Developmental potential of defined neural progenitors derived from mouse embryonic stem cells

Nicolas Plachta[1], Miriam Bibel[2], Kerry Lee Tucker[3] and Yves-Alain Barde[1,*]

[1]Biozentrum, University of Basel, Klingelbergstrasse 50/70, 4056 Basel, Switzerland
[2]Novartis Institutes for Biomedical Research, Neuroscience, CH-4002 Basel, Switzerland
[3]Interdisciplinary Center for Neurosciences, University of Heidelberg, Im Neuenheimer Feld 345, 69120 Heidelberg, Germany
*Author for correspondence (e-mail: yves.barde@unibas.ch)

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Summary

The developmental potential of a uniform population of neural progenitors was tested by implanting them into chick embryos. These cells were generated from retinoic acid-treated mouse embryonic stem (ES) cells, and were used to replace a segment of the neural tube. At the time of implantation, the progenitors expressed markers defining them as Pax6-positive radial glial (RG) cells, which have recently been shown to generate most pyramidal neurons in the developing cerebral cortex. Six days after implantation, the progenitors generated large numbers of neurons in the spinal cord, and differentiated into interneurons and motoneurons at appropriate locations. They also colonized the host dorsal root ganglia (DRG) and differentiated into neurons, but, unlike stem cell-derived motoneurons, they failed to elongate axons out of the DRG. In addition, they neither expressed the DRG marker Brn3a nor the Trk neurotrophin receptors. Control experiments with untreated ES cells indicated that when colonizing the DRG, these cells did elongate axons and expressed Brn3a, as well as Trk receptors. Our results thus indicate that ES cell-derived progenitors with RG characteristics generate neurons in the spinal cord and the DRG. They are able to respond appropriately to local cues in the spinal cord, but not in the DRG, indicating that they are restricted in their developmental potential.

Key words: Neural tube, Stem cells, Motoneuron, Radial glial cells, Neurotrophin receptors

Introduction

Unlike many other adult tissues, the nervous system of mammals has a limited ability to compensate for the loss of cells after lesion. While recent results suggest that exogenously administered growth factors can increase neurogenesis following neuronal death caused by focal ischemia (Nakatomi et al., 2002), large scale cell replacement based on the recruitment of endogenous progenitor cells does not seem to be sufficient to restore functional neuronal circuits when the cell losses are extensive. A number of neurodegenerative diseases dramatically illustrate the consequences of this situation.

In theory, there is no quantitative limit to cell replacement based on the implantation of in vitro generated neural progenitors, and previous studies have indicated that nestin-positive, ES-derived cells have the potential to integrate in the host nervous system (e.g. Brustle et al., 1997). However, experiments of this kind have been typically performed with heterogeneous cell populations (for a review, see Anderson, 2001). We recently found that the addition of retinoic acid (RA) to rapidly dividing mouse ES cells leads to the generation of a uniform population of neural progenitors that display the characteristics of RG cells found in the developing dorsal telencephalon (Bibel et al., 2004). This finding offered the possibility to test the differentiation potential of a homogenous cell population corresponding to progenitors participating in normal brain development.

RG cells are the first cell type that can be distinguished from neuroepithelial cells, and they have traditionally been considered to guide the migration of newly born neurons and to subsequently become astrocytes (for a review, see Rakic, 2003). Recently, they were also discovered to generate neurons (Malatesta et al., 2000), and it now appears that most pyramidal neurons in the developing telencephalon derive from RG cells (Malatesta et al., 2003). In the present study, we implanted Pax6-positive RG cells in place of a portion of the chick neural tube, and examined their fate several days after implantation.

Materials and methods

All reagents for cell culture were purchased from Invitrogen unless otherwise indicated.

Cell culture

Mouse ES cells were deprived of mouse embryonic fibroblasts and cultured on gelatine-coated dishes containing Dulbecco's Modified Eagle Medium and leukemia inhibitory factor (LIF, 1000 U/ml) (for details, see Bibel et al., 2004). To facilitate their detection in the host, we used ES cells engineered to express green fluorescent protein (GFP) from both *tau* alleles (for details, see Tucker et al., 2001; Bibel et al., 2004). Embryoid bodies (EBs) were formed in bacteriological dishes for a period of 8 days, with the addition of 5 μM all-trans RA (Sigma) during the last 4 days. In some experiments (see Results), EBs were used 36 hours after the beginning of their formation. EBs were fixed in 4% paraformaldehyde for 30 minutes, incubated in 30% sucrose for 12 hours, embedded in cryomedium (OCT, Sakura) and

stored at –80°C until cryosectioning. In some experiments, 10 μM bromo-deoxyuridine (BrdU, Sigma) was added to EB cultures 3 hours prior to fixation.

Chick embryo experiments

Fertilized chick eggs were incubated at 38.5°C and 80% humidity for approximately 42 hours, until they reached the 19-21 somite stage. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Two millilitres of albumen was removed from the egg and a portion of the upper eggshell was opened. To visualize the embryo, drawing ink (Pelikan, A17) was dissolved in PBS (16 μl/ml) and injected under the blastoderm. One neural fold was removed over a length of 4 somites, at the level of the forelimb bud, by tearing the tissue with glass needles. RA-treated EBs were incubated with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA) at 37°C for 10 minutes. EBs are typically heterogeneous in size and those corresponding approximately to the size of the gap to be filled were selected for the implantation experiments. RA-untreated EBs were trypsinized for 6 minutes. After incubation with trypsin, one EB was transferred, with a pipette tip, onto the top of the missing portion of the neural tube and implanted manually using a tungsten needle. By the end of these manipulations, the EB had become a loose cell aggregate, which helped to accommodate it in the appropriate position. Trypsin-treatment of EBs was found to be essential, as untreated EBs remained compact and did not integrate into the host environment. After sealing and incubation for 6 days, the embryos were removed from the eggs, examined for GFP fluorescence and fixed in 4% paraformaldehyde for 4 hours. Following incubation in 30% sucrose for 36 hours, they were embedded in cryomedium and stored at –80°C for cryosectioning.

Immunohistochemistry

Sixteen micrometre thick cross-sections were rinsed in PBS and incubated for 30 minutes in blocking solution containing 10% serum and 0.2% Triton in PBS (7% Triton was used for Oct3/4 staining). Sections were then incubated with primary antibodies in blocking solution for 12 hours at 4°C. The following antibodies were used at the indicated dilutions: Isl1 (1:500, gift from S. Arber, Biozentrum, University of Basel, Switzerland), Brn3a (1:10000, gift from E. Turner, UCSD, USA), pan-Trk C-14 (1:1000, Santa Cruz), Glast (1:1000, Chemicon), Oct3/4 N-19 (1:20000, Santa Cruz), Sox2 AB5770 (1:3000, Chemicon), BrdU (1:1000, Sigma), and a p75 serum raised against the bacterially expressed cytoplasmic domain of rat p75 (1:1000). The antibodies 40.3A4 (Isl1, 1:1500), 4F2 (Lim1/2, 1:500), 81.5 C10 (Mnr2, 1:500), Pax6 (1:1000), 74.5A5 (Nkx2.2, 1:50), Nestin (1:10), Rc2 (1:10), 50.5A5 (Lmx1, 1:50) and Pax7 (1:500) were obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa. In all cases, PBS was substituted for the primary antibodies to test for unspecific labelling of secondary antibodies. Sections were rinsed in PBS repeatedly and incubated with the following antibodies for 1 hour at room temperature: anti-rabbit Rhodamine Red X-conjugated antibody and anti-mouse Cy3 antibody (1:1000, Jackson), anti-guinea pig antibody (1:1000, gift from S. Arber). Secondary antibodies were combined with the nuclear stain Hoechst 33342 (10 μg/ml, Sigma). Sections were rinsed in PBS and mounted. Sections used for BrdU staining were previously incubated in 2 N HCl for 30 minutes at 37°C, then neutralized in 0.1 M sodium tetraborate for 30 minutes and rinsed in PBS. Pictures were collected with a Zeiss Axioplan2 Imaging fluorescent microscope and processed with Adobe Photoshop 7.0.

Results

Characterization of implanted RA-treated EBs

We first examined the expression of nestin, Sox2, Rc2, Glast and Pax6 in RA-treated EBs at the time of implantation (Fig. 1). The vast majority of the cells were found to be positive for

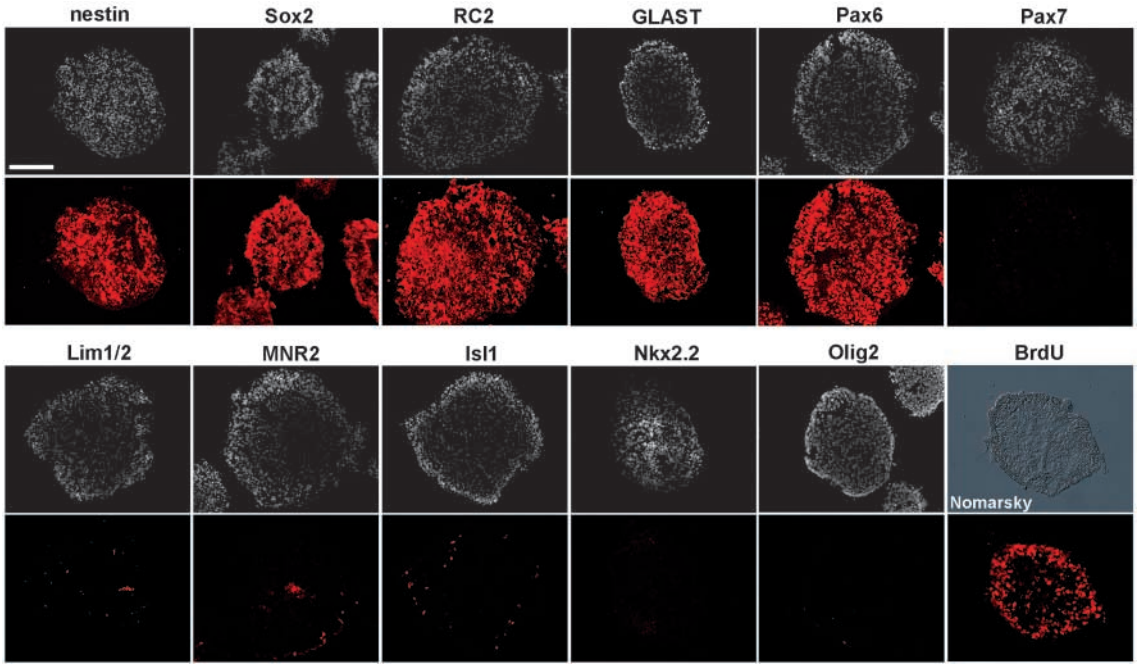

Fig. 1. Characterization of CNS progenitors in RA-treated EBs. Cryosections (12 μm thick) of EBs after 4 days of RA treatment (see Materials and methods), double labelled with a nuclear stain (upper rows) and with the indicated markers (lower rows). The majority of the cells express nestin, Sox2, Rc2, Glast and Pax6, a profile characteristic of neurogenic RG cells. Note that the expression of these markers is evenly distributed throughout the EB. RA-treated EBs contain very few cells (<1%) expressing the markers Lim1/2, Mnr2 and Isl1, and no cells expressing Pax7, Nkx2.2 or Olig2. A 3-hour pulse with BrdU shows the presence of proliferative cells in RA-treated EBs. At least five EBs from different experiments were analyzed for each marker. Scale bar: 100 μm.

Table 1. Integration of donor cells in operated embryos

Cell type implanted	Number of embryos operated	Number of embryos surviving operation	Number of embryos with GFP+ cells	Percentage of embryos containing GFP+ cells in VSC with axons	Percentage of embryos containing GFP+ cells in DRG
RA-treated cells	113	101	63	65.5±27.3 (*n*=24)	59.2±34.5 (*n*=27)
RA-untreated cells	86	71	56	74.9±27.6 (*n*=16)	86.1±16.4 (*n*=19)

Analyses were performed on embryos from 16 independent experiments (nine with RA-treated EBs and seven with RA-untreated EBs). Operated embryos were initially observed as wholemounts and those showing a GFP signal at the implant region (third column) were sectioned. All these embryos contained numerous GFP+ cells in several continuous sections, but the number of these varied. GFP+ cells were frequently detected in the ventral spinal cord (VSC) extending axons towards the periphery (fourth column), and in the DRG (fifth column). In few sections, donor cells were detected only in the DRG, suggesting that they also migrated along the anteroposterior axis. We failed to detect mouse nuclei in embryos that did not exhibit a GFP signal (~40%), suggesting that the donor cells did not survive in these animals. Results are ±s.e.m.; *n* represents the number of embryos analyzed.

these markers and they were evenly distributed throughout the EBs (Fig. 1). RA-treated EBs did not contain cells expressing the markers Pax7 or Nkx2.2, which in the neural tube define precursors located at more dorsal and ventral positions, respectively, of Pax6-positive cells (Jessell, 2000). Less than 1% of the cells in RA-treated EBs expressed Lim1/2, Mnr2, Isl1 or Olig2, which are normally expressed by differentiating neuronal precursors in the spinal cord and in the DRG. Also, no cells were positive for the sensory marker Brn3a (data not shown). The majority of the cells within RA-treated EBs were dividing, as assessed by a 3-hour BrdU pulse prior to fixation (Fig. 1). Taken together, these observations indicate that RA-treated EBs contain a uniform population of CNS progenitors with the known antigenic characteristics of RG cells found in the dorsal telencephalon (reviewed by Kriegstein and Gotz, 2003).

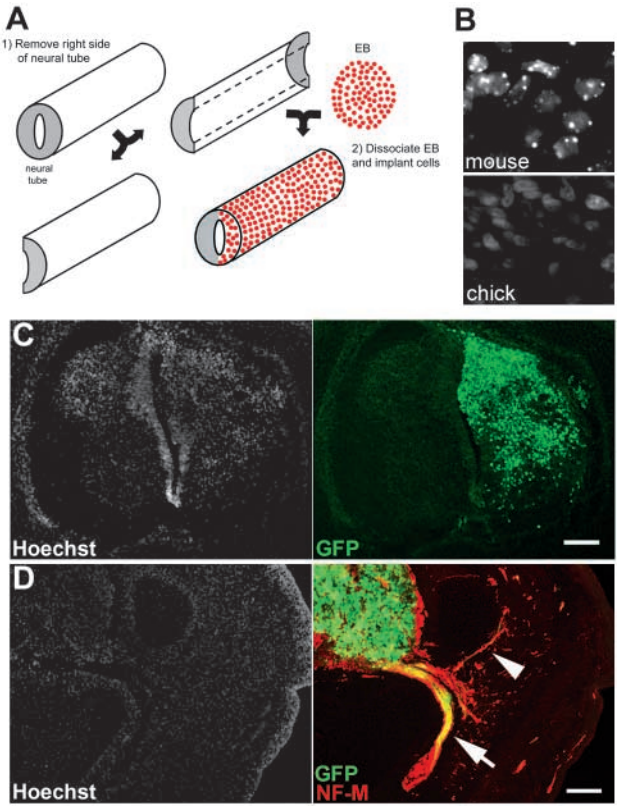

Fig. 2. Progenitors from RA-treated EBs integrate in the chick neural tube. (A) A segment of one neural fold (~400 μm long) was removed and discarded at the level of the forelimb bud of E2 chick embryos. EBs were dissociated with trypsin and implanted (see Materials and methods), and embryos were allowed to develop until E8. (B) Identification of donor cells by nuclear morphology. Nuclear staining with Hoechst of 16 μm-thick cryosections reveals differences in morphology between chick and mouse nuclei. (C,D) Transverse sections (16 μm thick) through chick embryos operated on as described in A. (C) Donor cells survive in large numbers and differentiate into GFP+ neurons throughout the operated side of the spinal cord. Left image shows nuclear staining revealing spinal cord morphology. Note that the operated side resembles the non-operated side in size and morphology. (D) GFP+ neurons populate the ventral spinal cord and extend axons towards the periphery. Axons can be observed projecting dorsally (arrowhead) and ventrally (arrow). GFP signal co-localizes with neurofilament (NF-M) staining (yellow). Scale bar: 10 μm in B; 100 μm in C,D.

Differentiation of RA-treated EBs after implantation in the neural tube

Following the removal of one half of the neural tube, one RA-treated, trypsinized EB was implanted in the gap (Fig. 2A). Embryos surviving the operation (see Table 1) were removed from the egg, washed with PBS, and observed under a fluorescent microscope. A GFP signal found at the implant region indicated that the donor cells survived and differentiated into neurons in the chick host (see Table 1). Serial transverse sections were then analyzed and stained with the nuclear stain Hoechst 33342, allowing mouse and chick cells to be unambiguously identified on the basis of their distinct nuclear morphologies (Fontaine-Perus et al., 1997) (see Fig. 2B). Mouse GFP neurons were detected in all consecutive sections of the implant area. Notably, the operated side of the spinal cord was abundantly populated by GFP neurons, closely resembling the adjacent non-operated side in size and morphology (Fig. 2C). Also, a bundle of GFP-labelled axons was often observed projecting from the ventral spinal cord towards the periphery, resembling a ventral root (Fig. 2D, Table 1). Cell counts indicated that the majority of the nuclei exhibiting mouse morphology co-localized to GFP-expressing cells (see Table 2). These observations show that donor cells survive in very large numbers for prolonged periods of time,

Table 2. Neuronal differentiation of donor cells

Cell type implanted	Percentage of mouse neurons in spinal cord	Percentage of mouse neurons in DRG
RA-treated cells	78.5±15.3	81.3±5.5
RA-untreated cells	72.8±19.2	65.4±17.1

The number of mouse nuclei was quantified in the spinal cord and in the DRG of embryos collected from 8 independent experiments. Results show the percentage±s.e.m. of Hoechst-stained mouse nuclei that expressed GFP, indicating their neural identity.

Table 3. Expression of neural markers by donor cells

Cell type implanted	LIM1/2 in SC	Mnr2 in VSC	Isl1 in VSC	Brn3a in DRG	Isl1 in DRG
RA-treated cells	39.3±10.2	73.6±23.5	80±26.4	0	0
RA-untreated cells	42.8±10.9	15.4±6.4	43.9±36.8	36.7±18.6	21.1±4.7

Analysis performed on sections from embryos implanted with RA-treated or RA-untreated cells, collected from five independent experiments. Results show percentage±s.e.m. of GFP+ cells that expressed Lim1/2 throughout the spinal cord (SC), Mnr2 or Isl1 in the ventral spinal cord (VSC), and Brn3a or Isl1 in the DRG.

and that they differentiate mostly into neurons in the chick spinal cord.

Because in the spinal cord some interneurons and motoneurons are generated from a progenitor pool expressing markers that we also find in our RA-treated EBs (Ericson et al., 1997; Graham et al., 2003), we next investigated whether these cell types could also be identified in the progeny of the implanted EBs. We found that GFP-expressing neurons located throughout the spinal cord expressed the interneuron marker Lim1/2 (Fig. 3A, Table 3), while they expressed Mnr2 and Isl1 in the ventral spinal cord (Fig. 3B,C). These cells also extended long GFP-positive axons towards the periphery (Fig. 3B). Importantly, GFP+/Lim1/2+ cells located dorsal to the motoneuron domain of the spinal cord did not express Mnr2 (Fig. 3J-K′). In the dorsal spinal cord, the GFP+ cells failed to express the dorsal interneuron marker Lmx1 (data not shown).

As previous studies revealed that when chick motoneurons differentiate and start to elongate axons they express both Trk and p75 receptors (McKay et al., 1996), we next investigated whether ventrally located donor cells also expressed these neurotrophin receptors. Fig. 3D-I shows labelling of ventrally located GFP+ neurons and their axons using pan-Trk and p75 antibodies.

Cells from RA-treated EBs colonize dorsal root ganglia but fail to differentiate into sensory neurons

In the chick embryo, neural crest cells delaminate from the dorsal neural tube and start to migrate to the periphery to form the PNS at E2 (Le Douarin and Kalcheim, 1999). As our

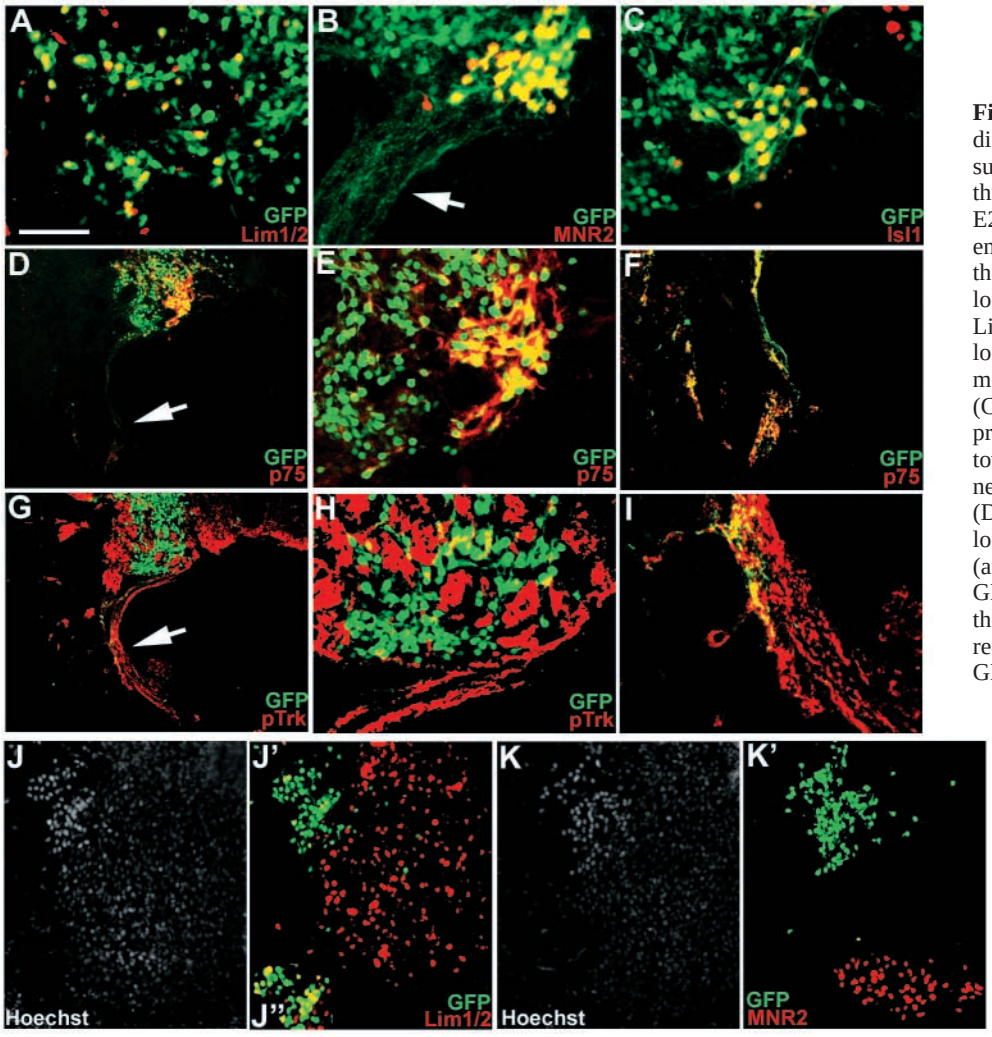

Fig. 3. Progenitors from RA-treated EBs differentiate into defined neuronal subtypes. (A-K) Transverse sections through E8 chick embryos operated on at E2, as described in Fig. 2, showing endogenous GFP signal and labelled for the indicated markers. (A) GFP+ neurons located at the middle spinal cord express Lim1/2 (merged yellow). Ventrally located GFP+ neurons express the motoneuron markers Mnr2 (B) and Isl1 (C). Note in B, the GFP+ axons (arrow) projecting from the MNR+/GFP+ cells towards the periphery. (D-I) Expression of neurotrophin receptors by donor cells. (D) p75 expression is detected in ventrally located GFP+ neurons and their axons (arrow). (E,F) Higher magnification of the GFP+ somata and the axons indicated by the arrow in D. (G) Expression of Trk receptors is detected in ventrally located GFP+ neurons using pan-Trk antibodies. (H,I) Higher magnification of the GFP+ somata and the axons indicated by the arrow in G. (J-K) Consecutive sections of the same embryo, double labelled by Hoechst (J,K), and by Lim1/2 (J′) or Mnr2 (K′). GFP+ cells located at the middle spinal cord express Lim1/2 (J′ shows a higher magnification of the same section), but do not express Mnr2 (K′). Scale bar: 50 μm in A-C,E,F,H,I; 100 μm in J-K′; 200 μm in D,G.

progenitors were implanted at these developmental stages, we next examined whether donor cells could colonize the PNS. GFP-positive cells were frequently found in the host DRG (Fig. 4A and Tables 1, 2). However, unlike their chick counterparts in the DRG, mouse neurons never expressed the transcription factors Brn3a (Fig. 4B) and Isl1 (Fig. 4C), which are markers that define most neurons in that structure (Anderson, 1999). Surprisingly, we never found mouse neurons elongating axons outside the DRG, even though GFP expression indicated their neuronal identity. These cells expressed p75 at high levels (Fig. 4D), but they failed to express detectable levels of Trk receptors (Fig. 4E).

RA-untreated EBs generate both spinal cord and DRG neurons in vivo

We next tested the prediction that in the absence of RA treatment, ES cells would generate both CNS and PNS neural progeny in vivo. Undifferentiated ES cells were allowed to form EBs for 36 hours in the presence of LIF and without RA, and subsequently were implanted in the chick neural tube following the same procedure as has been described for RA-treated embryos. Prior to implantation, ES cells expressed the transcription factor Oct3/4 (Fig. 5A), indicating their undifferentiated, pluripotent character (Niwa et al., 2000; Boiani et al., 2002). They failed to express nestin, Sox2, Pax6 or Pax7, Rc2, Glast, Lim1/2, Mnr2, Isl1, Nkx2.2, Olig2 or Brn3a (data not shown). Like RA-treated ES cells, they also survived in large numbers and differentiated into neurons in the host spinal cord (see Tables 1, 2). ES cell-derived GFP-positive neurons located throughout the spinal cord expressed Lim1/2 (Fig. 5B), suggesting their differentiation into interneurons. Surprisingly, and like the RA-treated cells, they failed to express Lmx1 in the dorsal spinal cord (data not shown). Ventrally located mouse neurons expressed Mnr2 and

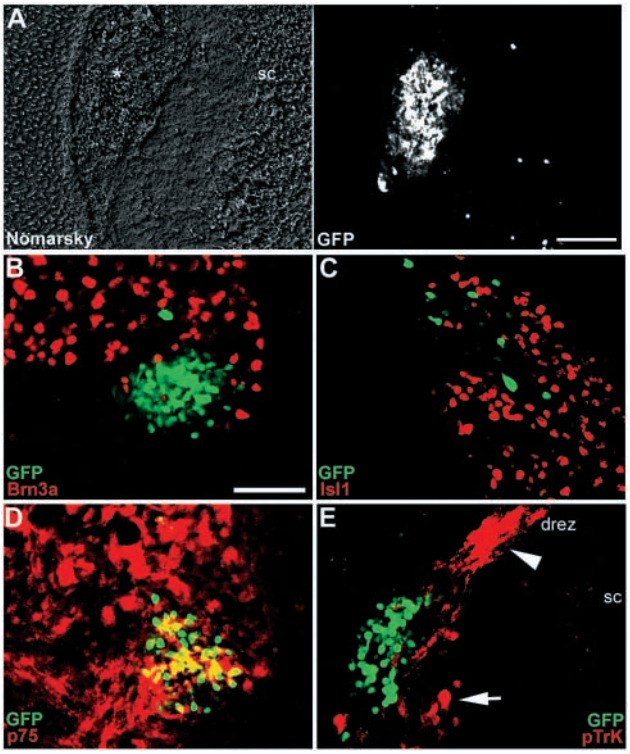

Fig. 4. Progenitors from RA-treated EBs fail to acquire sensory neuron characteristics. (A) Host DRG (asterisk, Nomarsky image, left) is shown to be colonized by GFP+ cells (right). Donor cells colonized much of the host DRG, and the majority expressed GFP, indicating their neural differentiation. Note the lack of GFP+ processes. In this section (see also Fig. 6), mouse donor cells have migrated longitudinally, away from the site of implantation, and the spinal cord segment at this level is occupied mostly by chick cells. Expression of Brn3a (B) and Isl1 (C) in DRGs colonized by GFP+ cells. Note the absence of Brn3a and Isl1 expression by GFP+ cells (no merged yellow). (D) GFP+ cells express high levels of the p75 neurotrophin receptor in the DRG (merged yellow). (E) Expression of Trk receptors is detected in chick cells in the DRG (arrow), and in their axons (arrowhead) projecting to the spinal cord, but not in GFP+ donor cells. Cells expressing Brn3a or Isl1 always exhibited chick nuclear morphology (not shown). drez, dorsal root entry zone; sc, spinal cord. Scale bars: 100 μm in A; 50 μm in B-E.

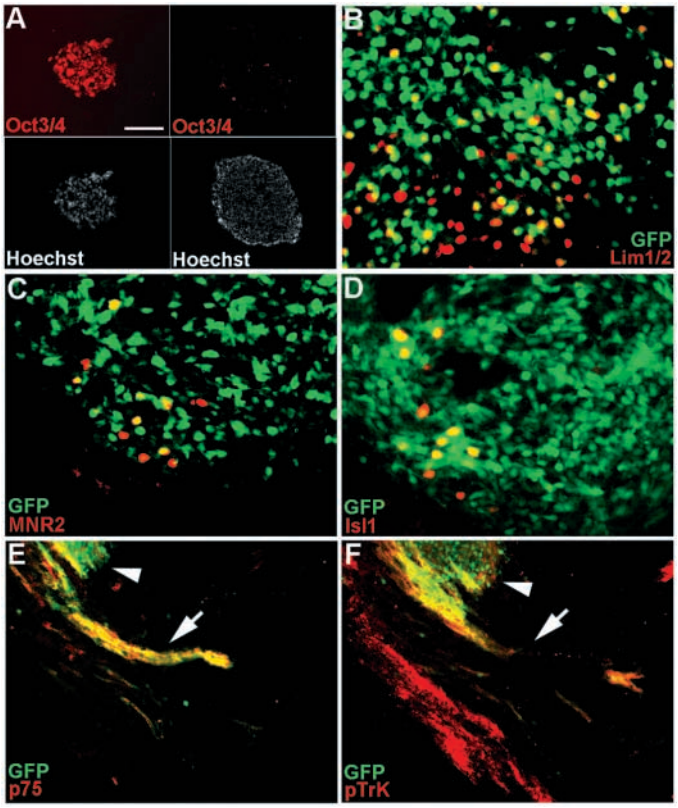

Fig. 5. Developmental potential of cells from RA-untreated EBs. ES cells were allowed to form EBs for 36 hours and then implanted into the chick neural tube as described in Fig. 2. (A) Cryosections (12 μm thick) through EBs double-labelled by Oct3/4 antibodies (upper row) and the nuclear staining Hoechst (lower row). After 36 hours, the majority of the cells within the EB express Oct3/4 (left column). After 8 days (4 without and 4 with RA), very few cells within the EB maintain Oct3/4 expression (right column). (B-F) Transverse sections through E8 chick embryos operated on as described previously, showing endogenous GFP signal and labelled for the indicated markers. (B) ES cell-derived GFP+ cells located at the middle spinal cord express the interneuron marker Lim1/2 (merged yellow). Ventrally located GFP+ cells express the motoneuron markers Mnr2 (C) and Isl1 (D). GFP+ cells in the ventral spinal cord elongate axons towards the periphery; both their somata (arrowheads) and axons (arrows) are labelled by p75 (E) and pan-Trk (F) antibodies. Scale bar: 100 μm in A; 25 μm in B-D; 100 μm in E,F.

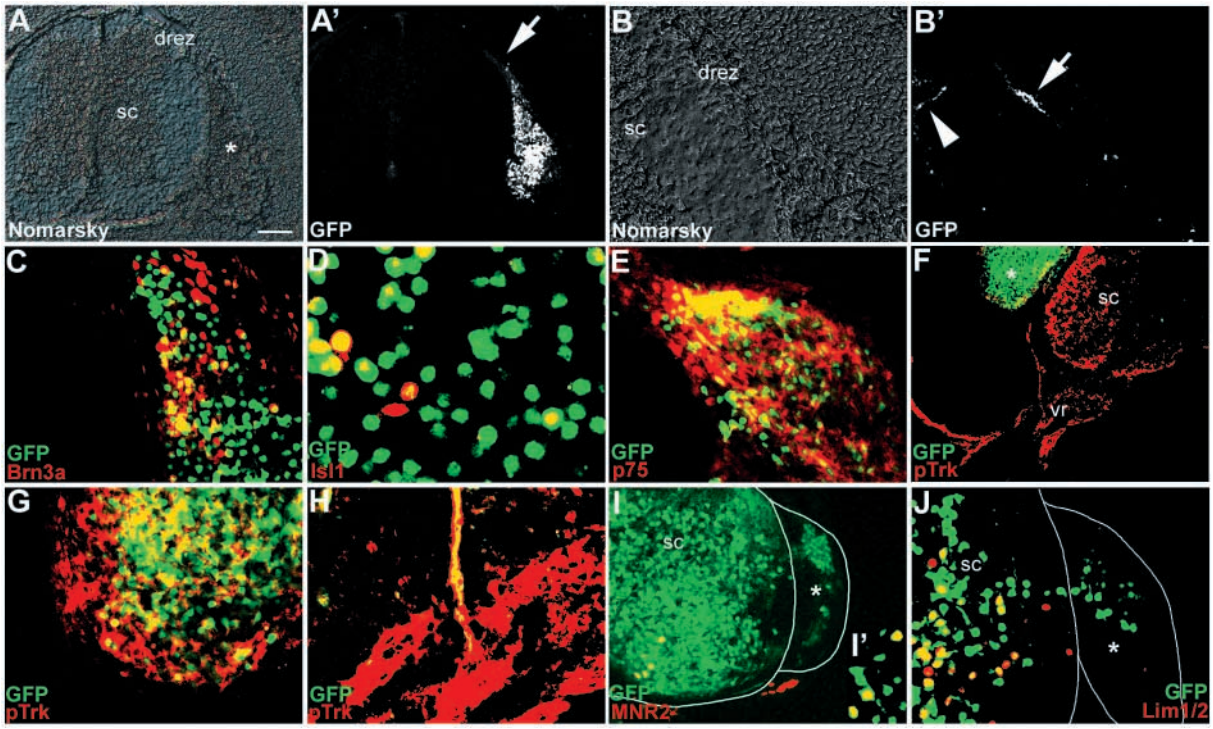

Fig. 6. Cells from RA-untreated EBs differentiate into DRG neurons and extend axons. (A) Host DRG (asterisk, Nomarsky image, A) is shown to be colonized by ES cell-derived GFP+ neurons (A′). Note GFP+ axons (arrow) projecting towards the spinal cord. (B,B′) Donor axons are detected by endogenous expression of GFP (B′) both in the DREZ (arrow) and in the spinal cord (arrowhead). (C-E) Expression of Brn3a (C), Isl1 (D) and p75 (E) by many GFP+ neurons (merged yellow) in host DRG. (F) Expression of pan-Trk in host spinal cord, ventral root, and in the DRG (asterisk), which is colonized by GFP+ cells. Higher magnification images of the same section show pan-Trk expression by GFP+ cells in the DRG (G), and also in their axons joining the ventral root (H). (I,J) GFP+ cells colonize both the spinal cord and the DRG (asterisk). I is stained for Mnr2; J for Lim1/2. GFP+ cells located only in the ventral spinal cord express Mnr2 (also shown in I′ as a higher magnification of the same section), while GFP+ cells located throughout the spinal cord express Lim1/2. Note that no GFP+ cells express these markers in the DRG (asterisk). White lines delineate the spinal cord and DRG limits. drez, dorsal root entry zone; sc, spinal cord; vr, ventral root. Scale bar: 100 μm in A,F,I; 50 μm in B; 25 μm in C,E,G,H,J; 12.5 μm in D.

Isl1 (Fig. 5C,D), and elongated long axons towards the periphery, suggesting that they differentiated into motoneurons. These cells also expressed Trk and p75 neurotrophin receptors in their somata and their axons (Fig. 5E,F). Like the progeny of RA-treated cells in the spinal cord, donor cells located dorsal to the motoneuron domain did not express Mnr2 (data not shown).

Numerous GFP-positive neurons were also found in the DRG (Fig. 6A). Notably, these cells expressed Brn3a (Fig. 6C) and Isl1 (Fig. 6D), and also elongated axons both towards the spinal cord and the periphery (Fig. 6A,B,H; see Table 3 for a quantitative comparison with RA-treated cells). ES cell-derived neurons expressed p75 (Fig. 6E), but, in contrast to RA-treated cells in the DRG, they also expressed high levels of Trk receptors in their somata, as well as in their axons (Fig. 6F-H). Neither ES cells nor RA-treated cells expressed Mnr2 (Fig. 6I) or Lim1/2 (Fig. 6J) when colonizing the DRG.

Discussion

In the RA-treated EBs used for implantation, most cells divided, and virtually all expressed nestin, Sox2, Pax6, Rc2 and Glast, whereas only very few expressed interneuron and motoneuron markers such as Lim1/2, Mnr2 or Isl1. No cells were found to be GFP-positive in intact EBs or during the first hours following EB dissociation (data not shown). These results indicate that RA-treated EBs contained a homogeneous progenitor population with the characteristics of cortical RG cells. Previous studies using similar markers indicated cellular heterogeneity of RA-treated EBs (Renoncourt et al., 1998; Wichterle et al., 2002). In particular, none of our progenitors expressed Pax7 or any neuronal markers to significant levels. We feel that these differences are most likely due to the methods used to culture ES cells, such as the absence of feeder cells at the time of EB formation, as well as the selection of rapidly dividing, presumably uncommitted ES cells for EB formation (Bibel et al., 2004). The apparent homogeneity of our RA-treated EBs prior to implantation is likely to explain why motoneurons are generated following grafting in the absence of pre-treatment with sonic hedgehog (see Wichterle et al., 2002). Indeed, as motoneurons constitute only a fraction of our progenitors, it can be expected that if the ES cells-derived population is heterogeneous at the time of implantation, it is likely to contain fewer cells that are able to enter the motoneuron differentiation pathway. Presumably in our case, sonic hedgehog or other signalling molecules delivered by the host drive the generation of motoneurons at the appropriate location following grafting of sufficient

numbers of competent progenitors able to interpret differentiation signals.

Large numbers of EB-derived cells differentiate into neurons in the host

Six days after implantation, the vast majority of the progenitors were found to differentiate in the spinal cord into Lim1/2+ interneurons and Mnr2+/Isl1+ motoneurons. The latter extended axons towards the periphery and expressed the neurotrophin receptors p75 and Trk, both in their somata and axons. In addition to the identification of donor cells using GFP, we also monitored, by nuclear staining, the fate of other cells that survived in the chick embryo but failed to differentiate into neurons. Both in the spinal cord and the DRG, about 80% of the mouse nuclei were found to belong to GFP+ cells. A previous study has indicated that there is a complete overlap between cells expressing GFP from the *tau* locus and those positive for the antibody TuJ1 that recognizes a neuron-specific form of tubulin (Tucker et al., 2001). In addition, we also found that, in the spinal cord, many GFP-negative mouse cells expressed either Lim1/2 or Mnr2 (data not shown), suggesting that they were on their way to become post-mitotic interneurons or motoneurons, respectively. Although it may seem surprising that our progenitor cells only give rise to few cells not belonging to the neuronal lineage, our experiments did not go beyond E8, which is before the time when large number of astrocytes are generated in the spinal cord. It is possible that some of the GFP-negative cells may later go on to differentiate into astrocytes and other cell types. Our results also indicate that the implanted progenitors are able to respond to patterning signals in the spinal cord, generating spinal cord interneurons and motoneurons in a time- and position-dependent manner. At present, in vivo cell lineage studies have not rigorously proven that Pax6-positive RG cells in the spinal cord generate motoneurons and subtypes of interneurons, but this appears quite likely. Indeed, the pattern of expression of RG markers and of Pax6 in the spinal cord, as well as the decrease of motoneuron numbers in the small eye mutant (Ericson et al., 1997), are compatible with this interpretation.

RA-treated EBs fail to elongate axons in the DRG

After implantation in the neural tube, the progenitors also exhibited a migratory behaviour and colonized the adjacent DRG. Strikingly, while most differentiated into GFP+ neurons in the host DRG, they failed to elongate axons or to express Brn3a and Isl1, which define most neurons in that structure. This is in contrast to donor-derived motoneurons in the spinal cord that were Isl1+ and extended axons to the periphery, a result that does not support an intrinsic limitation of the progenitors to express Isl1 or to elongate axons. Moreover, while the mouse motoneurons expressed both p75 and Trk receptors, donor neurons in the DRG did not express detectable levels of Trk receptors. By contrast, these neurons expressed p75 at relatively high levels. We previously showed that p75 intrinsically activates Rho and inhibits axonal elongation (Yamashita et al., 1999), and it is possible that p75 expression by donor neurons in the DRG in the absence of detectable expression of Trk receptors may account for their failure to elongate axons. During normal development, all sensory neurons that express p75 also express at least one of the three types of Trk receptor (Wright and Snider, 1995). Our results suggest, then, that neurons located in the DRG do not extend axons by default, even in the highly conducive environment provided by developing DRG. Although we do not know why Trk receptors are not expressed when mouse cells are located in the host DRG, we note that they fail to express the POU transcription factor Brn3a. It has been shown that the expression of all Trk receptors is compromised in the trigeminal ganglia of Brn3a$^{-/-}$ mice, while the expression of p75 is not affected (Huang et al., 1999). In addition, Brn3a has recently been shown to directly induce transcription of TrkA in the DRG (Ma et al., 2003). Thus, the absence of Brn3a expression by donor neurons in the DRG may be related to their failure to express Trk receptors. Why the expression of Brn3a is not turned on is unclear, but we note that in the DRG, the progenitors of Brn3a cells are not Pax6-positive. In view of these results with the DRG, we performed similar implantation experiments with RA-untreated ES cells. Virtually all ES cells at the time of implantation expressed Oct3/4, one of the markers correlating with pluripotency of ES cells (Niwa et al., 2000; Boiani et al., 2002). We found that these cells also survived in large numbers after implantation. Like RA-treated cells, they also differentiated into Lim1/2+ interneurons, and into Mnr2+ and Isl1+ motoneurons, extending long axons towards the periphery that were positive both for Trk and p75 neurotrophin receptors. We observed, however, that RA-untreated ES cells generated fewer motoneurons than RA-treated cells, as judged by the expression of Mnr2 and Isl1. Recent studies indicate that somite-derived RA plays an early role in the acquisition of a neural fate by neural tube cells (Diez del Corral et al., 2003), and that the same molecule can further promote these cells into a motoneuron differentiation pathway, even in the absence of sonic hedgehog (Novitch et al., 2003). It is therefore conceivable that pre-treatment with RA may not only induce neural differentiation of ES cells, but also brings these cells closer to a motoneuron fate. ES cells also colonized the host DRG, but, in contrast to RA-treated cells, they acquired expression of the markers Brn3a and Isl1. These cells elongated axons outside the DRG both towards the spinal cord and the periphery, and they expressed Trk receptors in addition to p75. The proportion of ES cells colonizing DRG and differentiating into neurons in that structure was similar to that observed for RA-treated cells. Thus, the failure of RA-treated cells to express DRG markers and to elongate axons did not result from a higher number of cells colonizing the DRG and differentiating into neurons. We also note that neither RA-treated nor RA-untreated cells colonizing the DRG ever expressed spinal cord markers.

Restricted developmental potential of RA-treated EBs

In vivo, RG cells expressing the markers Rc2, BLBP and Glast are widely distributed throughout the embryonic CNS (Kriegstein and Gotz, 2003). However, not all of them are neurogenic. For example, RG cells in the ganglionic eminence do not substantially contribute to the neuronal population found in the striatum, or to the interneuron population in the cerebral cortex, and the neurogenic potential of RG cells seems to correlate with their expression of Pax6 (Heins et al., 2002; Malatesta et al., 2003). Thus, RG cells from the dorsal telencephalon express Pax6, while RG cells located in the

ventral telencephalon are Pax6-negative and are essentially non-neurogenic. Recently, this conclusion was challenged by Anthony et al. (Anthony et al., 2004), who suggested that RG cells may be neuronal progenitors in most of the CNS. However, the BLBP promoter used by Anthony et al. to drive the expression of Cre and to mark RG cell derivatives seemed to be effective as early as E10.5, which may be before the time when BLBP is expressed in RG cells. Fewer neurons are found in the cortex of Pax6 mutant mice, and transfection of Pax6 into astrocytes seems to be sufficient to cause their differentiation into neurons (Heins et al., 2002). The RA-treated ES cells used in our study have the antigenic profile of RG cells found in the developing dorsal, but not in the ventral telencephalon, as at the time of implantation essentially all of them expressed Pax6. While previous work with these cells showed that, in vitro, they differentiated into neurons with the characteristics of pyramidal cells (Bibel et al., 2004), we now find that they can also respond to local cues, interpret them and differentiate according to their position in the embryo. However, their differentiation potential seems to be restricted. In particular, they cannot acquire the typical antigenic and morphological features of peripheral sensory neurons.

Conclusion

As neural progenitors with the characteristics of cortical RG cells can be generated from ES cells in virtually unlimited amounts, they may represent a useful source of defined cells to compensate for the loss of specific cell types in the CNS, including motoneurons. It will be interesting to examine the molecular determinants imposing developmental restrictions on such progenitors, as this knowledge may become important in the context of specific cell-replacement therapies.

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