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Improvement of Nerve Regeneration in Tissue-Engineered Skin Enriched with Schwann Cells

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The incorporation of Schwann cells in reconstructed skin (RS) could have a major role in achieving functional recovery of cutaneous sensory perception. We showed with a unique *in vitro* model of a tissue-engineered innervated reconstructed dermis that Schwann cells promoted a twofold increase in the number of sensory neurites migrating in the three-dimensional tissue as compared with the control. In addition, Schwann cells spontaneously colocalized along neurites and achieved the formation of myelin sheaths *in vitro* as assessed by transmission electron microscopy. We prepared RS samples enriched or not with Schwann cells and transplanted them on nude mice for 60–90 days. We demonstrated that Schwann cells induced a 1.8- and 1.7-fold increase in the number of nerve fibers migrating in the graft 60 and 90 days after transplantation, respectively. In addition, the RS sample enriched with Schwann cells had a current perception threshold similar to that of normal skin for the large and myelinated Aβ-sensory fibers, in contrast with the control. Thus, we showed that the addition of Schwann cells to tissue-engineered skin not only enhanced nerve migration but also promoted myelin sheath formation *in vitro* and nerve function recovery *in vivo*.

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INTRODUCTION

The major purpose of covering deep and extensive burns is the restoration of the barrier function of the skin to prevent fluid loss and bacterial infection. Meanwhile, burns also destroy the nerve components of the skin responsible for pain, temperature, and sensory perceptions. Peripheral nerves have the capacity to regenerate through nerve migration in healing tissue, but patients complain about poor discriminative sensibility and/or hyperesthesia and dysesthesia after a conventional split-thickness skin graft (Ward and Tuckett, 1991; Malenfant *et al.*, 1998; Ward *et al.*, 2004; Nedelec *et al.*, 2005).

The cutaneous sensory nerve fibers are broadly classified as Aβ-, Aδ-, or C-fibers on the basis of the degree of myelination and the speed at which action potentials travel along afferent fibers (Gardner *et al.*, 2000). The thicker myelinated Aβ-fibers innervating hair shafts and specialized mechanoreceptors such as Pacini's corpuscles mediate touch perception, whereas Aδ- and C-fibers are important thermoreceptors and nociceptors (Gardner *et al.*, 2000).

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Abbreviations: GFAP, glial fibrillary acidic protein; IRD, innervated reconstructed dermis; RS, reconstructed skin

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We developed a collagen–chitosan sponge, which was used as a scaffold to grow dermis and epidermis. We have previously shown that, once transplanted on nude mice, our biomaterial-based human reconstructed skin (RS) allowed for the migration of new sensory nerve fibers, detected by immunofluorescent staining of the neurofilament M, an intermediate filament of the cytoskeleton specific to neurons (Gingras *et al.*, 2003b). Linear arrangements of migrating Schwann cells were also observed in those experiments.

We further showed that the incorporation of recombinant laminin directly into the biomaterial enhanced cutaneous nerve regeneration (Caissie *et al.*, 2006). Laminin is an adhesion molecule that is normally produced by Schwann cells to create a basement membrane when axonal contact occurs (Clark and Bunge, 1989). Laminin is produced by neither neurons nor fibroblasts (Bunge *et al.*, 1980, 1982).

Schwann cells have been shown to promote nerve regeneration when embedded in high concentrations in specialized peripheral nerve conduits or used to treat spinal cord injuries (Hou *et al.*, 2006; Oudega and Xu, 2006). However, there is no evidence in the literature indicating whether Schwann cells can improve nerve regeneration when randomly distributed within a whole tissue.

Our goal was to investigate whether the incorporation of Schwann cells in an RS sample could improve global nerve regeneration *in vitro* and *in vivo*. First, we investigated the effect of Schwann cells on axonal migration and myelination *in vitro* in a model of innervated reconstructed dermis (IRD). Second, we analyzed the effect of Schwann cells on nerve regeneration and nerve function recovery *in vivo* via the transplantation of tissue-engineered RS on athymic mice.

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RESULTS

Schwann cells increased nerve migration *in vitro* in the IRD

Human dermal fibroblasts were cultured into collagen–chitosan sponges for 2 weeks. Sensory neurons alone or with Schwann cells were then seeded on the top of the construct. No difference was observed after 7 days of culture with neurons (Figure 1a vs b), but there were more nerve fibers 14 days after the addition of neurons in the IRD with Schwann cells, as assessed by NF160 staining (Figure 1d vs c). There were 2.15 times more nerve fibers per mm^2 in the IRD enriched with Schwann cells compared with the control without Schwann cells at day 14 (Figure 1e; $P<0.002$; $n=6$).

Myelin sheath formation around neurites in the IRD

We have previously demonstrated that incorporation of laminin in the sponge enhanced nerve regeneration in RS (Caissie *et al.*, 2006). Schwann cells are the only cell type known to secrete the neural adhesion molecule laminin in our model (Bunge *et al.*, 1980, 1982). Therefore, any laminin detected would originate from them. To detect the colocalization of laminin with nerve fibers, we performed a series of three double indirect immunofluorescent stainings: NF160 and glial fibrillary acidic protein (GFAP) (Figure 2a), laminin and GFAP (Figure 2b), and laminin and NF160 (Figure 2c). We found that Schwann cells were often closely associated with neurites (Figure 2a). They also deposited laminin into the surrounding extracellular matrix (Figure 2b), whereas nerve fibers were often colocalized with laminin deposits (Figure 2c). Schwann cells also formed myelin sheaths around some sensory neuron axons in the IRD, as assessed by transmission electron microscopy (Figure 2d and e, arrow). Panel e is an enlargement of panel d (white square).

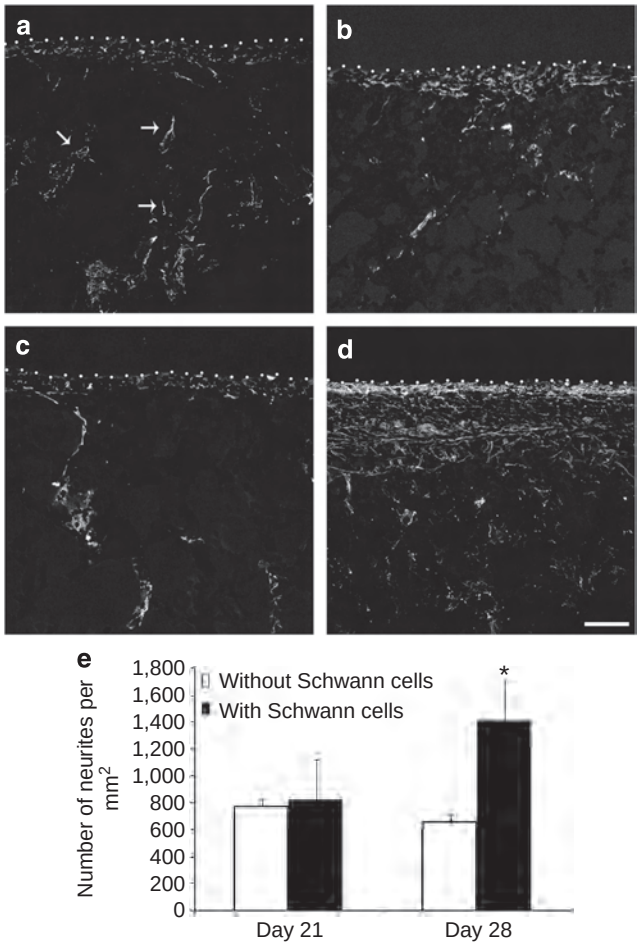

Figure 1. Quantification of axonal migration in a reconstructed connective tissue enriched with Schwann cells *in vitro*. Sensory neurons were seeded alone (**a** and **c**) or with Schwann cells (**b** and **d**) for 7 (**a** and **b**) or 14 days (**c** and **d**). Axonal migration was assessed by immunofluorescent staining of the 160 kDa neurofilament (**a**–**d**). The arrows in panel **a** indicate examples of nerve fibers. The dotted lines indicate the upper sides of the collagen sponges where the cells were seeded. (**e**) The number of neurites was expressed per mm^2. The graph is a representation of the mean results ± SD obtained for each condition (*$P\leqslant0.002$; $n=6$). Bar = 100 µm.

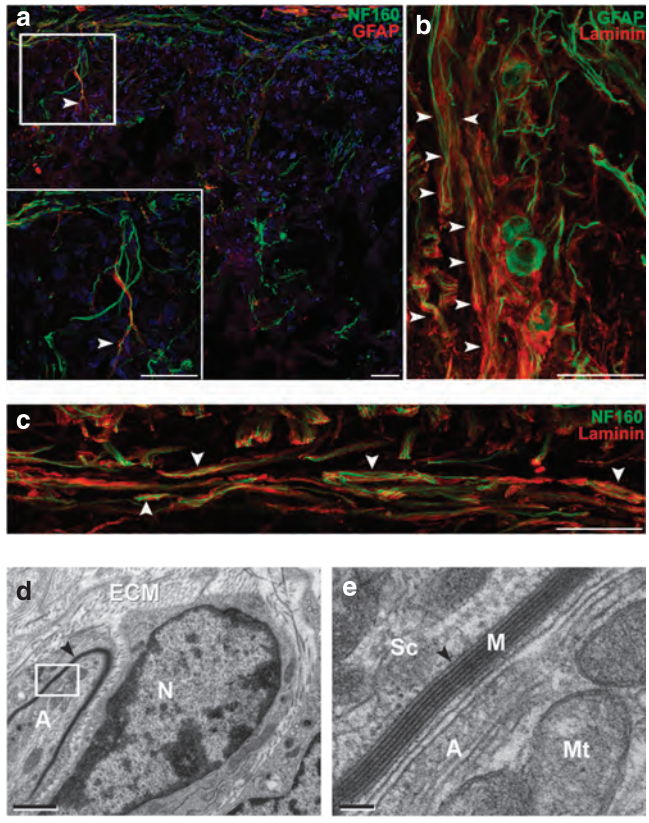

Figure 2. Detection of laminin and myelin sheaths in the reconstructed connective tissues enriched with Schwann cells. (**a**) Double immunofluorescent staining of neurons (160 kDa neurofilament, green) and Schwann cells (GFAP, red) on day 14. Schwann cells are colocalized with neurites (arrowheads). (**b**) Double immunofluorescent staining of Schwann cells (GFAP, green) and laminin (red). The GFAP staining is detected inside the laminin staining (arrowheads). (**c**) Double immunofluorescent staining of neurons (160 kDa neurofilament, green) and laminin (red). The 160-kDa neurofilament staining colocalized with the laminin staining (arrowheads). (**d**–**e**) The extent of myelination was analyzed by transmission electron microscopy (TEM). Myelin sheaths (arrowheads) were found around some sensory neurites. The box in panel **d** is enlarged in panel **e**. A, axon; ECM, extracellular matrix; M, myelin; Mt, mitochondria; N, nuclei; Sc, Schwann cell. Bar = 50 µm (**a**–**c**), bar = 1 µm (**d**), and bar = 0.1 µm (**e**).

Schwann cells enhanced axonal migration *in vivo*

To investigate whether Schwann cells have the potential to enhance cutaneous nerve regeneration *in vivo*, we reconstructed a human RS sample enriched with Schwann cells (Figure 3b) or not (Figure 3a) for 28 days in culture *in vitro*. The RS samples enriched (Figure 3d) or not (Figure 3c) with Schwann cells showed a thick and well-differentiated epidermis that persisted 90 days after grafting on athymic mice without signs of necrosis.

Nerve migration was monitored by the indirect immunofluorescent staining of the 160-kDa neurofilament in the RS samples enriched or not with Schwann cells 60 and 90 days after grafting (Figure 4). A 1.81-fold increase in the number of nerve fibers was detected 60 days after graft in the RS sample enriched with Schwann cells compared with the control (Figure 4e, b vs a; $P \leqslant 0.05$; $n = 4$). There were 1.71 times more nerve fibers in the RS with Schwann cells 90 days after grafting (Figure 4e, d vs c; $P \leqslant 0.005$; $n = 4$). However, a much smaller number of nerve fibers was observed in the RS after grafting compared with the IRD *in vitro*, because the number of neurites *in vitro* depends on the number of neurons added in the model, a large number being more convenient for imaging.

Schwann cells enhanced current perception recovery in the RS

A current perception threshold analysis in the RS was performed 90 days after grafting with a Neurometer as described in the Materials and Methods section. The current perception threshold for the Aδ- and C-nerve fibers did not show a significant difference between the normal mouse skin and the graft enriched or not with Schwann cells (Figure 5). The current perception threshold of the large and myelinated Aβ-fibers was significantly higher ($P < 0.05$; $n = 4$) in the graft without Schwann cells compared with normal skin, indicating a decreased functionality of these fibers. By contrast, no significant difference was observed in the current perception

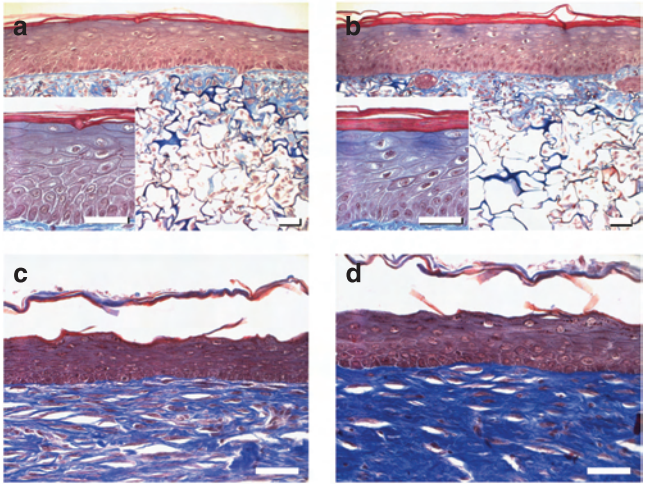

Figure 3. Histology of the reconstructed skin (Masson's trichrome staining). Tissue-engineered skin enriched (**b**) or not (**a**) with Schwann cells was cultured for 28 days *in vitro* and grafted on nude mice for 90 days (without Schwann cells (**c**) and with Schwann cells (**d**)). Bars = 50 μm (**a**–**d**).

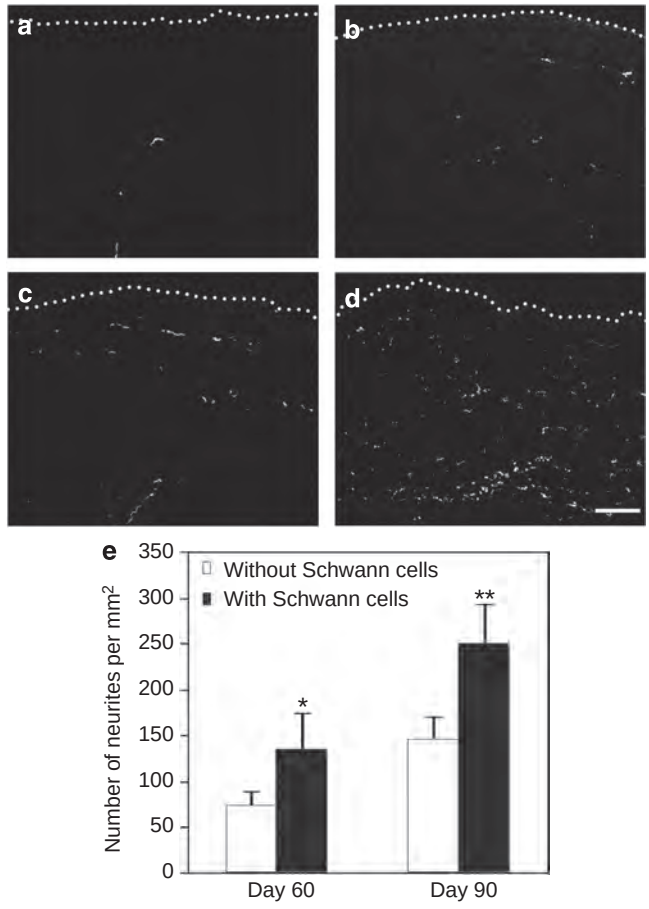

Figure 4. Quantification of the nerve migration in the reconstructed skin (RS) enriched with Schwann cells and grafted on mice. (**a**–**d**) The innervation was revealed by immunofluorescent staining of the 160-kDa neurofilament on biopsies taken from the reconstructed skin enriched (**b** and **d**) or not (**a** and **c**) with Schwann cells, 60 days (**a** and **b**) and 90 days (**c** and **d**) after the graft. The dotted lines indicate the upper sides of the graft. (**e**) Nerve fibers were counted and divided by the total area of the graft (in mm^2). The graph is a representation of the mean results ± SD obtained for each condition. (*$P \leqslant 0.05$, **$P \leqslant 0.005$; $n = 4$). Bar = 100 μm.

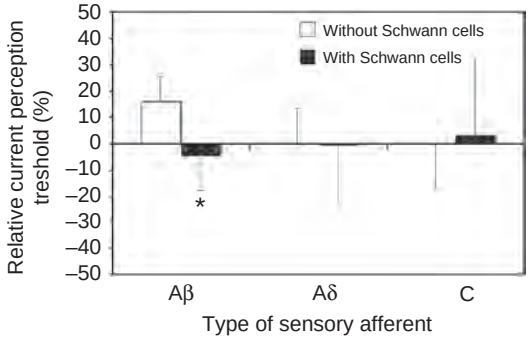

Figure 5. Current perception threshold analysis 90 days after the graft. The results are presented as the percentage of the relative current perception threshold of the graft with respect to the perception threshold for the corresponding normal skin of the same mouse, positive values indicating hypoesthesia, and negative values hyperesthesia. Aβ-, Aδ-, and C-nerve fibers were recruited using an electrical sine wave stimulus at 2,000, 250, and 5 Hz, respectively (*$P < 0.05$; $n = 4$).

threshold of Aβ-fibers in the graft enriched with Schwann cells compared with normal mouse skin (Figure 5).

DISCUSSION

Schwann cells have a major role in the promotion and organization of axonal migration in the peripheral nervous system. However, they have mostly been studied in large nerves after an injury, not in the regeneration of the thin nerve network of the whole target organ. Skin is a perfect organ in which to study this process because its sensory nerve network can be easily visualized and functionally tested *in vivo*.

First, we developed an *in vitro* model to study axonal migration through a three-dimensional reconstructed connective tissue. The major questions to be addressed in such a model concerned the ability of Schwann cells to enhance neurite migration, to migrate and organize around neurites, and finally to achieve *in vitro* the myelination and formation of myelin sheaths around neurites.

Using our unique IRD model, we showed that Schwann cells induced a 2.15 times increase in the number of neurites migrating through the tissue compared with the control without Schwann cells. In addition, these cells were shown to be colocalized with neurites, showing that they were able to migrate through the tissue and organize around neurites in a very physiological process. We also showed that Schwann cells deposited large amounts of laminin in their close vicinity that could participate in the promotion of axonal migration (Bunge *et al.*, 1980, 1982; Clark and Bunge, 1989). They are also known to secrete neurotrophic factors that enhance nerve migration and stabilize neurites (for a review, see (Boyd and Gordon, 2003; Lykissas *et al.*, 2007)). Moreover, Schwann cells produced myelin sheaths that wrapped around neurites *in vitro*. Myelin sheath formation is a crucial process for achieving nerve function recovery, as Aβ- and Aδ-nerve fibers need to be myelinated *in vivo* to propagate nerve impulses efficiently. This myelin sheath formation *in vitro* suggested that Schwann cells added to a tissue-engineered organ could participate in the myelination of migrating axons *in vivo*.

The second step was to investigate the *in vivo* effect on nerve migration of Schwann cells cultured in RS transplanted into nude mice. Our purpose was to analyze whether Schwann cells would survive for at least the 25 days of *in vitro* maturation of the RS before grafting and whether they would have any beneficial effect on nerve migration after grafting. We prepared an RS sample enriched or not with Schwann cells and grafted it onto the backs of athymic mice. We also incorporated into this RS sample a network of capillary-like tubes to promote rapid vascularization of the graft as a routine method to optimize graft take and integration into the wound (Tremblay *et al.*, 2005). The human epidermis reconstructed *in vitro* persisted after grafting with no signs of necrosis for up to 90 days. The addition of Schwann cells to the RS induced a 1.81- and 1.71-fold increase in the number of nerve fibers migrating in the graft 60 and 90 days after transplantation, respectively. Schwann cells could promote nerve regeneration through both the deposition of laminin in the extracellular matrix, which has previously been shown to enhance innervation (Caissie *et al.*, 2006), and the secretion of growth factors, or by a faster myelination of axons. We investigated whether Schwann cells could improve the perception threshold of the RS with regard to the normal mouse skin. We showed that our model of RS, with or without Schwann cells, promoted optimal regeneration of the Aδ- and C-nerve fibers, achieving a current perception threshold in the graft similar to that of normal mouse skin. By contrast, the current perception threshold of the Aβ-fibers was significantly higher than that of the normal skin in the RS without Schwann cells, suggesting a hypoesthesia of the graft compared with normal skin. This deficit of perception was not observed in the RS enriched with Schwann cells, indicating that these cells enhanced the regeneration of functional Aβ-fibers (those responsible for the sense of touch).

The improvement in the density of nerve fibers migrating in the graft, as well as its perception recovery, suggests that Schwann cells can promote faster and more efficient cutaneous nerve regeneration *in vivo*, even when they are randomly incorporated into tissue-engineered skin.

The major limitation in the use of Schwann cells from a clinical perspective to promote nerve regeneration in tissue-engineered organs is their availability. These cells can be isolated from a peripheral nerve biopsy, which may cause morbidity. However, recent studies have demonstrated that Schwann cells can be differentiated from human adult stem cells isolated from a skin biopsy (Biernaskie *et al.*, 2006, 2007). Skin biopsies can be harvested from patients with minimal morbidity and could be a valuable source of stem cells for tissue engineering.

Our work has demonstrated that Schwann cells randomly incorporated into a connective tissue promote nerve regeneration in the whole organ and enhance large myelinated nerve fiber regeneration and recovery of function. Schwann cell enrichment could be a valuable strategy for increasing nerve regeneration in any tissue-engineered organ that needs fast recovery of its nerve function after transplantation.

MATERIALS AND METHODS

Cell cultures

Fibroblasts and keratinocytes were isolated from human skin biopsies after breast reductive surgeries as previously described (Germain *et al.*, 1993; Auger *et al.*, 1995). The study was approved by the Laval University research ethical committee; participants gave their written informed consent. Fibroblasts used between the third and the sixth passages were grown in the Dulbecco–Vogt modification of Eagle's medium (Flow Lab., Mississauga, ON, Canada) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), 100 U ml^{-1} penicillin, and 25 mg ml^{-1} Gentamicin (Schering, Pointe-Claire, QC, Canada)—this culture medium is abbreviated as DME_c. Schwann cells were isolated from mouse sciatic nerves using a modified version of a previously described method (Komiyama *et al.*, 2003). Briefly, sciatic nerves were cut into 1-mm sections and cultured as explants in petri dishes for 3–4 weeks in DME_c. The explants were transferred to new petri dishes every week. To harvest Schwann cells, nerve explants were digested in DME_c containing 0.05% collagenase IA (Sigma-Aldrich, Oakville, ON, Canada),

0.1% dispase II (Roche, Laval, QC, Canada), and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) for 3 hours at 37 °C. The mixture was then triturated and centrifuged. Cells were suspended in DME_c supplemented with 10 μM forskolin (Sigma-Aldrich) and 10 ng ml^{-1} human neuregulin (NRG1-β1/HRG1-β1 EGF domain; R&D Systems, Minneapolis, MN) and cultured onto poly-D-lysine- (10 μg ml^{-1}; Sigma-Aldrich) and laminin (10 μg ml^{-1}; Sigma-Aldrich)-coated flasks.

Sensory neurons were extracted from the dorsal root ganglia of mouse embryos (E13) as described previously (Gingras *et al.*, 2003a). Sensory neurons were added fresh to the tissue-engineered models.

Human umbilical vein endothelial cells were obtained from healthy newborns by enzymatic digestion with 0.25 μg ml^{-1} thermolysin (Sigma-Aldrich) as previously described (L'Heureux *et al.*, 1998) and grown in endothelial growth medium 2 (Cambrex Bioscience, Baltimore, MD).

Preparation of the collagen–chitosan biomaterial

Collagen sponges were prepared as described previously (Berthod *et al.*, 1994, 1997) but without chondroitins 4–6 sulfates. Briefly, types I and III bovine collagen (Symatese, Chaponost, France) and chitosan (Kemestrie, Sherbrooke, QC, Canada) were dissolved in 0.1% acetic acid and mixed, and 1 ml per well of the final solution was poured into 12-well plates (for the *in vitro* experiments) or 2.5 ml per well into 6-well plates (for the *in vivo* experiments) (BD Biosciences, Mississauga, ON, Canada) and frozen at −80 °C for 1 h. The frozen plates were then lyophilized in a vacuum lyophilizer (Dura-Stop Microprocessor Controlled Tray Freeze-Dryer; FTS Systems, Stone Ridge, NY).

Preparation of the reconstructed tissues

Preparation of the IRD sample for in vitro testing. The IRD sample was prepared from a 3.8-cm^2 collagen–chitosan sponge seeded with human fibroblasts at 2.1×10^5 cells per cm^2 and cultured in DME_c supplemented with 100 μg ml^{-1} ascorbic acid. A suspension of sensory neurons was seeded at 2.1×10^5 cells per cm^2 14 days later, and the IRD sample was then lifted to the air–liquid interface and cultured in 3:1 ratio of DMEM–Ham's F-12 medium (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum, 0.4 μg ml^{-1} hydrocortisone (Calbiochem, La Jolla, CA), 5 μg ml^{-1} bovine insulin (Sigma-Aldrich), 10 ng ml^{-1} NGF (nerve growth factor)-2.5S (natural murine; Invitrogen), and 100 μg ml^{-1} ascorbic acid and antibiotics. A suspension of Schwann cells was seeded at 2.1×10^5 cells per cm^2 on the IRD at the same time as the neurons (day 14). The neurons were allowed to innervate the IRD in the presence or absence of Schwann cells for 7 or 14 additional days.

Preparation of the RS sample for in vivo testing. Collagen–chitosan sponges (9.6 cm^2) were first seeded with a 1:1 suspension of human fibroblasts and human umbilical vein endothelial cells at 4.2×10^5 cells per cm^2 and cultured in a 1:1 ratio of endothelial growth medium 2–DME_c supplemented with 25 μg ml^{-1} ascorbic acid. Three days later, a suspension of Schwann cells was seeded at 2.1×10^5 cells per cm^2 on half of the sponges. The sponges were then cultured in a 1:1 ratio of endothelial growth medium 2–DME_c supplemented with 25 μg ml^{-1} ascorbic acid, 5 μM forskolin, and 5 ng ml^{-1} human neuregulin. On day 7, a suspension of human keratinocytes (third passage) was seeded on all samples at 2.1×10^5 cells per cm^2. These samples were cultured for 7 additional days in DME–Ham's F-12 medium in a 3:1 ratio (Flow Lab.) supplemented with 24.3 μg ml^{-1} adenine, 10 ng ml^{-1} human EGF (Chiron, Emeryville, CA), 0.4 μg ml^{-1} hydrocortisone (Calbiochem), 5 μg ml^{-1} bovine insulin, 5 μg ml^{-1} human transferrin, 2×10^{-9} M 3,3′,5′-triiodo-L-thyronine, 100 μg ml^{-1} ascorbic acid, 10^{-10} M cholera toxin (Schwarz/Mann, Cleveland, OH), 5 μM forskolin, 5 ng ml^{-1} human neuregulin, antibiotics, and 10% newborn calf serum (Fetal Clone II, Hyclone, Logan, UT). The RS were then lifted to the air–liquid interface and cultured for 14 additional days without EGF.

Animals and surgical manipulations. Adult male athymic nu/nu mice (56 days old) (Charles River Laboratories, LaSalle, QC, Canada) were used as surgical recipients, as previously described (Gingras *et al.*, 2003b). The mice were injected with ceftazidime (140 mg per mouse; Glaxo, Toronto, ON, Canada) 24 and 48 hours before surgery. Penicillin G and gentamicin (100 U ml^{-1} and 25 mg ml^{-1}, respectively; Sigma, Mississauga, ON, Canada) were also added to their sterile drinking water. Animals were anesthetized via inhalation of 3% isoflurane with 1.5 l minute^{-1} oxygen (lowered to 2% for surgery). A 2.5×2.5 cm^2 full-thickness skin area was excised to the muscle, on the back of the mouse. A Fusenig chamber was installed in the wound and stitched to the mouse skin, to protect the RS after grafting. The Fusenig chamber was removed after 30 days, and the mouse skin was stitched to the graft margins. Mice were killed 60 and 90 days after grafting for histological and immunohistochemical analysis of RS biopsies. All the manipulations of the animals were performed according to the rules established by the Canadian Council on Animal Care.

Current perception threshold testing. Neurosensory recovery was evaluated by testing Aβ-, Aδ-, or C-fibers with a Neurometer (Neurotron, Baltimore, MD) 90 days after grafting. The Neurometer evaluation uses three different frequencies of an electrical sine wave stimulus: 2,000, 250, and 5 Hz for the specific stimulation of Aβ-, Aδ-, and C-fibers, respectively (Katims *et al.*, 1986; Masson *et al.*, 1989; Anon, 1999; Caissie *et al.*, 2007). The apparatus was connected to each animal by two self-sticking electrodes. One was placed on the graft site, the other on the proximal tail. Each animal was placed in a restraint system for easy manipulation and to prevent chewing of the electrodes. The current intensity (in mA) was increased until the mouse showed a tail flick, which defined the current stimulus threshold. This stimulation procedure was repeated until the same current intensity was obtained on three consecutive stimulations. The relative current perception threshold percentage was calculated as follows: ((graft current intensity/normal skin current intensity of the same mouse) × 100)−100.

Indirect immunofluorescence staining. Indirect immunofluorescence assays were performed on tissue sections that had been treated with formaldehyde (3.7%) followed by methanol (100%) and then frozen embedded in OCT (optimal cutting temperature) compound (Sukura; Finetek USA, Torrance, CA). The primary antibodies used were rabbit polyclonal anti-neurofilament M (160 kDa), mouse monoclonal anti-GFAP (both from Upstate International, Temecula, CA), and rat monoclonal anti-laminin (Abcam, Cambridge, MA). The secondary antibodies used were goat anti-rabbit IgG Alexa Fluor 488, goat anti-mouse IgG Alexa Fluor 594, and donkey anti-rat IgG Alexa Fluor 594 (all from Invitrogen). Cell nuclei were labeled with Hoechst reagent 33258 (Sigma-Aldrich). As a control, the primary antibody

was omitted (data not shown). Sections were viewed using a Nikon C1 laser scanning confocal microscope (Nikon, Mississauga, ON, Canada). Images were collected from the center of the RS dermis. The serial images collected throughout one section using the confocal microscope were summed to give a two-dimensional representation of the full depth of the field. Images were processed using Adobe Photoshop CS3 (Adobe Systems, San Jose, CA).

Electron microscopy. For electron microscopy analysis of myelin sheath formation, tissues were fixed overnight at 4 °C in 2.5% glutaraldehyde, washed with 0.1 M cacodylate buffer, and postfixed with 1% OsO_4 for 30 minutes. Regions of interest were identified and embedded in LRWhite (London Resin, Theale, Berkshire, UK). Ultra-thin sections stained with uranyl acetate were observed with a transmission electron microscope (JEOL JEM-1230; Soquelec, Montreal, Canada).

Quantification of innervation. For *in vitro* quantification, 160 kDa neurofilament-positive neurites detected in transversal sections of the IRD were counted in five representative fields and expressed per mm^2 . For each condition, a total of six IRD samples were assessed. For *in vivo* assessment of innervation, neurites detected in whole transversal sections of the graft were quantified as previously described (Caissie *et al.*, 2006). A total of four biopsies per condition were taken on day 60, and a total of five biopsies per condition were taken on day 90. Values are expressed as the means ± SD. Differences were tested by bilateral Student's *t*-test. The probability level was regarded as significant at $P<0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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