Cell Transplantation, Vol. 15, pp. 733–744, 2006
Printed in the USA.

0963-6897/06 $90.00 + .00
E-ISSN 1555-3892
www.cognizantcommunication.com

Establishment of Cocultures of Osteoblasts, Schwann Cells, and Neurons Towards a Tissue-Engineered Approach for Orofacial Reconstruction

Kirsten Haastert,*† Nicole Semmler,* Maike Wesemann,* Martin Rücker,‡ Nils-Claudius Gellrich,‡ and Claudia Grothe*†

*Department of Neuroanatomy, Hannover Medical School, Hannover, Germany
†Center of Systems Neuroscience (ZSN) Hannover, Hannover, Germany
‡Department of Oral and Maxillofacial Surgery, Hannover Medical School, Hannover, Germany

In orofacial reconstruction not only the osseous structures themselves but also neighboring cranial nerves need to be regenerated. To replace autologous bone implants, biocompatible tissue-engineered scaffolds are under investigation at least for bone replacement but until now these studies have not focused on parallel reconstruction of injured cranial nerves. The present study contributes to the development of optimized tissue-engineered products that will enable regeneration of both bone and nervous tissue. For the first time, cocultures of primary osteoblasts (rat or human) and primary Schwann cells (rat or human) were established. The suitability of monocultures of osteoblasts and cocultures of osteoblasts plus Schwann cells as substrate for sensory neurons as well as motoneurons was tested here. The results suggest that whereas osteoblasts provide a good substrate for sensory neurons, motoneurons depend on the presence of Schwann cells for survival and neurite outgrowth. For prolonged availability of regeneration-promoting growth factors at the site of the graft, those proteins should be delivered by the transplanted cells themselves. To enable this, we established electroporation-based nonviral transfection of osteoblasts as well as Schwann cells. Our new cell culture system will enable investigations of the effect of graft-derived growth factors on osteoblasts and Schwann cells as well as on neurite outgrowth from cocultured neurons of the sensory and motor system.

Key words: Orofacial reconstruction; Tissue engineering; Osteoblast; Schwann cells; Bone tissue; Nervous tissue

INTRODUCTION

Bone defects of the skull still pose a problem of significant clinical relevance. Craniofacial defects can be of posttraumatic origin or result from congenital deformations or even from ablative tumor surgery. Herein the fast surgical replacement of bone is necessary even if bone possesses an intrinsic repair capacity (34). Thus, not only the osseous structures themselves but also neighboring cranial nerves are frequently affected.

The "gold standard" for the reconstruction of bone tissue is autogenous bone transplantation (35). Harvesting of autogenous bone transplants results in an additional donor site morbidity, unfavorable scar formation, insufficient wound healing, or recurrent pain (34). Tissue-engineering strategies are promising to replace harvesting of autologous transplants by the development of innovative bone substitutes. This attempt combines harvesting, isolation, and culture of autologous and allogene cells as well as seeding of these cells on biocompatible materials that are substituted with extracellular matrix proteins (19,41,43). Osteoblasts accomplish rapid osteoid matrix production (34). With regard to bone repair it was shown that osteoblasts seeded on bioresorbable matrices or scaffolds increase reconstruction and healing of bony defects because of an enhanced osteogenic potency compared to common bone substitutes (21).

Similar to replacement and regeneration of bone where three main strategies may be used alone or in combination (34), also in peripheral nerve reconstruction and regeneration, matrix-based therapies as well as factor-based therapies and cell-based therapies are under investigation (24). Similar to bone reconstruction, the "gold standard" to perform tension-free reconnection of transected nerve stumps is the use of autologous nerve transplants (37). Availability of autologous nerve transplants is limited because harvesting of healthy nerves is

Received June 27, 2006; final acceptance August 7, 2006.
Address correspondence to Dr. med. vet. Kirsten Haastert. Department of Neuroanatomy, OE 4140, Hannover Medical School, Carl-Neuberg-Str.1, D-30623 Hannover, Germany. Tel: +49-511-532-2891; Fax: +49-511-532-2880; E-mail: haastert.kirsten@mh-hannover.de

needed for this technique and is accompanied by donor site morbidity (24). Synthetic nerve bridges can be modulated to assist peripheral nerve regeneration by addition of extracellular matrix proteins (4,5,26), neurotrophic factors (1,7,9), or Schwann cells (2,6,14,22,32). Schwann cells, the myelinating and nonmyelinating glial cells of the peripheral nervous system, are crucially involved in peripheral nerve regeneration processes by forming bands of Büngner to guide regenerating axons along the Schwann cell basal lamina and by producing a regeneration-promoting environment via expression of neurotrophic and other growth factors (3,24). Synthetic nerve conduits stimulated axonal regeneration in rat models after seeding the material with neonatal (18,28,29) or adult rat Schwann cells (2,14).

For the development of tissue-engineered constructs that will enable rapid bone reconstruction in parallel to enhanced regeneration of adjacent peripheral nerves, cell types should be used that are crucially involved in both processes. Nothing has been known so far about the suitability of osteoblasts used as a substrate for neurons and neurite outgrowth. In vivo, osteoblasts never get into direct contact to neurons or peripheral neurites or axons in particular, whereas neurons and peripheral neuronal processes are physiologically accompanied by Schwann cells. Furthermore, Schwann cells and osteoblasts are not physiological neighbors in vivo as well, because bone and peripheral nerves are at least separated by connective tissue. As a first attempt, neuron survival and neurite outgrowth in the case of direct contact to osteoblasts had to be investigated. As a second attempt the feasibility to culture and grow osteoblasts and Schwann cells side by side had to be tested because the presence of Schwann cells could positively modulate osteoblast effects on neurons and neurite outgrowth.To combine cell-based therapies with factor-based therapies for regeneration, it would be favorable if the transplanted cells could be genetically modified to produce regeneration-promoting proteins over prolonged periods at the implantation site.

The aim of the current study was to establish methods that will allow the development of a complex implant made of cell-seeded bone substitutes for bone and peripheral nerve regeneration. Therefore, osteoblastic cells and Schwann cells, alone and in mixed populations, were cocultured with neurons to investigate differential effects of the different cellular substrates on neurons of the sensory and the motor system. Furthermore, a method for nonviral transfection of adult human osteoblasts was evaluated to serve as a basis for genetic modification of these cells prior to implantation and to produce cells that will provide a source of biologically active regeneration-promoting proteins after implantation and a prolonged delivery of graft-derived growth factors.

MATERIALS AND METHODS

Culture Conditions for Monocultures of Human and Rat Osteoblasts

Rat osteoblast-like cells were grown from tibial bone of 3-month-old female Sprague-Dawley rats. Cells were cultured on noncoated culture dishes using Dulbecco's modified Eagle medium (DMEM)/Ham's F12 supplemented with 10% fetal calf serum (FCS) and 4 mmol L-glutamine and penicillin/streptomycin (100 U/ml/100 µg/ml) (all PAA, Germany).

Human osteoblast-like cells grown from maxillar bone of a 20-year-old male patient were cultured under the same conditions as described above. Sister cultures of rat and human cells were at times identified as osteoblasts by their production of alkaline phosphatase, which is exclusively produced by osteoblasts or by von Kossa staining of calcified extracellular matrix (33).

The use of rat and human tissue for initiating cell cultures was permitted by the Bezirksregierung Hannover or the ethics committee of the Hannover Medical School, respectively.

Conditions for Seeding of Sensory Neurons on Different Cellular Substrates

First, to compare the capacity of rat or human adult osteoblasts to promote survival and/or axonal outgrowth when used as a cellular substrate for sensory neurons, substrate cells were grown in monolayers and sensory neurons were seeded on top.

Therefore, dorsal root ganglia (DRG) of neonatal Sprague Dawley rats on postnatal day 1–3 (P1–P3) were collected and dissociated. Sensory neurons did not go through any enrichment procedure. After seeding dissociated DRG cells on top of osteoblastic cells and other cell types as below, the following medium suitable for cultures of sensory neurons was used: DMEM/Ham's F12 added with 0.25% BSA, 2 mmol L-glutamine, 1 mmol sodium pyruvat, penicillin/streptomycin (100 U/ml/100 µg/ml), N1-supplement (apo-transferrin, putrescine, progesterone, sodium selenit), and insulin. Sensory neurons were cultured on top of the different cell types for 2–5 days. Cultures were stopped by cell fixation with 4% paraformaldehyde (PFA) in PBS.

We then evaluated the effect of adult rat or human Schwann cells on sensory neuron survival and neurite outgrowth. For this purpose, Schwann cells from both species were either cultured in mixed populations with osteoblasts from the same species (as below) or in monocultures before sensory neurons were seeded on top.

Isolation, Enrichment, and Culture of Adult Schwann Cells

Adult rat Schwann cells were obtained from in vitro predegenerated sciatic nerves of adult female Sprague-

Dawley rats (8 weeks of age, 220 g body weight, Charles River Germany) as described previously (27).

Adult human Schwann cells were obtained from in vitro predegenerated sural nerve biopsies of male and female patients as described elsewhere (17). Adult rat and human Schwann cells were seeded on poly-ornithin (PORN)-laminin (Becton-Dickinson, Europe) double-coated culture dishes and cultured in melanocyte growth medium (PromoCell, Germany) supplemented with manufacturer supplements and additional 10 ng/ml FGF-2 (30), 5 μg/ml bovine pituitary extract, and 2 μm forskolin (Calbiochem, Germany) (17,27).

For enrichment of either adult rat or human Schwann cells we used Cold Jet, an easy and effective washing step based on different adhesion properties of Schwann cells and fibroblasts (17,27). To prove high purity of adult Schwann cell cultures, Schwann cells in sister cultures were characterized by an immunocytochemical staining for the low-affinity nerve growth factor receptor, $p75^{LNGFR}$, a cell surface marker for Schwann cells in vitro (15,17,27).

Mixed Cultures of Osteoblasts and Schwann Cells

For culturing osteoblastic cells and Schwann cells in mixed cultures the respective Schwann cell medium was found to maintain cocultured osteoblasts as well, even while slightly reducing the proliferation rate of this population in comparison to osteoblasts cultured in original osteoblast medium. Adjusting to the needs of Schwann cells in coating of culture dishes, cocultures of osteoblasts with adult Schwann cells were seeded on PORN-laminin-coated surfaces.

Evaluation of Sensory Neuron Survival and Neurite Outgrowth on Different Cellular Substrates

Sensory neuron survival and neurite outgrowth behavior were analyzed after immunocytochemical identification of DRG neurons with an antibody against neuron-specific β-III-tubulin [Upstate, USA, 1:140 in PBS + 1% BSA (Fraction V) (w/v) + 0.3% Triton X-100 (v/v); all Sigma, Germany]. Cy2-conjugated goat anti-mouse IgG (Jackson, USA) were used as secondary antibodies for detection (1:200 in 1% BSA/PBS). Green (Cy2) fluorescence of secondary antibodies was visualized using a fluorescent microscope (IX70, Olympus, Germany).

In a first attempt and to exclude species effects of the cellular substrates, rat sensory neurons were seeded on top of (I) rat osteoblast monocultures or (II) human osteoblast monocultures, respectively. For control, (III) PORN-laminin coating was used as a noncellular substrate.

In a second step beside rat osteoblast monocultures (IVa), mixed cultures of rat osteoblasts and adult rat Schwann cells (Va) or adult rat Schwann cell monocultures (VIa) were used as substrates for rat sensory neurons. Noncellular PORN-laminin coating (VIIa) served as control substrate. The same experiments (IVb–VIb) were done for substrate cells of human origin with (VIIb) noncellular PORN-laminin coating as control.

In all settings, neuron survival as well as neurite outgrowth behavior was analyzed in terms of (i) number of neuronal processes per soma, (ii) number of branches per neurite, and (iii) length of the longest branch (axon). The morphometric measurements were done using the AnalySIS (Soft Imaging Systems, Münster, Germany) computer software package. Numbers of branches per neurite were determined in an area of the approximately doubled cell diameter.

Conditions for Seeding of Motoneurons on Different Cellular Substrates

Rat embryonic spinal motoneurons grow and survive well when seeded on top of a neonatal rat Schwann cell feeder layer (15). As this cell culture system is well established in our lab, we decided to address the question whether an osteoblast cellular substrate will influence survival and/or axonal outgrowth of motoneurons, by modifying this system. Motoneurons were harvested and purified by density gradient centrifugation from ventral lumbal spinal cord preparations from rat embryos (gestational day 15) as described previously (15).

Motoneurons were then seeded on top of (VIII) rat neonatal Schwann cells, (IX) a mixed population of rat neonatal Schwann cells plus human osteoblasts, or (X) human osteoblast monocultures. After seeding motoneuron-rich cell fractions on top of the different cell types the following medium was used: neurobasal medium added with 2% (v/v) horse serum, 2% (v/v) B27-supplement (GIBCO Invitrogen, Germany), 0.5 mM L-glutamine, 25 μM β-mercaptoethanol, and 10 ng/ml rHu BDNF (PromoKine, Germany). Cultures were stopped after 3 days in vitro by cell fixation with 4% PFA in PBS. Besides counting of motoneurons, the morphology of the cells when cultured on different substrates was evaluated with regard to neurite outgrowth behavior. For visualizing differences in outgrowth behavior on the different cell layers, motoneurons were immunocytochemically identified using motoneuron-specific antibodies against nonphosphorylated neurofilament-H/M (npNF-H/M) [SMI32-R, Covance, USA, 1:1000 in PBS + 1% BSA (Fraction V) (w/v) + 0.3% Triton X-100 (v/v); all Sigma, Germany]. Cy3-conjugated goat anti-mouse IgG (Jackson, USA, 1:400 in 1% BSA/PBS) was used to detect bounded npNF-H/M antibodies. Cells were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI, Sigma, Germany), resulting in blue fluorescent nuclei in all cultured cells. Schwann cells could be identified by oval and smaller nuclei compared to the obviously bigger and paler nuclei of osteoblasts after counterstaining with

DAPI, resulting in blue fluorescent nuclei. And Schwann cells were as well identified by S100 immunostaining using rabbit anti-cow S100 antibodies (Dako, 1:200 in PBS + 1% BSA + 0.3% Triton-X-100). Cy2-conjugated goat anti-rabbit IgG (Union Carbide Chemicals and Plastics Co., Inc., 1:200 in PBS) was used for detection.

In order to exclude bias of this experiment due to difference in the developmental stage or age of the substratum cells we performed a control experiment. Therefore, similar to motoneurons, neonatal rat DRG cells were seeded on top of (XI) rat neonatal Schwann cells, (XII) a mixed population of rat neonatal Schwann cells plus human osteoblasts, or (XIII) human osteoblast monocultures.

Isolation, Enrichment, and Culture of Neonatal Rat Schwann Cells

Neonatal rat Schwann cells were obtained from sciatic nerves of Sprague-Dawley rat puppies on P1–P3. After digestion of the dissected nerves in an enzyme mixture [0.175% (v/v) trypsin + 0.03% (v/v) collagenase in Hank's balanced salt solution; all PAA, Germany] and primary seeding on poly-L-lysine (PLL)-coated plastic culture surfaces (Nunclon™ Surface, Nunc, Germany) most of contaminating fibroblasts were removed by addition of arabinoside C (1 mM) for 2 days. Remaining fibroblasts were removed from the Schwann cell cultures using immunopanning by incubation with anti-Thy1 antibody-coupled magnetic beads (Dynabeads, Dynal, Denmark). Occasionally, the latter was repeated once or twice and resulted in about 99% pure Schwann cell cultures. Adjusting to the needs of Schwann cells in coating of culture dishes, cocultures of osteoblasts with neonatal Schwann cells and highly enriched neonatal Schwann cells were seeded on PLL coated dishes.

Culture medium in theses cases was DMEM supplemented with 10% FCS, 2 mmol L-glutamine, 1 mmol sodium pyruvat, penicillin/streptomycin (100 U/ml/100 µg/ml) (all PAA, Germany) and 1 µm forskolin (Calbiochem, Germany). Schwann cell purity was confirmed in sister cultures by an immunocytochemical staining for calcium binding protein S100, an intracellular Schwann cell marker (15,17,27). Cy2-conjugated goat anti-mouse IgG (Jackson, USA) was used as secondary antibody for detection and was diluted 1:200 in 1% BSA in PBS. Green fluorescence of secondary antibodies was visualized using a fluorescent microscope (IX70, Olympus, Germany). Cells were counterstained with DAPI, resulting in blue fluorescent nuclei.

Genetic Modification of Human Osteoblasts

For establishment of the transfection of osteoblasts, we used the Nucleofector technology (Amaxa, Germany), which enables nonviral gene delivery directly to the cell nuclei, by using electrical parameters in combination with cell-specific solutions. In screening experiments we used the Rat Oligodendrocyte Nucleofector™ Kit, because there is no kit available for osteoblasts. The kit comes with Nucleofector™ solution optimized for oligodendrocyte precursor cells. We tested several transfection programs to check for high transfection efficiency accompanied by good cell survival. We used 1.5 µg plasmid DNA to transfect 1.5 million cells. As a reporter gene, pEGFP-N2 was used, resulting in the production of green fluorescent protein by the transfected cells. Using a fluorescent microscope (IX70, Olympus, Germany), the percentage of transfected cells was calculated after visualizing nuclei of all cells with DAPI.

Statistical Analysis

Statistical tests were performed using the GraphPad InStat Version 3.0 software (GraphPad Software, Inc., USA). For the different experiments different statistical tests were reasonable and will be mentioned in particular in the Results section. All data are presented as mean ± SD.

RESULTS

Table 1 summarizes all the different experiments and cell combinations examined throughout this study. All parts of the study were important for the establishment of our new coculture system but, to strengthen the contribution of our study to the field of cell transplantation and tissue engineering for orofacial bone and nervous tissue repair, not all results we be presented in detail.

In a first attempt we established monocultures of osteoblastic cells from human and rat origin. The impact of osteoblastic cells when used as a cellular substrate for neurons was tested with regard to sensory neuron survival and sensory neurite outgrowth. For the establishment of our new cell culture system neonatal rat sensory neurons could be easily obtained in high amounts from dorsal root ganglia (DRG) of newborn rats.

To test modulating effects of adult Schwann cells on neuron survival and neurite outgrowth, cocultures of osteoblastic cells and Schwann cells were established with regard to the different needs of the different cell types in vitro. Suitable culture conditions were determined that allowed proliferation and maintenance of both cell types, while at the same time preventing overgrowth of one cell type by the other. In parallel we also tested the effect of Schwann cell monocultures and the noncellular PORN-laminin as substrates for sensory neurons.

In another set of experiments we also evaluated the survival and morphology of neurons of the motor system. Therefore, we modified a well-established model of embryonic rat motoneurons that are cultured on a feeder layer of neonatal rat Schwann cells (15) and cul-

Table 1. Experimental Design (Refer to Materials and Methods)

Substratum	Neuron Type Seeded on Top: Sensory Neurons	Neuron Type Seeded on Top: Motoneurons	Comment
Rat adult osteoblasts	(I) + (IVa)		Evaluation of possible interferences due to species differences between substratum and seeded sensory neurons
Human adult osteoblasts	(II) + (IVb) + (XIII)	(X)	
Rat adult osteoblasts + *rat* adult Schwann cells	(Va)		
Human adult osteoblasts + *human* adult Schwann cells	(Vb)		
Rat adult Schwann cells	(VIa)		
Human adult Schwann cells	(VIb)		
Rat neonatal Schwann cells	(XI)	(VIII)	
Rat neonatal Schwann cells + *human* adult osteoblasts	(XII)	(IX)	Evaluation of possible interferences due to developmental stage or age of substratum cells
PORN-laminin	(III) + (VIIa) + (VIIb)		Noncellular substrate as control

tured the motoneurons as well on a mixed layer of neonatal rat Schwann cells plus human adult osteoblasts or on a layer of human adult osteoblasts, respectively. To evaluate the suitability of neonatal rat Schwann cells in comparison to adult Schwann cells we also performed a control experiment. Therefore, rat DRG cells were seeded on top of mixed cultures of human osteoblasts and rat neonatal Schwann cells and monocultures of both cell types as described for motoneurons.

To enable future genetic modification of osteoblasts to overexpress regeneration-promoting proteins, we evaluated the Nucleofector™ technology for transfection of osteoblasts with marker DNA to induce expression of green fluorescent protein (GFP).

Survival of Sensory Neurons on Different Cellular Substrates

First, survival of rat neonatal sensory neurons was examined after seeding them on top of osteoblast monolayers as well as PORN-Laminin coating as control. Within this part of the study we also examined a possible species effect. For this purpose rat neonatal sensory neurons were cultured on cellular substrates from rat (I) as well as from human (II) origin. There was no difference in sensory neuron survival after seeding the cells on top of rat or human osteoblast monocultures. However, sensory neuron survival was significant higher on these cellular substrates compared to (III) noncellular PORN-laminin substrate (data not shown).

In a second attempt the suitability of monocultures of osteoblasts as cellular substrate for sensory neurons was compared with that of mixed cultures of osteoblasts and adult Schwann cells as well as monocultures of adult Schwann cells and noncellular PORN-laminin coating.

As summarized in Figure 1, we first evaluated the survival of rat sensory neurons on rat cellular substrates (Fig. 1A). No significant difference in the number of β-III-tubulin-immunopositive neurons was found 5 days after seeding of 15,000 rat neonatal DRG cells on top of (IVa) rat osteoblast monocultures (1662 ± 621 cells), (Va) a mixed population of rat osteoblasts and adult rat Schwann cells (1498 ± 866 cells), or (VIa) adult rat Schwann cell monocultures (1135 ± 485 cells). Seeding of rat neonatal DRG cells on (VIIa) a noncellular PORN-laminin coating resulted in significantly less survival of β-III-tubulin-immunopositive neurons (609 ± 322 cells, $p < 0.05$, unpaired *t*-test Welch corrected).

Second, survival of rat sensory neurons on human cellular substrates was evaluated (Fig. 1B). Therefore, data from two preparations of three or two sister cultures, respectively, resulted in data from $n = 5$ culture wells per condition and were statistically analyzed by the Mann-Whitney test. Seeding of approximately 15,000 neonatal rat DRG neurons on top of (IVb) human osteoblast monocultures resulted in 14988 ± 2511 sensory neurons that could be visualized by β-III-tubulin-immunostaining after 2 days in cultures. (Vb) Mixed cultures of human osteoblasts and adult human Schwann cells provided significantly less survival of seeded sensory neurons (11695 ± 2173 β-III-tubulin-immunopositive neurons, $p < 0.05$). However, the mixed culture substrate provided still a significantly better survival of sensory neurons compared to the (VIb) monocultures of adult human Schwann cells (5946 ± 1282 β-III-tubulin-immunopositive neurons, $p < 0.005$). After seeding of dissociated

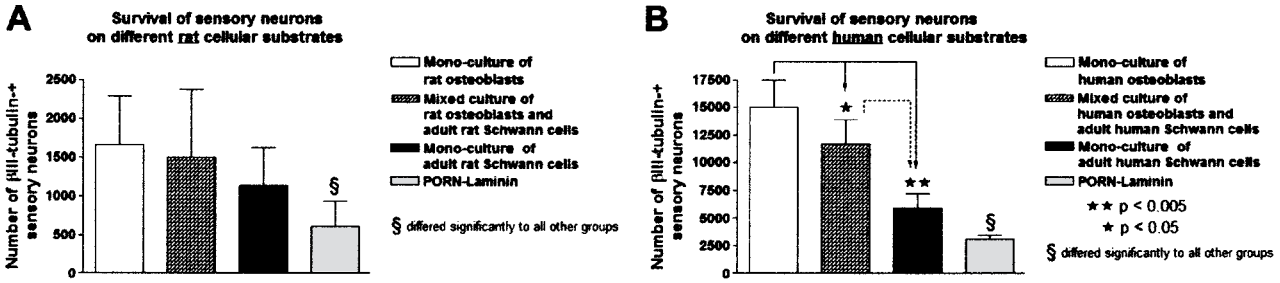

Figure 1. (A) Survival of sensory neurons cultured for 5 days on different rat cellular substrates and PORN-laminin as a noncellular control. Unpaired *t*-test with Welch correction was used for statistical analysis. (B) Survival of sensory neurons cultured for 2 days on different human cellular substrates and PORN-laminin. Mann-Whitney test was used for statistical analysis. Bars represent means ± SD.

DRG cells on top of (VIIb) PORN-laminin significantly less sensory neurons survived compared to all other conditions (3149 ± 268 sensory neurons, $p < 0.01$).

Morphological Evaluation of Rat Sensory Neurons Cultured on Different Rat Cellular Substrates

To avoid bias, we analyzed a restricted subpopulation of surviving sensory neurons with regard to morphological criteria. Therefore, sensory neurons providing a soma diameter of about 30 μm were further analyzed. Morphological data were statistically analyzed with an unpaired *t*-test with Welch correction.

First, β-III-tubulin-immunopositive neurons that had been cultured for 5 days on top of rat (I) versus human (II) osteoblast monocultures did not differ in their morphological appearance, such as (i) number of neurites extending from the cell somata, (ii) number of branches of each neurite within an area of about 60 μm around the cell soma (doubled cell soma size), and (iii) length of the longest neurite. Again, there were significant differences in all of theses criteria when both conditions were compared to (III) noncellular PORN-laminin substrate, where sensory neurons were morphologically less developed ($p < 0.005$).

Neonatal rat sensory DRG neurons were further analyzed with regard to morphological aspects after seeding them on top of (IVa) rat osteoblast monocultures, (Va) a mixed population of rat osteoblasts and adult rat Schwann cells, (VIa) adult rat Schwann cell monocultures, or (VIIa) noncellular PORN-laminin substrate. The morphological appearance of neonatal rat DRG neurons cultured on top of different rat cellular substrates is shown in Figure 2A–C. No difference was found in (i) number of neurites extending from the cell somata as a mean of three neurites extended from DRG neurons independently of the different rat cellular substrates used. Seeded on PORN-laminin with only about one process, significant less neurites extended from the soma of the analyzed β-III-tubulin-immunopositive neurons (1 ± 0.8 neurites, $p < 0.05$). The number of branches per neurite (ii) is summarized in Figure 2D; processes of sensory neurons cultured on top of (IVa) rat osteoblast monocultures showed significant more branching (2.5 ± 0.5 branches/neurite, $n = 88$ neurons analyzed) compared to neurons cultured on top of (Va) mixed populations of both rat cell types (1.6 ± 0.4 branches/neurite, $n = 89$ neurons analyzed, $p < 0.005$) or (VIa) adult rat Schwann cell monocultures (1.4 ± 1.1 branches/neurite, $n = 101$ neurons analyzed, $p < 0.05$). Branching of neurites was significantly less on (VIIa) PORN-laminin coating compared to a pure osteoblast substrate or a mixed substrate of osteoblasts and Schwann cells (0.6 ± 0.5 branches, $p < 0.005$). With regard to (iii) the lengths of the longest neuronal processes (axon, Fig. 2E) of the morphologically analyzed neurons, a significant difference was found for sensory axon outgrowth on (IVa) rat osteoblast monocultures (703.1 ± 74.3 μm); here it was significantly enhanced compared to sensory axon outgrowth on (Va) mixed populations of both rat cell types (325.6 ± 99.1 μm, $p < 0.0001$) or (VIa) adult rat Schwann cell monocultures (325.4 ± 198.3 μm, $p < 0.005$). A significant decrease in length of the longest axon on (VIIa) PORN-laminin coating (259.1 ± 273.3 μm) was as well found when compared to the osteoblast monosubstrate ($p < 0.05$).

Evaluation of Motoneuron Survival on Different Cellular Substrates

To analyze the impact of osteoblast cellular substrates on the survival of rat motoneurons in vitro, this paradigm was evaluated in a well-established model of motoneurons cultured on a neonatal Schwann cell feeder layer (15). In this particular part of the study rat embryonic motoneurons were seeded on top of (VIII) rat neonatal Schwann cells as the standard condition, (IX) on a mixed population of neonatal rat Schwann cells with human osteoblasts, and (X) on top of human osteoblast monocultures. Data from two preparations of three and

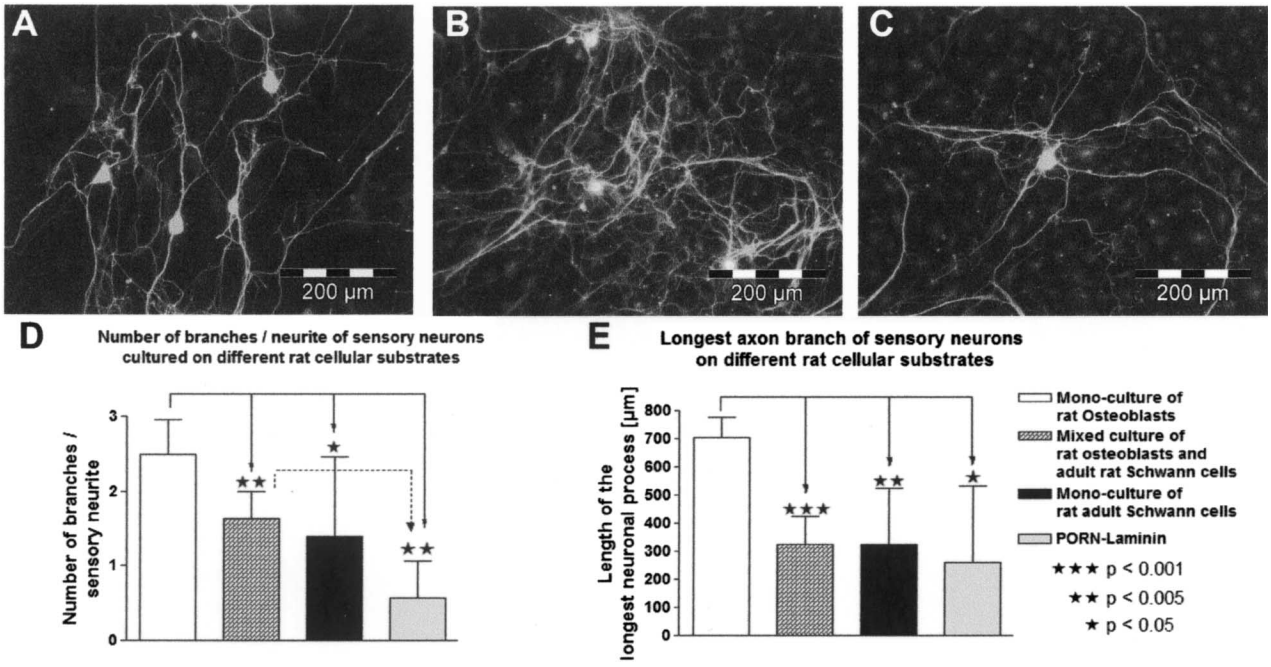

Figure 2. Morphology of β-III-tubulin-immunopositive rat sensory neurons cultured for 5 days on top of (A) rat osteoblast monocultures, (B) mixed cultures of rat osteoblast plus adult rat Schwann cells, or (C) rat adult Schwann cells. (D) Rat sensory neurons showed most prominent branching of their extending neurites when cultured on top of osteoblasts. (E) Farthest elongation of neurites/axons was observed after seeding of DRG neurons on top of osteoblasts. Bars represent means ± SD. Unpaired *t*-test with Welch correction was used for statistics.

six sister cultures each resulted in data from $n = 9$ culture wells per condition and were statistically analyzed by the Mann-Whitney test. Cells immunostained for nonphosphorylated (np)-neurofilament (NF)-H/M after 3 days in vitro were counted on a strip crossing the diameter of the cell culture well. Figure 3 depicts motoneuron survival, which was significantly higher on (VIII) monocultures of rat neonatal Schwann cells (1805 ± 355 cells) compared to survival on (IX) mixed cultures of both cell types (1246 ± 736 cells, $p < 0.05$) and (X) human osteoblast monocultures (406 ± 170 cells, $p < 0.001$). Even mixed cultures of Schwann cells and osteoblasts provided a significantly better cellular substrate for motoneurons when compared to an osteoblast monosubstrate ($p < 0.001$). To confirm that the herein chosen standard conditions with neurons cultured on neonatal Schwann cells compared to adult Schwann cells was without effect on neuron survival, we also analyzed sensory neuron survival under the same conditions as used for motoneurons (XI–XIII). Like before in experiments with the use of adult rat Schwann cells as cellular substrate, no significant differences between surviving sensory neuron numbers on different cellular substrates were found.

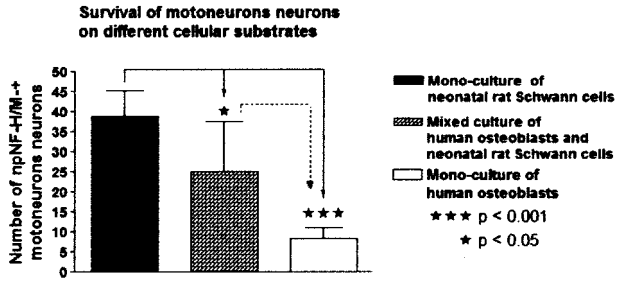

Figure 3. Survival of embryonic rat motoneurons 3 days after seeding on top of different cellular substrates of human osteoblasts and neonatal Schwann cells. Bars represent means ± SD. Mann-Whitney test was performed for statistical analysis.

Observations on the Neurite Outgrowth Behavior of Motoneurons on the Different Cellular Substrates

The cultured motoneurons (np-NF-H/M-immunopositive cells) showed a typical tripolar morphology with extending neurites to several directions when cultured on (VIII) Schwann cells (Fig. 4A). Extending neurites were mainly growing along the Schwann cells when motoneurons were cultured on a (IX) mixed monolayer of osteoblasts and Schwann cells (Fig. 4B). An untypical bipolar shape of cultured motoneurons was found after seeding on top of (X) osteoblasts alone (Fig. 4C). To

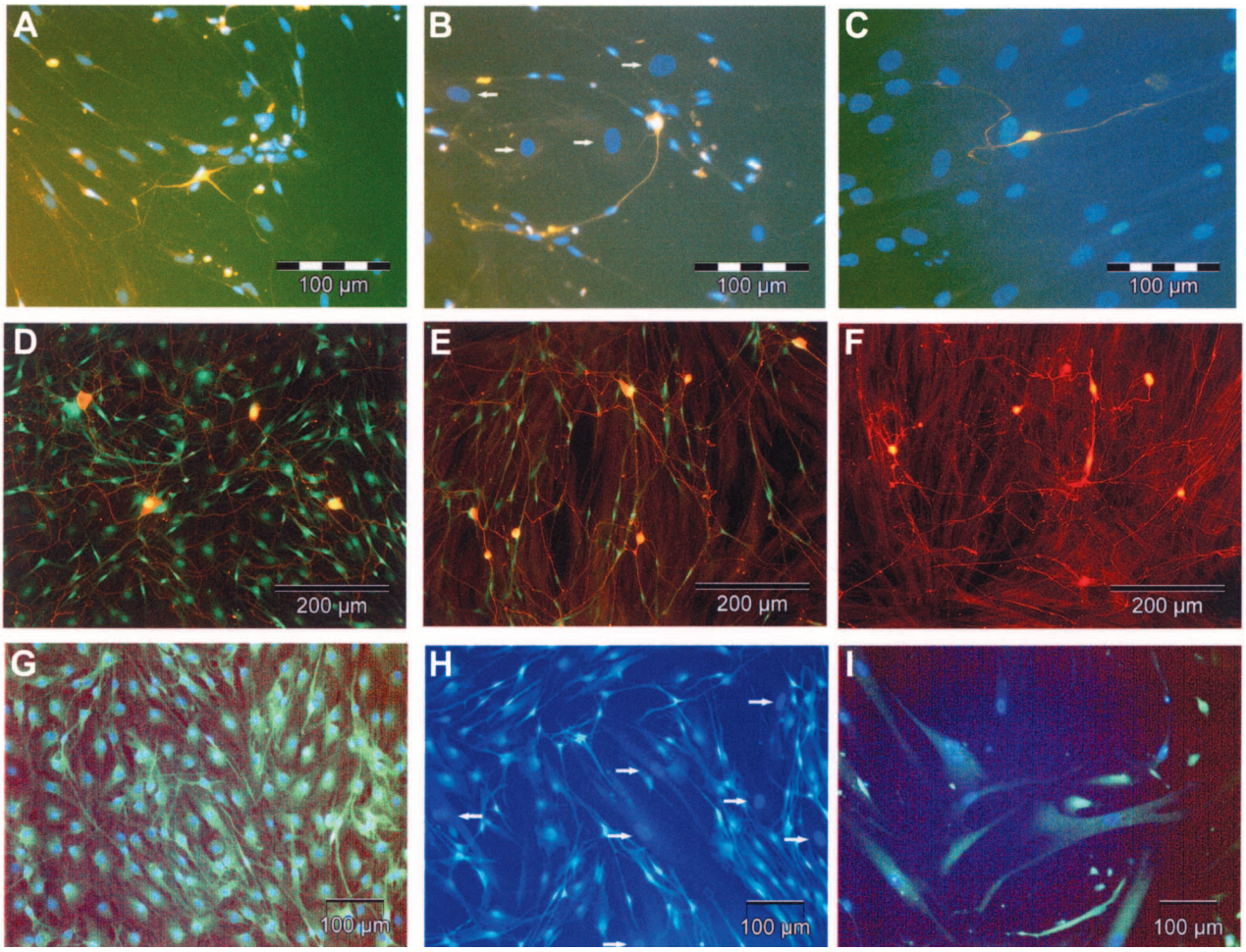

Figure 4. Neurite outgrowth behavior of embryonic rat motoneurons (A–C, red fluorescence of np-NF-H/M immunopositivity) and rat sensory neurons (D–F, red fluorescence of β-III-tubulin immunopositivity) cultured on neonatal rat Schwann cells (A, D), mixed cultures of neonatal rat Schwann cells plus human osteoblasts (B, E), or human osteoblasts alone (C, F). To distinguish osteoblasts from Schwann cells, the latter can be identified by intense S100 immunopositivity (D, E, G, H, green fluorescence) and small, oval nuclei in DAPI staining (D, E, G, H, blue fluorescence) while osteoblasts showed only slight background staining (H, I) and large, more pale nuclei (B, H, white arrows).

clarify if differences in fiber outgrowth could be due to species differences, motoneuron substrate conditions were additionally used for sensory neurons. Morphology of sensory neurons (β-III-tubulin-immunopositive cells) cultured for 5 days on either (XI) rat neonatal Schwann cells (Fig. 4D), (XII) mixed cultures of both cell types (Fig. 4E), or (XIII) human osteoblasts (Fig. 4F) did not show any preference of extending neurites to grow along a certain cell type. For this observation Schwann cells and osteoblasts could be distinguished by the sizes of their nuclei after DAPI staining. Osteoblasts provide larger and paler nuclei compared to the more oval, smaller, and more intense stained nuclei of Schwann cells (Fig. 4B, H). Schwann cells could be additionally identified by S100 immunostaining (Fig. 4D, E, G, H), although some osteoblasts showed a weak background staining (Fig. 4H, I).

Transfection of Human Osteoblasts

We screened the transfection efficiency of several electrical programs that could be used for transfection of primary cells with the help of the Nucleofector™ technology. In a first attempt each program was tested only once and analyzed with regard to transfection efficiency and cell survival of human osteoblasts. Combining the Rat Oligodendrocyte Nucleofector™ Kit with the use of program T30 resulted in 29.7% transfected cells and 56% cell survival after nucleofection. This combination was the most promising with regard to high cell numbers for cell expansion after transfection and num-

ber of transfected cells, compared to the use of program T20 (21.6% transfection, 93% survival), V20 (43% transfection, 37% survival), or X05 (20.8% transfection, 63.7% survival). In a second attempt we optimized the use of program T30 and found a transfection efficiency of $36.5 \pm 2.5\%$ ($n = 3$ sister cultures) in human osteoblasts (Fig. 5).

DISCUSSION

The aim of the current study was to establish a coculture system of osteoblasts and Schwann cells for future investigation of tissue-engineering approaches in reconstruction of bone tissue and adjacent peripheral nerves. Neither Schwann cells and osteoblasts nor neurons and osteoblasts are physiologically direct neighbors in vivo. However, sensory dendritic axons as well as motor axons build the nerve fibers of peripheral nerves that cross or penetrate maxillofacial bone tissue and thus can be destroyed by trauma or ablative tumor surgery together with the bone tissue itself. With regard to optimized conditions for reconstruction, not only fast remodeling of bone structures but also promoted outgrowth of regenerating nerve fibers from affected sensory neurons and motoneurons is crucial for complete functional recovery. This study shows for the first time differential effects of osteoblastic cells on sensory neurons and motoneurons, which have been seeded on top of bone cells.

With regard to neuron survival we show here that osteoblasts do not suppress but even promote sensory neuron survival. When cultured on human or rat cellular substrates rat sensory neurons showed a better survival on osteoblasts compared to adult Schwann cells. Osteoblasts even provided a rescue effect for sensory neurons when cultured in mixed populations with adult Schwann cells. Furthermore, neurite outgrowth of sensory neurons was promoted while growing on an osteoblastic cell monoculture compared to mixed cultures of adult osteoblastic cells and adult Schwann cells or adult Schwann cell monocultures.

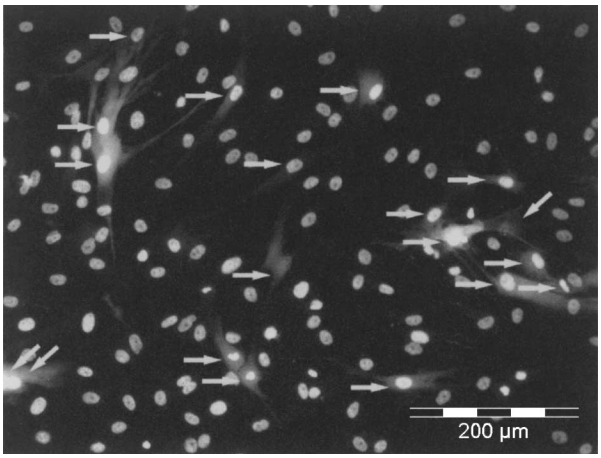

Figure 5. Genetically modified human osteoblasts expressing green fluorescent protein (arrows) as a marker protein for successful transfection, counterstained with DAPI (nuclei showing presence of untransfected cells as well).

In contrast, the opposite effect was seen for rat motoneurons. Survival of motoneurons was significantly reduced after seeding of these cells on top of mixed cultures of osteoblasts and Schwann cells or osteoblastic cell monocultures compared to Schwann cell monolayers. In addition, motor neurites showed a suppressed outgrowth on top of mixed cultures of osteoblasts and Schwann cells where the neurites extended preferentially along the Schwann cell surfaces.

We do not assume that the clear differential effects of rat neurons cultured on human cellular substrates were a consequence of species differences, because for sensory neurons similar effects were seen in survival and neurite outgrowth behavior after seeding on rat cellular substrates. Neurite outgrowth behavior of rat sensory neurons cultured on the same substrates from rat and human sources as motoneurons showed also no alteration. It is likely that osteoblasts provide a good substrate for sensory neurons. Ingrowth of neurites originating from calcitonin gene-related protein-immunopositive sensory DRG neurons in bone grafts in vivo, for example, has already been reported to possibly optimize the environment for bone regeneration (25). In contrast, it would not be reasonable to guide motor axons into regenerating bone, as bone is not a physiological target tissue for motor innervation. There is also a growing body of evidence supporting a cross-talk between sensory neurons and osteoblastic cells during development and bone repair via certain neuropeptides (10,12,23). During development neuropeptides released from sensory terminals seem to promote new bone formation by stimulating osteoblasts and inhibiting osteoclasts (10). A receptor-mediated interaction between neuronal mediators and osteoblasts that express the respective receptors such as neurokinin receptors may affect bone remodeling during tissue restoration in vivo (12). Furthermore, the local regulation of bone turnover at the fracture site is discussed to be elicited by a neuroendocrine response driven by information about local physical and chemical conditions in the healing fracture mediated via peripheral nerve endings to the central nervous system (23). Thus, given the fact of a physiological relationship between sensory neurite and regenerating bone tissue, our finding of well-growing sensory neurites on an osteoblast cellular substrate in vitro is not so surprising.

However, our investigations clearly demonstrate that an optimized implant for bone reconstruction and parallel regeneration of adjacent mixed or motor nerves will

need both osteoblasts and Schwann cells in a cell-based tissue-engineering therapy. The presence of Schwann cells will be particularly needed for reconstruction of bone and nervous tissue after substantial lesion of the mandible and the adjacent mixed mandibular nerve (inferior alveolar nerve and mylohyoideal nerve) or traumatic orbital apex syndrome where pure motor nerves are affected (43).

To further enhance the impact of tissue-engineered implants on regeneration processes, the combination of a cell-based with a factor-based therapy is favorable (34). Our paradigm aims in ex vivo gene therapy, where genetically modified cells will be produced in vitro prior to subsequent implantation at the site of injury (8). Seeding genetically modified osteoblasts and Schwann cells in reconstructive implants will provide a prolonged delivery of biologically active proteins at the site of injury. A drawback of factor-based therapies so far is the difficulty to induce the right dosage of a protein over prolonged periods (e.g., by the use of biocompatible scaffold materials loaded with certain proteins) that is due to short half-life of the proteins (36). The feasibility to transfect neonatal as well as adult Schwann cells to overproduce fibroblast growth factor-2 (FGF-2) with regard to cell- and factor-based therapies for peripheral nerve regeneration as well as its in vivo effects have been shown by our group previously (16,17,27,38). Furthermore, a rat model has been established to successfully demonstrate that the method of somatic gene transfer by implantation of genetically modified Schwann cells that overexpressed FGF-2 into the transected adult rat sciatic nerve increases peripheral nerve regeneration across long gaps (16,38).

Thus, at least for the peripheral nervous system, it has been shown already that the three main concepts of tissue-engineering can be combined by transplanting cells (cell-based therapy) that have been genetically modified to produce or overproduce certain regeneration-promoting proteins (factor-based therapy) into nerve bridges filled with extracellular matrix (matrix-based therapy) components. FGF-2 is also a promising candidate for enhancement of bone reconstruction as it is a key regulator of osteogenesis (31). FGF-2 is expressed in granulation tissue during fracture healing and it increases cell migration and angiogenesis at early stages of bone repair. Furthermore, there are studies indicating an increased bone repair rate after application of exogenous FGF-2 while endogenous FGF-2 is important for maintenance of bone mass (20). Thus, the delivery of exogenous FGF-2 at the lesion site increases integration of allogene bone transplants in rats in a dose- and time-dependent manner (40). Local infusion of FGF-2 in bone chamber experiments in rabbits did elucidate an important role for FGF-2 in balancing growth of new bone to resorption of overgrown bone tissue in bone healing (11). To enable somatic gene transfer by osteoblasts within tissue-engineered constructs a nonviral method for genetic modification was evaluated in this study. To our knowledge, the Nucleofector™ technique was used for the first time in adult human osteoblasts and resulted in high transfection rates accompanied by sufficient cell survival. Nonviral transfection of osteoblastic cells has so far been used for the investigation of transcriptional processes in these cells (39). Most gene therapy-related investigations used viral transduction (8), a highly effective method but from our experiences also a controversially discussed method with regard to its clinical application.

In summary, we present here a nonviral transfection protocol for human adult osteoblasts together with a new coculture system consisting of osteoblasts and Schwann cells that will be used in the future to investigate the effects of growth factor producing genetically modified osteoblasts and Schwann cells on bone formation and neuronal cell survival and neurite outgrowth behavior. For future investigations, besides the use of neonatal sensory neurons, the use of adult sensory neurons isolated from rat (13) or human DRG is also planned.

Our newly established system so far serves as a basis for the development of innovative tissue-engineered constructs for rapid bone reconstruction and parallel enhanced regeneration of adjacent peripheral nerves.

ACKNOWLEDGMENTS: We thank Dr. Andreas Thomsen for initial help in culture of human osteoblast-like cells. We are very grateful to Kerstin Kuhlemann and Hella Brinkmann for excellent technical assistance. We thank Dr. C. Matthies and her patients for providing sural nerve biopsies to isolate adult human Schwann cells. We also acknowledge the help of Dr. Christina Mauritz who isolated the adult human Schwann cells used in this study.

REFERENCES

1. Aebischer, P.; Guenard, V.; Brace, S. Peripheral nerve regeneration through blind-ended semipermeable guidance channels: Effect of the molecular weight cutoff. J. Neurosci. 9:3590–3595; 1989.
2. Ansselin, A. D.; Fink, T.; Davey, D. F. Peripheral nerve regeneration through nerve guides seeded with adult Schwann cells. Neuropathol. Appl. Neurobiol. 23:387–398; 1997.
3. Bunge, R. P. Expanding roles for the Schwann cell: Ensheathment, myelination, trophism and regeneration. Curr. Opin. Neurobiol. 3:805–809; 1993.
4. Ceballos, D.; Navarro, X.; Dubey, N.; Wendelschafer-Crabb, G.; Kennedy, W. R.; Tranquillo, R. T. Magnetically aligned collagen gel filling a collagen nerve guide improves peripheral nerve regeneration. Exp. Neurol. 158: 290–300; 1999.
5. Chen, Y. S.; Hsieh, C. L.; Tsai, C. C.; Chen, T. H.; Cheng, W. C.; Hu, C. L.; et al. Peripheral nerve regeneration using silicone rubber chambers filled with collagen, laminin and fibronectin. Biomaterials 21:1541–1547; 2000.
6. Feltri, M. L.; Scherer, S. S.; Wrabetz, L.; Kamholz, J.;

Shy, M. E. Mitogen-expanded Schwann cells retain the capacity to myelinate regenerating axons after transplantation into rat sciatic nerve. Proc. Natl. Acad. Sci. USA 89: 8827–8831; 1992.
7. Fine, E. G.; Decosterd, I.; Papaloizos, M.; Zurn, A. D.; Aebischer, P. GDNF and NGF released by synthetic guidance channels support sciatic nerve regeneration across a long gap. Eur. J. Neurosci. 15:589–601; 2002.
8. Franceschi, R. T. Biological approaches to bone regeneration by gene therapy. J. Dent. Res. 84:1093–1103; 2005.
9. Fujimoto, E.; Mizoguchi, A.; Hanada, K.; Yajima, M.; Ide, C. Basic fibroblast growth factor promotes extension of regenerating axons of peripheral nerve. In vivo experiments using a Schwann cell basal lamina tube model. J. Neurocytol. 26:511–528; 1997.
10. Gajda, M.; Litwin, J. A.; Cichocki, T.; Timmermans, J. P.; Adriaensen, D. Development of sensory innervation in rat tibia: Co-localization of CGRP and substance P with growth-associated protein 43 (GAP-43). J. Anat. 207: 135–144; 2005.
11. Goodman, S. B.; Song, Y.; Yoo, J. Y.; Fox, N.; Trindade, M. C.; Kajiyama, G.; et al. Local infusion of FGF-2 enhances bone ingrowth in rabbit chambers in the presence of polyethylene particles. J. Biomed. Mater. Res. A 65: 454–461; 2003.
12. Goto, T.; Kido, M. A.; Yamaza, T.; Tanaka, T. Substance P and substance P receptors in bone and gingival tissues. Med. Electron. Microsc. 34:77–85; 2001.
13. Grothe, C.; Unsicker, K. Neuron-enriched cultures of adult rat dorsal root ganglia: Establishment, characterization, survival, and neuropeptide expression in response to trophic factors. J. Neurosci. Res. 18:539–550; 1987.
14. Guenard, V.; Kleitman, N.; Morrissey, T. K.; Bunge, R. P.; Aebischer, P. Syngeneic Schwann cells derived from adult nerves seeded in semipermeable guidance channels enhance peripheral nerve regeneration. J. Neurosci. 12:3310–3320; 1992.
15. Haastert, K.; Grosskreutz, J.; Jaeckel, M.; Laderer, C.; Bufler, J.; Grothe, C.; et al. Rat embryonic motoneurons in long-term co-culture with Schwann cells-a system to investigate motoneuron diseases on a cellular level in vitro. J. Neurosci. Methods 142:275–284; 2005.
16. Haastert, K.; Lipokatic, E.; Fischer, M.; Timmer, M.; Grothe, C. Differentially promoted peripheral nerve regeneration by grafted Schwann cells over-expressing different FGF-2 isoforms. Neurobiol. Dis. 21:138–153; 2006.
17. Haastert, K.; Mauritz, C.; Matthies, C.; Grothe, C. Autologous adult human Schwann cells genetically modified to provide alternative cellular transplants in peripheral nerve regeneration. J. Neurosurg. 104:778–786; 2006.
18. Hadlock, T.; Sundback, C.; Hunter, D.; Cheney, M.; Vacanti, J. P. A polymer foam conduit seeded with Schwann cells promotes guided peripheral nerve regeneration. Tissue Eng. 6:119–127; 2000.
19. Hankemeier, S.; Keus, M.; Zeichen, J.; Jagodzinski, M.; Barkhausen, T.; Bosch, U.; et al. Modulation of proliferation and differentiation of human bone marrow stromal cells by fibroblast growth factor 2: Potential implications for tissue engineering of tendons and ligaments. Tissue Eng. 11:41–49; 2005.
20. Hurley, M. M.; Marie, P. J.; Florkiewicz, R. Z. Fibroblast growth factor (FGF) and FGF receptor families in bone. In: Bilezikian, J. P.; Raisz, L. G.; Rodan, G. A., ed. Principles of bone biology. Vol. 1, 2 ed. San Diego: Academic Press; 2002:825–851.
21. Johnson, P. C. The role of tissue engineering. Adv. Skin Wound Care 13:12–14; 2000.
22. Levi, A. D.; Bunge, R. P. Studies of myelin formation after transplantation of human Schwann cells into the severe combined immunodeficient mouse. Exp. Neurol. 130: 41–52; 1994.
23. Li, J.; Ahmad, T.; Spetea, M.; Ahmed, M.; Kreicbergs, A. Bone reinnervation after fracture: A study in the rat. J. Bone Miner. Res. 16:1505–1510; 2001.
24. Lundborg, G. Alternatives to autologous nerve grafts. Handchir. Mikrochir. Plast. Chir. 36:1–7; 2004.
25. Madsen, J. E.; Wang, J. S.; Hukkanen, M.; Nordsletten, L.; Konttinen, Y. T.; Santavirta, S.; et al. Sensory nerve ingrowth during bone graft incorporation in the rat. Acta Orthop. Scand. 67:217–220; 1996.
26. Matsumoto, K.; Ohnishi, K.; Sekine, T.; Ueda, H.; Yamamoto, Y.; Kiyotani, T.; et al. Use of a newly developed artificial nerve conduit to assist peripheral nerve regeneration across a long gap in dogs. ASAIO J. 46:415–420; 2000.
27. Mauritz, C.; Grothe, C.; Haastert, K. Comparative study of cell culture and purification methods to obtain highly enriched cultures of proliferating adult rat Schwann cells. J. Neurosci. Res. 77:453–461; 2004.
28. Mosahebi, A.; Fuller, P.; Wiberg, M.; Terenghi, G. Effect of allogeneic Schwann cell transplantation on peripheral nerve regeneration. Exp. Neurol. 173:213–223; 2002.
29. Mosahebi, A.; Woodward, B.; Green, C.; Martin, R.; Terenghi, G. Long-term effect of vital labelling on mixed Schwann cell cultures. Histochem. J. 32:337–343; 2000.
30. Muller-Ostermeyer, F.; Claus, P.; Grothe, C. Distinctive effects of rat fibroblast growth factor-2 isoforms on PC12 and Schwann cells. Growth Factors 19:175–191; 2001.
31. Praul, C. A.; Ford, B. C.; Leach, R. M. Effect of fibroblast growth factors 1, 2, 4, 5, 6, 7, 8, 9, and 10 on avian chondrocyte proliferation. J. Cell Biochem. 84:359–366; 2002.
32. Rodriguez, F. J.; Verdu, E.; Ceballos, D.; Navarro, X. Nerve guides seeded with autologous schwann cells improve nerve regeneration. Exp. Neurol. 161:571–584; 2000.
33. Romeis. Mikroskopische Technik. 17 ed. München/Wien/Baltimore: Urban & Schwarzenberg; 1989.
34. Rotter, N.; Haisch, A.; Bucheler, M. Cartilage and bone tissue engineering for reconstructive head and neck surgery. Eur. Arch. Otorhinolaryngol. 262:539–545; 2005.
35. Sailer, H. F.; Weber, F. E. [Bone substitutes]. Mund. Kiefer Gesichtschir. 4(Suppl. 1):384–391; 2000.
36. Schliephake, H.; Jamil, M. U.; Knebel, J. W. Experimental reconstruction of the mandible using polylactic acid tubes and basic fibroblast growth factor in alloplastic scaffolds. J. Oral Maxillofac. Surg. 56:616–627; 1998.
37. Schmidt, C. E.; Leach, J. B. Neural tissue engineering: Strategies for repair and regeneration. Annu. Rev. Biomed. Eng. 5:293–347; 2003.
38. Timmer, M.; Robben, S.; Muller-Ostermeyer, F.; Nikkhah, G.; Grothe, C. Axonal regeneration across long gaps in silicone chambers filled with Schwann cells overexpressing high molecular weight FGF-2. Cell Transplant. 12:265–277; 2003.
39. Towler, D. A.; Arnaud, R. S. Use of cultured osteoblastic cells to identify and characterize transcriptional regulatory complexes. In: Bilezikian, J. P.; Raisz, L. G.; Rodan, G. A., eds. Principles of bone biology. San Diego: Academic Press; 2002:1503–1527.
40. Wang, J. S.; Aspenberg, P. Basic fibroblast growth factor

enhances bone-graft incorporation: Dose and time dependence in rats. J. Orthop. Res. 14:316–323; 1996.
41. Wiedmann-Al-Ahmad, M.; Gutwald, R.; Gellrich, N. C.; Hubner, U.; Schmelzeisen, R. Search for ideal biomaterials to cultivate human osteoblast-like cells for reconstructive surgery. J. Mater. Sci. Mater. Med. 16:57–66; 2005.
42. Wiedmann-Al-Ahmad, M.; Gutwald, R.; Hubner, U.; Gellrich, N. C.; Schmelzeisen, R. Growth of human osteoblast-like cells on beta-tricalciumphosphate (TCP) membranes with different structures. J. Mater. Sci. Mater. Med. in press; 2006.
43. Yeh, S.; Foroozan, R. Orbital apex syndrome. Curr. Opin. Ophthalmol. 15:490–498; 2004.