BMP-2-dependent integration of adult mouse subventricular stem cells into the neural crest of chick and quail embryos

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Summary

Central nervous system (CNS) stem cells isolated from the subventricular zone (SVZ) show a remarkable differentiation potential into neural derivatives. Surprisingly adult SVZ cells can also be induced in vitro to differentiate into neural crest cell fates. This fate switch is dependent on the combination of fibroblast growth factor 2 (FGF2) and bone morphogenetic proteins (BMPs). Here we transplanted adult SVZ stem cells from GFP mice as neurospheres into the trunk neural tube of chick and quail embryos. Only neurospheres pre-exposed to BMP-2 and FGF2 formed close contacts with the dorsal neuroepithelium corresponding to the neural crest area. GFP-positive cells emigrated from the neurosphere and were identified in the roof plate, the dorsal neuroepithelium and among emigrating neural crest cells adjacent to the neural tube. Neurospheres not treated with BMP-2 did not integrate into the neuroepithelium. Our data demonstrate that adult CNS stem cells can be efficiently prepared in vitro for integration into the embryonic neural crest. BMP-2 treatment conveys the necessary morphogenetic capabilities to adult stem cells for future clinical transplantation strategies.

Supplementary material available online at
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Key words: BMP-2, SVZ cells, Neurospheres, Chick embryo, mL1, EMT

Introduction

The discovery of adult stem cells in the central nervous system (CNS) raised many hopes for stem cell therapy (Reynolds and Weiss, 1992). For therapeutic approaches stem cells have to be characterized with respect to proliferation potential, state of determination and morphogenetic behavior. To test the morphogenetic behavior of stem cells, specific experimental in vivo models are required. Our approach was to transplant adult mouse CNS stem cells into the microenvironment of the avian embryo.

Subventricular zone stem cells (SVZ cells) are well characterized in vitro. In general, after isolation from the subventricular zone cells are grown in coated culture dishes. After a phase of proliferation with the mitogen FGF2, stem cells are transferred into non-coated culture dishes, where free-floating neurospheres form. Neurospheres are highly dynamic structures with distinct radial gradients of cell proliferation, cell survival zones, apoptosis and phagocytosis (Bez et al., 2003). On FGF2 withdrawal SVZ stem cells leave the phase of proliferation and differentiate in vitro. SVZ cells generate CNS neurons, astrocytes, and oligodendrocytes (Johe et al., 1996; Reynolds and Weiss, 1996). BMP-2 and FGF2 treatment during proliferation induces neural crest cell fates (measured by expression of smooth muscle α-actin, SMA) upon withdrawal of growth factors (Shah et al., 1996; Mujtaba et al., 1998; Molne et al., 2000; Sailer et al., 2005). Whether their smooth muscle cells are identical to the smooth muscle in vivo or those derived from neural crest stem cells remains a matter of discussion (Shah et al., 1996; Tsai and McKay, 2000).

The role of BMPs was studied in the embryo and in SVZ cells in vitro. In the chick embryo, BMP-2 and BMP-4 mRNA is expressed in the dorsal neural tube between stages 12 and 20 (Sela-Donenfeld and Kalcheim, 1999). In the mouse embryo, BMPs are capable of inducing dorsal precursor fates in the neural tube, including roof plate and neural crest by inducing the expression of the helix-loop-helix protein MASH1 (Shah et al., 1996; Liem et al., 1997). BMPs expressed in the epidermal ectoderm and by dorsal neural cells appear to provide a secondary source of dorsalizing signals that might operate at a time when the epidermal ectoderm is no longer in contact with the neural epithelium (Liem et al., 1995; Sela-Donenfeld and Kalcheim, 1999). Myb is an important mediator in the BMP-4-induced formation of the neural crest (Karafiat et al., 2005).

Basch et al. (Basch et al., 2006) implanted beads coated with Wnt or BMP inhibitors into the presumptive *Pax7* expression domain of the stage 3-4 chick embryo. *Pax7* is an early marker required for neural crest formation in avian embryos. They were able to show a decrease in *Pax7* expression, indicating that Wnt and BMP proteins are required for induction of neural crest cell fates during gastrulation.

Sox9 is essential for BMP signal-mediated induction of *Snail2* and subsequent epithelial-mesenchymal transformation (EMT) in the avian neural crest (Sakai et al., 2006). Co-electroporation of *Sox9* and *SLUG* or a forced expression of

FoxD3 resulted in EMT, observed by ectopic HNK-1 expression in the chick embryo neural tube (Cheung et al., 2005). Both *SLUG* and *Snail* are repressors of E-cadherin and have been shown to cause EMT (reviewed by Hay, 2005).

Most of the known actions of BMPs during neural precursor development can be attributed to the distinct actions of two BMP receptors: BMPR1A and BMPR1B. This was demonstrated in the knockout caBmpr-1A transgenic mouse (Panchision et al., 2001). BMP-2 causes proliferation via BMPR1A in all proliferative zones of the neural ectoderm after gastrulation, regardless of location along the anterior-posterior or dorsal-ventral axis. The expression of BMP receptors BMPR1A and BMPR1B has been shown in adult mouse SVZ cells (Lim et al., 2000).

Brustle et al. (Brustle et al., 1995; Brustle et al., 1997; Brustle et al., 1998) injected human embryonic stem cells (ES cells) into the cerebral ventricles of embryonic rats. The cells incorporated individually at random into all major compartments of the brain (olfactory bulb, cortex, hippocampus, striatum, septum, tectum, thalamus and brain stem). After incorporation, the cells differentiated into neurons, astrocytes and oligodendrocytes, which populated the host fore-, mid- and hindbrain (Brustle et al., 1995; Brustle et al., 1997; Brustle et al., 1998). In these experiments, the possible fates of the injected cells were tested after specific homing in, in various parts of the brain. By contrast, the goal of our experiments was to directly observe the behavior of neurospheres in a defined micro-location.

The objective of the current study was to assess the effect of BMP-2 treatment of neurospheres from adult GFP mice on their behavior in the embryonic neural crest environment. Therefore we transplanted BMP-2-treated neurospheres into the neural tube of the 2-day-old chick embryo and compared them with untreated neurospheres of the same batch. After a further incubation period of 12 and 24 hours and explantation of the embryos from the windowed egg, we studied the integration of the neurospheres into the neuroepithelium by immunofluorescence studies of living cells. For characterization on the cellular level, immunohistochemical staining for anti-GFP, HNK-1 and nestin, and in situ hybridization of the mouse-cell-specific *mL1* sequence was performed. We were able to detect and evaluate the grafted mouse cells in the chick embryo. The experiments with quail embryos were conducted to exclude graft and host cell fusion by combining quail-selective immunostaining and *mL1* in situ hybridization.

Results

Neurospheres

Neurospheres from adult GFP mice were used for transplantation experiments. Under the chicken β-actin promoter and cytomegalovirus enhancer, 90% of the cells in neurospheres expressed the enhanced GFP (EGFP)-actin fusion protein (Okabe et al., 1997). Neurospheres were loose aggregates of apparently undifferentiated cells (Fig. 1A-F). Nestin was expressed in a minimum of 70% of cells in BMP-2 treated and non-treated neurospheres (mean ± s.d. 75±5%, evaluated by microscopical counting of ten sections of neurospheres using Analysis image-processing software), whereas *mL1* DNA sequence was detected in all cells (Fig. 1B-D). Staining of neurospheres with neural-crest-specific HNK-1 showed that SVZ neurosphere cells showed a strong reactivity, which has not been reported before. Surprisingly, there was no difference in HNK-1 staining between BMP-2-treated and untreated neurospheres (Fig. 1E,F). Neurospheres remaining after transplantation were cultured with FGF2 but without BMP-2. There was no difference in the growth properties (DAPI staining, GFP epifluorescence) between BMP-2-treated and untreated groups. Proliferation of cultured neurosphere cells was measured by BrdU uptake and also showed no difference between the two groups of neurospheres. Viability of SVZ cells without BMP-2 treatment 24 hours after transplantation was assessed with the TUNEL reaction (supplementary material Fig. S1). Only two TUNEL-positive SVZ cells were found in a parallel section of the non-integrated neurosphere aggregate shown in Fig. 3B. The TUNEL staining is included as supplementary material Fig. S1.

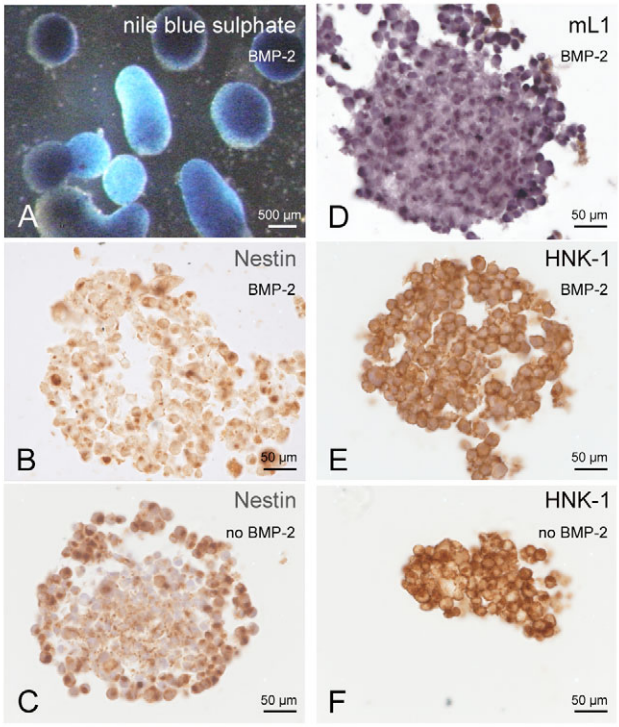

Fig. 1. (A) SVZ neurospheres stained with Nile Blue sulphate immediately before injection. (B) Paraffin section of BMP-2 pre-treated neurosphere: nestin expression was observed in 70% of the cells evaluated by microscopical counting of ten sections of neurospheres using image processing software (Analysis, SIS, Germany). (C) Untreated neurosphere: nestin expression was observed in 70% of the cells. (D) Neurosphere after hybridization with the mL1 probe. Strong HNK-1 reactivity was observed in both BMP-2-treated (E) and untreated (F) neurosphere cells.

Transplantation into the chick embryo

Neurospheres were transplanted into the neural tube. The positioning of the neurosphere transplant (a small entire neurosphere, or parts of larger ones per embryo) was evaluated by epifluorescence in the fenestrated egg using a stereomicroscope. The location was at the transition from the segmented to the non-segmented somite mesoderm. This

corresponded to the 16th and 19th pair of somites of the 2-day-old embryo equal to stage 12 to 13 (Hamburger and Hamilton, 1992; Graham and Meier, 1975).

The first group of embryos was harvested from the egg 12 hours after transplantation. The embryos had reached stage 13. Five embryos with BMP-2-treated transplants were compared with four embryos carrying untreated neurospheres from the same batch of transplants in the same experiment (Table 1). No difference was detected in the fluorescence pattern between the two groups. The transplants were located between the fully formed 17th to 19th pairs of somites (Fig. 2A). In serial paraffin sections, the BMP-2-treated neurospheres were closely attached to the dorsal neuroepithelium and the roof plate, but transplanted cells and host cells were not intermingled (Fig. 2C). Neurosphere cells were HNK-1 positive, whereas in this stage at the trunk level, autochthonous chick embryo neural crest cells did not yet express HNK-1 (Fig. 2B). Untreated neurospheres formed free aggregates in the lumen of the neural tube (not shown).

Seven experiments were performed in which the embryos were harvested 24 hours after transplantation with a total incubation time of 72 hours. Here, 22 embryos with BMP-2-treated neurospheres were compared with 20 embryos containing untreated neurospheres (Table 1). The embryos had reached stage 17 to 19. A clear difference in behavior between BMP-2-treated and untreated neurospheres was visible (supplementary material Fig. S2). In the inverted fluorescence microscope at low magnification, cellular emigration was evident in the BMB-2-treated group (Fig. 2D) whereas untreated neurospheres appeared unchanged even at higher magnification, compared with the 12-hour time point (Fig. 3A). When phase-contrast illumination was added, the borders of the neural tube of the embryos could be clearly distinguished from the background. Single emigrating cells with cytoplasmic fluorescence and no nuclear fluorescence were visible. Cells protruding from the neurospheres into the epithelium were found and single emigrated cells were easily discernible in the neural tube epithelium by their size and shape. Emigration of single cells from the transplant laterally above the projected border of the neural tube was visible (Fig. 2D).

A tangentially cut cryostat section in a stage 19 embryo stained with GFP antibody shows intermingling of mouse and chick cells (Fig. 2E) and single GFP-positive mouse cells in the region of the roof plate (Fig. 2E, upper insert). In situ hybridization of paraffin-embedded transverse sections of embryos showed *mL1*-positive emigrated cells in the dorsal roof plate, dorsal neural tube, in the mesenchyme adjacent to it or at the ventral root of the neural tube (Fig. 2F).

Fig. 3 shows a neurosphere without BMP-2 treatment. No

Table 1. Quantification of emigrated neurosphere cells 24 hours after transplantation

	Treatment	*n*	Emigration/ No emigration	*P**
Exp. 1 (Chick)	BMP-2	10	7 / 3	0.003
	No BMP-2	9	0 / 9	
Exp. 2 (Chick)	BMP-2	8	6 / 2	0.007
	No BMP-2	7	0 / 7	
Exp. 3 (Chick)	BMP-2	4	2 / 2	n.d.
	No BMP-2	4	0 / 4	
Exp. 4 (Quail)	BMP-2	4	4 / 0	n.d.
	No BMP-2	1	0 / 1	
	Total	47	19 / 28	

For statistical evaluation, transplanted neurospheres were rated for 'emigration' or 'no emigration'. Only neurospheres with emigration of single cells beyond the lumen of the neural tube were classified as 'emigration'. The correlation between BMP-2 treatment and emigration was statistically significant ($P<0.01$) in experiments 1 and 2. Owing to the low number of observations, experiments 3 and 4 could not be evaluated statistically, although these results were comparable to experiments 1 and 2. *Fisher's Exact Test, two-tailed; *n*, number of embryos.

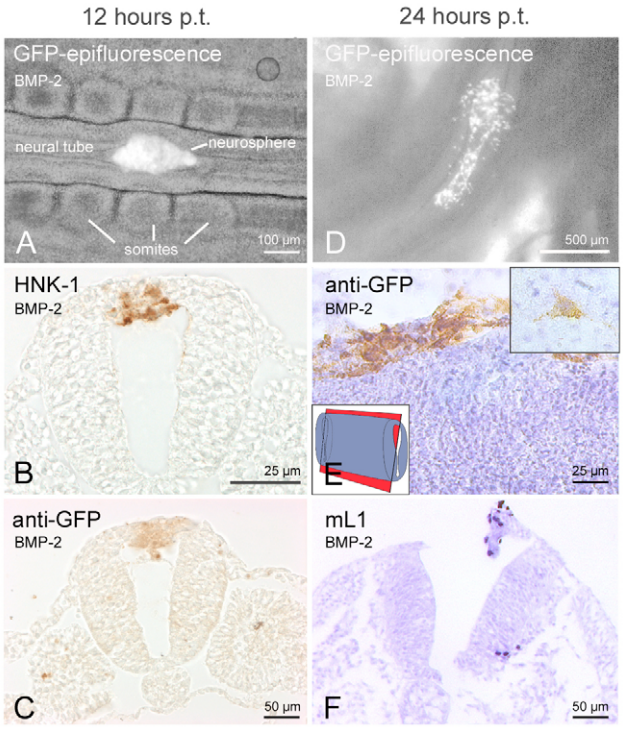

Fig. 2. (A) Neurosphere transplant (BMP-2 pre-treated) in the chick embryo neural tube 12 hours post transplantation (p.t.) (stage 13, HH) with visible fluorescence signal of the neurosphere. Transplant is located between the 18th pair of somites. (B) Enlargement of the same embryo as in A in paraffin section stained with HNK-1 antibody (brown reaction). SVZ neurosphere cells appear HNK-1 positive, whereas autochthonous chick neural crest cells are HNK-1 negative at this stage in the lower trunk region. (C) Same embryo as in A and B in parallel section. Anti-GFP staining shows integration of SVZ cells into the dorsal neuroepithelium and the roof plate, but transplanted mouse cells and host cells do not intermingle yet. (There is some nonspecific staining in somites.) (D) BMP-2 pre-treated neurosphere 24 hours p.t. in the chick embryo neural tube (stage 19, HH, total incubation time 72 hours). The transplant is located at the region of the upper appendage. GFP-epifluorescence shows bilateral emigration of single cells of the transplant laterally above the projected border of the neural tube. (E) The same embryo as in D. The tangentially cut cryostat section (see insert at lower left for orientation) stained with GFP antibody shows intermingling of mouse and chick cells and single GFP-positive mouse cells in the region of the roof plate (upper right insert). (F) Paraffin section of a different embryo of the same experiment. Emigrated SVZ cells visualized by in situ hybridization with the *mL1* probe. Reactive cells can be seen in the dorsal neural tube and at the ventral root.

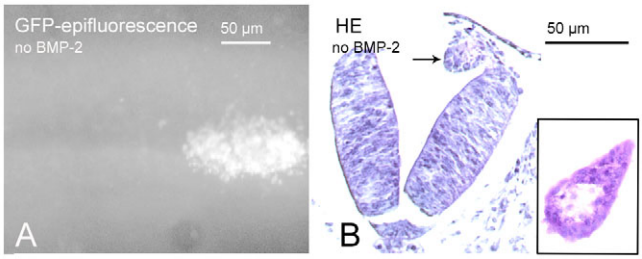

Fig. 3. Untreated neurosphere in the chick embryo neural tube 24 hours after transplantation (stage 19, HH). (A) GFP epifluorescence is restricted to the lumen of the neural tube. (B) The same embryo as in A, in transversally cut paraffin section. Neurosphere cells remaining in the lumen of the chick embryo neural tube can be seen with no or little attachment to the neural tissue (arrow). Part of the neurosphere fell out of the neural tube lumen during the histological processing (insert).

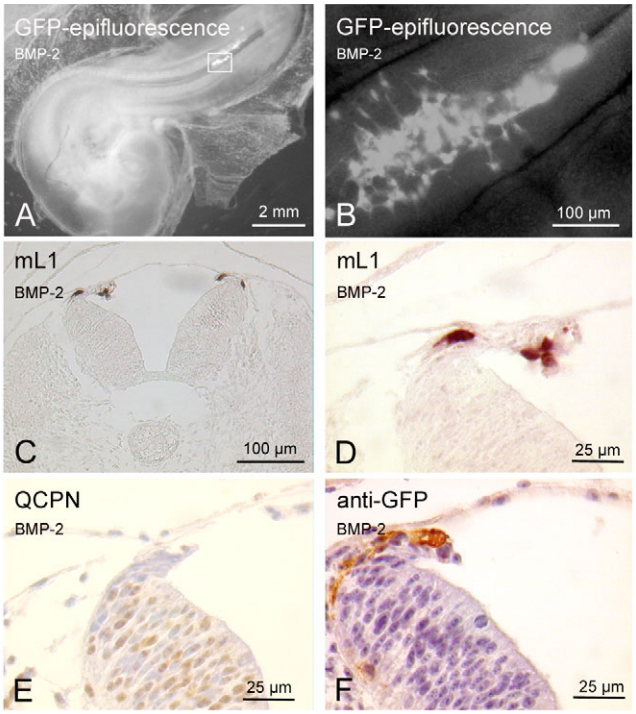

Fig. 4. BMP-2 pre-treated neurosphere 24 hours after transplantation into the quail embryo neural tube (equal to stage 19, HH, total incubation time 72 hours). (A) Fluorescence image. (B) Enlargement of the boxed region in A. Emigrating SVZ neurosphere cells are visible by their typically stretched cellular morphology. Bilaterally emigrating cells are contained within the neuroepithelium (black borders of the neural tube). (C) In situ hybridization of the same embryo. Emigrated mouse SVZ cells can be seen in the roof plate of the neural tube when visualized using the *mL1* probe.
(D) Enlargement of C shows that only cell nuclei are stained.
(E) Parallel section of embryo shown in C. Quail nuclei stained with the QCPN antibody, which recognises a species-specific antigen carried by all quail cells (brown colour), but not by mouse cells. A region of dorsal neural tube remains unstained.
Immunohistochemistry for anti-GFP (F) shows that transplanted cells in the dorsal neural tube are GFP positive (mouse cells). Comparison with parallel sections in C, D, and E reveals that there is no cross-reactivity between *mL1*, QCPN and anti-GFP.

fluorescent emigrating cells were visible, and fluorescent cellular debris was easily discernible by its size and shape (Fig. 3A). Non-treated neurospheres remained in the lumen of the neural tube with no contact with the neuroepithelium (Fig. 3B). In some cases, the neurospheres fell out of the neural tube lumen during the process of histological preparation (Fig. 3B, insert). The lack of contact between untreated neurosphere cells and the neuroepithelium underlines the importance of BMP-2 for cellular integration into the chick embryo neural crest. Untreated neurosphere cells remained viable after transplantation into the neural tube. BMP-2 did not have an effect on cell viability measured by GFP epifluorescence before or after transplantation.

To address the question of final differentiation of SVZ cells after transplantation into the neural tube, we performed immunohistochemistry of the neuron-specific marker TuJ1. Independently of BMP-2 pre-treatment, two cells in a neurosphere aggregate remaining in the lumen of the neural tube (without BMP-2 treatment) and two cells integrating into the roof plate (with BMP-2 treatment) showed positive reactivity for TuJ1, indicating a neuronal differentiation (supplementary material Fig. S3A-D). In the host chick embryo, TuJ1 stained the early differentiating motor neurons and axons emerging from the ventral root.

Transplantation into the quail embryo

Neurospheres were transplanted into quail embryos to check whether the transplanted cell nuclei would fuse with host cell nuclei. For this purpose, we compared 4 embryos with BMP-2-pre-treated neurospheres to one embryo with a non-treated neurosphere. BMP-2 had the same effect on SVZ cells in quail embryos as in the chick (Fig. 4A,B). Neurosphere SVZ cells that had become integrated into the host neural crest, or had emigrated, were clearly marked with the *mL1* probe (Fig. 4C,D). Emigrated cells were found in the same locations as in the chick: In the roof plate and dorsal wall of the neural tube, and in the mesenchyme adjacent to the neural tube at the region of the ventral root.

Quail nuclei were stained with the QCPN antibody, which recognises a species-specific antigen carried by all quail cell nuclei, but not by mouse cell nuclei. Mouse cells that specifically reacted with anti-GFP antibody (Fig. 4F) and were stained with the mouse specific *mL1* DNA probe (Fig. 4D) did not react with the QCPN antibody (Fig. 4E). QCPN-positive cells reacted with neither anti-GFP-antibody, nor the *mL1*-probe, thus suggesting that nuclei from the transplanted mouse cells had not fused with host cells.

Comparison with HNK-1-stained whole mounts

To compare the emigration pattern of BMP-2-treated neurosphere cells transplanted into the neural tube of the chick with normal neural crest cell migration, we stained whole mounts of chick embryos of equivalent stages with the neural-crest-specific marker HNK-1 (supplementary material Fig. S4). In accordance with the findings of Bronner-Fraser (Bronner-Fraser, 1986), in stage 12, the first neural crest cells appeared on the dorsal margin of the neural tube about six somites

cranial to the most recently formed somite (at stage 12 this corresponds to the 16th pair of somites). In caudal trunk regions corresponding to the site of neurosphere transplantation, no HNK-1-positive host cells performed migration (supplementary material Fig. S5A,B).

At this stage, neural crest cell migration is not yet restricted to the caudal part of the somite, behavior that can be observed in more cranial segments. The segmental migration pattern of neural crest cells can be distinguished 24 hours after transplantation, throughout the embryo up to about eight pairs of somites rostral to the most recently formed somite. At the location of the transplanted neurosphere (16th pair of somites), host neural crest cells have reached the para-aortic ganglia (sympathetic ganglia), whereas another population of neural crest cells is still emigrating from the borders of the neural tube.

Fig. 5 shows a stage 18 chick embryo (total incubation time 3 days) stained with HNK-1 antibody as a whole mount (Fig. 5A). An enlarged section in the area of the upper limb bud (site of transplantation of neurospheres in other embryos) shows an emigrating population of neural crest cells dorsal to the roof plate (Fig. 5B). These cells do not follow a specific migration pattern when still in the area of the neural tube. Emigrating neurosphere cells pre-treated with BMP-2 showed the same pattern of emigration as the HNK-1-stained host neural crest cells and can be seen in the same trunk location (Fig. 5C). At the level of transplantation, HNK-1-positive host neural crest cells perform migration at embryonic stage 18 (supplementary material Fig. S5C,D).

Discussion

The experiments show that in vitro BMP-2 pre-treatment of neurospheres prepared from SVZ cells changes the morphogenetic behavior of the chick embryo in the neural crest environment. After transplantation, treated neurospheres established close contacts with the dorsal neuroepithelium. Single cells emigrated from the transplant and invaded the dorsal neuroepithelium and the roof plate, as well as the region of the ventral root. Non-treated neurospheres did not integrate into the host tissue. They formed compact aggregates or nodules in the lumen of the neural tube.

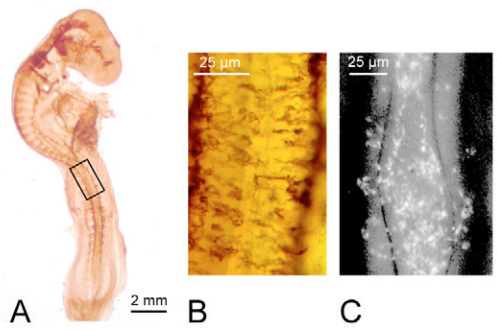

Fig. 5. (A) Stage 18 chick embryo (3 days total incubation time) stained with HNK-1 antibody as whole mount. Emigrating neural crest cells show a typical pattern for this specific stage. (B) An enlargement of the boxed region in A showing the area of the upper limb bud (site of transplantation of neurospheres in other embryos). An emigrating population of neural crest cells can be observed outside the dorsal neural tube. These cells do not follow a segmental migration pattern. (C) Corresponding patterns of bilaterally emigrating neurosphere cells after BMP-2 pre-treatment can be seen in the same trunk location of a transplanted embryo.

From in vitro experiments it is known that BMP-2 and FGF2 treatment changes the state of determination of the neurospheres. Upon withdrawal of both growth factors, the SVZ cells in the neurosphere differentiate. Pre-treatment with BMP-2 results in the expression of smooth muscle actin when the neurospheres expand at the surface of a culture dish. Without such pre-treatment neurons and glial cell fates arise (Sailer et al., 2005). Expression of BMP receptors, BMPR1A and BMPR1B, has been shown in adult mouse SVZ cells (Lim et al., 2000).

In the neural tube environment, BMP-2 pre-treatment results in integration of mouse SVZ cells into the host neural crest. The SVZ cells participate in neural-crest-specific epithelial-mesenchymal transformation (EMT). Our experiments thus show a BMP-2-dependent change in morphogenetic competence that is otherwise not evident in the in vitro experiments. In the chick embryo, determination of autochthonous trunk neural crest cells into pseudounipolar spinal ganglia neurons, sympathicoblasts, Schwann cells and melanocytes occurs at later stages not included in the present study. The question of final differentiation of mouse neurospheres after integration into the neural crest of the chick embryo requires long-term observations, which are under way in our lab.

EMT is a crucial step in neural crest development. EMT is defined as loss of epithelial traits of the dorsal neuroepithelium and acquisition of mesenchymal characteristics, such as expression of vimentin, myosin and invasive motility (Hay, 2005). These traits represent the activation of genes that create the mechanisms, which cells use to migrate through a 3D extracellular matrix. According to Hay, outgrowth of fibroblasts in the culture dish does not necessarily correspond to EMT but might represent an artefact created by culturing on a 2D substrate. The cells flatten out and lose their elongated migratory morphology. Most, if not all, of the myosin and actin molecules needed for cell locomotion, polymerize within the so-called stress fibers. It is important not to define the mesenchymal or migrating neural crest cell on the basis of stress fibers (Hay, 2005). In the neural tube transplantation model, BMP-2 pre-treatment induces EMT-behavior in SVZ cells.

In the embryo, BMPs and members of the Wnt family of secreted glycoproteins were described to induce EMT (Aybar and Mayor, 2002). EMT of neural crest cells in vivo is characterized by HNK-1 expression and downregulation of E-cadherin (Cheung et al., 2005). In our experiment, HNK-1 was expressed in all neurospheres independently of BMP-2 treatment before and after transplantation. This suggests that either neurosphere cells had already adopted a neural crest fate without the presence of BMP-2, or neurosphere cells remained in an undifferentiated stem cell state in which cells often express HNK-1 [e.g. non-seminomatous germ cell tumours of the testis (Navarro et al., 1997) and avian primordial germ cells (Anstrom and Tucker, 1996)]. With respect to the epithelial character, we observed no difference between BMP-2-treated and untreated neurospheres before transplantation. In the neural tube environment untreated neurospheres formed

compact epithelial aggregates indicating high E-cadherin expression.

The transcription factors *PAX3*, *PAX7*, *Sox9*, *FoxD3*, *SLUG*, *Snail*, *Snail2*, *MSX* and *DSL1*, as well as other factors such as MMP-2, Myb and E-cadherin have been implicated in neural crest determination and differentiation (Basch et al., 2006; Sakai et al., 2006; Karafiat et al., 2005; Cheung et al., 2005; Hay, 2005; Duong and Erickson, 2004; Sela-Donenfeld and Kalcheim, 1999; Liem et al., 1995). The expression of these factors in SVZ neurospheres before and after integration into the embryonic neural crest has not yet been studied. Inhibition of BMP-2 activity by noggin misexpression within the developing chick gut inhibits normal migration of enteric neural crest cells (Goldstein et al., 2005). In the same way, neurospheres that had not been treated with BMP-2 were unable to perform emigration.

When compared with the physiological neural crest cell migration pattern in chick embryos we observed that 24 hours after transplantation, emigrating SVZ cells from neurospheres show a strikingly similar pattern to host neural crest cells. At this stage, a population of host neural crest cells is still emigrating from the ventral borders along the entire neural tube and can be identified by using the HNK-1 antibody. These cells can be detected in the dorsal neuroepithelium and show no specific segmental emigration pattern, which occurs once the neural crest cells have entered the somites. This segmental migration pattern is caused by ephrin-related receptor tyrosine kinases and their ligands. In this respect, EphB3 localises to the rostral half-sclerotome, including the neural crest, and the ligand EphB1 has a complementary pattern of expression in the caudal half-sclerotome (Krull et al., 1997).

Emigrating neurosphere cells seem to join host neural crest cells on their migration. Our observations demonstrate that BMP-2 has an effect not only on cell fate, which has been shown in vitro before (Sailer et al., 2005), but also on the morphogenetic capabilities of SVZ stem cells to perform EMT in vivo.

To address the question of possible cell fusion between graft and host cells, we stained 5 μm parallel sections with the quail-specific antibody QCPN and anti-GFP, and performed in-situ-hybridization with the mouse specific mL1 probe. Double immunohistochemical staining is usually conducted to exclude cell fusion for in vitro cell studies. In our case, the conclusive double staining corresponding to in vitro protocols would be the combination of mL1 with QCPN. However, both methods result in extensive nucleic staining, and the in situ hybridization requires the heating of the sections to 85°C, which makes further immunohistochemical procedures extremely difficult by altering cell surface epitopes. With the available methods double stained nuclei would not be discernible. Considering the limitations of the methods and our interpretation of the results, we concluded that cell fusion between graft and host cells could be excluded to a high level of probability.

The ability of BMP-2 to alter the morphogenetic behavior of CNS stem cells is only visible in the embryonic in vivo environment. Therapeutic strategies have to consider not only the correct state of determination, but also the morphogenetic capabilities of transplanted stem cells. Further studies are under way in our lab to clarify the interaction between BMP-2 and EMT in vitro and in vivo and to determine any clinical relevance.

Materials and Methods

Tissue preparation and neurosphere culture

The GFP-actin transgenic mice used were described by Heimrich et al. (Heimrich et al., 2002). The mice express the *EGFP* (S65T+F64L) transgene in the entire body. Adult male mice (younger than 2 months) were killed with CO_2 and the brain was harvested in sterile, ice-cold HBSS (Gibco, Life Technologies, Grand Island, NY). With a tissue chopper (McIlwain), 400 μm sections were cut coronally. Using a dissection microscope (Zeiss, Oberkochem, FRG), the lateral subventricular zone was cut out. The stripes of tissue were minced using bent scissors. The tissue from four animals was collected and washed once with sterile ice-cold HBSS. The HBSS was removed and replaced with HBSS containing trypsin (1.5 mg/ml; 10,000 BAEE U/mg, Sigma) and DNase (0.35 mg/ml; 3000 U/mg, Amersham Biosciences, Freiburg, Germany). The tissue was incubated for 10 minutes at room temperature (Johe et al., 1996). After centrifugation for 3 minutes at 1000 rpm, the pieces of tissue were mechanically dissociated by trituration and centrifuged for 5 minutes at 600 rpm. The supernatant was removed, the pellet re-suspended and rinsed twice in Dulbecco's minimum essential medium (DMEM)/F12-based medium (GIBCO) supplemented with trypsin inhibitor (0.7 mg/ml, Gibco). This was followed by two washes with ice-cold HBSS; the supernatant was discarded, and the cells were resuspended in (DMEM)/F12-based medium, supplemented with N2 (Gibco), plus 20 ng/ml of recombinant human FGF2 (R&D Systems, UK).

Cells (0.5×10^6 per ml) were plated in six-well plates (Becton Dickinson, Heidelberg, Germany) precoated with 15 μg/ml poly-L-ornithine and 2 μg/ml fibronectin (Sigma). FGF2 was added daily, and media was replaced every 2 days. Cells were passaged at 50% confluency every 3-5 days by mechanical dissociation. After the third passage, cells were plated as described in non-coated six-well plates to promote the growth of neurospheres (Reynolds and Weiss, 1992; Uchinda et al., 2000).

The cells and neurospheres were maintained at 37°C with 95% air and 5% CO_2. Neurospheres were prevented from attachment by gently shaking the six-well plates every other day. Viable neurospheres used for transplantation had been passaged three times. Before transplantation, half of the neurospheres were pre-exposed with BMP-2 (R&D Systems) for 24 hours at 20 ng/ml, in addition to FGF2, whereas other neurospheres were maintained in the presence of FGF2 alone. All animal work was conducted in accordance with local ethical guidelines and approved animal care.

Breeding and embryo harvesting

Fertilized eggs of white leghorn chickens (*Gallus Gallus domesticus*) and Japanese Quails (*Coturnix coturnix japonica*) were obtained from a hatchery and incubated at 37.5°C in a temperature-controlled brooder (Ehret, Esslingen, FRG). After transplantation, eggs were sealed with Super33+ electrical adhesive tape (3M, St Paul, MN).

Transplantation

Immediately before transplantation, neurospheres were removed from the six-well plates and kept on ice. Neurospheres were drawn up into a pointed micropipette (one at a time) made from borosilicate glass (World Precision Instruments, Sarasota, FL). One neurosphere, or a part thereof, was transplanted into the neural tube, located between the 16th and 19th pair of somites of the embryo, which is about equal to the site of the anterior appendages at chick embryos stage 12-13 HH (Patten, 1948). After transplantation, the neurosphere remained at the site of injection and embryos were incubated for 12 or 24 hours. Embryos were excised with intact circulation from the fenestrated egg and transferred into a petriperm dish in a temperature-controlled chamber under the inverted fluorescence microscope (Olympus). Outgrowth of cells from the neurospheres was evaluated by GFP epifluorescence with a 20× objective. The vitality of the embryo was monitored by counting the heart rate. During observation, the heart rate did not fall below 120 beats/minute. The overall survival rate was 45/47.

Immunohistochemistry

After fixation in 4% buffered paraformaldehyde for 2-4 hours, depending on the size, embryos were embedded in paraplast and incubated with the first antibody (HNK-1 (Serotec) 1:1000; Nestin (gift from R. D. McKay, NIH, Bethesda, MD) 1:1000. For GFP immunohistochemistry (1:500, Molecular Probes, Invitrogen, Karlsruhe, Germany), cryostat sections were used. Tissues were incubated with the first antibody overnight at 4°C, followed by goat anti-mouse IgG (Linaris, Wertheim, FRG) at room temperature for 1 hour. Binding was visualized by biotin-streptavidin-HRP complex for 1 hour and HRP substrate (Vector Laboratories) for 1 hour.

Quail cells were detected using the QCPN-antibody (the monoclonal antibody QCPN was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA) (Selleck and Bronner-Fraser, 1995). Staining procedures were carried out as described (Männer, 1999). Chick embryo whole mounts for visualisation of normal crest cell migration were stained with HNK-1 antibody following the protocol of Lumsden (Lumsden and Keynes, 1989). Neuronal fate of the transplanted cells was tested using the neuron-specific TuJ1 antibody (Convance, Richmond, CA, USA).

Tissues were incubated with the first antibody (1:500) overnight at 4°C, followed by goat anti-mouse IgG (Linaris, Wertheim, Germany) at room temperature for 1 hour. Binding was visualized by incubation with biotin-streptavidin-HRP complex for 1 hour and HRP substrate (Vector Laboratories) for 1 hour.

To detect apoptotic cells in untreated neurospheres, we conducted the TUNEL assay on parallel sections using a commercially available kit (TUNEL apoptosis detection kit, Chemicon, Temecula, CA) using the procedure described by the manufacturer.

In situ hybridization

In situ hybridization for *mL1* sequences was carried out with a digoxigenin (DIG)-labeled, 416 bp antisense DNA probe corresponding to the consensus sequence of mouse *mL1* (Hatano et al., 1998). Probe labeling was performed by PCR with the PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany). The in situ hybridization protocol is a modification of a method described for the *hALU* sequence (Just et al., 2003). Sections were incubated with TE buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8) containing 2 μg/ml proteinase K for 10 minutes at room temperature. To reduce nonspecific background, slides were acetylated with TEA buffer (0.1 M triethanolamine, pH 8.0) containing 0.25% (v/v) acetic anhydride (Sigma) twice for 5 minutes. After pre-hybridization with hybridization buffer [50% formamide (Sigma), 10% dextran sulfate, 5 mM EDTA, 20 mM Tris-HCl pH 8, 10 mM DTT, 1× Denhardt's solution, 0.05% tRNA, 300 mM NaCl] for 3 hours at 85°C, slides were incubated with fresh hybridisation buffer containing the denatured DIG-labeled DNA-L1 probe (100 ng/ml) for 2 hours at 85°C. Slides were immediately transferred to ice for 10 minutes and then incubated overnight at 37°C. After hybridization, slides were briefly rinsed in 2× SSC at room temperature and three times in 0.1× SSC for 15 minutes at 37°C. Detection of DIG-labeled RNA probe was performed according to the protocol of the DIG nucleic acid detection kit (Roche). The tissues were blocked for 30 minutes with blocking buffer (1% blocking reagent (Roche) in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5), and then incubated with alkaline phosphatase-conjugated antibody solution [anti-DIG antibody (1:2500 Roche) in blocking buffer containing 0.1% Triton® X-100] for 1 hour. Following four washes with maleic acid buffer for 15 minutes, slides were equilibrated for 5 minutes in Tris buffer pH 9.5 (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$). The color development was carried out with substrate solution [nitro blue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) (Roche) in Tris buffer pH 9.5]. After three washes with PBS, slides were rinsed in distilled water, dried and coverslips were sealed with Kaiser's gelatine (Merck, Darmstadt, Germany).

Statistical analysis

Fisher's Exact Test was applied for statistical analysis. Detected emigration of neurosphere cells into the surrounding tissue was categorized into 'emigration' or 'no emigration'. The criterion was the observation of fluorescent neurosphere cells extending beyond the neural tube lumen (supplementary material Fig. S2). Statistical evaluation of all experiments is shown in Table 1. We found that in two of the four experiments performed, emigration was significantly ($P<0.01$) observed in embryos that had been transplanted with BMP-2-treated neurospheres.

In experiment 1, BMP-2-treated and untreated neurospheres were transplanted in experiments on different days, but the same batch of neurospheres was used. In experiments 2, 3 and 4, BMP-2-treated and untreated neurospheres from the same batch were transplanted on the same day.

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