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The instability of the neural crest phenotypes: Schwann cells can differentiate into myofibroblasts

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ABSTRACT In the vertebrate embryo, the neural crest cells (NCCs) that migrate out from the neural primordium yield multiple phenotypes, including melanocytes, peripheral neurones and glia and, in the head, cartilage, bone, connective cells and myofibroblasts / vascular smooth muscle cells (SMCs). The differentiation of pluripotent NCCs is mainly directed by local growth factors. Even at postmigratory stages, NC-derived cells exhibit some fate plasticity. Thus, we reported earlier that pigment cells and Schwann cells are able *in vitro* to interconvert in the presence of endothelin 3 (ET3). Here, we further investigated the capacity of Schwann cells to reprogram their phenotype. We show that purified quail Schwann cells in dissociated cultures produce α smooth muscle actin (αSMA)-expressing myofibroblasts through the generation of a pluripotent progeny. This transdifferentiation took place independently of ET3, but was promoted by transforming growth factor β1 (TGFβ1). Moreover, when implanted into chick embryos, the Schwann cells were found to contribute with host cephalic NCCs to perivascular SMCs. These data provided the first evidence for the acquisition of an NC-derived mesenchymal fate by Schwann cells and further demonstrate that the differentiation state of NC-derived cells is unstable and capable of reprogramming. The high plasticity of Schwann cells evidenced here also suggests that, as in the CNS, glial cells of the PNS may function as NC stem cells in particular circumstances such as repair.

KEY WORDS: *transdifferentiation, quail embryo, in vitro culture, transplantation, pluripotency*

Introduction

During vertebrate embryogenesis, a number of cell types originates from a unique transitory structure, the NC. NCCs arise from the dorsal aspect of the neural primordium, undergo extensive migration as they proliferate and then differentiate in various organs and tissues (for references see, (Le Douarin, 1982, Le Douarin and Kalcheim, 1999). These cells give rise to skin melanocytes, some endocrine cells and the neural cells of the peripheral nervous system (PNS), including sensory, autonomic and enteric neurones and associated glial cells, as well as Schwann cells lining the peripheral nerves. In addition, the cranial and cervical NCCs yield several mesenchymal cell types (the so-called «mesectoderm»), which form most of the head dermis, cartilages and bones in the skull and facial skeleton (Couly *et al.*, 1993, Le Lievre and Le Douarin, 1975). The cephalic NCCs also provide forebrain meninges, connective tissue cells in glands and muscles, the cardiac outflow tract as well as the myofibroblasts/SMCs that are associated with the vessels derived from the aortic arches and those irrigating the face and forebrain (Etchevers *et al.*, 1999, Etchevers *et al.*, 2001, Kirby and Waldo, 1995, Le Lievre and Le Douarin, 1975).

How these diverse NC-derived phenotypes are specified early and maintained later in the differentiated tissues, begins to be understood. The final phenotype adopted by NCCs depends mainly on local cues encountered by the cells during and at the end of their journey (reviewed in (Le Douarin and Dupin, 2003). Single cell studies, essentially carried out *in vitro*, have revealed that migratory NCCs include highly pluripotent as well as oligopotent (i.e., with a more limited developmental potential) progenitors and

Abbreviations used in this paper: CNS, central nervous system; d, culture day; E, embryonic day; ET1, endothelin 1; ET3, endothelin 3; ETRB, endothelin receptor B; NC, neural crest; NCCs, neural crest cells; NF, neurofilament; PNS, peripheral nervous system; αSMA, α smooth muscle actin; SMCs, smooth muscle cells; TGFβ, transforming growth factor beta; TH, tyrosine hydroxylase.

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fate-restricted precursors (Baroffio *et al.*, 1988, Baroffio *et al.*, 1991, Dupin *et al.*, 1990, Sieber-Blum, 1991, Trentin *et al.*, 2004). Some of the pluripotent and bipotent progenitors in the early NC are stem cells endowed with self-renewal capacity (Stemple and Anderson, 1992, Trentin *et al.*, 2004).

At postmigratory stages, resident pluripotent stem cells were identified in the PNS (in nerves, dorsal root ganglia and gut) (Bixby *et al.*, 2002, Iwashita *et al.*, 2003, Kruger *et al.*, 2002, Morrison *et al.*, 1999). These *in vitro* findings confirm and further document a notion previously revealed by *in vivo* transplantations of quail PNS ganglia from quail into the NCC migration pathway of chick embryos at embryonic day 2 (E2) (for a review (Le Douarin and Kalcheim, 1999). Therefore, a certain degree of differentiation plasticity and regeneration capacities characterize the NC-derived cells long after PNS organogenesis is completed.

Besides the possible recruitment and differentiation of resting stem cells, another way to replenish organs is by converting already differentiated cells of one type to a different functional phenotype. Such «transdifferentiation» has been described long ago in vertebrates. One of the classical examples is the regeneration of the amphibian urodele tail, which proceeds by cell de-differentiation into blastemal cells capable to re-differentiate along various lineages (for references, (Brockes and Kumar, 2002). In higher vertebrates, conversion between cell types that develop in close relationship can occur, such as in the chick eye, between cells of the retinal pigmented epithelium, the neural retina and the lens (Eguchi and Okada, 1973, Okada, 1991). Otherwise, although rare in mammals, phenotype switch can be induced *in vitro* by growth factors, chemicals or by manipulating gene expression, as recently documented for myotubes (McGann *et al.*, 2001, Odelberg *et al.*, 2000).

In the quail NC, we have described recently two examples of phenotype conversion. Pigment cells and glial Schwann cells *in vitro* can convert into each others when exposed to the mitogenic signal of ET3, generating an intermediate cell type which express markers specific for both lineages (Dupin *et al.*, 2000, Dupin *et al.*, 2003). The Schwann cell and pigment cell progeny could also recapitulate the bipotentiality of their immediate common ancestor, the glial-melanocytic (GM) progenitor, previously identified in clonal cultures of migrating NCCs (Lahav *et al.*, 1998). These data thus have suggested that differentiation of NCCs can be reversed, resulting eventually in phenotype conversion between NC lineages. To further investigate this possibility, we have examined here whether the isolated Schwann cells are able to switch to alternative NC phenotypes, in addition to the melanocytic phenotype. We have particularly focused our attention on the so-called «myofibroblasts», characterized by the expression of αSMA and which correspond to muscle cells of NC origin associated *in vivo* with the blood vessels in the cephalic region. The present study shows that myofibroblasts arise from the Schwann cells *in vitro* independently of ET3. In addition, we find that this phenotype switch can also occur *in vivo*, since Schwann cells grafted into chick embryos yield vascular SMCs. This work suggests that NC-derived cell types may retain (or recover) the potential to differentiate along other NC lineages and it further supports the instability of NC phenotypes.

Results

Isolated Schwann cells generate myofibroblasts in vitro

The Schwann cells from E10.5 quails were immunopurified by FACS using the monoclonal antibody (Mab) against the Schwann cell myelin protein (SMP) (Dulac *et al.*, 1988, Dulac *et al.*, 1992) and grown in mass cultures in control or in ET3-supplemented medium as described (Dupin *et al.*, 2003). The cultures were then analysed, at different time points, using several NC lineage-specific markers: SMP and P0 protein for Schwann cells, αSMA for myofibroblastic cells and neurofilament (NF) proteins and tyrosine hydroxylase (TH), to identify neurones and adrenergic cells, respectively.

In control cultures (without ET3 supplementation) after overnight

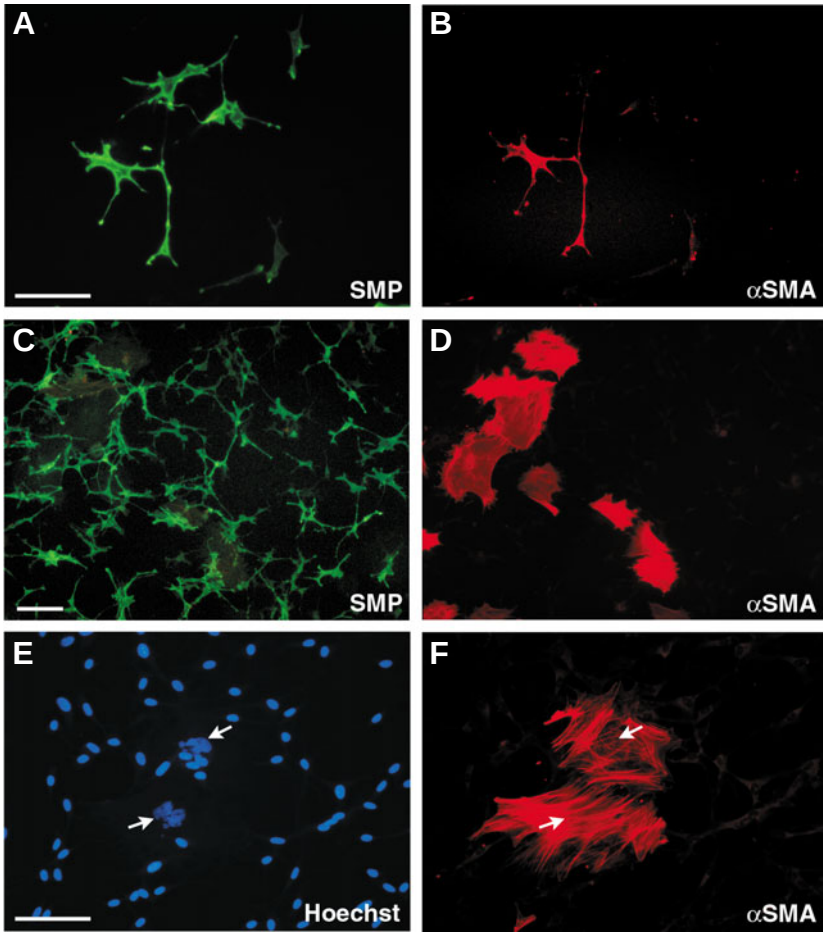

Fig.1. Expression of αSMA and SMP in Schwann cell control cultures. *Expression of SMP and αSMA, in the same optic field, at d3* **(A,B)** *and d7* **(C-F)**. *Notice in (F) that at d7, the αSMA protein is assembled in filaments. Nuclei visualization by Hoechst staining* **(E)** *and expression of αSMA* **(F)** *at d7. The αSMA-expressing cells exhibit a much larger nucleus than the other cultured cells (arrows). All detected by immunofluorescence. Bar, 100 μm in A,B and E,F; 300 μm in C,D.*

incubation (culture day 1-d1), virtually all cells expressed the glial proteins SMP and P0 as well as p75 NGF low-affinity receptor (not shown); neither αSMA nor neuronal markers were detected, thus supporting the initial glial identity of the plated cells. Later on, the glial markers were progressively down regulated by a subpopulation of the cultured cells. At d3 and concomitantly with the extinction of glial markers, αSMA-expressing cells emerged. At this time point, the majority of the αSMA$^+$ cells still expressed SMP and maintained the same elongated morphology as SMP$^+$ (αSMA$^-$) glial cells (Fig. 1A, B). At d7, most of the αSMA$^+$ cells had lost the expression of SMP and exhibited a large and flattened morphology as well as a high level of αSMA immunoreactivity in cytoplasmic filaments (Fig. 1C, D). These cells also possessed a much larger nucleus than SMP$^+$ cells (Fig. 1E, F). Relying on all these features, we designated those αSMA$^+$ cells "myofibroblasts".

In ET3-treated cultures, the transition from SMP$^+$ Schwann cells to myofibroblasts occurred similarly to non-treated cultures. However, the presence of ET3 increased the total number of cells, but maintained the proportion of myofibroblasts (about 0.1% of total cells). In both culture conditions at d7, neither neurones nor melanoblasts/melanocytes were detected. However, melanocytic cells differentiated in cultures maintained in the presence of ET3 for a longer culture time (see below), as described previously (Dupin *et al.*, 2003).

These data therefore show that Schwann cells developing in culture de-differentiate and are able to generate myofibroblasts in their progeny, independently of the presence of ET3. The finding of an intermediate phenotypic state, in which cells co-express glial (SMP) and myofibroblastic (αSMA) proteins, suggests that the transition from glial to myofibroblastic cells is progressive.

Marker expression in the transition of Schwann cells to myofibroblasts

In order to further characterise the Schwann cell-myofibroblast phenotype transition, we have verified the expression, by differentiating αSMA$^+$ cells, of additional markers, expressed by both NCCs and differentiated Schwann cells, that is, the transcription factor gene *Sox10* (Cheng *et al.,* 2000) and the HNK1 surface carbohydrate epitope (Abo and Balch, 1981, Tucker *et al.*, 1984). Moreover, we examined *Slug* expression in the cultures. *In vivo*, this transcription factor is required by early avian NCCs for emigration from the dorsal neural tube (Nieto *et al.*, 1994) and is re-expressed later by differentiated vascular SMCs (Marin and Nieto, 2004).

These experiments were performed in control and ET3-supplemented media, which gave similar results. In d1-cultures, virtually all cells expressed, together with SMP, HNK1, *Sox10* and the gene encoding the endothelin receptor B (ETRB) (not shown). However, at d3, some cultured cells, including part of the αSMA$^+$ cells, had lost these markers. The αSMA$^+$ cells were thus heterogeneous; some were negative for HNK1 immunoreactivity (Fig. 2A, B) and others no longer expressed *Sox10* transcripts (Fig. 2C, D). At d7, the αSMA$^+$ myofibroblasts had completely extinguished both HNK1 (Fig. 2E, F) and *Sox10* (Fig. 2G, H). However, these two markers were occasionally present in a minor fraction of the αSMA$^+$ cells that were still small and elongated as d3-cultured cells are (Fig. 2G, H-arrowheads).

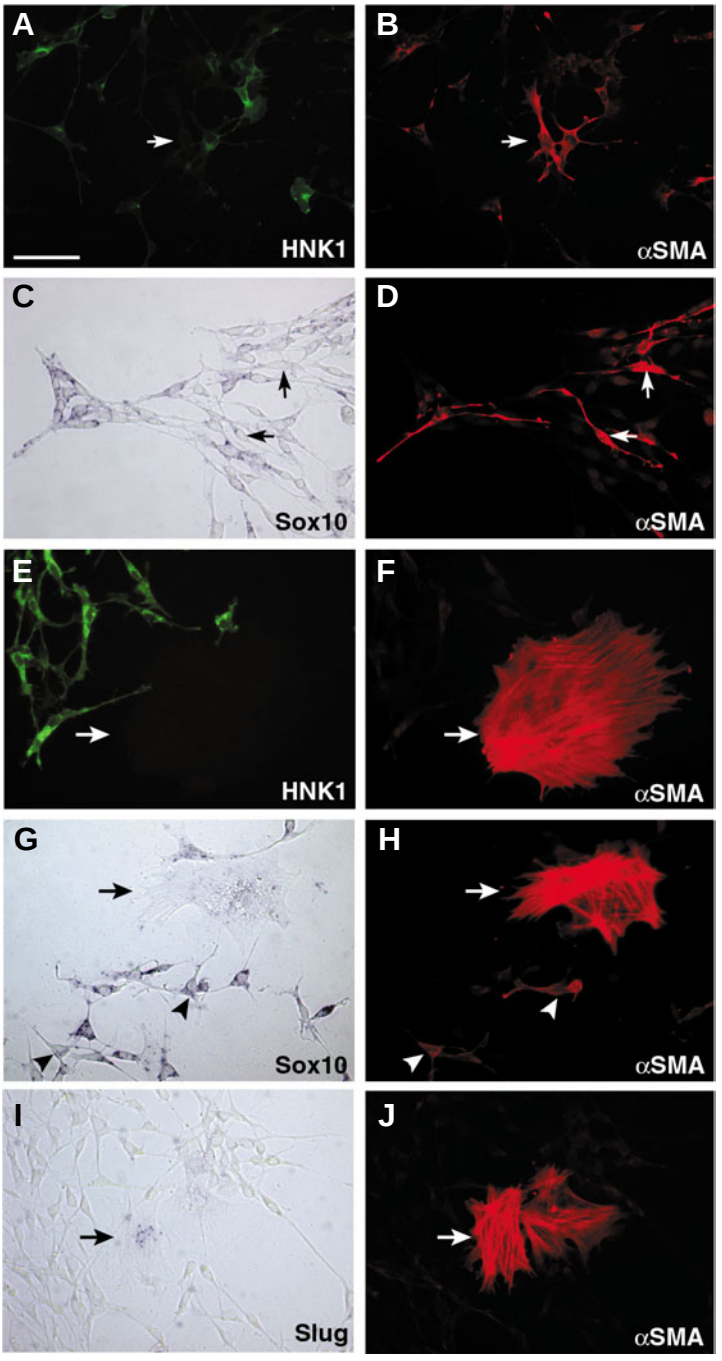

Fig. 2. Expression of HNK1, *Sox10* and *Slug* by αSMA-positive cells in d3 and d7-Schwann cell cultures. *In d3-Schwann cell cultures (A-D): Immunofluorescence with HNK1* **(A)** *and αSMA* **(B)**, *which is the same field as (A) and detection of both* Sox10 *mRNA* **(C)** *(bright field) and αSMA* **(D)**, *which is the same field as (C). A subset of αSMA$^+$ cells has lost the HNK1 epitope (arrow in A,B) and does not express* Sox10 *(arrows in C,D). In d7-Schwann cell cultures (E-J): double labelling with αSMA and HNK1* **(E,F)**, *αSMA and* Sox10 **(G,H)** *and αSMA and* Slug **(I,J)**. *(E,F), (G,H) and (I,J), same optic fields. The αSMA$^+$ large fibroblastic cells are HNK1-negative (arrow in E,F) and do not express* Sox10 *(arrow in G,H).* Sox10 *transcripts are detected in small elongated αSMA$^+$ cells (arrowheads in G,H). Some αSMA$^+$ fibroblastic cells express* Slug *(arrow in I,J). Bar, 100 μm.*

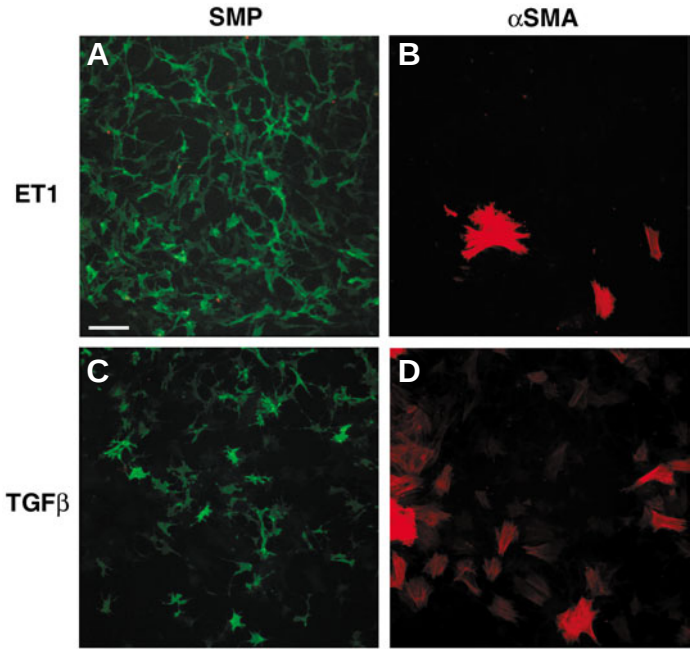

Fig. 3. Expression of SMP and αSMA in d7 Schwann cell cultures treated with ET1 (A,B) and TGFβ1 (C,D). *SMP and αSMA expression was detected by immunofluorescence. (A, B) and (C,D) same optic field. Bar, 300 μm.*

Slug transcripts were never detected in d3-cultures. By contrast, the expression of this gene was identified at d7 and exclusively in some myofibroblasts (Fig.2I, J).

Therefore, the Schwann cells which start synthesising αSMA loose progressively the expression of *Sox10* and HNK1 from d3 onward, although this is not the case for the expression of SMP. Later in the transition from glial to myofibroblastic phenotypes, all the Schwann cell markers tested here, including SMP, are extinguished in differentiated myofibroblasts. In addition, some of these cells activate the *Slug* transcription factor gene.

Effect of growth factors on myofibroblast production by cultured Schwann cells

The present results have shown that differentiation of myofibroblasts from Schwann cells did not require the presence of ET3 in the culture medium. We therefore tested whether the addition of other factors, implicated in the development of perivascular smooth muscle cells *in vivo* and *in vitro*, could stimulate the generation of myofibroblasts by cultured Schwann cells. Cultures prepared as above were grown in control medium further supplemented with either TGFβ1 or ET1. They were analysed at d3 and d7 for the presence of glial and myofibroblastic cells and compared with control and ET3-treated cultures at the same time points.

Treatment with ET1 increased the number of SMP^+ glial cells and both types of αSMA^+ cells (co-expressing or not SMP), as compared to control medium (Fig. 3A, B). Such overall growth stimulation by ET1 in Schwann cell cultures was thus similar to the effect of ET3 (Dupin *et al.,* 2003).

Exposure to TGFβ1 did not stimulate Schwann cell growth as compared to control cultures. However, this factor strongly enhanced the morphological and phenotypic differentiation of myofibroblastic cells. As soon as d3, most of the αSMA^+ cells had differentiated to large and flattened SMP^- cells, while smaller cells expressing both αSMA and SMP were rarely found. Moreover, when these cultures were exposed to TGFβ1 until d7, the percentage of myofibroblasts increased significantly as compared to control and ET3-treated cultures (4% versus 0.1%) (Fig. 3C, D).

Therefore, among the growth factors tested here, TGFβ1 has the unique ability to promote differentiation of the myofibroblasts arising from cultured Schwann cells. In the present conditions (i.e., in serum and embryo extract-containing medium), addition of ET1 does not alter the emergence of the myofibroblast phenotype.

Myofibroblast differentiation potential by Schwann cell clonal progeny

To analyse the developmental potential of the Schwann cells that convert into myofibroblasts, we performed clonal cultures of SMP^+ nerve cells and compared the cellular composition of the clones obtained in the absence and presence of ET3. This factor, although not necessary for the generation of myofibroblasts in mass cultures, was previously shown to promote clone survival and melanocyte outcome in long-term single Schwann cell cultures (Dupin *et al.*, 2003).

Clones were thus maintained until d17 and analyzed for cell survival, growth and presence of glial, melanocytic and myofibroblastic cells. Table 1 shows the detailed distribution of the different types of clones. In control medium, the majority of the clones generated by Schwann cells contained both glial cells and myofibroblasts (GF clones). The others were composed only of glial cells (G clones), myofibroblasts (F clones) or lineage marker-negative cells (unidentified, U clones). In presence of ET3, besides clones of the G, F, GF and U types already identified in control cultures, two additional clone types were recorded which contained melanocytic cells, i.e., GM clones (with both glial and melanocytic cells) and GMF clones (with glial, melanocytic and myofibroblastic cells). Although it allowed generation of melanocyte-containing progeny, the treatment with ET3 did not significantly modify the overall proportion of the clones containing myofibroblasts (37.8% versus 60.5% in controls; $P = 0.5$).

TABLE 1

PHENOTYPIC ANALYSIS OF CLONAL CULTURES DERIVED FROM SCHWANN CELLS

Clone types	control	ET3	P
G	13 (30.2 %)	17 (32.1 %)	*ns*
F	3 (7 %)	11 (20.7 %)	*ns*
GF	23 (53.5 %)	3 (5.7 %)	*< 0.05*
GM	0	11 (20.7 %)	-
GMF	0	6 (11.4 %)	-
U	4 (9.3 %)	5 (9.4 %)	*ns*

Purified SMP^+ Schwann cells plated individually and grown in control and ET3-supplemented medium, gave rise to similar numbers of d1-colonies (66% and 69%, respectively from 210 plated cells in each medium). At d17, the clones were analyzed for cell phenotypes using lineage-specific markers. The absence or presence of SMP^+ glial cells (G), $MelEM^+$ melanocytic cells (M) and αSMA^+ myofibroblastic cells (F), led to classify the clones in 6 different categories. Those referred as to unidentified clones (U) contained exclusively marker-negative (SMP^-, $MelEM^-$, αSMA^-) cells. The number (and percent of total clones) is given for each clone type. P values from statistical analysis of control and ET3-treated cultures; ns, not significant.

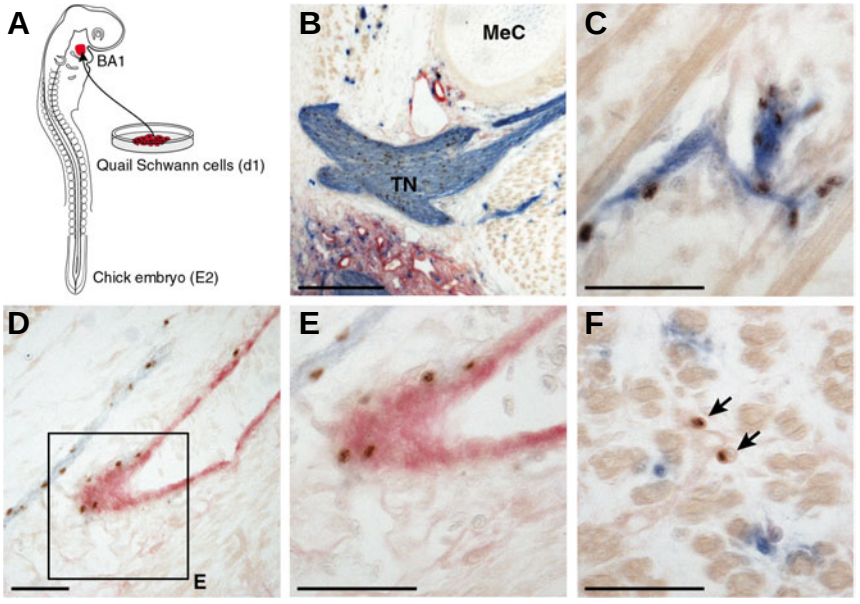

Fig. 4. Fate of Schwann cells when back-transplanted into chick embryos. *Unilateral implantation of d1-cultured Schwann cells in the first branchial arch of a chick embryo* **(A)**. *At E9, histological sections of the host mandible were stained with QCPN Mab (in brown), HNK1 Mab (in blue) and αSMA Mab (in red). The QCPN-positive cells had integrated HNK1⁺ trigeminal nerve, TN in* **(B)** *and nerve fibers innervating the depressor facial muscle* **(C)**. *They also homed to the vascular smooth muscle layer where they express αSMA* **(D)** *which is amplified in* **(E)**. *Other engrafted cells which expressed neither SMP nor αSMA, were dispersed in facial muscles; see arrows in* **(F)**. *Bar, 300 μm in (B) and 50 μm in (C-F). Abbreviations: BA1, first branchial arch; MeC, Meckel's cartilage.*

Taken together, these data further argue for the ability of embryonic Schwann cells *in vitro* to reprogram their phenotype along NC-derived melanocyte and myofibroblast lineages. In most cases, myofibroblastic cells arise from bipotent (GF) and pluripotent (GMF) progeny, thus indicating that conversion of Schwann cells to myofibroblasts likely involves reversal to a pluripotent state.

Transplanted Schwann cells generate perivascular SMCs in vivo

In order to investigate whether Schwann cells isolated from embryonic sciatic nerves could give rise to myofibroblasts *in vivo* as they did in culture, we examined if these cells would integrate into the smooth muscle layer of developing blood vessels in younger embryos. For this purpose, Schwann cells issued from d1-control cultures were implanted unilaterally into the first branchial arch (BA1) of E2-chick embryos, that is, at the time when myofibroblast precursors, derived from the host cephalic NC, colonise the head vascular network (Etchevers *et al.*, 2001) (Fig. 4A). The host embryos were re-incubated and analysed at E9. Cells derived from the implant were then identified in histological sections by using the quail-specific QCPN Mab. Simultaneously, the vascular SMC phenotype was revealed using αSMA and the PNS component cells using HNK1. The engrafted cells were found in the BA1-derived host mandibular process 7 days post-grafting (Fig. 4 B-F). Some of these cells lined the host cranial peripheral nerves, including the trigeminal nerve and the fibres that innervate the facial muscles (Fig. 4B, C). In these locations, the graft-derived cells exhibited HNK1 immunoreactivity, indicative of their glial identity. Another large subset of the implanted cells had homed to host blood vessels and integrated the periendothelial smooth muscle layer, as shown by co-expression of QCPN and αSMA (Fig. 4D, E). The vascular smooth muscle layer was also colonised by HNK1⁺ αSMA⁻ quail cells, which very likely represent graft-derived Schwann cells that accompany the host terminal autonomic innervation of the vascular mural cells (not shown). In addition, other quail cells, located in the vicinity of blood vessels or within facial muscles, did not express either HNK1 or αSMA (Fig. 4F).

Therefore, Schwann cells isolated from embryonic nerves and back-transplanted into a younger host embryo are able to re-populate host peripheral nerves, maintaining their initial Schwann cell phenotype. More importantly, as in culture, they also generate myofibroblasts expressing the αSMA protein *in vivo*. Furthermore, these graft-derived myofibroblasts are capable to contribute to normal development of the muscular wall of the developing host blood vessels.

Discussion

We have previously shown that Schwann cells isolated from embryonic nerves are capable of changing their lineage program and generate pigment cells in long-term cultures supplemented with ET3 (Dupin *et al.,* 2003). Here we describe that, in addition to melanocytes, these cells give rise to another NC-derived phenotype, the myofibroblasts, which are obtained earlier in culture than melanocytes and independently of the cytokine ET3. Moreover, by using transplantation experiments, we show that the Schwann cells also generate myofibroblasts *in vivo*, which are recruited by blood vessel primordia to participate in the formation of the perivascular smooth muscle.

Characterisation of the transition from Schwann cells to myofibroblasts/SMCs

In this work, we have focused on the phenotype plasticity of Schwann cells that were isolated from quail sciatic nerves (as described in (Dupin *et al.*, 2003), using the surface glycoprotein SMP (Dulac *et al.*, 1988, Dulac *et al.*, 1992, Dupin *et al.*, 1990). The expression of SMP is detected from E5 in quail Schwann cells, when those have already contacted the nerve fiber (Dulac *et al.*, 1988). The SMP⁺ sorted cells from E10.5 quail nerves also express the myelin protein P0 characteristic of Schwann cells (Bhattacharyya *et al.*, 1991). In addition, they express the transcription factor *Sox10* (Cheng *et al.,* 2000) and the HNK1 epitope (Tucker *et al.*, 1984). These markers are present *in vivo* in early NCCs and later in PNS cells, including Schwann cells, but are lost by mesenchymal and melanocytic NC derivatives. Here we identified the myofibroblasts by morphological criteria and expression of αSMA and *Slug*. The myofibroblasts, together with the pericytes and SMCs, are the components of the periendothelial muscular wall in maturing blood vessels. They have been shown to derive from NCCs in the cephalic region (Etchevers *et al.*, 2001, Le Douarin

and Kalcheim, 1999) and, in the other body regions, from the mesoderm or from the epicardium in the case of coronary vessels (for references, (Owens, 1995). The αSMA cytoskeletal protein is the early and widest used marker for myofibroblasts/SMCs differentiating either *in vivo* or *in vitro*. In particular, this marker led to the characterisation of SMCs derived from avian and mammalian NCCs (Ito and Sieber-Blum, 1991, Jain *et al.*, 1998, Shah *et al.*, 1996, Trentin *et al.*, 2004, Youn *et al.*, 2003). The transcription factor Slug, which is required for the emigration of the early NCCs (Nieto *et al.*, 1994), has been shown recently to label avian vascular SMCs/pericytes (Marin and Nieto, 2004).

We report here that, when purified Schwann cells develop and divide in culture, they generate a subpopulation of myofibroblasts. By analysing the developmental potentials of individual Schwann cells *in vitro*, we show that, first, the ability to switch phenotype is independent of Schwann cell density. Moreover, we provide evidence that the differentiation of myofibroblasts from Schwann cells involves common progenitor cells for glial and myofibroblastic cell types. The multilineage myofibroblast-containing clones derived from Schwann cells also contained glia or both glia and melanocytes and therefore were of the GF or GMF types, which are similar to those previously obtained from trunk and cephalic quail NCC cultures (Trentin *et al.*, 2004). This demonstrates that Schwann cells, or their immediate progeny, behave as pluripotent NC-like cells.

The analysis of the dynamics of SMP, HNK1, *Sox10* and αSMA expression in the cultures, allows us to distinguish two phases during the generation of myofibroblasts from Schwann cells. The first, i.e. the induction phase, takes place between d0 and d3 and corresponds to the emergence of αSMA expression. This phase is characterised by the production of multiple cell states, intermediate between the Schwann cell and myofibroblast phenotypes, where $SMP^+\alpha SMA^+$ cells also express or not HNK1 and/or *Sox10* and maintain the morphological features of the parental glial cells. In the subsequent differentiation phase, between d3 and d7, the αSMA^+ cells loose their glial (SMP, P0, HNK1 and *Sox10*) features and acquire SMC characteristics, as defined by fibroblastic morphology, filamentous αSMA and, in some cases, expression of *Slug*.

The above data thus reveal that the Schwann cells (or their immediate progeny) are able to switch their initial phenotype and generate myofibroblasts, another NC-derived cell type. However, they do not allow to identify with certainty the myofibroblast progenitor in Schwann cell descendance. Either it is a SMP^+ Schwann cell that can directly convert in a myofibroblast, or it is a multipotent precursor that needs first to be produced by Schwann cell de-differentiation. Our results argue for the first possibility, since no dedifferentiation state (particularly no downregulation of SMP) was found to precede the onset of αSMA expression. Among the growing number of studies showing transdifferentiation in vertebrates, only a few were able to unambiguously characterise a de-differentiation process, by using clonal analysis and available precursor-specific markers (Brockes and Kumar, 2002, Odelberg *et al.*, 2000, Tsonis, 2004).

We have shown that Schwann cells can give rise to myofibroblasts/SMCs and melanocytes (Dupin *et al.*, 2003 and the present work), revealing their high level of cell plasticity. In the central nervous system (CNS) of adult vertebrates, some glial cells were also identified as pluripotent precursor cells. Astrocytes and radial glial cells behave as neural stem cells, generating neurons and glial cells *in vivo* and *in vitro* (for references, Doetsch, 2004). Moreover, the Müller glial cells of the retina can be recruited after injury to regenerate neurones (Fischer and Reh, 2001). Therefore, it is conceivable that, as in CNS, glial cells of the PNS may function as NC stem cells in particular circumstances such as repair.

Influence of growth factors on phenotypic conversion of Schwann cells

Attempting to determine whether the switch from Schwann cells to myofibroblasts is influenced by growth factors, we first found that the progression of Schwann cells along the myofibroblast differentiation pathway is independent of ET3 and ET1. The addition of these peptides did not significantly influence the generation of myofibroblastic cells in d7-Schwann cell cultures, although it stimulated the overall cell division. The ET1 peptide interacts with both ETRB and endothelin receptor A (ETRA) reviewed by (Masaki, 2004) and the disruption of ET1/ETRA pathway in mice induces malformations of the cephalic and cardiac NC derivatives, including the blood vessels (Clouthier *et al.*, 1998, Kempf *et al.*, 1998, Kurihara *et al.*, 1994). Our results suggest that ET1, similarly to ET3, acts through ETRB, to enhance cell proliferation. Accordingly, in longer-term clonal cultures (d17), the presence of ET3 did not change the frequency of Schwann cells yielding myofibroblasts, as shown previously for the myofibroblastic precursors of the quail NC (Trentin *et al.*, 2004). Instead, ET3 triggers the differentiation of melanocytes in Schwann cell and NCC cultures, as previously reported (Dupin *et al.*, 2003). The present data therefore reveal that myofibroblasts and melanocytes can arise from the same GMF, initially derived from Schwann cells, in the presence of ET3.

We further show that, in contrast to ET3 and ET1, TGFβ1 is able to promote myofibroblast differentiation from isolated Schwann cells in culture. *In vivo*, this factor induces mesenchymal cell differentiation into pericytes/SMCs (Rossant and Howard, 2002) by increasing the activity of specific SMC genes, such as *αSMA* (reviewed in (Kumar and Owens, 2003). In addition, TGFβ1 favours the differentiation *in vitro* of SMCs from rat embryonic NC stem cells (Mann *et al.*, 2004, Shah *et al.*, 1996) and from a pluripotent mouse NC cell line (Chen and Lechleider, 2004, Jain *et al.*, 1998). Here we found that TGFβ1 triggers premature differentiation of myofibroblasts in d3 cultures and increases the myofibroblast number at d7. Whether TGFβ1 favours the production of myofibroblasts in the induction and/or the differentiation phases, is presently unclear. One possibility is that TGFβ1 influences the decision to activate αSMA synthesis in the induction phase, resembling its instructive effect on the differentiation of myofibroblasts from NC stem cells (Shah *et al.*, 1996).

It is noteworthy that all culture media tested here, including control medium, have permitted the emergence of myofibroblasts in the Schwann cell cultures. This might be due to the activation by the serum of potent transcriptional inducers of smooth muscle protein genes (including αSMA), like serum response factors (SRFs) (Kumar and Owens, 2003, Oettgen, 2001). Indeed, Schwann cells cultured in “low-serum medium” (containing 1% foetal calf serum only) maintained their phenotype, did not divide and produced no αSMA-expressing cells (data not shown).

Schwann cells can give rise to SMCs in vivo

We also show that the same phenotypic switch, from Schwann cells to myofibroblasts, occurs *in vivo*. Moreover, when the SMP^+

Schwann cell population is grafted into early chick embryos, the engrafted cells generate vascular SMCs that integrate the wall of the host cranial vessels. Another subset of graft-derived cells, which do not express glial or SMC markers and are located in striated muscles, may correspond to fibroblasts of the connective tissues, an alternative derivative of cephalic NCCs (see Le Douarin and Kalcheim, 1999). The signals, which promote homing of engrafted Schwann cells to the blood vessel wall and their conversion *in vivo* into vascular myofibroblasts, are still to be identified. It is however to be noted that, in the grafting experiments, none of the Schwann cell descendants contributed to the pigment cell lineage, although this occurs *in vitro*. One possibility to account for such failure to produce melanocytes *in vivo* may be that the post-grafting period (7days) is insufficient to trigger pigment cell differentiation from the Schwann cells, as in culture, the latter requires at least 13 days of exposure to ET3 (Dupin *et al.*, 2003). Alternatively, the branchial arch environment could restrict the melanogenic potential of the grafted cells, as observed for NCCs (Ciment and Weston, 1985, Jacobs-Cohen *et al.*, 2002).

An interesting characteristic of the vascular and the peripheral nervous systems is that blood vessels and nerve fibres develop in close relationship and share a number of signalling molecules, which influence the differentiation of their respective cell types and direct their growth (Bates *et al.*, 2003, Carmeliet, 2003, Mukouyama *et al.*, 2002). Taken all these informations together, we suggest that, in case of injury wherein both blood vessels and nearby nerves have been damaged, some Schwann cells, which become separated from the axon and escape nerve cues (Fawcett and Keynes, 1990), might be induced, under the influence of signals issued from the neighbouring blood vessels, to change their phenotype and eventually take part in the regenerating perivascular muscular wall.

In conclusion, the present *in vitro* and *in vivo* experiments have revealed the ability of Schwann cells, isolated from the sciatic nerve, to reprogram toward mesenchymal fates that consist of myofibroblasts/SMCs of the vascular system and connective tissue cells. The myofibroblasts, which in normal development derive from cephalic NCCs, arise here in cultures of Schwann cells of trunk origin. In fact, trunk NCCs possess a myofibroblastic cell potential, which is not expressed *in vivo* but which can be revealed by *in vitro* culture (Hagedorn *et al.*, 1999, Morrison *et al.*, 1999, Shah *et al.*, 1996, Trentin *et al.*, 2004) or by heterotopic grafting (Nakamura and Ayer-le Lievre, 1982). Therefore, it can be concluded that the embryonic Schwann cells are able, not only to recover a developmental pathway specific of their NC precursors (melanocytic differentiation), but also to recapitulate some mesenchymal potential, expressed *in vivo* by the cephalic NCCs.

Materials and Methods

Cultures of purified quail Schwann cells

Cultures were prepared essentially as described previously (Dupin *et al.*, 2003). Briefly, sciatic nerves of E10.5 quails were dissociated to single cells and labelled with the Mab against SMP, a glycoprotein expressed by Schwann cells in myelinated and unmyelinated avian peripheral nerves (Dulac *et al.*, 1988, Dulac *et al.*, 1992). The SMP^+ cells were purified by FACS and then transferred to mass cultures ($5x10^3$ cells per 20 µl culture medium) or plated as single cells by micro-manipulation (Dupin *et al.*, 2003). Cells were grown on rat tail collagen substratum (Biomedical Technologies Inc.) in "cloning medium" containing 10% fetal calf serum (Dutscher), 2% chick embryo extract and various hormones and growth factors as described (Dupin *et al.*, 2000, Dupin and Le Douarin, 2003, Dupin *et al.*, 2003, Lahav *et al.*, 1998, Trentin *et al.*, 2004). After overnight incubation, medium was added to the cultures and changed every 3 days thereafter.

When indicated, the culture medium was supplemented with 100 mM human ET3 or ET1 (Sigma), or with human TGFβ1 (1 ng/ml; R&D Systems). All cultures were incubated at 37ºC in a humidified 5% CO2, 95% air atmosphere and maintained until d3 and d7, for mass cultures and until d17, for clonal cultures.

Immunocytochemical analysis of the cultures

The cell phenotypes in the cultures were identified using lineage-specific markers after fixation with 4% paraformaldehyde. Glial cells were immunostained with Mabs against SMP (Dulac *et al.*, 1988, Dulac and Le Douarin, 1991, Dulac *et al.*, 1992) or protein P0 (Bhattacharyya *et al.*, 1991) (1E8 clone supplied by Developmental Studies Hybridoma Bank (DSHB); University of Iowa, Iowa City, IA) using "Tyramide Signal Amplification" (TSA fluorescence systems, PerkinElmer) (Dupin *et al.*, 2003). Neurones and adrenergic cells were labelled using rabbit serum against 200 KD NF protein (Sigma) and anti-quail TH Mab (Fauquet and Ziller, 1989), respectively. Pigment cells were recognised by melanin granules and unpigmented melanocytes by staining with the melanoblast/melanocyte early marker (MelEM) Mab (Nataf *et al.*, 1993). Myofibroblastic cells were visualized by immunoreactivity for αSMA (1:400, 1A4 clone; Sigma). When indicated, immunostaining with rabbit polyclonal anti-p75 low-affinity NGF receptor (1:200, Chemicon) and HNK1 Mab (Abo and Balch, 1981) (undiluted hybridoma supernatant) was also carried out.

Secondary antibodies were purchased from Southern Biotechnologies Associates. Detailed staining procedures are described elsewhere (Dupin *et al.*, 2000, Dupin *et al.*, 2003, Lahav *et al.*, 1998, Trentin *et al.*, 2004). Fluorescence was observed under an Olympus (Melville, NY) X70 inverted microscope.

The average percentage of myofibroblastic cells in the cultures was determined as the ratio between the number of αSMA-labelled cells and the total number of cell nuclei counted after staining with Hoeschst bisbenzimide (Trentin *et al.*, 2004) in at least four microscopic fields.

Differences in colony numbers between ET3-treated and control clonal cultures were analysed by X^2 test (GraphPad InStat, San Diego) and considered to be statistically significant when $P<0.05$.

In situ *hybridisation*

Antisense RNA probes to chick transcription factor genes *Sox10* and *Slug* (gifts from P. Scotting and M.A. Nieto, respectively) were generated according to Cheng *et al.* (2000) and Nieto' *et al.* (1994). *In situ* hybridisation on cell cultures was performed as described previously (Lahav *et al.*, 1998). Briefly, the cultures were fixed with 4% formaldehyde, dehydrated and then kept at -20ºC. After rehydration and pre-hybridisation steps, they were hybridised with digoxigenin-labelled probes. Hybrids were revealed using alkaline phosphatase (AP)-anti-digoxigenin Mab (Roche) and colour staining was developed in nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) substrate. The stained cultures were then processed for immunofluescence labelling with HNK1 and αSMA as described above.

Transplantation of quail Schwann cells into chick embryos

Quail SMP^+ Schwann cells, immunopurified as described above, were used for constructing quail-chick chimeras. After overnight incubation in control medium, about 10^3 cells were scraped off the culture dish and implanted into the mesenchyme of BA1 in E2-chick embryos (stage 15-16 of Hamburger and Hamilton, 1951), using a fine-tipped glass micropipette. The hosts were further incubated and those surviving at E9 (n=2/6) were sacrificed and treated by standard histological procedures. After fixation, dehydration and paraffin embedding, sections of the host embryos were treated for antigen retrieval by microwave exposure (twice

5 min, in 0.01 M citrate buffer at 700 W). Then, the sections were incubated overnight at 4°C with the following primary antibodies: anti-quail QCPN Mab for detection of the grafted quail cells (mouse IgG1 hybridoma from DSHB), together with anti-αSMA (mouse IgG2a; Sigma) and HNK1 (mouse IgM), to identify perivascular myofibroblasts and PNS cells, respectively. The secondary antibodies (all from Southern Biotechnology Ass.) were peroxidase-conjugated anti-mouse IgG1, AP-conjugated anti-mouse IgM and AP-conjugated anti-mouse IgG2a. The immunostainings were colour-developed using diaminobenzidine (for QCPN) and different chromogenic AP substrates (Vector) (for αSMA and HNK1).

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