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Impairment of PMP22 transgenic Schwann cells differentiation in culture: implications for Charcot-Marie-Tooth type 1A disease

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Charcot-Marie-Tooth type 1A (CMT1A) is a hereditary demyelinating neuropathy due to an increased genetic dosage of the peripheral myelin protein 22 (PMP22). The mechanisms leading from PMP22 overexpression to impairment of myelination are still unclear. We evaluated expression and processing of PMP22, viability, proliferation, migration, motility and shaping properties, and ability of forming myelin of PMP22 transgenic ($PMP22_{tg}$) Schwann cells in culture. In basal conditions, $PMP22_{tg}$ Schwann cells, although expressing higher PMP22 levels than control ones, show normal motility, migration and shaping properties. Addition of forskolin to the media induces an additional stimulation of PMP22 expression and results in an impairment of cells migration and motility, and a reduction of cell area and perimeter. Similarly, co-culturing transgenic Schwann cells with neurons causes an altered cells differentiation and an impairment of myelin formation. In conclusion, exposure of $PMP22_{tg}$ Schwann to the axon or to axonal-mimicking stimuli significantly affects the transition of transgenic Schwann cells to the myelinating phenotype.

Keywords: CMT1A; Hereditary neuropathy; PMP22; Schwann cell; Axon; Motility; Migration; Proliferation; Myelin; Dysmyelination

Introduction

Charcot-Marie-Tooth type 1A (CMT1A) disease is a human hereditary demyelinating neuropathy due to a duplication on chromosome 17p11.2, containing the *peripheral myelin protein 22* (*PMP22*) gene (Lupski et al., 1991; Matsunami et al., 1992). Rarely, CMT1A may be caused by mutations in *PMP22* (Roa et al., 1993; Valentijn et al., 1992). *PMP22* mRNA and protein are overexpressed in CMT1A nerve biopsies, suggesting that an increased dosage of *PMP22* is the most likely disease mechanism in duplicated patients (Roa and Lupski, 1993; Vallat et al., 1996; Yoshikawa et al., 1994).

PMP22 is a tetraspan glycoprotein mainly expressed in myelinating Schwann cells and in the compact peripheral myelin (Snipes et al., 1992). It also regulates the proliferation, differentiation, shaping and apoptosis of Schwann cells (Brancolini et al., 1999; Fabbretti et al., 1995; Suter and Snipes, 1995). Axonal contact tightly controls PMP22 expression and intracellular trafficking (Pareek et al., 1997). Even if functions and processing of PMP22 have been extensively studied, the molecular mechanisms underlying CMT1A are unclear (Hanemann and Muller, 1998).

To study the effects of altered PMP22 expression on the myelination process, animal and in vitro models of the disease have been developed (Huxley et al., 1996; Nobbio et al., 2001; Sereda et al., 1996). Dysmyelination is a prominent feature of experimental CMT1A, at least in the early stages of the disease (Nobbio et al., 2001; Robataglia-Schlupp et al., 2002; Sereda et al., 1996). At birth, Schwann cells, after reaching a 1:1 association with axons, begin myelination by elongating and spiraling their cytoplasm and plasma membrane around the axon (Scherer, 1997). Therefore, the movements and shaping capacities of Schwann cells are critical for myelin formation (Mezei, 1993). Cultured Schwann cells, induced to express extremely high PMP22 levels, show reduced proliferation and abnormalities in cell shape and spreading (Brancolini et al., 1999; Zoidl et al., 1995). In homozygous CMT1A rats, Schwann cells, although switching on the myelination process at the molecular level, fail to proceed from the promyelinating to the myelinating phenotype (Niemann et al., 2000). We established primary Schwann cell cultures from this animal model of CMT1A, to evaluate viability, proliferation, motility, migration and shaping properties of PMP22 transgenic ($PMP22_{tg}$) Schwann cells. Moreover, as previous in vitro and in vivo experiments did not consider the role of neuron/axon complex in the pathogenesis of CMT1A, we also studied the phenotype of

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$PMP22_{tg}$ Schwann cells after co-culturing with neurons or exposing cells to forskolin (FSK), which mimics the presence of the axon in primary cultures of Schwann cells (Morgan et al., 1991). Co-cultures of transgenic Schwann cells and normal neurons were also grown for 30 days in myelinating conditions to test their ability of forming myelin around axons.

In basal conditions, homozygous and hemizygous $PMP22_{tg}$ Schwann cells, although expressing higher levels of PMP22 than control ones, show a normal cell motility and migration capacity. Exposure of $PMP22_{tg}$ Schwann cells to FSK, further stimulating PMP22 expression, determines a significant decrease in cell migration and motility, and a reduction of cell area and perimeter. Co-culturing hemizygous transgenic Schwann cells with neurons causes a similar impairment of Schwann cells ability to elongate and determines myelin abnormalities resembling those previously observed in CMT1A organotypic dorsal root ganglia (DRG) cultures (Nobbio et al., 2001). As expected, homozygous Schwann cells, which produce highest levels of PMP22, completely fail to myelinate peripheral axons.

Materials and methods

Cell cultures

Schwann cells were isolated from sciatic nerves of 30-day-old $PMP22_{tg}$ homozygous and hemizygous CMT1A rats (Sereda et al., 1996) according to a technique optimized for adult animals. Wild-type rats from the corresponding genetic background were used to obtain control cultures. We used adult animals as homozygous $PMP22_{tg}$ rats can be easily distinguished from the hemizygous ones, starting from about 4 weeks of age, by their more severe and early-onset phenotype (Niemann et al., 2000; Sereda et al., 1996). Homozygous rats were selected by their phenotype and, to avoid them further stress, were not genotyped. Instead, routine identification of hemizygous and normal rats was performed by DNA polymerase reaction (PCR) (Nobbio et al., 2001; Sereda et al., 1996) on the high-molecular-weight DNA extracted, using standard techniques, from rat tails. Hemizygous rats, carrying three copies of the PMP22 transgene, are a more appropriate model of CMT1A than homozygous ones (Sereda et al., 1996). However, we also used homozygous Schwann cells to evaluate whether the effects of PMP22 on cells function are truly sensitive to the protein prevalence.

Sciatic nerves were sterilely dissected from the rats, minced into small pieces, centrifuged and transferred to RPMI 1640 medium (Invitrogen, Srl, Italy) containing 15% fetal bovine serum (FBS) (Invitrogen, Srl), 1.25 units/ml Dispase (crude, Boehringer Mannheim, Germany), 0.05% collagenase (Sigma-Aldrich, Srl, Italy) and 0.1% hyaluronidase (Sigma-Aldrich, Srl) for 3 h at 37°C. Digestion products were further mechanically dissociated, rinsed with serum containing medium and plated on collagen-coated dishes. Schwann cells were grown for 5 days in DMEM/F12 containing 10% FBS (basal medium) in the presence of cytosine arabinoside (Ara-C) at a final concentration of 10^{-5} M to produce cultures that were 95% pure (Brockes et al., 1979). Following treatment with Ara-C, the medium was replaced with basal medium and, 24 h later, the cells were rinsed, trypsinized, counted and used alone or in co-culture with DRG's neurons. Pure Schwann cell cultures were grown in basal medium or in the presence of 8 μM FSK as described in the following sections.

Co-cultures of normal sensory neurons and transgenic or control Schwann cells were established according to published methods. Briefly, following a 3-week treatment of DRG neurons with a medium supplemented with nerve growth factor (NGF) 5 ng/ml final dilution, ascorbic acid 0.5 mg/ml final dilution and antimitotic agents (10^{-5} M 5-fluoro-2′-deoxyuridine), 2×10^5 Schwann cells were added to each neuronal culture. To evaluate changes in cell morphology and morphometry, Schwann cells were kept for 24 h in presence of the axons. Instead, to study myelination properties, the co-cultures were maintained 30 days in neurobasal medium (Invitrogen, Srl), supplemented with 15% newborn calf serum (NCS) (Invitrogen, Srl), 5 ng/ml NGF (Invitrogen, Srl) and 0.5 mg/ml ascorbic acid (Sigma-Aldrich, Srl).

Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR)

PMP22 and *myelin protein zero* (*MPZ*) mRNA expression was estimated in Schwann cells grown for 24 h in the presence or absence of 8 μM FSK. After rinsing with cold sterilized PBS, Schwann cells were scraped from ACLAR dishes and recovered by centrifugation. Total RNA was extracted by a single-step method (TriPure Isolation Reagent; Boehringer Mannheim). Semiquantitative RT-PCR was performed according to a previously published method for detection of PMP22 and MPZ mRNA levels (Schenone et al., 1997). Briefly, first-strand cDNA was synthesized from 225 ng of total RNA. To detect PMP22 mRNA expression, PCR reaction was performed by co-amplification of endogenous plus transgene-derived PMP22 cDNA using primers corresponding to exon 3 (5′-CTGTACCACATCCGCCTTGG-3′) and exon 5 (5′-TCAACACGAGGCTGACGGTC-3′) of the mouse PMP22 gene and of 18S ribosomal RNA (rRNA) (5′-GGGGCCCGAAGCGTTTACT-3′; 5′-GGTCGGAACTACGACGGTTATC-3′) as an endogenous standard sequence. MPZ mRNA expression was evaluated by co-amplification of MPZ cDNA using mouse-specific primers (5′-GCTCCCAACACCACCCCATA-3′; 5′-GTCCAGTGAATGGGTCTCAG-3′) and 18S rRNA. Preliminary experiments ensured that quantitation was done in the exponential phase of the amplification process for both sequences and that expression of the reference gene was uniform in the different culture conditions (Schenone et al., 1997). The following PCR conditions were then used: amplification after a hot start (5 min at 95°C) was done for 25 cycles consisting of 1 min at 95°C, 1 min at 52°C (55°C for the MPZ sequence) and 1 min at 72°C; an additional 10 min at 72°C was added at the end of the 25 cycles. PCR products were resolved on 2% agarose gel and band intensity was measured with a Gel Doc 1000 imaging system (Bio-Rad). Band volume was calculated from the number of pixels inside the area of the spot and from individual pixel intensity, and the amount of each mRNA was expressed as a ratio between PMP22 or MPZ and 18S rRNA.

Western blot analysis and deglycosylation assay

Lysates of normal and $PMP22_{tg}$ Schwann cells, grown for 24 h in the presence or absence of 8 μM FSK, were prepared directly in 40 μl of denaturation buffer Laemmli 2× (4% SDS, 20% glycerol, 120 mM Tris–HCl, pH 6.8, 2% β-ME). For endoglycosidase digestions, PNGase F and Endo H were used as previously described (Fabbretti et al., 1995). Schwann cell lysates with and without enzyme were incubated for 3 h at 37°C. Proteins were then separated on 15% SDS gels and transferred to 0.2-μm-pore-sized

nitrocellulose membranes (Amersham, Arlington Heights, IL) using a semidry blotting apparatus (Bio-Rad). Positive cleavage by Endo H and PNGase F was monitored by Coomassie blue staining of parallel gels and by Ponceau S staining of the nitrocellulose before Western blotting. After washing with TBST-T (0.05% Tween 20 TBS) and blocking with 5% skim milk in TBS-T, blots were incubated with anti-rat PMP22 antiserum (1:800) (gift of Dr. Brancolini) overnight and with β-tubulin antibody (1:200) (Sigma-Aldrich, Srl) for 1 h. Blots were then rinsed with TBS-T and incubated with peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma-Aldrich, Srl) for 1 h at room temperature. Bound antibodies were detected using the ECL detection method (Amersham). Band intensity was measured on the film by a Gel Doc 1000 imaging system (Bio-Rad). The amount of PMP22 was expressed as a ratio between the 22-kDa glycosylated form and the β-tubulin.

Viability and proliferation assays

A colorimetric assay, based on the cleavage of the yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) to purple formazan crystals by dehydrogenase activity in active mitochondria, was used for the quantitative determination of Schwann cell viability and proliferation (Heeg et al., 1985); 1.5×10^4 normal and $PMP22_{tg}$ Schwann cells were grown in microtiter plates (tissue culture grade, 96 wells, flat bottom) in a final volume of 100 μl basal medium per well. Five time points (recorded every 24 h) were chosen and for each time point six wells per condition were established. Schwann cells were incubated with the yellow MTT solution (final concentration 0.5 mg/ml) for 4 h. After this incubation period, purple formazan salt crystals were formed, in living and metabolically active Schwann cells. Formazan crystals were then solubilized by adding the solubilization solution (10% SDS in 0.01 mol/l HCl) to the cells overnight. The resulting colored solution was quantified with a scanning multiwell spectrophotometer (ELISA reader). An increase in the number of living cells results in an increase in the overall activity of mitochondrial dehydrogenases in Schwann cells. This increase directly correlates to the amount of the purple formazan crystals formed, as monitored by the evaluation of absorbance.

The proliferation rate of normal, hemizygous and homozygous $PMP22_{tg}$ Schwann cells was also estimated by 5-bromo-2′-deoxyuridine (BrdU) labeling for 1 h using the BrdU labeling and detection kit I (Roche Diagnostics GmbH, Mannheim, Germany). Purified wild type and transgenic Schwann cells were seeded on 10-mm round glass coverslips at a density of 1×10^4 cells/coverslip and processed following the manufacturer's instructions. Four slides per condition were established and mounted with the mounting medium Citifluor containing DAPI (Citifluor Ltd., London, UK) to test the total number of Schwann cells towards the proliferating ones. $BrdU^+$ and $DAPI^+$ cells were counted in 15 random fields per slide for a total of 54 fields per condition with a 20× objective using our image system for nerve morphometry. Results were expressed as total number of viable cells counted and percentage of proliferating cells.

Motility study

Schwann cell motility was measured using a gold particle motility assay as previously described (Silletti et al., 1991). A uniform carpet of gold particles was prepared on bovine serum albumin-coated 12-mm glass coverslips in 35-mm tissue culture plates. Colloidal gold-coated coverslips were rinsed and then seeded with 5×10^4 Schwann cells in 2 ml of either basal medium or basal medium supplemented with 8 μM FSK. After 24 h, cells were rinsed with cacodylate buffer and fixed with 3.5% glutaraldehyde in cacodylate buffer, pH 7.4, air dried and mounted on glass slides with Permount (Fisher Scientific, Fair Lawn, NJ). The cell area and the gold particle-free clear zone surrounding cells (halo area), which is representative of Schwann cell motility (Cheng et al., 2000), were measured using our imaging system for nerve morphometry. At least 50 cells, originating from three different culture dishes, were analyzed for each condition.

Migration assay

To test the ability of $PMP22_{tg}$ Schwann cells to migrate in the presence or absence of FSK, we followed a recently described protocol (Meintanis et al., 2001). Schwann cells were plated on poly-L-lysine-coated 12-mm coverslips at a density of 6×10^4 cells/coverslip in a 40-μl drop. After 6 h, an additional 200 μl of basal medium was added, and cells were left for 24 h without any further treatment. The medium was then replaced with either basal medium or basal medium supplemented with 8 μM FSK; 12 h later, a cell-free area was generated by gently scratching the cell monolayer with a sterile yellow Gilson-pipette tip, thus resulting in the formation of an approximately 1-mm-wide gap. Immediately after scratching, the media were replaced with the same fresh ones and the migration of Schwann cells within the gap was monitored microscopically. We considered time zero (T_0) the time in which the scratch was created, and the cells were firstly acquired. Twelve hours later (T_1), the same Schwann cell cultures were newly monitored and images saved. Migration distance was assessed by subtracting the average gap width at T_1 from the average gap width at T_0. Schwann cell migration was monitored for a maximum of 12 h to avoid interference in the evaluation due to Schwann cell proliferation rather than migration (Meintanis et al., 2001). Counting of migration distance was performed on 10 Schwann cell cultures for each condition, using our imaging system for nerve morphometry.

Morphological studies

To investigate axon-induced changes in $PMP22_{tg}$ Schwann cells, primary Schwann cell cultures supplemented for 24 h with 8 μM FSK and co-cultures of Schwann cells and DRG's neurons were used. Schwann cells were labeled with 12 μg/ml dioctadecylindocarbocyanine (DiI, Molecular Probes Inc., Eugene, OR) according to the manufacturer's protocol. After rinsing, 5×10^4 labeled normal and $PMP22_{tg}$ Schwann cells were plated on poly-L-lysine-coated coverslips and grown for 24 h in the presence or in the absence of FSK. Changes in Schwann cell morphology were documented with fluorescence microscopy (Zeiss Axiovert 200 microscope equipped with a fluorescence source). For each cell, perimeter and area were calculated using a specific software for nerve morphometry (Image Pro-Plus; Immagini e Computer, Milan, Italy). At least 60 cells, originating from three different culture dishes, were measured for each condition. The same morphological and morphometrical evaluations were also performed in co-cultures of Schwann cells and DRG neurons. After rinsing, 1×10^5 labeled Schwann cells were added to each DRG culture dish for 24 h; three co-cultures of Schwann cells and

sensory neurons were established for each condition and the morphometrical study carried out as previously described.

To detect myelin formation, 30-day-old co-cultures of sensory neurons and Schwann cells were washed in PBS, fixed in Trump's fixative at 4°C overnight, postfixed the following day in 2% osmium tetroxide and stained with 2% Sudan black in 70% ethanol. After dehydration in alcohol, cultures were mounted on slides with glycerol and analyzed with light microscopy. Each culture was assessed for the presence or absence of myelin and for the morphologic appearance of all observed fibers.

Electron microscopy

For electron microscopic (EM) examination, Schwann cell cultures were established from sciatic nerves of 30-day-old homozygous $PMP22_{tg}$ or wild-type rats, according to the method described above. After 5 days in culture, cells were rinsed, trypsinized and fixed in 2.5% glutaraldehyde in cacodylate buffer for 30 min. Finally, cells were postfixed for 1 h in 2% osmium tetroxide, dehydrated in alcohol and embedded in epoxy resin. Ultrathin sections, stained with 5% uranyl acetate and lead citrate, were then prepared and examined under a Zeiss EM 109.

Statistical analysis

Since three different conditions have been evaluated (hemizygous $PMP22_{tg}$, homozygous $PMP22_{tg}$ and wild-type Schwann cells), results were analyzed using a one-way analysis of variance (ANOVA), followed by a Dunnett post-test to compare the two groups of $PMP22_{tg}$ Schwann cells with the wild-type cells. When appropriate (comparison of FSK-treated vs. cells grown in basal medium within a single condition), a two-tailed *t* test for unpaired samples was used. Frequency histograms were calculated for cell perimeters. Measurements of Schwann cells with light and fluorescence microscopy were performed blindly by examiners who did not know the origin of the cultures. A general linear model (GLM) for repeated measurements was used to compare the proliferation rates between the three different conditions.

Results

Expression and trafficking of PMP22 in cultured $PMP22_{tg}$ Schwann cells

We used primary Schwann cell cultures, grown in basal medium or in the presence of FSK, to test whether axonal-mimicking stimuli were able to interfere with the expression and processing of PMP22 in transgenic cells. *MPZ* levels were also tested as a marker of Schwann cell differentiation in response to FSK treatment (Lemke and Chao, 1988; Morgan et al., 1991). Homozygous and hemizygous $PMP22_{tg}$ Schwann cells express higher levels of *PMP22* mRNA than normal ones (0.81 vs. 0.59 vs. 0.09) already in basal medium (Fig. 1). As previously shown in sciatic nerves of transgenic rats (Niemann et al., 2000), *MPZ* mRNA is also upregulated in $PMP22_{tg}$ Schwann cells in basal conditions (2.4 vs. 1.9 vs. 0.52). As expected, FSK treatment increases *PMP22* (0.19) and *MPZ* (1.2) mRNA expression in control cells, thus suggesting that the concentrations used were adequate to stimulate differentiation of Schwann cells (Morgan et al., 1991). Interestingly, FSK addition to homozygous and hemizygous $PMP22_{tg}$ Schwann cells results in an enhanced expression of *PMP22* (1.56 and 0.93, respectively) and *MPZ* (3.02 and 2.2, respectively) as judged by mRNA levels (Fig. 1). Since in CMT1A neuropathy the duplication of the PMP22 gene leads to protein overexpression (Vallat et al., 1996), we also analyzed protein levels in our culture system. In homozygous $PMP22_{tg}$ Schwann cells, and at a lesser extent in hemizygous cells, basal levels of PMP22 are higher than in normal controls (11.8 vs. 4.19 vs. 3.57) and FSK treatment enhances this difference (13.5 vs. 4.64 vs. 4.09) (Fig. 1).

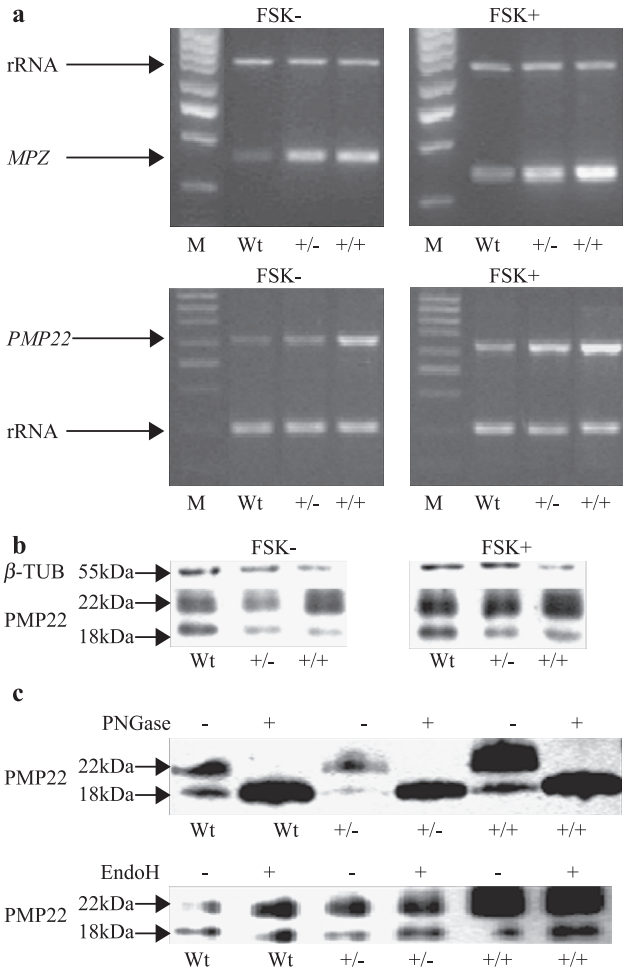

Fig. 1. Expression of PMP22 mRNA and protein (see Results for values). (a) Semiquantitative RT-PCR: in basal conditions (FSK−), homozygous (+/+) and hemizygous (+/−) $PMP22_{tg}$ Schwann cells already show higher levels of *PMP22* and *MPZ* mRNA than normal ones (wt); FSK treatment (FSK+) increases both *PMP22* and *MPZ* mRNA expression in control as well as in $PMP22_{tg}$ Schwann cells. (b) Western blot: also at the protein level, homozygous (+/+) $PMP22_{tg}$ Schwann cells, and at a lesser extent hemizygous (+/−) cells, show higher relative amounts of PMP22 (PMP22kDa/β-tubulin) than control cells (wt), in basal conditions (FSK−); FSK treatment (FSK+) enhances this difference. (c) Intracellular processing of PMP22. PMP22 is sensitive to PNGase F digestion both in normal and $PMP22_{tg}$ Schwann cells. On the contrary, treatment of the same extracts with Endo H reveals that PMP22 is resistant to the enzyme. These results suggest that the protein is mature and has reached post-Golgi compartments, also in transgenic Schwann cells.

Since it is known that mature PMP22 accumulates overtime in the Golgi compartment of normal Schwann cells co-cultured with DRG neurons (Pareek et al., 1997) and overexpressed PMP22 is

diffusely Endo H resistant in sciatic nerves of $PMP22_{tg}$ rats (Niemann et al., 2000), we investigated the protein trafficking in cultured $PMP22_{tg}$ Schwann cells by treating cell lysates with Endo H and PNGase F endoglycosidases. The PMP22 steady state is largely Endo H resistant in $PMP22_{tg}$ and normal Schwann cells grown for 24 h in the presence of FSK (Fig. 1) or in basal medium (data not shown), suggesting that, in our culture system, PMP22 is not retained in the endoplasmic reticulum. Moreover, positive PNGase F digestion of the protein in $PMP22_{tg}$ Schwann cells treated for 24 h with FSK suggests that PMP22 is mature and has reached post-Golgi compartments (Fig. 1).

Viability and proliferation assays

We determined the viability and proliferation rates of $PMP22_{tg}$ and normal Schwann cells using a colorimetric assay and BrdU analysis. Absorbance values of homozygous $PMP22_{tg}$ Schwann cells are significantly (GLM, $P < 0.05$) lower than normal controls and hemizygous transgenic Schwann cells at all time points examined (Fig. 2A). Instead, no significant differences (GLM, P = NS) have been observed between normal controls and hemizygous $PMP22_{tg}$ Schwann cells. To test whether these changes in cells viability are due to differences in the rate of cells proliferation, we also used BrdU analysis. Although the total number of living cells/frame appears lower in homozygous Schwann cells (8.5) than in hemizygous (24.7) and normal (19.4) ones, the percentage of BrdU positive cells/living cells is clearly decreased in homozygous Schwann cells (10.5%) compared to hemizygous (14%) and normal (20%) ones (Fig. 2B).

Motility and migration assays

To test $PMP22_{tg}$ Schwann cells ability to move in presence of axonal-mimicking stimuli, we used a gold particle motility assay (Silletti et al., 1991). Schwann cell motility was monitored both in basal medium and after a 24-h FSK treatment. Cell area and the gold

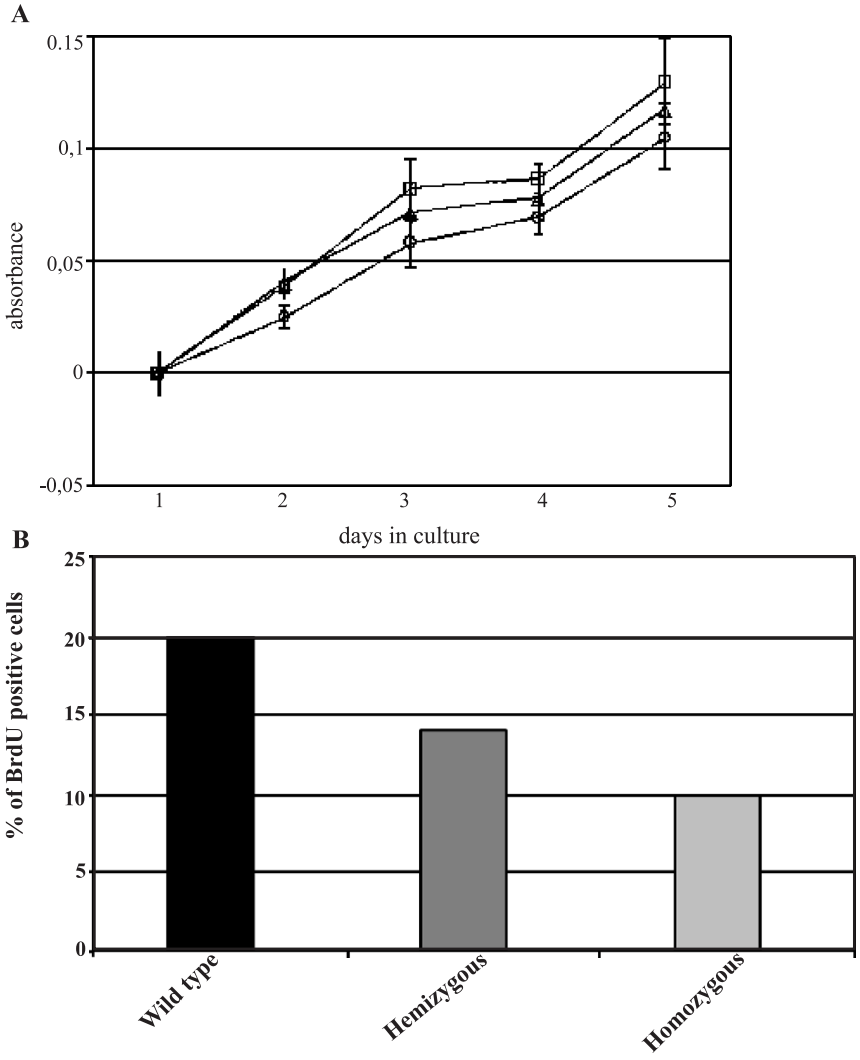

Fig. 2. Viability and proliferation assays. (A) MTT assay: after 2 days in culture, in basal medium, homozygous $PMP22_{tg}$ Schwann cells (○) show significantly (GLM, $P < 0.05$) lower absorbance rates than hemizygous (□) and control (△) Schwann cells. No significant differences have been observed between normal and hemizygous Schwann cells. (B) BrdU assay: homozygous $PMP22_{tg}$ Schwann cells show a lower percentage of proliferating cells than wild-type ones. Hemizygous Schwann cells display intermediate proliferation between normal and homozygous cells.

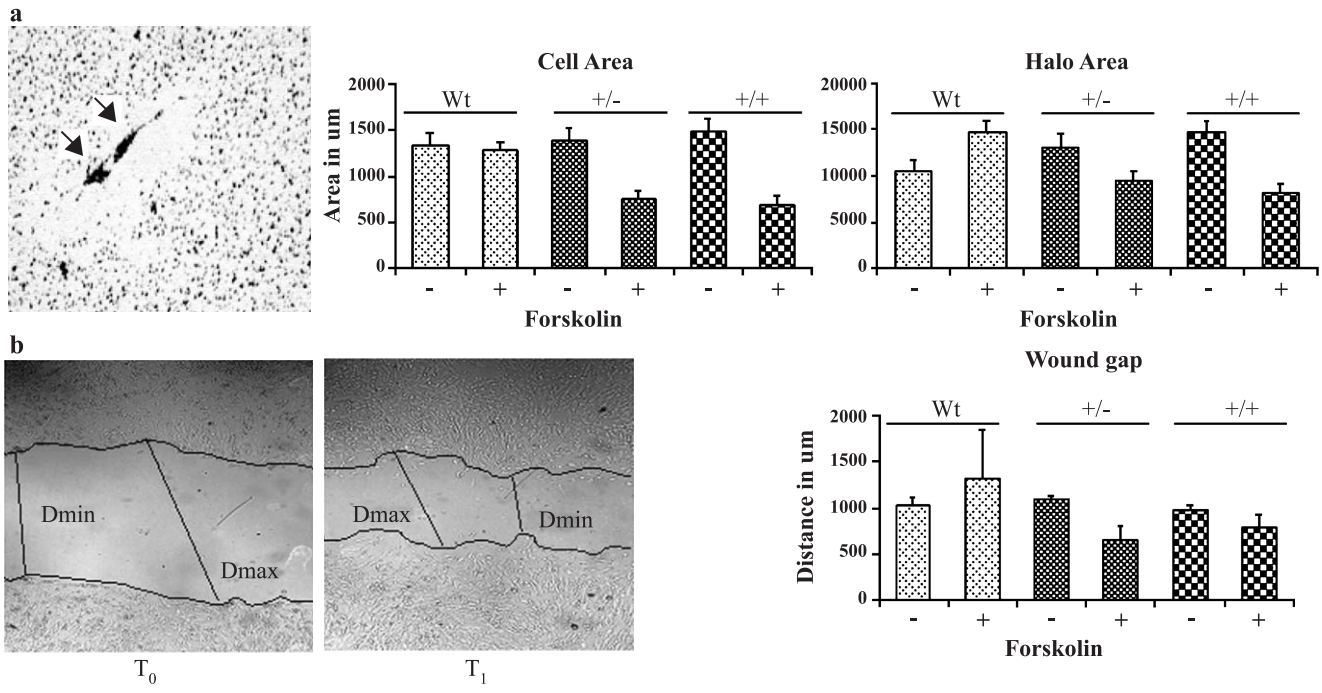

Fig. 3. Motility and migration assays (see Table 1 for values). (a) Schwann cell motility was monitored both in basal medium and after FSK treatment using a gold particle motility assay. Moving Schwann cells (arrow) remove gold particles and leave clear tracks. In basal conditions, cell area and the gold particle-free clear zone surrounding cells (halo area) do not significantly change among homozygous (+/+), hemizygous (+/−) and normal (wt) Schwann cells. FSK treatment produces a significant decrease of the same parameters in homozygous and hemizygous transgenic Schwann cells, thus suggesting that impairment of cell motility is due to the extremely high PMP22 levels reached in affected cultures in the presence of FSK. (b) To test cell migration, Schwann cell movement within a 1-mm-wide gap created on cells monolayer was monitored by time (T_0 and T_1). In agreement with the results of the gold particle motility assay, cells migration is only affected, in transgenic Schwann cells, after exposure to FSK. On the contrary, normal cells in the presence of FSK increase this property.

particle-free clear zone surrounding cells (halo area) were assessed (Fig. 3). In basal conditions, we do not observe changes in the cell area among homozygous, hemizygous and normal Schwann cells. In agreement with the morphometric study on primary Schwann cells and co-cultures of Schwann cells and sensory neurons, FSK treatment causes a significant (ANOVA, $P < 0.0001$) reduction of cell area in transgenic cells compared to normal ones. Halo area has been used as a measurement of Schwann cell ability to move in culture (Cheng et al., 2000). In the presence of FSK, the halo area is significantly lower (ANOVA, $P < 0.0001$), in both homozygous and hemizygous cells compared to control ones (Fig. 3, Table 1).

Migration of Schwann cells along the axon and cell motility properties are fundamental for myelin sheath assembly and maintenance. To test cell migration, primary Schwann cell cultures were established on poly-L-lysine coverslips and after the creation of a 1-mm-wide scratch on the cells monolayer, Schwann cells movement within the gap was monitored by time (Meintanis et al., 2001). After 12 h in basal medium, both homozygous and hemizygous $PMP22_{tg}$ Schwann cells do not show any significant difference in terms of migration properties compared to control ones. After FSK treatment, homozygous (976.1 ± 57.3 vs. 790.7 ± 137.4μm, two-tailed t test, $P <$

Table 1

Condition		Morphometry		Motility		Migration
		Area (μm²)	Perimeter (μm)	Area (μm²)	Halo (μm²)	Gap (μm)
Wt (A)	Fsk−	1364 ± 101.5* (n:61)	250.2 ±16.9**(n:61)	1327 ± 80.9* (n:56)	10450 ± 1137* (n:56)	1027 ± 88.86* (n:10)
	Fsk+	1283 ± 82.65*** (n:85)	298.4 ± 15.8*** (n:85)	1287 ± 80.94*** (n:50)	14660 ± 1248*** (n:50)	1318 ± 529.5* (n:10)
+/− (B)	Fsk−	1392 ± 115.5* (n:70)	230.3 ± 11.9** (n:70)	1398 ± 126.7* (n:56)	13010 ± 1435* (n:56)	1085 ± 38.11* (n:10)
	Fsk+	1033 ± 99.1*** (n:100)	217.9 ± 14.2*** (n:100)	759.7 ± 84.13*** (n:57)	9577 ± 844.7*** (n:57)	643.6 ± 155.8* (n:10)
+/+ (C)	Fsk−	1026 ± 92.85* (n:71)	196.7 ± 14** (n:71)	1480 ± 142.6* (n:51)	14730 ± 1201* (n:51)	976.1 ± 57.3* (n:10)
	Fsk+	637 ± 55.5*** (n:148)	143.3 ± 14*** (n:148)	697.1 ± 99.33*** (n:51)	8155 ± 971.8*** (n:51)	790.7 ± 137.4* (n:10)
ANOVA		A−/B−, NS	A−/B−, NS	A−/B−, NS	A−/B−, NS	A−/B−, NS
*NS		A−/C−, $P < 0.05$	A−/C−, $P < 0.05$	A−/C−, NS	A−/C−, NS	A−/C−, NS
**$P < 0.05$		A+/B+, NS	A+/B+, $P < 0.01$	A+/B+, $P < 0.01$	A+/B+, $P < 0.01$	A+/B+, $P < 0.01$
***$P < 0.0001$		A+/C+, $P < 0.01$	A+/C+, $P < 0.01$	A+/C+, $P < 0.01$	A+/C+, $P < 0.01$	A+/C+, $P < 0.01$

A−: wild type, FSK−; B−: hemizygous, FSK−; C−: homozygous FSK−; A+: wild type, FSK+; B+: hemizygous, FSK+; C+: homozygous FSK+.
A one-way analysis of variance (ANOVA) was used to compare the three different conditions (A, B, C) in basal conditions (FSK−) and after FSK addition (FSK+), followed by a Dunnett post test to compare affected cultures to control ones. Results are expressed as means ± standard error.

Table 2

Co-cultures	Morphometry	
	Area (μm^2)	Perimeter (μm)
Wt (A)	1294 ± 67.22*** (n:115)	314.5 ± 13.95*** (n:115)
+/− (B)	1137 ± 64.64*** (n:99)	292.3 ± 14.29*** (n.99)
+/+ (C)	879.2 ± 63.75*** (n:86)	230.9 ± 13.29*** (n:86)
ANOVA	A/B, NS	A/B, NS
***$P < 0.0001$	A/C, $P < 0.01$	A/C, $P < 0.01$

A: wild type; B: hemizygous; C: homozygous. A one-way analysis of variance (ANOVA) was used to compare the three different conditions (A,B,C), followed by a Dunnett post-test to compare affected co-cultures to control ones. Results are expressed as means ± SD.

0.001) and hemizygous (1085 ± 38.11 vs. 643.6 ± 155.8 μm, two-tailed t test, $P < 0.0001$) $PMP22_{tg}$ Schwann cells decrease their migratory ability; on the contrary, FSK increases the migration of normal Schwann cells (1027 ± 88.86 vs. 1318 ± 529.5 μm, two-tailed t test, P = NS) (Fig. 3, Table 1).

Morphological studies in $PMP22_{tg}$ Schwann cells exposed to FSK or in presence of the axons

It has been recently shown that PMP22 overexpression leads to cell spreading alterations which could interfere with the correct enwrapping of the axon by Schwann cells (Brancolini et al., 2000). To define morphological features of $PMP22_{tg}$ Schwann cells and to determine their behavior in the presence of the axon or of axonal-mimicking stimuli, we used both pure primary Schwann cells and co-cultures of Schwann cells and DRG's sensory neurons.

DiI-labeled Schwann cells grown for 24 h in the presence of 8 μM FSK were compared with Schwann cells grown in basal medium. The cellular perimeter and area were measured (Table 1).

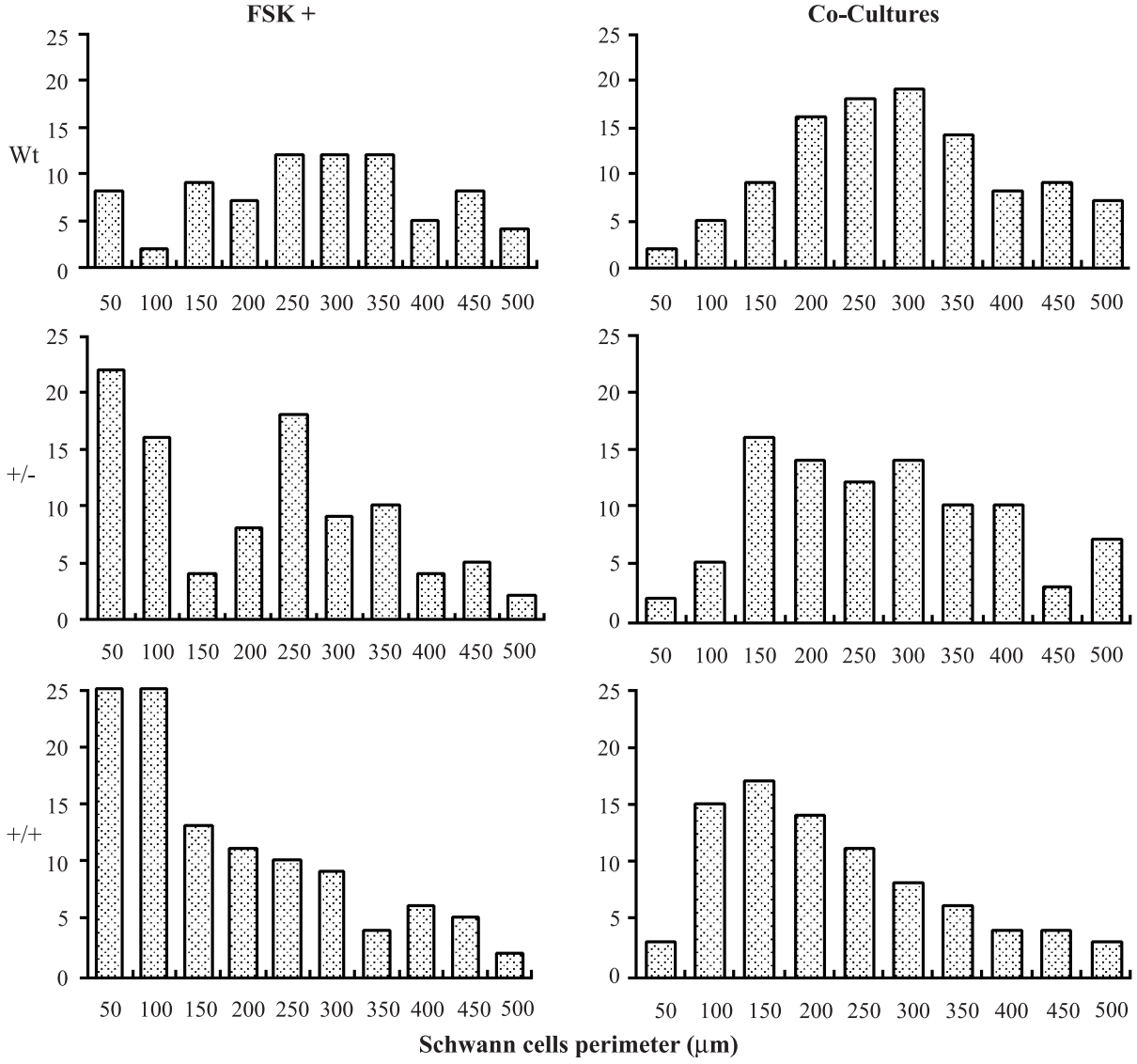

Fig. 4. Frequency histograms of cell perimeter in primary cultures of homozygous (+/+), hemizygous (+/−) and control (wt) Schwann cells after the exposure to FSK, and in co-cultures of transgenic cells with sensory neurons. The presence of the axon induces a rather homogeneous increase of the cell perimeter in normal controls. Instead, homozygous $PMP22_{tg}$ Schwann cells show a shift to the lower perimeter distribution compared to control cells. Interestingly, hemizygous transgenic Schwann cells display a bimodal distribution, both after FSK treatment and when co-cultured with sensory neurons, suggesting that, in this condition, a population of cells differentiate normally.

Since Schwann cells relating to axons elongate to start the myelination process (Mezei, 1993) and Schwann cell differentiation in the presence of FSK is characterized by flattening and increased complexity of the cell cytoplasm (Morgan et al., 1991; Sobue et al., 1986), we considered the increase of the perimeter as an indicator of Schwann cell differentiation. In fact, when co-cultured with neurons, normal Schwann cells do show a lengthening of their cytoplasm along the axons before starting the myelination process (Bunge et al., 1989). Moreover, we measured the cell area to evaluate the spreading ability of $PMP22_{tg}$ Schwann cells. Homozygous $PMP22_{tg}$ cells show, in basal conditions, a slightly reduced perimeter compared to hemizygous $PMP22_{tg}$ and normal ones, but the difference is statistically significant only for homozygous vs. normal Schwann cells (ANOVA, $P < 0.05$). After FSK treatment, homozygous and hemizygous $PMP22_{tg}$ Schwann cells react by significantly (ANOVA, $P < 0.0001$) decreasing their perimeter compared to wild-type cells, which show the expected tendency to increase their perimeter and to assume a more complex shape. The same trend is observed when we compare the cell area before and after FSK treatment. Again, homozygous Schwann cells show lower values than hemizygous and normal ones, the difference being statistically significant only for homozygous vs. normal cells (ANOVA, $P < 0.05$). FSK treatment, significantly decreasing the area of homozygous and hemizygous Schwann cells (ANOVA, $P < 0.0001$), enhances these differences.

To determine whether the observations made on pure Schwann cells in the presence of FSK could be also reproduced in co-cultures of Schwann cells and DRG's neurons, DiI-labeled Schwann cells were added to purified sensory neurons and 24 h later assessed for their perimeter and area. For each analyzed parameter, we found a complete overlapping of results between primary Schwann cell cultures and co-cultures of Schwann cells and sensory neurons (Tables 1 and 2). Homozygous $PMP22_{tg}$ cells in the presence of the axon show a significantly (ANOVA, $P < 0.0001$) lower perimeter and area compared to wild-type cells. Interestingly, Schwann cells from hemizygous rats, which more closely reproduce the human CMT1A phenotype, show intermediate values of cell perimeter and area between normal and homozygous ones. However, the difference between hemizygous and normal cells is not statistically significant.

Frequency histograms of cell perimeter (Fig. 4), in co-cultures of Schwann cells and sensory neurons, show a peak of distribution at 300 μm in control cells, whereas homozygous ones peak at 150 μm. Hemizygous Schwann cells demonstrate a tendency to form two peaks, at 150 and at 300 μm. A similar distribution pattern can be observed after the exposure to FSK, at least in normal Schwann cells, which peak between 250 and 350 μm. Homozygous $PMP22_{tg}$ Schwann cells show a peak at 50 μm, whereas hemizygous transgenic Schwann cells display again a bimodal distribution, peaking at 50 and 250 μm.

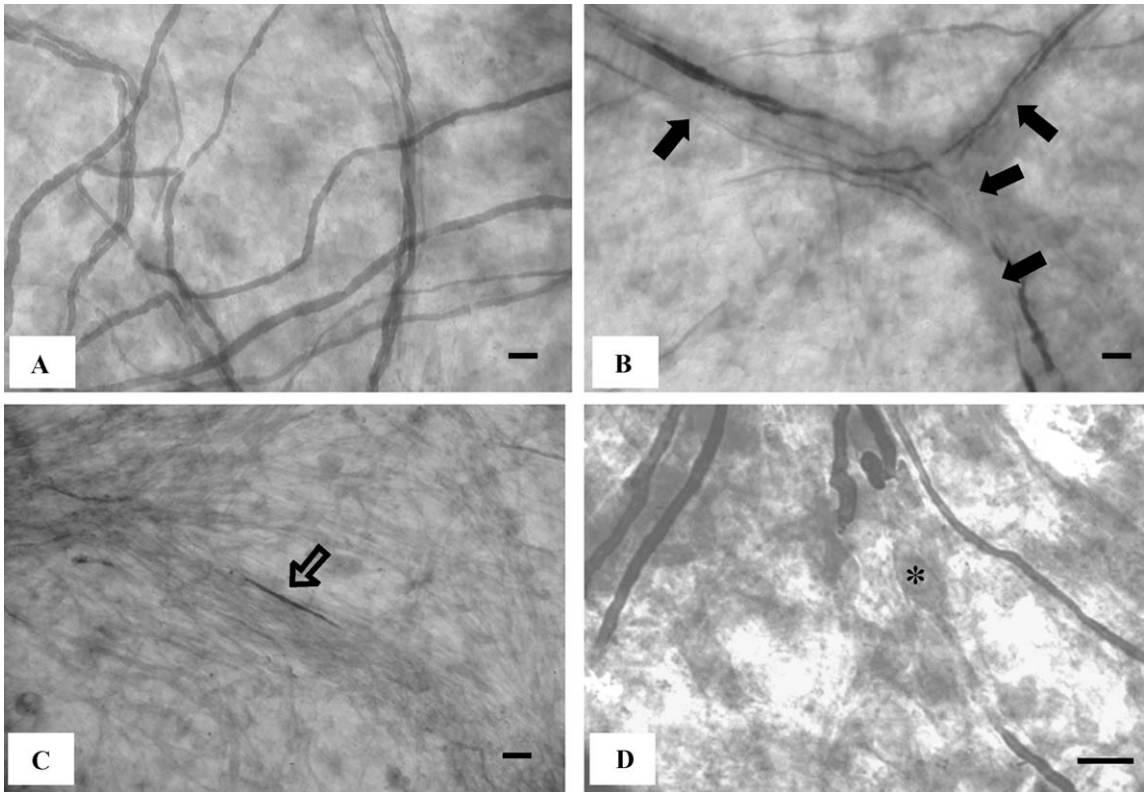

Fig. 5. Co-cultures of Schwann cells and sensory neurons. (A) After 30 days in culture, normal Schwann cells are able to correctly provide myelin sheaths for the axons; several myelinated internodes may be seen. (B) In co-cultures of hemizygous $PMP22_{tg}$ Schwann cells and sensory neurons, most fibers show axonal segments devoid of myelin (filled arrows) along with normally myelinated internodes. (C) Homozygous $PMP22_{tg}$ Schwann cells fail to produce any myelin sheath around normal axons; occasionally, a single myelinated internode may be observed (open arrow). (D) At higher magnification, in co-cultures of hemizygous $PMP22_{tg}$ Schwann cells and sensory neurons, the segments devoid of myelin are usually associated with the nucleus of a spindle-shaped cell (asterisk), suggesting that a Schwann cell has aligned with the axon, but is unable to produce myelin. [Osmium tetroxide and Sudan Black; scale bars: 30 μm (A, B, C) and 10 μm (D)].

Fig. 6. Ultrastructural analysis of $PMP22_{tg}$ cells. In basal conditions, some homozygous $PMP22_{tg}$ Schwann cells, cultured from 30-day-old rats, show intracytoplasmic periodic, myelin-like structures (asterisk). Similar figures were never observed in wild-type Schwann cells. Scale bar, 0.2 μm.

Since after 24 h in the presence of the axons transgenic Schwann cells show a tendency to de-differentiate, co-cultures of normal neurons and hemizygous, homozygous or control Schwann cells were assessed for their ability to form myelin. Accordingly to our previous results, hemizygous $PMP22_{tg}$ cultures demonstrate an intermediate phenotype characterized by the presence of several internodes devoid of myelin together with normally myelinated ones, along the same fiber. At high magnification, the segments devoid of myelin in $PMP22_{tg}$ cultures usually are associated with the nucleus of a spindle-shaped cell, suggesting that a Schwann cell has aligned with the axon but does not produce myelin (Nobbio et al., 2001). Instead, widespread myelination is obtained in normal Schwann cells co-cultured with neurons. As expected, co-cultures of homozygous Schwann cells and neurons are virtually unable to produce myelin (Fig. 5).

Since intracellular myelin figures have been observed in sciatic nerves of homozygous $PMP22_{tg}$ animals (Niemann et al., 2000), we examined by EM cultured Schwann cells obtained from homozygous rats. Intracytoplasmic periodic structures are also present in cultured $PMP22_{tg}$ Schwann cells (Fig. 6). This observation suggests that transgenic cells show similar ultrastructural features both in vitro and in vivo.

Discussion

The functions of PMP22 as a structural protein of peripheral myelin and a regulator of the proliferation, differentiation, shaping and apoptosis of Schwann cells are well known (Brancolini et al., 1999; Fabbretti et al., 1995; Snipes et al., 1992; Zoidl et al., 1995). However, the effects of its overexpression on the behavior of Schwann cells and on their ability of producing myelin, in CMT1A, are still unclear (Muller, 2000). We studied the molecular and morphological phenotype of cultured Schwann cells obtained from a previously published rat transgenic model of CMT1A (Sereda et al., 1996).

We firstly evaluated the expression and processing of PMP22 in transgenic and wild-type Schwann cells. Hemizygous and homozygous Schwann cells, in basal conditions, retain the ability to express higher levels of PMP22 and MPZ than control ones, as previously observed in vivo (Niemann et al., 2000). PMP22 expression is highest in homozygous cells. As expected, FSK enhances expression of PMP22 and MPZ in normal controls. Interestingly, also $PMP22_{tg}$ Schwann cells retain the ability to strongly increase their production of PMP22 and MPZ, in the presence of FSK, both at the mRNA and protein levels. Moreover, deglycosylation assay shows that, in transgenic Schwann cells, after 24 h of culturing in the presence of FSK, PMP22 is largely Endo H resistant and PNGase F sensitive, as previously observed in normal Schwann cell cultures and in sciatic nerves of homozygous CMT1A rats (Niemann et al., 2000; Pareek et al., 1997). This observation suggests that exposure to axonal-mimicking stimuli, in Schwann cells constitutionally overexpressing PMP22, does not affect the short-term processing and transport of the protein to the intermediate compartment between ER and Golgi.

The transition from precursor to immature Schwann cells is accompanied by an intense cell proliferation (Mezei, 1993; Scherer, 1997). Then, at the promyelinating stage, Schwann cells reach a 1:1 relationship with the axon, stop dividing and differentiate into myelin and non-myelin-forming cells (Mezei, 1993). Reduced proliferation has been observed in primary Schwann cell cultures from sural nerve biopsies of adult CMT1A patients and in rodent Schwann cells induced to express high levels of PMP22 (Hanemann et al., 1998; Zoidl et al., 1995), whereas increased cells death due to PMP22 overexpression has been reported in fibroblasts (Fabbretti et al., 1995). Our data on the viability and proliferation of $PMP22_{tg}$ Schwann cells confirm previous results showing that the effects of PMP22 on cell growth and proliferation are highly sensitive to the protein prevalence (Zoidl et al., 1995). In fact, the lower viability of homozygous $PMP22_{tg}$ Schwann cells may be explained by a decrease in the rate of cells proliferation, as demonstrated by BrdU analysis. However, our finding that hemizygous cells, which more closely resemble human CMT1A Schwann cells, show viability and proliferation rates comparable to normal controls, and the observations that young CMT1A patients have a normal number of Schwann cells in sural nerve biopsies and Schwann cells in animal models do reach the promyelinating stage (Hanemann et al., 1997; Niemann et al., 2000), suggest a more complex pathogenetic mechanism for CMT1A, than a simple defect in cells growth or proliferation (Muller, 2000). In particular, important cells features, like motility and migration, which regulate the ability of a Schwann cell to elongate along the axon, produce myelin and establish future internodes (Webster, 1971), have not been studied yet in experimental CMT1A. An impairment of Schwann cells spreading, which could ultimately reflect a defect in cell motility, has been recently observed after microinjection of cells with retroviral vectors carrying PMP22 cDNA (Brancolini et al., 1999).

Our results suggest that hemizygous and homozygous $PMP22_{tg}$ Schwann cells, in basal conditions, although expressing higher levels of PMP22 than control ones, do not show significant changes in migration and motility properties. Exposure of transgenic Schwann cells to FSK induces a significant impairment of cell migration and motility properties. Instead, both these features are stimulated by FSK, in cultures of normal Schwann cells. Therefore, the presence of axonal-mimicking stimuli seems to be necessary for producing changes in the physiology of $PMP22_{tg}$ Schwann cells, which may in turn affect the myelination process.

As motility and migration are, in concept, strictly related to the shaping properties of a cell and as a significantly negative effect of

PMP22 overexpression on Schwann cells spreading has been recently shown (Brancolini et al., 1999), we also performed a morphometric evaluation of $PMP22_{tg}$ Schwann cells, in basal conditions and in the presence of FSK. In basal medium, the area and perimeter of hemizygous transgenic Schwann cells, more closely resembling human CMT1A, is similar to those of wild-type cells; exposure to FSK induces a significant reduction in $PMP22_{tg}$ Schwann cells area and perimeter, compared to normal controls. The homozygous situation enhances these differences, suggesting that increasingly high levels of PMP22 are needed to affect the shaping properties of a Schwann cell.

Whether the overexpressed protein has to reach the plasma membrane to exert this effect, is, however, not clear. The observation that PMP22 is not aberrantly retained in the ER, even in the presence of extremely high levels of PMP22, and that some Schwann cells overproducing PMP22 accumulate intracytoplasmic myelin-like figures, as previously found in homozygous CMT1A rats (Niemann et al., 2000), may suggest that increased PMP22 levels also impair myelin formation at the plasma membrane level, eventually through the involvement of some endosomal recycling pathways (Brancolini et al., 2000; Chies et al., 2003).

To demonstrate that these results on Schwann cells morphology are truly related to the presence of the axon and that the negative effects of PMP22 overexpression on Schwann cells motility, migration and shaping properties lead to a failure in myelin formation, we also used co-cultures of transgenic Schwann cells and normal sensory axons.

In agreement with the observation on cultures exposed to FSK, $PMP22_{tg}$ Schwann cells co-cultured for 24 h with sensory neurons showed a lower cells area and perimeter compared to wild-type ones. Furthermore, after 30 days in culture, a failure in myelination, resembling our previous findings in organotypic DRG cultures from the same animal model (Nobbio et al., 2001), may be observed in co-cultures of hemizygous transgenic Schwann cells and normal sensory neurons. Homozygous $PMP22_{tg}$ Schwann cells are virtually unable to start the myelination process, as previously observed in the CMT1A rat (Niemann et al., 2000). Since in our culture system, as well as in human CMT1A sural nerves and in sciatic nerves of hemizygous $PMP22_{tg}$ rats, normal myelinated internodes and demyelinated ones alternate (Dyck et al., 1993; Nobbio et al., 2001; Sereda et al., 1996), it is possible that, in the hemizygous situation, only some Schwann cells reach levels of PMP22 which are sufficient to arrest cell differentiation and myelin formation. Accordingly, hemizygous Schwann cells grown for 24 h in the presence of FSK or co-cultured with sensory neurons, always show morphometric features which are intermediate between homozygous and control cells. This observation may be explained by the ability of hemizygous Schwann cells to express levels of PMP22 higher than control cells but lower than homozygous ones. Although highly speculative, it is tempting to interpret these results by the presence of two populations of Schwann cells. The analysis of the frequency distribution of cell perimeters supports the latter hypothesis. In fact, frequency histograms clearly demonstrate that hemizygous transgenic Schwann cells after exposure to the axon or to FSK show a bimodal distribution, suggesting that a population of cells has a tendency to differentiate whereas another one shifts toward an undifferentiated phenotype. Instead, homozygous $PMP22_{tg}$ Schwann cells remain all in an undifferentiated stage, and control ones show a general tendency to increase their perimeter as in normal differentiation in vitro (Morgan et al., 1991; Sobue et al., 1986).

In conclusion, we propose that hemizygous $PMP22_{tg}$ Schwann cells, in the early phases of their development, still behave as normal cells in terms of motility, migration and shaping properties. Then, exposure to axonal-derived stimuli induces, in transgenic cells, a further increase of PMP22 production, leading to significant changes in the behavior of the Schwann cell, which is unable to correctly differentiate and switch on the myelination process. All these features are exasperated in the homozygous situation, which, although not representative of the human CMT1A genotype and phenotype, is important to study the general effects of PMP22 on cell function. Knowing early changes in the behavior of Schwann cells overexpressing PMP22 could be of help, in the future, to assess possible pharmacological approaches to prevent these abnormalities and to treat CMT 1A in humans.

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