GENE-SUPPLEMENTED COLLAGEN SCAFFOLDS FOR NON-VIRAL GENE DELIVERY FOR BRAIN TISSUE ENGINEERING

By

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ABSTRACT

Recent advances in tissue engineering, combining an extracellular matrix (ECM)-like vehicle with therapeutic molecules, cells and/or genes has yielded promising results for brain injury repair. The purpose of this thesis was to develop a collagen scaffold for the non-viral delivery of the gene encoding for Glial Cell-Derived Neurotrophic Factor (GDNF); hence to provide a local, long-term release and overexpression of GDNF via transfection of cells seeded into the scaffold or endogenous cells.

The first part of the thesis aimed to investigate the *in vitro* transfection of marrow stromal stem cells (also referred to as mesenchymal stem cells, MSCs) in monolayer with plasmid GDNF (pGDNF). Several parameters were evaluated: the choice of a transfer reagent (GenePorter2 versus Lipofectamine 2000), the doses of plasmid incorporated in the liposomes (ranging from 0.2μg to 2μg), the post-transfection medium (Medium 1: DMEM low glucose, 20% FBS and 1% antibiotic versus Medium 2: DMEM low glucose, 20% FBS, 1% antibiotic and 10ng/ml FGF-2) and the culture environment during transfection (static versus dynamic). The objective of the second part was to determine the conditions, including the design of the scaffold and the method of seeding, under which MSCs could attach and grow on the scaffold. Collagen scaffolds were made by a freeze-drying technique and prepared with various amounts of collagen, cross-link densities, and freezing temperatures. The effect of gene supplementation on the cross-link density was evaluated using the swelling ratio. Finally, the aim of the third part was to evaluate different parameters to optimize the transfection of cells grown in the scaffolds. The profile of production of GDNF was studied for different cross-link density, initial plasmid dose (2 and 10 μg) and plasmid-transfection reagent ratio. Finally the effect of the pore diameter and static and dynamic culture environments were tested to optimize the *in vitro* conditions for the plasmid uptake and expression by the MSCs.

The results demonstrated the possibility of using non-viral transfection conditions *in vitro* to enable MSCs to express a selected neurotrophic factor, GDNF, in therapeutic doses. MSCs were shown to over-express GDNF for at least a two-week period of time. Lipoplexes loaded with as little as 0.2 μg could result in a significant production of GDNF by MSCs for several days, before falling off to control levels after one week. For the highest loading of plasmid (2 μg), the level of GDNF production was still above the

control after 2 weeks. Dynamic transfection had a dramatic effect on the production of GDNF. The accumulated amount of GDNF during the 2-week period reached 65 ng/ml compared to 20 ng/ml produced in static conditions. The growth factor bFGF, which is used in transdifferentiation of MSCs for a neuronal phenotype, was shown to promote a high level of cell death when used in the post-transfection medium. Collagen scaffolds can be prepared to incorporate the plasmid DNA-lipid complexes for subsequent release. Also, gene and subsequent cross-link density have an effect on the mechanical behavior of scaffolds. Finally, the gene-supplemented collagen scaffolds can serve as a carrier for lipoplexes and modified MSCs and provide a long-term overexpression of GDNF. The level of gene expression in the collagen constructs was lower than those obtained in MSC monolayers but high enough to result in therapeutic doses previously found *in vitro*. The cross-linking treatment did not affect significantly the release profile of GDNF. The application of orbital shaking during the 4 hours of transfection had a positive effect on the production of GDNF but not as strong as reported in monolayer studies. The load of plasmid DNA is a prominent parameter in the three-dimensional (3-D) transfection. In this study, the highest level of GDNF expression was observed for 10 μg of plasmid DNA and 6 days after transfection.

Overall, these results demonstrated that the combination of tissue engineering and non-viral transfection of MSCs for the over-expression of GDNF was a promising approach for the long-term production of selected neurotrophic growth factors. This approach could provide benefits in the treatment of conditions involving the loss of brain tissue.

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1 GENERAL INTRODUCTION

1.1 PROBLEM STATEMENT

There are several types of injuries and disease processes that result in the loss of brain tissue. Currently there are no proven methods for treating such defects in such a way as to facilitate a reparative response and to reduce the conditions that promote additional degradation of brain tissue.

Traumatic brain injury (TBI), also called head injury, occurs when a sudden trauma causes damage to the brain. This major public health problem affects approximately 1.4 million people each year in the United States. Transportation accidents are the main cause of TBI (50%), followed by violence (20%) and sports injury (3%) [1]. There are distinctive types of TBI converging toward a common endpoint: the death of the nerve cells. For example, shaking of the brain within the confines of the skull, after a high-speed stop in a car accident, can cause damage to individual nerve cells and loss of connections within the neuronal network.

Defects in the brain can also result from removal of tumors, leaving a defect filled with cerebrospinal fluid (CSF). Damage to one of the major arteries leading to the brain as a result of trauma or disease can result in a stroke, with the formation of a hematoma and cell necrosis.

The health complications following TBI include seizures, post-traumatic ventricular enlargement, CSF leaks, infections, vascular injuries, multiple organ system failure and polytrauma. About 25 percent of patients with hematomas and about 50 percent of patients with penetrating head injuries will develop seizures [1]. Other long-term problems which can develop after TBI include Parkinson's disease and other motor problems, and Alzheimer's disease.

The overall goal of the ongoing project is to develop an implantable device which combines cell and gene therapy with a tissue engineering scaffold, for the long-term production of selected neurotrophic growth factors and antagonists to neurite outgrowth

inhibitors. This approach could provide benefits in the treatment of conditions involving the loss of brain tissue.

1.2 THE NERVOUS SYSTEM

The central nervous system (CNS) tissues, brain and spinal cord, are composed of two main cell types: nerve cells - neurons- and the support cells - glial cells (Figure 1.1). Neurons have a cell body, the soma, and neurites (dendrites and axons). Nerve cells are characterized by their ability to respond to stimuli with an electric signal transmitted in a few milliseconds. Glial cells are mainly responsible for isolating neuronal processes and controlling the environment of neurons.

Glial cells can be divided into different types, each with a specific function: astrocytes, oligodendrocytes, microglia, and ependymal cells [2].

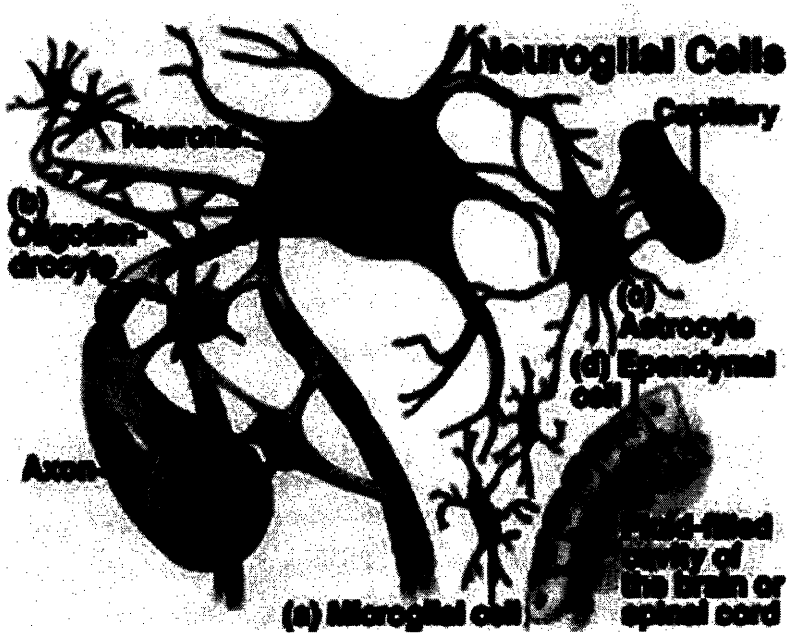

Figure 1.1: Cells of the Nervous System[3]

- The astrocyte maintains an appropriate chemical environment for the generation of nerve impulses, nourishes neurons, takes up the excess neurotransmitters, assists in brain development, and forms the blood-brain barrier.
- The oligodendrocyte produces the myelin sheath.
- The microglia cell phagocytoses dead/injured nerve tissue.

- The ependymal cell participates in the formation and circulation of the CSF, and serves as neural stem cell.

1.3 REGENERATION PROCESS IN THE CENTRAL NERVOUS SYSTEM (CNS)

Tissues can respond to injury in one of three ways: there may be no healing, resulting in the persistence of the defect as a fluid-filled space, *e.g.*, glial cyst; healing may result in a tissue identical to the original tissue, *i.e.*, regeneration; or healing may result in the formation of scar, which in musculoskeletal tissue, epithelia, and muscle is composed of fibrous tissue, and in the neural system is composed of glia and entangled axons, neuroma.

In contrast to other self-regenerating tissues such as the liver and the epidermis, the brain lacks the regenerative potential to compensate adequately for neuronal and glial cell death [4]. A number of significant barriers preclude recovery after CNS injury, including: the formation of glial scarring, which acts as a physical barrier to nerve growth; the release of tissue-degrading pro-inflammatory cytokines and free radicals during inflammation; the presence of nerve growth inhibitory molecules; the inefficient removal of cellular debris; the limited supply of neurotrophic factors; and the programmed cell death [5].

The regeneration of the CNS is a complex process which involves a cascade of sequential events. The transected axons would need to survive in order to sprout and bridge the lesion site to re-establish the proper connections. This regeneration could be regulated in a specific environment, in the presence of neurotrophic factors which support the survival, growth and differentiation of neurons. Another significant factor is the need for an extracellular microenvironment to support the axonal outgrowth [6, 7].

1.4 CURRENT PROGRESS IN BRAIN TISSUE REPAIR

The reparative process in several types of tissues may benefit from the implantation of a biomaterial scaffold alone. Regarding the limited self-reparative potential of the CNS, the brain tissue repair strategy would likely need to include different agents of regeneration including: cellular replacement by means of cell transplantation or endogeneous stem cell activation; neurotrophic factor delivery;

manipulation of intracellular signaling; and perhaps modulation of the immune response [8].

The infusion of therapeutic molecules such as blockers of myelin-associated growth inhibitor/receptor [9], [10],[11] is a simple way to deliver therapeutic molecules, but has limitations in brain tissue repair. These molecules, if delivered systemically, can undergo metabolic degradation by the liver prior to reaching the brain. Even if delivered locally the molecules have a short half-life, which would require a continuous administration of the drug. In addition, the blood-brain barrier, which limits the entry of substances into the brain, may only be able to be overcome by transient pemeabilization. Finally, there are some difficulties in targeting a specific cell population such as neurons [12].

Cell transplantation strategies for the injured brain have been performed using a variety of cell types and tissues. In Parkinson's disease, human tissue grafts have been employed, but have resulted in only minor clinical improvement due to limited graft survival [13]. Other studies of transplantation of embryonic tissue in animal models [14] have shown promising clinical outcome but would need a substantial amount of tissue to be effective in human subjects, and have raised ethical issues [15]

Cell-based therapy in the brain can be used in multiple ways such as a direct replacement of lost cells or as a vehicle for growth factor or gene delivery. For achieving a localized therapeutic effect, a broad range of cells has been studied [12]. including: olfactory ensheathing cells [16], [17], multipotent marrow stem cells [18] [19], [20], neural stem cells [21], [22], [23]; and astrocytes [24].

A number of vectors have been developed to introduce proteins or genes and provide a high level and localized delivery of specific neurotrophic factors such as: brain derived neurotrophic factor (BDNF); glial cell-derived neurotrophic factor (GDNF); fibroblast growth factor (FGF); and neurotrophin 3 (NT3) [25], [26].

Finally, recent advances in tissue engineering, combining an extracellular matrix (ECM)-like vehicle with pharmaceuticals, cells and/or genes has yielded promising results for brain injury repair. In a first study, a poly[N-(2-hydroxypropyl) methacrylamide] (PHPMA) hydrogel immobilized with the neurite-promoting peptide sequence of xIKVAVx has been reported by Tian et al. [27] to support axonal outgrowth

in the injured cerebral cavity of adult rats, and to promote tissue regeneration. In another recent study by this group, Tian et al. showed that a controlled release of antagonists to the receptors inhibiting the neurite outgrowth has some therapeutic potential[28]. Recently, Ellis-Behnke et al. reported that a self-assembling peptide nanofiber scaffold creates a permissive environment for axons to regenerate through the site of an injury and "knits" the brain tissue together. Using the mammalian visual system as a model, they showed that regenerated axons reconnect to target tissues with sufficient density to promote functional return of vision [29].

In light of the prior work, the implant to be developed in this thesis could facilitate endogenous neuronal cell infiltration, thus restoring a certain degree of neuronal contiguity, and act as a delivery vehicle for neural stem cells, and therapeutic molecules such as neurotrophic factors and their genes, antagonists to inhibitors of neurite outgrowth, and anti-inflammatory agents, with a controlled and localized release from weeks to months. Moreover, the implantable device could serve as a delivery vehicle for anti-cancer and anti-microbial drugs.

1.5 BACKGROUND

1.5.1 *Collagen Scaffolds for CNS Repair*

Biomaterial scaffolds can be of use in: supporting cells migration and proliferation; serving as a vehicle for growth factor and gene delivery; and serving as a regulatory of cell behavior to enhance the growth of a functional tissue [30], [31], [32]. Porous scaffolds have a much larger surface area compared to the types of two-dimensional surfaces on which cells are grown in monolayer culture; therefore porous scaffolds have the capability to yield high cell densities. Scaffolds can be prepared with specific properties that allow for programmed biodegradability, mechanical behavior and cell attachment, regulation, and gene expression.

There are numerous advantages in using collagen biomaterials for the fabrication of scaffolds. As a natural polymer contained within the extracellular matrix of most tissues, collagen can bind to array of matrix molecules and cells. It can be prepared in such a way as to suppress problems related to toxicity, chronic inflammatory reaction and lack of recognition by cells; collagen is generally a weak immunogen relative to the

majority of proteins. Finally, collagen has been extensively studied in various tissue engineering and regenerative medicine applications such as in the central [33], [34], [35] and peripheral nervous system [36], the connective tissues [37, 38], the meniscus [39], and the articular cartilage [40], [41]. In a recent study, a type I collagen gel has been used to support the growth and differentiation of neural stem and progenitor stem cells and has been reported to display characteristic properties of neuronal circuits [42].

1.5.2 *Role of Growth Factors*

Numerous studies have shown the importance of growth factors to enhance the repair potential in the injured CNS. Recent findings have demonstrated the positive effects of selected growth factors such as fibroblast growth factor (FGF), brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3) [43], [44], [45].

While numerous combinations of these growth factors can be investigated for brain tissue engineering, glial cell-derived neurotrophic factor (GDNF) alone has shown potent actions on damaged and diseased neurons [46], [47], [48], [49], [50]. GDNF has been reported to: be able to reverse some aspects of aging in monkeys [51]; have significant effects on the symptoms of Parkinson disease when directly infused in the monkey brain [52]; and prevent nigral cell death when recombinant human GDNF is injected either before or after lesions produced in the rat substantia nigra[53]. Finally, GDNF is expressed in many tissues and by many cell types throughout the body, including neurons [54].

1.5.3 *Rationale for Gene Transfer in Brain Tissue Engineering*

The aim of the gene therapy is to deliver a gene encoding a specific protein into cells for exerting the intended therapeutic effect. A number of DNA delivery methods using viral and non-viral vectors have been investigated to improve gene transfer and expression.

The viral methods for gene transfer generally provide for substantially higher efficiencies of gene transfer and have demonstrated promising results when using GDNF for the rescue of nerve cells in the CNS [13], [55], [56-58]. The disadvantage of viral

vectors is that they contain strongly antigenic genes and envelop proteins, which may elicit a strong immunogenic response in the host.

Thus, for safety concerns, non-viral vectors have attracted much attention. The main non-viral vectors are: cationic lipids (lipoplexes); cationic polymers, such as block copolymers (polyetherimide, polyhistidine); dendrimers and natural polymers (gelatin, chitosan). Moreover, physical methods to enhance the entry of naked plasmid into the cells are also undergoing active investigation: electroporation, laser beam induction, ultrasound, and shock waves. Many cationic lipids have been developed and optimized in the last past years, and have been largely used for their efficiency and simplicity of use [59]. In a recent study, Hoelters et al. demonstrated that human MSC, when transfected with a non-viral vector, can maintain their proliferation capacity paired with the ability to differentiate into different mesodermal lineages (bone, cartilage, and fat) without loss of gene expression [60].

1.5.4 *Rationale for Multipotent Marrow Stromal Cells (MSCs)*

Cells vary in their potential to be transfected non-virally. Autologous multipotent marrow stromal cells (also referred to as mesenchymal stem cells, MSCs) are being investigated widely for use in cell therapies, for their differentiation and proliferation capacities as well as their accessibility. Also, they present advantages compared to allografts and xenografts because autologous MSCs do not harbor potentially infectious agents and do not result in the need for immunosuppressive therapies. Hence, the transfection of MSCs could ensure a localized, high dose and long-term release of a targeted therapeutic molecule.

Bone marrow-derived stem cells are progenitors to skeletal tissues such as bone, cartilage and fat. Some studies have shown the therapeutic potential of direct injection of MSCs in the CNS. Chen et al. proved that following intravenous infusion of MSCs, after cerebral ischemia in rats, these cells can enter the brain and provide neurological functional gains [18]. In another study, Mahmood et al. demonstrated that an intravenous injection of human MSCs provides long-term functional benefits after TBI in rats [20]. Finally, it has been showed that MSCs can be differentiated into neurons, both under

specific experimental conditions *in vitro*[61], [62], [63] and *in vivo*, in response to the nervous system environment [64], [19].

1.6 SPECIFIC AIMS

In the context of the overall goal of the ongoing project, this Master's thesis investigated a collagen scaffold for non-viral gene delivery of GDNF plasmid for brain tissue repair. The implant would act as a vehicle for delivery of modified MSCs, which express the GDNF, and for lipoplexes containing the plasmid GDNF. The biomaterial system would induce a localized over-expression of GDNF for reparative processes in defects in brain tissue. The specific aims of the thesis included:

1. Comparison of various cationic lipids with GDNF plasmid for non-viral transfection of rat MSCs.
2. Evaluation of the GDNF plasmid-lipid complex dose response in MSCs in monolayer.
3. Identification of an optimal culture environment for the transfection of MSCs.
4. Determination of the time course of the over-expression of the plasmid DNA after non-viral transfection of the MSCs in monolayer.
5. Incorporation of the plasmid in the collagen implant for the development of a gene-supplemented scaffold.
6. Determination of the effect of the plasmid-lipid complex on the 'mechanical behavior' of the scaffold (reflected in the swelling ratio).
7. Determination of the conditions (pore diameter and culture environment) under which MSCs could attach and grow on the collagen scaffold.
8. Investigation of the effects of the pore size and the cross-link density of the scaffolds on the transfection and the expression of the plasmid GDNF in MSCs.
9. Identification of the effect of culture environment on 3-D transfection.
10. Determination of the effect of the plasmid load on 3-D transfection.

1.7 HYPOTHESES

Three principal hypotheses were tested in this thesis through achieving the nine specific aims:

1. Rat MSCs in monolayer can be transfected non-virally with the plasmid DNA encoding for GDNF. This hypothesis has been tested in achieving the aims 1 to 4.
2. Collagen scaffolds can be prepared to incorporate plasmid DNA-lipid complexes for subsequent release. This hypothesis has been tested in achieving the aims 5 to 7.
3. Gene-supplemented collagen scaffolds can serve as a carrier for lipoplexes and modified MSCs and provide a long-term overexpression of GDNF. This hypothesis has been tested in achieving the aims 8 to 10.

2 NON-VIRAL GDNF GENE TRANSFER TO MESENCHYMAL STEM CELL MONOLAYERS

2.1 INTRODUCTION AND EXPERIMENTAL DESIGN

Several studies have demonstrated the potential of cells used as vehicles for delivery of neurotrophic factors in CNS regenerative medicine [65], [66], [67]. As a carrier, transfected cells allow for the over-expression and a localized delivery of a selected growth factor. Particularly, MSCs ([68], [69] offer numerous advantages such as accessibility, proliferative abilities and differentiation potential. As for genetically engineering cells to increase the production of growth factors, diverse methods have been studied for gene uptake and expression such as: viral vectors, electroporation, cationic lipids, and polymers. Among those techniques, non-viral methods have been preferred for safety reasons. Particularly, liposome-mediated transfection provides a relatively high efficiency of gene transfer and has been widely used and optimized for various types of cells [70]; [71], [72], [73]. Several easy-to-use transfection reagents are commercially available and among those GenePorter® 2 in particular has demonstrated abilities to enhance gene transfection in diverse studies [74], [75]. Lipofectamine 2000, another transfection reagent which has been previously studied for the transfection of MSCs, [76], [60] was also used in this thesis for comparison.

The medium for monolayer expansion and differentiation of MSCs requires a specific combination of growth factor. Basic fibroblast growth factor (bFGF; also referred to as FGF-2) has been reported to enhance cell density, which could be an advantage for reaching confluence faster. bFGF is also a mitogen for MSC and stimulates angiogenesis and other wound-healing functions by collagen synthesis, wound contraction, epithelialization, and fibronectin and proteoglycan synthesis. It also has been recently used to induce transdifferentiation of MSCs to cells with neuronal phenotype. In this experimental part of the thesis, bFGF was used as a medium supplement for the transfection of the MSC monolayer.

The objective of this study was to investigate the *in vitro* transfection of plasmid GDNF (pGDNF) in MSC monolayers. Several parameters were evaluated (n=6): the

choice of a transfer reagent (GenePorter2 versus Lipofectamine 2000), the doses of plasmid incorporated in the liposomes (ranging from 0.2μg to 2μg), the post-transfection medium (Medium 1: DMEM low glucose, 20% FBS and 1% antibiotic versus Medium 2: DMEM low glucose, 20% FBS, 1% antibiotic and 10ng/ml FGF-2) and the method of transfection (static versus dynamic). This chapter achieved the specific aims 1 to 4.

Four experiments were performed to achieve the objectives. In the first experiment MSCs were transfected in culture using GP2 with a dose of 2 μg of pGDNF, and L2000 with a dose of 1.6 μg pGDNF and 2 different media. In the second experiment the release of GDNF was measured after the transfection of MSCs with different loads of pGDNF (0.5μg, 0.2μg and 2μg) and a constant ratio pGDNF: GP2 (μg: μl) equal to 5. In the third experiment, transfection of MSCs was carried out in static culture and in an orbital shaker using 2μg pGDNF in DMEM low glucose and 20% FBS medium. The fourth experiment evaluated the effect of bFGF during MSCs transfection. The GFP production was estimated for each experiment. In the various monolayers studies, passage 2 and 3 MSCs were used.

2.2 MATERIALS AND METHODS

2.2.1 *Rat Mesenchymal Stem Cells Isolation and Expansion*

Rat MSCs were isolated from both the femur and the tibia of young rats (<6 weeks old). The detailed protocol of MSC harvesting can be found in Appendix A. Cells from only one animal were used to eliminate variability related to inter-animal differences.

Isolated MSCs were then plated in 150 cm^2 flasks at 5000 cells/cm^2 and expanded in the expansion media prepared with low glucose Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Cat#11885-084), 20% of FBS and 1% of antibiotics – Penicillin/Streptomycin. Once the cells in P0 reached about 90% confluency, they were detached, resuspended and replated to P1 before being frozen at -80C (Appendix B). One day prior to the transfection, the cells were counted by hematocymetry and plated at 8000 cells/cm^2 in well-plates.

2.2.2 *Isolation and Bacterial Transformation of the Plasmid Rat Glial Derived Neurotrophic Factor (pGDNF)*

2.2.2.1 *Plasmid Expansion*

pGDNF (Figure 2.1) which contains the Green Fluorescent Protein (GFP) gene under the control of the Internal Ribosome Entry Site (IRES) immediately early promoter, was a gift from Professor M. Bohn (Northeastern University, Chicago).

The plasmid DNA was amplified in competent Escherichia coli (DH5 from Invitrogen) and isolated from the bacteria with the Mega QIAfilterTM Plasmid kit (Qiagen). Briefly, grown bacteria were harvested by centrifugation at 6000g at 4°C and the bacterial pellet was resuspended in a buffer containing 50mM Tri.Cl, 10nM EDTA, 100 μg/ml Rnase A (pH 8). The preparation was then lysed in an alkaline solution (200 nM NaOH) containing 1% (w/v) of sodium dodecyl sulfate (SDS). The lysate was neutralized by the addition of 3M potassium acetate (pH 5.5). After separation of the nonsoluble portion with a Qiafilter Cartridge (Qiagen), the lysate was passed through an equilibrated Qiagen-tip (anion-exchange resin) by gravity flow. The cleared lysate was washed with a buffer containing 1M NaCl, 50 mM MOPS, 15% isopropanol (v/v, pH 7) to remove the traces of RNA and protein. Plasmid DNA was eluted with an elution buffer containing 1.25 M NaCl (pH 8.5) and precipitated with 2-propanol. Precipitated plasmid DNA was centrifuged at 15,000g for 10min at 4C and washed with 70% ethanol solution. After centrifugation (15,000g, 10min, 4C), the resulting plasmid DNA was air dried and dissolved in TE buffer (10mM Tri.Cl, 1mM EDTA, pH 8). (Appendix C)

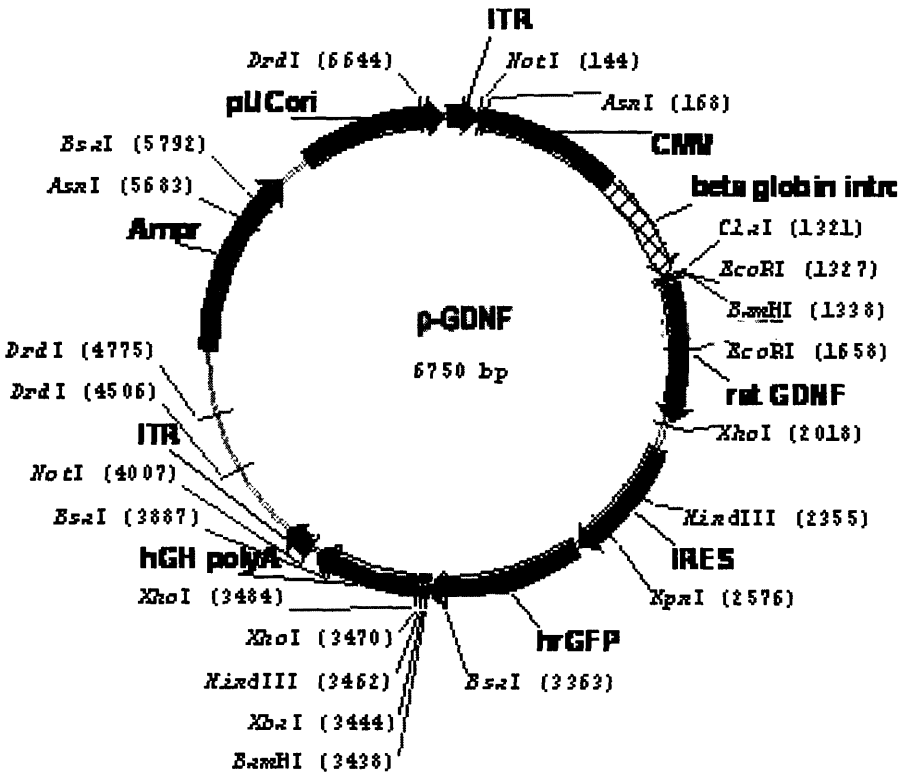

Figure 2.1: Sequence map of pGDNF

(from M. Bohn, Northwestern Univesity)

2.2.2.2 *Quantification of Isolated Plasmid*

When measured to assess the purity of plasmid DNA obtained, the ratio of absorbance at wavelengths of 260 and 280nm ranged around 1.75 and 1.8.

2.2.2.3 *Integrity of Isolated Plasmid*

The integrity of the amplified plasmid was determined by agarose gel electrophoresis, the isolated pGDNF was thus compared uncut and cut with Not I and Xho I, to the original plasmid. The size of pGDNF was 6.75kb. The bands demonstrated the integrity of the plasmids (Appendix D).

2.2.3 *Non-Viral Gene-Transfer in Monolayer Culture*

The most common synthetic lipid components of liposomes developed for gene delivery have an overall net positive charge at physiological pH and are often mixed with a neutral lipid such as L-dioleoyl phosphatidylethanolamine (DOPE). The cationic portion of the lipid molecule associates with the negatively charged nucleic acids, resulting in compaction of the nucleic acid in a liposome/nucleic acid complex (Figure 2.2) [77], [78].

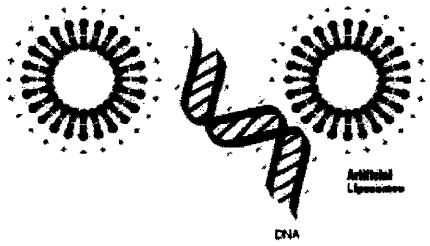

Figure 2.2: liposome/DNA complex

The entry of the liposome complex into the cell occurs by the processes of endocytosis or fusion with the plasma membrane via the lipidic part of the liposome [79]. Following cellular internalization, the plasmids are released in the cytoplasm and gain entry into the nucleus where they are expressed. In this study, two commercially available transfection reagents were used: Gene Porter 2 (GP2) and Lipofectamine 2000 (L2000).

Passage 3 rat MSCs were seeded at a concentration of 8.10^3 cells/cm^2 in 12-well plates and cultured in the expansion media containing DMEM low glucose, 20% FBS, 1% antibiotic. The transfection procedures were based on the manufacturer recommendations. Following are the experimental groups.

GenePorter2 (GP2): For the GP2 experiment, 0.2, 0.5, and 2μg pGDNF was loaded in the plasmid/lipid complex, where a ratio between the plasmid DNA and the liposome (μg:μl) was fixed at 5 (n=6). Plasmid DNA was transfected at 1 week after cell seeding at about 70% confluency using GenePorter 2 (Genlantis, Cat #T202007) according to a

standard protocol. The amount of DNA was varied whereas the ratio DNA: GP2 was maintained at the constant value 5. Briefly, GP2 was hydrated at room temperature with the hydration buffer and a plasmid DNA solution was prepared with the DNA diluent. The hydrated GP2 reagent was diluted with serum-free medium (DMEM low glucose) and incubated at room temperature for 5 minutes. Then pGDNF solution was added to the diluted GP2 reagent, and incubated at room temperature for 10 minutes to form GP2/DNA complexes.

Before the transfection, the expansion medium was replaced with 0.5ml of serum-free medium (DMEM low glucose). The mixture of GP2/DNA complex was added directly to the cells growing in serum-free media and incubated at 37°C for 4 hours, both in static and dynamic conditions (orbital shaker, 160rpm). After 4 hours, 0.5ml of Medium 1 (80% DMEM low glucose, 20% FBS) or 0.5ml of Medium 2 (80% DMEM low glucose, 19% FBS, 1% antibiotic + 10ng/ml FGF-2) was added to the wells.

Two control group received 2 μg of the naked plasmid or no plasmid (n=6).

Lipofectamine 2000 (L2000): In the L2000 experiment, 1.6 μg was employed at a ratio of 1.6 μg plasmid to 4 μl L2000 (n=6). In order to compare the two systems, the cumulated release of GDNF in the medium was also normalized to the amount of pGDNF initially incorporated in the lipoplexes. Plasmid DNA was transfected at 9 days after cell seeding at about 95% confluency using Lipofectamine 2000 CD Reagent (Invitrogen, Cat# 12566-014). Two different transfection media were compared: Opti-Pro (GIBCO, Cat# 12309) with 4mM L-Glutamine and DMEM low glucose (GIBCO, Cat#11885-084). The mixture complex was added directly to the cells in serum-free media (DMEM low glucose) and incubated at 37°C for 24 hours.

Two control group received 1.6 μg of the naked plasmid or no plasmid (n=6).

2.2.4 *Fluorescence Microscopy of the MSCs Transfected with the Lipoplexes*

Micrographs of MSCs expressing GFP were taken in a fluorescence microscope, with every change of the media. Determination of transfection efficiency was performed after 24 hours and on days 3, 6, 9, 12, 14 post-transfection by fluorescence microscopy.

2.2.5 *Biochemical Analysis: GDNF Enzyme Linked Immunosorbent Assay (ELISA)*

The media were collected (0.3ml) on days 3, 6, 9, 12, and 14 post-transfection for ELISA analysis (Duo-Kit ELISA for the human GDNF protein; R&D Systems) and stored at -20°C until the ELISA was performed. Chemiluminescent ELISA assays (R&D Systems) were run in a multilabel Victor 3 microplate counter and analyzed using Wallac 1420 software (Perkin Elmer Life Sciences). The amount of GDNF released in the medium was monitored by reading absorbance at 450nm minus absorbance at 540nm to correct for optical aberrations. (Appendix E)

2.2.6 *DNA Assay for Cell Number*

Cells were obtained at the day of the transfection and at the end of the experiment (14 days) to evaluate for cell number. Briefly, the medium was removed from the culture, the cells were washed with phosphate-buffered saline (PBS), and then the cells were detached by incubation with collagenase followed by incubation with 0.25% trypsin/EDTA. Rat MSCs were recovered by centrifugation and resuspended in PBS. The number of cells was determined following the DNA quantification of the samples with a Pico-Green Assay (Appendix F).

2.2.7 *Statistical Analysis*

Data were analyzed by one and two-factor analysis of variance (ANOVA) and Fisher's protected least squares differences (PLSD) post-hoc testing using the software StatView (SAS Institute, Cary, NC). Data are presented as mean ± standard error of the mean (SEM). The significance level (type I error) was set up at 5% (α=0.05).

2.3 RESULTS

2.3.1 *Effect of the Transfection Reagent on the Production of GNDF and on MSCs Morphology.*

In the first experiment GDNF expression was observed for all monolayers transfected with a cationic reagent. Control MSC monolayers (n=6 per condition), cultured without the addition of the plasmid and with the naked plasmid, displayed small

amounts (< 1 ng/ml) of GDNF released into the medium at the collection time points (Figure 2.7). The results of the cumulated release of GDNF (Figure 2.3) showed some noticeable differences in the expression profile of GDNF among the transfection reagents. During the first 12 days, the production of GDNF was linear ($R^2 \approx 0.99$) and reached about 1.2 ng/ml per day in the GP2 study (2 μg plasmid load), whereas the use of L2000 (1.6 μg plasmid load) gave about 0.2 ng/ml per day for both media.

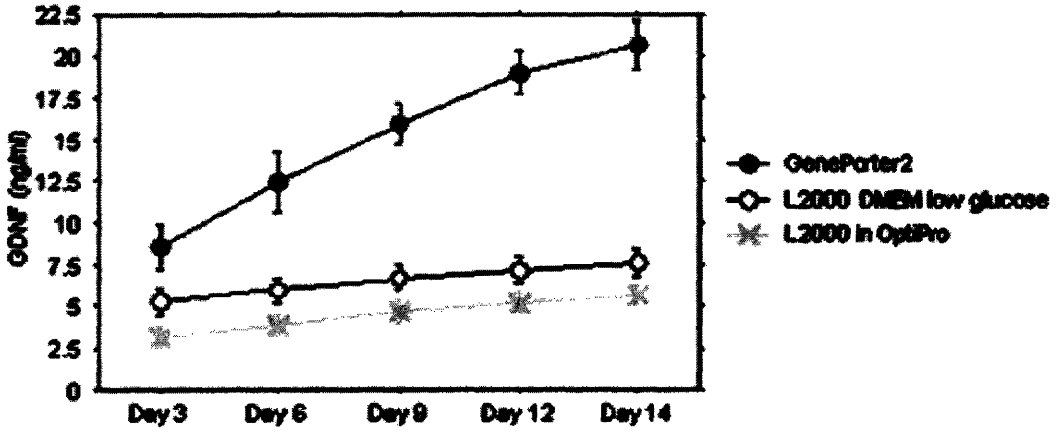

Figure 2.3: Cumulated release of GDNF (ng/ml) into the medium after transfection of rat MSCs, using different transfection reagents. n=6; mean±SEM

When considering the total cumulated and normalized GDNF released at the end of the two week period, the result with GP2 is about 3-fold higher than L2000 (Figure 2.4). Two-factor ANOVA showed a significant effect of both time ($P<0.0001$; power=1) and transfection reagent/medium ($P<0.0001$; power=1) on the cumulative GDNF release. Two-factor ANOVA was also performed to determine the effects of medium (for the L2000 data) and time on the accumulated GDNF release. The statistical analysis showed a significant effect of the transfection medium ($P<0.0001$; power=1) and a moderate effect of the time ($P<0.008$; power=0.88) on GDNF production.

The effect of the transfection reagent on the release of GDNF was also analyzed with one-factor ANOVA and Fisher's PLSD, for each time period. The post-hoc test revealed a significance difference in GDNF release between GP2 and L2000 in both medium for any time period ($P<0.0001$). The effect of time on the release of GDNF for each condition was also analyzed with a one-factor ANOVA. There was a significant

effect of time on GDNF release for GP2 ($P<0.0001$, power=1) whereas no significant effect was observed for L2000 in DMEM low glucose ($P<0.22$, power=0.4) and L2000 in OptiPro ($P<0.06$, power=0.6). To fully examine the statistical differences between the two media in the transfection of the MSCs with L2000, a Fisher's PLSD test was conducted. The results of the post-hoc test between Day 3 and Day 14 showed a moderate statistical significance difference for the release of GDNF for both L2000 in DMEM low glucose ($P<0.04$) and L2000 in OptiPro ($P<0.01$).

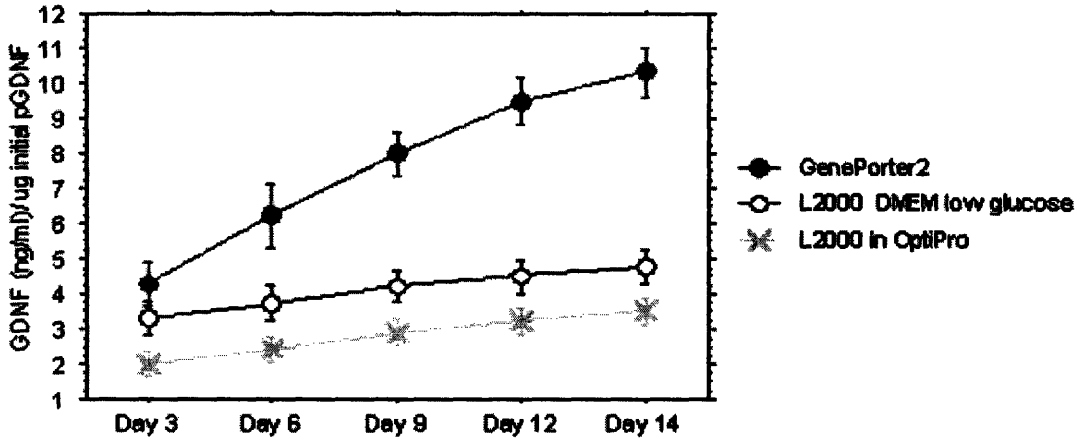

Figure 2.4: Accumulated release of GDNF (ng/ml) normalized to the initial amount of pGDNF (μg) for different transfection reagents and medium. n=6; mean±SEM

The type of the transfection reagent as well as the medium had a prominent effect on the morphology of MSCs during the transfection process. Light microscopy showed a small decrease in the cell density in GP2 experiments (Figure 2.5) relatively to the L2000 experiments. At the day of transfection, MSCs showed high density (95% confluency) and high biosynthetic activity but 6 days after transfection with L2000 in serum-free medium, cells appeared rounded and swollen and an obvious decrease of matrix synthesis. After 11 days, the cells decreased significantly in size and density (fig 2.6).

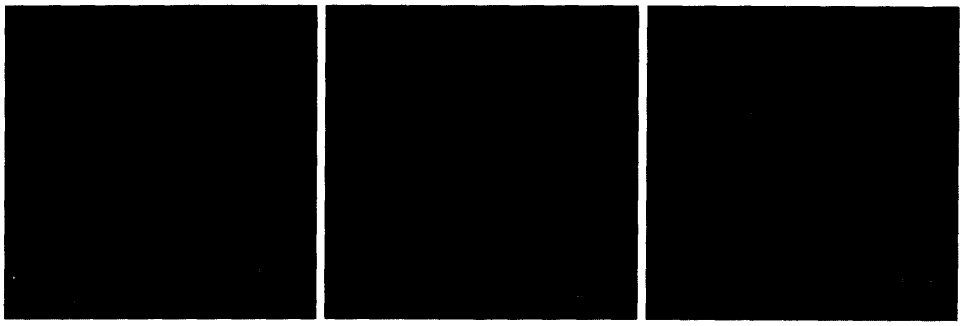

Figure 2.5: Light Microscopy of Rat MSCs at 3 hours (left) 24 hours (middle) and 3 days (right) after static transfection with GP2 (2μg pGDNF: 10μl GP2)

Figure 2.6: Light Microscopy of Rat MSCs, at 3 days (left), 6 days (middle) and 11 days (right) after static transfection with L2000 (1.6 μg pGDNF: 4 μl GP2)

A PicoGreen Assay was performed for each condition (n=6) at Day 14, and the DNA content was compared to a control (n=6) where MSCs were grown in the same medium conditions, but not transfected. The DNA content for the GP2 experiment was 460ng/well whereas its control was 530ng/well. In static conditions, there were 13% fewer cells after transfection than for the control. The DNA content for the L2000 experiment was 130ng/well for both media whereas the control was 520ng/well in OptiPro, and 250ng/well in DMEM; these results showed respectively 75% and 50% fewer cells for the transfected cells than the control.

This experiment demonstrated the importance of the selection of the transfection reagent and the expansion medium, which affect the cells morphology and density.

2.3.2 *Effect of the Doses of pGDNF : GenePorter2 on the Synthesis of GDNF*

In the second experiment, production of GDNF could be noted for loading of plasmid DNA as low as 0.2μg per well. The ELISA assay also showed that the expression of pGDNF decreased with time for any initial load contained in the lipoplexes (Figure 2.7). The maximal transfection was obtained with the highest initial dose of plasmid reaching more than 8ng/ml at Day 3 and maintaining the highest levels of expression throughout the 2-week period of the experiment.

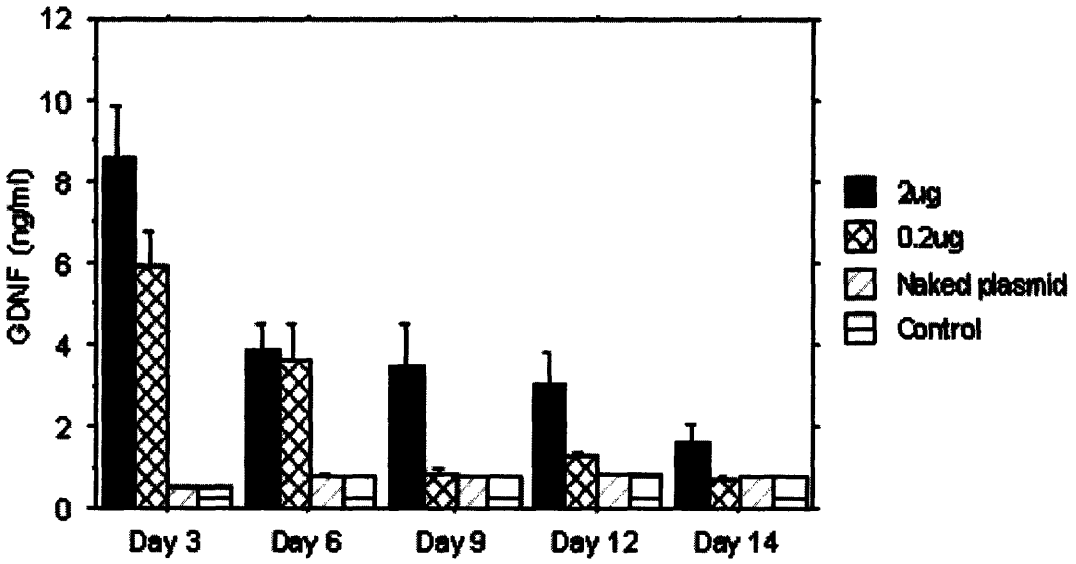

Figure 2.7: Release of GDNF (ng/ml) collected in the medium after transfection of rat MSC monolayers and using different loads of plasmid in the lipoplexes. n=6; mean±SEM

The accumulated release of GDNF for an initial 2 μg load of pGDNF showed a production of 22ng/ml after a 2-week period (Figure 2.8). Two-factor ANOVA of the cumulative release of GDNF demonstrated significant effects of both time and plasmid amount on the release of GDNF ($P<0.0001$; power=1). Further statistical examination with Fisher's PLSD post-hoc test showed no significant difference between 0.5μg and 0.2μg at any period of time but showed a significant effect between 2μg and both 0.2 μg and 0.5 μg after Day9 ($p<0.0001$).

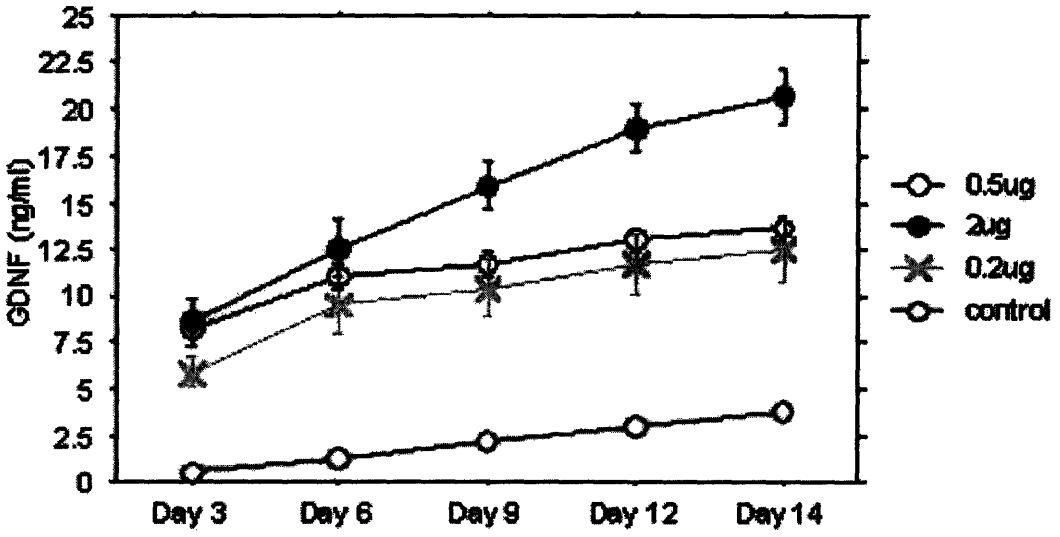

Figure 2.8: Accumulated release of GDNF (ng/ml) collected in the medium for various amounts of plasmid incorporated into the lipoplexes. n=6; mean±SEM

2.3.3 *Effect of the Transfection Method on the Cell Morphology and GDNF Synthesis*

In the third experiment, the transfection method (static culture versus orbital shaker) showed a prominent effect on the release of GDNF (Figure 2.9). The orbital shaking during the transfection (4 hours, 160 rpm) demonstrated an increased production of GDNF throughout the time of the experiment whereas in static condition, the production of GDNF clearly decreased with time.

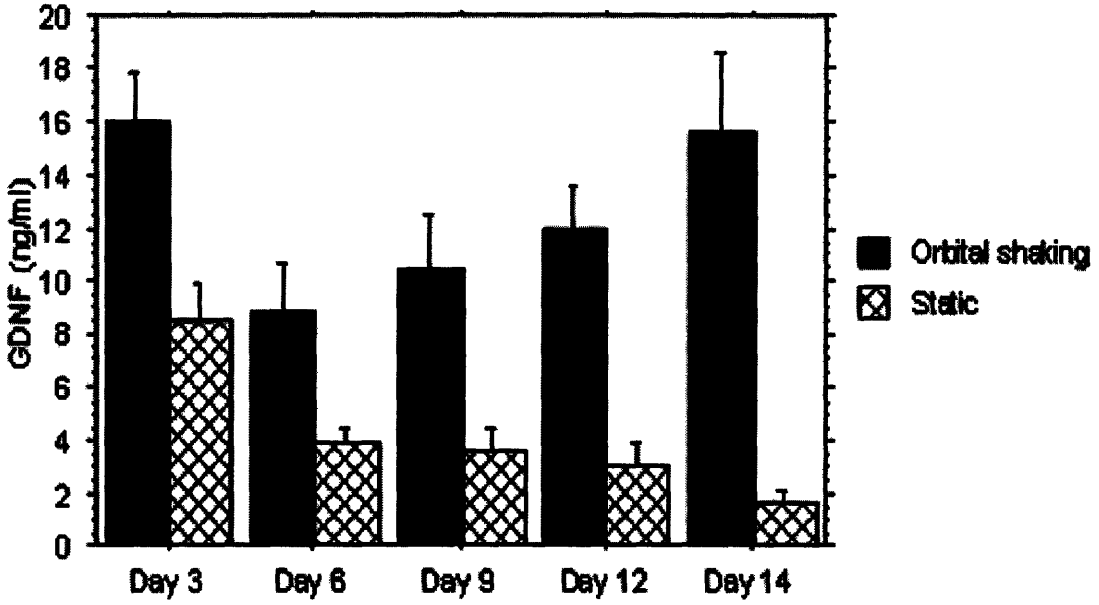

Figure 2.9: Comparison of the release of GDNF (ng/ml) collected at different times regarding the method of transfection employed (static versus orbital shaking). n=6; mean±SEM

For a similar initial pGDNF load, the results showed three-fold higher GDNF production in dynamic experiments to reach about 65ng/ml after 2 weeks (Figure 2.10). Two-factor ANOVA of the accumulated results showed significant effects of time ($P<0.0001$, power=1) and transfection method ($P<0.0001$, power=1) on the release of GDNF. Fisher's PLSD test, comparing the transfection methods for each time point confirmed statistical significance of static and orbital shaking during the 2 weeks period.

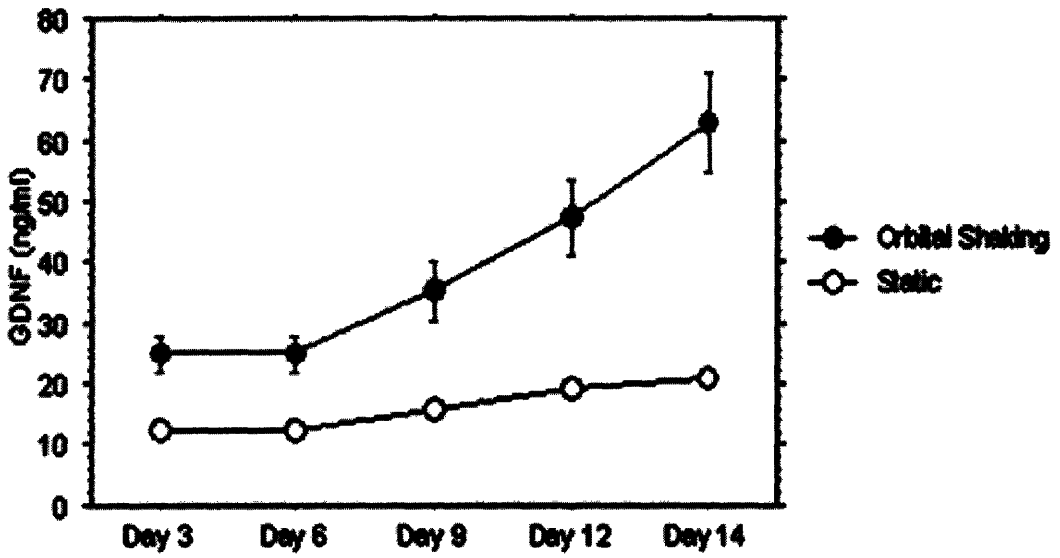

Figure 2.10: Accumulated release of GDNF (ng/ml) for the 2 culture conditions used for transfection (static versus orbital shaking). n=6; mean±SEM

Light Microscopy showed a very dense cellular arrangement and elevated level of matrix synthesis only after 4 hours of dynamic transfection at 160 rpm (Figure 2.11). At Day 14, PicoGreen Assay was performed for each condition (n=6) and the DNA content was compared with a control (n=6) where MSCs were grown in similar media conditions but without transfection. The DNA content in static conditions was 460ng/well (control at 530ng/well). However, the DNA value in dynamic conditions was 600ng/well (control was 560ng/well). There was a 20% difference in cell density between the two techniques. This result may partly explain the difference in the expression level of pGDNF.

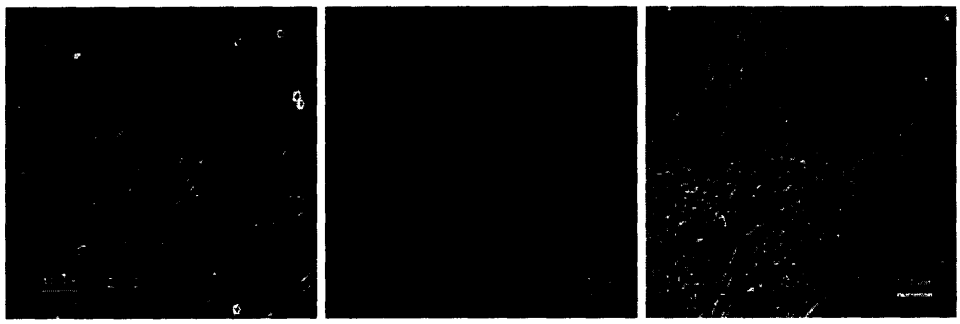

Figure 2.11: Light Microscopy of rat MSCs, at the day of transfection (left), at 24 hours (middle) and 12 days (right) after dynamic transfection (2μg pGDNF: 10μl GP2)

2.3.4 *Effect of the Medium on Cells Morphology and Expression of pGDNF (DMEM versus DMEM+bFGF)*

Light microscopy showed qualitatively the effect of bFGF on the cell morphology. Only 4 hours after transfection with GP2, the cells were arranged like a neuronal network (Figure 2.12, left). However, a rapid decrease of cell density was observed throughout the 2-weeks period (Figure 2.12, right).

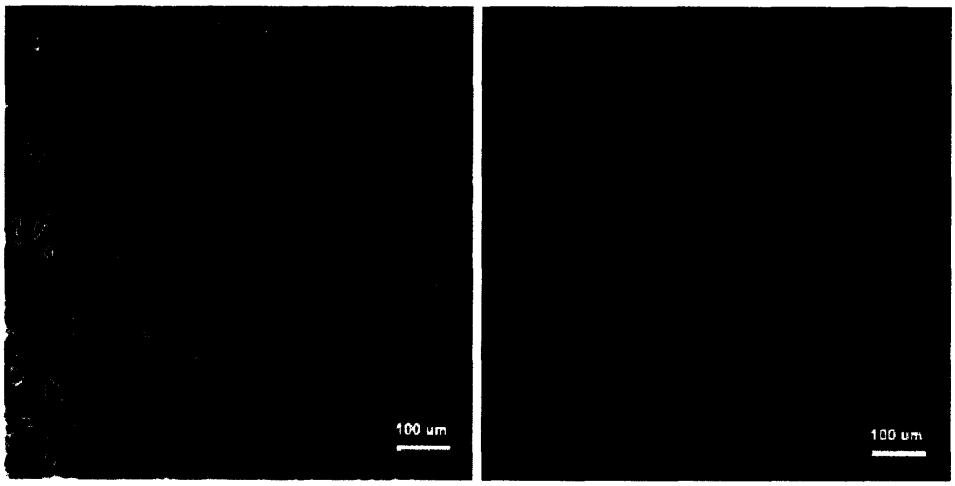

Figure 2.12: Light Microscopy of rat MSCs in Medium 2 at 4h (left) and 9 days (right) after transfection in static conditions

After 9 days of culture, the dynamic transfection (4 hours in orbital shaking at 160rpm) in Medium 1 showed higher cell density (Figure 2.13) than static transfection. The experiment also underscored the combined effect of bFGF and transfection reagent on cell death.

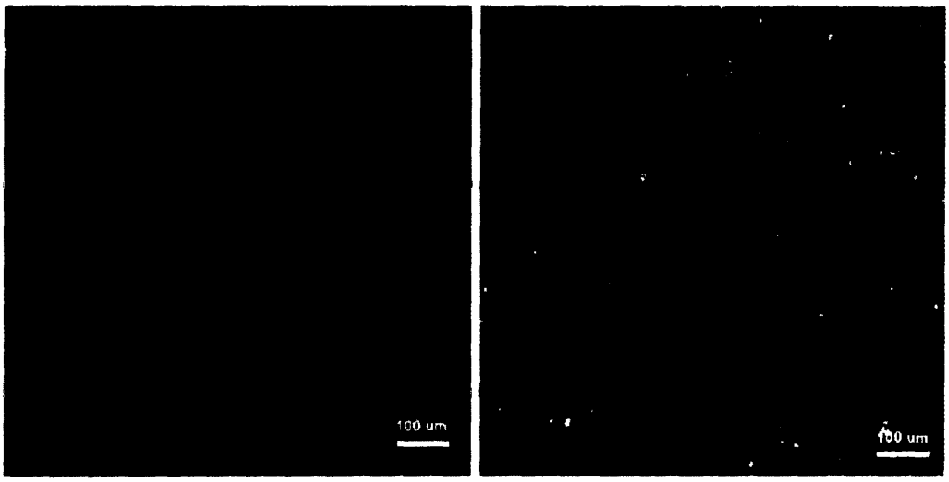

Figure 2.13: Light Microscopy of Rat MSCs in Medium 1 at 4h (left) and 9 days (right) after transfection in dynamic conditions

2.3.5 *Fluorescent Microscopy: GFP Production and pGDNF Expression.*

The plasmid was engineered such as it contained the gene encoding for the GFP which is commonly used as a reporter for gene expression. Florescent microscopy is used in this experiment as a quick technique to qualitatively evaluate the transfection efficiency. The superposition of light and fluorescent micrographs (Figure 2.14) showed the cells expressing GFP, therefore GDNF.

Figure 2.14: Superposition of Light Microscopy and Fluorescent Microscopy of Rat MSCs in Medium 1 at Day3 after transfection with GP2 in static conditions (2μg:10μl)

2.4 DISCUSSION

The results in this chapter demonstrated the possibility of using non-viral transfection conditions *in vitro* to enable MSCs to express a selected neurotrophic factor, GDNF, in therapeutic doses (ref). Price et al.[80] showed that GDNF, at 1 and 100 ng/ml, significantly increased by nearly 100% the number of trigeminal ganglion sensory neurons in culture at 5 days post-plating. Clarkson et al. [81] demonstrated that GDNF (10 ng/ml) in human embryonic dopamine neurons cultures nearly doubled dopamine neuron survival and reduced the rate of apoptosis from 6% to 3%.

In the current work MSCs were shown to over-express the plasmid encoding for GDNF for at least a two-week period of time. A notable finding of the experiment was that lipoplexes loaded with as little as 0.2μg could result in a significant production of GDNF by MSCs for several days, before falling off to control levels after one week. For the highest loading of plasmid, the level of GDNF production was still above the control after 2 weeks.

An important finding in this part of the thesis was the dramatic effect of the dynamic transfection on the production of GDNF. Whereas the synthesis of GDNF has been shown to decrease over the time in static conditions, GDNF released in the medium started to increase again after 6 days. The accumulated amount of GDNF produced during the 2-week period reached 65 ng/ml compared to 20 ng/ml produced in static conditions. Dynamic transfection seemed to also have stimulated cells growth. Higher cell population may explain the difference in level of production of GDNF found between static and dynamic conditions. Hoelters et al.[60] reported an optimized non-viral transfection of human MSCs resulting in transfection efficiency of up to 50% and a survival rate of more than 85% whereas other studies showed much lower transient transfection rates in stem cells including multipotent adult progenitor cells (11–13%)[82], neural stem cells (11–15%) [83] and human MSCs (14–16%)[84].

GenePorter2 yielded higher GDNF production compared to Lipofectamine 2000. The observation of the cell morphology and density throughout the transfection revealed an important cell death in serum-free medium (Lipofectamine 2000) whereas

GenePorter2 maintained significant cell viability. Future studies are needed to optimize the serum-free medium by the mean of growth factors and complements to be used with Lipofectamine 2000 transfection. Several studies, reported the prominent effect of cell death in non-viral transfection. Hamm et al. [85] showed that using Nucleofector technology in human MSCs could achieve transfection efficiencies of up to 45%, but viability rates were markedly reduced (16.5%).

The growth factor bFGF, which is used in transdifferentiation of MSC for a neuronal phenotype, was shown in the experiments in this chapter to promote a high level of cell death when used in the post-transfection medium.

The gene expression of the GDNF plasmid containing the gene for GFP gave visual evidence of the success of the transfection, but showed in general only a small population of cells expressing the fluorescent protein. However, for the application being addressed in this thesis, high transfection efficiency may not be required for a local therapeutic effect in defects in the brain.

In conclusion, the results demonstrated clearly that a therapeutic level of GDNF could be reached using non-viral transfection of MSCs with pGDNF. Two-week overexpression GDNF by MSCs was possible *in vitro*. This is an important fundamental basis for further experiments using these cells in gene-supplemented scaffolds for 3-D transfection.

Future work needs to include a systematic study of the toxicity of the transfection reagent on the cells, and monitoring of the cell viability throughout transfection process. Also, it would be of importance to estimate the transfection efficiency by immunofluorescence. Finally, it would be interesting to investigate whether the differentiation capacity and long-term stability of GDNF-expressing MSCs is affected either by the transfection and/or the expansion procedure

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3 GENE-SUPPLEMENTED COLLAGEN SCAFFOLDS FOR NON-VIRAL GDNF GENE DELIVERY

3.1 INTRODUCTION AND EXPERIMENTAL DESIGN

Many strategies have been explored for CNS repair procedures including: the injection of therapeutic molecules; the transplantation of cells [13]; and gene therapy [25], [26]. However, none of these methods has yet been shown to have a long-term beneficial effect. In cell transplantation, the efficacy is limited by apoptosis and the migration of the cells away from the defect. In procedures delivering neurotrophic factors, a localized therapeutic action is limited by the short half-life of the proteins *in vivo* and their dissolution. The inability to maintain therapeutic levels for a prolonged period could be overcome by the use of a scaffold as a carrier for genes [30], [31], [32]. The structural and material properties of the biomaterial scaffold are of importance for insuring a targeting action. For pDNA release, a porous structure with high surface area could provide a sustained, localized and high concentration of the plasmid. Furthermore, the degradation of the scaffold, controlled by the cross-linking density, could provide a prolonged release of a selected gene as the scaffold is replaced by the tissue ingrowth. A scaffold could also provide support for the migration of endogenous cells into the defect, and the migration of the transfected cells, seeded into the scaffold prior to implantation, from the defect site into surrounding tissue.

The objective of this part of the thesis was to develop a gene-supplemented collagen scaffold to have a long shelf life, an optimized release of lipoplexes for the endogenous cells and the delivery of transfected cells to the defect over a period of several weeks when implanted.

Collagen scaffolds were made by a freeze-drying technique and prepared with various amounts of collagen, various cross-link densities, and with various freezing temperatures. The effect of gene supplementation on the cross-link density was evaluated using a swelling ratio. Finally, cell seeding and incorporation into the scaffolds were investigated for 2 different scaffold conditions, Pilot 1 and 2. Pilot #1: Scaffolds were made using a 1% collagen concentration with an freezing temperature of -40°C. Scaffolds were seeded

with MSCs using different techniques and were evaluated after 24 hours. Pilot #2: Collagen scaffolds were made with various pore sizes and seeded with MSCs under static conditions, and evaluated after 1 week to determine the most promising scaffold for cell incorporation. For both experiments, DNA content of the scaffold was measured with a PicoGreen Assay, cell localization within the scaffold was visualized by histology and cell-mediated contraction was evaluated with the effective diameter of the scaffolds. This chapter achieved the specific aims 5 to 7.

3.2 MATERIALS AND METHODS

3.2.1 *Type I Collagen Scaffold Fabrication*

3.2.1.1 *Freeze-drying*

The porous collagen sheets were fabricated from type I/III collagen derived from porcine (Biogide powder, Geistlich Biomaterials, Wolhusen, Switzerland). Various concentrations of collagen were explored, ranging from 0.5% to 2% (w/w), Appendix G. Briefly, the collagen suspensions (pH=3) were prepared by mixing the collagen with a 0.001 N hydrochloric acid solution (Mallinckrodt Chemicals). This step induced the swelling of the collagen fibrils and the conversion of about 90% of banded collagen fibers to an unbanded structure. The slurries were first cooled at 0°C to prevent the denaturation of the collagen fibrils during blending and then mixed with a blender at 15,000 rpm for a total of 30 min. The final suspension was degassed by spinning at 1000 RCF (Relative Centrifugal Force) for 3 min. The collagen suspension was poured into plastic molds 15 cm x 3.7 cm and equilibrated to 20°C in the freeze-dryer for 15 min. Then the temperature of the freeze-dryer chamber (VirTis, Gardiner, NY) was decreased at a controlled rate of freezing to -40°C or -10°C. Previous studies have shown that the ramping process makes more homogeneous pores in the fabrication of the scaffolds [86]. Once the final temperature was reached, the temperature was annealed for a minimum period of 60 min. to allow the nuclei of ice crystals to form. After the solidification step, the suspension was converted to a dry foam through the process of lyophilization, by which the ice is removed by sublimation, leaving an empty pore in the place of every ice crystal. Hence, the lower the final temperature and the higher the rate of freezing, the

smaller the final pore diameter. After this freezing period, the ice crystals were sublimated under vacuum (<200 mTorr) at 0°C for 17 hours to leave behind a homogeneous and highly porous solid collagen scaffold. (Appendix H). Similar scaffolds have been previously reported in previous studies [87, 88] to have pore sizes of 151 um for 0.5% collagen with an freezing temperature of -10C and 96 um for 0.5% collagen with an freezing temperature of -40C. Furthermore, Doillon et al.[89] studied the effect of collagen concentration on pore size and showed that an increased concentration of collagen resulted in smaller average size of pores.

3.2.1.2 *Cross-linking Methods*

i. **Dehydrothermal Treatment**

Dehydrothermal (DHT) treatment through drastic dehydration forms inter-chain peptide bonds between amino acid residues [37]. DHT results in a decrease in the free amine groups and water-binding capacity, and it increases the tensile strength [90]. Moreover, it has the effect of sterilizing the collagen scaffold. Immediately after the freeze-drying process, the porous sheets were cross-linked by DHT treatment under a vacuum of 50 mTorr and a temperature of 105°C for 17 hours.

Following the DHT treatment, 8-mm-diameter disks were cut from the porous sheets using a sharp-edged punch. Additional cross-linking was obtained by treatment with a water-soluble carbodiimide.

ii. **Carbodiimide Cross-Linking**

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) is a water-soluble agent which catalyzes collagen cross-linking. Since it is not incorporated into the amide cross-links it can readily be washed out of the scaffold. EDAC treatment results in collagen-collagen bonds. Carboxylic groups of glutamic and aspartic acid residues are activated and form amide bonds in the presence of lysine or hydroxylysine residues (Figure 3.1). N-hydroxysuccinimide (NHS) increases the rate and amount of cross-linking [91], [92]. Olde Damink et al. [92] found little benefit is derived beyond 2 hours in the EDAC/NHS solution.

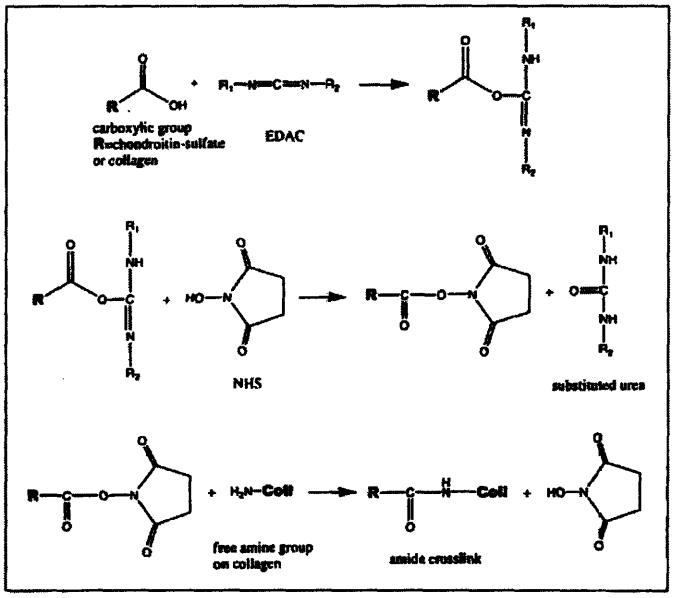

Figure 3.1: Mechanism of EDAC crosslinking

The 8-mm-diameter disks were immersed in a solution containing EDAC (Sigma Chemicals) and N-hydroxysuccinimide (NHS, Sigma) at various molar ratio of EDAC:NHS:carboxylic acid (Table 3.1). The excess of EDAC was removed by rinsing in PBS (Appendix I). In this study, the following molar ratios were used:

Table 3.1: Nomenclature of the different cross-linking treatments used in this study.

Treatment	EDAC:NHS:COOH
DHT	24hr, 105C, 30 mTorr
EDAC 1	1 :0.4 :1
EDAC 2	5 : 2 :1
EDAC 3	14 : 5.5 :1

3.2.2 *Physical Characterization of the Scaffolds*

3.2.2.1 *Swelling Ratio*

The cross-link density for randomly coiled polymer networks is inversely related to the swelling ratio. The density of cross-links formed by different cross-linking

techniques was determined as described by Weadock et al.(1983). Collagen Scaffolds were gelatinized in water at 90°C for 2 min., placed between sheets of filter paper under a 1 kg weight for 20 sec. to expel excess water remaining in the pores, and then the scaffolds were weighed to determine the wet mass (M_W). Matrices were then dried in a oven at 110°C overnight and weighed to determine the dry mass (M_D), Appendix M.

$$r^* = 1/Vf = [(M_D/\rho c) + ((M_W-M_D)/\rho water)]^*\rho c/M_D$$

r^* = swelling ratio

Vf = volume fraction of dry collagen

ρc = density of collagen = 1.32 g/cm3

ρwater = density of water = 1 g/cm3

3.2.3 *Plasmid Incorporation into Collagen Scaffolds*

Solutions of the plasmid-lipid complex were pipetted into the collagen scaffolds according the following plan (Figure 3.2):

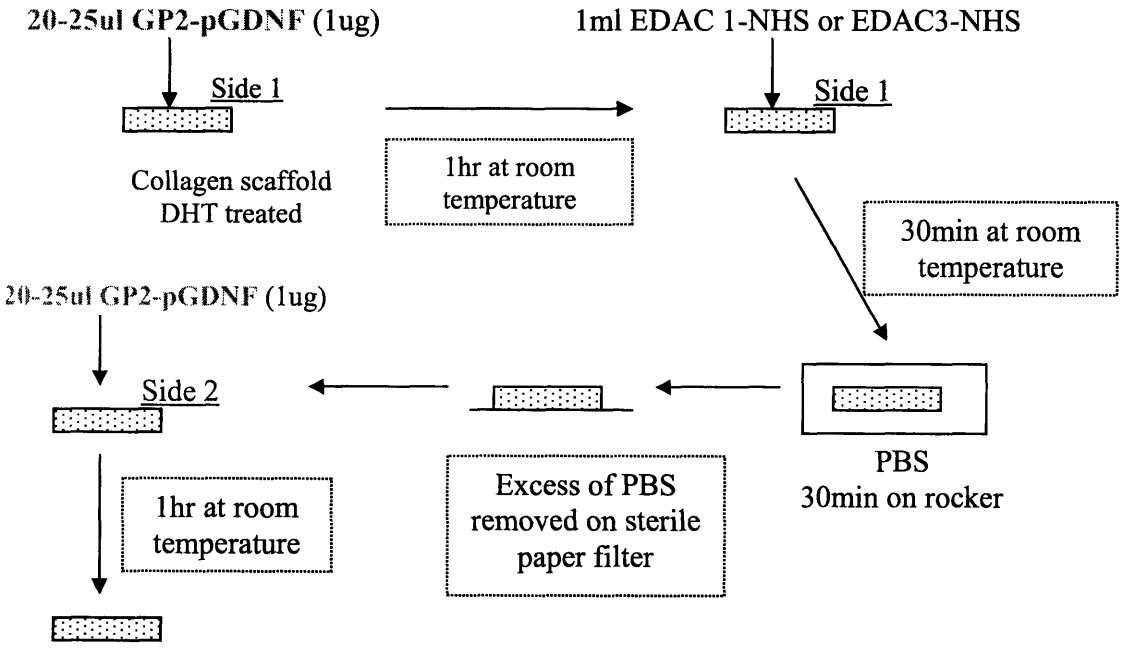

Figure 3.2: Method of incorporation of the lipoplexes in the collagen scaffolds

3.2.4 *Cell Seeding of the Collagen Scaffolds*

Previous studies demonstrated that pore size and cross-linking density affect the incorporation of MSCs into collagen scaffolds [93], [87]. Two pilot studies for the cell seeding experiments were conducted. The first study (Pilot 1) aimed to optimize the incorporation of the rat MSCs after 24 hours in static and dynamic conditions. Scaffolds were made at a concentration of 1% collagen and had a subsequent EDAC treatment (EDAC 1 and EDAC 3). The cell density was 1 million cells per 8-mm-diameter disk. The objective of the second study (Pilot 2) was to evaluate the incorporation of the rat MSCs after 1 week of static seeding. Four different pores size were evaluated and the scaffolds were made at 0.5% and 1% collagen with T (freezing) = -10°C and -40°C and had a subsequent EDAC treatment (EDAC 1). The cell density was 500,000 cells per 8-mm disk.

Both pilot studies followed the same cell-seeding method (Figure 3.3). At the day of seeding, the excess of water in the hydrated scaffolds was quickly removed on sterile filter paper (Fisherbrand, Fisher Scientific Co) and the scaffolds were placed onto the 24 well-plates previously coated with 500ul of 3% agarose (m/w) (Appendix N). The scaffolds were subsequently cross-linked with 1 ml of EDAC solution for 30 min. Then the scaffolds were rinsed for 30 min in PBS. After the cross-linking step, 10 µl of MSC suspension was pipetted onto one surface. After 10 min, the scaffolds were flipped over and an additional 10 µl of the cell suspension was added, and the cell-seeded scaffold left for 10 min at room temperature. Finally 1 ml of DMEM low glucose, 20% FBS and 1% antibiotic-antimycotic solution was added to each well.

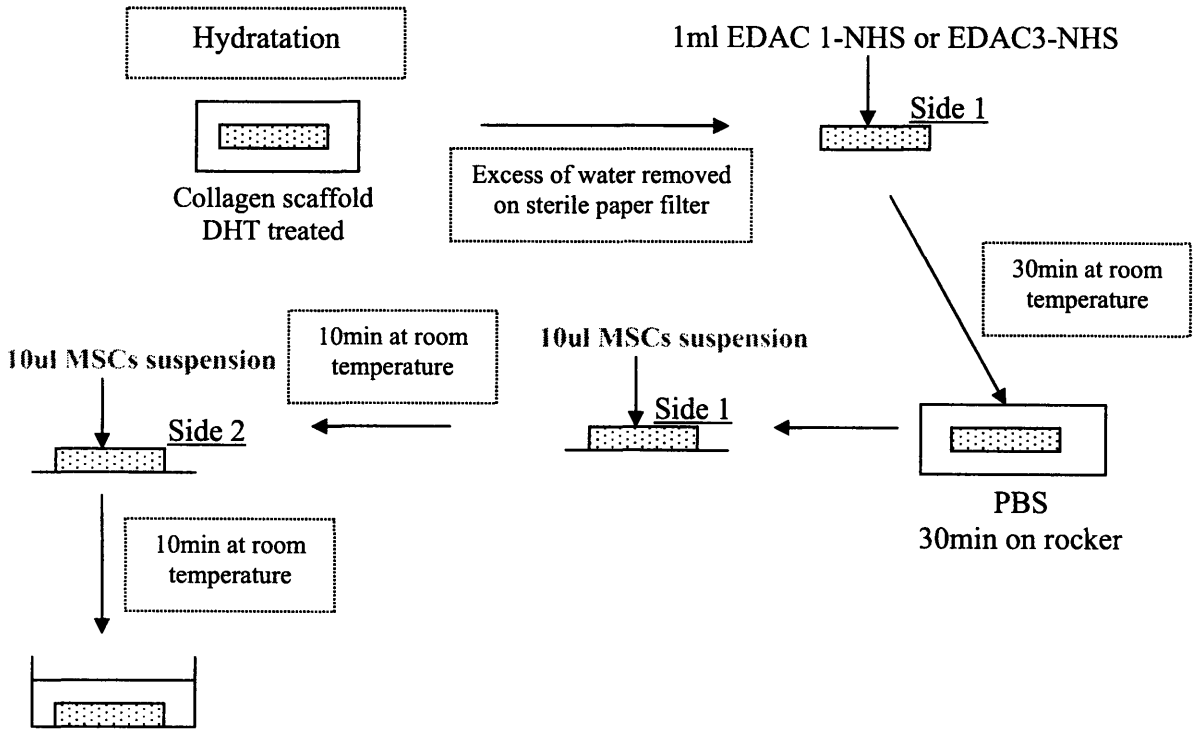

Figure 3.3: Method of incorporation of the MSC suspension in the collagen scaffolds

The cell-seeded scaffolds were cultured in an incubator at 37°C in a 5% CO_2 atmosphere and 95% humidity. For long-term seeding, the medium was changed every 3 days.

3.2.5 *Contraction Measurement of the Scaffolds*

The diameter of the scaffolds was measured with every change of medium. Briefly, a diameter template (Appendix O) was placed under the 24-well plates to provide a measure of the diameter. If the difference in the two orthogonal diameters appeared to be greater than 10%, both diameters were measured. The effective diameter was then taken as the root mean square of the two diameters. Cell-mediated contraction was calculated by dividing the average seeded effective diameter at a specific time point by the initial diameter of the matrix at Day 0.

3.2.6 *DNA Content*

The cell-seeded scaffolds were terminated at 24 hours and 1 week after seeding, and the DNA content was determined with a PicoGreen Assay. Briefly, at the day of the termination, the medium was removed from the culture, and the scaffolds were rinsed twice with a phosphate-buffered saline solution (PBS) and lyophilized overnight. The dried matrix samples were then digested overnight at 60°C with a Proteinase K solution (500 μg/ml) (Appendix P). The number of cells was estimated from the DNA quantification of the samples (n=5) (Appendix F). Previous studies showed a DNA concentration in mammalian cells of 7-10 pg DNA/cell [94]. In the assay in this thesis, a sample with a known number of cells (n=5) was run to determine the DNA content of the rat MSCs. The DNA content of the rat MSCs was 10 pg/cell.

3.2.7 *Histology*

Two disks per condition were terminated at the end of the experiment (after 24 hours for Pilot 1 and after 7 days for Pilot 2) for Hematoxylin and eosin (H&E) staining. The scaffolds were initially examined macroscopically and the stiffness assessed with a forceps. Then, the scaffolds were placed in 10% buffered formalin at 4°C for at least two days. Samples were fixed using a tissue processor (Hypercenter XP, Tissue Processor, ThermoShandon, Houston, TX) (Appendix Q), embedded in paraffin, and sectioned at 6-μm thickness with a microtome. The sections were then mounted on glass slides and stained with H&E (Appendix R) to determine the cell morphology and distribution into the scaffolds. After being stained, coverslips were applied to the slides, and the slides were left drying overnight.

3.2.8 *Statistical Analysis*

Data were analyzed by 1-, 2-, and 3-factor ANOVA and Fisher's PLSD post-hoc testing using the software StatView (SAS Institute, Cary, NC). Data are presented as mean ± standard error of the mean (SEM). The significance level (type I error) was set up at 5% (α=0.05).

3.3 RESULTS

3.3.1 *Effect of Gene-supplementation on Cross-link Density*

The relative cross-link density/ stiffness of the scaffolds was determined indirectly by means of the swelling ratio. The present study compared the effect of the amount of collagen (0.5% and 1%, w/w), the cross-link technique (DHT and three different concentrations of EDAC), and the presence of lipoplexes, on the swelling ratio. The significance of the effects of these variables on the cross-link density was determined by ANOVA.

3.3.1.1 *Effects of the presence of the Lipoplexes on the Cross-link Density of the Collagen Scaffolds at 0.5% and 1%. (Figure 3.4)*

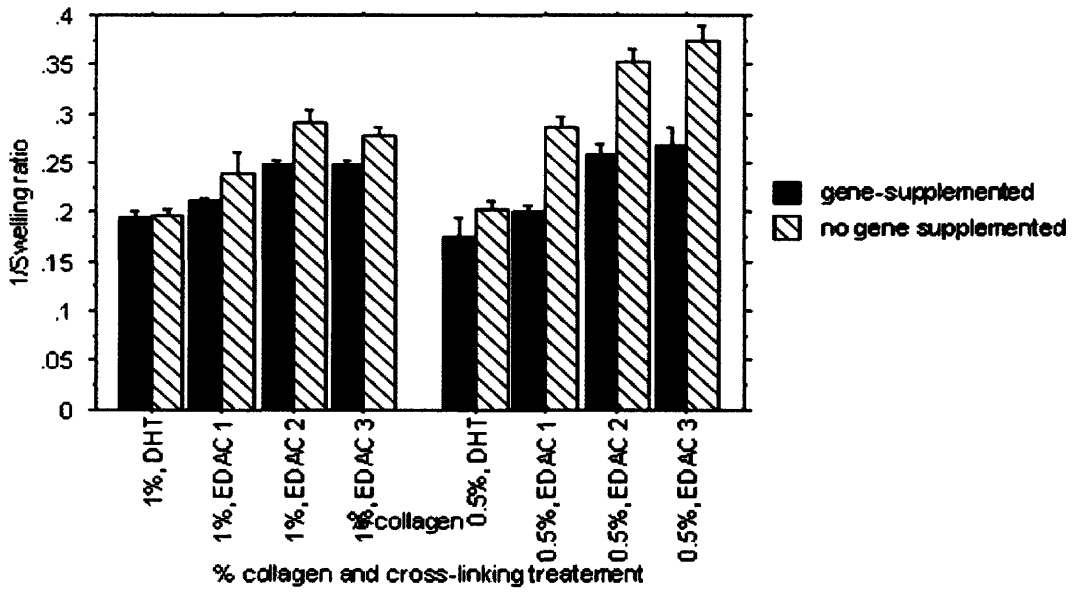

Figure 3.4: Inverse of the swelling ratio for gene-supplemented scaffolds and control for various collagen concentration and cross-linking treatments. Mean±SEM; n=5.

Three-factor ANOVA shows significant effect of the collagen concentration ($P<0.0001$, power= 0.997), cross-linking density ($P<0.0001$, power=1) and lipoplex incorporation ($P<0.0001$, power=1) on the inverse of swelling ratio. Further analysis with Fisher's PLSD test confirmed a significant effect of the lipoplex load on the cross-linking density.

3.3.1.2 *Effects of the Cross-linking Treatment on Collagen Scaffolds at 0.5% and 1%.*

The swelling ratio of the collagen scaffolds, calculated as the inverse of the volume fraction of collagen ranged for scaffolds at 1% collagen varied from 5.0 ± 0.2 (mean ± standard deviation) for the DHT scaffolds to 3.6 ± 0.2 for the EDAC 3 scaffolds, and for scaffolds at 0.5% collagen from 5.0 ± 0.5 (mean ± standard deviation) for the DHT scaffolds to 2.7 ± 0.2 for the EDAC 3 scaffolds (Figure 3.5). Taking the cross-link density to be proportional to the inverse of the swelling ratio, the density of the cross-links increased with the different cross-linking methods as follows: DHT< EDAC 1< EDAC 2< EDAC 3 (Figure 3.5).

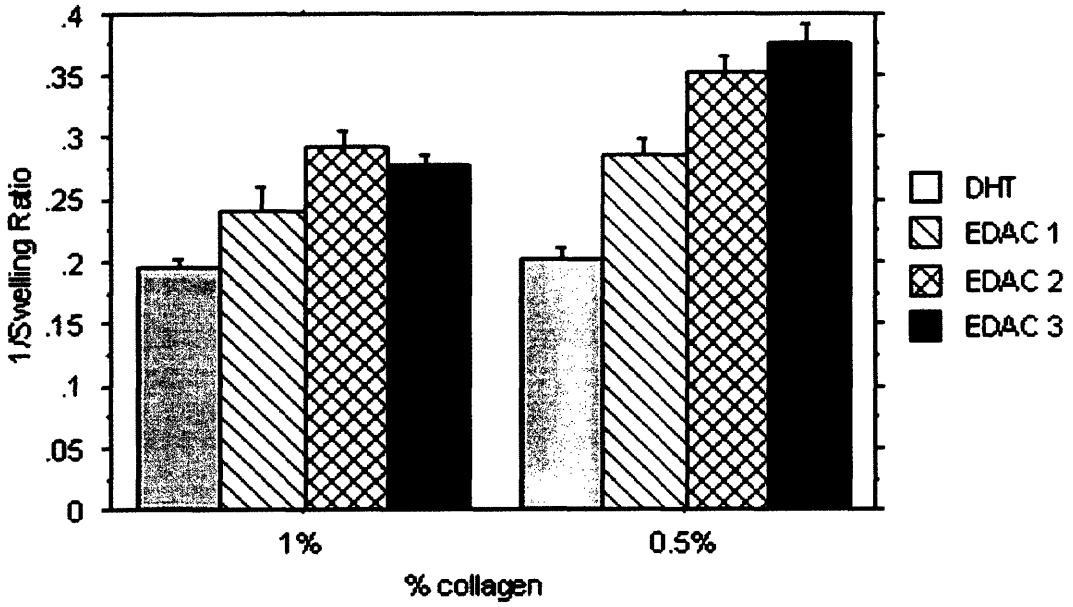

Figure 3.5 : Inverse swelling ratio of cross-linked scaffolds with different concentrations of collagen. Mean±SEM; n=5.

Two-factor ANOVA showed significant effects of both the collagen concentration and the cross-linking treatment on the swelling ratio ($P<0.0001$; power=1). Further analysis with Fisher's PLSD demonstrated that the effect of EDAC 2 and 3 was not significantly different. Also, according to the results, the cross-link density increased as the amount of collagen in the scaffold decreased.

3.3.1.3 *Effects of the Cross-linking Treatment on pDNA loaded Collagen Scaffolds at 0.5% and 1%.*

The swelling ratio of the collagen scaffolds calculated as the inverse of the volume fraction of collagen ranged for scaffolds at 1% collagen from 5.6 ± 0.4 (mean ± standard deviation) for the DHT scaffolds to 4.0 ± 0.2 for the EDAC 3 scaffolds and for scaffolds at 0.5% collagen from 5.9 ± 1.2 (mean ± standard deviation) for the DHT scaffolds to 3.8 ± 0.6 for the EDAC 3 scaffolds (Figure 3.6). The inverse of the swelling ratio followed the same trend as seen with the unloaded collagen scaffolds but with a moderate impact: the density of the cross-links increased with the different cross-linking methods as follows: DHT< EDAC 1< EDAC 2< EDAC 3 (Figure 3.6).

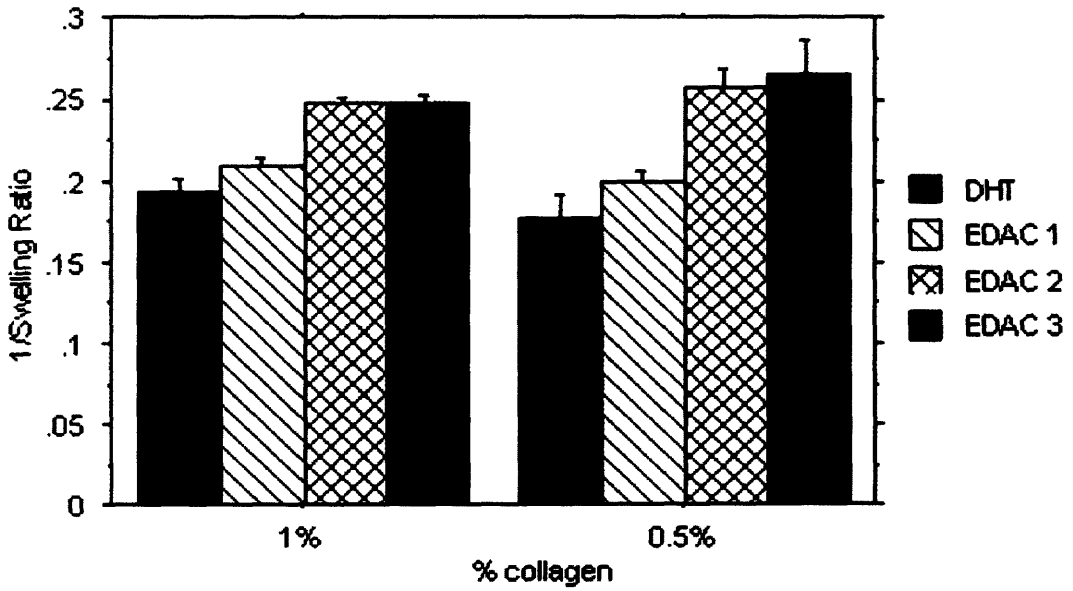

Figure 3.6: Inverse swelling ratio of gene-supplemented scaffolds with different concentrations of collagen and cross-link densities. Mean±SEM; n=5.

Two-factor ANOVA showed that there were a significant effect of the cross-linking treatment ($P<0.0001$; power=1) and no significant effect of the collagen concentration on the swelling ratio (power=0.5). Further analysis with Fisher's PLSD test demonstrated that the effect of EDAC 2&3 was not significantly different as well as DHT and EDAC 1 for the 0.5% collagen scaffolds.

3.3.2 *Effect of the Seeding method and Pore Sizes on Cell Incorporation into the Scaffolds.*

3.3.2.1 *Experimental Matrix*

The aim of the following studies was to evaluate the cell incorporation into the scaffolds. The experimental conditions are presented in the table below (Table 3.3). The variables studied were the collagen concentration, freezing temperature and cross-link density of the scaffolds and the method of seeding (static or dynamic).

Table 3.2: Experimental matrix for cell seeding experiments in collagen scaffolds.

VARIABLES	Experience #	Collagen concentration (%)	T freezing (°C)	EDAC: NHS:COOH	Experimental condition	# scaffolds
Cells incorporation in scaffolds with different seeding and cross-linking methods (1 day)	1-1	1%	[redacted]	1:0.4:1	static	4
	1-2	1%	[redacted]	[redacted]	static	4
	1-3	1%	[redacted]	1:0.4:1	[redacted]	4
	1-4	1%	[redacted]	[redacted]	[redacted]	4
	1-5	1%	[redacted]	1:0.4:1	[redacted]	4
	1-6	1%	[redacted]	[redacted]	[redacted]	4
Cells incorporation in scaffolds with different pore sizes (1 week)	2-1	0.5%	[redacted]	1:0.4:1	static	8
	2-2	0.5%	[redacted]	1:0.4:1	static	8
	2-3	1%	[redacted]	1:0.4:1	static	8
	2-4	1%	[redacted]	1:0.4:1	static	8

3.3.2.2 *Main Results*

The following table (Table 3.4) presents the main results obtained 24 hours and 1 week after cell seeding. DNA content, number of cells and relative number of cells were

obtained after a PicoGreen Assay and the effective diameter was measured with a template. The results are discussed in the following parts.

Table 3.3: Main results after cell seeding experiments in collagen scaffolds.

Experience #	DNA content (ng)	Number of cells	Final amount cells/initial (%)	Effective diameter (%)
1-1	7900±1700	790,000	79	NA
1-2	6400±2600	640,000	64	NA
1-3	5300±2100	530,000	53	NA
1-4	4300±2300	430,000	43	NA
1-5	7100±85	710,000	71	NA
1-6	4400±627	440,000	44	NA
2-1	1160±190	116,000	23	56±0.1
2-2	1660±290	166,000	33	92±0.1
2-3	2340±300	234,000	47	90
2-4	2330±320	233,000	47	100

3.3.2.3 *Effect of the Method of Seeding on the Cells Incorporation into the Scaffolds (Pilot 1)*

i. **DNA Content**

The number of cells attached to the scaffolds after 1 day (n=2), based on the DNA contents (Table 3.4; Figure 3.7), varied from about 40-70% of the number of cells seeded into the samples (*i.e.*, 1,000,000). The implants cultured in static conditions contained about 790,000 cells/scaffold for EDAC 1 and 640,000 cells/scaffold for EDACS 3, whereas the scaffolds cultured in dynamic conditions contained about 530,000 cells/scaffold (EDAC 1) and 430,000 cells/scaffold (EDACS 3) for the rocking plate and about 710,000 cells/scaffold (EDAC 1) and 440,000 cells/scaffold (EDACS 3) for the

orbital shaking. The small sample size of the pilot experiment precluded statistical analysis.

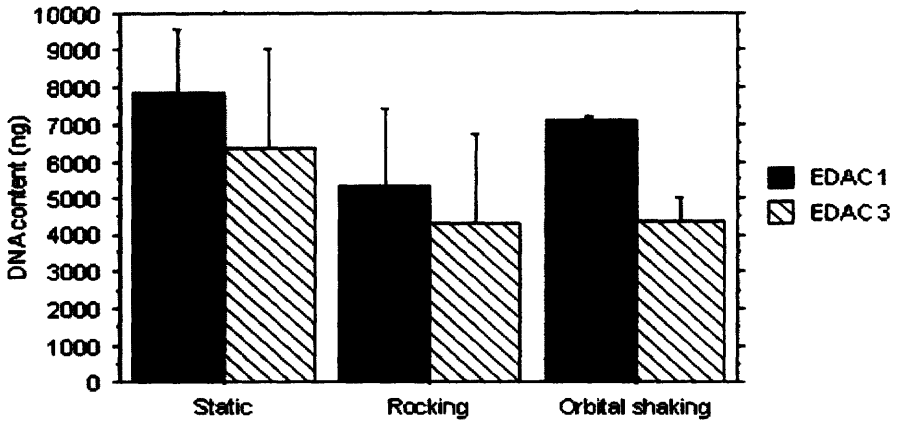

Figure 3.7: DNA content (ng) per scaffold after 24 hours of culture in static and dynamic conditions, for two different cross-link densities. Collagen scaffolds were seeded with 1E6 rat MSCs per scaffold. Mean ± SEM; n=2.

ii. **Histology**

H&E staining revealed that, after 24 hours of culture under dynamic condition, rat MSCs tend to stay and cluster on the surface of the scaffolds whereas in static condition (Figure 3.8 a), cells seem better incorporated into the scaffolds (Figure 3.8 b)

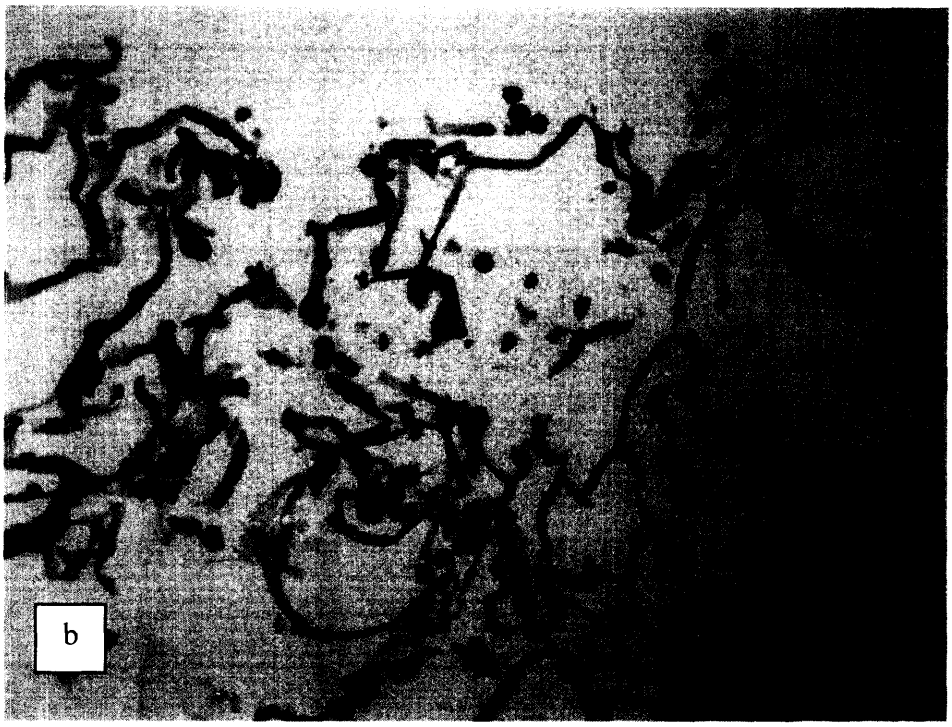

Figure 3.8: Hematoxylin and eosin staining of 1% collagen scaffolds (Tfreezing= -40C, EDAC 1) after 24 hours of culture in static (a) and dynamic (b) conditions, transversal section, scale bar 100μm. The scaffolds were seeded with rat MSCs at 1 million cells/scaffold.

3.3.2.4 *Effect of the Pore Size on Cell Incorporation into the Scaffolds.*

i. **DNA Content**

After one week of culture, there was a pronounced trend in the DNA content per scaffold versus the pore size (n=6). As the size of the pores decreased, the DNA content in the scaffold increased (Figure 3.9). The implants with the smallest pore size (1% collagen scaffolds with $T_{freezing}$=-40°C), contained about 233,000 cells/scaffold, whereas the scaffolds with a larger pore size (0.5%collagen scaffold with $T_{freezing}$=-10°C) contained about 116,000 cells/scaffolds. The intermediate pore sizes (0.5% collagen scaffolds with $T_{freezing}$=-40C; 1% collagen scaffolds with $T_{freezing}$=-10C) contained about 166,000 and 234,000 cells/scaffolds, respectively. The number of cells attached to the scaffold after one week of culture, going from the smallest to the largest pore size, counted for 47%, 47%, 33% and 23% of the initial number of cells.

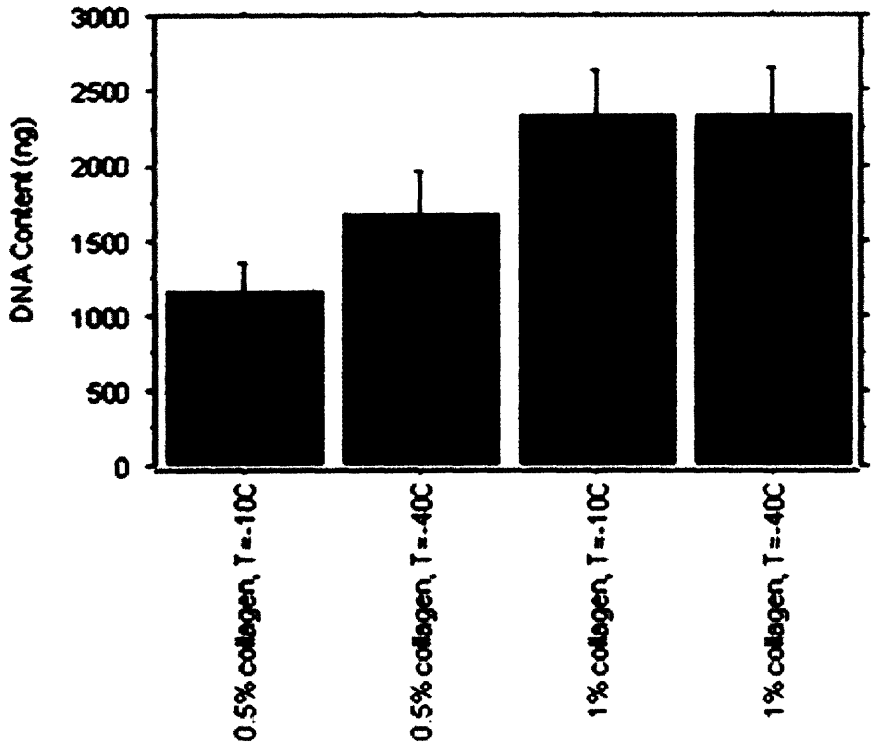

Figure 3.9: DNA content (ng) per scaffold after one week of culture for four different pore sizes. The collagen scaffolds were seeded with 500,000 rat MSCs per scaffold. Mean ± SEM; n=6.

One-factor ANOVA showed a significant effect of the pore size of the scaffold ($P<0.03$, power= 0.73) on the number of cells attached to the scaffold after 1 week. A post-hoc test, showed a significant difference in the number the cells in the scaffolds ($P<0.011$) for the 0.5% collagen scaffold and $T_{freezing}$=-10°C and with both the 1% collagen scaffolds and $T_{freezing}$= -40°C and the 1% collagen scaffolds and $T_{freezing}$=-40°C.

ii. **Matrix Contraction**

Not all the cell seeded scaffolds underwent a reduction in diameter by the end of the 1-week period of culture. The scaffolds with the smallest pore sizes maintained their diameter during the entire duration of the experiment whereas the scaffolds with the biggest pore sizes contracted to about half of their size (Figure 3.10).

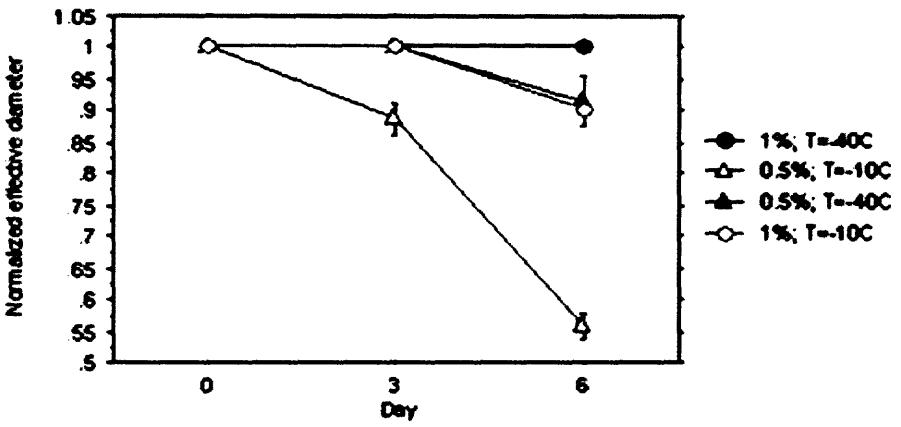

Figure 3.10: Cell-mediated contraction of collagen scaffolds versus culture time for four different pore sizes. The scaffolds were seeded with Rat MSCs (5E5 cells/scaffold). Mean ± SEM; n=7.

Two-factor ANOVA showed a significant effect of both culture time ($P<0.0001$, power= 1) and pore size ($P<0.0001$, power= 1) on the contraction of the scaffolds

iii. **Histology**

H&E staining showed that cells could be incorporated more easily inside the scaffolds with the biggest pores (fig 3.11 a) whereas cells tend to stay on the surface of the scaffolds with smaller pore sizes (fig 3.12 a). After 2 weeks of culture, MSCs infiltrated entirely the lipoplex-supplemented scaffolds at 0.5% collagen and $T_{freezing}$=-10°C. MSCs seemed to have kept their rounded morphology inside the pores (fig 3.11 b) but showed an elongated and fibroblast-like morphology in the periphery of the scaffold (fig 3.11 c).

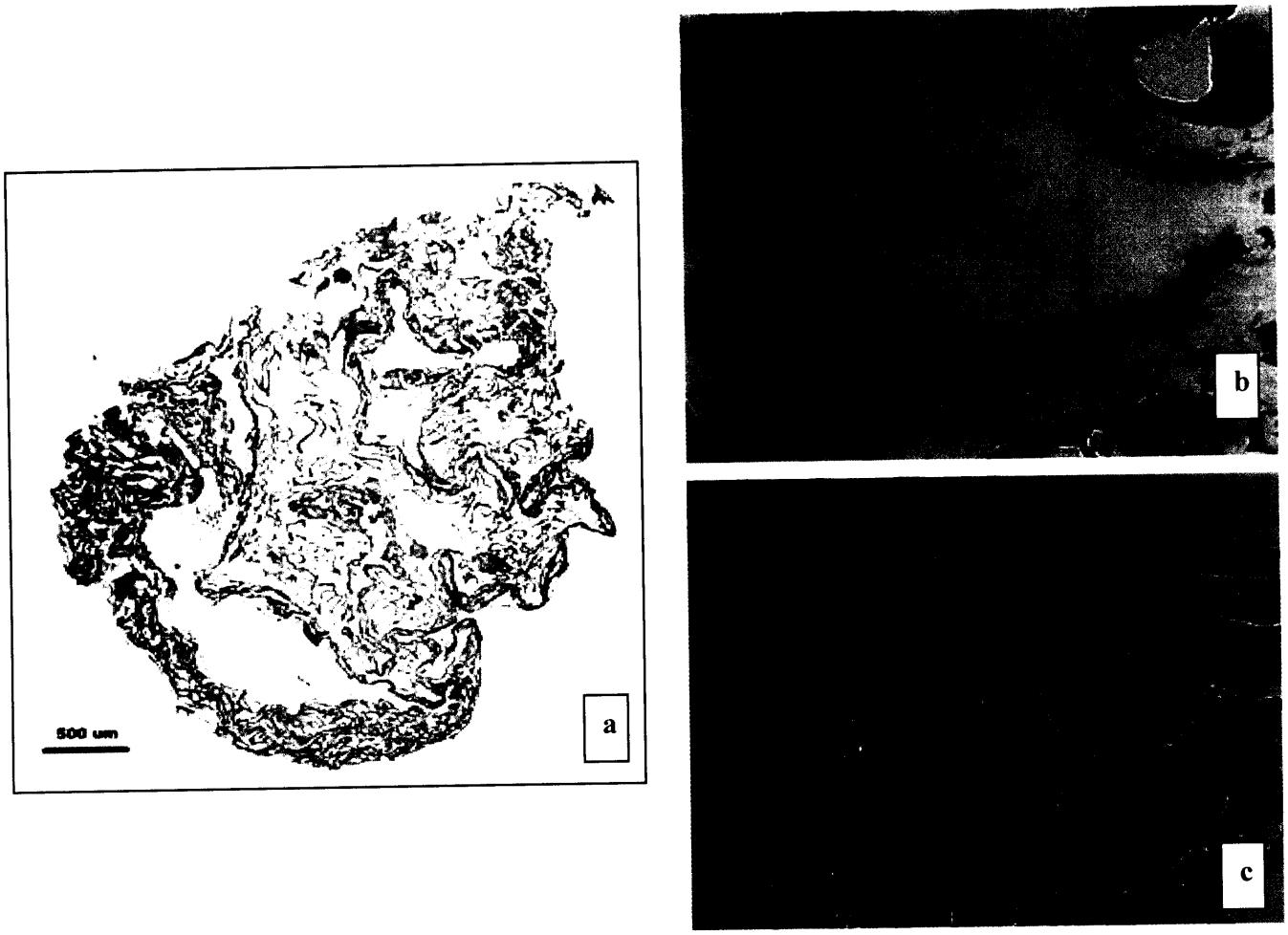

Figure 3.11: Hematoxylin and Eosin staining of 0.5% collagen scaffolds Tfreezing= -10C after 1 week of culture (a), inner parts of the scaffold at higher magnification (b), surface of the scaffolds at higher magnification (c). The scaffolds were seeded with Rat MSCs at 5E5 cells/scaffold.

However, after 2 weeks of culture, MSCs did not infiltrate significantly the gene-supplemented scaffolds at 1% collagen and $T_{freezing}$=-40°C (fig 3.12 a). Cells were mainly localized onto the surface of the scaffolds where they kept their rounded morphology (fig 3.12 c). Some cells could be noted inside few very large pores (fig 3.12 b). No contraction was observed after the 2-week experiment.

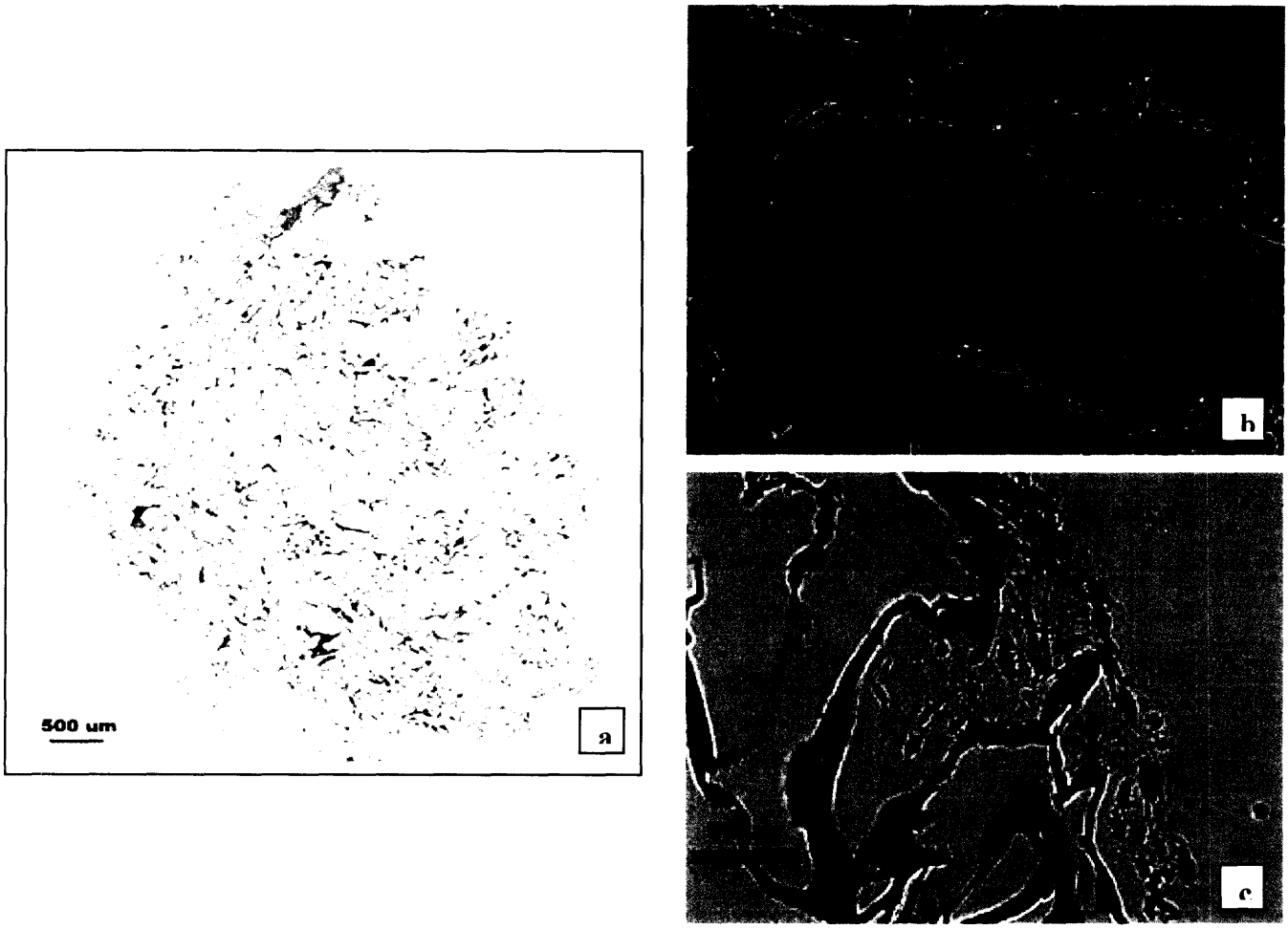

Figure 3.12: Hematoxylin and eosin staining of 1% collagen scaffolds Tfreezing= -40°C after 1 week of culture (a), inner parts of the scaffold at higher magnification (b), surface of the scaffolds at higher magnification (c). The scaffolds were seeded with Rat MSCs at 5E5 cells/scaffold.

3.4 DISCUSSION

Biomaterial scaffolds used in tissue engineering should be biocompatible, biodegradable and facilitate cell attachment and proliferation. Collagen has been widely used because of the favorable compatibility with cells and its processability. However one potential drawback of collagen sponge as a scaffold for cell proliferation and differentiation for certain applications is its low mechanical strength. Collagen has been used combined to other materials or cross-linked to increase the resistance to compression *in vitro* and *in vivo*. The combination of DHT and carbodiimide treatment

has the advantage of not incorporating foreign agents into the construct, and these procedures can be easily controlled. Weadock et al. evaluated the physical, mechanical, biological behaviors of collagen sponges cross-linked by physical (DHT) and chemical (EDAC) methods. The results revealed that the combination of these cross-linking treatments of collagen significantly reduced the swelling ratio and increased the collagenase resistance time and low- and high-strain modulus compared with other treatments.

In the present study, the swelling ratio obtained for the scaffolds processed differently showed a trend in the results in accordance with previous findings. The results showed that an increased concentration of carbodiimide and a lower concentration of collagen provided a lower swelling ratio, hence a higher cross-link density. Lee reported a similar trend for scaffolds treated with different cross-linking techniques. The fact that highest cross-link density could be observed for the scaffolds with the lowest collagen concentration may be related to a more open structure where possible sites for cross-links initiation would be more accessible.

Attachment of the cells to the matrix is a major factor which controls cell behavior, function and cell cycle. The 3-D collagen scaffold provides a greater surface area for cell attachment and proliferation than a 2D surface. In addition, 3-D scaffold surface affects cell adhesion, spreading and proliferation and controls the spatial arrangement of cells and their transmission of biochemical and mechanical signals.

The present seeding studies showed that the number of MSCs attached to the scaffold did not change significantly with the method of seeding and the cross-link density in the first 24-hour period after seeding. However, histology demonstrated that the dynamic culture conditions, particularly orbital shaking, could help with the incorporation of the MSCs in the scaffolds.

Another important finding in this chapter was the incorporation of the MSCs in scaffolds with different pore sizes. The results showed an increased number of cells attached to the scaffold with smaller pore size after 1 week. However, histology confirmed that only the scaffolds with the biggest pore size would actually allow the cells to reach the inner portions of the scaffold. It seems that the cells were able to migrate through the scaffolds with the biggest pore sizes.

The cell-mediated compression was effectively prevented during the two-week experiment in the scaffolds with the highest cross-link density.

In conclusion, this chapter demonstrated the effect of the lipoplex supplementation on the mechanical behavior of the collagen scaffolds, and the effect of the scaffold design on the cell incorporation. Cell adhesion and proliferation are two important parameters which trigger the signaling cascade that control gene expression. The question raised by the results of the cell seeding relates to the importance of the localization of the cells in the scaffolds (surface versus inner parts) for an optimized 3-D transfection. This point is addressed in the fourth chapter.

Future work should include the study of the release of the lipoplexes from the scaffolds. This could be done by labeling the plasmid DNA with rhodamine isothiocyanate (RITC). The signal from the labeled plasmid within the lipoplexes could be monitored by fluorometry. Another important study would be the determination of the mechanical properties of the gene-supplemented scaffold to match the brain mechanical properties. Finally, it would be of interest to have a quantitative analysis of the pore sizes of the scaffolds used in this study.

4 NON-VIRAL GDNF TRANSFER TO MSCS VIA GENE-SUPPLEMENTED COLLAGEN SCAFFOLD

4.1 INTRODUCTION AND EXPERIMENTAL DESIGN

Stem cells have been extensively studied in numerous tissue engineering applications [12], [18], [19], [20] due to their proliferative and differentiation potential. Among them, MSCs have been widely investigated [18], [19], [20] either used by themselves or combined with a scaffold. The interest of a combinational therapy with scaffolds, genes and MSCs as a vehicle carrying target genes is to obtain a localized and stable release of a specific neurotrophic factor for an extended time period. Also, endogeneous cells could migrate through the scaffold and participate in the process of tissue repair. Furthermore, MSCs have been shown to demonstrate neuronal phenotype under specific *in vitro* conditions [61],[62], [63] as well as *in vivo*[64], [19]. This last fact is of importance because of the potential for transdifferentiation of MSCs to contribute to tissue repair.

The objective of this part of the thesis was to investigate the effect of the properties of a 3D gene-supplemented scaffold on the enhancement of the gene transfection of MSCs.

The specific aim of this study was to use a lipoplex-supplemented collagen scaffold for the non-viral delivery of p-GDNF to MSCs. Type I/III porcine collagen scaffolds incorporated the gene encoding for GDNF. GP2 was selected as the transfection reagent for the experiments because it has been showed, in the monolayer studies (Chapter 2), to provide higher gene expression than Lipofectamine 2000 therefore facilitating the uptake by the cells. MSCs were seeded into the scaffolds to facilitate the DNA expression for an extended time period. Chapter two demonstrated the elevated and long-term release of GDNF after non-viral transfection of MSCs in monolayer and under dynamic culture. Chapter three showed that scaffolds slightly cross-linked by carbodiimide (EDAC 1) facilitated the incorporation of the cells into

the scaffold and that dynamic seeding favored the incorporation of the cells in the scaffold.

The following studies evaluated different parameters to optimize the transfection in 3D. The profile of production of GDNF was studied for different cross-link density (1:0.4:1 and 14:5.5:1), and GDNF synthesis was evaluated relative to the initial plasmid doses (2 and 10 μg) and plasmid-transfection reagent ratio (5 and 10). Finally the effect of the pore size and static and dynamic culture methods were tested to optimize the *in vitro* conditions for the plasmid uptake and expression by the MSCs. One of the experimental conditions was used as the reference (group 1). Other conditions were modified by only one variable such that the results could always be compared to the reference group. For all experiments: GDNF production, DNA content, matrix contraction and H&E staining were performed. This chapter achieved the specific aims 9 to 11.

4.2 MATERIALS AND METHODS

4.2.1 *Incorporation of pGDNF/GP2 Complexes into Collagen Scaffolds*

At the day of the 3-D transfection, the excess of water in the hydrated matrices (Appendix S) was quickly removed on sterile filter papers (Fisherbrand, Fisher Scientific Co) and placed onto the 24-well-plates previously coated with 500μl of 3% agarose (m/w) (Appendix N). The pGDNF-GP2 complex (20-25μl) was subsequently pipetted onto the surface of the scaffold and left at room temperature for 1 hour. After the complex was soaked into the scaffold, 1 ml of EDAC solution was added for 30 min. at room temperature. Then, the EDAC solution was aspirated and replaced by a solution of PBS. The scaffolds were rinsed for 30 min. After the cross-linking step, the scaffolds were flipped over and an additional 20-25 μl of pGDNF-GP2 solution was pipetted onto the surface of the scaffold and left at room temperature for 1 hour. The scaffolds were then ready for cell seeding.

4.2.2 *Transfection of Rat MSCs in Gene-Supplemented Scaffolds*

The gene-supplemented scaffolds were seeded with rat MSCs (500,000 cells/scaffold). Briefly, 10 µl of a suspension containing 250,000 cells in DMEM low glucose was pipetted onto the surface of the scaffolds. After 10 min. of incubation at room temperature, the scaffolds were flipped over and another 10 µl of a suspension containing 250,000 cells in DMEM low glucose was pipetted onto the surface. The scaffolds were left at room temperature for 10 min. Then, 0.5 ml of serum-free medium (DMEM low glucose) was added to the 24-well plates and incubated at 37°C. After 4 hours of transfection, 0.5 ml of MSCs medium (DMEM low glucose, 20% FBS, 1% antibiotic-antimycotic) was added. Finally another 0.5 ml of MSCs medium was added after 24 hours.

4.2.3 *Biochemical Analysis: GDNF Enzyme Linked Immunosorbent Assay (ELISA)*

The media were collected (1 ml) on days 3, 6, 9, 12, and 14 post-transfection for ELISA analysis (Duo-Kit ELISA for the human GDNF protein (R&D Systems) and stored at -20°C until ELISA was performed. Chemiluminescent ELISA assay (R&D Systems) was run in a multilabel Victor 3 microplate counter using Wallac 1420 software (Perkin Elmer Life Sciences). The amount of GDNF released in the medium was then monitored by reading absorbance at 450 nm minus absorbance at 540 nm to correct optical aberrations (Appendix E).

4.2.4 *DNA Content*

The gene-supplemented scaffolds were terminated 2 weeks after seeding and the DNA content was determined with a PicoGreen Assay. Briefly, at the day of the termination, the medium was removed from the culture, then the scaffolds were rinsed twice with PBS and lyophilized overnight. The dried matrix samples were then digested overnight at 60°C with a Proteinase K solution (500 µg/ml) (Appendix P). The number of

cells was determined following the DNA quantification of the samples (n=6) (Appendix F).

4.2.5 *Histology*

Two discs per condition were allocated at the end of the experiment for the H&E staining. The scaffolds were examined macroscopically and the firmness assessed with a forceps. Then, the scaffolds were placed into 10% buffered formalin at 4°C for at least two days. Samples were fixed using a tissue processor (Hypercenter XP, Tissue Processor, ThermoShandon, Houston, TX) (Appendix Q), embedded in paraffin and sectioned to 6 μm thickness with a microtome. After being mounted on glass slides the sections were stained with H&E (Appendix R) to determine the cell morphology and distribution into the scaffolds. The slides were coverslipped and left to dry overnight.

4.2.6 *Statistical Analysis*

Data were analyzed by one- and two-factor ANOVA and Fisher's PLSD post-hoc testing using StatView (SAS Institute, Cary, NC). Data are presented as mean ± standard error of the mean (SEM). The significance level was p=0.05.

4.3 RESULTS

4.3.1 *Experimental Matrix*

The experimental matrix (Table 4.1) for the optimization for the transfection in the gene-supplemented scaffolds was designed such that any experiment could be compared with a reference (group 1). The variables studied were: the collagen concentration; freezing temperature; cross-link density of the scaffolds; doses of plasmid and transfection reagent; and the method of transfection (static or dynamic).

Table 4.1: Experimental matrix for the 3D transfection in the gene-supplemented scaffolds. Implants were seeded with 500,000 rat MSCs/scaffold.

VARIABLES	Experiment #	Collagen concentration (%)	T freezing (°C)	pDNA: GenePorter2	EDAC: NHS:COOH	Experimental condition	# scaffolds
Static versus dynamic	**1 (reference)**	0.5%	-10	2ug:10ul	[redacted]	Static	8
	2	0.5%	-10	2ug:10ul	[redacted]	[redacted]	8
Cross-link density	**3**	0.5%	-10	2ug:10ul	[redacted]	Static	8
pDNA: GP2	**4**	0.5%	-10	[redacted]	[redacted]	Static	8
	5	0.5%	-10	[redacted]	[redacted]	Static	8
Pore size	**6**	0.5%	[redacted]	2ug:10ul	[redacted]	Static	8
	7	1%	-10	2ug:10ul	[redacted]	Static	8
	8	1%	[redacted]	2ug:10ul	[redacted]	Static	8

4.3.2 *Main Results*

The following table (table 4.2) presents the main results obtained after 2 weeks of 3D transfection. Accumulated GDNF was evaluated by an ELISA assay. DNA content and the related calculation of the number of cells were obtained using a PicoGreen Assay. The effective diameter was measured with a template with every change of medium. The results are discussed in the following a section.

Table 4.2: Main results of the 3D transfection over a 2-week period with various conditions

Experience #	Cumulated GDNF (ng/ml)	DNA content (ng)	Number of cells	Final amount cells/initial (%)	Scaffold contraction (%)
1	5.7±0.4	296±18	300E3±18E3	59	34±4
2	6.7±0.4	281±30	280E3±30E3	56	37±5
3	4.3±0.6	733±86	735E3±86E3	147	15±3
4	16±2.7	534±42	535E3±42E3	107	34±7
5	5.3±0.9	604±81	605E3±81E3	121	58±7
6	3.1±0.4	365±79	365E3±79E3	73	13±1
7	5.1±1.2	402±94	400E3±94E3	80	34±11
8	2.5±0.6	531±57	530E3±57E3	106	20±9

4.3.3 *Effect of the Transfection Method (Experiments #1 and 2)*

4.3.3.1 *GDNF Synthesis*

Cultures of MSC-seeded gene-supplemented grown under static (Group 1) and dynamic (Group 2) conditions demonstrated a monotonic increase in the accumulated GDNF over the 2-week time course of the experiment to approximately 5.5-6.5 ng/ml (figure 4.1). Group 2 samples, cultured under dynamic conditions, showed a higher GDNF production, and similar synthesis profile, compared to the static cultures (figure 4.1). At the end of the 2-week period, the total of accumulated GDNF for the group in dynamic conditions was about 18% higher than the group cultured in static conditions.

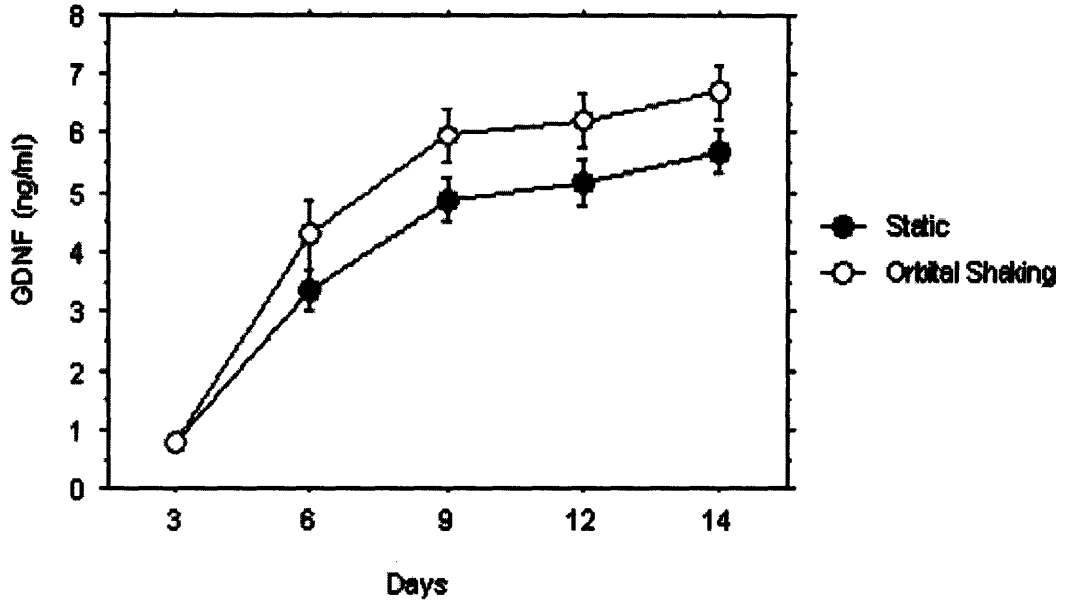

Figure 4.1: Accumulated GDNF (ng/ml) released in the medium versus culture time for two different transfection methods (dynamic and static). The gene-supplemented scaffolds were seeded with 500,000 rat MSCs per scaffold. Mean ± SEM; n=8.

Two-factor ANOVA showed a significant effect of time in culture ($p<0.0001$, power= 0.997), and a significant effect of the transfection method ($p<0.002$, power=0.92), on the synthesis of GDNF by rat MSCs. Additional analysis with a Fisher's PLSD test demonstrated that, after 2 weeks, the effect of the transfection method on the GDNF expression significant ($p=0.0017$)

4.3.3.2 *DNA Content*

DNA content at the end of the 2-week period of culture in dynamic conditions showed no noticeable difference in the results compared to the reference group (figure 4.2). The gene-supplemented scaffolds cultured in static conditions contained about 300,000 cells/scaffold and the scaffolds cultured in dynamic conditions contained about 280,000 cells/scaffolds. The number of cells at the end of the experiment accounted for, respectively, 59% and 56% of the initial number of cells seeded into the scaffold.

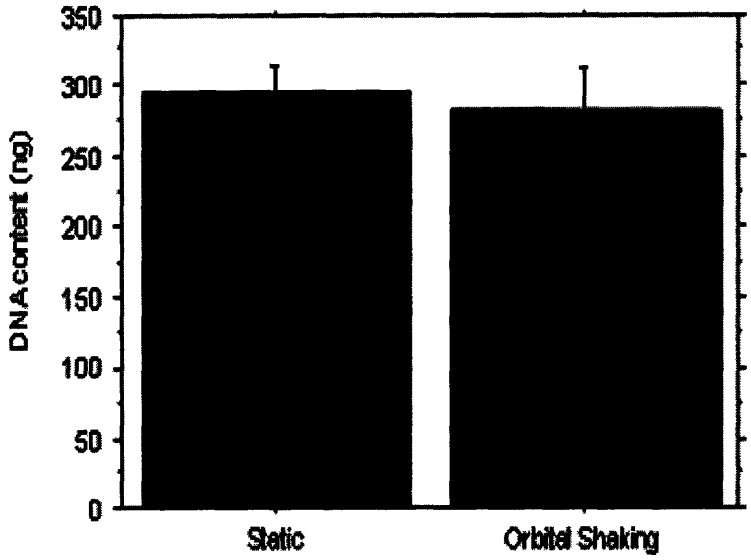

Figure 4.2: DNA content (ng) per scaffold after two weeks of culture in static and dynamic conditions in gene-supplemented implants. Collagen scaffolds (0.5% collagen; $T_{freezing}$= -10C, 2ug pGDNF: 10ul GP2) were seeded with 5E5 rat mesemchymal stem cells per scaffold. Mean ± SEM; n=6.

One-factor ANOVA did not show a significant effect of the transfection method ($p<0.69$, power=0.066) on the incorporation and growth of the rat MSCs within the scaffolds during the 3D transfection.

4.3.3.3 *Cell-Mediated Contraction of Gene-supplemented Scaffolds*

The cell-seeded scaffolds underwent a minimum of 35% reduction in diameter by the end of the 2-week period of culture (figure 4.3). The contraction profiles for the scaffolds cultured in static and dynamic conditions were similar.

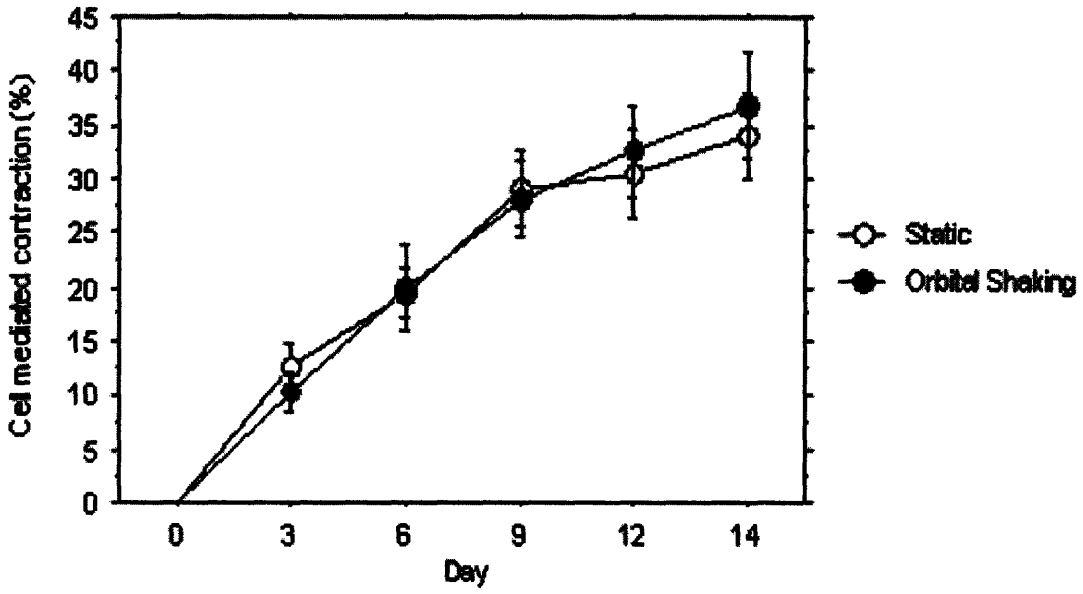

Figure 4.3: Cell-mediated contraction of gene-supplemented scaffolds versus culture time for two different transfection methods (dynamic and static). Collagen scaffolds (0.5% collagen; $T_{freezing}$= -10C, EDAC 1, 2ug pGDNF: 10ul GP2) were seeded with Rat MSCs (5E5 cells/scaffold) and cultured in static or dynamic conditions. Mean ± SEM; n=8.

Two-factor ANOVA showed a significant effect of culture time ($p<0.0001$, power= 1) and no significant effect of the transfection method ($p<0.86$, power=0.054) on contraction of the scaffold during the 3D transfection.

4.3.4 *Effect of the Cross-link Density (Experiments # 1 and 3)*

4.3.4.1 *GDNF Synthesis*

Group 1 samples, cross-linked with EDAC 1, showed a noticeable elevation in GDNF expression throughout the duration of the experiment compared to group 3 (figure 4.4 a). At the end of the 2-week period, the total accumulated for group 1 was about 33% higher than group 3. The profiles of production of GDNF were similar for both cross-link conditions, with a peak in GDNF synthesis on day 6 (Figure 4.4b). However, the

concentration of GDNF found in the medium was much higher for group 1 with about 2.6 ng/ml than group 3 (1.6ng/ml), on day 6.

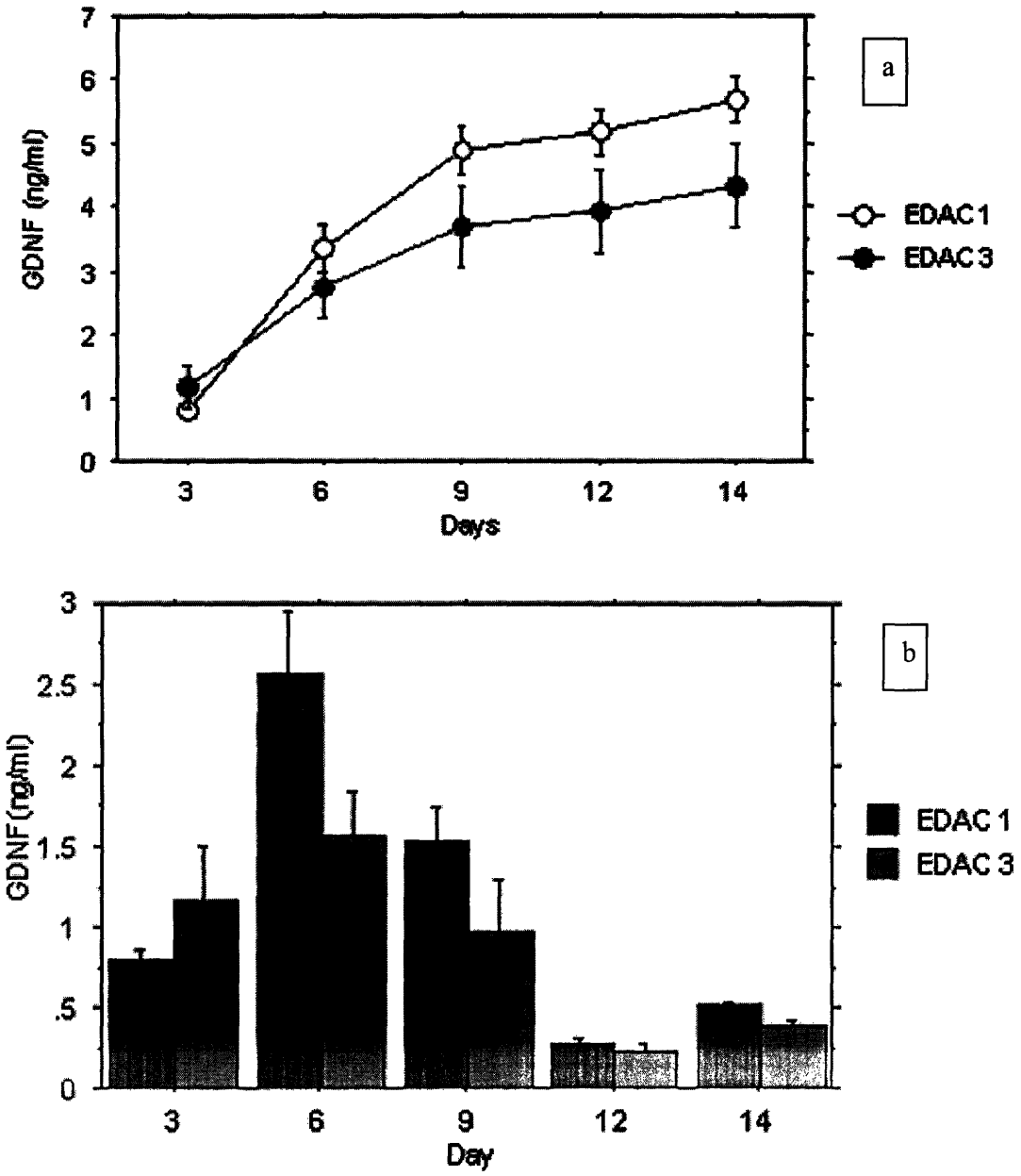

Figure 4.4: Accumulated GDNF (ng/ml, a) and GDNF produced at the medium collection periods (ng/ml, b) versus culture time for two different cross-link densities (EDAC 1= 1:0.4:1 and EDAC 3= 14: 5.5: 1). The gene-supplemented scaffolds were seeded with 500,000 rat MSCs per scaffold. Mean ± SEM; n=8.

Two-factor ANOVA showed a significant effect of culture time ($p<0.0001$, power= 1) and a significant effect of the cross-link density ($p=0.0071$, power=0.793) on the accumulated synthesis of GDNF by the rat MSCs. Further analysis with Fisher's PLSD test on the collection of GNDF at specific time point showed that there was 1) a significant effect of the cross-link density in the production of GDNF on day 6 ($p=0.046$) and 2) no significant difference in GDNF synthesis after 9 days.

4.3.4.2 *DNA Content*

After 2 weeks of culture in static condition, the group with the highest cross-link density showed clearly higher values in DNA content compared to the reference group (figure 4.5). The gene-supplemented scaffolds with the lowest cross-link density (EDAC 1) contained about 300,000 cells/scaffold whereas the scaffolds cross-linked with a higher dose of EDAC contained about 735,000 cells/scaffolds. The number of cells at the end of the experiment counted for, respectively, 59% and 147% of the initial amount of cells seeded into the scaffolds.

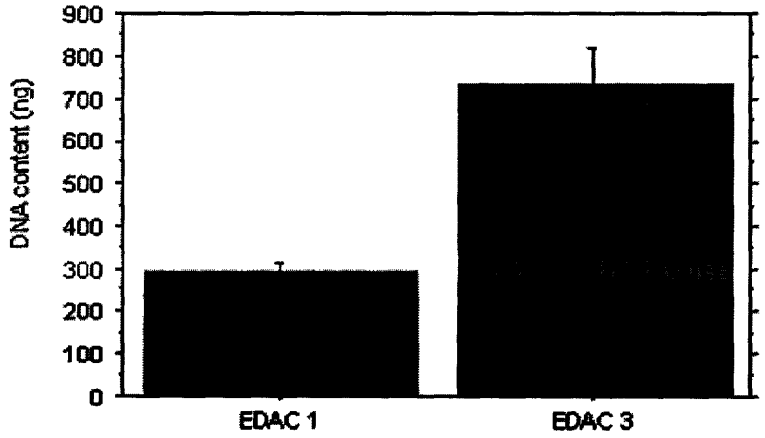

Figure 4.5: DNA content (µg) per scaffold after two weeks of culture in gene-supplemented implants for two different cross-link densities (EDAC 1= 1:0.4:1 and EDAC 3= 14: 5.5: 1). Collagen scaffolds (0.5% collagen; $T_{freezing}$= -10C, 2ug pGDNF: 10ug GP2) were seeded with 5E5 rat MSCs per scaffold. Mean ± SEM; n=6.

One-factor ANOVA showed a significant effect of the cross-link density ($p<0.0005$, power= 0.997) on the number of cells in the scaffolds during the 3D transfection.

4.3.4.3 *Cell-Mediated Contraction*

After a two-week experiment, the scaffolds with the lowest cross-link density (EDAC 1) had contracted about twofold more than the more heavily cross-linked scaffolds (EDAC 3) (figure 4.6). The higher stiffness of the higher cross-link density likely provided more resistance to the cell-mediated contraction.

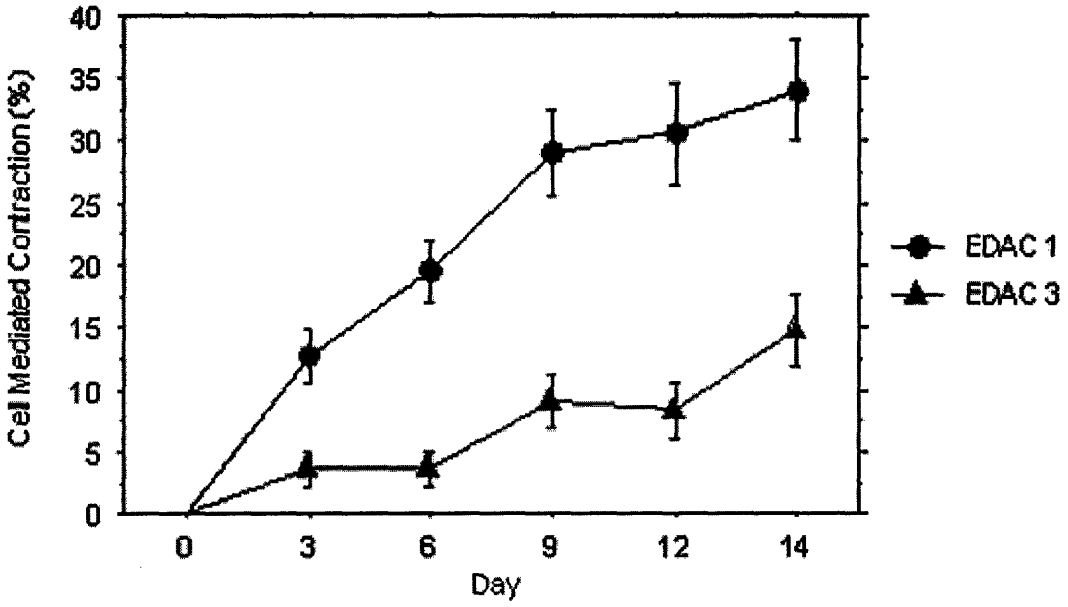

Figure 4.6: Cell-mediated contraction of gene-supplemented scaffolds versus culture time for two different cross-link densities (EDAC 1= 1:0.4:1 and EDAC 3= 14: 5.5: 1). Collagen scaffolds (0.5% collagen; $T_{freezing}$= -10C, 2ug pGDNF: 10ul GP2) were cross-linked and seeded with rat MSCs (500,000 cells/scaffold) in static conditions. Mean ± SEM; n=8.

Two-factor ANOVA showed a significant effect of time of the experiment ($p<0.0001$, power= 1) and the cross-link density ($p<0.0001$, power= 1) on the contraction of the scaffolds during the 3D transfection.

4.3.4.4 *Histology*

H&E staining of the EDAC 3 scaffolds showed that MSCs tend to stay near the surface of the scaffolds and inside the widest pore sizes (fig 4.7 a). Cells seemed to have kept their rounded morphology inside the pores (fig 4.7 b).

Figure 4.7: Histology of gene-supplemented scaffolds (a: 4X, b: 40X) after 2 weeks of 3D transfection (500,000 rat MSCs per scaffold). Scaffolds 0.5% collagen and EDAC 3 cross-linked.

4.3.5 *Effect of the Doses pGDNF-GenePorter2 (Experiments # 1, 4 and 5)*

4.3.5.1 *GDNF Synthesis*

Group 4 specimens, with the highest plasmid load, showed a noticeable elevation in GDNF expression whereas the group with the highest ratio of plasmid to the transfection reagent (group 5) showed production of GDNF similar to the control group (figure 4.8). At the end of the 2-week period, the total accumulated of GDNF for the group 4 was about 180% higher than the group 1 whereas for group 4, it was about 7% lower than the control group. The profiles of GDNF production were similar for the three conditions, with a peak at day 6 (Figure 4.8 b). On day 6, the amount of GDNF released bys group 4 samples was more than 3-fold higher than the GDNF produced in the two other groups. However, the rate of GDNF synthesis decreased after day 9, and was not significantly different for all three groups after day 12.

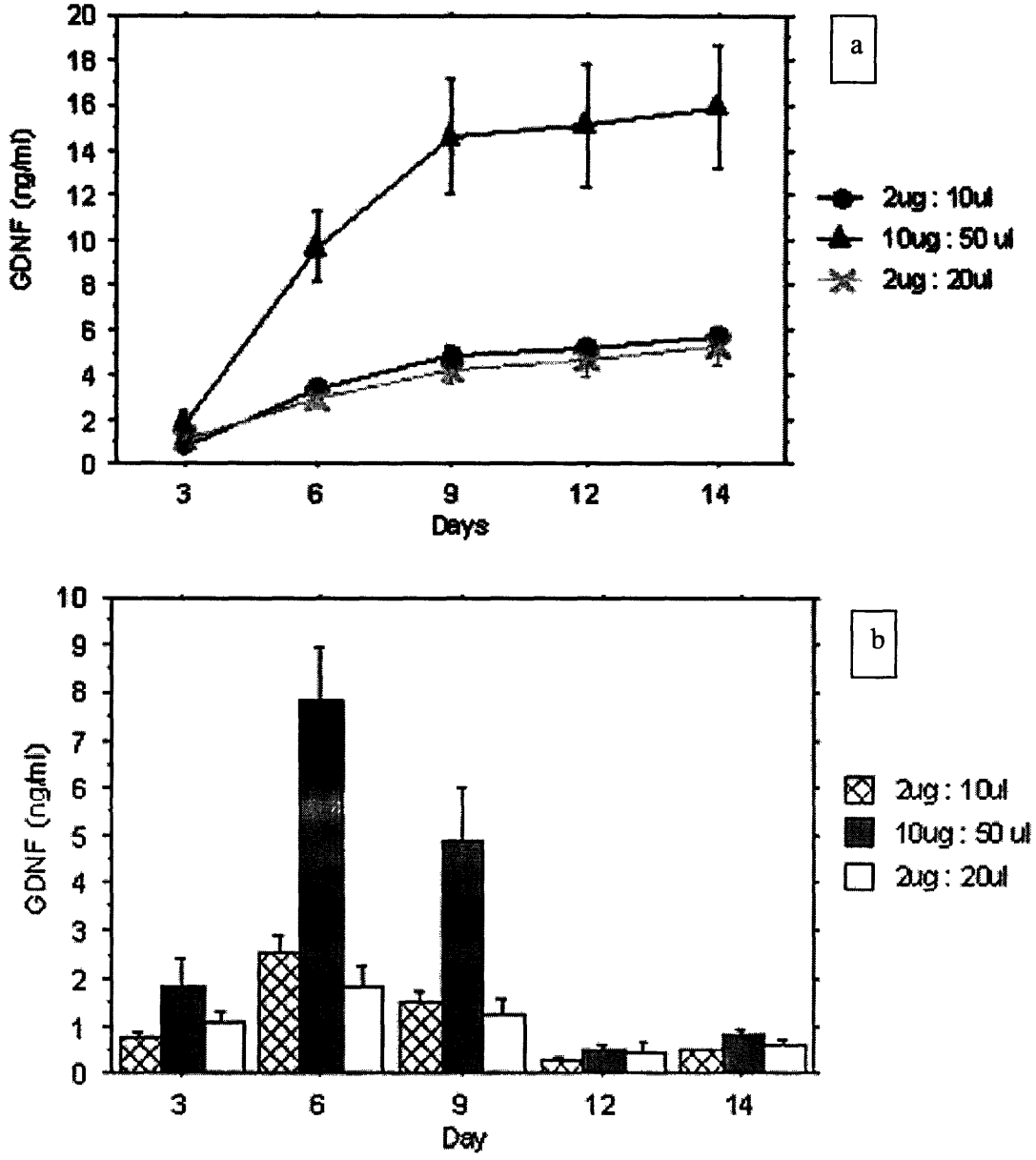

Figure 4.8: Acumulated GDNF (ng/ml, a) and GDNF produced per collection period (ng/ml, b) versus culture time for three different doses of lipoplexes (pGDNF (μg): GenePorter2 (μl)). The gene-supplemented scaffolds were seeded with 500,000 rat mesemchymal MSCs per scaffold. Mean ± SEM; n=8.

Two-factor ANOVA showed a significant effect of both the duration of the experiment ($p<0.0001$, power= 1) and the dose of plamid:transfection reagent ($p<0.0001$, power= 1) on the synthesis of GDNF by the rat MSCs. Further analysis with Fisher's

PLSD test on the temporal effect showed that, after two weeks, there was a prominent effect of the amount of plasmid in the lipoplexes ($p<0.0001$) but no significant effect of the ratio plasmid load: transfection reagent ($p=0.68$) in the production of GDNF.

4.3.5.2 *DNA Content*

After 2 weeks of culture, both groups with the highest plasmid load and highest ratio plasmid to transfection reagent showed higher values in DNA content than the reference group (figure 4.9). The gene-supplemented scaffolds cross-linked with the lowest load of plasmid and ratio to the transfection reagent (2ug pGDNF: 10ul GenePorter2) contained about 300,000 cells/scaffold, whereas the scaffolds with a highest amount of plasmid (10 µg pGDNF: 50 µl GenePorter2) and a highest ratio to the transfection reagent (2 µg pGDNF: 20 µl GenePorter2) contained about 535,000 and 605,000 cells/scaffolds, respectively. The number of cells at the end of the experiment counted for 60%, 105 and 120%of the initial amount of cells incorporated into the scaffold, respectively.

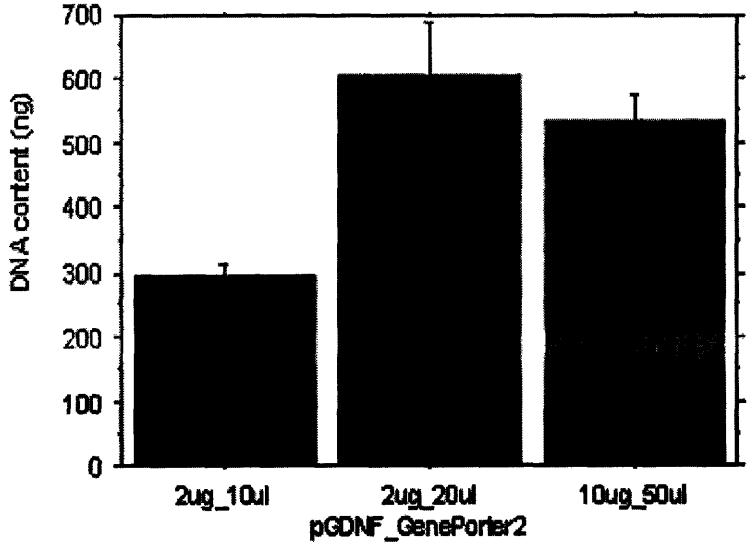

Figure 4.9: DNA content (ng) per scaffold after two weeks of culture in gene-supplemented implants for three different doses of lipoplexes (pGDNF (µg): GenePorter2 (µl)). The scaffolds were seeded with 500,000 rat MSCs per scaffold. Mean ± SEM; n=6.

One-factor ANOVA showed a significant effect of the dose of plasmid and transfection reagent ($p<0.0001$, power= 1) on the number of rat MSCs in the scaffolds during the 3D transfection.

4.3.5.3 *Cell-Mediated Contraction*

After two weeks of culture, the scaffolds with the highest ratio of plasmid to transfection reagent had been contracted about two-fold more than the two others groups (figure 4.10). These results need to be regarded relative to the number of cells found in the scaffolds. On day 14, the contraction normalized to the number of cells (Table 4.3) showed similar results for both 2ug:10ul and 2ug:20ul conditions (C.N≈0.1 %/ng), whereas, the condition with the highest plasmid load demonstrated lower results (C.N≈0.06 %/ng).

Table 4.3: Contraction normalized to the DNA content (%/ng) of the scaffolds for three different doses of lipoplexes (pGDNF (µg): GenePorter2 (µl)).

Experimental condition (ug pGDNF: ul GP2)	Contraction (%)	DNA content at day 14/scaffold (ng)	Contraction normalized to the DNA content (%/ng)
2ug:10ul	34	296	0.11
2ug:20ul	58	604	0.1
10ug:50ul	34	534	0.06

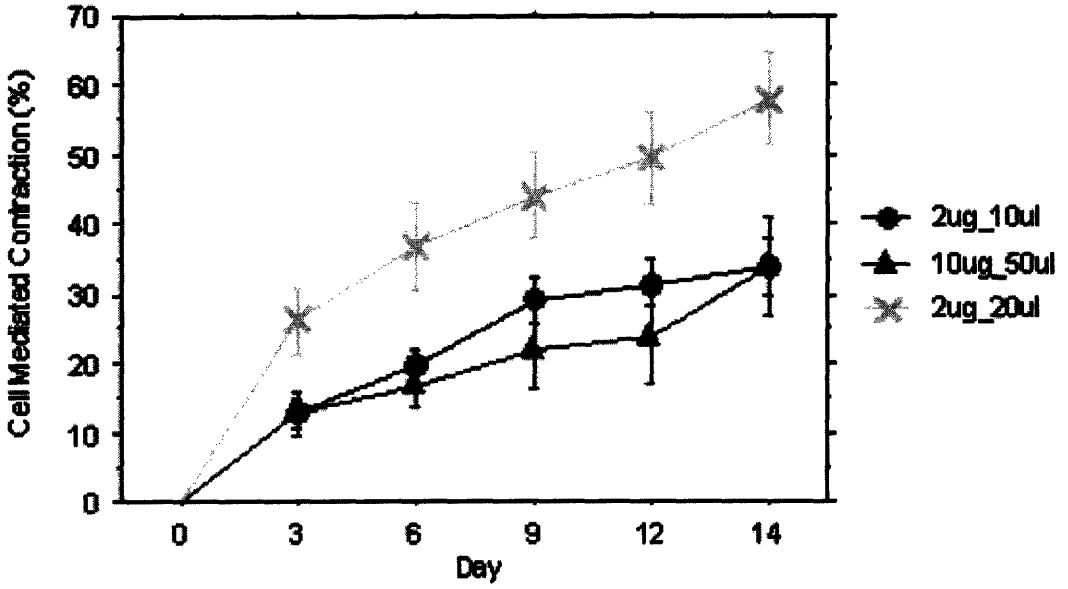

Figure 4.10: Cell-mediated contraction of gene-supplemented scaffolds versus culture time for three different doses of lipoplexes (pGDNF (µg): GenePorter2 (µl)).. Collagen scaffolds (0.5% collagen; $T_{freezing}$= -10C, EDAC 1, 2ug pGDNF: 10ul GP2) were seeded with rat MSCs (500,000 cells/scaffold). Mean ± SEM; n=8.

Two-factor ANOVA showed a significant effect of time in culture ($p<0.0001$, power= 1) and the dose of plasmid:transfection reagent ($p<0.0001$, power=1) on the contraction of the scaffolds during the 3D transfection. Further information with a post hoc test confirmed no significant difference in the results between 2 µg pGDNF: 10 µl GenePorter2 and 2 µg pGDNF:20 µl GenePorter2 after two weeks.

4.3.5.4 *Histology*

H&E staining showed an important infiltration of the cells into the scaffold for all conditions after 2 weeks of experiment (Figure 4.11 a, 4X objective lens magnification). The morphology of the MSCs in the pores of the scaffolds previously supplemented with 10 µg pGDNF: 50 µl GP2 seemed to have been affected by the transfection conditions compared with the conditions without transfection (Figure 3.11 b).

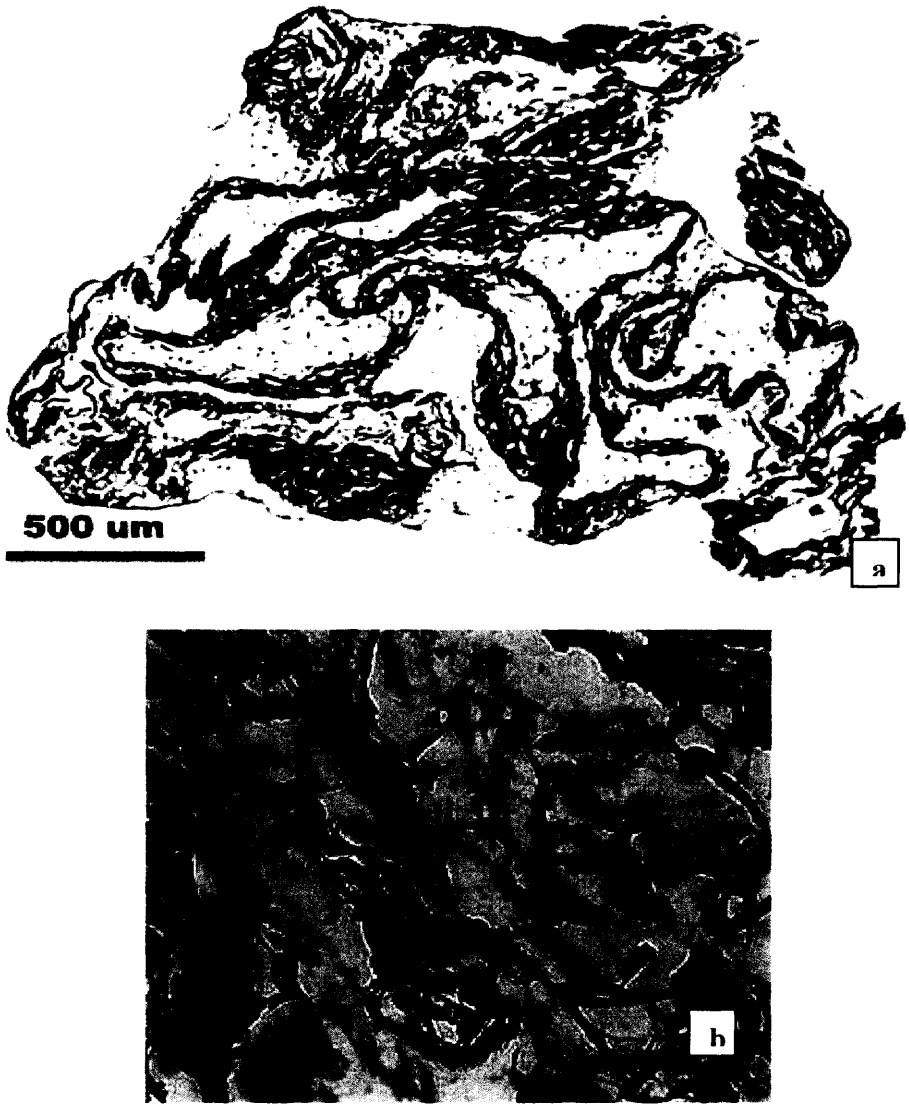

Figure 4.11: Histology of gene-supplemented scaffolds at 10 μg pGDNF: 50 μl GP2 (4X,a and 40X, b) after 2 weeks of 3D transfection (500,000 rat MSCs per scaffold).

4.3.6 *Scaffold Design (experiments #2, 6, 7 and 8)*

4.3.6.1 *GDNF Synthesis*

Group 1, with the biggest pore size (group 1) showed a prominent elevation in GDNF expression compared to the groups with smaller pore sizes (group 7 and 8) (figure 4.12). At the end of the 2-week period, the total accumulated GDNF for the group 6 was about

11% lower, group 7 about 46% lower and group 8 about 56% lower than the samples in group 1. The profiles of production of GDNF per collection period were different for all four groups (Figure 4.4b). The peak in GDNF synthesis was visible on day 6 for group 1, on day 3 for group 6 and on day 9 for group 7. The level of GDNF production was maintained constant for about 9 days for group 8.

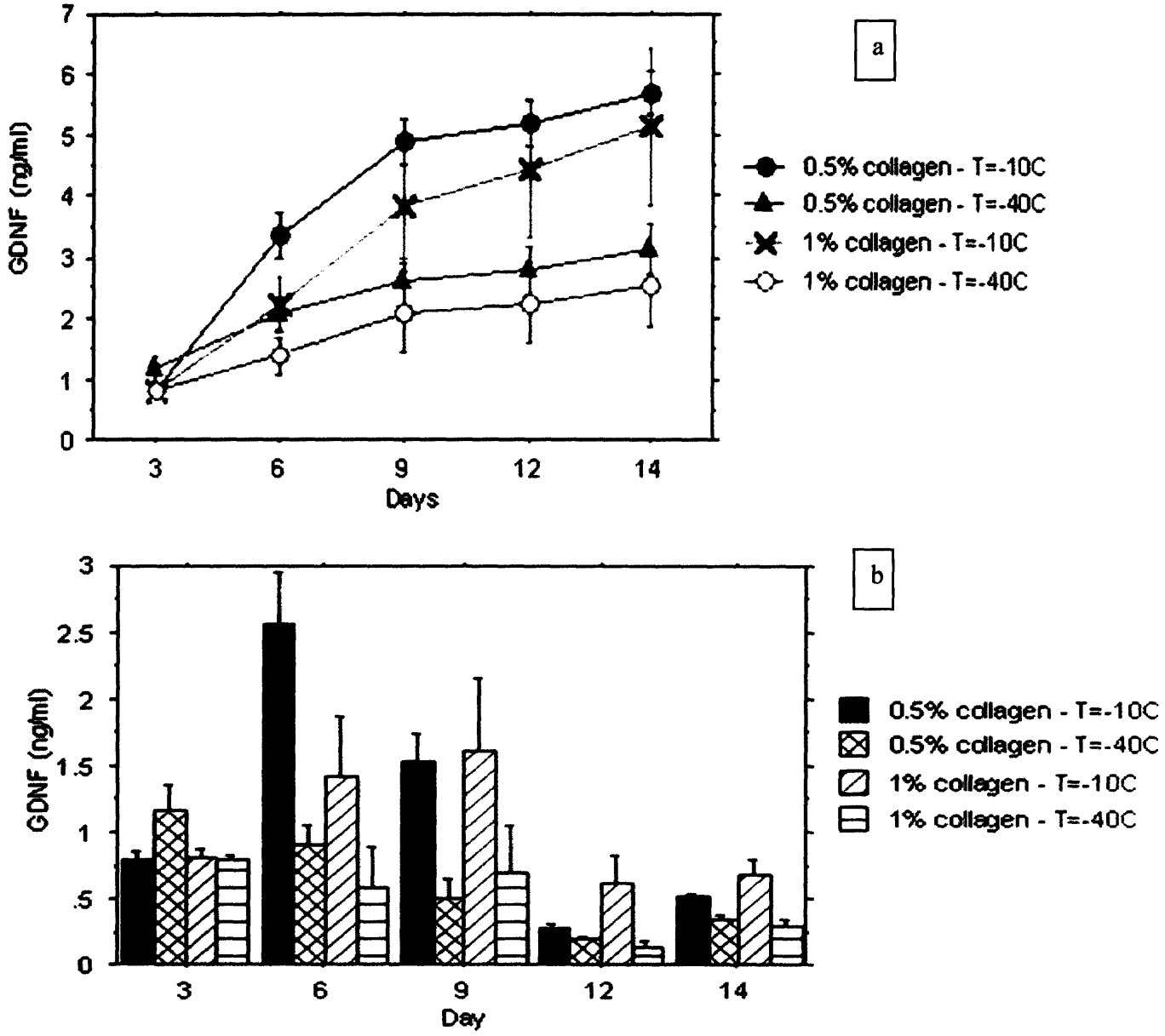

Figure 4.12: Accumulated GDNF (ng/ml, a) and GDNF produced per collection period (ng/ml, b) versus culture time for four different pore sizes. The gene-supplemented scaffolds were seeded with 500,000 rat MSCs per scaffold. Mean ± SEM; n=8.

2-factor ANOVA showed a significant effect of both the time in culture ($p<0.0001$, power= 1) and the scaffold design ($p<0.0001$, power= 1) on the synthesis of GDNF by the rat MSCs. Further analysis with Fisher's PLSD test showed that, after two weeks, there was no significant effect of the pore size between groups 1 and 6 ($p=0.59$) and between groups 7 and 8 ($p=0.56$).

4.3.6.2 *DNA Content*

After 2 weeks of culture, there was a pronounced trend in the DNA content per scaffold as the pore size changed (figure 4.13). As the size of the pores decreased, the DNA content in the gene-supplemented increased. The implants with the smallest pore size (1% collagen scaffolds with $T_{freezing}$=-40C) contained about 530,000 cells/scaffold, whereas the scaffolds with a bigger pore size (0.5% collagen scaffold with $T_{freezing}$=-10C) contained about 300 000 cells/scaffolds. The intermediate pore sizes (0.5% collagen scaffolds with $T_{freezing}$=-40C; 1% collagen scaffolds with $T_{freezing}$=-10C) contained about 365 000 and 400 000 cells/scaffolds. The number of cells at the end of the experiment accounted for, going from the biggest to the smallest pore sizes, 60%, 73%, 80% and 105% of the initial amount of cells seeded into the scaffold.

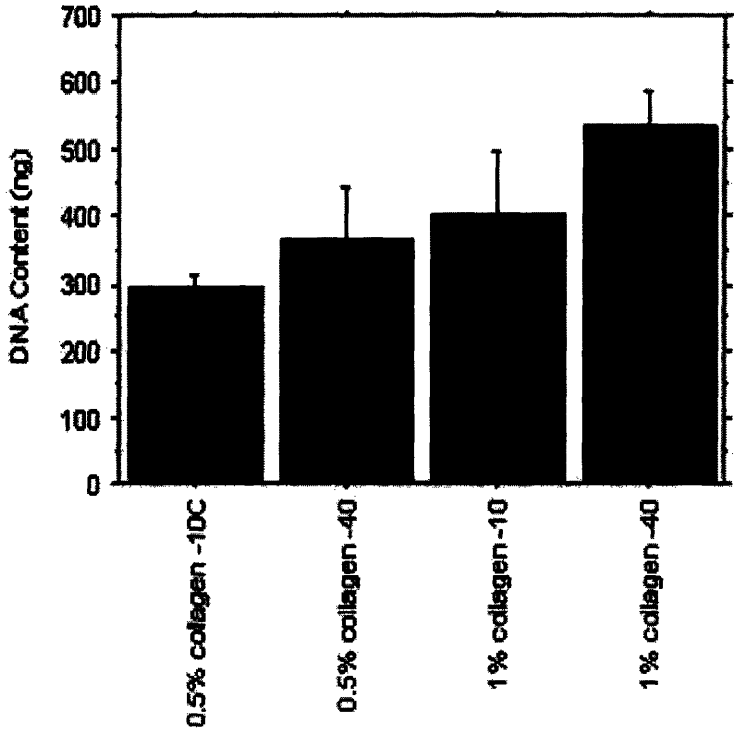

Figure 4.13: DNA content (ng) per scaffold after two weeks of culture in gene-supplemented implants for four different pore sizes. The scaffolds were seeded with 500,000 rat MSCs per scaffold. Mean ± SEM; n=6.

One-factor ANOVA showed no significant effect of the pore size of the scaffold ($p<0.14$, power= 0.44) on the number of cells in the scaffolds during the 3D transfection. A post-hoc test, showed a significant difference in the incorporation and survival of the cells into the scaffolds ($p<0.025$), comparing the 0.5% collagen scaffold with $T_{freezing}$=-10°C and the 1% collagen scaffolds with $T_{freezing}$=-40°C.

4.3.6.3 *Cell-Mediated Contraction*

Scaffolds with 0.5% collagen/ $T_{freezing}$= -10°C and 1% collagen/ $T_{freezing}$= -10°C have the highest contraction and a similar profile. Further analysis with a post-hoc test showed that after two weeks, there was no significant difference between the following conditions: 0.5% -10C (group 1) vs1% -10C (group 7) and 0.5% and -40°C (group 6) vs 1% and -40°C (group 8).

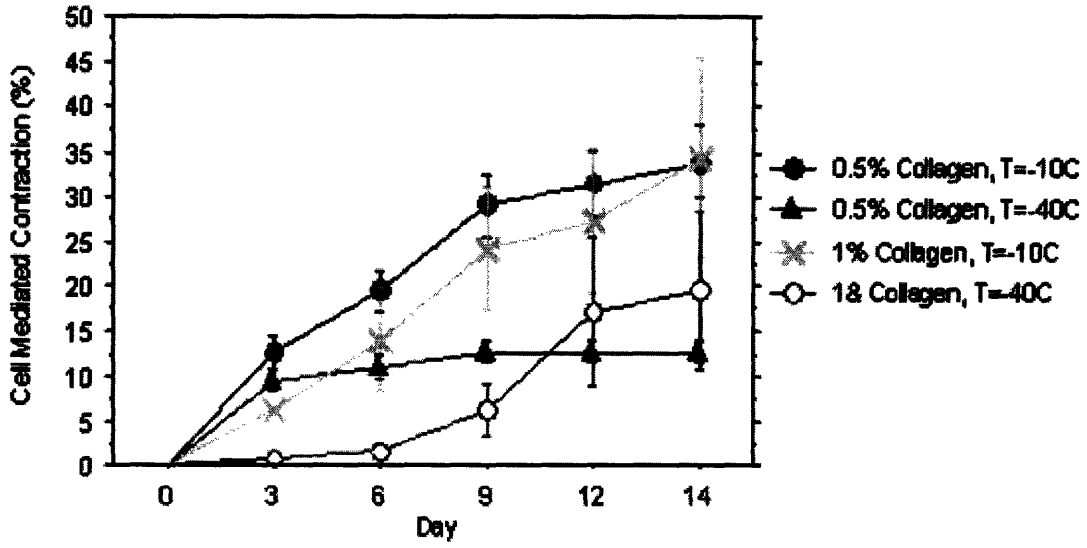

Figure 4.14: Cell-mediated contraction of gene-supplemented scaffolds versus culture time for for four different pore sizes. Collagen scaffolds (EDAC 1, 2ug pGDNF: 10ul GP2) were seeded with Rat MSCs (5E5 cells/scaffold). Mean ± SEM; n=8.

Two-factor ANOVA showed a significant effect of the culture time ($p<0.0001$, power= 1) and the pore size ($p<0.0001$, power= 1) on the contraction of the scaffolds during the 3D transfection.

4.4 DISCUSSION

Gene transfection in 3D constructs is a promising new field with many questions yet to be answered. The combinational strategy of tissue engineering principles with gene and cell therapy involves the selection of a stable transfection reagent, the introduction of foreign genes into cells grown *in vitro* and seeding cells (modified or not) into a scaffold. Xie et al [95], demonstrated that cells transfected with a plasmid encoding for β-galactosidase in a polymer scaffold retain their ability to express the gene for longer than cells grown on 2D surface. Thus, a 3D construct may offer a superior method for the transient expression of a selected plasmid compared to more conventional methods such as the direct transplantation of modified cells into the host.

In this chapter, the level of gene expression in the collagen constructs was lower than those obtained in MSC monolayers but high enough to result in therapeutic doses

previously found *in vitro*. These results demonstrated that the combination of tissue engineering and non-viral transfection of MSCs for the over-expression of GDNF was a promising approach.

In general, lipoplexes released from collagen scaffolds are governed by two mechanisms. The first is the simple diffusion of the complexes through the pores of the sponge scaffold. The second mechanism involves the degradation and water solubilization of the scaffold material. Some parameters of the scaffolds were altered during the fabrication process (collagen concentration and freezing temperature) and the cross-linking treatment to study the effect of the pore size and the cross-linking density on the 3D transfection. The cross-linking treatment did not affect significantly the release kinetics of GDNF which could be explained by a mechanical entrapment of the lipoplexes in the collagen fibrils and not a direct cross-linking of the lipoplexes to the scaffold.

The application of orbital shaking during the 4 hours of transfection had a positive effect on the production of GDNF but less dramatic than previously reported in monolayers studies (chapter 2).

As expected, the load of plasmid DNA was a prominent parameter in the 3D transfection. In this study, the highest level of GDNF expression was observed for 10 μg of plasmid DNA and 6 days after transfection.

In conclusion, this chapter showed that gene-supplemented collagen scaffolds can serve as a carrier for lipoplexes and modified MSCs and provide a long-term overexpression of GDNF. The overall study in this last chapter demonstrated that better results of 3D transfection were obtained on constructs with less cross-link density and bigger pore sizes. This could be related to a better accessibility of the plasmids to the cells and higher cell density as the scaffold starts contracting.

Future work could include a systematic study in vitro on how the increase of the number of cells and the initial dose of plasmid would affect the 3D transfection. The dynamic transfection could be studied further with a bioreactor.

5 SUMMARY

The overall goal of this Master's thesis was to develop an implantable device which combines cell and gene therapy with a tissue engineering scaffold, for the long-term production of selected neurotrophic growth factors and antagonists to neurite outgrowth inhibitors. This approach could provide benefits in the treatment of conditions involving the loss of brain tissue.

The present study investigated a scaffold-based gene transfer approach employing type I/III collagen scaffold as non viral gene delivery vehicle to provide local, elevated, and long-term release of a selected neurotrophic growth factors, GDNF. The main variables investigated for developing the gene-supplemented constructs were: 1) transfection reagent for an optimal non-viral transfection of GDNF in MSC monolayers, 2) GDNF plasmid/lipid complex dose in MSCs in monolayer and collagen scaffolds, 3) transfection method of MSCs in monolayer and collagen scaffolds, 4) seeding method and pore sizes on cell incorporation in the scaffolds, 5) scaffold design (pore size and cross-linking treatment) for an optimal 3D transfection of MSCs.

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Chapter 2 studied various variables for an optimal non-viral gene transfection of MSCs in monolayer. The variables included: 1) the transfection reagent, 2) the doses of pGDNF: GP2, 3) the transfection method, and 4) the post-transfection medium. The main findings of this part are:

- Lipoplexes loaded with as little as 0.2 μg resulted in a significant production of GDNF by MSCs for several days and for the highest loading of plasmid (2 μg), the level of GDNF production was still above the control after 2 weeks.
- Dynamic transfection had a dramatic effect on the production of GDNF. The synthesis of GDNF has been shown to decrease over the time in static conditions whereas GDNF released in the medium after 4 hour-transfection in

an orbital shaker started to increase again after 6 days. Also, dynamic transfection seemed to also have stimulated cell growth.

- GenePorter2 yielded higher GDNF production compared to Lipofectamine 2000.
- bFGF was shown to promote a high level of cell death when used in the post-transfection medium.

The results of this first part support the first hypothesis that rat MSCs in monolayers can be transfected non-virally with the plasmid DNA encoding for GDNF.

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First, Chapter 3 evaluated the effect of the lipoplexes supplementation on the swelling ratio of the scaffold. Second, this part evaluated various scaffolds properties (collagen concentration, freezing temperature and cross-linking treatment) and conditions (static and dynamic) under which MSCs could attach and grow on the implant. The study of the incorporation of MSCs in different seeding conditions was done with Pilot #1, where the scaffolds were made using a 1% collagen concentration with a freezing temperature of -40°C. Scaffolds were seeded with MSCs using different techniques and were evaluated after 24 hours. The study of the incorporation of MSCs was investigated with Pilot #2, where the collagen scaffolds were made with various pore sizes and seeded with MSCs under static conditions, and evaluated after 1 week. The main findings are the following:

- Swelling ratio experiments showed that:
  - An increased concentration of carbodiimide and a lower concentration of collagen provided a lower swelling ratio, hence a higher cross-link density.
  - Gene-supplementation increased the swelling ratio of the scaffolds
- Seeding studies demonstrated that:

- The number of MSCs attached to the scaffold did not change significantly with the method of seeding and the cross-link density in the first 24-hour period after seeding.
- However, histology showed that the dynamic culture conditions, particularly orbital shaking, could help with the incorporation of the MSCs inside the scaffolds.

- Studies on the effect of the pore sizes on the incorporation of the MSCs showed that:
  - An increased number of cells attached to the scaffold with smaller pore size after 1 week.
  - However, histology confirmed that only the scaffolds with the biggest pore size would actually allow the cells to reach the inner portions of the scaffold.
  - Cell-mediated compression was effectively prevented during the two-week experiment in the scaffolds with the highest cross-link density.

This second series of experiments support the second hypothesis that collagen scaffolds can be prepared to incorporate the plasmid DNA-lipid complexes for subsequent release.

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Based on the results obtained in the two previous chapters, Chapter 4 investigated the effects of the pore size and the cross-linking density of the scaffolds on the 3-D transfection and the expression of the plasmid GDNF in MSCs. The main findings of this part are:

- Gene expression in the collagen constructs was lower than obtained in MSCs monolayers but high enough to provide therapeutic doses previously found in vitro.
- Cross-linking treatment did not significantly affect the release profile of GDNF. This may be explained by a mechanical entrapment of the

lipoplexes in the collagen fibrils and not a direct cross-linking of the lipoplexes to the scaffold.

- Application of orbital shaking during the 4 hours of transfection had a slight positive effect on the production of GDNF but not as strong as previously reported in monolayers studies (chapter 2).
- Load of plasmid DNA was a prominent parameter in the 3D transfection. In this study, the highest level of GDNF expression was observed for 10 μg of plasmid DNA and 6 days after transfection.

The findings in this last part corroborate the third hypothesis that gene-supplemented collagen scaffolds can serve as a carrier for lipoplexes and modified MSCs and provide a long-term overexpression of GDNF.

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6 CONCLUSION

The main conclusions of this Master's thesis are the following:

- Rat MSCs in monolayer can be transfected non-virally with a plasmid DNA complexed with a cationic transfection reagent. Furthermore, the protein production can be dramatically increased under dynamic conditions.
- Collagen scaffolds can be prepared to incorporate the plasmid DNA-lipid complexes for subsequent release. Also, supplementation of the collagen scaffold with a plasmid-lipid complex, in conjunction with carbodiimide cross-link treatment, affects its mechanical behavior. Cells can be efficiently incorporated in the scaffolds using static conditions and the drop method.
- Gene-supplemented collagen scaffolds can serve as a carrier for lipoplexes and modified MSCs and provide a long-term overexpression of GDNF

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8 APPENDICES

APPENDIX A: RAT MSCS HARVESTING

GROWTH MEDIUM

- 400 ml low-glucose DMEM
- 100 ml FBS
- 5 ml antibiotics – penicillin/Streptomycin

BONE MARROW HARVEST AND CULTURE:

1. Sacrifice young rat (< 6 weeks old) with CO_2
2. Shave rat: Lower back and entire leg area
3. Place rat on surgery board in prone position
4. Sterilize skin with beta-dyne solution
5. Using a size 15 blade, cut through the skin of the legs from the dorsum of foot to above the hip
6. Remove both tibias and femurs
7. Place in sterile PBS in 50ml centrifuge tube and take to sterile hood
8. Thoroughly clean attached muscle, periosteum, cartilage, and ligaments
9. Wash bones with sterile PBS
10. Gently break bones apart with bone cutters or scissors
11. Place bones in mortar and add 2ml complete medium
12. Gently crush bones with pestle then add another 10ml of medium
13. Filter solution from mortar twice with 40 micrometer filters.
14. Spin the solution at 1500 rpm for 10 min
15. Discard supernatant
16. Resuspend the cells in 2ml PBS (using small pipette to gently break down pellet) then add 13ml of PBS to rinse the cells

17. Centrifuge again at 1500 rpm for 10 min
18. Discard supernatant
19. Resuspend the cells in 2ml growth medium (using small pipette to gently break down pellet) then add an additional 13ml growth medium
20. Seed the cells from one rat onto a $75cm^2$ flask
21. Incubate at 37°C and 5% CO_2.
22. Remove non-adherent cells after 48 hours by medium change
23. Change culture medium every other day
24. Culture until cells reach approximately 90% confluence

APPENDIX B: DETACHING, FREEZING AND THAWING MSCs:

DETACHING MSCS:

1. Split cells when they're about 80-90% confluent
2. Warm media, PBS and the Trypsin-EDTA in the water bath
3. Remove medium from culture in T flask
4. Wash cells with PBS (10ml for a T25, ~15ml for a T75, ~ 30ml for a T150)
5. Aspirate off the PBS
6. Add collagenase 0.0015g/ml (5ml for T75, 10ml for T150), for 5 min, 37C
7. Remove collagenase and place in 50 ml tube. (some cells here)
8. Add Trypsin/EDTA (5ml for T75, 10ml for T150)
9. Incubate in the 37-degree incubator for 5-7 minutes (usually 5 min.); don't do it much longer as it will damage the cells
10. Take out dish from incubator and bang the sides of the dish to help dislodge cells

11. Add 2-4 fold volume (i.e. 2-4 times the amount of trypsin) of medium (containing FBS) to attenuate the action of the trypsin
12. Remove suspension and place in 50ml tube with collagenase
13. Centrifuge cells in 50 ml tube for 10 min at 1500 rpm (~500g)
14. Aspirate off the supernatant
15. Resuspend in 10ml of medium (first resuspend cells in only 2ml as it is easier to break up clumps using a small volume of media)
16. Remove 100µl of cell suspension for cell counting and add 100µl Trypan Blue for D=2
17. Centrifuge cells for 10 min at 1500 rpm (~500g)
18. Aspirate off the supernatant
19. Resuspend in necessary amount of media for desired cell concentration

PROCEDURE FOR FREEZING MSCS

1. Filter Dimethylsulfoxide (DMSO) through a 0.2 µ syringe filter.
2. Prepare freeze medium containing 10% of DMSO in growth medium
3. Centrifuge the appropriate volume of cells and discard the supernatant.
4. Resuspend the cells in the DMSO freeze medium to give the desired freeze cell density (1million cells per 1 ml of medium)
5. Make aliquots of 0.5 - 0.7 ml of cell suspension in cryovials

(This should be done quickly since it is not advisable to leave the cells in the liquid solution containing DMSO for more than 20 minutes).

6. Place cryovials in freeze control container and place in -80C freezer.
7. After 4-24 hours transfer vials to a liquid nitrogen freezer for long term storage.

PROCEDURE FOR THAWING MSCS

1. Before thawing the cells, prepare and warm the growth medium.
2. Pipette warmed growth medium (10 times the thawed cell suspension volume) into a sterile centrifuge tube. Repeat for the number of vials being thawed.
3. Thaw each vial of cells quickly in a 37°C water bath. As soon as the ice has melted (40-60 seconds) remove the vial from the water bath.
4. Transfer the contents of each cryovial into the previously prepared sterile centrifuge tube containing growth medium.

APPENDIX C: BACTERIAL TRANSFORMATION AND PLASMID ISOLATION

MATERIALS:

- **LB Broth Base** (LENNOX L BROTH BASE) (From Invitrogen, Cat# 12780-052)
- **LB Agar** (LENNOX L AGAR) (From Invitrogen, Cat# 22700-025)
- **Antibiotic** (see chart below)
- **Plasmid**
- **Mega QIAfilter™ Plasmid kit** (Qiagen, Cat #12281)
- **TE Buffer for plasmid storage:**
    - 1ml 1M Tris (pH=8) (UltraPure Tris from Invitrogen, Cat# 15504-020, FW=121.1
    - 0.2 ml 0.5M EDTA (pH=8) from Invitrogen, Cat# 15575-038)
    - 98.8 ml dH_2O
- **S.O.C Medium** (Invitrogen, Cat#15544-034) (per liter: 2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulfate, 20 mM glucose)

PREPARATION :

- Water Bath 60C (Rm 110)
- **LB Broth Base** (LENNOX L BROTH BASE) (From Invitrogen, Cat# 12780-052): follow instructions on bottle
    - Prepare 1L solution (20g/L in dH20) in 2L flask (Rm 110)

- Autoclave 121C for 15min (Program Liquid II)

- **LB Agar** (LENNOX L AGAR) (From Invitrogen, Cat# 22700-025): follow instructions on bottle
  - Prepare 500ml solution (32g/L in dH20) in 1L flask (Rm 108)
  - Autoclave 121C for 15min (Program Liquid II)
- **Ampicillin**
  - Cool down LB Broth on bench and Agar in water bath at 60C
  - Add 1mL Ampicillin 100mg/mL in LB Broth
  - Add 0.5mL Ampicillin 100mg/mL in LB Agar
- Plate LB Agar in Petri dishes
  - Label 18 Petri dishes (Rm 108)
  - Surround LB Agar flask with tissue previously sprayed with Ethanol
  - Pour LB Agar+Ampicillin in Petri dishes
  - Evacuate air bubbles in quickly passing flame of burner on surface of the dish
- Store Petri dishes and LB Broth Base overnight in cold room.

TRANSFORMATION PROTOCOL USING HEAT SHOCK

(Place LB agar plates in warm room)

1. Take competent *E.coli* cells (DH5α—from Invitrogen, Cat# 18258-012) from –80C freezer (3rd floor) and thaw them on ice (~20min).
2. Prepare bench + material needed (2 racks, TE buffer in 50ml tube, timer, 1.5 ml tube)
3. Pre-warm SOC Medium in water bath (37C)
4. Turn on heat block to 42C.
5. Put 50ul competent cells in 1.5 ml tube (Eppendorf or similar) for transforming a DNA construct.
6. Keep tubes on ice.
7. Add 1-10 ng of circular DNA into *E.coli* cells. Never exceed (1/10) of the total volume. Ideally add 5ul of construct to 50 ul of competent cells. Mix gently by swirling pipette tip in solution
   (DO NOT pipette up and down).
8. Incubate on ice for 30 min.
9. Put tube(s) with DNA and *E.coli* into water bath at 42C for 45 seconds.
10. Put tubes back on ice for 2 minutes to reduce damage to the *E.coli* cells.

11. Add 500 ul of S.O.C Medium.
12. Incubate tubes for 1 hour at 37C and shake at 225 rpm.
13. Dry Agar plates surfaces in hood under blower (Rm 110)
14. Label Agar plates
15. Spread about 100 ul (can include also a different spread volume on another plate—20 to 200ul) of the resulting culture on LB plates (with appropriate antibiotic added – usually Ampicillin or Kanamycin.)
    a. Pipette appropriate volume
    b. Spread on plate using glass stick previously bathed in ethanol and sterilized with burner + spinning plateau
16. Put plates up-side-down in the warm room (37C). Grow overnight.
17. Pick and grow colonies about 12-16 hours later (or isolate in cold room – stop growth)

BACTERIAL GROWTH AND ISOLATION

1. Place 5 ml of LB medium w/ Ampicillin (100ug/ml) in culture tube.
2. Pick isolated colony w/ a sterile stick and place it in the tube.
3. Spin at max revolutions in the warm room for 6-8hrs (medium should look cloudy when ready).
4. For mega prep, transfer whole contents of tube into a sterilized 3000ml flask containing 1000ml of LB medium w/ antibiotic.
5. Put on shaker (~260rpm—max to prevent severe shaking of bench) overnight.
6. Isolate plasmid w/ Qiagen Plasmid Purification kit (Mega QIAfilter™ Plasmid kit, Cat. 12281).
7. Check amount of plasmid isolated w/ spectrophotometer and integrity w/ gel electrophoresis (cutting w/ appropriate restriction enzymes).

Antibiotics Used for GSCG Scaffolds

Antibiotic	Concentration	Storage	Working concentration (dilution)
Ampicillin (sodium salt) (From Sigma Cat# A-8351)	100 mg/ml in water	–20°C	100 µg/ml (1/1000)
Kanamycin Sulfate (From Invitrogen Cat# 11815-024)	50 mg/ml in water	–20°C	50 µg/ml (1/1000)

** Stored in 1ml aliquots at -20C and used 1 tube per 1L LB medium,*

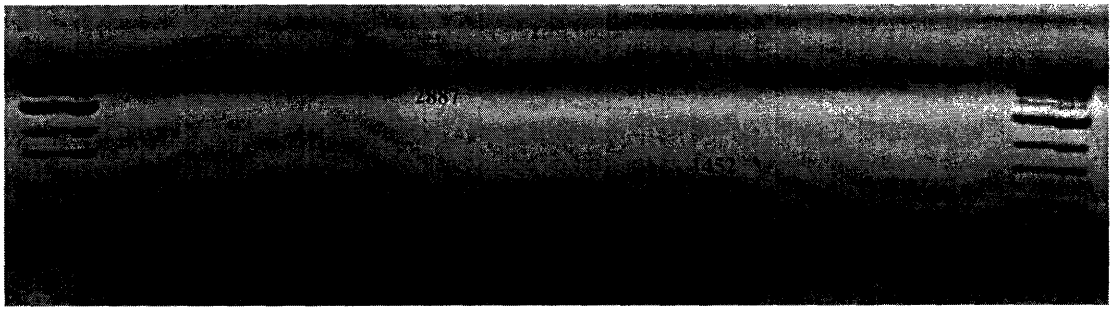
Concentrations of Commonly Used Antibiotics

*Ampicillin (sodium salt)	50 mg/ml in water	–20°C	100 µg/ml (1/500)
Chloramphenicol	34 mg/ml in ethanol	–20°C	170 µg/ml (1/200)
*Kanamycin	10 mg/ml in water	–20°C	50 µg/ml (1/200)
Streptomycin	10 mg/ml in water	–20°C	50 µg/ml (1/200)
Tetracycline HCl	5 mg/ml in ethanol	–20°C	50 µg/ml (1/200)

(From: http://www1.qiagen.com/plasmid/BacterialCultures.aspx?)

APPENDIX D: INTEGRITY OF ISOLATED PLASMID GDNF

	non cut R	cut R / Not I	cut R / Xho I
RAT pGDNF	6750	3863	5284
		2887	1452
			14
		1858	340
		569	



APPENDIX E: Human GDNF - ELISA PROTOCOL (R&D systems)

MATERIALS PROVIDED

Bring all reagents to room temperature before use.

- Capture Antibody (Part 840189, 1 vial) = mouse anti-human GDNF (360 ug/mL)

- Dilute Capture Antibody in 1mL syringe filtered PBS
- Mix until dissolved completely (now at 360 µg/mL)
- After initial reconstitution allow component to sit 15min w/ gentle agitation (rocking plate)
- Dilute a working concentration of 2ug/ml in filtered PBS 1X
- Store at 2 - 8C (up to 60 days) or aliquot and store at -20 to -70C in a manual defrost freezer(up to 6 months)

- Detection Antibody (Part 840190, 1 vial) = biotinylated goat anti-human GDNF (18 ug/mL)

- Reconstitute Detection Antibody with 1.0 mL of Reagent Diluent (see Solutions Required section).
- Store at 2- 8C (up to 60 days) or aliquot and store at -20to -70C in a manual defrost freezer (up to 6 months)
- After initial reconstitution allow component to sit 15min w/ gentle agitation (rocking plate)
- Dilute to a working concentration of 100 ng/mL in Reagent Diluent. Use immediately.

- Standard (Part 840191, 1 vial) = recombinant human GDNF (80 ng/mL)

- Reconstitute with 0.5 mL of Reagent Diluent (see Solutions Required section).
- Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.
- Store reconstituted standard 2 - 8° C (up to 60 days) or aliquot and store at -70° C (up to 6 months).
- Make a seven point standard curve using 2-fold serial dilutions in Reagent Diluent, and a high standard of 2000 pg/mL

- Streptavidin-HRP (Part 890803, 1 vial) = 1.0 mL of streptavidin conjugated to horseradish-peroxidase.

- Store at 2 - 8° C (up to 6 months).**DO NOT FREEZE.**
- Prepare a solution of Streptavidin in Reagent Diluent (1:200)
- Dilute to the working concentration specified on the vial label using Reagent Diluent (see Solutions Required section).

SOLUTIONS REQUIRED

PBS - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2 - 7.4, 0.2 μm filtered.

Wash Buffer - 0.05% Tween20 in PBS, pH 7.2 - 7.4 (R&D Systems Catalog # WA126) filtered

Reagent Diluent1 - 1% BSA (Cat #82-045) in PBS, pH 7.2 - 7.4,0.2 μm filtered (R&D Systems Catalog # DY995).

Substrate Solution - 1:1 mixture of Color Reagent A (H2O2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems Catalog # DY999).

Stop Solution - 2 N H2SO4 (R&D Systems Catalog # DY994).

Preparation solutions:

- Wash Buffer: Prepare PBS 20X. Mix 2 packs of PBS powder (Sigma, Cat #) with 100ml dH2O, filter and add 1ml of Tween 20. For 4* 96 well-plates, mix 100ml of 20X in 1.9L dH2O.
- Reagent Diluent: Prepare PBS 10X. Mix 1 pack of PBS powder with 100ml dH2O, filter and add 1g of BSA (Cat #82-045). For 4*96 well-plates, mix 15ml of 10X PBS in 135ml dH2O.
- Streptavidin-HRP: Pipette 200ul of Streptavidin and add to 40ml of Reagent Diluent 1X, cover the 50ml tube with aluminum to protect from the light (use only the quantity needed for the analysis)
- Capture Antibody (working concentration 2ug/ml): for 4* 96well-well plates, add 257 ul (from 360ug/ml) in 45.8ml of PBS 1X
- Detection Antibody (working concentration 100ng/ml): for4* 96well-well plates, add 257 ul (from 18ug/ml) in 45.8ml of Reagent Diluent 1X

GENERAL ELISA PROTOCOL

1) **Plate Preparation**

Day 1: - Dilute the Capture Antibody to the working concentration in PBS without carrier protein.

- Immediately coat a 96-well microplate with 100 μL per well of the diluted Capture Antibody.

- Seal the plate and incubate **overnight** at room temperature.

Day 2: - Aspirate each well and wash with Wash Buffer

- repeat the process for a total of three washes.

- Wash by filling each well with Wash Buffer (400 µL) using a multi-channel pipette
(Complete removal of liquid at each step is essential for good performance).

- After the last wash, remove any remaining Wash Buffer by aspirating and by inverting the plate and blotting it against clean paper towels.

- Block plates by adding 300 µL of Reagent Diluent to each well.

- Incubate at room temperature for a minimum of **1 hour**.

- Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

2) Assay Procedure

- Add 100 µL of sample or standards in Reagent Diluent per well.

- Cover with an adhesive strip and incubate **2 hours** at room temperature.

- Repeat the aspiration/wash as in step 2 of Plate Preparation.

- Add 100 µL of the Detection Antibody, diluted in Reagent Diluent, to each well.

- Cover with a new adhesive strip and incubate **2 hours** at room temperature.

- Repeat the aspiration/wash as in step 2 of Plate Preparation.

- Add 100 µL of the working dilution of Streptavidin-HRP to each well.

- Cover the plate

- Incubate for **20 minutes** at room temperature **Avoid placing the plate in direct light**

- Repeat the aspiration/wash as in step 2.

- Add 100 µL of Substrate Solution to each well.

- Incubate for **20 minutes** at room temperature **Avoid placing the plate in direct light**.

- Add 50 uL of Stop Solution to each well.

- Gently tap the plate to ensure thorough mixing.

- Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

APPENDIX F: PICOGREEN DSDNA QUANTITATION ASSAY

MATERIALS:

Quant-iT PicoGreen dsDNA Assay Kit (Molecular Probes, Cat# P7589) contains:

- PicoGreen dye reagent (1mL solution in DMSO)—light sensitive (keep covered)
- 20X TE (25mL of 200mM Tris-HCl, 20mM EDTA, pH 7.5)
- Lambda DNA standard (1mL of 100 mg/mL in TE)

TE buffer for diluting samples:

- 1ml 1M Tris (pH=8) (UltraPure Tris, Invitrogen, Cat# 15504-020, FW=121.1; or use 1M Tris (pH=8.0), Ambion, Cat# 9855G)
- 0.2 ml 0.5M EDTA (pH=8) (Disodium Ethenediamine Tetraacetate, Fisher, Cat#S311, FW=372.24; or use 0.5M EDTA (pH=8.0), Invitrogen, Cat# 15575-038)
- 98.8 ml dH_2O

96-well plate—Black Isoplate (Clear bottom plates, Wallac, Cat# 1450-571)

PROCEDURE:

1. Assay Buffer Preparation: Add 25ml (20X TE) + 500 ml sterile, distilled, DNase-free water
2. DNA std working solution (2mg/ml): Add ***294ml of TE buffer*** + ***6ml DNA stock*** (100 mg/mL)
3. Dilute all digested samples ***1:10*** with TE buffer (not from kit): ***180ul TE + 20ul digest*** (can be diluted in microcentrifuge tubes or 96-well plate)
4. Prepare the DNA standards as follows in the first 8 wells of Picogreen 96-well plate:

Standard No.	Standard (ng/ml)	TE buffer (ul)	DNA solution (ul)
Blank	0	100	0
1	10	99	1
2	50	95	5
3	100	90	10
4	250	75	25
5	500	50	50
6	750	25	75
7	1000	0	100

5. Add 20ul of diluted digested sample to each well—***vortex samples before adding***
6. Add 80ul TE buffer (from kit) to each well (for total of 100ul solution per well)
7. Dilute PicoGreen dye stock with TE buffer (from kit), ***200X***—*Prepare just before use*
   --100 ul of working dye solution needs to be added to well used
   --Make more working dye then needed (i.e. add solution for 5 additional wells)
   For example, if there are ***96 wells*** to fill, make enough dye for 110 wells:
   110 x 100ul = 11000ul (or 11ml); 11000/200 = ***55ul of PicoGreen stock + 11ml TE***
8. Dispense 100 ul PicoGreen working dye solution to each well being used
9. Take fluorescence reading on microplate reader (WALLAC VICTOR^2 1420 Multilabel Counter, Perkin Elmer Life Sciences)): Protocol assigned in computer program under DNA Assay—"Fluorescein (485nm/535nm, 1.0s)" (includes a 5 min incubation period & shake)

* *If samples end up having a reading greater than the highest standard, samples need to be diluted more and re-run*

APPENDIX G: PROTOCOL COLLAGEN (PORCINE I/III) SLURRY PREPARATION (1%)

MATERIALS
HCl (6N)
Biogide Powder
dH_2O
ice

Be sure to clean equipment before use

1- Prepare 200ml HCl solution at 0.001N

a. *Add 50ul of 6N HCl to 3ml dH_20 to make 0.1N HCl*
b. *Add 2ml of 0.1N HCl to 198ml dH_2O to make 0.001N HCl*
c. *Stir*
d. *Adjust pH=3 in adding drops 6N HCl*

2- Add progressively 2g Biogide collagen powder while stirring
   a. *Add 100ul 6N HCl to adjust pH=3*

3- Blend at 15,000 rpm for 15min
   a. *Remove stir bar*
   b. *Surround beaker with ice water*
   c. *Place weigh on top of beaker*

4- Add 50ul 6N HCl to adjust pH=3

5- Blend at 15,000rpm for 15min (same as #3)

6- Place in 4*50ml tubes

7- Spin at 1000 rcf for 3 min to degas

8- Remove bubbles on top with spatula

9- Store in fridge (4C)

10- Pour solution in plastic mold

APPENDIX H: TYPE I/III COLLAGEN MATRIX FABRICATION PROTOCOL – CONSTANT COOLING

MATERIALS

Type I/III collagen slurry suspension
150mm*37mm plastic mold

PROCEDURE

1. Turn on the freeze-dryer:
   » Check that the vacuum oil level is at least 2/3, the oil appears clean, and that the vacuum pump is properly vented (either outside or into a fume hood)

   » Plug the drain valve leading from the condenser and close the condenser door
   » Turn the main ***Power*** switch on
   » Turn the ***Condenser*** switch on
   » Set the ***SV*** gauge to 20°C and turn on the ***Freeze*** and ***Heat*** switches

You need to leave approximately 60 minutes for the freeze-dryer temperature to stabilize and for the condenser to reach a cold enough temperature to continue.

2. Spin collagen suspension to degas (15000rpm, 10min).

3. Clean the plastic mold with ethanol or 0.05M acetic acid and wipe the inside with Kim-Wipes to removal all dust and remaining collagen. When cleaning and handling the pan, do not touch the inside of the pan with your bare hands. Use gloves. Allow the pan to air dry.

4. Pipet 16.65ml of the collagen suspension into the pan.

5. Remove an air bubbles introduced into the pan using a 200µl pipette tip. Drag the bubbles to the edge of the pan, allowing them to stick to the edge. Place the pan into the freeze-dryer.

6. Check the appropriate program is selected using the wizard controller. Press the button under ***Program X*** (X = 1 – 12) (Button #1) on the digital display. Select the appropriate program number using the ***Up*** and ***Down*** keys and then press the ***Edit*** key. Check the program following the appropriate progression. If the progression is incorrect, correct the values. Use the outer two buttons to scroll left or right through the program and the inner two keys to change the value of the selected criteria to match the program.

Program 2: Ramp to –40°C. Total time from start to sublimation: ~135 minutes
Average pore size: 95.9 ± 12.3µm.
Percent Porosity: 0.995
Specific Surface Area (S.A./Vol): 0.00748 μm^{-1}

Freezing Step	Temperature, °C	Time, min	R/H
1 (start)	20	5	H
2 ("ramping")	-40	15	R
3 ("freezing")	-40	> 60	H

Program 5: Ramp to –10°C. Total time from start to sublimation: ~135 minutes
Average pore size: 95.9 ± 12.3µm.
Percent Porosity: 0.995
Specific Surface Area (S.A./Vol): 0.00748 μm^{-1}

Freezing Step	**Temperature, °C**	**Time, min**	**R/H**
1 (start)	20	5	H
2 ("ramping")	-10	5	R
3 ("freezing")	-10	> 60	H

NOTE: In all cases, step 3 should run for 60 minutes, but it is given a longer time in case you are delayed in returning. It is acceptable for that step to run longer than 60 minutes, as you are just canceling the program after 60 minutes to start the vacuum.

7. After confirming the program, press the two middle keys on the Wizard together to cancel out of the program. Select the ***Save*** option from the menu. The Wizard screen should return to the original screen seen at start-up.

8. Turn off the ***Freeze*** and ***Heat*** buttons, turn the ***Auto*** button on, and press the ***Start*** key. The program should start running. Leave the program to run for the specified length of time.

9. At the end of the 60 minute freezing period, cancel the program. Press the middle two buttons on the wizard controller together, and then when prompted press the outer two keys. Turn off the ***Auto*** switch, then turn on the ***Freeze*** and ***Heat*** switches and set the ***SV*** to the appropriate temperature of freezing (-10, -20, -30, or –40°C).

10. Turn on the ***Vacuum*** switch. Make sure the seal on the condenser and chamber doors in tight and put pressure on the door to the condenser until a vacuum pressure registers on the pressure reading in the wizard control screen (typically ~1900mTorr).

11. When the vacuum pressure reaches below 300mTorr, raise the temperature in the ***SV*** display to 0°C. Allow the freeze-dryer to run for 17 hours at a temperature of 0°C and a pressure <300mTorr.

12. After 17 hours, raise the value of the ***SV*** control to 20°C. Wait for the chamber temperature to equilibrate to 20°C (temperature displayed in the ***PV*** display). Turn off the ***Vacuum*** switch and turn on the ***Chamber Release*** switch. Wait for the pressure to be equilibrated. Remove the pan.

13. Turn off the freeze-dryer:
    - » Turn off the ***Chamber Release*** switch
    - » Turn off the ***Freeze*** and ***Heat*** switches
    - » Turn off the ***Condenser*** Switch
    - » Turn off the ***Power*** Switch

    » Open the condenser door, unplug the condenser drain line and place it into the drain bucket.

14. Remove the Collagen scaffold from the pan with gloved hands. Place the scaffold into an aluminum foil packet and store in a dessicator.

15. Wash the pan with ethanol or 0.05M acetic acid and wipe down with Kim-Wipes to remove any portion of the scaffold that may have torn during removal.

APPENDIX I: CROSS-LINKING COLLAGEN-GAG SCAFFOLDS BY CARBODIIMIDE TREATMENT

References

Olde Damink, *et al.*, Biomaterials, 1996; 17: 765–73.
Lee, *et al.* Biomaterials, 2001; 22: 3145-3154.

MATERIALS

- 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC)
  MW = 191.7 g/mol (Sigma #E-7750; stored in desiccator at -20°C)
- N-hydroxysuccinimide (NHS)
  MW = 116g/mol (Sigma #H-7377; stored in dessicant at room temp)
- Sterile, distilled water
- 50 ml centrifuge tubes or smaller if only cross-linking few matrices
- Phosphate Buffered Saline (PBS; Invitrogen #4190-250)
- Sterile filter (0.22□m) and sterile container
- Sterile pipettes and pipetteman

PROCEDURE

1. Let EDAC warm to room temperature for about 30 minutes before use in order to prevent condensed moisture inside bottle.

2. Hydrate matrices using sterile, distilled water.

3. Calculate the amounts of EDAC and NHS required for desired molar ratios of EDAC:NHS:COOH, according to the following equations:

$$\#\,\text{Scaffolds} \times \frac{\text{g collagen}}{\text{scaffold}} \times \frac{\text{mol COOH}}{\text{g collagen}} \times \frac{\text{mol EDAC}}{\text{mol COOH}} \times \frac{\text{g EDAC}}{\text{mol EDAC}} = \text{g EDAC}$$

$$\#\,\text{Scaffolds} \times \frac{\text{g collagen}}{\text{scaffold}} \times \frac{\text{mol COOH}}{\text{g collagen}} \times \frac{\text{mol NHS}}{\text{mol COOH}} \times \frac{\text{g NHS}}{\text{mol NHS}} = \text{g NHS}$$

Notes: We make the assumptions that the scaffolds are primarily collagen (i.e., the mass of other components is negligible), and that there are 1.2mmol COOH per mole of collagen (see Olde Damink). Typical values for mass of 8mm scaffolds is 2-3 mg.

Typical calculations for molar ratios of 5:2:1 would be as follows:

$$50\,\text{scaffolds} \times \frac{0.002\text{g collagen}}{\text{scaffold}} \times \frac{0.0012\text{mol COOH}}{\text{g collagen}} \times \frac{5\,\text{mol EDAC}}{1\,\text{mol COOH}} \times \frac{191.7\text{g EDAC}}{\text{mol EDAC}} = 0.115\text{g EDAC}$$

$$50\,\text{scaffolds} \times \frac{0.002\text{g collagen}}{\text{scaffold}} \times \frac{0.0012\text{mol COOH}}{\text{g collagen}} \times \frac{2\,\text{mol NHS}}{1\,\text{mol COOH}} \times \frac{116\text{g NHS}}{\text{mol NHSC}} = 0.278\text{g NHS}$$

Notes: The amounts of EDAC and NHS required are frequently very small and difficult to weigh out, especially when only a small number of scaffolds are required. It is often easier to weigh out a larger amount of the chemicals, dissolve in dH2O, and dilute to the desired concentration.

4. Dissolve EDAC and NHS in dH_2O.
   The final volumes should be 1ml solution per scaffold.

5. Sterile filter EDAC/NHS solutions into sterile containers.

6. Transfer hydrated matrices to EDAC/NHS solutions.
   Hydrated scaffolds will retain a small volume of water within the pores, which will cause the final concentration of the EDAC/NHS solution to be very slightly diluted. If this is a concern, hydrate the scaffolds in half of the final volume (i.e. 0.5ml dH_2O per scaffold), prepare the EDAC/NHS solution at 2X the final desired concentration, and add the solution directly to the water in which the scaffolds being hydrated.

7. Pipette solution over matrices to ensure good mixture.

8. Cross-link at room temperature for desired time (usually 30 minutes).

9. After cross-linking, transfer matrices to 50ml centrifuge tubes. Rinse matrices in sterile PBS. Pipette out PBS and put fresh PBS and allow removal of residual NHS/EDAC solution by rocking for about 1 hr.

10. Remove PBS and rinse in sterile dH_2O for 10 minutes twice.

11. Store matrices in sterile dH20 at 4°C for up to one week (effects of longer storage unknown, usually used within 1 days of cross-linking).

APPENDIX J: JB-4 EMBEDDING AND SECTIONING FOR PORE ANALYSIS

REFERENCE: (Freyman, 2001; O'Brien, Harley, et al., 2004; O'Brien, Harley, et al., 2005)

MATERIALS:

JB-4 A monomer solution (Cat. No. 0226A-800, Polysciences)
JB-4 A catalyst, (Benzoyl Peroxide, plasticized, Cat. No. 02618-12, Polysciences)
JB-4 B embedding solution (Cat. No. 0226B-30, Polysciences)

SOLUTIONS:

- Infiltration solution, 0.625 g of catalyst dissolved in 50 ml JB-4 solution A.
- Equilibration solution: 50:50 solution of infiltration solution and 100% ethanol

- Embedding solution: 2 ml of JB-4 solution B dissolved in 50 ml catalyzed solution A.

PROCEDURE:

1) Dehydration
Collagen matrix samples are dehydrated in 100% alcohol overnight at 4C.

2) Equilibration
Following dehydration, samples are equilibrated for 12 hours at 4°C in a solution 50% ethanol/50% catalyzed JB-4 solution A.

3) Infiltration
Infiltrate in 100% catalyzed JB-4 solution A, 1-4 days at 4°C. Change solution every 12 hours.
To ensure a high degree of infiltration and especially with larger samples, this step should be carried out on a rotator..
When infiltration is complete, tissue usually appears translucent and will sink to the bottom of container.

4) Embedding
Combine JB-4 catalyzed solution A : JB-4 solution B at a ratio of 25:1.
Mix well and pipet into plastic molds.
Place samples face down in plastic molds.
Ensure that sample orientation is maintained.
Solution begins to harden in approximately 30 minutes.
After the JB-4 mixture becomes viscous enough that the samples do not float, place labeled metal or plastic block holders onto each well and place the plastic mold tray in a refrigerator (4°C) and wait overnight.
Pop the blocks from the mold
Let the samples dry at room temperature for 1 day

5) Sectioning
Section very slowly with the microtome at 5um
Place the section in water bath + few drops ammonium hydroxyde
Set sections on glass slides
Let dry overnight before staining with aniline blue

APPENDIX K: ANILINE BLUE STAINING PROTOCOL

MATERIALS:

- Aniline Blue (Fisher Cat. # A-967)
- Glacial acetic acid (Fisher Cat. # A A507-500)
- Cytoseal 60 (Electron Microscopy Sciences Cat. # 18006)

SOLUTIONS:

Aniline Blue

2.5 g aniline blue
2 mL glacial acetic acid
100 mL distilled water
Filter before use.

Acetic Acid, 1 % (v/v)

1 mL acetic acid
99 mL distilled water

STAINING PROCEDURE:

1. Dip in aniline blue solution for 2 min
2. Place in 1% acetic acid solution for 1 min.
3. Dip 5-10X in 95% alcohol until most of background staining goes away.
4. Dip 5-10X in 100% alcohol.
5. Mount with Cytoseal 60 and coverslip. Try not to get air bubbles.
6. Dry in hood for 1 hour

APPENDIX L: PORE SIZE ANALYSIS PROTOCOL: IMAGE ACQUISITION AND LINEAR INTERCEPT ANALYSIS

REFERENCE: (Freyman, 2001; O'Brien, Harley, et al., 2004; O'Brien, Harley, et al., 2005)

PROCEDURE

Image Acquisition

1. Visualize the embedded, sectioned, and stained scaffold samples using an optical microscope at 4X objective lens magnification.
2. take TIF image with microfire

Image Preparation with Irfanview Program

1. Select images to
2. Start batch process
3. Output format = tiff, choose advanced options and click set advanced options
4. Check box to convert to greyscale, select resize "1024*768"
5. Hit start

Image Editing with Scion image program

1. Open Scion Image. Open each prepared scaffold image
2. Under the *Options* menu, select "*Threshold.*" Change the threshold values until an optimal image of struts is visible.
3. Clean up any remaining spots with the erase tool. The pore analysis macro will not count any artifact under 5 pixels across, so it is not necessary to remove every stray spot.
4. Under *Process* menu, select "*Make Binary*" under the "*Binary*" sub-menu. This will transform the thresholded image into a permanent binary image. Save the image as an edited *.TIF file.
5. Under *Edit*, select *Invert* to invert pores to black and background to white.
6. Under *Analyze*, choose *Set scale*. Units are in mm. Determine the number of pixels for 1mm of the scale bar.
7. Select an area of the image to be analyzed using the oval drawing tool. Try to get as much of the viable image enclosed within the curve.
8. Under the "*Special*" menu, run *Load Macrot*. Choose pore analysis macro (cf program below)
9. Under the "*Special*" menu, run *Linear Intercept*. The distance between the pore walls along lines at various angles emanating from the center of the selected region will be calculated.
10. Run the *Plot Intercepts* macro. This macro will transform the average distance between struts along each line into a best-fit ellipse and will calculate linear intercept coefficients C0, C1, and C2 for that ellipse.
11. Transfer CO, C1, and C2 data to an Excel spreadsheet for each scaffold sample. Calculate the minor (a) and major (b) axes of the best-fit ellipse describing the average CG scaffold pore as well as the aspect ratio using the following equations:

$$a = \frac{1}{\sqrt{C_0 + \sqrt{C_1^{\,2} + C_2^{\,2}}}}$$

$$b = \sqrt{\frac{\sqrt{C_1^{\,2} + C_2^{\,2}}}{C_0\sqrt{C_1^{\,2} + C_2^{\,2}} + C_2^{\,2} - C_1^{\,2}}}$$

$$\text{Aspect ratio} = \frac{a}{b}$$

12. Calculate the mean pore diameter d, from the major and minor axes of the best fit ellipse. To account for the effects of pores that were not sectioned through their maximal crosssection but rather at an arbitrary angle, the ellipse major and minor axes were corrected by multiplying by 1.5 (Gibson and Ashby, 1997). The mean pore size was calculated from the average radius by multiplying by a factor of 2.

$$d=1.5\times 2\times\sqrt{\frac{a^2+b^2}{2}}$$

Macro Code:

```
macro 'Linear Intercept'
{This macro measures the linear intercept distance over a giver ROI at intervals of angle
"ThetaStep"}
var
left,top,width,height,MinDim,nx,ny,i,j,k:integer;
ThetaStep,NSteps,PI,x1,x2,y1,y2,dy,dx:real;
- 332 -
Theta,valx,valy,plength,scale,AspectRatio:real;
IntLength,LineSum,dummy:real;
Intercepts:integer;
switch,indicator:boolean;
unit:string;
begin
SetOptions('User1;User2');
GetRoi(left,top,width,height);
if width=0 then begin
PutMessage('Selection required.');
exit;
end;
if width<height then MinDim:=width
else MinDim:=height;
PI:=3.141592654;
GetScale(scale,unit,AspectRatio);
NSteps:=18;{GetNumber('Enter # steps between 0 and 90 deg.',3,0);}
ThetaStep:=PI/(2*NSteps);
{block out next line when doing cumulative measurements}
SetCounter(2*NSteps);
SetUser1Label('Theta(rad)');
SetUser2Label('Lx10^3');
for j:=0 to 2*NSteps-1 do begin
LineSum:=0;
Intercepts:=0;
x1:=left;
y1:=top;
Theta:=j*ThetaStep;
nx:=10*sin(Theta)*width/height;
ny:=10*abs(cos(Theta));
for i:=0 to nx do begin
if Theta=0 then begin
x1:=left;
x2:=x1+width;
end else begin
x1:=left+(width*i/(nx+1))+width/(2*(nx+1));
```

```
x2:=x1+(height*cos(Theta)/sin(Theta));
end;
y2:=top+height;
if x2>=left+width then begin
x2:=left+width;
y2:=y1+(x2-x1)*sin(Theta)/cos(Theta);
end else if x2<left then begin
x2:=left;
- 333 -
if Theta>PI/2 then y2:=y1+(x2-x1)*sin(Theta)/cos(Theta);
end;
{plength is the length of the line to be drawn in pixels}
plength:=sqrt(sqr(x2-x1)+sqr((y2-y1)/AspectRatio));
valx:=x1;
valy:=y1;
dx:=(x2-x1)/plength;
dy:=(y2-y1)/plength;
switch:=true;
if plength>=MinDim then begin
LineSum:=LineSum+(plength/scale);
for k:=0 to plength do begin
if GetPixel(x1+k*dx,y1+k*dy)>0
then indicator:=true
else indicator:=false;
if (switch=true) and (indicator=true) then begin
Intercepts:=Intercepts+1;
switch:=false;
end;
if (indicator=false) then switch:=true;
end;
end;
end;
for i:=1 to ny do begin
if Theta<=PI/2 then begin
x1:=left;
x2:=left+width
end else begin
x1:=left+width;
x2:=left;
end;
y1:=top+height*i/(ny+1);
y2:=y1+(width*sin(Theta)/abs(cos(Theta)));
if y2>top+height then begin
y2:=top+height;
x2:=x1+((y2-y1)*cos(Theta)/sin(Theta));
end;
```

```
{plength is the length of the line to be drawn in pixels}
plength:=sqrt(sqr(x2-x1)+sqr((y2-y1)/AspectRatio));
valx:=x1;
valy:=y1;
dx:=(x2-x1)/plength;
dy:=(y2-y1)/plength;
switch:=true;
if plength>=MinDim then begin
LineSum:=LineSum+(plength/scale);
- 334 -
for k:=0 to plength do begin
if GetPixel(x1+k*dx,y1+k*dy)>0
then indicator:=true
else indicator:=false;
if (switch=true) and (indicator=true) then begin
Intercepts:=Intercepts+1;
switch:=false;
end;
if (indicator=false) then switch:=true;
end;
end;
end;{i}
IntLength:=LineSum/Intercepts;
dummy:=rUser2[j+1];
rUser1[j+1]:=180*Theta/PI;
{to do cumulative measurements, type in 'dummy+ before Intlength in the next line}
rUser2[j+1]:=IntLength*1000;
end; {j}
ShowResults;
end;
Macro 'Plot Intercepts'
{This macro plots the linear intercept distance as a function of angle
in cylindrical coordinates
It then finds the best-fit ellipse to a set of linear intercept distance vs. angle data
using multiple linear regression of the equation Y=C0+C1*X+C2*Z, where
Y=1/L^2 , where L is one half the linear intercept distance at Theta
X=cosine(2*Theta), Z=sine(2*Theta)
C0=(Mii+Mjj)/2 , C1=(Mii-Mjj)/2 , C2=Mij.
The objective is to solve for M11, Mjj, and Mij
The best-fit ellipse it then plotted on top of the linear intercept measurements}
var
left,top,width,height,X0,Y0,X1,Y1,i,n:integer;
pscale,aspectRatio,dx1,dx2,dy1,dy2,maxdim:real;
unit:string;
sumX,sumY,sumZ,sumXZ,sumXY,sumYZ,sumZsqr,sumXsqr:real;
C0,C1,C2,Mii,Mjj,Mij,Y,X,Z,PI,Theta1,Theta2,L1,L2:real;
```

```
begin
PI:=3.141592654;
SaveState;
SetForegroundColor(255);
SetBackgroundColor(0);
- 335 -
width:=400;
height:=400;
maxdim:=0;
for i:=1 to rCount do begin
if rUser2[i]>maxdim then maxdim:=rUser2[i];
end;
pscale:=.8*(width+height)/(2*maxdim);
SetNewSize(width,height);
MakeNewWindow('Linear Intercepts vs. Theta');
SetLineWidth(1);
X0:=(width/2);
Y0:=(height/2);
MakeLineROI(0,Y0,width,Y0);
Fill;
MakeLineROI(X0,0,X0,height);
Fill;
for i:=1 to rCount do begin
dx1:=pscale*0.5*rUser2[i]*cos(rUser1[i]*PI/180);
dy1:=pscale*0.5*rUser2[i]*sin(rUser1[i]*PI/180);
if i<rCount then begin
dx2:=pscale*0.5*rUser2[i+1]*cos(rUser1[i+1]*PI/180);
dy2:=pscale*0.5*rUser2[i+1]*sin(rUser1[i+1]*PI/180);
end else begin
dx2:=-pscale*0.5*rUser2[1]*cos(rUser1[1]*PI/180);
dy2:=-pscale*0.5*rUser2[1]*sin(rUser1[1]*PI/180);
end;
MoveTo(X0+dx1,Y0+dy1);
LineTo(X0+dx2,Y0+dy2);
MoveTo(X0-dx1,Y0-dy1);
LineTo(X0-dx2,Y0-dy2);
end;
n:=rCount;
sumX:=0;
sumY:=0;
sumZ:=0;
sumXY:=0;
sumYZ:=0;
sumXZ:=0;
sumZsqr:=0;
sumXsqr:=0;
```

```
for i:=1 to n do begin
Y:=1/(sqr(rUser2[i]/2));
X:=cos(2*PI*rUser1[i]/180);
Z:=sin(2*PI*rUser1[i]/180);
sumX:=sumX+X;
sumY:=sumY+Y;
- 336 -
sumZ:=sumZ+Z;
sumXY:=sumXY+(X*Y);
sumYZ:=sumYZ+(Y*Z);
sumXZ:=sumXZ+(X*Z);
sumZsqr:=sumZsqr+sqr(Z);
sumXsqr:=sumXsqr+sqr(X);
end;
C1:=((sumXY*sumZsqr)-(sumXZ*sumYZ))/((sumXsqr*sumZsqr)-sqr(sumXZ));
C2:=((sumYZ*sumXsqr)-(sumXY*sumXZ))/((sumXsqr*sumZsqr)-sqr(sumXZ));
C0:=(sumY/n)-C1*(sumX/n)-C2*(sumZ/n);
NewTextWindow('Results');
writeln('C0 = ',C0);
writeln('C1 = ',C1);
writeln('C2 = ',C2);
for i:=1 to rCount do begin
Theta1:=rUser1[i]*PI/180;
if i<rCount then Theta2:=rUser1[i+1]*PI/180
else Theta2:=rUser1[1]*PI/180;
L1:=1/sqrt(C0+C1*cos(2*Theta1)+C2*sin(2*Theta1));
L2:=1/sqrt(C0+C1*cos(2*Theta2)+C2*sin(2*Theta2));
dx1:=pscale*L1*cos(Theta1);
dy1:=pscale*L1*sin(Theta1);
if i<rCount then begin
dx2:=pscale*L2*cos(Theta2);
dy2:=pscale*L2*sin(Theta2);
end else begin
dx2:=-pscale*L2*cos(Theta2);
dy2:=-pscale*L2*sin(Theta2);
end;
MoveTo(X0+dx1,Y0+dy1);
LineTo(X0+dx2,Y0+dy2);
MoveTo(X0-dx1,Y0-dy1);
LineTo(X0-dx2,Y0-dy2);
end;
end;
```

APPENDIX M: SWELLING RATIO PROTOCOL

MATERIALS:

Distilled water
Hot plate
100 ml beaker
Tweezers
Thermometer
Filter paper sheets (Whatman #2)
1.0 kg weight

Procedure:

1. Fill beaker w/ distilled water and heat to 90°C on hot plate. Place thermometer inside beaker to adjust the hot plate over time.
2. Place matrix in the hot water bath for 2 min. in order to denature the collagen and allow it to swell w/ water--samples will shrink in size
3. Expel water from pores by placing hydrated matrix between sheets of filter paper w/ the 1.0 kg weight placed on top for 20 seconds. Need sufficient number of filter paper so that there is no water visible on the outer layers of the filter paper when the weight is taken off. Usually 7 pieces of filter paper on the bottom and 4 pieces on top of the matrix is sufficient
4. Immediately weigh sample after being pressed and record mass as the wet mass (WM)
5. Dry samples in the DHT oven overnight at 110°C
6. Weigh samples after taken out of the oven and record mass as dry mass (DM)
7. Calculate the swelling ratio (which is the inverse of the volume fraction of dry collagen, Vf) using this equation:

$$r^* = 1/Vf = [(DM/\rho c) + ((WM-DM)/\rho water)] \cdot \rho c/DM$$

where $\rho c = 1.32\ g/cm^3$ (density of collagen) and $\rho water = 1.00 g/cm^3$ (density of water)

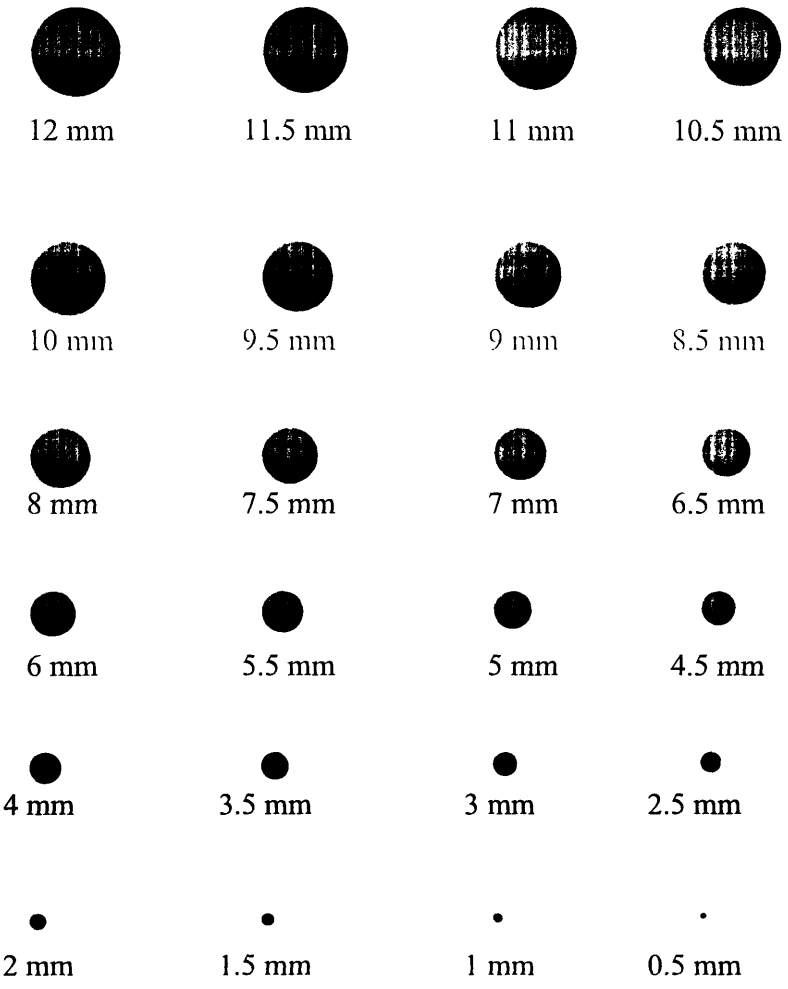
APPENDIX N: AGAROSE COATING

Agarose coating prevents cells from attaching and growing on the bottom of the well plates. Each well of a 24-well plate is to be coated with 500 µl of liquid agarose.

1. In a 100 ml glass bottle, place 1g of agarose low melt per 25 ml of distilled water.

2. Place the bottle in the microwave *(no cover!)* power 50 until the solution appears homogeneous *(careful! The solution tends to boil quickly).*
3. Coat the wells with 500 µl of the liquid agarose.
4. Place parafilm around the well plates and put them in the cold room for at least 4 hours. *Do not use the plates after more than one day in the cold room because the agarose will crack.*
5. Warm the plates in the incubator 1-2 hours prior to use.
6. Change agarose-coated well plates every two weeks because the agarose breaks down.

APPENDIX O: MATRIX MEASUREMENT TEMPLATE



APPENDIX P: PROTOCOL FOR PROTEINASE K MATRIX DIGESTION

(Lyophilize samples prior to digestion)

Digest matrix samples (8mm) overnight (12-24 hours) at 60C in a 500 ug/ml proteinase K solution:

For 100 ml solution: 100 ml Tris-HCl buffer (see below)

50 mg proteinase K powder (Roche #745-723)

** Keep left over proteinase K solution at 4C*

Tris-HCl buffer (1L): 0.05M Tris HCl (7.88 g/L)

1mM $CaCl_2$ (147 mg/L)

Dissolve above salts in 900 ml distilled water

Adjust pH to 8.0 with 1N NaOH

Bring to volume with distilled water

APPENDIX Q: TISSUE PROCESSOR, PARAFFIN EMBEDDING AND CUTTING WITH THE MICROTOME

1. Flush tissue processor (Thermp Shandon: Hypercenter XP, P11889) if necessary.
2. Clean tissue processor with paper towels; remove remaining paraffin/ETOH in outlet with transfer pipette.
3. Set up tissue processor by setting finishing Time (Button "Set Finish") you might choose to run the machine over night.
4. Select Module 1 (Button "1" in Module Section)
5. Select Program 1: Normal samples (Button "1" in Program Section)
6. Set Finishing time by using Arrows and press Quit when finished
7. Start the program by selcting Module 1 (Button "1" in Module Section) and Program 1: Normal samples (Button "1" in Program Section)
8. Pressing Start will run the machine and flush the chamber with ETOH 70%.

9. The machine will not start the whole program until the selected time is reached, this will let you time to place your samples in the chamber.

10. Put all cassettes into Tissue Processor

11. For interrupting the program press "Stop" to hold the program. To start from the same point press "Start". To Abort a program press "Stop" and than "Abort" in the program section.

Paraffin embedding protocol:

1. Switch on heating – and cold plate at Histocentre 2; Thermo Shandon
2. After the tissue processor program is finished, leave samples in chamber filled with paraffin.
3. When "Histocenter" is ready remove one or several samples from the chamber and put it into paraffin basin
4. Switch on light at working station
5. Open tissue cassette on hot plate and remove the blue sponge.
6. Take small embedding mould out of storage basin with pick-up (hot!) and place on hot plate
7. Dry of paraffin, leaving a small amount at the bottom of the mould
8. Place samples on the bottom, with the desired surface facing downwards (Take care for the proper orientation of the sample, since the cutting of the sample will start at the bottom)
9. Transfer the mold to the cold plate in order to cool and harden the small amount of paraffin. This will keep the specimens in place.
10. Place the labeled cassette onto the mould
11. Quickly transfer the mould under the paraffin outlet and fill it slowly from a corner up to the level of the cassette.
12. Place on cold plate for 30 min and transfer into -20 for 60min before taking off the mold.

Microtomy protocol:

1. Switch "power on" of Microtome (Finesse, Thermo Shandon)
2. Take paraffin block out of -20 and trim paraffin/cassette with blade. This will ensure a correct fitting into the sample holder.
3. Change single use blade if necessary, clean the holder.
4. Place cassette into holder and tighten the top screw. If to tight this may crack the paraffin!
5. Unlock the cutting wheel on the right side of the microtome and bring the sample close to the blade by using the arrow keys at the control board.
6. Check from above the parallel alignement of the specimen and the blade.
7. Switch to "trim" mode (10μm) and start cutting the paraffin block by turning the wheel with a constant speed.
8. Check at the beginning that the cutting includes the surface of the whole block if necessary adjust orientation by using the screws of the specimen holder.
9. When reaching the region of interest switch to "cut" mode (6μm)
10. At the beginning the section will be curled!
11. Take a small forceps and pick up one paraffin section, leaving it in contact with the cutting edge.
12. The next section will stick to this on and will allow you to get a continuous number of sections at once.
13. Transfer 5-15 sections to the water bath by using a small pencil.
14. The warm water will smoothen the sections by warming the paraffin. Check now your section and cutting level.
15. Dissect your sections in groups by 3-5 with a small pencil and/or pick-up.
16. Pick up you section with superfrost plus gold slides up to 5 sections. Place slides vertically to a wall and let run of water.
17. Label after each block and put onto hot plate (60°C) for 1-2 hours.

APPENDIX R: HEMATOXYLIN AND EOSIN (H & E) STAINING

Formalin fixed, paraffin embedded specimens

SOLUTIONS

Hematoxylin	Harris Hematoxylin Solution, Sigma Cat# HHS-128.	Filter 200ml
	of stock solution into staining dish.	
Eosin	Eosin Y Solution Aqueous, Sigma Cat# HT110-2-128	
Acid Alcohol	0.5 % in 80% alcohol (99.5ml of 80% alcohol + 0.5 ml HCl)	

OTHER MATERIALS

Cytoseal	Cytoseal 60, Cat# 18006, Electron Microscopy Sciences.

PROCEDURE:

1. Deparaffinize and Rehydrate

Xylene (or substitute)	2 x 5 min.
100% alcohol	2 x 3 min.
95% alcohol	2 x 2min.
80% alcohol	1 min.
Wash in tap water	5 min.

2. Hematoxylin, 3 min. Note: be sure to filter hematoxylin prior to use!

3. Wash in tap water for 5 min.

4. One quick dip in acid alcohol.

5. Wash in tap water for 5 min.

6. Eosin, 3 quick dips.

7. Dehydrate

100% alcohol	2 x 3 min.
Xylene (or substitute)	2 x 3 min.

8. Coverslip with Cytoseal

APPENDIX S: HYDRATATION SCAFFOLD

1. 100% Reagent alcohol (histology), 30 min, rocker

2. 80% reagent alcohol, 30 min, rocker

3. 50% reagent alcohol, 30 min, rocker

4. Rinse 2x with sterile water.

5. Leave in sterile water in 50ml tubes (30-40 scaffolds/tube) for 48 hours on rocker