/noggin significantly less than pCS2, $P \leq 0.05$; ** indicates pCS2/chordin significantly less than pCS2, $P \leq 0.05$.

RESULTS

Noggin expression. Exposure of ES cells to noggin protein—whether by transfection of a noggin expression plasmid or in conditioned medium—resulted in the rapid differentiation of small, immature neurons. ES cells transfected with the control plasmid remained largely undifferentiated and maintained an ES morphology throughout the culture period (Figs. 1A–1C). Growth in defined medium for 72 h resulted in some differentiation of control ES cells, the mean number of neurons increased gradually from 0.45 (24 h) to 4.4% (48 h) to a high of 16.6% at 72 h (Table 1). However, as early as 24 h after transfection with pCS2/noggin, 21.9% of the ES cells adopted a neuronal morphology (Fig. 1E). Neuronal differentiation increased to 71.6% by 48 h; by 72 h, 91.7% of the cells in noggin cultures exhibited a neuronal phenotype (Figs. 1F and 1G). As they differentiated, ES cells gradually lost their polygonal shape and prominent nucleolus, becoming ovate and extending processes (Figs. 1F and 1G), and staining with anti-neurofilament antibodies (Fig. 1H).

Noggin transfectants expressed low levels of early neuroepithelium markers N-CAM and nestin, did not express GFAP, vimentin, keratin, or a marker of early somite, alpha-actin (Devlin and Emerson, 1978), or neurotransmitter markers of more mature neurons. Growth of control ES cells in defined medium on laminin substrates resulted in scattered cell staining with anti-neurofilament (Fig. 1D) or anti-TUJ1 antibodies and widespread expression of nestin and N-CAM.

TABLE 2
Cell Proliferation

	Hours *in vitro*			
	24	48	72	96
pCS2	6.4 ± 0.4 (1090)	4.7 ± 1.5 (1250)	5.1 ± 2.4 (1340)	5.6 ± 1.9 (1441)
pCS2/noggin	2.1 ± 0.2* (1210)	1.9 ± 0.5* (1140)	1.7 ± 0.3* (1060)	1.1 ± 0.7* (1025)
pCS2/chordin	4.8 ± 1.1** (1160)	4.2 ± 0.4 (1190)	4.5 ± 0.8 (1330)	4.3 ± 0.2 (1249)

Note. Mean number of dividing cells (anti-H3 +) per field ± SEM, determined from five microscope fields (10×) from four replicate cultures. Mean number of cells (stained and unstained) per field in parentheses. * indicates pCS2/noggin significantly less than pCS2, $P \leq 0.05$; ** indicates pCS2/chordin significantly less than pCS2, $P \leq 0.05$.

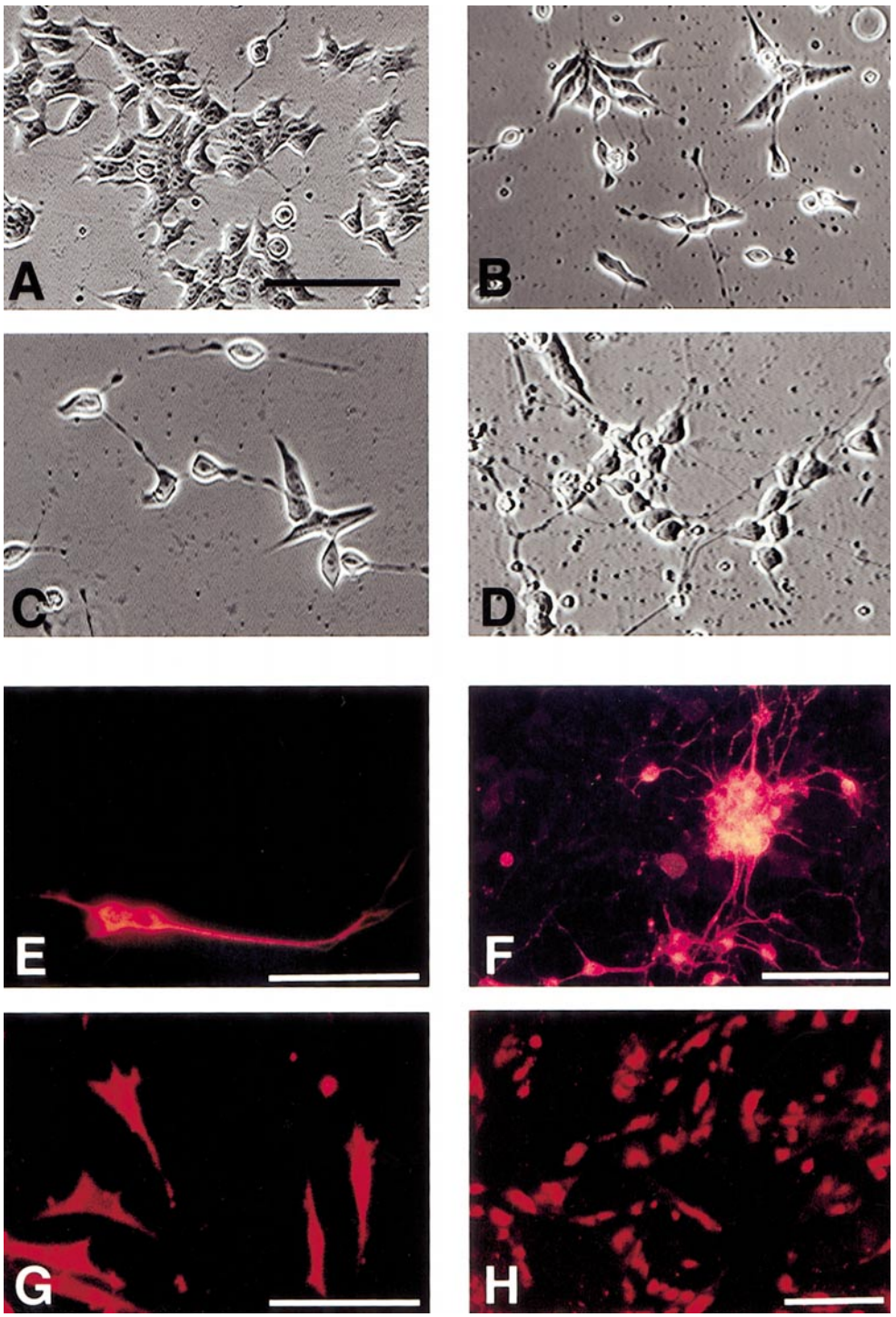

FIG. 3. Differentiation of ES cells following chordin exposure. Neuronal differentiation was observed in cultures transfected with pCS2/chordin as early as 24 h after transfection (A), but throughout the culture period, additional flattened mesenchyme like cells were present in the cultures, as were clumps of cells retaining an ES cell morphology (B–D). After as long as 7 days *in vitro*, unlike noggin-exposed cultures, neuronal process outgrowth was considerable, and cells had a much more mature appearance (E). To determine the nature of the cell differentiation, cultures were fixed in methanol or paraformaldehyde and exposed to cell type-specific intermediate filament protein antibodies including anti-neuronal tubulin (TUJ1; E, F), anti-vimentin (G, mesenchyme), and anti-nestin (H, neural stem cells). (A–D) Phase contrast images; (E–H), Cy3-conjugated secondary antibody. Scale bars, 100 μm.

In noggin-transfected cultures, there was very little cell division as evidenced by the lack of expression of stem cell marker genes (see Fig. 6 below), assessment of differentiation, cell number, as well as staining with the cell proliferation markers anti-PCNA-Cyclin or anti-phospho-histone H3 (Fig. 2). Cell proliferation in noggin-transfected cultures was significantly less than in control or pCS2/chordin cultures at all time points examined (Table 2), supporting the conclusion that selective proliferation/survival did not differentially affect these results.

Chordin expression. Unlike the rapid and widespread neuronal differentiation of ES cells exposed to noggin, fewer neurons formed in response to chordin exposure. After 24 h in culture, scattered neuronal cells were present in these cultures (3.1%; Fig. 3A), increasing to 21.6% at 48 h (Fig. 3B), and 54.7% after 72 h (Fig. 3C) *in vitro*. After longer culture periods (7-10 days), clumps of neurons were often observed (Fig. 3D). These neurons were typically present in small aggregates, and extended significantly longer processes than the small immature neurons which differentiated in response to noggin exposure, or in control cultures after similar times *in vitro*.

In chordin cultures, unlike ES cells exposed to noggin, there were cells that retained an undifferentiated, ES-like appearance, and continued to divide slowly. After 24 h *in vitro*, there were significantly fewer dividing cells in chordin-exposed cultures compared with controls. In addition to neuronal cells (anti-TUJ1, or anti-neurofilament positive; Figs. 3E and 3F), additional cells stained with anti-vimentin antibodies (Fig. 3G), or antibodies to the neural intermediate filament protein nestin (Lendahl *et al.*, 1990; Fig. 3H), as well as with anti-N-CAM and scattered cells with anti-SSEA1 antibodies. Few cells stained with antibodies to cytokeratin or to muscle actin, indicating the presence of stem cells, mesenchymal cells, as well as neural progenitors.

Coculture and conditioned medium experiments. It is clear from these experiments that noggin was expressed and the recombinant protein secreted by ES cells in an active form. When untransfected ES cells were grown either in defined medium conditioned for 24 h by pCS2/noggin transfected ES cells or on coverslips placed in coculture with noggin-transfected ES cells, robust neuronal differentiation of the control cells occurred after an additional 24-72 h in culture (Table 1). Not surprisingly, exposure to noggin CM resulted in more rapid neuronal differentiation than coculture (46.3 and 21.8% at 24 h). By 72 h, however, the differences in extent of neuronal differentiation between exposure to noggin CM and coculture were no longer significant (88.2 and 89%).

Because chordin was not expressed by ES cells as highly as noggin, a number of approaches were taken to increase chordin present in the medium. First, several different plasmid preparations were tested, with only slight differences in chordin expression levels. Second, medium from COS cells transfected with the pCS2/chordin construct using the Fugene 6 reagent (Roche) was concentrated 10 and 100× and applied directly to ES cultures. At the highest concentration, differentiation proceeded slightly more rapidly (Table 1), but the outcome of differentiation remained mixed, with additional cells with a fibroblast or mesenchymal morphology, but fewer undifferentiated ES cells present in these cultures. Finally, when control ES cells were grown in coculture with ES cells transfected 24 h before with pCS2/chordin, there was less obvious differentiation than in cultures directly transfected with the chordin expression construct (Table 1). The remaining cells retained an ES appearance and staining pattern, or took on a flattened mesenchymal morphology.

BMP-4 exposure. Addition of BMP-4 to these cultures inhibited noggin induced differentiation at 10 and 100 ng/ml (Fig. 4). Cells took on both a polygonal stem cell morphology and a very flattened appearance, with no stimulation in cell division. When added to chordin-transfected cells at 10 ng/ml, cells rapidly adopted a flattened, mesenchymal morphology, with very few additional cell types present in the cultures. Many stained with anti-vimentin antibodies, but not with other cell type-specific antibodies.

Aggregation mediated differentiation. For comparison with the neuronal differentiation protocols currently in use which rely on an aggregation step ± retinoic acid exposure (e.g., Bain *et al.*, 1995), the extent of neuronal differentiation was also assessed in aggregate cultures of transfected ES cells. Neurons were present in 51% of control cultures (Figs. 5A–5C). Exposure to noggin had the strongest effect on neuronal differentiation, with 88% of aggregates containing TUJ1-positive cells (Figs. 5D–5F), while exposure to chordin resulted in an intermediate number of aggregates containing neurons—67% (Figs. 5G–5I). Control aggregates often contained isolated neuronal cells (e.g., Figs. 5A and 5C), while in both noggin (Figs. 5D–5F) and chordin (Figs. 5G–5I) transfectants, clusters of cells were neuronal. Neurons often differentiated at the edge of these aggregates, and migrated onto the laminin-1 substrate (Fig. 5D), consistent with the presence of a secreted factor. There was widespread expression of nestin and of N-CAM, and scattered expression of GFAP as well.

Gene expression. The pattern of stem cell, neuronal, mesenchymal, and epidermal gene expression was examined by RT-PCR at 24, 48, and 72 h. Undifferentiated ES cells and blastocysts express the stem cell transcription factors *Rex1* (Rogers *et al.*, 1991; Fig. 6, lanes 1 and 2) and *Oct4* (Niwa *et al.*, 2000; data not shown), and low levels of *BMP4*, but do not express *Brachyury (T)*, *noggin*, *chordin*, *Sox1* (neuroepithelium marker; Pevny *et al.*, 1998) or the bHLH transcription factors *Neurod1*, *Neurod2* (not shown), or *Neurod3/ngn1* (McCormick *et al.*, 1996). Control transfectants (pCS2) continue to express the ES markers *Rex1* (lanes 3 and 4) and *Oct4* (not shown), and after 48 h in

defined medium, low levels of *chordin* and *Sox1* (lane 4). Following transfection, *noggin* expression peaked at 24 h (lane 5) and remained high at 48 (lane 6) and 72 h (not shown). Clearly, the noggin cDNA was expressed efficiently, as the amount of *noggin* mRNA synthesized exceeded even the control *β-actin* mRNA (lanes 5 and 6). In noggin-transfected cells, *Sox1* was expressed at high levels at 24 h, and remained high throughout the 72-h culture period. Sustained expression of *noggin* resulted in the expression of the early neuronal determination genes *Neurod3/ngn1,* as well as *Neurod1* and *Neurod2* (not shown), and rapid down-regulation of *Rex1,* as well as *Oct4* (not shown). Addition of BMP-4 to noggin-transfected cultures did not affect *noggin* expression, but induced *BMP4* expression as well as expression of *Rex1.*

Chordin was not expressed in either blastocysts or in control ES cells (lanes 1 and 2), but was expressed at low levels in pCS2 transfectants after 48 h (lane 4) and 72 h (not shown) *in vitro. Chordin* was expressed at moderate levels throughout the culture period (lanes 7 and 8) following transfection of ES cells with pCS2/chordin. The pan-neural marker *Sox1* was expressed in chordin-transfected cells, but *Neurod3 /ngn1* was not induced. Consistent with the presence of cells with an undifferentiated morphology in these cultures, chordin transfectants continued to express *Rex1* (lanes 7 and 8), as well as *Oct4* (not shown). Other lineage markers, e.g., *Brachyury* and *BMP4,* were also expressed in chordin-transfected cells (lanes 7 and 8) unlike in noggin cultures.

DISCUSSION

Exposure of pluripotent mouse ES cells to noggin results in the rapid differentiation of these cells to a primitive neuronal phenotype, while exposure to chordin was more complex, producing mesenchymal as well as pan-neural differentiation. Consistent with previous reports, culture of control ES cells in defined medium alone resulted in low levels of neural differentiation (Wiles and Johansson, 1999) and expression of the intermediate filament protein / neural stem cell marker nestin (Lendahl *et al.,* 1990) as well as a marker of early neuroepithelium *Sox1* (Pevny *et al.,* 1998), while exposure to BMP-4 strongly inhibited neural differentiation (e.g., Finley *et al.,* 1999). The downstream genes activated by noggin expression were pan-neural—members of the SRY family of transcription factors (*Sox1, 2*), the bHLH factor/neurogenic gene *Neurod3/ngn1,* followed by expression of later neural differentiation genes, *Neurod1* and *Neurod2*. Of these, *Neurod3/ngn1* may be of particular importance as it has been suggested to be a "molecular switch" between neuronal and glial cell differentiation (Sun *et al.,* 2001). Thus, neural differentiation of ES cells initiates cascades of gene expression similar to those observed in *Xenopus* (*Sox, Zic, Iroqouis*; Mizuseki *et al.,* 1998; Sasai, 1998), and models the critical bridge between induction and neural differentiation (review, Sasai, 1998). Interestingly, expression of chordin induced low levels of *Sox1,* as well as expression of *Brachyury* and of *Wnt 3a,* which may play a role in mesoderm differentiation (Liu *et al.,* 1999). Alternatively, it has been suggested that Sox proteins may regulate Wnt signaling pathways (e.g., Zorn *et al.,* 1999).

Precise control of BMP signaling in the extracellular space appears to play a critical role in multiple, crucial events in development including: neural induction (Harland, 2000), tissue patterning (e.g., McMahon *et al.,* 1998), epithelial–mesenchymal interactions critical to organogenesis (e.g., Kulessa *et al.,* 2000), lineage selection (Mabie *et al.,* 1999), and in creating stem cell "niches" during development and in adult organs (review, Watt and Hogan, 2000). The local microenvironment is presumably maintained by the high affinity binding of these secreted signaling molecules to extracellular matrix proteins (e.g., Larrain *et al.,* 2000), which present them to cells and protect them from inactivation by matrix metalloproteases.

Although structurally unrelated, chordin, follistatin, noggin, Xnr3, and cerebrus act extracellularly to prevent the activation of BMP receptors (Wilson and Hemmati-Brivanlou, 1997). All are expressed in Spemann's organizer in the amphibian embryo, and considerable data support the conclusion that inhibition of BMP signaling by these antagonists is required for neural induction (Harland, 2000). The situation is likely to be complex, as many diverse pathways rescue the uv-ventralized frog embryo (DeRobertis *et al.,* 2000), and somewhat surprisingly, CNS tissue DOES form following mutation of genes encoding BMP inhibitors in *Drosophila* (the *chordin* homolog *sog,* Francois *et al.,* 1994), in zebrafish (e.g., *chordino*; Hammerschmidt *et al.,* 1996), and in mouse embryos with mutations in *noggin* (Brunet *et al.,* 1998; McMahon *et al.,* 1998), *cerberus-related* (Shawlot *et al.,* 1998), or *follistatin* (Matsuk *et al.,* 1995), as well as in embryos double null for *chordin* and *noggin* (Bachiller *et al.,* 2000).

While these experiments have been interpreted to suggest that multiple, overlapping signaling molecules are required for induction, data which suggest that CNS tissue forms in the absence of mesoderm in *Xenopus* (Wessley *et al.,* 2001) and in *cripto* mutants (Ding *et al.,* 1998), following deletion of the organizer either surgically in zebrafish (Shih and Fraser, 1996; Saude *et al.,* 2000) and mouse (Davidson *et al.,* 1999) embryos, or genetically (*HNF-3β* mutants; Klingensmith *et al.,* 1999), have forced a reevaluation of the role of these vertical signals in induction. Careful study has also indicated that noggin, follistatin, Xnr3 and chordin are expressed in the blastula *prior* to the formation of Spemann's organizer, where they are reexpressed (Wessley *et al.,* 2001).

This accumulating evidence suggests that neural induction may begin earlier than previously thought and, in amphibia, requires signals mediated by the β-catenin path-

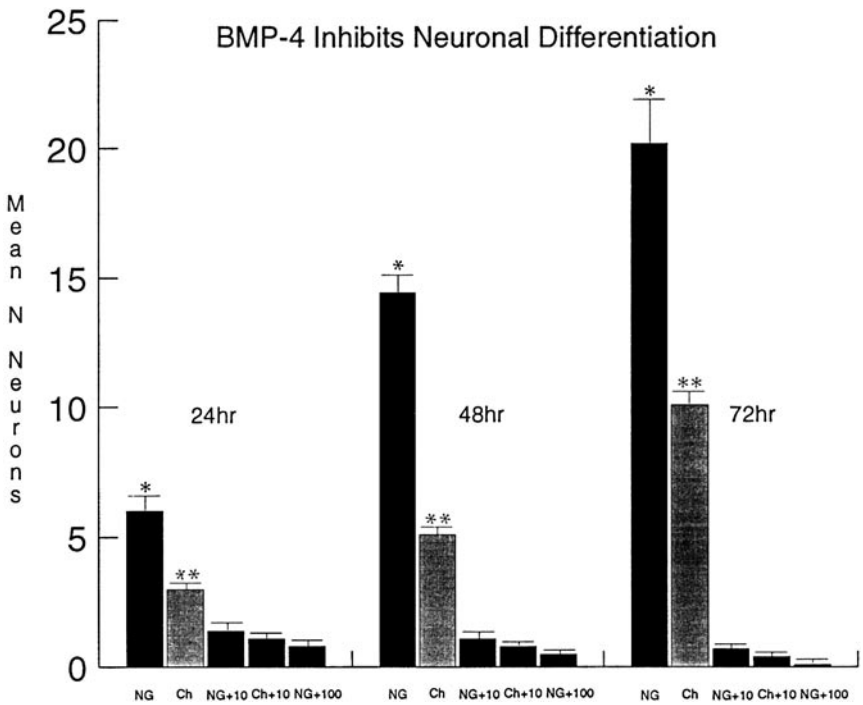

FIG. 4. BMP-4 inhibits neuronal differentiation of noggin-and chordin-transfected ES cells. Bars indicate mean ± SEM. *, Indicates noggin transfectants (no BMP-4) significantly greater number of neurons than BMP-4 treated (10, 100 ng/ml). **, Indicates chordin transfectants (no BMP-4) significantly greater number of neurons than BMP-4 exposed (10 ng/ml), $P \leq 0.01$, Student's *t* test. NG, noggin-transfected cells; Ch, chordin transfectants; NG + 10, noggin transfectants + 10 ng/ml BMP-4; Ch + 10, chordin-transfected cells + 10 ng/ml BMP-4; NG + 100, noggin-transfected cells + 100 ng/ml BMP-4.

way to inhibit *BMP4* transcription (Baker *et al.*, 1999) and activate expression of extracellular antagonists. The mechanisms by which down-regulation of BMP gene transcription is effected may vary between species; in the avian embryo, FGF family members may be primarily responsible (Wilson *et al.*, 2000; Streit *et al.*, 2000); alternatively, Wnt signaling may contribute to the inhibition of BMP expression in the ectoderm (Baker *et al.*, 1999, McGrew *et al.*, 1999). Unlike other reports that exposure to rh-noggin, or mouse cerberus-related protein (Tropepe *et al.*, 2001), or to mouse chordin (Kawasaki *et al.*, 2000) were less effective inducers of neuronal differentiation, the high sustained expression of noggin obtained in the current investigation was sufficient to inhibit *BMP-4* transcription, with widespread neuronal differentiation. These results are similar to those of Lim *et al.* (2000) who demonstrate that expression of mouse noggin by adult ependymal cells strikingly promotes neuronal differentiation of neural stem cells resident in the subventricular zone.

The pluripotent embryonic stem cell is a valuable model for the study of lineage decisions during mammalian development. The *in vitro* differentiation of mouse embryonic stem cells has recently been suggested (Wiles and Johansson, 1999; Sasai, 2001) to be the functional equivalent of the dorsal ectoderm (animal cap) explant model employed so efficiently to study neural induction in *Xenopus* embryos. ES cells are ideal for studies of this type because they express genes typical of the early ICM/ectoderm, including BMPs (e.g., Winnier *et al.*, 1995), and can be transfected to express a number of candidate signaling/lineage determination genes. Despite the potential of ES cells to provide cells for transplantation and gene delivery, the ES differentiation field has been hampered by the lack of methods to derive specific cell types; aggregation and retinoic acid based differentiation typically produces mixed populations of cells rather than single lineages (review, O'Shea, 1999). Although a variety of growth and differentiation factors have been added to cultures of ES cells to promote cell type-specific differentiation, they have produced mixed results, likely due both to growth factors produced by the aggregated ES themselves and to the presence of serum (e.g., Johansson and Wiles, 1999) or undefined factors (Kawasaki *et al.*, 2000) in the culture medium of many of these experiments. The present study provides both a model system for studying lineage segregation during differentiation and a source of primitive neuronal cells for transplantation.

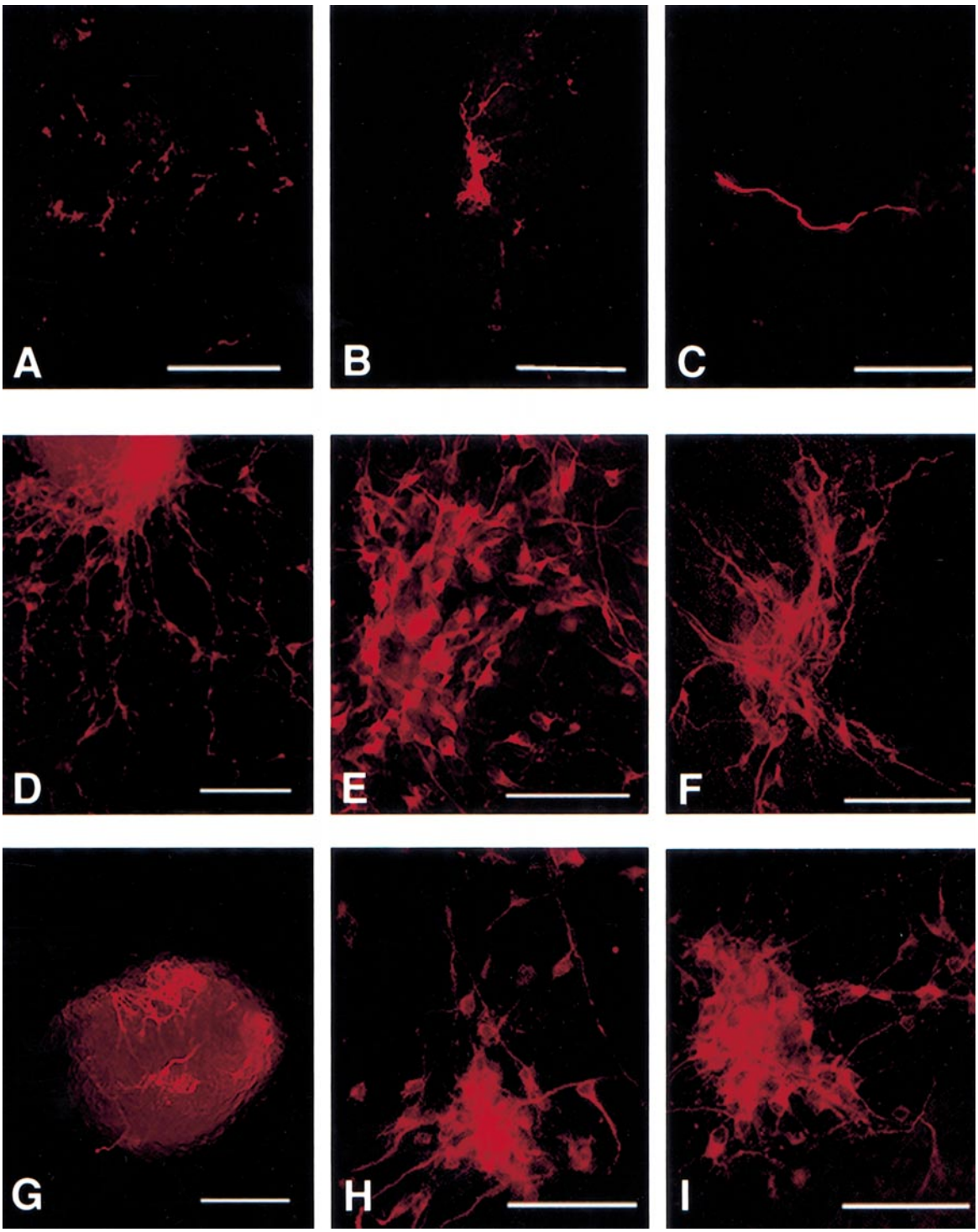

FIG. 5. Neuronal differentiation in aggregate cultures of pCS2 (A–C), pCS2/noggin (D–F), or pCS2/chordin (G–I)-transfected cells. Primary antibody (TUJ1, 1:100), secondary antibody-Cy3. Neuronal differentiation was present in all three groups, but was most extensive in the noggin-transfected cultures. In control cultures, isolated neurons were common (A, C), while clusters of neurons were present in noggin- and chordin-transfected cells. Cells exposed to noggin or chordin often migrated from the aggregate to the laminin-coated substrate (D). Scale bars, 100 μm.

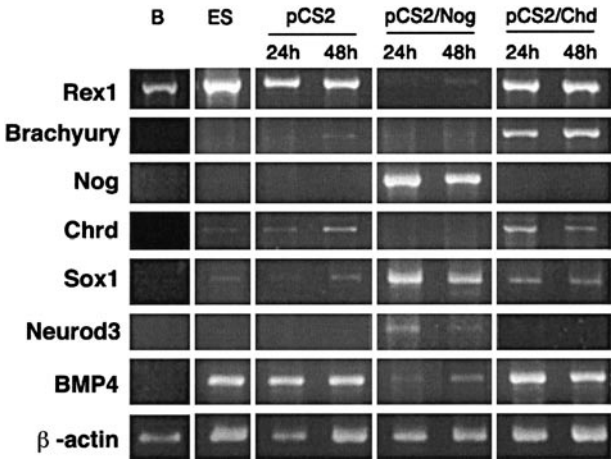

FIG. 6. Gene expression patterns. Lane 1 indicates expression of each gene in RNA isolated from pooled blastocysts; lane 2 from undifferentiated ES cells; lanes 3 and 4, gene expression in pCS2 (control) transfectants after 24 and 48 h *in vitro*. Lanes 5 and 6 indicate gene expression in the pCS2/noggin cultures at 24 and 48 h, and lanes 7 and 8, gene expression in pCS2/chordin cultures at 24 and 48 h.

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