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Regional Specification of Neurosphere Cultures Derived from Subregions of the Embryonic Telencephalon

Malin Parmar,[1] Charlotta Skogh, Anders Björklund, and Kenneth Campbell[2]

Wallenberg Neuroscience Center, Division of Neurobiology, Lund University, Solvegatan 17, BMC A11, S-221 84 Lund, Sweden

We have studied the molecular specification of precursor cells in expanded neurosphere cultures derived from distinct subregions of the embryonic mouse telencephalon. These regionally derived cultures exhibited differential responses to the mitogens EGF and bFGF, suggesting that the precursors in these cultures were differentially specified as is the case *in situ.* To examine this further, cultures from each of the telencephalic subregions were expanded in both EGF and bFGF before differentiation. The neurons produced displayed molecular phenotypes similar to those normally derived from each of these regions *in vivo.* Moreover, analysis of gene expression in the undifferentiated cultures showed that the regionally derived neurospheres express many of the same developmental control genes as their *in vivo* counterparts. Taken together, the present findings suggest that precursor cells in neurosphere cultures, derived from distinct subregions of the embryonic telencephalon, maintain at least certain aspects of their molecular specification, even after significant expansion *in vitro.*

INTRODUCTION

The generation of neuronal diversity in the vertebrate nervous system is known to depend on the temporally and spatially restricted expression of developmental control genes in neural precursor cells (for reviews, see Jessell, 2000; Rubenstein *et al.,* 1998). At spinal cord levels, combinatorial codes of transcription factors have been shown to control the generation of distinct neuronal subtypes (see, e.g., Briscoe *et al.,* 2000). The molecular specification of neural precursor cells has been suggested to be a progressive process with a transition from an early requirement for extrinsic signals to intrinsic mechanisms (Edlund and Jessell, 1999). This intrinsic specification is likely regulated by the coordinate expression of transcription factors ultimately controlling cell fate decisions. To date, most studies on the molecular control of regional neuronal specification have been carried out *in vivo*. *In vitro* approaches would be useful to determine the degree of intrinsic specification present in neural precursors at different stages of neurogenesis.

A well-established and currently widely used method for expanding neural precursor cells *in vitro* is to grow them as neurosphere cultures (Reynolds *et al.,* 1992). Using this culture system, precursor cells are grown serum-free in defined medium with either epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) or both. Cells expanded under these conditions have been shown to exhibit stem cell characteristics *in vitro,* including self-renewal and multipotency (i.e., producing all three cellular subtypes typical of the nervous system: neurons, astrocytes, and oligodendrocytes) (Reynolds and Weiss, 1996). The multipotency of neurosphere cultures led to the general view that neural stem cells contained in these cultures are capable of generating nearly all of the neuronal subtypes of the nervous system. While earlier transplantation studies of neurospheres derived from the mouse telencephalon did not support this notion (Winkler *et al.,* 1998), recent transplantation studies of neurospheres derived from

[1] To whom correspondence should be addressed. E-mail: malin.parmar@mphy.lu.se.

[2] To whom correspondence should be addressed at present address: Division of Developmental Biology, Children's Hospital Research Foundation, 3333 Burnet Ave., Cincinnati, OH 45229-3039. Fax: (513) 636-4317. E-mail: kenneth.campbell@chmcc.org.

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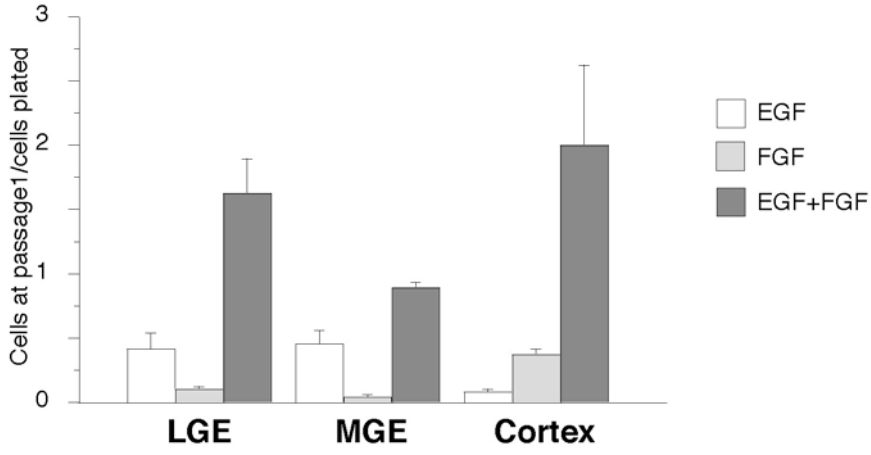


FIG. 1. Differential response of MGE-, LGE-, and cortex-derived neurospheres to EGF and bFGF. Bar graphs illustrating the numbers of cells at the first passage after expansion in either or both of the mitogens. Note that precursors in the MGE and LGE respond better to EGF than bFGF, while the cortical precursors grow better in bFGF than EGF. The addition of both mitogens dramatically potentiates the growth response of precursors from each of the telencephalic subregions.

the human embryonic telencephalon have indeed demonstrated a broad developmental potential for these cells (Englund *et al.*, 2002; Rosser *et al.*, 2000).

The goal of this study was to examine the degree of molecular specification, if any, of neurosphere cultures derived from distinct subregions of the embryonic mouse telencephalon at midneurogenesis (i.e., E13.5) stages. A previous study has shown that immortalized cell lines derived from the embryonic rat telencephalon retain aspects of their regional identity, *in vitro,* despite the fact that many of their normal environmental influences are not present (Nakagawa *et al.*, 1996). The subregions we studied were the lateral ganglionic eminence (LGE), which is known to give rise to striatal projection neurons (Deacon *et al.*, 1994; Olsson *et al.*, 1997, 1998a; Wichterle *et al.*, 2001) and interneurons of the olfactory bulb (Wichterle *et al.*, 1999, 2001); the medial ganglionic eminence (MGE), which contributes to the pallidum (Olsson *et al.*, 1997, 1998a) as well as numerous interneurons which migrate tangentially to populate the striatum and cortical structures (Anderson *et al.*, 2001; Lavdas *et al.*, 1999; Pleasure *et al.*, 2000; Wichterle *et al.*, 1999); and the developing cortex, which produces, at least, the cortical projection (i.e., pyramidal) neurons (Bayer and Altman, 1991). Our results show that regionally derived neurosphere cultures from the embryonic mouse telencephalon do appear to maintain certain aspects of their molecular identity and, when differentiated, give rise to appropriate neuronal phenotypes, even after numerous passages *in vitro*.

RESULTS

Precursors in the LGE, MGE, and Cortex Respond Differentially to EGF and bFGF

Previous studies have shown that EGF and bFGF have different effects on the establishment of neurosphere cultures dissected from the embryonic telencephalon (Ciccolini and Svendsen, 1998; Tropepe *et al.*, 1999; Martens *et al.*, 2000). We generated neurosphere cultures from the LGE, MGE, and cortex of E13.5 mouse embryos in the presence of either or both of these mitogens. The cells were plated at a density of 250 cells/μl in T25 tissue culture flasks. After 7 days *in vitro,* the spheres that had formed were mechanically dissociated and the total number of live cells were counted using the trypan blue dye exclusion method. The LGE and MGE were found to have a higher total cell number at passage 1 when expanded in EGF compared to bFGF. The opposite was true for the cortex, which had more cells at passage 1 when expanded in bFGF compared to EGF. For all three areas, the combination of both EGF and bFGF was found to generate the highest number of cells (Fig. 1). Since the combination of these two mitogens generated the most cells for all three areas, we used this culture condition for the remainder of this study. Precursor cells expanded under both EGF and bFGF stimulation produced neurons with similar frequency after differentiation regardless of what area they were isolated from. Under our differentiation conditions, LGE, MGE, and cortical spheres displayed sim-

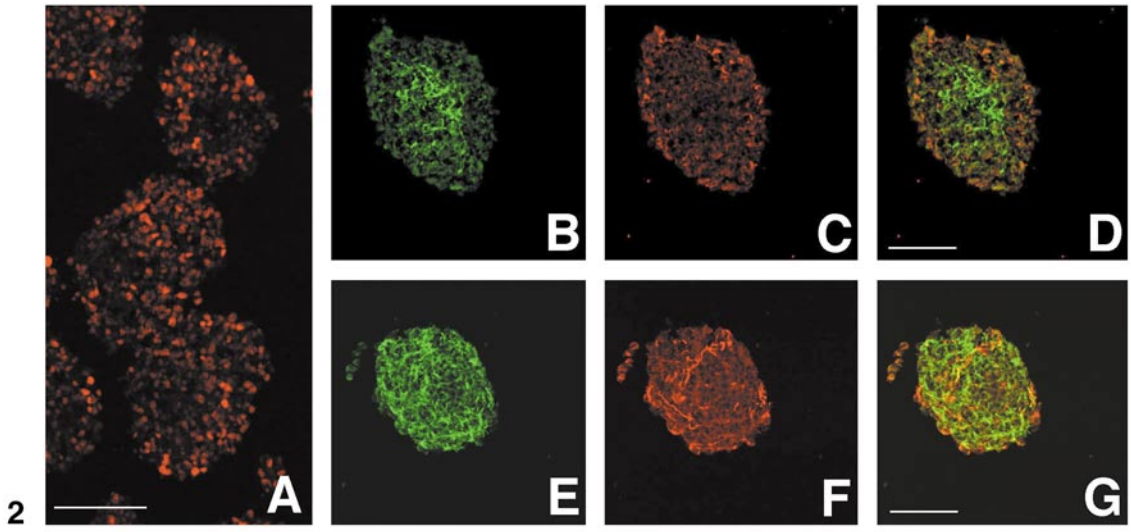
ilar neurogenic potentials, producing around 12% neurons each (data not shown).

Cellular Composition of Neurospheres

Neurospheres generated from the LGE, MGE, and cortex were passaged six times and fixed 7 days after the last passage. To determine whether cells proliferate in localized regions of the spheres, we stained the sections of the spheres with an antibody against Ki67, which is expressed by cells throughout most of the cell cycle (i.e., late G1/S/G2/M) (Schlüter *et al.*, 1993). While Ki67$^+$ cells were observed in all areas of the spheres, in most cases, Ki67-expressing cells were more numerous in the outer portions (Fig. 2A). To analyze the cellular composition of the spheres, we assayed for the expression of proteins that are characteristic of immature cells, such as the neural precursor and radial glial marker nestin (Hockfield and McKay, 1985), and the radial glia protein recognized by the RC2 antibody (Misson *et al.*, 1988). In addition, we also analyzed the expression of glial fibrillary acidic protein (GFAP), a protein expressed in astrocytes (Bignami *et al.*, 1972), and the neuron-specific β-III-tubulin (Lee *et al.*, 1990). Regardless of their origin (i.e., MGE, LGE, or cortex), all spheres were found to contain cells of different subtypes, expressing one or more of the four cell type-specific proteins studied. Many of the cells in the neurospheres exhibited glial protein expression. Cells expressing GFAP (Fig. 2B) were found predominantly in the core of the sphere, while those labeled by the RC2 antibody (Fig. 2C) were distributed in the outer regions (Fig. 2D). The most predominantly expressed molecule in the spheres was nestin, which did not show any regional restriction (Figs. 2E and 2G). Nearly all of the cells labeled with the RC2 antibody coexpress nestin (data not shown). Interestingly, in almost every sphere studied, at least some cells were found expressing β-III-tubulin (Figs. 2F and 2G), suggesting that even under expansion conditions, some neurons are generated in the spheres. Indeed, the β-III-tubulin-positive cells sometimes had quite mature neuronal morphologies. Unlike the Ki67-, RC2-, and GFAP-positive cells, the β-III-tubulin-positive neurons were distributed randomly within the different spheres. These results indicate that the MGE-, LGE-, and cortex-derived spheres contain cells at different stages of maturation, and that these cells are somewhat compartmentalized.

Molecular Identity of Differentiated Neurons

To study the molecular identity of neurons differentiated from regionally derived neurosphere cultures, we differentiated passage 6 spheres derived from the MGE, LGE, or cortex for 7 days and subsequently studied the expression of transcription factors known to be regionally expressed in developing neurons of the embryonic ventral telencephalon. DLX proteins are expressed by precursors as well as differentiating neurons in the ventral telencephalon (Eisenstat *et al.*, 1999) (Fig. 3A). Accordingly, cells in the LGE (Fig. 3B) and MGE cultures (Fig. 3C) were observed to express DLX proteins. Recent studies have shown that a subpopulation of DLX-positive cells migrates into the cortex from the ventral telencephalon (reviewed in Marin and Rubenstein, 2001). Interestingly, differentiating cells in the cortically derived spheres also exhibited DLX protein expression (Fig. 3D); however, these were significantly fewer than in LGE or MGE cultures. We also looked at the expression of MEIS2, which is normally expressed at moderate to low levels in the telencephalic ventricular zone, but greatly enriched in differentiating and mature striatal neurons (Toresson *et al.*, 1999, 2000a) (Fig. 3E). In the differentiating cultures, MEIS2 is expressed at much higher levels in cultures originating from the LGE (Fig. 3F) than from the MGE (Fig. 3G) or cortex (Fig. 3H). In the developing brain, nuclear PBX has been found to coincide with *Meis* gene expression (Toresson *et al.*, 2000a) (Fig. 3I). This was the case also *in vitro* where nuclear PBX is found mainly in the differentiated LGE cultures (Fig. 3J) compared to those derived from the MGE and cortex (Figs. 3K and 3L). To ensure that the expression of these transcription factors is in the neurons, the cultures were double-stained with β-III-tubulin. In all cases, we found that it was the neurons that expressed high levels of MEIS2 (Fig. 4A), PBX (Fig. 4B), and DLX (Fig. 4C). Low-level expression of each of these proteins was seen in cells with large nuclei (likely differentiating glial cells) (Fig. 4). Next, we quantified the number of neurons expressing each of these transcription factors and found that 55.4 ± 4.8% of the neurons from the LGE and 58.4 ± 3.8% of the neurons from the MGE cultures express the ventrally enriched protein DLX, compared to only 13.2 ± 2.6% of the neurons differentiated from cortical spheres (Fig. 5). The *in vivo* LGE-enriched proteins, MEIS2 and nuclear PBX, were also found to be expressed at the highest level in the cultures originating from the LGE neurospheres (Fig. 5). In fact, 84 ± 5.8% of the LGE-derived neurons express MEIS2, and 72 ± 6.8% contain nuclear PBX. In the differentiated MGE cultures, only 24 ± 4.8% expressed MEIS2, while 10 ± 4.3% displayed nuclear PBX. For the cortical cultures, MEIS2 and nuclear PBX were found in 15.6 ± 4.9% and 9.4 ± 1.5% of neurons, respectively. These results show that the neurons gen-



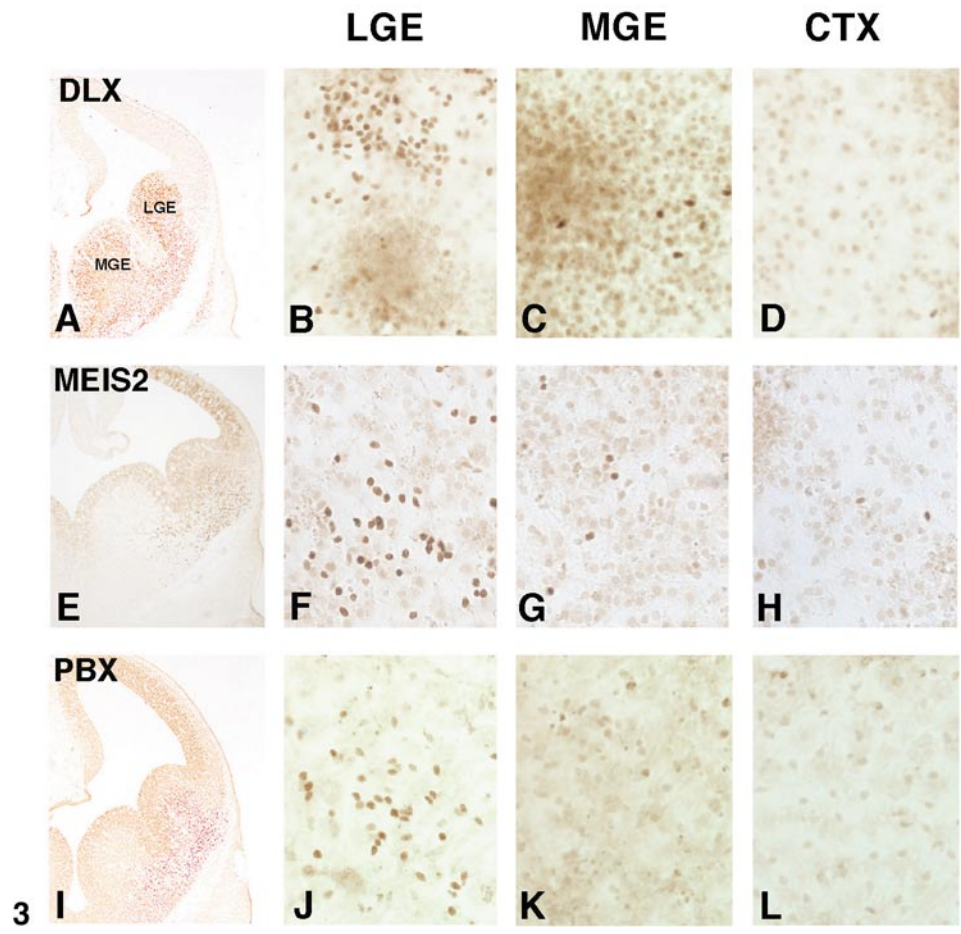


FIG. 2. Cellular composition of telencephalic neurospheres grown in both EGF and bFGF. Sectioned spheres stained for Ki67 (A), GFAP (B and D, green), RC2 (C and D, red), nestin (E and G, green), and β-III-tubulin (F and G, red). Note that Ki67 is higher in outer regions of the spheres (A). (D) Confocal image showing GFAP (green) expression in the core of the sphere and RC2 staining (red) in the outer portions. (G) Confocal image showing uniform nestin expression (green) and β-III-tubulin-staining neurons and processes (red) in a sphere. Scale bar, 100 μm.

FIG. 3. Molecular identity of differentiating cells in regionally derived telencephalic neurosphere cultures expanded in both EGF and bFGF. (A) DLX proteins are expressed in the ventral telencephalon (i.e., the LGE and MGE). DLX proteins are observed in many differentiating cells of the LGE (B) and MGE (C) cultures, while fewer cells are positive for this marker in the cortex cultures (D). (E) MEIS2 and PBX (I) proteins are highest expressed in the LGE. This relationship is also maintained in differentiating cultures from the LGE spheres (F, J) compared to MGE (G, K) and cortical (H, L) spheres.

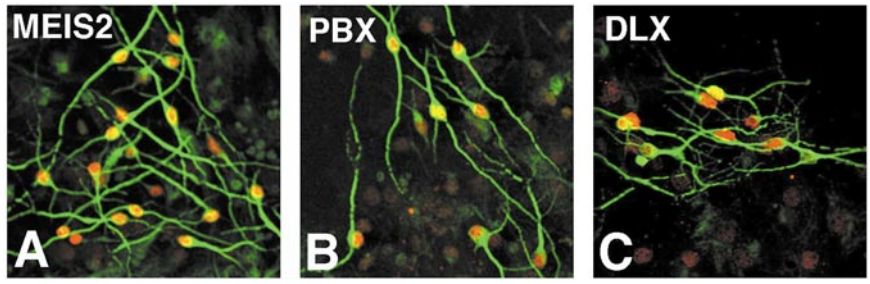


FIG. 4. Double-staining of MEIS2 (A, red), PBX (B, red), and DLX (C, red) with β-III-tubulin (green in A–C) in differentiated LGE sphere cultures expanded in both EGF and bFGF. Essentially all nuclei highly expressing MEIS2, PBX, or DLX proteins are colabeled for β-III-tubulin and exhibit neuronal morphology. Note also the weak expressing nuclei, which are larger than in the neurons.

erated in the differentiated LGE cultures exhibit molecular phenotypes similar to those of LGE-derived neurons *in situ*.

The homeobox protein NKX2.1 has also been shown to mark subpopulations of neurons in the ventral telencephalon that are thought to derive from the MGE, including striatal interneurons (Marin *et al.*, 2000). In the MGE cultures, NKX2.1 was indeed expressed in the differentiating neurons. The proportion of neurons expressing NKX2.1 was higher in the MGE cultures that had been differentiating for 3 days (Fig. 6A) compared 7 days (Fig. 6B). Few, if any, neurons differentiating in the LGE cultures expressed NKX2.1 at either 3 or 7 days of differentiation (Figs. 6C and 6D). Thus, as is the case in LGE cultures, at least some neurons derived from the differentiated MGE cultures can express an appropriate molecular phenotype.

Previous studies have shown that the LIM homeobox protein Islet1 is expressed in the telencephalon (Thor *et al.*, 1991), particularly in LGE progenitors and differentiating striatal neurons (Toresson *et al.*, 2000b; Toresson and Campbell, 2001; Wang and Liu, 2001). We were unable to detect expression of Islet-1 in the differentiating LGE cultures or in the MGE and cortex cultures, either by immunocytochemistry or by RT-PCR (data not shown). Moreover, the LGE is known to give rise to DARPP-32-expressing striatal neurons (Deacon *et al.*, 1994; Olsson *et al.*, 1998a); however, we were also unable to detect DARPP-32 expression in the *in vitro* differentiated LGE cultures (data not shown).

Molecular Specification In Undifferentiated Neurosphere Cultures

Despite the fact that the sphere cultures from the MGE and LGE had been passaged as many as six times, they were still capable of differentiating into neurons with at least some molecular similarities to the neurons

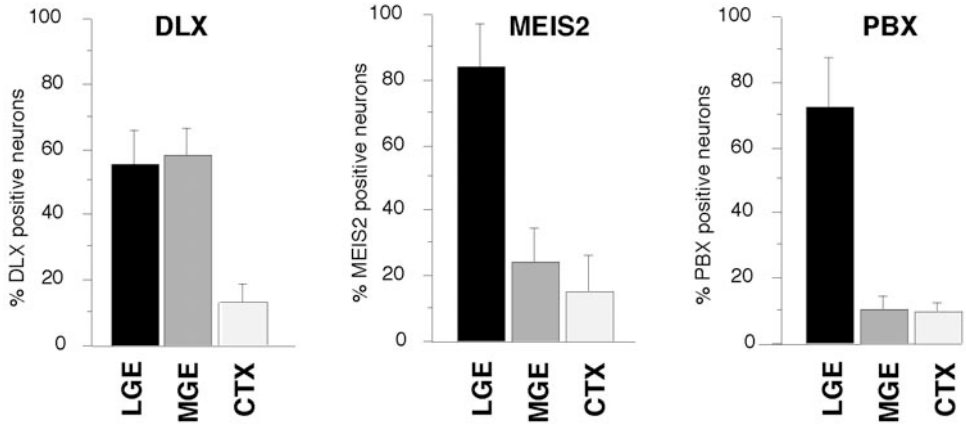


FIG. 5. Quantification of neurons expressing DLX, MEIS2, or PBX in differentiated cultures from each of the telencephalic subregions expanded in both EGF and bFGF. Bar graphs indicate the percentage of neurons positive for each of the markers in the different neurosphere cultures.

normally generated from these regions *in vivo*. This finding suggests that precursor cells in the neurosphere cultures maintain certain aspects of their molecular specification even after significant expansion *in vitro*. To analyze this further, RNA was harvested from the E13.5 MGE, LGE, or cortex as well as from neurosphere cultures derived from each of these regions after six passages, and the molecular specification of the cells in the spheres was studied by gene chip and RT-PCR analysis. We studied the expression of 15 developmental control genes regionally expressed in undifferentiated/differentiating cells of the embryonic telencephalon that were found on the Affymetrix U74Av2 murine genome chips. All of these genes showed differential expression between the different telencephalic regions (Table 1), which was in accordance with previously published *in situ* and/or immunohistochemical data (see alphabetical references in Table 1). The undifferentiated MGE spheres appropriately expressed 8 of the 15 genes compared to the normal MGE, which expressed 12 (Table 1). The levels of expression varied greatly, however, from those in the normal MGE (Table 1). The ectopic expression of *Pax6* in MGE spheres (Table 1) was confirmed by RT-PCR (Fig. 7). This ectopic expression may be due to slight variations in the dissections leading to contamination of the MGE cultures with LGE cells, which have expanded through the different passages together with the MGE cells. For the LGE spheres, 9 of the 15 genes were expressed and no ectopic expression was observed (Table 1). Again, the level of expression was often different in the sphere cultures compared to the normal LGE. Interestingly, *Dlx* genes, which are expressed in neurons of both MGE and LGE cultures after differentiation (see above), were not highly expressed in either of the undifferentiated MGE or LGE cultures (Table 1). *Gsh2*, a gene required for normal LGE development (Corbin *et al.*, 2000; Toresson *et al.*, 2000b; Yun *et al.*, 2001), is not on the Affymetrix U74Av2 chip. Thus, to analyze this gene in our neurosphere cultures we performed RT-PCR. *Gsh2* is normally expressed in the MGE and LGE but not the cortex (Fig. 7). In the neurosphere cultures, *Gsh2* is detected in both LGE and MGE spheres; however, it is also detectable in the cortical spheres (Fig. 7).

Cortical spheres appropriately expressed 6 of the 15 genes, while their *in vivo* counterparts expressed 9 (Table 1). To confirm that the low level of *Ngn2* expression that was detected on the gene chips was true, we did RT-PCR and found that a band could be amplified only in the cortex cultures (Fig. 7). In addition to *Gsh2*, *Er81* was also ectopic in cortical cultures. As suggested for the ectopic *Pax6* in the MGE cultures, the *Er81* and *Gsh2* expression in the cortex cultures may, at least in part, be due to variations in the cortex dissections that included small numbers of LGE cells. Moreover, at later stages of development Er81 is found in the ventral portion of the cortical ventricular zone (Stenman *et al.*, 2002). In any case, our gene expression analysis suggests that at least certain aspects of the molecular specification of precursor cells in the MGE, LGE, and cortex are maintained during expansion *in vitro* as neurosphere cultures.

DISCUSSION

The results of this study indicate that neurosphere cultures obtained from distinct subregions of the embryonic telencephalon retain aspects of their molecular specification, even after significant expansion (approximately 10,000-fold) *in vitro*. Moreover, after differentiation *in vitro*, regionally derived neurosphere cultures (at least those from the MGE and LGE) generated neurons with molecular identities that were similar to those derived from their *in vivo* counterparts.

We have previously described a culture system for progenitors derived from the LGE wherein the cells are grown as attached cultures in the presence of serum, EGF, and a defined hormone mix similar in composition to N2 supplement (Skogh *et al.*, 2001). In undifferentiated cultures, the cells express many molecular characteristics of glial cells; however, upon removal of the EGF and serum many neurons are formed and at least some of these are derived from GFAP-expressing cells. Remarkably, these cells can be passaged as many as 30 times and when differentiated they produce neurons bearing molecular characteristics typical of LGE-derived neurons *in situ* (Skogh *et al.*, 2001). Since these cells are grown in the presence of serum, however, unknown signals/factors may be controlling the apparent maintenance of regional identity in these cultures.

The neurosphere paradigm provides a more controlled situation to assess the intrinsic specification of precursors in expanded cultures since they are grown in defined medium (i.e., serum-free). A number of recent studies (Hitoshi *et al.*, 2002; Ostenfeld *et al.*, 2002; Yamamoto *et al.*, 2001; Zappone *et al.*, 2000) have assessed regional specification in neurosphere cultures derived from distinct regions of the embryonic brain and spinal cord, including the telencephalon or subdivisions thereof. Zappone *et al.* (2000) showed that neurosphere cultures derived from the embryonic telencephalon retained their molecular specification compared to those derived from the spinal cord after three passages *in vitro*. The telencephalic-derived cul-

tures were found to express a telencephalic-specific enhancer for the *Sox2* gene as well as the developmental control genes *BF-1* (*Foxg1*), *Otx1,* and *Tbr2,* each of which is known to be expressed in telencephalic precursor cells. Likewise, Hitoshi *et al.* (2002) also demonstrated that neurospheres derived from the cortex, MGE, or mid-hindbrain region retain aspects of their molecular identity after two passages. In their study, the cortical spheres exclusively expressed *Emx1,* while the MGE and mid-hindbrain cultures were *Dlx2*- and *En1*-positive, respectively. The present results are in general agreement with these previous studies and extend them by showing that certain aspects of regional identity are maintained even after as many as six passages *in vitro.*

Does the fact that precursor cells in neurosphere cultures maintain, at least in part, their molecular specification mean that these cells are irreversibly committed to specific fates? Hitoshi *et al.* (2002) have addressed this question and found that cortical or mid-hindbrain neurospheres can adopt molecular characteristics of the MGE-derived spheres if cocultured on ventral telencephalic explants. Interestingly, they also showed that new neurosphere cultures generated from the cocultured cortical or mid-hindbrain spheres exhibited molecular characteristics of ventral telencephalic neurospheres (i.e., *Dlx2* expression), rather than the regions that they were originally obtained from. This finding supports previous transplantation studies *in vivo,* which have shown respecification of primary precursors (Brüstle *et al.,* 1995; Campbell *et al.,* 1995; Fishell, 1995; Olsson *et al.,* 1997), and *in vitro,* showing expanded neural precursor/stem cell cultures (Fricker *et al.,* 1999; Shihabuddin *et al.,* 2000; Suhonen *et al.,* 1996) after ectopic localization. Taken together, these findings indicate that precursor cells in the neurosphere cultures are not committed to specific fates. In the absence of environmental cues, however, the cells appear to retain aspects of the molecular identity imparted on them before removal from their *in vivo* locations.

The results of the coculture and transplantation studies described above implicate environmental factors as strong determinants of regional identity. Although precursor cells do seem to be capable of maintaining aspects of their molecular specification through numerous cell divisions in the absence of external signals, it is clear that normal development of specific neuronal subtypes requires instruction until or even after the cells exit from the cell cycle. For example, motor neuron progenitors have been shown to require high levels of Sonic Hedgehog until their last cell division (Ericson *et al.,* 1996). This might explain some of the apparent deficiencies in our differentiation system reported here. The LGE has recently been shown to contain two distinct progenitor pools, one of which expresses Islet1 and gives rise to striatal neurons and another which expresses the ETS transcription factor Er81 and contributes interneurons to the olfactory bulb (Stenman *et al.,* 2002). In the differentiating LGE cultures, we have not observed Islet1 expression in the neurons. It may be that the expression of this developmental regulator may normally depend on a factor in the LGE that is not maintained in the neurosphere cultures. However, it is also possible that the subpopulation of LGE cells specified to express Islet1 is not expanded under the culture conditions used here. As striatal neurons mature they begin to express the phosphoprotein DARPP-32 (Gustafsson *et al.,* 1992). We were also unable to detect any DARPP-32 expression in the differentiated neurons from the LGE spheres. This was the case as well in a previous culture paradigm of expanded LGE cells in which the differentiated neurons also exhibited molecular similarities to those of LGE-derived neurons *in vivo* (i.e., DLX and MEIS2 expression) but no DARPP-32 expression (Skogh *et al.,* 2001). DARPP-32 expression in LGE derived neurons (i.e., striatal neurons) has been shown to depend on the external signals retinoic acid (Toresson *et al.,* 1999) and BDNF (Ivkovic and Ehrlich, 1999). Thus DARPP-32 expression might not be expected in the differentiated LGE cultures since our differentiation conditions did not contain either of these factors.

Interestingly, the undifferentiated MGE and LGE neurospheres were found to express very low levels of *Dlx* genes compared to their *in vivo* counterparts. Nevertheless, upon differentiation, these genes were seen to up-regulate significantly as evidenced by the high levels of DLX protein expression. This result might point to the source of cells that were expanded in the MGE and LGE cultures. In fact, Eisenstat *et al.* (1999) have shown that only a subpopulation of ventricular zone cells express DLX proteins, while most subventricular zone cells are DLX-positive. These authors proposed that DLX-negative ventricular zone cells progress through the differentiation process by sequentially activating *Dlx* gene expression starting with *Dlx2* and following with *Dlx1, Dlx5,* and *Dlx6* (Eisenstat *et al.,* 1999). Since the *Dlx* gene expression in our undifferentiated MGE and LGE spheres is so low compared to these regions *in situ,* we may have expanded a population of very undifferentiated ventricular zone cells which are specified to express *Dlx* genes upon further differentiation. Indeed, the MGE and LGE spheres express *Gsh2* and

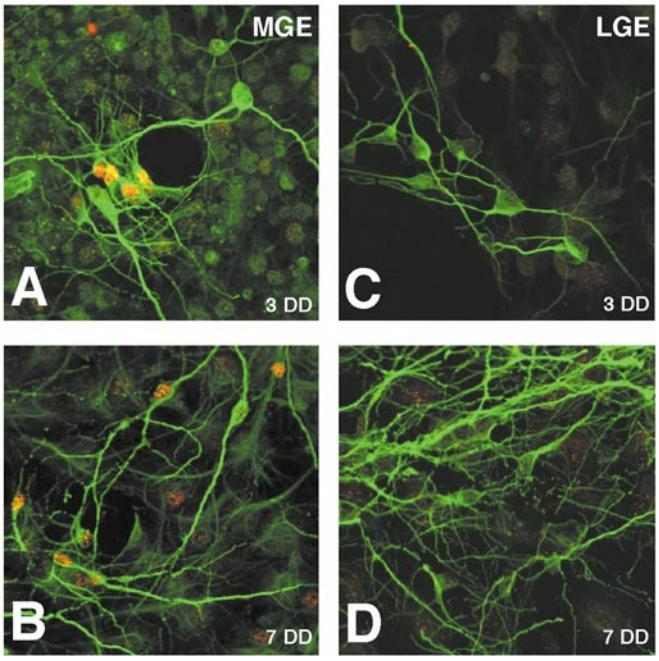


FIG. 6. NKX2.1 expression in differentiated neurons from MGE but not LGE neurosphere cultures expanded in both EGF and bFGF. NKX2.1 expression in neurons from the MGE-derived neurospheres after 3 (A) or 7 (B) days of differentiation (DD). Few if any neurons in the LGE cultures express NKX2.1 after either 3 (C) or 7 (D) days of differentiation (DD).

Mash1, which are normally found in many cells of the MGE and LGE ventricular zones.

Despite the lack of *Dlx* gene expression in undifferentiated cortex neurospheres, differentiation resulted in the appearance of significant numbers of DLX-positive neurons, albeit fewer than in the MGE or LGE cultures. These cells could be the result of LGE cells that were inadvertently included in the dissections of the cortex or due to the expansion of cells destined to differentiate into cortical interneurons that were caught migrating into the cortex from the ventral telencephalon (reviewed in Marin and Rubenstein, 2001). The latter possibility seems unlikely, since these migrating cells are believed to be postmitotic at this developmental stage (Anderson *et al.*, 2001). Another intriguing possibility is that these DLX-positive cortical neurons are derived from an expanded subpopulation of cortical precursors normally fated to generate DLX-positive neurons. In fact, Letinic *et al.* (2002) have recently shown that a MASH1-positive population of human cortical precursors gives rise to GABAergic cortical interneurons which also express DLX proteins. Previous studies have shown that ectopic expression of *Mash1* in mouse cortical precursors can induce both *Dlx* and glutamic acid decarboxylase (an enzyme involved in GABA production) gene expression (Fode *et al.*, 2000). Moreover, *Mash1* is known the be expressed in the cortical ventricular zone of the mouse but at significantly lower levels than in the MGE and LGE (Guillemot and Joyner, 1993). Our gene chip analysis shows that cortical spheres

TABLE 1
Developmental Control Gene Expression in the Embryonic Telencephalon and Neurosphere Cultures at E13.5

	E13.5 Telencephalon			E13.5 NS cultures		
	MGE	LGE	Cortex	MGE	LGE	Cortex
Pax6 a	A	P (0.36)	P (1.00)	P (0.22)	P (0.41) [0.93]	P (1.00) [0.81]
Ngn2 b	A	P (0.10)	P (1.00)	A	A	P [0.08]
Fez c	A	A	P (1.00)	A	A	A
c-kit d	P (0.34)	P (0.24)	P (1.00)	P (1.00) [3.39]	P (0.13) [0.64]	P (0.55) [0.64]
Islet1 e	P (0.21)	P (1.00)	A	A	A	A
Meis2 f	P (0.25)	P (1.00)	P (0.66)	P (0.96) [0.40]	P (0.87) [0.08]	P (1.00) [0.15]
Six3 g	P (0.40)	P (1.00)	A	P (1.00) [2.29]	P (0.94) [9.26]	A
Mash1 h	P (1.00)	P (0.91)	P (0.17)	P (0.65) [0.19]	P (1.00) [0.33]	P (0.56) [1.00]
Dlx1 i	P (1.00)	P (0.75)	A	A	A	A
Dlx2 i	P (1.00)	P (0.68)	P (0.03)	P (1.00) [0.05]	P (0.22) [0.01]	A
Dlx5 i	P (1.00)	P (0.92)	P (0.01)	A	A	A
Dlx6 i	P (1.00)	P (0.90)	A	P (0.99) [0.17]	P (1.00) [0.19]	A
Spalt1 j	P (1.00)	P (0.77)	P (0.28)	P (0.56) [2.56]	P (1.00) [6.01]	P (0.32) [5.26]
Er81 k	P (1.00)	P (0.63)	A	P (1.00) [2.06]	P (0.62) [2.02]	P (0.67)
Vax1 l	P (1.00)	P (0.95)	A	A	A	A

Note. A, absent; P, present. Data were obtained from the Affymetrix U74Av2 murine genome chips. The neurosphere RNA was obtained from spheres grown in both EGF and bFGF. Numbers in parentheses are normalized to the highest expressing region for each of the wild-type and neurosphere (NS) dissections. Numbers in brackets indicate the level of expression compared to the same region *in vivo*. References to support expression patterns in the embryonic telencephalon: a, Walther and Gruss, 1991, Sussel *et al.*, 1999; b, Sommer *et al.*, 1996, Gradwohl *et al.*, 1996; c, Matsuo-Takasaki *et al.*, 2000; d, Motro *et al.*, 1991; e, Toresson *et al.*, 2000a; f, Toresson *et al.*, 1999; g, Oliver *et al.*, 1995; h, Guillemot and Joyner, 1993; i, Liu *et al.*, 1997; j, Ott *et al.*, 2001; k, Yun *et al.*, 2001, Stenman *et al.*, 2002; l, Hallonet *et al.*, 1998.

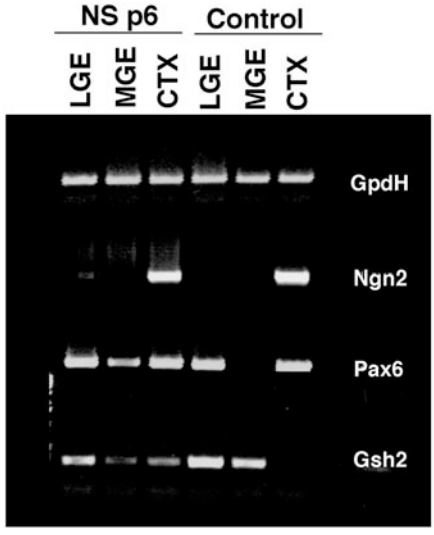


FIG. 7. RT-PCR results for *Ngn2, Pax6,* and *Gsh2* on RNA derived from the LGE, MGE, or cortex (CTX) of E13.5 embryos or from undifferentiated neurosphere cultures derived from each of these regions and passaged six times in the presence of EGF and bFGF.

maintain levels of *Mash1* that are equal to that in the normal cortex (see Table 1). Thus, at least some of the DLX-positive cortex neurons may be due to an expansion of an endogenous subpopulation of cortical interneuronal precursors.

The results of this study together with those of other recent reports (Hitoshi *et al.,* 2002; Ostenfeld *et al.,* 2002; Yamamoto *et al.,* 2001; Zappone *et al.,* 2000) indicate that stem cell/precursor cell cultures do maintain aspects of their molecular specification after expansion *in vitro*. This finding is important when considering the use of these expanded cells for brain repair. Although factors present in immature environments have been shown to redirect the fate of these cells, a damaged adult brain may not express these factors. Thus, a better approach to specific neuronal replacement in the damaged brain (such as dopamine neurons in Parkinson's disease) may be to isolate the precursor cells from the embryonic region that is specified to develop into the particular cell type requiring replacement.

EXPERIMENTAL METHODS

Dissection and Culturing

Cultures were gerenated from timed pregnant wild-type NMRI mice, and in some cases also from NMRI mice expressing GFP under the β-actin promoter (courtesy of A. Matus). No differences between the wild-type and GFP-expressing cultures were observed. All animal-related procedures were conducted in accordance with local ethical guidelines and approved animal care protocols. LGE, MGE, and cortex were differentially dissected from Embryonic Day E13.5 wild-type NMRI or GFP-expressing mouse embryos (Olsson *et al.,* 1998b). The tissue pieces were incubated in 0.1% trypsin and 0.05% DNase in DMEM for 15 min at 37°C followed by mechanical dissociation. The cells were spun down and resuspended at a concentration of 250 cells/μl in DMEM/F12 supplemented with a defined hormone and salt mixture (Reynolds *et al.,* 1992), containing 10 ng/ml bFGF and 20 ng/mlEGF (expansion medium). Cells were passaged once a week by mechanical dissociation and replated at a density of 50 cells/μl. Cells were frozen at passage 4, defrosted, and used for experiments at passage 6.

Differentiation

Seven days after passage 6, the spheres were plated on PLL-coated plastic chamber slides or PLL-coated T12.5 tissue-culture flasks. EGF and bFGF were removed from the expansion medium and 1% serum was added (differentiation medium) (Reynolds and Weiss, 1996). The spheres were maintained under differentiating conditions for 7 days before fixation and/or RNA isolation.

RNA Isolation

RNA was isolated from E13.5 mouse brain (control), differentially dissected E13.5 LGE, MGE, and cortex, and from passage 6 undifferentiated neurospheres originating from the E13.5 LGE, MGE, and cortex. Total RNA was prepared from cells or tissue using RNAqueous-4PCR (Ambion). For RT-PCR, RNA isolation was followed by two rounds of DNaseI treatment.

Gene Chip Analysis

RNA was further purified by ethanol extraction before labeling with biotinylated CTP and UTP. Labeled probes were hybridized to Affymetrix U74Av2 chips overnight before washing thoroughly and detecting with streptavidin R–phycoerythrin. Chips were read in the Affymetrix gene array scanner. All gene expression analysis was performed using the Affymetrix software Microarray Suite (MAS).

RT-PCR

One microgram of DNA-free total RNA was used to synthesize cDNA with oligo(dT)$_{30}$ primers and superscript-II- RT (GIBCO, Life Technologies). An RT-negative control was always included for each sample to exclude genomic DNA contamination. cDNA was amplified in a thermal cycler using the following program for all primer pairs: denaturation at 94°C for 1 min, annealing at a primer-specific temperature (see below) for 1 min, followed by extension at 72°C for 1 min. PCR products from the different genes were amplified using 25–35 cycles. The primers, Mg^{2+} concentrations, and annealing temperatures used were *G3PDH*, sense 5′-ACCACAGTCCATGCCATC, antisense 5′-TCCACCACCCTGTTGCTGTA, 1.5 mM Mg^{2+}, 56°C; *Pax6*, sense 5′-AGTCACAGCGGAGT5GAATCAG, antisense 5′-AGCCAGGTTGCGAAGAACTCT, 1.5 mM Mg^{2+}, 56°C; *Ngn2*, sense 5′-CGTCAAATCTGAGACTCTGG, antisense 5′-ATCTTCGTGAGCTTGGCATC 0.5 mM Mg^{2+}, 52°C; *Gsh2*, sense 5′-CAGCTTTCCGGACAGTGCTC, antisense 5′-GGTAGCAGAAGGAGCCTCTG, 1.5 mM Mg^{2+}, 60°; *Islet1*, sense 5′-GCAGCATAGGCTTCAGCAAG, antisense 5′-GTAGCAGGTCCGCAAGGT, 1.5 mM Mg^{2+}, 56°C. The RT-negative control from each sample was included in every PCR reaction. These controls never resulted in amplification.

Immunocytochemistry

Intact spheres or cell cultures were fixed in cold 4% PFA, for 15 min at room temperature, followed by three rinses in PBS. Intact, undifferentiated spheres were then mounted in tissue tech, and cryostat-sectioned at a thickness of 14 μm. Before immunostaining, the sections and cultures were rinsed in KPBS and preincubated in 10% normal serum and 0.25% Triton X-100 for 1 h at room temperature. Primary antibodies used were rabbit anti-distalless (i.e., DLX, 1:500, provided by G. Panganiban), rabbit anti-GFAP (DAKO, 1:1000), rabbit anti-Meis2 (1:5000, provided by A. Buchberg), rabbit anti-nestin (1:500, provided by R. McKay), rabbit anti-PBX1/2/3 (1:400, Santa Cruz), rabbit anti-Nkx2.1 (1:1250, provided by R. DiLauro), and mouse anti-β-III-tubulin (1:250, Sigma). RC2 (generated by M. Yamamoto, used at 1:4) was obtained from the Developmental Studies Hybridomas Bank, University of Iowa. Incubations in primary antisera were carried out overnight at room temperature. Detection with secondary antibodies was carried out using the standard protocols. Both fluorescent (1:400) and biotin-conjugated (1:200) antibodies were diluted in the preincubation solution and incubated for 2 h at room temperature. The biotinylated antibodies were detected using the ABC method (Vector Labs) with diaminobenzidine as the final chromogen.

Quantification

To assess the total cell number, nuclei were stained with DAPI and counted. Using these numbers, the proportion of total cells expressing β-III-tubulin, MEIS2, PBX, and DLX was determined. All quantification was done on cultures differentiated in five separate wells, using LGE, MGE, and cortical spheres from two different dissections. All results are reported as mean $\pm$ 1 standard deviation.

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