Dental Pulp Stem Cells for Tissue Engineering

STRO-1 selection and transfection strategies

Xuechao Yang

***Dental Pulp Stem Cells for Tissue Engineering;
STRO-1 selection and transfection strategies***

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STRO-1 selection and transfection strategies***

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on the authority of the Rector Magnificus, prof. dr. S.C.J.J. Kortmann

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牙髓干细胞在组织工程中的应用;

STRO-1 分选及基因转染策略

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Chapter 1

General introduction

1. INTRODUCTION

Tissue engineering is the field of functional restoration of tissue structure and physiology for impaired or damaged tissues. The three key ingredients for tissue engineering are signals for morphogenesis, stem cells responding to these morphogens, and a scaffold to represent the extracellular matrix (ECM) (Figure 1).¹

Tissue engineering procedures have been developed for many tissues and organs such as bone, heart, liver, and kidney. In recent years, researchers have also isolated cells from postnatal dental pulp, and found that these populations exhibited the two basic properties of stem cells, i.e., (1) self-renewal and (2) multilineage differentiation.³⁻⁵ This fact provides the possibility to repair damaged teeth with the patient's own tissue, when dental tissue engineering procedures can be developed. In the next chapters, attention is given to the necessary morphogens, cells, scaffolds, and methods to develop such tissue engineering procedures.

Figure 1: The key elements of tissue engineering and dentin regeneration. The triad consists of stem cells, a scaffold and signals of morphogens.²

2. MORPHOGENS

Morphogens are inductive signals that function as growth/differentiation factors. The morphogenetic signaling networks exhibit redundant and reiterative properties, each with distinct temporal and spatial expression during initiation, patterning formation and morphogenesis, and cytodifferentiation.⁶ Although many morphogens are involved in embryonic tooth development, bone morphogenetic proteins (BMPs) appear to be sufficient for tooth regeneration in adults.⁷

2.1 Bone Morphogenetic Proteins

In 1965, demineralized bone matrix was discovered to induce ossification in intramuscular sites in rabbits.⁸ The responsible molecule was found to be a protein called bone morphogenetic protein.⁹ Today we know that the human genome encodes 20 BMPs.¹ BMPs are dimeric molecules critically dependent on the single intermolecular disulfide bond for biological activity. The monomeric subunit has about 120 amino acids, including seven conserved cysteine residues. The BMP family can be divided into four distinct subfamilies: first, BMP2 and 4; second, BMP3 and 3B, the latter also known as growth/differentiation 10 (GDF10); third, BMP5, 6, 7, and 8; and fourth, GDF5, 6, and 7, also known as cartilage-derived morphogenetic protein 1, 2, and 3. BMP1 is not a member of the BMP family but rather a metalloprotease involved in the proteolytic processing of soluble procollagen.^{10,11}

A single recombinant BMP can have pleiotropic effects on different steps in bone morphogenesis depending on its concentration: femtomolar concentrations promote chemotaxis; high concentrations promote mitogenesis and differentiation.¹ The BMPs play pivotal roles in the development of the brain, eyes, heart, kidneys, skin, bones and teeth. In the postnatal skeleton, BMPs are intimately associated with the collagenous extracellular

matrix and are localized in periosteal cells and in mesenchymal cells of marrow stroma during fracture repair.^{12,13}

BMPs also play a critical role in tooth morphogenesis.^{7,14} BMP family members are used sequentially and repeatedly throughout embryonic tooth development, initiation, morphogenesis, cytodifferentiation and matrix secretion. Six different BMPs (BMP2–7) are coexpressed temporally and spatially.¹⁵ In craniofacial development, they are involved in the inductive interactions between dental epithelium and mesenchyme in a stage-dependent, reiterative manner. BMP2, 4 and 7 are expressed in dental epithelium, and recombinant BMP2 and 4 can be used as a substitute for dental epithelium, to induce odontogenic mesenchyme differentiation.^{10,16} BMPs are also expressed in the enamel knot and there are associated with the differentiation of odontoblasts and ameloblasts. BMP2, 4 and 7 signals from the enamel knot influence both epithelial and mesenchymal cells and are responsible for the maintenance of the enamel knot and for the subsequent morphogenesis of epithelium.⁶ These signals also regulate the patterning of the tooth crown by influencing the initiation of the secondary knots together with mesenchymal signals such as BMP4.¹⁷⁻¹⁹ BMP3 and 7 have also been immunolocalized in developing PDL, cementum and alveolar bone.^{20,21} However, BMP2 is localized only in alveolar bone during root morphogenesis.¹⁸ A role for BMP3 in the cementoblast lineage has been suggested by its localization in root-lining cells.²²

As for in vitro research, BMP2, 4 and 6 have been identified in human primary culture of dental pulp cells.²³ Ten BMP family members (BMP2, 4, 6, 7 and 8, growth/differentiation factor(GDF)1, 5, 6, 7 and 11, and glial cell line-derived neurotrophic factor (GDNF)) have been cloned from rat incisor pulp tissue.^{7,24} Recombinant human BMP2 stimulates differentiation of adult pulp cells into odontoblasts in monolayer cultures and in three dimensional pellet cultures.^{25,26} Recombinant human BMP2 and 4, and GDF11 soaked in agarose beads also stimulated odontoblast differentiation in organ cultures of dental papillae

cells.^{16,27,28} Recombinant human BMP2 and 4, and 7 can induce reparative/regenerative dentin formation *in vivo*.²⁹⁻³² BMP-induced formation of tubular dentin is critically dependent on the scaffold. Odontoblast differentiation and tubular dentin formation were seen directly on a demineralized dentin matrix, which was inactivated by 4M guanidine hydrochloride extraction of BMPs.³³

BMP signaling networks are complex during morphogenesis. Three levels by which BMP signaling pathways are regulated can be distinguished: intracellular domains, membrane site, and extracellular sites. The BMP antagonists noggin, chordin, follistatin, FSRP, DAN, Cerberus and Gremlin modulate the bioavailability of the morphogen.³⁴ Two transmembrane receptors, type I and type II with serine–threonine kinase activity, are expressed in dental pulp.³⁵⁻³⁸ BMP signals are transduced from the plasma membrane to the nucleus through a limited number of Smad proteins, receptor-activated Smads (R-Smads), common mediator Smads (co-Smads) and inhibitory Smads (I-Smads). Many Smad-interacting proteins have been detected and determine the outcome of the signaling.³⁹ The BMP signals are frequently present for communication and signaling between epithelium and mesenchyme. The same signals in different tissues and at different times result in various cellular responses, because the history of the cells is determining their competence to respond to the signals.⁶

2.2 Transforming Growth Factor Beta

Transforming Growth Factor Beta (TGF- β) was discovered as a product of murine sarcoma virus transformed cells.⁴⁰ In mammals there are three main types (TGF- β 1 to 3), all showing similar activities *in vitro* though with some quantitative differences. The effect of TGF- β is dependent on cell type, differentiation stage, growth conditions and the presence of other growth factors.^{41,42}

TGF- β molecules are involved as key participants in odontoblast differentiation and dentin mineralization. Differentiated odontoblasts secrete and deposit TGF- β into the dentin matrix, and then respond to it in an autocrine action.⁴³ Most studies so far focused on the function of TGF- β 1 because of its higher expression compared with TGF- β 2 and 3. TGF- β 1 is known to stimulate the synthesis of extracellular matrix and initiate odontoblast differentiation in vivo and in vitro.⁴⁴ Further, teeth contain collagen type I, as well as non-collagenous proteins such as osteocalcin (OC) and dentin sialoprotein (DSP). The expression of these matrices is regulated by TGF- β 1. Odontoblasts and dental pulp cells show the presence of TGF- β receptors I and II.⁴⁵ Activation of TGF- β 1 expression in sub-odontoblast cells after injury has been suggested to be associated with a differentiation pathway leading to odontoblast-like cells.⁴⁶ The regulatory mechanisms of TGF- β 1 as an external stimulus may be due to upregulating the secretion of extracellular matrix (ECM) by odontoblasts, and mitogenic ability to dental pulp cells in vitro and in vivo.⁴⁷⁻⁴⁹ Recent results have also confirmed that TGF- β 1 could induce the expression of an odontoblast-like phenotype and the formation of dentin-like structures in dental pulp cells.^{48,50}

2.3 Dentin Sialophosphoprotein

Odontoblasts and dental pulp cells synthesize and secrete several collagenous and non-collagenous proteins (NCPs) to form a unique dentin ECM. Dentin sialophosphoprotein (DSPP), the major NCP in dentin, is a phosphorylated parent protein that is cleaved post-translationally into two products: dentin sialoprotein (DSP) and dentin phosphoprotein (DPP).^{51,52} DSP and DPP contain high levels of carbohydrate and sialic acid as well as aspartic acid and phosphoserine, suggesting a function related to dentin mineralization and mineral nucleation.⁵³ DPP appears to be secreted via odontoblastic processes to the mineralization front. DPP binds to collagen and plays important roles for the nucleation and

modulation of hydroxyapatite crystal formation.⁵⁸ DSP in dentin and bone is very similar in nature but different in quantity, as expression in bone is 400 times lower.⁵⁵ The function of DSP is largely unknown.

Studies have shown that DSPP is expressed predominantly in odontoblasts, transiently in preameloblasts, and at a low levels in bone.^{54,55} This suggests that the functional role of DSPP is mainly involved in tooth formation and mineralization. DSPP is also regarded as the specific marker for odontoblasts. The spatial-temporal expression of DSPP is largely restricted during differentiatinal stages of dental cells, and plays a vital role in tooth development. The dentin of DSPP knockout mice shows dentinogenesis imperfecta and dentin dysplasia.^{56,57}

2.4 Dentin Matrix Protein 1

Dentin matrix protein 1 (DMP1), a non-collagenous protein expressed in mineralized tissues, is an acidic phosphorylated extracellular matrix protein originally cloned from the mineralized dentin matrix.⁵⁹ DMP1 has recently been proposed to belong to a group of proteins called SIBLINGs, for small, integrin-binding ligand, N-linked glycoproteins. The members of this family also include bone sialoprotein, osteopontin, osteocalcin, osteonectin, and dentin sialophosphoprotein. These proteins are known to have an important role in matrix mineralization through the regulation of crystal size and morphology.^{58,60,61} However, Dmp1 knockout mice have no major phenotypic abnormality in dentin and bone during early development. Only with progression of age, defects in bone and dentin are noted. In other words, these results suggest that DMP1 might be involved in the late development of bone and dentin.⁶²

Apart from its role in development, DMP1 has a putative function in modulating gene expression. DMP1 is an essential factor in the terminal differentiation of mesenchymal stem

cells. DMP1 is able to upregulate the expression of specific marker proteins in cells of the osteoblast and odontoblast lineage, such as OC, ALP and DSPP, and can enhance the mineralization process. Conversely, blocking translation of DMP1 by antisense techniques inhibited the expression of these genes and the mineralization process.⁶³ More recently, DMP1 has been shown to be involved in more complex biological processes. DMP1 can be localized in the nucleus during early differentiation of odontoblasts, and function as a transcriptional regulator of the DSPP gene by binding to the DSPP promoter to activate its transcription.⁶⁴ Moreover, DMP1 could act as a morphogen on dental pulp stem cells, and induce cytodifferentiation of these cells into odontoblasts.⁶⁵

3. DENTAL PULP STEM CELLS

3.1 Adult Stem Cells

Pluripotent stem cells can be isolated from the embryonic inner cell mass. However, ethical concerns about the requirement of human embryos and the potential danger of teratoma formation, have contributed to the limited use of these cells. In the adult organism, most tissues generally contain a small subpopulation of cells, adult stem cells, with the innate ability to maintain a stem-cell pool by self-replication, and generate more committed progenitors.⁶⁶ Adult stem cells are known to be present in bone marrow, pancreas, liver, skin, etc.⁶⁷⁻⁶⁹ Moreover, they also have been isolated from other adult tissues of which previously was thought no self-repair was possible, such as the brain.⁷⁰ Stem cells are relatively quiescent, yet respond strikingly to tissue damage.⁷¹

When stem cells were first discovered, scientist generally accepted that the differentiation potential of adult stem cells was restricted to the tissues in which they resided. Later, more and more evidence supported that they could even differentiate into specific cell types of non-related tissues. This property is called “plasticity” or “transdifferentiation”.⁷²⁻⁷⁶

3.2 Dental Pulp Stem Cells

3.2.1 Stem Cell Properties

When dental pulp tissue is injured and exposed, damaged odontoblasts degenerate and are replaced by undifferentiated mesenchymal cells that migrate to the injured site from the deeper regions of the pulp tissue. These cells differentiate into new odontoblasts, and produce so-called reparative dentin.^{77,78}

As pioneers in the field, Gronthos et al. have isolated odontogenic stem cell populations from postnatal human dental pulp, and found that these populations shared the two basic properties of stem cells, (1) self-renewal and (2) potency for multilineage differentiation. Transplantation of human dental pulp stem cells (DPSCs) in immunocompromised mice resulted in the formation of a dentin-pulp-like complex structure.³⁻⁵ DPSCs appeared also to be able to differentiate into adipocytes, neural-like cells, myocytes, and chondrocytes when suitable inductions were used.^{4,5,79,80}

In contrast to bone marrow stem cells (BMSCs), dental stem cells did not form haematopoietic marrow or adipocytes at the transplantation site.³ When the gene expression profile of human dental pulp stem cells was compared with bone marrow stromal stem cells, a few genes, including collagen type XVIII α 1, insulin-like growth factor-2 (Igf2), discordin domain tyrosine kinase 2, and cyclin-dependent kinase 6, were more highly expressed in DPSCs. Furthermore, the study revealed that the expression levels for several genes, which belong to cell division, cell signal, cell structure, and metabolism, were regulated differently between both cell types, upon prolonged culturing.^{81,82}

3.2.2 Subpopulations of DPSCs

There is ample evidence showing that DPSCs have the potential to be applied for tissue engineering. However, the behavior of various types of DPSCs is quite different from each other, even when derived from the same source. The majority of the progeny (80%) derived from single colonies was only able to proliferate for less than 20 population doublings. Only 67% of the more highly proliferative single-colony-derived DPSC strains formed the same amount of dentin as multi-colony derived dental pulp cells on a HA/TCP carrier *in vivo*.^{3,4} These results suggest a hierarchy of subpopulations in adult dental pulp, including a minor population of self-renewing, highly proliferative, multipotent stem cells, among a larger compartment of perhaps more committed progenitors.⁴ The opinion of a hierarchy of cellular differentiation has also been found in other stem cell populations, such as in BMSCs.⁸³

STRO-1 is the most extensively used antibody that appears to recognize stage-and/or lineage-specific stromal antigens. The monoclonal antibody STRO-1 reacts with an unidentified cell surface antigen expressed by a minor subpopulation of BMSCs.^{84,85} Some data demonstrates that the STRO-1 fraction in adult bone marrow are eventually the stem cells with the capacity for differentiation into smooth muscle cells, adipocytes, osteoblasts and chondrocytes.⁸⁴⁻⁸⁷ Previous studies showed that human dental pulp also contained STRO-1 positive cells. Like in other adults tissues, these cell populations always existed in the stem cell niches, identified as highly vascularized sites.^{4,5,88,89} The STRO-1 positive cells could be purified from dental pulp cells by fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS).⁸⁹⁻⁹¹ Although STRO-1 selection usually is aimed at human cell populations, a recent study also showed that rat derived dental pulp cells contained STRO-1 positive cells.⁹²

Other cell markers have also been explored to sort functional subpopulations from dental pulp cells, such as CD146 which is expressed by endothelial cells, smooth muscle cells and

pericytes, α -smooth muscle actin which is expressed by smooth muscle cells and pericytes, and a pericyte-associated antigen (3G5). Transplantation of expanded CD146 positive cells with hydroxyapatite/tricalcium phosphate scaffold resulted in ectopic dentin formation.⁸⁹ Furthermore, a side population could be isolated from dental pulp based on the exclusion of the DNA binding dye (Hoechst 33342) by FACS. These subpopulation cells maintained the self-renewal capacity, and multipotency for dentinogenesis, chondrogenesis, adipogenesis, and neurogenesis, and stimulated the reparative dentin formation.^{80,93,94}

4. SCAFFOLDS

Scaffolds provide a physicochemical and biological three-dimensional micro- environment for cell growth and differentiation, promoting cell adhesion, and migration. The scaffold can also serve as a carrier for the morphogen. Scaffolds should be effective for the transport of nutrients, oxygen, and waste. Preferably, a scaffold should be gradually degraded and replaced by the regenerated tissue, while retaining the features of the final tissue structure. Furthermore, it should be biocompatible, non-toxic, and have proper mechanical strength.⁹⁵

Various scaffold materials have been employed in recent studies on tooth regeneration, ranging from long-lasting porous hydroxyapatite ceramics, naturally occurring molecules capable of degrading at intermediate duration (e.g. collagen and chitosan), to relatively short-lasting polymers such as polyglycolic acid (PGA), poly(L-lactic acid) (PLA) and poly(D,L-lactic-co-glycolic acid) (PLGA).⁹⁶⁻⁹⁸

Natural polymers, such as collagen and fibronectin, have the advantage of good cytocompatibility and bioactivity. Fibronectin can mediate the binding of signaling molecules and play a role in the interaction between extracellular matrix and cells, to reorganize the cytoskeleton of polarizing preodontoblasts in the pulpal wound healing process.⁹⁹ The RGD site in fibronectin is critical for cell adhesion.¹⁰⁰ Collagen shares similar chemical and

biological characteristics compared to natural tissues, and has a low antigenicity. Collagen type I is the predominant component of the dentin matrix as well as the dental pulp. The presence of collagen type I in dentin is considered to offer initiation sites for calcification.¹⁰¹ Collagen also allows the arrangement of preodontoblasts, and binds newly formed odontoblasts to pulp tissue, supporting a reparative dentinogenesis framework.¹⁰² Therefore, many researchers have concentrated on this type of scaffold material. Finally, collagen has been used as a capping material to achieve the regeneration of the dentin-pulp complex.^{103,104}

Synthetic extracellular matrices have also been developed as potential scaffolds for reparative dentinogenesis.¹⁰⁵ Alginate hydrogel facilitates pulpal wound healing and can deliver growth factors, like TGF β 1 to enhance the natural regenerative capacity of the dental pulp.¹⁰⁶ Mineral trioxide aggregate (MTA), a powder consisting of fine hydrophilic particles of tricalcium silicate, tricalcium aluminate, tricalcium oxide, and silicate oxide has recently been investigated as a potential restorative material. MTA sets in the presence of moisture, prevents microleakage, is biocompatible, and promotes reparative dentin formation.¹⁰⁷

Calcium phosphate ceramic is also a generally used material for hard tissue regeneration because of its highly biocompatible nature and the ability to support the differentiation of osteogenic cells. In dentistry, calcium phosphate-related materials are widely applied as pulp-capping agents.¹⁰⁸ Moreover, calcium phosphate ceramics have been used to manufacture dental implants.¹⁰⁹ Especially, porous ceramics consisting of hydroxyapatite (HA) and tricalcium phosphate (TCP), have been utilized for ectopic bone formation. These also show clear hard tissue formation in vivo when seeded with DPSCs, and exhibited abundant ingrowth of tissue and vasculature.^{3-5,104}

Titanium is a highly biocompatible and mechanically strong material. These properties make titanium one of the most popular materials for bone regeneration and dental implantation.^{110,111} Although non-degradable, porous and fibrous titanium scaffold designs

harbor great potential for hard tissue engineering purposes. Titanium fibrous mesh supported the differentiation of DPSCs, and induced the formation of calcified nodules *in vitro*.^{92,112} Furthermore, the beneficial *in vivo* behavior of this scaffold was also confirmed.¹¹³

An alternative to the use of a synthetic matrix is the use of three-dimensional cultures with an assembly of endogenous extracellular matrix. Recently, the scaffold-free culture system named “cell-pellet engineering” has been developed.^{26,114} This system involves formation of cell pellets or aggregates, allowing for three-dimensional interaction between the neighboring cells favoring the synthesis of extracellular matrix in the pellet.

5. REGENERATION OF DENTIN-PULP COMPLEX

The dentin-pulp complex protects teeth from caries and trauma by maintaining the hydration of the extracellular matrix. The goal of endodontics and conservative dentistry is to restore or regenerate the dentin-pulp complex to maintain the vitality and function of teeth. The ideal therapy for repairing a pulp wound would be to stimulate proliferation of dental pulp stem cells, and induce their differentiation into odontoblasts to enhance healing potential and rapid dentin formation.¹⁰ To date, different approaches have been developed, including stem cell-based therapy, and gene therapy.

5.1 Stem Cell Therapy

For cell therapy, stem cells can be derived from various tissues. The choice of the tissue source is governed by availability, as well as by the degree of characterization of the stem/progenitor cells in terms of surface markers and differentiation pathway. The generally weak immunogenicity of mesenchymal stem cells has broad advantages and implications for allogeneic cell therapy.¹⁰

Dentin repair/regeneration in adult animals takes place through the activity of odontoblasts that differentiate from a stem/progenitor cell population existing in postnatal dental pulp tissue. Highly proliferative dental pulp stem cells (DPSCs) have the ability to generate a dentin/pulp-like complex including a mineralized matrix when grafted into the dorsal surface of immunocompromised mice.^{3-5,104} Complex tooth-like structures have been generated by seeding DPSCs onto biodegradable polymer scaffolds.⁹⁶⁻⁹⁸

Using the cell pellet engineering approach, the autogenous transplantation of DPSCs onto an amputated pulp stimulated reparative dentin formation.²⁶ DPSCs pellets, when transplanted into the renal capsule of immunocompromised mice, resulted in the generation of a regular-shaped dentin-pulp complex containing distinct dentinal tubules and predentin.¹¹⁴ This method avoids several obstacles associated with biodegradable scaffolds and provides some significant advantages in dental tissue engineering. First, cell-cell and cell-matrix interactions in cell pellet are more sufficient and original than those in scaffold-based tissue engineering. Second, the cell movement and selective cell adhesion inside the pellets are more native than those in scaffolds. Additionally, because there are no artificial materials used, host response to graft material can be avoided.¹¹⁵ However, the optimization of this pellet culture system still needs further investigations, including the *in vivo* culture time, pellet size, right culture medium, and so on.

5.2 Gene Therapy

In tissue engineering there is always the need for the application of morphogens. However, there are some limitations for local delivery of proteins, including short half-life, large dose requirement, high cost, the need of repeated applications, and poor distribution.¹¹⁶ Gene therapy is an alternative approach to conquer the limitations of protein therapy. Gene therapy is a means of delivering genes for growth factors, morphogens, transcription factors,

extracellular matrix molecules, etc, locally to somatic cells of individuals with a resulting therapeutic effect. The genes applied can stimulate or induce natural biological processes by expressing bioactive molecules that are involved in regenerative response for the tissue.¹¹⁷ Precise delivery and efficient transfer of genes into target tissue cells, appropriate gene expression at required times and levels, and minimal toxicity *in vivo* are essential for a successful gene therapy. Both *in vivo* and an *in vitro* approaches can be used for gene therapy (Figure 2). The choice for an *in vivo* or *in vitro* approach depends on morphological and physiological characteristics of target tissue, the vector used, nature of affected disease and the safety of the procedure.¹¹⁸

Either viral or non-viral vectors are used to enable the cellular up-regulation and expression of genes. Viral vectors are genetically altered to eliminate the ability of causing disease, without losing infectious capacity. The viruses can replicate genes of interest together with their own genome through the use of the host cell genetic machinery. At present, gene vectors have been developed from adenovirus, retrovirus, adeno-associated virus, herpes simplex virus and lentivirus.^{10,118} Non-viral delivery systems have also been described, e.g. plasmids, cationic liposomes, DNA-ligand complex, gene gun, electroporation, and sonoporation, to address safety concerns such as immunogenicity and insertional mutagenesis.^{119,120}

Figure 2: Two main strategies for pulp therapy to regenerate dentin: *in vivo* and *ex vivo* methods by application of BMP gene²

5.2.1 In Vivo Gene Therapy

For the in vivo approach, the gene is delivered into the blood or target tissue by injection or implantation.¹¹⁸ Recently, genes for vascular endothelial growth factor (VEGF), angiopoietin, fibroblast growth factor (FGF), and hepatocyte growth factor (HGF), all have been directly transfected in tissues. The efficacy for stimulating angiogenesis to improve cardiac and limb vascular insufficiency has been demonstrated preclinically.¹²⁰

BMPs are important growth/differentiation factors in dental tissue engineering. Both viral and non-viral gene transfer have been used to express genes, and produce endogenous BMPs in pulp tissue. BMP7 protein therapy is known to be effective to induce reparative/regenerative dentin, but not in inflamed pulp tissue.³¹ However, a recombinant adenovirus containing the full-length BMP7 gene induced a small amount of dentin formation after direct transduction in experimentally inflamed pulp.¹²¹

Although the efficiency of gene delivery is relatively low, non-viral approaches have also been used in pulp tissue. There are some advantages for non-viral gene therapy, such as stable production of plasmids with a high level of purity, easy manipulation, minimal risk of replication or incorporation, and weakly immunogenicity.^{27,119,122} The GDF11 plasmid was transfected efficiently by electroporation into DPSCs to induce the expression of DSPP, and this approach resulted in some level of reparative dentin formation.²⁷ A study using ultrasound-mediated GDF11 gene transfer together with microbubbles also induced differentiation of pulp stem cells into odontoblasts in vitro, and gave a more homogenous and complete reparative dentin formation in vivo.¹²²

5.2.2 In Vitro Gene Therapy

The in vitro gene therapy approach involves the genetic manipulation or decoration of somatic cells in vitro, which are subsequently transplanted to the regeneration site. For

example, mesenchymal cells have been transfected with morphogens or growth factors such as BMPs genes, and implanted into a defect site in cartilage or bone.^{118,123,124} The cells play a role not only in the repair process, but also in secretion of growth factors to stimulate host cells at the local site.¹²⁵

Some studies have shown that in vivo gene therapy is ineffective for reparative dentin formation, if there is a severe inflammation and only few stem/progenitor cells are present in the pulp tissue.¹²¹ An alternative in vitro gene therapy approach, using the autogenous transplantation of BMP gene-transfected DPSCs into the exposed pulp, might be clinically useful. This approach can initially induce osteodentin. It can be followed by a lasting tubular dentin formation in the exposed pulp by transplantation of GDF11-transfected pulp stem/progenitor cells in pellet.²⁷ The transplantation of dermal fibroblasts, transfected with BMP7 gene mediated by adenoviral vector, also was shown to induce reparative dentin formation in the exposed pulp with reversible pulpitis.¹²¹ BMP gene decorated cells can directly contribute to reparative/regenerative dentin formation, and the various growth/differentiation factors retained in the matrix of the transplantation might be gradually released and induce host pulp cells differentiation. The great potency of the BMP genes to provoke differentiation of pulp cells, demonstrates the utility of in vitro gene therapy in clinical treatment. The present challenges for gene therapy will be to demonstrate that gene therapy can provide cost-effective and safe long-term treatments for conditions that would otherwise lead to significant pulp necrosis.¹¹⁹

Furthermore, recent studies have revealed that adult fibroblasts can be driven into functional pluripotent stem cells by introducing four factors: Oct3/4, Sox3, c-Myc, and Klf4.^{126,127} Therefore, it seems possible to obtain embryonic dental epithelial/mesenchymal cells and initiate a bioengineered odontogenesis by delivering specific genes or growth factors into adult dental cells.

6. PROSPECTS

With the improvement of dental care, deciduous (primary) and permanent (secondary) teeth can be preserved much longer than before. However, trauma and old age still can cause tooth loss. Although tooth-loss is not a lethal disease, it influences mastication and esthetics, and decreases the quality of life of individuals.¹²⁸ Stem cell-based dental tissue engineering may be the answer to one day achieve a “tertiary dentition”. Further systematic studies with stem/progenitor cells, morphogens, and novel scaffolds are required for such regenerative dental tissue engineering.

7. OBJECTIVES OF THIS THESIS

In this thesis, the following research questions will be addressed:

1. Can a more homogeneous cell subpopulation be sorted from rat dental pulp cells using STRO-1 selection?
2. What is the multilineage differentiation potential of STRO-1 selected rat dental pulp stem cells *in vitro*?
3. Do STRO-1 selected dental pulp stem cells have the ability for hard tissue formation *in vivo*, when seeded in porous HA/TCP ceramic scaffolds?
4. What is the influence of adenoviral-mediated *Bmp2* transfection on dental pulp stem cells *in vitro*?
5. How is the *in vivo* behavior of adenoviral-mediated *Bmp2* transfected dental pulp stem cells?
6. Can calcium phosphate nanoparticles be applied as a non-viral vector for *Bmp2* transfection into dental pulp stem cells?

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Chapter 2

The Odontogenic Potential of STRO-1 Sorted Rat Dental Pulp Stem Cells

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1. INTRODUCTION

Several studies have isolated the highly proliferative dental pulp cells (DPCs) from adult pulp tissue of various species, and have shown the potential of cells to differentiate towards odontoblasts and mineralize *in vitro*.¹⁻⁴ Recent studies proved the existence of stem cells in human dental pulp tissue, and characterized the properties of such cells.^{5,6} The dental pulp stem cells (DPSCs) demonstrated self-renewal capability, and could differentiate towards odontoblasts which formed a pulp-dentin complex *in vivo* when transplanted into immunocompromised mice, even after long-term cryopreservation.⁷ A recent study showed that DPSCs, when treated with conditional medium from tooth germ cells, could differentiate into regular-shaped dentin-pulp complexes.⁸ Furthermore, DPSCs were also capable of trans-differentiating into adipocytes and neural-like cells by conditional inductions.^{6,9} However, the behavior of DPSCs was quite variable, even for cells derived from the same source. These results suggest a hierarchy of progenitors in adult dental pulp, including a minor population of self-renewing, highly proliferative, multipotent stem cells, among a larger compartment of perhaps more committed progenitors. The opinion of a hierarchy of cellular differentiation has also been found in other stem cell populations, such as in bone marrow stem cells (BMSCs).¹⁰

Although the isolation of DPSCs can provide a potential cell source for hard tissue engineering in the future, previous *in vivo* research has showed variable results, which results in a low therapeutic potential.^{5,6} Fluorescence-activated cell sorting (FACS) with antibodies against specific cell receptors can form a more standardized way of retrieving and culturing DPSCs.

The monoclonal antibody STRO-1 reacts with an unidentified cell surface antigen expressed by a minor subpopulation of BMSCs. Some data demonstrate that the STRO-1 fraction in adult bone marrow is eventually stem cells with the capacity for differentiation into smooth muscle cells, adipocytes, osteoblasts and chondrocytes.¹¹⁻¹⁴ Previous studies also

demonstrated that DPSCs contained a STRO-1 positive cell population and showing positively of 9% in DPSCs.^{6,9} A STRO-1 positive populations were sorted from human DPCs.¹⁵ Further, recent study showed that also rat derived DPCs contained STRO-1+ cells.¹⁶ In this study, we will investigate if a STRO-1 positive cell population can be isolated from rat DPCs, and if, such still exhibits odontogenic potential.

In the current study, we hypothesize that a STRO-1 positive cell sorting can result in a more homogenous cell population, which will harbor an improved odontogenic differentiation capacity compared to STRO-1 negatives or the non-sorted cell population.

2. MATERIALS & METHODS

2.1 Cell Culture

The animal ethics committee of the Radboud University Nijmegen Medical Centre approved this research, and all national guidelines were taken into consideration. Maxillary incisors of Wistar rats (40 days old, male) were dissected, and primary rat dental pulp cells were acquired from these dental pulp tissues.¹⁶

2.2 Fluorescence-activated Cell Sorting

For cell sorting experiments, 10×10^6 dental pulp cells of the third passage were resuspended in 1 mL PBS/1%BSA and preincubated for 15 min on ice. Subsequently, cells were incubated with 200 μ L PBS/1%BSA with STRO-1 monoclonal antibody (20 μ g/mL, R&D Systems, Minneapolis, MN) for 15 min on ice. After centrifugation and washing, samples were incubated in 200 μ L PBS/1%BSA with phycoerythrin (PE)- conjugated goat anti-mouse IgG antibody (1:50; Sigma) for an additional 15 min on ice, washed, resolved, and kept on ice until sorting. Cells were sorted using a FACStar Plus flow cytometer (Beckton Dickinson & Co., Mountain View, CA). The samples were maintained at 4°C through the

cell-sorting procedures. Positivity was defined as a level of fluorescence greater than 99% of the blank (without 1st and 2nd antibody reaction) and negative (without 1st antibody reaction) controls. STRO-1 positive (+) cells were collected and expanded. Non-sorted cells and STRO-1 negative (-) cells were also used as controls for osteogenic cell culture.

To prove the efficacy of the applied FACS sorting, three types of cells obtained (STRO-1⁺ / STRO-1⁻ / non-sorted cells) were also assessed for STRO-1 positive cell content by immunofluorescence. Subsequently, the three cell populations were seeded onto 24-well plates or on coverslips (Thermanox®) for scanning electron microscopy (SEM) analysis at a density of 2×10^4 cells/well in α -MEM supplemented with 10% FCS, 10 mM sodium β -glycerophosphate, 10^{-8} M dexamethasone, L-ascorbic acid (50 μ g/mL), and gentamycin (50 μ g/mL). Culture medium was changed at 2- to 3- day intervals.

2.3 Cell Proliferation, Alkaline Phosphatase and Calcium Content

On day 1, 2, 4, 8, 16 and 24, the proliferation rates of cells, as measured by total DNA content, were assessed with a PicoGreen dsDNA quantification kit (Molecular Probes, Leiden, The Netherlands). The same supernatants as used above were also used to measure alkaline phosphatase (ALP) activity. Twenty μ L of 0.5 M 2-amino-2-methyl-1-propanol buffer was added to 80 μ L of the samples or standards,. Next, 100 μ L of substrate solution (p-nitrophenyl phosphate) was added and the mixtures were incubated at 37°C for 1 h. ALP activity was measured at 405nm, using an ELISA microplate reader (Bio-Tek Instruments Inc., USA). On day 8, 12, 16 and 24, the calcium content of the samples was measured by the *o*-cresolphthalein complexone (Sigma) method.¹⁶

2.4 Scanning Electron Microscopy

On day 8, 12, 16 and 24, samples were rinsed with filtered PBS, and fixed in 2% glutaraldehyde for 5 min. They were then dehydrated in graded series of ethanol, and dried in tetramethylsilane (Merck, Germany). Gold was sputtered on the samples just before SEM analysis. The specimens were examined with a JEOL 6310 Scanning Electron Microscope.

2.5 Real-Time Quantitative PCR

On day 1, 8 and 16, total RNA of every sample was prepared with RNEasy kit (Qiagen, The Netherlands). Isolated RNA was measured with the spectrophotometer. One μ g RNA was used to make cDNA. Five markers monitored the differentiation of cells: ALP, osteocalcin (OC), collagen type I (Col I), dentin sialophosphoprotein (DSPP), and dentin matrix protein-1 (DMP-1). GAPDH was used as house-keeping gene to normalize RNA expression (Table.1). Real-time PCR was performed using SYBR Green PCR kit (Eurogentec, Liege, Belgium) and controlled in a spectrofluorimetric thermal iCycler® (BioRad, The Netherlands). After the real-time PCR run, a Ct-value showed how many PCR cycles were necessary to obtain a certain level of fluorescence. Amplification efficiency of different genes was determined relative to GAPDH as an internal control (Δ Ct = Ct gene – Ct GAPDH). Interested mRNA in each sample was calculated by a comparative $\Delta\Delta$ Ct (Δ Ct gene – Δ Ct control) value method. Each sample was assessed in triplicate.

Table 1: Rat specific primers for real-time PCR

	Sense primer	Antisense primer
ALP	5'-GCTTCACGGCATCCATGAG-3'	5'-GAGGCATACGCCATGACGT-3'
OC	5'-GGCTTCCAGGACGCCTACA-3'	5'-CATGCCCTAAACGGTGGTG-3'
Col I	5'-TGGAATCTTGGATGGTTTGGA-3'	5'-GCTGTAAACGTGGAAGCAAGG-3'
DSPP	5'-CGGTCCCTCAGTTAGTC-3'	5'-TACGTCCTCGCGTTCT-3'
DMP-1	5'-CGTTCCTCTGGGGGCTGTCC-3'	5'-CCGGGATCATCGCTCTGCATC-3'
GAPDH	5'-GAAGGGCTCATGACCACAGT-3'	5'-GGATGCAGGGATGATGTTCT-3'

2.7 Statistical Analysis

Statistical significance was evaluated using one way-ANOVA with post-hoc Tukey testing, and $P < 0.05$ was considered significant. Error bars represent means $\pm$ standard deviation (SD).

3. RESULTS

3.1 Isolation of Dental Pulp Stem/Precursor Cells

Compared with the blank and negative controls, approximately 0.5% of the total dental pulp cells from the third passage were sorted as STRO-1⁺ population (Figure 1 A, B). All isolated cells had a typical spindle-shaped morphology with extending cytoplasmic processes, and no apparent differences were seen between STRO-1⁺ and STRO-1⁻ fractions by visual inspection. The immunofluorescence results however confirmed the efficacy of the sorting process, i.e. much more positively stained cells could be found in STRO-1⁺ cell population, compared to non-sorted cells. For the STRO-1⁻ cell population, positively stained cells were almost absent (Figure 1 C-E).

Figure 1: Isolation of *STRO-1*⁺ dental pulp cell population by FACS: (A) The living cells were selected for sorting by forward scatter (FSC); (B) FACS of *STRO-1*⁺ (PE) expression cell population showed a approximately 0.5% of the total dental pulp cell population of the third passage. Fluorescent micrographs of three cell populations after immunofluorescent staining: (C) *STRO-1*⁺ group; (D) *STRO-1*⁻ group; (E) non-sorted cells (original magnification 10 X).

3.2 Cell Proliferation, ALP Activity, and Calcium Content Measurement

The cell proliferation rates showed exponential growth of all three populations during 24 days, although there were some significant differences. Cells of the *STRO-1*⁺ and *STRO-1*⁻ fractions grew more rapidly than of the non-sorted cells, especially for *STRO-1*⁻ population (Figure 2 A). Considering ALP activity, all three groups kept increasing during the 24 days of incubation, but *STRO-1*⁻ population always showed significantly lower levels (Figure 2 B). The changes of calcium content in these fractions were always reflecting the ALP, and the *STRO-1*⁻ group retained significantly lower values during the 24 days (Figure 2 C).

A

B

Figure 2: Growth characteristics of three cell populations (A) The proliferation characteristics; (B) ALP activity; (C) Calcium content. The bars represent the means $\pm$ SD ($n=6$). The STRO-1+ group (S+) compared to the STRO-1- group (S-)* $P<0.05$, ** $P<0.01$; STRO-1+ group compared to non-sorted cells (P3): # $P<0.05$, ## $P<0.01$

3.3 SEM Analysis

On day 8, SEM analysis showed that all cells had spread well on the surface of the cover slips. Most of cells were spindle-shaped and interconnected. For the STRO-1⁺ and control cells, a mineralized extracellular matrix was observed on the surface after day 12. On day 24, matrix deposition had increased, and numerous calcified nodules were observed. At that time some cells were fully covered with mineralized deposits. In contrast, the STRO-1⁻ cells also formed a confluent layer, but there was no obvious mineralization visible (Figure 3).

Figure 3: Scanning electron micrographs of three cell populations on day 24. (A),(B) STRO-1+ group; (C),(D) STRO-1- group; (E),(F) non-sorted cells.

3.4 Real-time PCR

Using quantitative real-time PCR, expression of mRNA for ALP, OC, Col I, DSPP and DMP-1 was detected in all three cell populations on days 1, 8 and 16. The mRNA expression levels of non-sorted cells on day 1 were selected as baseline control (set as 100%), and were subsequently compared to the other groups and days. For ALP, gene expression level in STRO-1+ cells was significantly up-regulated at the beginning and down-regulated after that. In contrast, ALP expression peaked in non-sorted cells on day 8. For OC and collagen type I, gene expression level in STRO-1+ and non-sorted cells kept increasing from day 1 to 8, and decreased after that. However, mRNA levels in STRO-1+ cells were significantly higher than that in non-sorted cells from day 1 to day 8. The expression of DSPP and DMP-1, two dentin-specific markers, showed almost similar profiles in STRO-1⁺ and non-sorted cells. Results showed that DSPP and DMP-1 were gradually up-regulated from day 1 to 16, and peaked on day 16. STRO-1+ cells also showed significant up-regulation, compared to non-sorted cells.

However, for STRO-1⁻ cells, these genes of interest were only expressed at significantly low levels, and a completely different profile was formed (Figure 4).

Figure 4: Representative curves of the mRNA levels of ALP (A); collagen type I (B); OC (C); DSPP (D); DMP-1 (E); quantified during 16 days. The bars represent the means $\pm$ SD ($n=3$). The STRO-1⁺ group (S+) compared to the STRO-1⁻ group (S-): * $P<0.01$; STRO-1⁺ group compared to non-sorted cells (P3): # $P<0.05$, ## $P<0.01$

4. DISCUSSION

DPSCs harbor the capacity to form a dentin-pulp complex in vivo as well as the potential to be used in hard-tissue engineering. However, DPSCs acquired by single-colony-derived methods always show a hierarchy of differentiation ability and hard-tissue formation.^{5,6,17} This implies that clonogenic methods cannot provide a cell population with standardized

differentiation potential to be used in tissue engineering. Therefore, a method is needed to isolate appropriate populations of DPSCs.

Of course, we have to be very cautious in the description of our “unsorted cells”. Although we did not actively select these cells by the FACS technique, they do not fully represent the entire pulp tissue either. In fact, they have underwent some sort of sorting, as the cell culture process discriminates on adhesion criteria, i.e. non-adherent cells from the pulp were removed as a result of the culturing. This of course is very similar to the selection criteria that have been widely used before, to select differentiating cells from the bone marrow.

For FACS as a cell separation method, STRO-1 as specific cell receptor was selected in the current study because STRO-1 is the mesenchymal stem marker and is also expressed in some populations of DPSCs.^{6,9,15} Successful experiences for using FACS linked with STRO-1 antibody in BMSCs have been reported.^{13,14,18} The present study focused on the odontogenic potential of the STRO-1⁺ population, and the difference between STRO-1⁺ and STRO-1⁻ populations in cell phenotype. For the stem cells isolated from dental pulp tissue, these issues are still not fully understood.

Earlier experiments showed that DPSCs, isolated from the pulp of human exfoliated deciduous teeth by clonogenic method, exhibited a ~ 9% positively for STRO-1.⁹ In our study, a much lower percentage (0.5%) was positive for STRO-1⁺ cell population obtained from third passage dental pulp cells. This difference can have occurred because we used rat material, with a human marker. Still, our post-selection assessment proved undoubtedly that cell selection was affirmative. Other explanations for the low number might lie in the fact that the cell population was sorted from the complete primary culture cells rather than from the single-colony-derived cell populations. Compared with the clonogenic methods, which necessitate visual inspection and personal selection,⁵ FACS requires a relatively standardized skill and can be more helpful to isolate a stable and homogeneous population. Further, the

very low percentage of STRO-1⁺ cells can be due to the long time in primary culture before selection was applied.^{11,19}

On the smooth tissue culture surfaces, the ability of cells to form multilayers seems to be the basis of mineralization, because single-cell layers cannot produce mineralized matrix.²⁰ Similar to cells retrieved from bone marrow,^{12,13} our sorted STRO-1⁺ rat dental pulp cells showed the capacity to form multilayers and mineralized nodules when cultured in conditional medium. The STRO-1⁻ population from BMSCs showed a lack of osteogenic potential.²¹ Our study corroborated such data and also showed that the STRO-1⁻ fraction lack of the capability to form mineralized nodules in vitro, although possessing more proliferation ability, compared to STRO-1⁺ and non-sorted cells. This fact can be due to the inverse relationship between proliferation and differentiation, i.e. when cells are more proliferative then the differentiation of the cells is declined, and vice versa. ALP is a prerequisite for functional differentiation of BMSCs, and DPCs in vivo and in vitro.^{22,23} In our study, STRO-1⁺ cells showed outstanding ALP activity, analogous to that of DPCs under the same conditions.¹⁶ The results of the calcium content measurement mirrored the findings for ALP, and were consistent with quantity and scale of mineralized nodules as seen in SEM microscopy. These findings suggest that STRO-1⁺ cells possess the capacity to differentiate into odontoblast-like cells in vitro, similar to BMSCs.²³

In the current study, ALP, OC, Col I, DSPP, and DMP-1 were chosen as differentiation markers for real-time PCR. The mRNA confirmed the up-regulation of ALP expression in STRO-1⁺ cells, and reached a peak at day 1. However, the expression of mRNA is always prior to protein expression. ALP gene expression was down-regulated in STRO-1⁺ cells from day 1 to day 16. Furthermore, the STRO-1⁺ cell population, because of its more homogeneous stem cell population, would result in more sensitive reaction for odontogenic induction, in contrast to peak expression level at day 8 of non-sorted cells. Collagen type I is

the predominant collagen in dentin and bone matrix. OC, a non-collagenous protein, is also present in dentin and bone matrix. DSPP is the major part of the dentin noncollagenous proteins, and plays a crucial role during dentin mineralization.²⁴ DMP-1 is another specific protein in dentin, and is a candidate gene for dentinogenesis imperfecta.²⁵ Expression of these mRNAs can prove that these cells exhibit odontogenic potential. Our study confirmed that STRO-1⁺ cells possess relatively high expression levels of these genes, and showed the same results as previously described for BMSC and primary DPCs.^{16,26} Interestingly, all the genes investigated maintained a significantly low expression level in STRO-1⁻ cell population, similar to cells cultured without the conditional medium.¹⁵ For the results of real-time PCR, the STRO-1⁺ cell population showed a more sensitive reaction and odontogenic potential under conditional induction, compared to the STRO-1⁻ population, which was analogous to the primary DPCs.^{2,16}

BMSC were already confirmed to be heterogeneous,²⁷ and in vitro expanded STRO-1⁺ and STRO-1⁻ cells were shown to have differences in cell-phenotype and capacity for gene delivery. The STRO-1⁺ population from BMSC is more homogenous and therefore a better substrate for gene delivery than the STRO-1⁻ population.¹⁸ In our study, these two cell populations also showed differences in cell growth and odontogenic differentiation, although derived from the same primary cell populations.

Based on our results, we conclude that our hypothesis can only partially be proven. Indeed, removal of STRO-1⁻ population was possible in order to obtain a STRO-1⁺ cell population which is more homogenous. This positive cell fraction was capable of differentiating into the odontogenic pathway, whereas the negative fraction was not. However, the effect was not always advantageous, when compared to non-sorted cells.

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Chapter 3

Multilineage Potential of STRO-1+ Rat Dental Pulp Cells In Vitro

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1. INTRODUCTION

Stem cells generally are defined as colony-derived populations, which exhibit capacity for self-renewal and differentiation into a variety of tissue lineages.¹⁻³ Stem cells have been found in various adult tissues, including bone marrow, neural tissue, muscle, and fat.⁴⁻⁸ Recently, stem cells were also found in adult dental pulp cell (DPCs) populations. It was demonstrated that the stem cells in DPCs possessed the capacity for multilineage differentiation into cell types like osteoblasts, adipocytes, and neural-like cells.⁹⁻¹¹ Therefore, DPCs may represent a novel cell population that has potential for tissue engineering in the future.

Some pioneering studies on bone marrow stromal cells (BMSCs) showed that these cells were considerably heterogenous in terms of size, morphology, enzyme histochemistry, proliferation profile and differentiated potential.¹²⁻¹⁴ Analogous results were also found in DPCs. The behavior of DPCs and BMSCs was quite variable, even for cells derived from the same source. This suggests a need for a method that can isolate especially the stem cells fraction from the other cells present in dental pulp. The availability of monoclonal antibodies recognizing subpopulations is providing means for such selection. STRO-1 is the most extensively used antibody, and recognizes cell surface antigen expressed by a small subset of mesenchymal stem cells. Previous studies have shown that the enriched STRO-1 positive fraction from human bone marrow stromal cells had the capacity for self-renewal and could differentiate into smooth muscle cells, adipocytes, osteoblasts and chondrocytes.^{12,15-17} Human DPCs also contained STRO-1 positive cells, and these cells had the potential for differentiation into osteoblasts and adipocytes.^{10,18,19} Although STRO-1 selection usually is aimed at human cell populations, a recent study also showed that rat derived DPCs contained STRO-1⁺ cells.²⁰ In our previous study, a STRO-1 positive fraction was successfully isolated from rat incisor dental pulp cells with the use of fluorescence-activated cell sorting (FACS).

These cells showed self-renewal, osteogenic potential, and generally were more homologous than non-sorted cells.²¹

As an extension, in the current study, we investigated the multilineage potential of STRO-1-selected DPCs, again using rat cells. We hypothesized that STRO-1 sorting can have an effect on multilineage differentiation of rat dental pulp cells, in other words that STRO-1⁺ cells are more homologous and thus have a higher efficacy and a lesser variability for differentiation compared to non-sorted cells. If successful, the rat can be a good model for future use, to study tissue regeneration with stem cells derived from e.g. the pulp of extracted third molars.

2. MATERIALS & METHODS

2.1 Cell Culture

In our previous study, a STRO-1⁺ cell population was obtained from rat incisor dental pulp cells (3rd passage) through FACS (STRO-1 monoclonal antibody, R&D Systems, Minneapolis, MN).²¹ Approximately 0.5% of the total dental pulp cells were sorted as STRO-1 population. Cells were expanded in minimal essential medium- α (α -MEM; GIBCO-BRL) supplemented with 10% fetal calf serum (FCS; GIBCO-BRL) and gentamycin (50 μ g/mL; Sigma).

2.2 Multilineage Induction In Vitro

After expansion, STRO-1⁺ cells were seeded into 24-well plates at a density of 2×10^4 cells/well in α -MEM supplemented with 10% FCS. 48 hours later, the medium was substituted with four inductive media, inducing multiple differentiation pathways (Table 1).

For the induction of neurogenesis, firstly, pre-neurogenic medium was added to the cells, and incubated for 24 hr, followed by neurogenic medium for 5 hr. For the induction of

adipogenesis or myogenesis, cells were cultured for 4 weeks in adipogenic or myogenic medium. For the induction of chondrogenesis, approximately 250,000 cells were placed in a 15-ml polypropylene tube (Falcon), and centrifuged to pellet. Then, 0.5 ml of chondrogenic medium (PT-3003; Cambrex Bio Science, Verviers, Belgium) was added, freshly supplemented with 10 ng/ml TGF- β 3.

The conditioning media were refreshed at 3-day intervals. The first control group was non-sorted rat dental pulp cells (3rd passage) with the same conditioning media as the sorted cells, and the negative control group was the STRO-1⁺ cell population cultured in non-conditional medium.

Table 1: Lineage-specific differentiation inducing media

Medium	Media	Serum	Supplement
Pre-neurogenic	α -MEM	10% FCS	1mM DMSO
Neurogenic (NM)	α -MEM	None	10mM β -Mercaptoethanol (BME), 2% DMSO, 200 μ M butylated hydroxyanisole (BHA)
Adipogenic (AM)	α -MEM	10% FCS	0.5 mM isobutylmethylxanthine (IBMX), 1 μ M dexamethasone, 10 μ M insulin, 200 μ M indomethacin, 50 μ g/ml gentamycin
Myogenic (MM)	α -MEM	10% FCS, 5% Horse serum	0.1 μ M dexamethasone, 50 μ M hydrocortisone, 50 μ g/ml gentamycin
Chondrogenic (CM)	PT-3003 (Cambrex Bio Science, Verviers, Belgium), 10 ng/mL TGF- β 3		
Control	α -MEM	10% FCS	50 μ g/ml gentamycin

2.3 Real-Time Quantitative PCR

Total RNA of the samples was prepared with the RNeasy kit (Qiagen, Leusden, The Netherlands). Purified RNA was measured with the spectrophotometer, and 1 μ g of RNA was used to produce cDNA. The multilineage differentiation capacity of the cells was monitored by specific markers (Table 2); i.e. neurofilament and NSE (neurogenesis); PPAR γ 2 and lipoprotein lipase (LPL) (adipogenesis); myosin and myoD (myogenesis); collagen type II and aggrecan (chondrogenesis). GAPDH was used as the house-keeping gene to normalize the RNA expression levels. Real-time PCR was performed using the SYBR Green PCR kit (Eurogentec, Liege, Belgium) and controlled in a spectrofluorimetric thermal iCycler®

(BioRad, The Netherlands). After the real-time PCR run, a Ct-value was obtained for each well. This Ct value showed how many PCR cycles were necessary to obtain a certain level of fluorescence. Amplification efficiency of different genes was determined relative to GAPDH as an internal control ($\Delta Ct = Ct_{\text{gene}} - Ct_{\text{GAPDH}}$). Interested mRNA in each sample was calculated by a comparative $\Delta\Delta Ct$ ($\Delta Ct_{\text{gene}} - \Delta Ct_{\text{control}}$) value method.²² The fold change in gene expression relative to the control was calculated by $2^{-\Delta\Delta Ct}$. The samples from STRO-1⁺ cells before induction were the control, set as 1.0-fold expression level. Each sample was assessed in six-fold. Statistical analysis was performed by unpaired t-testing and $P < 0.05$ was considered significant. Error bars represent means $\pm$ standard deviation (SD).

Table 2: Rat specific primers for real-time PCR

	Sense primer	Antisense primer
Neurofilament	5'-TCGCCGCATATAGGAAACTACTG-3'	5'-GAGGGCTGTCGGTGTGTGTAC-3'
NSE	5'-TGATGACCTGACGGTGACCA-3'	5'-CAAACAGTTGCAGGCCTTCTC-3'
PPAR γ 2	5'-CGCTGATGCACTGCCTATGA-3'	5'-GGGCCAGAATGGCATCTCT-3'
LPL	5'-GCAAGGCATACAGGTGCAATT-3'	5'-GGCAGAGCCCTTTCTCAAATG-3'
Myosin	5'-AAATGTTCCCGCTTCCTCAAT-3'	5'-TCTGAAGCAGCTGTGAGGCA-3'
MyoD	5'-GGAGACATCCTCAAGCGATGC-3'	5'-AGCACCTGGTAAATCGGATTG-3'
Collagen type II	5'-GAACAACCAGATCGAGAGCA-3'	5'-CTCTCCAAACCAGATGTGCT-3'
Aggrecan	5'-CTGGAGACAGGACTGAAATC-3'	5'-CTCCATTGAGACAAGGGCTT-3'
GAPDH	5'-GAAGGGCTCATGACCACAGT-3'	5'-GGATGCAGGGATGATGTTCT-3'

2.4 Immunohistochemistry

The cells induced into the neurogenic, adipogenic, and myogenic differentiation were fixed in 4% paraformaldehyde for 15 min. For the chondrogenic culture, samples were fixed in 4% formalin, embedded in paraffin, and cross-sectioned (5 μ m thick). After blocking with 5% BSA/PBS, the samples were incubated with specific primary antibodies for 1 h, and subsequently incubated with biotinylated goat or donkey secondary antibodies (1:500 dilution; Jackson ImmunoResearch, UK) for 45 min. For enzymatic immunohistochemical staining, a peroxidase-conjugated avidin-biotin complex (Vectastain ABC staining kit; Vector Laboratories, CA) was used. Subsequently, the cells were incubated in DAB solution and intensified with 0.5% CuSO₄. Haematoxylin was used to counter-stain the nuclei. The

following antibodies were used; neuronal nuclei (NeuN, 1:50 dilution ; Chemicon, Temecula, CA, USA); neuron specific enolase (NSE, 1:50 dilution; Santa Cruz, USA); glucose transporter-4 (GLUT-4, 1:25 dilution; Santa Cruz, USA); myoblast determination protein 1 (MyoD, 1:25 dilution; PharMingen, USA); myosin heavy chain (MHC, 1:2 dilution; Development Studies Hybridoma Bank, USA); collagen type II (1:50 dilution; Development Studies Hybridoma Bank, USA).

2.5 Histochemistry

To identify the accumulation of lipid-rich vacuoles in adipogenesis, adipogenic induced cells were stained by the Oil red O (Sigma) as follows: cells were fixed with 4% paraformaldehyde, treated with 60% isopropanol for 5 min, stained with the working solution of Oil red O (0.18%) for 10 min, and then counter-stained with hematoxylin.

To determine the presence of sulfated glycosaminoglycans (GAGs) in chondrogenesis, pellet samples were stained with Alcian blue as follows: the samples were pre-treated with 3% acetic acid for 3 min, stained with 1% Alcian blue (pH 1.0) for 30 min, and then counter-stained with 0.1% nuclear fast red.

3. RESULTS

3.1 Neurogenic Differentiation

During neurogenic induction, real-time PCR analysis indicated that the expression levels of NSE and neurofilam mRNA in STRO-1⁺ cells were significantly higher than those in non-sorted cells with 3.74- and 12.78-fold versus 2.17-and 5.47-fold at hour 2 and 5, compared to STRO-1⁺ cells without induction (Figure 1 A,B).

STRO-1⁺ cells underwent a morphological change from fibroblast-like morphology to compact neuronal-like cells with thin branches. The cells were fixed and immuno-stained for

proteins specific for neural phenotypes, 5 hr past the initial neurococktail incubation. Neural differentiation was confirmed by the positive staining with antibodies to NeuN and NSE (Figure 2 A-D). In contrast, the STRO-1⁺ cells that did not receive the inductive medium showed negative for the applied staining.

*Figure 1: Representative columns of the mRNA levels (2-ΔΔCt) of (A) NSE and (B) neurofilament (neurogenesis); (C) PPARγ2 and (D) LPL (adipogenesis); (E) myosin and (F) myoD (myogenesis); (G) collagen type II and (H) aggrecan (chondrogenesis). STRO-1+ cells were cultured in inducing media (●) or control medium (▼). Non-sorted cells (3rd passage) were also cultured in inducing media (○). The STRO-1+ group (●) compared to the non-sorted group (○): * $P < 0.05$, ** $P < 0.01$; STRO-1+ group (●) compared to control group (▼): # $P < 0.05$, ## $P < 0.01$.*

Figure 2: Results of neurogenic differentiation. (A) morphological changes of STRO-1+ cells after induction (original magnification 10 X); (B) negative control (STRO-1+ cells in control medium); (C) immunohistochemical staining for STRO-1+ cells after induction (NeuN); (D) immunohistochemical staining for STRO-1+ cells after induction (NSE). (original magnification 20 X)

3.2 Adipogenic Differentiation

For the adipocyte-specific transcripts, real-time PCR analysis showed that the mRNA expression levels of PPARγ2 and LPL were significantly up-regulated with 7.89- and 15.79-fold after 4 weeks; as for non-sorted cells, only 5.79- and 6.77-fold up-regulation were detected, compared to STRO-1⁺ cells without induction (Figure 1 C, D).

During 4 weeks of adipogenic induction, the accumulation of lipid-rich vacuoles was observed within cells, 2 weeks past initial stimulation. The lipid vacuoles continued to develop over time, and were found even more after 4 weeks (Figure 3A). After 4 weeks of culture, Oil red O-positive lipid clusters were also demonstrated in STRO-1⁺ cells (Figure 3 B). Furthermore, induced cells were found to express GLUT-4, a specific marker of adipocytes (Figure 3 C). For the control STRO-1⁺ cells, the cells showed fibroblast-like morphology, and no positive immunostaining was detected (Figure 3 D).

Figure 3: Results after adipogenic differentiation. (A) morphological changes of STRO-1⁺ cells after induction; (B) Oil red O staining; (C) immunohistochemical staining for GLUT4; (D) negative control (STRO-1⁺ cells in control medium). (original magnification 20 X)

3.3 Myogenic Differentiation

For the myocyte-correlative transcripts, real-time PCR analysis demonstrated that the expression levels of myosin and myoD were increased with 16.67- and 12.80- fold in STRO-1⁺ cells after 4 weeks, and 7.89- and 7.34-fold in non-sorted cells, compared to STRO-1⁺ cells without induction (Figure 1 E, F). During 4 weeks of myogenic culture, no clear morphological changes were found in STRO-1⁺ cells, and some cells continued to form cytoplasmic branches and also showed fibroblast-like morphology. Two weeks after induction, cells were found to express MyoD and myosin, specific markers of the myocyte, and staining was even more pronounced after 4 weeks (Figure 4 A-D). For the control STRO-1⁺ cells, no positive immunostaining was detected.

Figure 4: Immunohistochemical staining after myogenic differentiation. (A) myoD (2 wk); (B) MHC (2 wk); (C) myoD (4 wk); (D) MHC (4 wk). (original magnification 20X)

3.4 Chondrogenic Differentiation

Real-time PCR analysis confirmed that the mRNA expression levels of collagen type II and aggrecan were obviously up-regulated in STRO-1⁺ after 4 weeks with 6.90- and 10.28-fold. At the same timepoint, there were 4.12- and 8.15-fold expression levels in non-sorted cells, respectively compared to control (STRO-1⁺ cells without induction) (Figure 1 G, H).

Figure 5: Results after chondrogenic differentiation. (A) STRO-1⁺ sample in 4 wk (Alcian blue staining); (B) negative control (STRO-1⁺ cells in control medium) in 4wk (Alcian blue staining); (C) STRO-1⁺ sample in 4 wk (immunohistochemical staining for collagen type II); (D) negative control in 4wk (immunohistochemical staining for collagen type II). (original magnification 20 X)

4. DISCUSSION

In a previous study, we have successfully isolated STRO-1⁺ cells from rat incisor dental pulp, and confirmed that a STRO-1⁺ cell population is more homogenous and it capable of differentiating into the osteogenic pathway.²¹ However, no systematic research has been performed to evaluate the multilineage potential of this tooth-derived STRO-1⁺ population, and whether the more homogeneous STRO-1⁺ cell population results in more standardized results.

The present study showed, considering the neurogenic differentiation, a clear morphological difference between treated groups and controls. The treated cells showed long extensions with multiple filopodia. The expression of NeuN and NSE proteins was also detected. Generally, NeuN is known to have expression in most neuronal cell types throughout the nervous system, including cerebral cortex, spinal cord, and peripheral nervous

system.²³ NSE is also localized in the cytoplasm of neurons, and is supposed to serve as a growth factor in neurons.²⁴ Under conditional inducement, NeuN and NSE were present in STRO-1⁺ DPCs, analogous to bone marrow stem cells.²⁵ The expression of specific neuronal phenotype markers and genotype together with the observed morphological changes in our study confirmed the process of neurogenic differentiation. However, these results do not mean that these cells possess the full function as neuron. Animal models are required to explore the usage of STRO-1⁺ cells in this field. Simultaneously, the real-time PCR assays can quantify and compare the expression levels between different samples. During the whole neurogenesis, the non-sorted rat dental pulp cells showed some up-regulation in neurogenic-specific gene expression, analogous to hDPSCs.²⁶ However, the gene expression in STRO-1⁺ cells indicated a higher up-regulation compared to the non-sorted cells. These results suggested that the STRO-1⁺ population was more potent in delivering neurogenic cells.

For the adipogenic differentiation, the protein expression of GLUT-4, and the mRNA expression of PPAR γ 2 and LPL were detected. GLUT-4 and LPL are the widely used adipogenic-specific markers.²⁷ PPAR γ 2 is also a marker for adipogenesis because it is mainly expressed in fat tissue and can activate adipocyte differentiation.²⁸ The achievement of true adipogenesis however always takes prolonged periods of time.^{12,19,26} On basis of specific gene expression levels, the STRO-1⁺ group also showed more potential for adipogenesis compared to non-sorted cells, during the late period of induction. The trans-differentiation of the induced cells was apparent by the accumulation of lipid-rich fields in the cytoplasm in the Oil red O staining, which is corroborated by other researchers.^{5,19,29}

The presence of multiple nuclei is an important characteristic of the terminal mature myocyte. However, no classical multinucleated cells could be observed in our study. In contrast, both myoD and myosin proved to be positive by immunohistochemistry, and by real-time PCR after induction. The reason of not finding classical multinucleated cells may be due

to the limited culture time in vitro, or due to the absence of a co-culture with a myogenic cell line.¹¹ MyoD plays a key role in the early myogenesis by activating muscle-specific genes in response to extracellular growth factors,^{30,31} whereas myosin is regarded as a late marker for myogenic differentiation.³² For the control groups, there were no positive markers for myocytes, as reported by others.⁹

To initiate chondrogenesis, the differentiation was successfully induced when cells were cultured as aggregates, as reported earlier in literature.^{5,33,34} The requirement for this high cell density suggests that cell interactions are very important in the chondrogenic system. Also the addition of TGF- β 1 or TGF- β 3 is necessary to induce the chondrogenesis.³³⁻³⁵ The alcian blue staining showed that only STRO-1⁺ cells supplemented with TGF- β 3 were able to form a structure with an increased sulfated proteoglycan-rich extracellular matrix. This was also confirmed by the specific cartilage matrix markers collagen type II (immunohistochemistry and real-time PCR) and aggrecan (real-time PCR). However, in our samples some matrix degradation or dead cells were found. On the other hand, the differences in specific gene expression levels between STRO-1⁺ cells and non-sorted cells still existed, though less than for the other differentiation pathways. This may be due to the excessive number of cells in the pellet, which might obstruct the exchange of nutrients; consequently, a three-dimensional culture system may be useful for this pellet culture method.

Based on the results mentioned above, we conclude that the STRO-1⁺ cell population has higher rate of multilineage differentiation than the non-sorted cells. In our second hypothesis, we claimed that the STRO-1⁺ cell population, because of its more homogeneous appearance, would result in more standardized results. This was tested by performing the real-time PCR in a statistical manner ($n = 6$, each n consisted of a pool of 3 wells). However, our PCR results showed a similar large standard deviation, which indicated comparable variation in results

between the 6 samples. Based on that result, we conclude that a higher level of differentiation were obtained by cell sorting, but not with more standardized results.

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Chapter 4

Hard Tissue Formation of STRO-1 Selected Rat Dental Pulp Stem Cells

In Vivo

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1. INTRODUCTION

Dental pulp stem cells (DPSCs) show mesenchymal stem cell properties, and might be a candidate cell type for dental tissue engineering. Previous studies have already demonstrated the self-renewal potential of DPSCs and their capacity to differentiate towards odontoblasts *in vivo*,^{1,2} but the efficacy of DPSCs for practical applications is still under investigation. Previous studies have shown a large variability in results of proliferation and differentiation phenotypes for DPSCs *in vitro* as well as *in vivo*.¹⁻³ Furthermore, our preceding study reported that mineralization and bone-like tissue formation in subcutaneously implanted DPSC-loaded constructs did not occur.⁴ This observation questions the potential of DPSCs for clinical applications, and illustrates the necessity to optimize stem cell technology.

Previous *in vitro* studies have already shown that a more homogeneous strain of DPSCs can be derived from pulp biopsies by STRO-1 cell selection.⁵⁻⁷ STRO-1 is described as a marker of human mesenchymal stem/precursor cells,⁸ but selection with this marker also increases the ability of odontogenic differentiation of rat DPSCs, as well as development towards other differentiation pathways.^{7,9,10} The addition of growth factors is another strategy that can enhance the differentiation of stem cells. For example, recombinant human BMP-2 is known to stimulate the odontoblastic differentiation of dental pulp cells.¹¹ The odonto/osteoinductive potential of BMP-2 has also been confirmed in clinical studies.¹²

Finally, previous studies indicated that *in vitro* culture time after seeding of the stem cells into a porous scaffold is important for the final tissue formation. In similar studies, using titanium fiber mesh and osteogenic cells, ectopic bone formation increased when after seeding the culturing of cells was continued for a short period of time (1-4 days) before implantation.^{13,14} Evidently stem cells exposed for a longer period to differentiation supporting conditions show enhanced differentiation into osteoblasts and, therefore, less

proliferation and migratory ability.¹³ However, all aforementioned knowledge has never been considered for dental pulp cell-based tissue engineering.

Therefore, in this study, we aimed to load STRO-1 selected rat DPSCs onto porous ceramic scaffolds (HA/TCP), after treatments with various odonto-inductive media and pre-culture times. After implanting subcutaneously into immunocompromised mice, ectopic hard tissue formation was evaluated. We hypothesized that (1) STRO-1 selected DPSCs can form abundant hard tissue in this in vivo model, and (2) the addition of BMP2 accelerates or enhances these results obviously. Further, we hypothesize that (3) the addition of BMP-2 can shorten the pre-culture period needed before implantation, which will facilitate final clinical application.

2. MATERIALS & METHODS

2.1 Cell Culture

Rat DPSCs were selected on the stem cell marker STRO-1 by fluorescence activated cell sorting (FACS) as described before.⁷ Briefly, 10 x 10⁶ cells of the third passage were incubated with STRO-1 monoclonal antibody (20 µg/mL, R&D Systems, Minneapolis, MN) and phycoerythrin (PE)- conjugated goat anti-mouse IgG antibody (1:50; Sigma). Cells were sorted using a FACStar Plus flow cytometer (Beckton Dickinson & Co., Mountain View, CA) with positivity defined as a level of fluorescence greater than 99% of the blank (without 1st and 2nd antibody reaction) and negative (without 1st antibody reaction) controls. To obtain enough cells for the experiments, cells were expanded to the 10th passage in minimal essential medium- α (α -MEM; GIBCO-BRL, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS; GIBCO-BRL) and gentamycin (50 µg/mL; Sigma). Although not quantified, immunofluorescence microscopy was used to confirm the STRO-1 positive cell content after the expansion step.

2.2 Three-Dimensional Scaffold

Sintered HA/TCP Camceram® ceramic discs of a 6mm diameter and 3 mm thickness with a HA/TCP ratio of 60/40 were kindly provided by CAM Implants (Leiden, the Netherlands). The volumetric porosity of the discs was 90%. The discs were sterilized by autoclaving.

2.3 In Vitro Analysis

2.3.1 Cell Seeding

All scaffolds were pre-wetted in α -MEM medium with 10% FCS and 50 μ g/ml gentamycin overnight. After that, STRO-1 selected DPSCs were seeded onto the scaffolds by submerging the scaffolds in a suspension of 5.0×10^6 cells/ml while gently rotating at 6 rpm for 3 hrs. For every 6 scaffolds, 1 ml cell suspension was used. Subsequently, the scaffolds were put in 24-well plates with 1 ml of medium, which was replaced at 3-day intervals. Three different media were used:

- (1) “BMP-plus”, i.e. α -MEM supplemented with 10% FCS, 10 mM sodium β -glycerophosphate, 10^{-8} M dexamethasone, L-ascorbic acid (50 μ g/mL), gentamycin (50 μ g/mL), and 100ng/mL of rhBMP-2 (R&D Systems).¹⁵
- (2) “odontogenic” medium, i.e. α -MEM supplemented with 10% FCS, 10 mM sodium β -glycerophosphate, 10^{-8} M dexamethasone, L-ascorbic acid (50 μ g/mL), and gentamycin (50 μ g/mL) similar to our previous study.⁴
- (3) “control” non-odontogenic medium, i.e. α -MEM supplemented with 10% FCS and 50 μ g/mL gentamycin only.

2.3.2 Cell Numbers and Alkaline Phosphatase Activity

On day 1, 4, and 8 post-seeding, the cell number was determined by total DNA content ($n = 3$), using the PicoGreen assay as described previously.¹⁶ The same samples were also used to

measure alkaline phosphatase (ALP) activity. Twenty μL of 0.5 AMP buffer (Sigma) was added to 80 μL of the samples or standards. Next, 100 μL of substrate solution was added and the mixtures were incubated at 37 °C for 1 h. ALP activity was measured at 405nm, using an ELISA microplate reader (Bio-Tek Instruments Inc., USA).¹⁶

2.3.3 Scanning Electron Microscopy (SEM)

On day 1, 4, and 8 post- seeding, cell/scaffold complexes were rinsed with filtered PBS, and fixed in 2% glutaraldehyde for 5 min, dehydrated in graded series of ethanol, and dried in tetramethylsilane (Merck, Germany). Gold was sputtered on the samples just before SEM analysis. The specimens were examined with a JEOL 6310 SEM.

2.4 In Vivo Analysis

For the *in vivo* analysis, cells were seeded as described above. Besides the three media, also three different post-seeding incubation times were studied, i.e. 1, 4, or 8 days, resulting in 9 study groups. In total, 8 samples of each group were used for *in vivo* analysis. Eighteen 10-week-old BALB/c immunocompromised nude mice were used after approval from the Radboud University Nijmegen Animal Ethics Committee (RU-DEC 2006-134). National guidelines for the care and use of laboratory animals were obeyed. Surgery was performed under general anaesthesia (isoflurane 1.5-3%). The backs of the mice were washed and disinfected with povidone-iodine. On each flank, parallel to the spinal column, two small incisions of about 10 mm were made through the skin. Using blunt dissection, subcutaneous pockets were created. To avoid cross over effects, each mouse received always 4 implants of the same culture medium and pre-culture time group. After implant placement the wounds were closed using staples.

2.4.1 Light Microscopical Assessment

After 12 weeks of implantation, the samples were retrieved. Four samples of each group were fixed in formalin, dehydrated in a series of ethanol, and embedded in methylmethacrylate (MMA). After polymerization of the MMA, at least three thin transversal sections (10 μ m) of each tissue block were made using a modified sawing microtome technique.¹⁷ The sections were stained with methylene blue/basic fuchsin and analyzed using light microscopy.

Furthermore, image analysis was performed to evaluate the quantity of hard tissue formation in different specimens. Three tissue sections per implant were digitized at low magnification (2.5 x), allowing coverage of the entire implant. Using the Leica® Qwin Pro image analysis system (Leica, Wetzlar, Germany), the areas of the ceramic scaffold and the novel bone-like tissue were detected. The outline of the implant area was designed as the region of interest (ROI). Then, with the use of the image analysis software, the surface area of bone-like tissue (BSA) and the percentage of bone-like area (BSA/ROI) were calculated.^{13,18,19}

2.4.2 Real-time Quantitative PCR

The remaining 4 samples of each group were used for real-time PCR analysis. Fibrous tissue capsules and other surrounding tissues were removed immediately after the retrieval of the samples. The calcium phosphate scaffolds together with the interstitial tissues were cut into small pieces and transferred to liquid nitrogen. Before use, samples were defrosted and total RNA of each sample was prepared with a Qiagen RNEasy kit (RNeasy kit, QIAGEN, Venlo, The Netherlands). Then, 1 μ g of total RNA was used to perform the reverse transcriptase (RT)-reaction. The obtained cDNA was used as a template in PCR. The odontogenic differentiation of cells was monitored by analyzing the specific markers

osteocalcin (OC), bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), and dentin matrix protein 1 (DMP-1). GAPDH was used as house-keeping gene to normalize RNA expression (Table 1).

Real-time PCR was performed using the SYBR Green PCR kit (Eurogentec, Liege, Belgium) and controlled in a spectrofluorimetric thermal iCycler® (BioRad, The Netherlands). After the real-time PCR run, the Ct-value showed how many PCR cycles were necessary to obtain a certain level of fluorescence. Amplification efficiency of different genes was determined relative to GAPDH as an internal control ($\Delta C_t = C_t \text{ gene} - C_t \text{ GAPDH}$). The mRNA in each sample was calculated by the comparative $\Delta\Delta C_t$ ($\Delta C_t \text{ gene} - \Delta C_t \text{ control}$) value method. Each measurement was assessed in triplicate.

Table 1: Rat specific primers for real-time PCR

	Sense primer	Antisense primer
<i>OC</i>	5'-GGCTTCCAGGACGCCTACA-3'	5'-CATGCCCTAAACGGTGGTG-3'
<i>DSPP</i>	5'-GTGAGGACAAGGACGAATCTGA-3'	5'-CACTACTGTCACTGCTGTCACT-3'
<i>DMP-1</i>	5'-GCGCGGATAAGGATGA-3'	5'-GTCCCCGTGGCTACTC-3'
<i>BSP</i>	5'-ACTTCCCTTCGCAAGCTTAGG-3'	5'-AAACTTCCCGCGTATGTTGG-3'
<i>GAPDH</i>	5'-GAAGGGCTCATGACCACAGT-3'	5'-GGATGCAGGGATGATG TTCT-3'

2.5 Statistical Analysis

Statistical significance was evaluated using one way-ANOVA with post-hoc Tukey testing, and $P < 0.05$ was considered significant. Error bars represent mean $\pm$ standard deviation (SD).

3. RESULTS

3.1 In Vitro Analysis

3.1.1 Cell Proliferation and ALP Activity

The total DNA measurement revealed that there were significant differences between the three medium groups (BMP-plus, odontogenic, and control) (Figure 1A). Supplementation of BMP-plus medium affected proliferation, as the cell number decreased from day 1 to day 8, and consistently showed a significantly lower level compared to the other two groups. On the

other hand, the absence of dexamethasone and BMP in the control group resulted in increased proliferation. The ALP activity of cells is depicted in Figure 1B. The ALP activity gradually increased from day 1 to 8 in all three groups. Still the BMP-plus group always showed significantly higher levels compared to the other two groups, with exception of BMP-plus vs. odontogenic at day 8.

Figure 1: (A) The proliferation characteristics; (B) ALP activity. The bars represent the means $\pm$ SD ($n=3$). BMP-plus medium compared to odontogenic medium: * $P<0.05$, ** $P<0.01$; BMP-plus medium compared to control medium: # $P<0.05$, ## $P<0.01$; odontogenic medium compared to control medium: † $P<0.05$, ‡ $P<0.01$.

3.1.2 SEM Morphology

One day post-seeding, SEM examination showed that cells had adhered well and spread fully on the surfaces of the HA/TCP ceramic scaffolds (Figure 2A-C). There were no obvious morphological differences among three medium groups. With increasing culture time, a noticeable amount of mineralized globular accretions were observed on cell/scaffold complexes in BMP-plus medium on day 4 (Figure 2D). For odontogenic culture group, only sparse mineralized deposits were found (Figure 2E). The control group only showed cells growing on the scaffolds (Figure 2F). On day 8, more numerous and larger mineralized nodules covered the surfaces of cell/scaffold complexes in BMP-plus and odontogenic medium groups (Figure 2G,H), but the latter also showed the presence of cells on top of the

mineralized matrix. For the control group, an intact cell-layer was present without obvious mineralized deposits (Figure 2I).

Figure 2: Scanning electron micrographs of three groups with different pre-culture periods. (A) BMP-2 plus group on day 1; (B) odontogenic group on day 1; (C) control group on day 1; (D) BMP-2 plus group on day 4; (E) odontogenic group on day 4; (F) control group on day 4; (G) BMP-2 plus group on day 8; (H) odontogenic group on day 8; (I) control group on day 8. Arrows indicate some of the calcified nodules.

3.2 In Vivo Analysis

All mice appeared to be in good health throughout the test period except for one, which suffered from a viral contamination not related to the experiment, and was excluded from further evaluation. This mouse contained four samples of constructs treated with odontogenic medium and 4 days of in vitro pre-culture time. After sacrifice, all other implants were retrieved with the surrounding soft tissues intact.

3.2.1 Light Microscopic Evaluation

Histological observation indicated that all HA/TCP ceramic implants after the 12 week implantation period were encapsulated with a thin fibrous tissue capsule, closely following

the outer perimeter of the scaffold. Inflammatory cells were only sporadically present. Tissue ingrowth was found in all the implants and macro-pores of scaffolds were filled with fibrous tissue.

For the implants with one-day pre-culture, obvious hard tissue formation had occurred in both the BMP-plus and odontogenic medium groups (Figure 3A,B). The newly formed hard tissue was stained deep red by the basic fuchsin and was present mainly along the surface of the porous ceramic material. Furthermore, at higher magnification (Figure 4A,B), distinct concentric lamellae were observed with entrapped osteocyte-like cells and osteoblast-like cells lying orderly arranged at the surface. Bone-marrow-like hematopoietic tissue was also seen together with the newly formed hard tissue. The constructs pre-cultured in control medium, showed no evident hard tissue formation (Figure 3C). At higher magnification, an orderly arranged thick collagen-like extracellular matrix (ECM) with multiple-layers of cells was seen along the pore surface (Figure 4C).

Also the 4 days of pre-culture samples in odontogenic, and especially in BMP-plus medium, showed upon visual inspection enhanced hard tissue formation compared to control medium (Figure 3D,E,F).

After 8 days of pre-culture, hard tissue was formed only sporadically in samples that had been grown in odontogenic or BMP-plus medium (Figure 3G,H). At higher magnification, these specimens revealed the presence of a granular-like mineral deposit accumulated on the surface of the scaffold (Figure 4D). Another difference with the one- or four-day pre-culture groups was that this deposit lacked a typical bone-like structure, and osteocyte-like and osteoblast-like cells were absent. On the other hand, the implants treated with control medium showed a small amount of hard tissue formation (Figure 3I).

Figure 3: General overview of the implants of 12 weeks (methylene blue basic/fuchsin staining) with different pre-culture periods. (A) BMP-2 plus group with 1 day pre-culture; (B) odontogenic group with 1 day pre-culture; (C) control group with 1 day pre-culture; (D) BMP-2 plus group with 4 days pre-culture; (E) odontogenic group with 4 days pre-culture; (F) control group with 4 days pre-culture; (G) BMP-2 plus group with 8 days pre-culture; (H) odontogenic group with 8 days pre-culture; (I) control group with 8 days pre-culture. Scale bar is 400 μm .

Figure 4: Histology and morphology of implants of 12 weeks at a high magnification (methylene blue basic/fuchsin staining). (A) BMP-2 plus group with 4-day pre-culture (original magnification 20X), bar size is 50 μm ; (B) BMP-2 plus group with 4-day pre-culture, the section showed the orderly array of osteoblast-like cells on the surface of bone-like matrix (arrow) (original magnification 40X), bar size is 25 μm ; (C) control group with 4-day pre-culture, the section showed multiple-layers of cells orderly arranged

along the pore surface (arrow) (original magnification 20X), bar size is 50 μm ; (D) BMP-2 plus group with 8-day pre-culture, the section showed the presence of a mineralized matrix however lacking osteocyte-like cells (arrow) (original magnification 20X), bar size is 50 μm .

3.2.2 Histomorphometry

In Figure 5 the percentages of bone formation in the various scaffold groups are depicted. Comparison of the different media groups of the 1-day pre-culture samples revealed that significantly more bone-like tissue formation had occurred in the BMP- plus group compared to the odontogenic medium, as well as to the control medium group. The odontogenic group showed also significantly more bone formation than the control group. Similar significant differences were found for the 4 day pre culture specimens. On the other hand, in the 8 days of pre-culture time group, a very different response was seen. Here, the BMP-plus and odontogenic group both revealed significantly less bone formation than the control medium group. Mutual comparison of the different pre-culture time points showed that bone formation in the BMP-plus and odontogenic groups peaked with four days of pre culture and was significantly higher compared to 1- and 8-days. In contrast, for the control group, the values at 8 days of pre-culture were significantly higher than for 1 or 4 days of pre-culture, but still significantly lower compared with BMP-plus and odontogenic medium groups at 4 days of pre-culture.

Figure 5: Histomorphometrical results showing the percentage of bone-like tissue formed in the implants of the different groups. The bars represent the mean $\pm$ SD ($n = 4$). BMP-plus medium compared to odontogenic medium: * $P < 0.05$; BMP-plus medium compared to control medium: ## $P < 0.01$; odontogenic medium compared to control medium: Δ $p < 0.05$, $\Delta\Delta$ $P < 0.01$; BMP-plus medium (4 days) compared to BMP-plus medium (1 or 8 days): ‡‡ $P < 0.01$.

3.2.3 Real-Time PCR

Expression levels of mRNA for *OC*, *Bsp*, *Dspp*, and *Dmp1* were compared among the three groups. mRNA expression levels of samples of the control group at 1-day pre-culture were set as baseline control (relative expression value, 100%). It was striking that the expression of these four genes showed similar profiles to each other (Figure 6). The most noticeable differences were that BMP-plus treated constructs demonstrated significant up-regulation of expression of all 4 measured genes in the 4 days pre-culture group compared to both 1 and 8 days of pre-culture. The odontogenic group also revealed the same expression profiles for these genes, although generally significantly lower values were found compared to BMP-plus. In the control medium-treated constructs, low gene expression levels were seen in the samples with 1-day or 4-day pre-culture, however these significantly had increased at 8 days of preculture time.

E

	OC			BSP			DSPP			DMP1		
	B-O	O-C	B-C	B-O	O-C	B-C	B-O	O-C	B-C	B-O	O-C	B-C
1	-	**	**	-	**	**	*	**	**	*	**	**
4	*	**	**	*	*	**	**	**	**	*	**	**
8	*	-	*	-	*	**	-	*	*	-	*	**
	B	O	C	B	O	C	B	O	C	B	O	C
1-4	**	-	**	**	-	**	*	*	**	**	**	**
4-8	**	*	*	**	*	*	**	**	**	**	**	*

Figure 6: Curves of the mRNA levels of OC (A); BSP (B); DSPP (C); DMP-1 (D); quantified for the *in vivo* specimens. The bars represent the mean $\pm$ SD ($n = 3$). (E) The statistics of (A)-(D). * $P < 0.05$; ** $P < 0.01$ – $P > 0.05$. B=BMP-plus medium; O=Osteogenic medium; C=Control medium; 1=one day of pre culture time; 4=four days of pre culture time; 8=eight days of pre culture time. B-O= BMP plus medium compared to Osteogenic medium, and so on.

4. DISCUSSION

The use of dental pulp stem cells is an essential component in dental tissue engineering. The aim of the current study was to evaluate the efficacy of STRO-1-selected DPSCs for the regeneration of (dentin-like) hard tissue, especially regarding the effect of BMP-2 treatment and pre-culture time.

Here, a porous calcium phosphate ceramic carrier was selected as scaffold material. Such ceramic also is used in other studies for hard tissue regeneration, because of its bone-compatible character and the ability to support the differentiation of osteogenic cells.²⁰ However, in our previous study using similar material but non-homogeneously selected DPSCs, only poor amounts of hard tissue were formed under the same condition as our 1 day/osteogenic group.⁴ Our current study demonstrated abundant hard tissue formation in the STRO-1 positive DPSCs/HA/TCP implants. The STRO-1 selection evidently resulted an enriched subpopulation with improved mesenchymal stem cell-like properties of more committed progenitors.⁷⁻⁹ These more homogeneous DPSCs loaded and implanted subcutaneously then resulted in enhanced hard tissue formation *in vivo*. This method therefore

seems advantageous for therapeutical applications, compared to the use of non-selected primary cells as described before.

Further, to enhance the hard tissue regeneration potential of the cell/scaffold construct several additional cell culture parameters can be modified during and after seeding, e.g. by optimizing the number of loaded cells.^{4,14,19,21} A high cell density was used in the current study. High cellularity is known to enhance cell-to-cell contact and communication between cells, and has a positive effect on cell attachment and differentiation.^{14,22} Moreover, relatively high numbers of cells loaded onto a scaffold previously were shown to benefit ectopic hard tissue formation.³ To achieve such high cell numbers, repeated passaging of cells was necessary. Apparently, such an extended culture protocol did not negatively influence the differentiation properties of the STRO-1-selected cells as was observed before for non-selected cells.^{4,7,9}

Enhancement in the odontogenic potential of our DPSCs/scaffold constructs can also be achieved by pre-culturing the complexes in the presence of stimulatory media supplements, which allows the differentiation of cells towards the odontoblast phenotype, and the secretion of dentin-like extracellular matrix (ECM).^{13,14,19} During this pre-culture period, such ECM has been reported to encourage the odontoblast differentiation *in vitro*.^{23,24} However, there is an inverse relationship between cell proliferation and differentiation. Therefore it has to be emphasized that during this preculture period, the occurrence of a mismatch in too much differentiation and not sufficient proliferation has to be prevented. Previous studies have indicated already that bone marrow cells (BMCs) only require short culture period (1 or 4 days) to show bone-like tissue formation after implantation in an orthotopic site. Furthermore, BMCs-loaded in titanium fiber mesh, with prolonged culturing of 8-16 days before implantation, resulted in a very limited amount of bone-like tissue formation after implantation.^{13,14,19,25} Our current study also showed that the hard tissue formation increased

with pre-culture times from 1 to 4 days, but significantly decreased when prolonging the pre-implantation culture time to 8 days. ALP is regarded as an early marker of osteogenic differentiation, which indicates the ability of hard tissue formation.^{16,27} We suppose that DPSCs, when implanted need to be at a very immature stage where the ALP activity is still at a basal level and cells still have a strong proliferation potential.^{19,26} With longer incubation time (i.e. 8 days), the differentiation of DPSCs towards a mature stage eventually occurred, with deposition of considerable amounts of mineralized ECM *in vitro*.¹⁹ Evidently, matured cells lose their proliferative potential, which explains the poor hard tissue formation *in vivo*. In conclusion, the *in vitro* cell phenotype or developmental stage definitely affects the *in vivo* performance. Four days of odontogenic stimulation drives DPSCs to the preferable stage, with a balance between proliferation and differentiation, and is most suitable for therapeutic applications *in vivo*.

As part of the current study, BMP-2 was supplemented in odontogenic media ('BMP-plus'). The addition of BMP-2 can induce odontoblastic differentiation of DPSCs in cell culture and induces the formation of reparative dentin *in vivo*.^{11,28} In the current study it was shown that such BMP-plus medium enhanced the differentiation level of DPSCs *in vitro* and significantly increased the amount of hard tissue formation *in vivo*. Moreover, we hypothesized that the addition of BMP-2 can reduce the pre-culture period compared to control medium, which was proven true by our results. One remark that must be made however is that no full randomization was applied. Since growth factor-loaded samples might influence neighboring samples placed in the subcutaneous area of a small animal like the mouse, we here decided to only use one pre-treatment type per mouse to avoid cross-over effects. Multiple mice were used for each group, without noticeable differences in reaction from mouse to another. Still, some additional influence of the host animal cannot totally be excluded.

The mRNA expression of *OC*, *BSP*, *DSPP*, and *DMP-1* were chosen as markers for the odontogenic phenotype. *OC* and *BSP* are usually regarded late markers for osteoblast differentiation and play an important role in hard tissue regeneration.²⁹ *DSPP* is the major dentinal non-collagenous protein and plays a crucial role during dentin mineralization.³⁰ *DMP-1* is another specific protein for dentin and is mostly known as the candidate gene for the disorder dentinogenesis imperfecta.³¹ The expression of these specific markers was most effectively enhanced by the addition of BMP-2 to the medium, compared to the dexamethasone containing odontogenic medium alone. The *in vivo* gene expression confirmed the dentin-like hard tissue formation *in vivo*. Furthermore, the expression of *DSPP* suggested a distinct developmental functionality *in vivo*,³² although the implants did not show the typical dentin-like appearance. Improved scaffold designs in composition as well as morphology can facilitate the formation of more natural tissue constructs, and have to be investigated next.

Interestingly, the DPSC/scaffold constructs pre-cultured in our control medium showed a gradual increase of hard tissue formation, but still at a low amount compared to the BMP-plus and dexamethasone-treated groups. This may be partially due to the HA/TCP ceramic. A previous study demonstrated that this scaffold material did not only support abundant ingrowth of tissue and vasculature, but also induced calcification [4]. Moreover, cells growing in multilayers also showed evidence for odontogenic differentiation, after a long *in vitro* culture time.³³

Still the overall result should be regarded critically. Although the supplements used in this and other dental tissue engineering studies are often described as "odontogenic" neither dexamethasone nor BMP-2 are, strictly speaking, odontogenic. As said, such formulations have also been widely used as an osteogenic supplement to promote osteoblast differentiation from bone marrow mesenchymal stem cells. So far, we have not been able to truly define

odontogenic conditions. The results of the study reflect this problem, as the observed hard tissue is very bone-like, with distinct concentric lamellae, osteocyte-like cells, and bone marrow-like hematopoietic tissue, even though the dentin-specific proteins Dspp and Dmp-1 were upregulated as observed by the real-time PCR. Still, to actually progress to potential therapeutic applications towards dentinal restoration either the development of a better suitable scaffold, or direct implantation into dentinal tissue as we performed before seems essential.³⁴

5. CONCLUSIONS

Based on our results, we conclude that STRO-1-selected rat dental pulp stem cells can form abundant hard dentin-like tissue after subcutaneous implantation on a ceramic carrier, and thus exhibit great potential for therapeutic application. Furthermore, the culture period between cell seeding and implantation needs to be optimized to maximize the efficacy of tissue regeneration. After 4 days of dexamethasone and/or BMP-2 induced stimulation, DPSCs formed the highest amounts of hard tissue *in vivo*. The addition of BMP2 further accelerated the odontoblastic differentiation of DPSCs and overall enhanced the amount of hard tissue formation.

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Chapter 5

STRO-1 Selected Rat Dental Pulp Stem Cells Transfected with Adenoviral-Mediated Human Bone Morphogenetic Protein 2 Gene Show Enhanced Odontogenic Differentiation

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1. INTRODUCTION

Tissue engineering requires three key elements: morphogenetic signals including growth and differentiation factors, responding stem cells, and a biocompatible scaffold.¹ Stem cells have been isolated from many tissues, including bone marrow, neural tissue, skin, fat, and muscle. Currently, mesenchymal stem cells have also been found in dental pulp tissue.²⁻⁴ Such dental pulp-derived adult stem cells (DPSCs) exhibited multilineage potential, and were able to differentiate towards odontoblast-like cells, adipocytes, myocytes, and neural cells.²⁻⁶ Interestingly, these cells were relatively easy to expand in vitro, and could be passaged for more than 25 times.³ However, as a new stem cell source for tissue engineering, the in vivo transplantation of such cells in porous calcium phosphate, collagen, or titanium fibre mesh did not show adequate hard-tissue formation.^{5,7} These results suggest a hierarchy of progenitors in adult dental pulp, including a minor population of self-renewing, highly proliferative, multipotent stem cells, among a larger compartment of probably more committed progenitors.⁵

For successful tissue engineering strategies, stem cell technology needs to be optimized. Previous studies have already shown that dental pulp cells could be derived from human or even rat biopsies by STRO-1 cell selection.^{4,6,8,9} STRO-1 is described as a marker of mesenchymal stem/precursor cells,^{9,10} and selection with this marker increases the ability of osteo/odontogenic differentiation, as well as development towards other differentiation pathways.^{6,9-13}

Another strategy to enhance the efficacy of stem cells could be the addition of growth factors. Recombinant human BMP-2 can stimulate differentiation of dental pulp cells into odontoblasts.¹⁴ The osteoinductive potential of recombinant human BMP-2 has also been confirmed in clinical studies.¹⁵ However, there are some limitations for local delivery of BMP, including short half-life, large dose requirement, high cost, the need of repeated

applications, and poor distribution.¹⁶ Possibly, transfer of the *Bmp2* gene into DPSCs could solve this problem. Recently, several studies showed that adenoviral-mediated bone morphogenetic protein-2 (AdBMP-2) transfected cells in bone implants were capable of stable expression of BMP-2 and revealed exciting results in ectopic osteoinduction animal models.¹⁷ Furthermore, adenoviruses are well-known for efficient delivery of therapeutic genes, and have already been applied for clinically relevant gene therapy.¹⁸

In this study, the differentiation characteristics of transfected STRO-1 selected rat DPSCs with adenoviral-mediated human *Bmp2* gene were evaluated. We hypothesized that *Bmp2* transfected cells have an increased odontogenic differentiation potential, compared to non-transfected cells, even when cultured in medium without dexamethasone or other stimuli.

2. MATERIALS & METHODS

2.1 Cell Culture

STRO-1 selected rat DPSCs (10th passage), as obtained by FACS,⁹ were expanded in minimal essential medium- α (α -MEM; GIBCO-BRL, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS; GIBCO-BRL) and gentamycin (50 μ g/mL; Sigma). The multilineage potential of this cell population was confirmed in a previous study.¹⁹

2.2 Adenovirus Gene Transfer

A replication-deficient adenoviral vector carrying the human *Bmp2* gene, AdBMP-2, was used in the current study. Firstly, to acquire effective gene transfer, an optimum multiplicity of infection (MOI) was evaluated. Cells were seeded in a 24-wells plate with a density of 2×10^4 cells/well. AdBMP-2 was added to the cells with different MOI (0, 5, 10 and 100) ($n = 4$). 48 hours after transfection, the presence of BMP-2 in the culture supernatants was tested using a modified BMP-selective reporter bioassay (BRE-luc) inducing luciferase expression.

Luciferase activity was quantified using the Bright-Glo™ Luciferase Assay System (Promega, Madison, WI, USA). For the standard curve, serial dilutions of rhBMP-2 (R&D Systems, Abingdon, United Kingdom) were used. The lower detection limit of this assay was 1.6 ng/ml BMP-2.

Secondly, after achieving the optimum MOI, the cells were plated in the same density in 24-well plates. 24 hours after seeding, cells were infected with AdBMP-2 (dilution in α -MEM) at the optimum MOI. The transfected and non-transfected cells were cultured for 24 days in α -MEM supplemented 10% FCS and 50 μ g/mL gentamycin. Culture medium was changed at 3-day intervals.

2.3 Cell Proliferation and ALP Activity

1, 4, 8, 12, 16 and 24 days after gene transfer, the proliferation rates of the cells, as measured by total DNA content (n=6 for each time point), were assessed with a PicoGreen dsDNA quantification kit (Molecular Probes, Leiden, The Netherlands).⁴ 1 mL of Milli-Q water (Millipore, Billerica, MA) was added to each well to lyse the cells. 100 μ L of PicoGreen working solution was added to 100 μ L supernatants of samples. After incubation, the fluorescence of each sample was measured with a fluorescence microplate reader (Bio-Tek Instruments Inc., USA), with excitation filter 365 nm and emission filter 450 nm, and the DNA amounts were calculated from a standard curve.

The same supernatants as used for the PicoGreen assay were also used to measure cellular ALP activity (Sigma). 20 μ L of 0.5 M 2-amino-2-methyl-1-propanol (AMP) buffer was added to 80 μ L of the samples or standards. Next, 100 μ L of substrate solution (p-nitrophenyl phosphate) was added and the mixtures were incubated at 37°C for 1 h. ALP activity was measured at 405nm using an ELISA microplate reader (Bio-Tek Instruments Inc, USA).

2.4 Calcium Content

4, 8, 12, 16 and 24 days after gene transfer, the rate of calcified extracellular matrix deposition was assessed by measuring the calcium content of the samples ($n = 6$), using a ortho-cresolphthalein complexone (OCPC, Sigma) method. 1 mL of 1 M acetic acid was added to each well overnight. Working solution was prepared: 5 mL of OCPC solution was added to 5 mL of 14.8 M ethanolamine-boric acid buffer (pH 11), 2 mL of 8-hydroxyquinoline, and 88 mL of Milli-Q water. 10 μ L of sample or standard was added to 300 μ L working solution. Serial dilutions of CaCl_2 were used to generate a standard curve. The plate was incubated at room temperature for 10 minutes then read at 575 nm (Bio-Tek Instruments Inc, USA).

2.5 Scanning Electron Microscopy (SEM)

Cells were cultured on coverslips (Thermanox®) ($n = 2$), and fixed in 2% glutaraldehyde (5 min) on 1, 8, and 16 days after gene transfer. They were then dehydrated in graded series of ethanol, and dried in tetramethylsilane (Merck, Germany). Gold was sputtered on the samples just before SEM analysis. The specimens were examined with a JEOL 6310 Scanning Electron Microscope.

2.6 Real-time Quantitative PCR

At day 1, 4, 8, and 16 after gene transfer, total RNA of every sample was isolated with the RNEasy kit (Qiagen, Venlo, The Netherlands). Quantity and quality of the RNA was measured with the spectrophotometer (SmartSpecs® Plus, Biorad Laboratories, Veenendaal, The Netherlands). 1 μ g RNA was used to make cDNA with the RT-PCR method (Invitrogen, Breda, The Netherlands). To perform quantitative PCR, a SYBR® Green PCR kit was used (Eurogentec, Liege, Belgium), and reactions were controlled in a spectrofluorimetric thermal

iCycler® (Biorad, Richmond, CA, USA). The odontogenic differentiation was monitored by six markers: alkaline phosphatase (ALP), osteocalcin (OC), collagen type I (Col I), bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), and dentin matrix protein-1 (DMP-1). GAPDH was used as housekeeping gene to normalize RNA expression (Table 1).

DNA was PCR-amplified under the following conditions: 2 minutes at 50 °C and 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C, with data collection in the last 30 seconds. After the real-time PCR run, a Ct-value showed how many PCR cycles were necessary to obtain a certain level of fluorescence. Amplification efficiency of different genes was determined relative to GAPDH as an internal control ($\Delta C_t = C_{t_{\text{gene}}} - C_{t_{\text{GAPDH}}}$). mRNA in each sample was calculated by a comparative $\Delta\Delta C_t$ ($\Delta C_{t_{\text{gene}}} - \Delta C_{t_{\text{control}}}$) value method. The fold change in gene expression relative to the control was calculated by $2^{-\Delta\Delta C_t}$.²⁰ Each sample was assessed in triplicate.

Table 1: Rat-specific primer sequences used for real-time PCR

	Sense primer	Antisense primer
<i>Alp</i>	5'-GCTTCACGGCATCCATGAG-3'	5'-GAGGCATACGCCATGACGT-3
<i>OC</i>	5'-GGCTTCCAGGACGCCTACA-3	5'-CATGCCCTAAACGGTGGTG-3'
<i>Col I</i>	5'-TGGAATCTTGATGGTTTGGA-3'	5'-GCTGTAAACGTGGAAGCAAGG-3'
<i>Dspp</i>	5'-CGGTCCCTCAGTTAGTC-3'	5'-TACGTCCTCGCGTTCT-3'
<i>Dmp1</i>	5'-CGTTCCTCTGGGGGCTGTCC-3'	5'-CCGGGATCATCGCTCTGCATC-3'
<i>Bsp</i>	5'-ACTTCCCTTCGCAAGCTTAGG-3'	5'-AAACTTCCCGCGTATGTTGG-3'
<i>Gapdh</i>	5'-GAAGGGCTCATGACCACAGT-3'	5'-GGATGCAGGGATGATGTTCT-3

2.7 Oligo microarray

RNA samples of day 0, and 8 were analyzed with the Oligo GEArray Rat osteogenesis microarray (Cat. No. ORN-026; SuperArray, Bioscience Corporation, Frederick, USA). According to the instructions by the manufacturer, an annealing mix was made with 1 µg RNA to synthesize cDNA. The Amplification Master mix was then added to the cDNA synthesis mix and incubated overnight at 37°C to produce cRNA, respectively. After prehybridizing the array membrane with the GEAhyb Hybridisation solution, 10 µg of cRNA in 750 µl Hybridisation buffer was added in the membrane to allow hybridisation overnight at

60°C. Afterward, the membrane was blocked for 40 minutes by GEAblocking solution. Subsequently, 2 ml AP-streptavidin (1:7500) was added to the membrane and incubated for 10 minutes. Finally, the membrane was incubated with 1 ml of CDP-star chemiluminescence substrate at room temperature for 5 minutes. Then, the membrane was exposed to a X-ray film for detection. The images were analyzed by the GEArray Expression Analysis Suite (GEASuite).

2.8 Statistical Analysis

Statistical significance in this study was evaluated using paired-t testing and $P < 0.05$ was considered significant. Error bars represent means $\pm$ standard deviation (SD).

3. RESULTS

3.1 Gene Transfer and Cell Proliferation

The test for MOI showed that cells could be transfected with BMP-2 adenovirus. After 48 hours, the transfected cells secreted 4.3, 5.0, 34.5, and 101.9 ng/ml hBMP-2 at MOI 0, 10, 50, and 100 respectively. Therefore, in the main study, cells were infected with AdBMP-2 at a MOI of 100. After gene transfer, both populations showed exponential growth for 24 days. Cells grew more rapidly in the non-transfected group than in the transfected group ($P < 0.05$) (Figure 1 A).

3.2 ALP Activity and Calcium Content

The transfected cells showed increasing ALP activity until day 16. At day 24, the ALP activity decreased. The transfected cells showed significantly higher levels of ALP activity compared to non-transfected cells ($P < 0.01$) (Figure 1 B). Finally, significantly higher levels of Ca were observed at day 12, 16 and 24 for the transfected cells ($P < 0.01$) (Figure 1 C).

Figure 1: Growth characteristics of transfected and non-transfected cells cultured in non-osteogenic medium. (A) The Proliferation characteristics (B) ALP activity, and (C) Calcium content. The bars represent the means $\pm$ SD ($n=6$). * $P<0.05$, ** $P<0.01$.

3.3 SEM Analysis

One day after transfection, SEM analyses showed that all cells had spread well on the surface of the coverslips. Mostly all cells had multiple filopodia, and nuclei and nucleoli could be observed clearly. There were no differences between the two groups (Figure 2 A, B). Although no external source of phosphate (no β -glycerophosphate) was added to the cultures,

Figure 2: Scanning electron micrographs of two cell populations. (A) the transfected cells on day 1; (B) the non-transfected cells on day 1; (C) the transfected cells on day 16; (D) the non-transfected cells on day 16

still at day 16, a noticeable amount of mineralized extracellular matrix was observed in the cultures of transfected cells (Figure 2 C). In contrast, the control cells only formed a confluent layer without any obvious sign of mineralization (Figure 2 D).

3.4 Real-Time Quantitative PCR

Expression levels of mRNA for *Alp*, *ColI*, *OC*, *Bsp*, *Dspp*, and *Dmp1* were compared on day 1, 4, 8 and 16. mRNA expression levels of cells before gene transfer (day 0), were set as baseline control (relative expression value, 100%). Results showed that *Alp* was gradually up-regulated in transfected cells, about 0.46-, 2.1-, 7.8-, and 2.9-fold on day 1, 4, 8, and 16. In contrast, non-transfected cells showed no up- or down-regulation of *Alp* at these days (Figure 3 A). *Col I* showed up-regulation for the transfected cells only at day 4 (3-fold) (Figure 3 B). *OC* showed some up-regulation at day 8 (7.4-fold), and 16 (24-fold) in transfected cells, and minimal increase in non-transfected cells (Figure 3 C). *Bsp* also revealed upregulation of transfected cells at day 4 (2.1-fold), 8 (3.9-fold), and 16 (2.7-fold) (Figure 3D). The expression of *Dspp* and *Dmp1*, two dentin specific markers, showed almost similar profiles. They were down-regulated on day 1 (0.19- and 0.26-fold). At day 4, *Dmp1* showed upregulation in transfected cells, while *Dspp* showed no differences. At day 16, both genes, *Dspp* and *Dmp1*, were up-regulated, about 4.6- and 2.4-fold (Figure 3 E,F).

Figure 3: Representative curves of the mRNA levels of *Alp* (A); collagen type I (B); *OC* (C); *Bsp* (D); *Dspp* (E); *Dmp1* (F) quantified during 16 days. Cells were from two cell fractions.

3.5 Gene Expression Profiles in Oligo-Microarray

The Oligo GEArray Rat osteogenesis microarray profiled the expression of 113 key genes involved in osteogenic differentiation. This system was used to compare the multiple gene expression profiles representative of the transfected cells *vs.* non-transfected cells at day 8, compared to the control cells at day 0. Table 2 showed the up- or down-regulation of the different functional genes. Only a 2-fold change (or more) in signal density was defined to be a relevant change in gene expression level. The number of functional genes in that was upregulated in the transfected cells compared to non-transfected cells, was 36 (Table 3). For the downregulated genes, this number was 15. Several genes, belonging to the categories of transcription factors and regulators, growth factors, extracellular matrix, and cell proliferation,

i.e. *Twist2*, *Csf3*, *Fgf3*, *Col5a1*, *Itga2*, *Smad4*, and *Vcam1*, were down-regulated in the transfected cells. The results showed that genes involved in skeletal development, bone mineral metabolism, cell growth and differentiation, ECM molecules, cell adhesion molecules, and transcription factors and regulators were more up-regulated in transfected cells compared to control and non-transfected cells (Table 2). Especially, *Bglap2* (Bone gamma-carboxyglutamate protein 2), *Bmpr1a* (Bone morphogenetic protein receptor, type 1A), *Ib*, and 2, *Biglycan*, *Anxa5*, *Bmp1* to 7, *Col4a1*, *Matrix Gla protein*, *Msx2* (Msh homeo box homolog 2), and *Ameloblastin* showed high upregulation in transfected cells compared to non-transfected cells (Table 3).

TABLE 2. FUNCTIONAL GENE EXPRESSION IN TRANSFECTED AND NON-TRANSFECTED CELLS ON DAY 8, COMPARED WITH CONTROL CELLS ON DAY 0

Major category	Subcategory	Transfected		Non-transfected	
		Up	Down	Up	Down
Skeletal development	Bone mineralization	7			1
	Cartilage condensation	4		2	1
	Ossification	9		2	
	Osteoclast differentiation	1		1	1
	Other genes involved	7		2	1
Bone mineral metabolism	Calcium ion binding and homeostasis	9		3	3
	Phosphate transport	5	1		3
Cell growth and differentiation	Regulation of the cell cycle	5	1	5	
	Cell proliferation	8	1	5	
	Growth factor and receptors	21	2	7	4
	Cell differentiation	7	1	3	
Extracellular matrix (ECM) molecules	Basement membrane constituents	2			1
	Collagens	1	1		1
	ECM protease inhibitors	2			
	ECM proteases	5	1	3	1
	Structural constituents of tooth enamel	2		1	
	Other ECM molecules	24	3	7	4
Cell adhesion molecules	Cell-cell adhesion			1	
	Cell-matrix adhesion	1	1		
	Other cell adhesion molecules	5	1		2
Transcription factors and regulators	Transcription factors and regulators	7	2	4	

4. DISCUSSION

Gene therapy has shown potential as a strategy for dental tissue engineering. In previous studies, *Bmp7* and *Bmp11*, two members of BMP family, have been applied for *in vitro* gene therapy, and resulted in reparative dentin formation *in vivo*.^{21,22} However, no study so far investigated the differentiation efficacy of *Bmp2* gene transfected dental pulp stem cells. Our

TABLE 3. UPREGULATION-EXPRESSION GENES IN TRANSFECTED CELLS

<i>Ratio</i>	<i>Symbol</i>	<i>GenBank</i>	<i>Unigene</i>	<i>Description</i>	<i>Functional Category</i>
23.2	<i>Bglap2</i>	NM_013414	9722	Bone gamma-carboxyglutamate protein 2	Bone mineralization, Calcium ion binding and homeostasis, Osteoclast differentiation, Cell adhesion
18.19	<i>Bmpr1a</i>	NM_030849	88925	Bone morphogenetic protein receptor, type 1A	Receptor, Extracellular matrix
10.94	<i>Bmpr1b</i>	XM_227759	124805	Bone morphogenetic protein receptor, type 1B	Cartilage condensation, Receptor
10.47	<i>Bgn</i>	NM_017087	783	Biglycan	Extracellular matrix
9.35	<i>Anxa5</i>	NM_013132	3318	Annexin A5	Calcium ion binding and homeostasis
7.88	<i>Bmp1</i>	XM_573814	9305	Bone morphogenetic protein 1	Calcium ion binding and homeostasis, Growth factor, ECM protease
7.4	<i>Bmp7</i>	XM_342591	18030	Bone morphogenetic protein 7	Skeletal development, Phosphate transport, Growth factor, Extracellular matrix
5.9	<i>Col4a1</i>	XM_214400	53801	Procollagen, type IV, alpha 1	Phosphate transport, Collagen, Basement membrane constituent
4.14	<i>Map</i>	NM_012862	2379	Matrix Gla protein	Bone mineralization, Ossification, Calcium ion binding and homeostasis
3.89	<i>Mx2</i>	NM_012982	10414	Msh homeo box homolog 2 (Drosophila)	Transcription factors and regulator
3.44	<i>Amba</i>	NM_012900	11370	Ameloblastin	Bone mineralization, Ossification, Structural constituent of tooth enamel
3.4	<i>Bmp3</i>	NM_017105	53974	Bone morphogenetic protein 3	Growth factor
3.3	<i>Bmp2</i>	NM_017178	90931	Bone morphogenetic protein 2	Skeletal development, Growth factor, Cell differentiation, Extracellular matrix
3.29	<i>Madh7</i>	NM_030858	29980	MAD homolog 7 (Drosophila)	Transcription factors and regulator
3.02	<i>Cd36</i>	XM_216076	102418	Cd36 antigen	Cell adhesion
2.88	<i>Bmpr2</i>	XM_217409	59276	Bone morphogenetic protein receptor, type II	Receptor, Extracellular matrix
2.83	<i>Spp1</i>	NM_012881	8871	Secreted phosphoprotein 1	Bone mineralization, Ossification, Growth factor, Cell proliferation and differentiation, Extracellular matrix, Cell-matrix adhesion
2.81	<i>Ahsa</i>	NM_012898	32083	Alpha-2-HS-glycoprotein	Bone mineralization, ECM protease inhibitor
2.72	<i>Bmp6</i>	NM_013107	40476	Bone morphogenetic protein 6	Skeletal development, Growth factor, Cell differentiation, Extracellular matrix
2.7	<i>Col2a1</i>	XM_243912	11218	Procollagen, type XII, alpha 1	Phosphate transport, Extracellular matrix, Cell adhesion
2.7	<i>Bmp4</i>	NM_012827	10318	Bone morphogenetic protein 4	Skeletal development, Growth factor, Cell differentiation, Extracellular matrix
2.66	<i>Sparc</i>	NM_012656	98989	Secreted acidic cysteine rich glycoprotein	Calcium ion binding and Homeostasis, Basement membrane constituent
2.63	<i>Igf2</i>	NM_031511	118681	Insulin-like growth factor 2	Growth factor
2.6	<i>Fgf2</i>	XM_341940	12732	Fibroblast growth factor receptor 2	Bone mineralization, Cell proliferation, Extracellular matrix
2.6	<i>Smad2</i>	NM_019191	2755	MAD homolog 2 (Drosophila)	Cell differentiation, Transcription factors and regulator
2.58	<i>Smad3</i>	NM_013095	10636	MAD homolog 3 (Drosophila)	Cell proliferation, Transcription factors and regulator
2.49	<i>Cabr</i>	NM_053816	10062	Calcitonin receptor	Calcium ion binding and Homeostasis, Extracellular matrix
2.46	<i>Smad1</i>	NM_013130	10635	MAD homolog 1 (Drosophila)	Bone mineralization, Transcription factors and regulator
2.45	<i>Col9a1</i>	XM_223124	90726	Procollagen, type IX, alpha 1	Phosphate transport, Extracellular matrix, Cell adhesion
2.43	<i>Alpl</i>	NM_013059	82764	Alkaline phosphatase, tissue-nonspecific	Ossification
2.31	<i>Ibsp</i>	NM_012587	9721	Integrin binding sialoprotein	Ossification, Extracellular matrix, Cell adhesion
2.31	<i>Bmp5</i>	XM_236415	7994	Bone morphogenetic protein 5	Skeletal development, Phosphate transport, Growth factor, Extracellular matrix
2.21	<i>Dspg</i>	NM_012790	21392	Dentin sialophosphoprotein	Ossification, Extracellular matrix
2.16	<i>Mmp10</i>	NM_133514	9946	Matrix metalloproteinase 10	ECM protease
2.1	<i>Dmp1</i>	NM_203493	19340	Dentin matrix protein 1	Ossification, Extracellular matrix
2.04	<i>Fgfr3</i>	NM_053429	23671	Fibroblast growth factor receptor 3	Cell differentiation, Extracellular matrix

current study showed that STRO-1 selected rat DPSCs could be transfected with AdBMP-2, and that the transfected cells secreted human BMP-2 in high levels (≈ 102 ng/ml) after 48 hours. Evidently, BMP-2 accumulated in this study to a functional concentration to induce cell differentiation *in vitro*.^{14,23,24} Although, only the BMP-2 protein expression at 48 hours was tested, other studies showed that the adenovirus had also the ability to mediate high levels of gene expression in cells for incubation times of more than 3 weeks.²⁵

Previous studies indicated that an adenovirus dose of 10^7 particle units or an MOI of over 50 can provoke a cytotoxic response to the infected cells.^{26,27} In our study, the transfected cells showed an exponential growth, although there was a small delay for cell proliferation in the early phase. This delay can be due to a hampering effect of the transfection, or due to the inverse relationship between proliferation and differentiation. When cells are more proliferative then the differentiation of the cells is declined, and vice versa.²⁸

ALP is a prerequisite for the differentiation of dental pulp *in vivo*. In our study, transfected DPSCs showed significantly higher ALP activity rates compared to non-transfected cells, which was analogue to cells under the influence of a standard osteogenic medium.⁴ The real-time PCR results confirmed the up-regulation of ALP expression. However, the expression of mRNA is always prior to protein expression, and thus it is well explainable that mRNA expression of ALP reached a peak on day 8, whereas ALP activity in the biochemical assay showed a peak value at day 16. Both calcium content and SEM observation demonstrated more calcified matrix deposition for transfected cells. The transfected cells showed the ability to form multilayers and mineralized nodules on a smooth surface *in vitro*, even without the addition of an external source of phosphate.

Collagen type I is the predominant protein and the basis for dentin repair. Some noncollagenous proteins in dentin or bone include OC and BSP. Besides, DSPP is the major part of noncollagenous proteins in dentin, and plays a crucial role during dentin

mineralization.²⁹ DMP-1 is another specific protein in dentin, and is a candidate gene for dentinogenesis imperfecta.³⁰ Although the different genes showed different mRNA expression profiles, their expression was more or less increased after AdBMP-2 transfection compared to non-transfected cells. This change was comparable to that of DPSCs under osteogenic culture or treated with rhBMP-2 protein.^{2,4,14} The expression of these markers offers strong evidence that DPSCs differentiate into odontoblasts by the presence of BMP-2 protein originated from the adenoviral-mediated BMP-2 transfer.

In previous studies, representative gene expression profiles and functional classifications were compared between human DPSCs and mesenchymal stem cells from bone marrow (MSCs) by using a cDNA microarray system containing over 4000 or 12814 human genes.^{31,32} Human DPSCs and MSCs were found to have similar levels of gene expression for more than 4000 known genes. A few genes, including Collagen type XVIII $\alpha 1$, insulin-like growth factor-2 (*Igf2*), discordin domain tyrosine kinase 2, and cyclin-dependent kinase 6, were more highly expressed in DPSCs.³¹ Further, the studies revealed that the expression levels for several genes, which belong to cell division, cell signal, cell structure, and metabolism, are regulated differently between both cell types, upon prolonged culturing.³² In our present study, the results of the functional oligo-microarray for rat osteogenesis showed that the transfected cells contained higher expression levels in functional genes. These genes were divided into several functional categories, including skeletal development, bone mineral metabolism, cell growth and differentiation, extracellular matrix (ECM) molecules, cell adhesion molecules, and transcription factors and regulators. The high expression levels of *Alp*, *Dspp* and *Dmp1* in the transfected cells confirmed the PCR and ALP activity results of our study. Furthermore, BMP-2, -4, -6, and -7, which are strong promoters for osteogenesis, mineralization formation, and tooth morphogenesis,^{17,33} showed markedly increased expression levels compared to the non-transfected cells. The higher expression levels for the

BMP receptors (*Bmpr1a*, *Bmpr1b* and *Bmpr2*) in transfected cells are probably a logical result of the increase in secreted BMP-2. Further, the increase in protein expression of BMP-2 results in signals from the plasma membrane to the nucleus requiring several SMAD proteins.³⁴ This could explain the upregulation for *Smad 1*, *2*, *3*, and *7* in the transfected cells. In the present study, also *Msx2* was upregulated after transfection. This is noticeable since Shi *et al.* described that *Msx2* was expressed at a low levels by both DPSCs and BMSCs.³⁵ The BMP-Msx pathway mediates reciprocal interactions between the epithelium and mesenchyme during early bone and tooth development. BMP-4 is known to induce *Msx2* expression in cultured dental mesenchyme.³⁶

Two final genes that were interestingly upregulated were *Bglap2* and *Ambn*. First, *Bglap2*, encoding a highly conserved protein associated with mineralized bone matrix and secreted by calcified tissues, was highly expressed in transfected cells. Second, *Ambn* encodes ameloblastin, that acts as a structural component of tooth enamel matrix.³⁵ This gene manifested up-regulation only in transfected cells. Generally, more active odontogenesis-related genes were undoubtedly found in the transfected cells, meaning that these cells were more directed towards the odontogenic pathway.

In summary, rat STRO-1 selected dental pulp stem cells were successfully transfected with AdBMP-2. Transfected cells underwent effective induction towards the odontoblast phenotype compared to non-transfected cells, even in medium without externally added substitutes (dexamethasone, β -glycerophosphate). The results of this study were in accordance with our hypothesis, and the here established combination of more homogeneous cell population with gene transfection could be a strategy to improve the clinical efficacy of DPSCs for hard tissue engineering. To prove that presence of BMP is a denominator for odontogenic differentiation, and thus to reconcile previous discordant reports, animal studies

are necessary. Therefore, further evaluation should be performed in three-dimensional scaffolds *in vitro* as well as *in vivo*.

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Chapter 6

Hard Tissue Formation by BMP2-transfected Pulp Stem Cells

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1. INTRODUCTION

It is generally assumed that dental pulp stem cells (DPSCs) are the optimal candidate for dental tissue regeneration.¹⁻⁴ However, various studies as performed in our laboratory showed that obvious hard tissue formation in subcutaneously installed synthetic scaffolds loaded with DPSCs did not occur.^{5,6} This observation means a limitation of the application of DPSCs for clinical purposes, and implies the necessity to optimize the applied stem cell technology. In view of this we have to notice that earlier studies indicated that a more homogeneous strain of DPSCs can be isolated by immunoselection using the mesenchymal stem cell marker STRO-1.^{4,7-9} STRO-1 selected cells possess enhanced ability to form mineralized extracellular matrix (ECM), as well as for development towards other differentiation pathways.^{4,9-11}

An additional strategy to enhance the efficacy of stem cell application can be to transfect the cells with certain genes. Especially the gene for bone morphogenetic protein 2 (*Bmp2*) seems relevant, as *in vivo* studies have already shown the BMP-2 protein to effectively promote hard tissue formation by DPSCs and bone cells.¹²⁻¹⁴ Gene therapy abolishes the need of using external stimulating growth factors or hormones like dexamethasone.¹⁵ Adenovirus, well known as gene vector, have already been applied in clinically relevant gene therapy.¹⁶ An adenoviral-mediated human *Bmp2* gene has been transfected into STRO-1 selected rat DPSCs, and also showed its ability to effectively promote odontogenic action.¹⁷ Thus, as an optimized approach, the combined application of cell selection with gene therapy may be most attractive for dental pulp cell-based tissue engineering.

Therefore, in this study, STRO-1-selected DPSCs transfected with *Bmp2* gene were loaded onto ceramic scaffolds. After subcutaneous implantation, hard tissue formation was evaluated. We hypothesized that only *Bmp2*-transfected cells have hard tissue formation capacity in this *in vivo* model, when no other stimulating culture supplements are used.

2. MATERIALS & METHODS

2.1 Cell and Gene Transfer

Rat DPSCs were selected on STRO-1 as described before.⁹ The selected cells were expanded to the 10th passage in minimal essential medium- α (α -MEM; GIBCO-BRL, Breda, The Netherlands) with 10% fetal calf serum (FCS; GIBCO-BRL) and gentamycin (50 μ g/mL; Sigma). STRO-1 positivity after the expansion step was confirmed.

A replication-deficient adenoviral vector carrying the human *Bmp2* gene, AdBMP-2 was used.¹⁷ STRO-1-positive cells were seeded at 3×10^4 /cm² and after 24 hrs, Ad-BMP2 was added at multiplicity of infection of 100 for 4 hrs.

2.2 Cell Seeding

Sintered ceramic discs (Camceram®, CAM Implants BV, Leiden, the Netherlands) were used of diameter 6mm, thickness 3mm, hydroxyapatite/tricalcium phosphate (HA/TCP) ratio 60/40, and volumetric porosity 90%. The discs were sterilized at 121°C for 15 min, and pre-wetted in culture medium overnight.

Two days post transfection, scaffolds were submerged in a suspension of 5.0×10^6 cells/ml while gently rotating at 6 rpm for 3 hrs. For every 6 scaffolds, 1 ml cell suspension was used. Subsequently, the scaffolds were transferred to 24-well plates. Non-transfected cells were used as control.

2.3 In Vitro Study

Cell-scaffold complexes (n=3) were assessed on day 1, 4, and 8 post-seeding on total DNA content, and alkaline phosphatase (ALP) activity as described before.¹⁸ For scanning electron microscopy (SEM, n=2) samples were fixed in 2% glutaraldehyde, dehydrated in ethanol, dried in tetramethylsilane (Merck, Germany), and sputtercoated with gold.

2.4 Implantation Study

Cells were seeded as above, and cultured for 4 days. Eight samples of each group and implantation time were used. Twelve 10-week-old BALB/c immunocompromised nude mice were used (approval DEC2007187). National guidelines for the care of laboratory animals were obeyed. Surgery was performed under general isoflurane anaesthesia. The backs of the mice were washed and disinfected. On each flank two subcutaneous pockets were created. To avoid cross-over effects of the virus matter, each mouse received always 4 implants of the same group (transfected or non-transfected). The skin was closed using staples. Samples were retrieved at 1, 4, and 12 weeks post-implantation.

2.5 Light Microscopical

Half of the samples for each group were fixed in formalin, dehydrated in a series of ethanol, and embedded in methylmethacrylate (MMA). Three thin transversal sections (10µm) of each tissue block were made and stained with methylene blue/basic fuchsin.¹⁹

Image analysis was performed using Leica® Qwin Pro (Leica, Wetzlar, Germany). The areas of the ceramic and hard tissue were detected. The outline of the implant area minus the area of the ceramic was designated as the region of interest (ROI). Then, the ratio of hard tissue to the ROI was calculated (Castano-Izquierdo et al., 2007).²⁰

2.6 Real-time Quantitative PCR

The remaining 4 samples of each group were used for PCR analysis. After retrieval the fibrous tissue capsules and other surrounding tissues were removed. The scaffolds and interstitial tissues were cut into small pieces, and transferred to liquid nitrogen immediately. Total RNA of each sample was prepared with a Qiagen RNEasy kit (QIAGEN, Venlo, The Netherlands). Then, 1 µg of total RNA was used to perform the reverse transcriptase reaction.

The obtained cDNA was used as a template in PCR. The odontogenic differentiation of cells was monitored by analyzing the specific markers osteocalcin (OC), bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), and dentin matrix protein 1 (DMP-1). GAPDH was used as house-keeping gene to normalize RNA expression. The primers were described previously.¹⁷ Real-time PCR was performed using the SYBR Green PCR kit (Eurogentec, Liege, Belgium) and controlled in a spectrofluorimetric thermal iCycler® (BioRad, The Netherlands). After the PCR run, the Ct-value showed how many cycles were necessary to obtain a certain level of fluorescence. Amplification efficiency of different genes was determined relative to GAPDH ($\Delta Ct = Ct \text{ gene} - Ct \text{ GAPDH}$). The mRNA in each sample was calculated by the comparative $\Delta\Delta Ct$ ($\Delta Ct \text{ gene} - \Delta Ct \text{ control}$) method.²¹ Each measurement was assessed in triplicate.

2.7 Statistical Analysis

Statistical significance was evaluated at $p < 0.05$ using one way-ANOVA and Tukey testing.

3. RESULTS

3.1 *In Vitro* Analysis

Significant differences in total DNA content was observed between the two groups; The non-transfected cells increased in number over 8 day period. In contrast, the transfected group showed lower cell numbers for all incubation times, and a decrease in DNA content from day 4 to 8. The ALP activity gradually increased from day 1 to 8 for both groups. However, the transfected group showed significantly higher levels at all incubation times (Figure 1A,B).

SEM observation of the day one specimen showed that cells had adhered and spread on the surfaces of the scaffolds. There were no obvious morphological differences between the two groups. At day 4, as confirmed by additional energy dispersive spectroscopy, a noticeable

amount of mineralized nodules appeared on cell/scaffold complexes in the transfected group only (Figure 2).

Figure 1: (A) The proliferation characteristics; (B) ALP activity. The bars represent the means $\pm$ SD ($n=3$). Transfected compared to the non-transfected at the same implantation period: * $P<0.05$, ** $P<0.01$; transfected on day 4 or 8 compared to day 1: # $P<0.05$, ## $P<0.01$; non-transfected on day 4 or 8 compared to day 1: # $P<0.05$, ## $P<0.01$; transfected on day 8 compared to day 4: Δ $P<0.05$, $\Delta\Delta$ $P<0.01$; non-transfected on day 8 compared to day 4: Δ $P<0.05$, $\Delta\Delta$ $P<0.01$.

Figure 2: Scanning electron micrographs of both groups. (A) transfection group on day 1; (B) non-transfection group on day 1; (C) transfection group on day 4; (D) non-transfection group on day 4. Arrows indicate some of the calcified nodules.

3.2 In Vivo Analysis

All mice appeared to be in good health throughout the test period. After sacrifice, all implants were retrieved successfully. There were no symptoms of inflammation at the implantation sites.

3.3 Light Microscopic Evaluation

Histological observation indicated that all implants were surrounded by a very thin fibrous tissue capsule composed of 4-6 cell layers, closely following the perimeter of the scaffold. Inflammatory cells were only sporadically present in this capsule.

For the 1-week implants, the macro-pores of scaffolds were filled with fibrous tissue. No evident hard tissue formation occurred in both groups (Figure 3A,B). However, at higher magnification, the transfected group showed large amounts of a red-stained granular deposit on the surface of the scaffold pores (Figure 4A).

At 4 weeks of implantation, all implants seeded with transfected DPSCs showed obvious hard tissue formation (Figure 3C). The newly formed hard tissue was stained deep red by the basic fuchsin and resided mainly along the surface of the pores. At higher magnification (Figure 4B,C), distinct concentric *lamellae* of mineralized matter were observed with entrapped osteocyte-like cells, and osteoblast-like cells lying orderly arranged at the pore surface. In addition always a bone-marrow-like hematopoietic tissue was associated with the newly formed hard tissue. The newly formed tissues seemed distributed evenly throughout the scaffold. In the samples loaded with non-transfected DPSCs, no hard tissue formation was found (Figure 3D). Only an orderly arranged thick fibril-like ECM with multiple-layers of cells was seen, organized parallel to the pore surface (Figure 4D).

After 12 weeks of implantation, the transfected group upon visual inspection showed enhanced hard tissue formation compared to the 4-week group (Figure 3E). The non-transfected group still showed only small amounts of hard tissue (Figure 3F).

3.4 Histomorphometry

Both at the 4 and 12-week implantation periods significantly more hard tissue formation had occurred in the transfection group ($17 \pm 4.2\%$ and $33 \pm 7.3\%$ of pore area, respectively)

Figure 3: General overview of the implants of 1, 4, and 12 weeks (methylene blue basic/fuchsin staining) from transfection or non-transfection group. (A) transfected at 1 week; (B) non-transfected at 1 week; (C) transfected at 4 weeks; (D) non-transfected at 4 weeks; (E) transfected at 12 weeks; (F) non-transfected at 12 weeks; Scale bar is 400 μm (original magnification 2.5X).

Figure 4: Histology and morphology of implants at a high magnification (methylene blue basic/fuchsin staining). (A) transfected at 1 week, note that the section showed large amounts of a granular-like mineral deposit around the osteoblast-like cells, as seen at the surface of the macro-pores of the scaffolds (arrow) (original magnification 40x), bar size is 25 μm ; (B) transfected at 4 weeks, (original magnification 20X), bar size is 50 μm ; (C) transfected at 4 weeks, note the orderly array of osteoblast-like cells on the surface of bone-like matrix (arrow) (original magnification 40X), bar size is 25 μm ; (D) non-transfected at 4 weeks, note the multiple-layers of cells orderly arranged along the pore surface (arrow), however lacking bone-like tissue formation (original magnification 40X), bar size is 25 μm .

compared to the non-transfected group (0 and $2 \pm 1.8\%$, respectively). Within the transfected group hard tissue formation increased significantly from the 4 to the 12-week period.

3.5 Real-Time PCR

Strikingly, the expression of the four measured genes always showed similar profiles (Figure 5A-D). Both groups revealed the same expression profiles for the measured genes, with overall expression increasing significantly in time. The transfected group without exception demonstrated significant up-regulation of expression compared to the non-transfected group.

Figure 5: Curves of the mRNA levels of OC (A); BSP (B); DSPP (C); DMP-1 (D); quantified for the *in vivo* specimens. The bars represent the mean $\pm$ SD ($n = 3$). Transfected compared to non-transfected with the same implantation period: * $P < 0.05$, ** $P < 0.01$; transfected at week 4 or 12 compared to week 1: #

$P < 0.05$, ## $P < 0.01$; non-transfected at week 4 or 12 compared to week 1: # $P < 0.05$, ## $P < 0.01$; transfected at week 12 compared to week 4: $\Delta P < 0.05$, $\Delta\Delta P < 0.01$; non-transfected at week 12 compared to week 4: $\Delta P < 0.05$, $\Delta\Delta P < 0.01$.

4. DISCUSSION

In our previous study, the differentiation efficacy of BMP-2 gene transfected DPSCs was approved *in vitro*.¹⁷ The purpose of this study was to evaluate their potential for *in vivo* hard tissue regeneration. In general, Bmp transfection proved to be an extremely effective strategy to promote hard tissue formation. However, the tissue that was formed upon visual inspection did not appear to be dentin, but based on its morphological appearance more resembled bone.

In our study set-up, specifically cell-culturing conditions were chosen without the external addition of dexamethasone, as the biological reaction of the osteogenic supplements may partially mask the benefit of BMP-2 produced by the genetically modified cells.¹⁵ In previous research, *Bmp2* transfected DPSCs underwent effective induction towards the odontoblast phenotype *in vitro*, even in non-osteogenic medium.¹⁷ Results of the *in vitro* part of our current study corroborated these findings. Further, ALP activity showed significantly enhanced rates. These results were analogous to the influence of a standard dexamethasone-containing medium on the growth and differentiation of DPSCs.^{5,6,17}

A porous calcium phosphate ceramic carrier was selected as scaffold material. This material was also used in other studies for hard tissue regeneration, because of its biocompatible character and the ability to support the differentiation of bone and dentin forming cells.^{5,11,22} Also the pre-culture time before implantation was based on our previous experience. There is an inverse relationship between cell proliferation and differentiation.^{20,23} Inadequate pre-implantation culture periods result in very limited amounts of hard tissue formation after implantation, whereas 4 days of odontogenic stimulation drives DPSCs to the preferable stage, with an optimal balance between proliferation and differentiation.¹¹ A final

technical remark regarding our study design is that no full randomization was applied for the *in vivo* implantation. Adenoviral-transfected samples might influence neighboring samples placed in the subcutaneous area of a small animal like the mouse. Thus, only one pre-treatment group per mouse was used to avoid cross-over effects. Still, multiple mice were used for each group, without noticeable intra individual differences in reaction.

For our mRNA expression analyses *OC*, *BSP*, *DSPP*, and *DMP-1* were chosen as markers for the odontogenic phenotype. OC and BSP are usually regarded late markers for osteoblast differentiation.²⁴ DSPP is the major dentinal non-collagenous protein and essential for dentin mineralization.²⁵ DMP-1 is also specific for dentin and is mostly known as candidate gene for *dentinogenesis imperfecta*.²⁶ Previously it was shown that upon subcutaneous implantation of DPSCs all these four genes were found highly expressed, however without tissue mineralization actually occurring.^{5,6} The expression levels could be increased by adding BMP-2 protein, and dexamethasone in the cell culture phase, which upon implantation elicited $36 \pm 9\%$ of newly formed hard tissue in the pore area.¹¹ This is statistically comparable to our current result with the transfected cells ($33 \pm 7\%$ of pore area).

The expression of DSPP suggests a distinct developmental functionality *in vivo*.²⁷ Previous literature has shown that DPSCs-mixed with HA/TCP powder can regenerate a dentin-pulp-like structure in ectopic implantation sites. DPSC transplants are characterized by a well-defined layer of aligned odontoblast-like cells, with their processes oriented in the same direction and extending into tubular structures within newly generated dentin.^{1,2} However, in the current study only a bone-like hard tissue formation was observed, with distinct concentric lamellae, osteocyte-like cells, and bone marrow-like hematopoietic tissue, although *Dspp* and *Dmp1* were upregulated. The absence of dentin-like features in favor of a bone-like morphology is striking, since the loaded cells are derived from dental pulp, a tissue which *in situ* forms dentin only. This can be result of the inductive supplements as used. In dental

tissue engineering research the “odontogenic” medium formulations are chosen similar to the osteogenic supplements known to promote osteoblast differentiation from bonemarrow. Hence, neither dexamethasone nor BMP-2 are strictly to be defined as “odontogenic” factors. A second explanation for the absence of dentin morphological features can be the consequence of the micro-morphology and/or chemical characteristics of the used HA/TCP ceramic scaffold.⁵ Improved scaffold designs in composition as well as morphology can facilitate the formation of more natural looking dentinal tissue constructs.²⁸

5. CONCLUSIONS

STRO-1-selected dental pulp stem cells can be genetically modified with the hBMP-2 gene. Compared with non-transfected cells, more effective induction towards the odontoblast phenotype, and abundant hard tissue after subcutaneous implantation is achieved, even without supplements like dexamethasone. The combination of homogeneously STRO-1 selected DPSCs with *Bmp* transfection shows great potential for therapeutic application. However, a remaining challenge is how to obtain a more biomimetic dentin-like appearance.

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Chapter 7

Non-Viral Bone Morphogenetic Protein 2 Transfection of Rat Dental Pulp Stem Cells Using Calcium Phosphate Nanoparticles as Carriers

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1. INTRODUCTION

In tissue engineering, gene therapy is used as a means of delivering genes locally to somatic cells encoding for proteins like growth factors, transcription factors, or extracellular matrix molecules with a resulting therapeutic effect. The applied genes can stimulate or induce a natural biological process by expressing bioactive molecules that are involved in the regenerative response of the tissue.¹ Gene therapy can be a valuable tool to avoid the limitations of local delivery of growth factors, including short half-life, large dose requirement, high cost, a need of repeated applications, and poor distribution.² In our previous study, an adenoviral- mediated human *Bmp2* gene has been transfected into STRO-1 selected rat dental pulp stem cells (DPSCs), and showed its ability to effectively promote odontogenic action.³ However, the clinical application of an adenovirus is not advisable as it results in endogenous recombination, oncogenic effects, and toxic immunological reactions leading to potential serious complications.^{4,5} For this reason, a number of non-viral vectors have been explored. Two types of classic non-viral vectors are liposomal formulations, and cationic polymers. Unfortunately it has been observed that, in addition to cytotoxicity, these vectors do not always lead to satisfactory efficacy of gene expression in the cells. The reasons may be due to low endosomal escape, no protection of DNA from nuclease degradation, and inefficient nuclear uptake.⁶⁻⁸

The application of inorganic nanoparticles with entrapped DNA is a new field for non-viral transfection, and is expected to overcome the limitations mentioned above. The advantages of these vectors are the ease of manufacture, low costs, stability, low immunogenicity, and low risk for the plasmid to be inserted into the host cell genome. In literature, plasmid DNA (pDNA)-loaded calcium phosphate nanoparticles have already been demonstrated to be highly efficient for transfection in the HeLa cell line.^{9,10}

The osteoinductive potential of recombinant human BMP2 has already been confirmed in a clinical study.¹¹ In our study recombinant human BMP-2 gene was used for the transfection because it can stimulate differentiation of adult dental pulp cells into odontoblasts, and it also plays a role during the various stages of the formation of hard skeletal and dental tissue in bone and teeth.¹²⁻¹⁴ Furthermore, for our dental tissue engineering purposes, dental pulp-derived adult stem cells (DPSCs) were used as these responding stem cells in nature. A hierarchy of DPSCs causes the variable behavior of these cells *in vitro*, and results in a low therapeutic potential *in vivo*.^{15,16} STRO-1⁺ DPSCs were chosen because selection with this marker increased the ability of osteo/odontogenic differentiation, as well as development towards other differentiation pathways.^{3,17,18}

In this study, we intended to transfect STRO-1 selected rat DPSCs with pDNA entrapped into calcium phosphate nanoparticles. We hypothesized that calcium phosphate nanoparticles would result in a relatively high transfection efficacy, and that the secreted BMP-2 would be effective in odonto-inductive action.

2. MATERIALS & METHODS

2.1 Preparation of Plasmid DNA Loaded Calcium Phosphate Nanoparticles

A recombinant green fluorescent eukaryotic cell expressing plasmid carrying human *Bmp2* gene (pEGFP-BMP2), was used in the current study. The preparation of pDNA-loaded calcium phosphate nanoparticles followed the protocol of Bisht *et al.*¹⁰ The basic method was as follows: 0.1 M AOT (Surfactant, Aerosol OT, i.e. bis (2-ethylhexyl) sulposuccinate) solution in hexane (Sigma) was prepared. In 25 ml of AOT in hexane, 70 µl aqueous solution of 1.36 M calcium chloride and aqueous solution of 2.94 µg of pDNA, were added by continuous stirring overnight to form a micro-emulsion “A”. In another 25 ml of AOT solution, 50 µl of 0.2 M Tris–HCl buffer (pH 7.4), 70 µl aqueous solution of 0.35 M Na₂HPO₄

and an aqueous solution of 2.94 μg of pDNA, were dissolved by continuous stirring overnight to form micro-emulsion “B”. Before stirring both micro-emulsions, excess water was added to make the total volume of water to 450 μl to adjust w_o (the molar ratio of water to AOT) at 10. Then, micro-emulsion “B” was added to “A” at an extremely slow rate (5 ml per hour) with continuous stirring at 4 $^{\circ}\text{C}$, and was kept in the same condition overnight. Subsequently, hexane was carefully removed from the resulting solution after centrifuging 30 min at 5000 rpm at 4 $^{\circ}\text{C}$. The sediment was dissolved in 10 ml of absolute ethanol by vortexing, followed again by centrifugation. After three of such cycles, the pelleted nanoparticles were dispersed in Milli-Q water at 4 $^{\circ}\text{C}$ by vortexing. Finally, the dispersed nanoparticles were lyophilized to dry powder (yield $\sim$ 1 mg).

2.2. Morphology and Characteristics of the Nanoparticles

2.2.1. TEM

To evaluate the size of the nanoparticles, one drop of aqueous dispersion of the particles was put on a formvar coated copper grid and air dried at room temperature. The dried grid was examined under a JEOL 1101 transmission electron microscope (TEM).

2.2.2. Gel electrophoresis

In order to check the protection of DNA in the nanoparticles from external Dnase degradation, 20 μl aqueous dispersion of calcium phosphate/DNA nanoparticles (containing 1 μg pDNA) prepared by the above method was incubated with 5 μl of DNaseI (5 mg/ml) at 37 $^{\circ}\text{C}$ for different time intervals (0, 0.5 and 1h), and then loaded in a 1% agarose gel. As positive and negative controls, 10 μl free pDNA was either loaded alone or after incubation with DNaseI.

2.2.3. Entrapment Efficiency

The entrapment efficiency of DNA attached to the nanoparticles was evaluated by dissolving the particles in acidic buffer (pH 3.0) and incubating overnight. The amount of pDNA released from the nanoparticles ($[pDNA]_r$) was estimated spectrophotometrically by measuring the OD at $\lambda = 260$ nm. The entrapment efficiency was then calculated from the amount of DNA originally added in the solution ($[pDNA]_0$), using the equation $E\% = [pDNA]_r/[pDNA]_0 \times 100$.

2.3. In Vitro Transfection Studies

2.3.1 Cell Culture

Rat DPSCs (10th passage) were selected on the stem cell marker STRO-1 by FACS as described before,¹⁷ and expanded in minimal essential medium- α (α -MEM; GIBCO-BRL, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS; GIBCO-BRL) and gentamycin (50 μ g/mL; Sigma).

2.3.2. Determination of Transfection Efficiency

The cells were seeded in 24-well plate at a density of 2×10^4 cells in 1 ml of culture medium per well. Upon reaching 80% confluency, cells were used for transfection. Then, 0.3 ml of pEGFP-BMP2-loaded calcium phosphate nanoparticles in α -MEM without FCS and antibiotics (total amount of pDNA $\sim 0.5 \mu$ g) was added in each well. After incubation at 37°C for 4 hrs, the transfection complex was replaced with fresh α -MEM containing 10% FCS and antibiotics. Forty-eight hrs post-transfection, cells were detached to detect green fluorescence with fluorescence microscopy to calculate the temporary transfection rate. Simultaneously, the supernatants were collected on day 2, 4, 6, 8, and 10 after transfection. These samples were used to measure the concentration of recombinant human BMP-2 by using a BMP-2

ELISA kit (Quantikine®, R&D System, Inc. Minneapolis, MN) ($n = 6$). Also, at day 1, 4, 8, and 16 after transfection the proliferation rates of the cells, as measured by the total DNA content ($n = 6$), were assessed with a PicoGreen dsDNA quantification kit (Molecular Probes, Leiden, The Netherlands).¹⁹ The commercial transfection reagent Lipofectamine™ 2000 (Invitrogen, Gaithersburg, MD, USA) was used as control.

The bioactivity of hBMP-2 produced from transfected cells was assessed by ALP activity upregulation using the cell line W20-17 as reported previously.²⁰ Briefly, 48 h post-transfection, the supernatants were collected and filtered. Then, these conditioned media were transferred to 80% confluent W20-17 cells ($n = 6$). After additional 48 hrs incubation, a lysate was prepared to measure ALP activity of the W20-17 cells.²⁰

2.4. Three-Dimensional Scaffold Culture

Titanium fibre mesh (Bekenit, SaitamaKen, Japan) was used as a 3-D scaffold. The fibre diameter was 45 μm . The volumetric porosity of the total mesh was 86%, and the weight was 400 g/m^2 . A titanium fibre mesh sheet was cut into discs of 6 mm in diameter and 1 mm in thickness, and then scaffolds were sterilized by autoclaving.

2.4.1. Cell Seeding

All scaffolds were pre-wetted in α -MEM medium with 10% FCS and 50 $\mu\text{g}/\text{ml}$ gentamycin overnight. Forty-eight hrs post- transfection, STRO-1 selected DPSCs were seeded onto the scaffolds by incubating the scaffolds in a suspension of 1.0×10^6 cells/ml while gently rotating at 6 rpm for 3 hrs. For every 6 scaffolds, 1 ml cell suspension was used. Subsequently, the scaffolds were put in 24-well plates for culturing with α -MEM supplemented with 10% FCS, 10 mM sodium β -glycerophosphate, 10^{-8} M dexamethasone, L-ascorbic acid (50 $\mu\text{g}/\text{mL}$), and gentamycin (50 $\mu\text{g}/\text{mL}$). The non-transfected cells were used as control, and cultured in the

same osteogenic medium as above, or in non-osteogenic medium (α -MEM supplemented with 10% FCS and 50 μ g/mL gentamycin.). Culture media were replaced at 3-day intervals.

2.4.2. Cell Proliferation, Alkaline Phosphatase Activity and Calcium Content

On day 1, 4, 8, 16 and 24 post-seeding, the proliferation rates of cells, as measured by total DNA content ($n = 3$), were assessed. The same samples as used above were also used to measure alkaline phosphatase (ALP) activity. Twenty μ L of 0.5 AMP buffer (Sigma) was added to 80 μ L of the samples or standards. Next, 100 μ L of substrate solution was added and the mixtures were incubated at 37 °C for 1 h. ALP activity was measured at 405nm, using an ELISA microplate reader (Bio-Tek Instruments Inc., USA). On day 4, 8, 16 and 24 after seeding, the calcium content of the samples ($n = 3$) was measured by the *o*-cresolphthalein complexone (Sigma) method.¹⁹

2.4.3. Scanning Electron Microscopy (SEM)

On day 1, 8, and 24 after cell seeding, scaffolds were rinsed with filtered PBS, and fixed in 2% glutaraldehyde for 5 min, dehydrated in graded series of ethanol, and dried in tetramethylsilane (Merck, Germany). Gold was sputtered on the samples just before SEM analysis. The specimens were examined with a JEOL 6310 SEM, at an accelerating voltage of 10 kV.

2.4.4. Real-Time Quantitative PCR

On day 1, 4, 8, and 16 after cell seeding, total RNA of every sample was prepared with Qiagen RNEasy kit (RNeasy kit, QIAGEN, Venlo, The Netherlands). Then, 1 μ g of total RNA was used to perform the reverse transcriptase (RT)-reaction. The obtained cDNA was used as a template in PCR. The odontogenic differentiation of cells was monitored by specific

markers, ALP, collagen type I (Col I), osteocalcin (OC), dentin sialophosphoprotein (DSPP) and dentin matrix protein 1 (DMP-1). GAPDH was used as house-keeping gene to normalize RNA expression (Table 1).

Real-time PCR was performed using SYBR Green PCR kit (Eurogentec, Liege, Belgium) and controlled in a spectrofluorimetric thermal iCycler® (BioRad, The Netherlands). After the real-time PCR run, the Ct-value showed how many PCR cycles were necessary to obtain a certain level of fluorescence. Amplification efficiency of different genes was determined relative to GAPDH as an internal control ($\Delta Ct = Ct \text{ gene} - Ct \text{ GAPDH}$). The mRNA in each sample was calculated by comparative $\Delta\Delta Ct$ ($\Delta Ct \text{ gene} - \Delta Ct \text{ control}$) value method. Each sample was assessed in triplicate.

Table 1: Rat specific primers for real-time PCR

	Sense primer	Antisense primer
ALP	5'-GCTTCACGGCATCCATGAG-3'	5'-GAGGCATACGCCATGACGT-3'
OC	5'-GGCTTCCAGGACGCCTACA-3'	5'-CATGCCCTAAACGGTGGTG-3'
Col I	5'-TGGAATCTTGATGGTTTGGA-3'	5'-GCTGTAAACGTGGAAGCAAGG-3'
DSPP	5'-CGGTCCCTCAGTTAGTC-3'	5'-TACGTCCTCGCGTTCT-3'
DMP-1	5'-CGTTCCTCTGGGGGCTGTCC-3'	5'-CCGGGATCATCGCTCTGCATC-3'
GAPDH	5'-GAAGGGCTCATGACCACAGT-3'	5'-GGATGCAGGGATGATGTTCT-3'

2.5. Statistical Analysis

Statistical significance was evaluated using one way-ANOVA with post-hoc Tukey testing and $P < 0.05$ was considered significant. Error bars represent mean $\pm$ standard deviation (SD).

3. RESULTS

3.1. pDNA Loaded Calcium Phosphate Nanoparticles

After continuous stirring overnight, both the micro-emulsion “A” and “B” showed optically clear solutions. In the resulting solution (mixture of “A” and “B”), development of translucency implicated nanoparticle formation in the aqueous core of the micro-emulsion droplets. The TEM investigation revealed the formation of dense particles with a spherical

morphology. The particle size, while well dispersed in water, was in the range of 75-100 nm diameter (Figure 1). The resistance of pDNA-loaded calcium phosphate complexes to external aggressive DNase attack was demonstrated in gel electrophoresis (Figure 2). Although free plasmid DNA could move to the usual position (~ 6 kb) and could also be completely digested by external DNase, the plasmid-loaded complexes had hardly moved and still stayed at the top of the lanes. One mg of pDNA loaded nanoparticles, in which the original amount of applied DNA was 5.88 μ g, was used to evaluate the entrapment efficiency of DNA inside the nanoparticles. After overnight incubation, the entrapment efficiency (E%) was calculated as $97.15\% \pm 2.04\%$ ($n = 4$).

Figure 1: Transmission electron micrograph (TEM) of pDNA encapsulated calcium phosphate nanoparticles.

*Figure 2: Agarose gel (1%) electrophoresis. **Lane 1:** DNA marker. **Lane 2:** free pEGFP-BMP2 DNA (6 kb). **Lane 3:** pEGFP-BMP2 DNA treated with DNase for 0.5 h. **Lane 4:** pEGFP-BMP2 loaded calcium phosphate nanoparticles. **Lane 5:** pEGFP-BMP2 loaded calcium phosphate nanoparticles treated with DNase for 0.5 h. **Lane 6:** pEGFP-BMP2 loaded calcium phosphate nanoparticles treated with DNase for 1h.*

3.2. In Vitro Transfection Studies

The successful transfection could be easily traced by green fluorescence protein (GFP). Forty-eight hrs post-treatment of nanoparticles, the DPSCs showed the expression of green fluorescence, visually demonstrating a similar positive rate to that of cells transfected by LipofectamineTM 2000 (Figure 3). The amount of hBMP-2 secreted by transfected rat DPSCs was quantitated by ELISA (Figure 4 A). With LipofectamineTM 2000 transfection, the cells produced significantly more hBMP-2 on day 2, however on day 8 and 10, the expression of hBMP-2 was significantly lower. The DNA results showed significantly higher cell numbers in the nanoparticle group for all time points, however the subsequent proliferation was similar to the cells transfected with LipofectamineTM 2000 (Figure 4 B). The bioactivity of hBMP-2 was assessed by the ability of conditioned media to upregulate the ALP activity of the BMP-2-sensitive W20-17 cell line (Figure 4 C). The medium from rat DPSCs transfected with nanoparticle indeed demonstrated a significant increase in W20-17 ALP activity relative to the control group of non-transfected cells ($P < 0.01$).

Figure 3: Fluorescent micrographs of GFP expression in pEGFP-BMP2 transfected rat selected DPSCs. (A) calcium phosphate nanoparticles group; (B) LipofectamineTM group, (original magnification 10 X).

3.3. 3-D Scaffold Culture

The cell proliferation rates are depicted in Figure 5 A. Analysis of the data showed that the proliferation of non-transfected rat DPSCs in non-osteogenic medium was significantly faster compared with transfected cells and non-transfected rat DPSCs in osteogenic medium.

Figure 4: (A) ELISA for hBMP-2 concentration in three groups. The bars represent the means $\pm$ SD ($n=6$). The calcium phosphate nanoparticle group (NP) compared to LipofectamineTM group (Lipo): * $P<0.05$, ** $P<0.01$; NP group compared to non-osteogenic group (control): ## $P<0.01$. (B) The proliferation characteristics of two groups. The bars represent the mean $\pm$ SD ($n=6$). NP group compared to lipo group (Lipo): * $P<0.05$, ** $P<0.01$. (C) The bioactivity of hBMP-2 produced from transfected rat DPSCs mediated with nanoparticles 48 h post transfection. Bioactivity was assessed by upregulation of ALP activity in W20-17 cells induced by conditional medium incubated with rat DPSCs. The bars represent the mean $\pm$ SD ($n=6$). NP group compared to non-osteogenic group (control): ** $P<0.01$.

However, no significant differences were found between these latter two groups. The ALP activity of both cell groups cultured in osteogenic medium increased during 24 days, but transfected DPSCs showed significantly higher levels from day 4 compared to non-transfected cells (Figure 5 B). The non-transfected cultured in non-osteogenic medium always demonstrated significantly lower levels of ALP activity ($P < 0.01$), and only started to increase after day 8. The calcium content increased significantly between day 8 and 24 for two cell groups cultured in osteogenic medium ($P<0.01$), but the transfected cells showed

significantly higher levels from day 16 compared to non-transfected cells ($P<0.01$) (Figure 5 C).

Figure 5: (A) The proliferation characteristics; (B) ALP activity; (C) calcium content.

The bars represent the mean $\pm$ SD (n=3). The calcium phosphate nanoparticle group (NP) compared to the group in osteogenic medium (O+) * $P<0.05$, ** $P<0.01$; NP group compared to the group in non-osteogenic medium (O-): # $P<0.05$, ## $P<0.01$.

SEM observation showed that cells already attached and spread on the surface of Ti-fibers after 1 day of seeding, and proliferated along the culture. The rat DPSCs in non-osteogenic culture grew faster compared to the two groups from osteogenic culture, and almost completely filled the Ti-mesh voids on day 8 (Figure 6 A-C). By day 8, the two cultures in osteogenic medium began to produce collagen and to form mineralized nodules. After 24 days of incubation, more numerous and larger mineralized globular accretions always associated with collagen bundles were observed (Figure 6 D,E). In the non-osteogenic cell culture, the formation of a mineralized extracellular matrix was never observed (Figure 6 F).

Expression levels of mRNA for ALP, Col I, OC, DSPP and DMP-1 were compared on day 1, 4, 8 and 16. mRNA expression levels of non-osteogenic cell cultures on day 1, were set as baseline controls (relative expression value, 100%). Results showed that ALP was

significantly increased in transfected cells (Figure 7 A). Col I showed up-regulation for all three groups, and was significant higher in cells cultured in non-osteogenic medium (Figure 7 B). OC showed some up-regulation at two groups cultured in osteogenic medium at day 8 and 16, but the transfected cells was significantly higher compared to non-transfected cells at day 8 (Figure 7 C). The expression of DSPP and DMP-1 revealed significant increase in cells cultured in osteogenic medium at day 8 and 16. However, the transfected cells obviously had a higher expression levels compared to the non-transfected cells (Figure 7 D,E).

Figure 6: Scanning electron micrographs (SEM) of three cell populations on day 8: (A) the transfected DPSCs, (B) the DPSCs in osteogenic medium, (C) the DPSCs in non-osteogenic medium; and on day 24: (D) the transfected DPSCs, (E) the DPSCs in osteogenic medium, (F) the DPSCs in non-osteogenic medium.

Figure 7: Representative curves of the mRNA levels of ALP (A); collagen type I (B); OC (C); DSPP (D); DMP-1 (E); quantified during 16 days. The bars represent the mean $\pm$ SD ($n=3$). The calcium phosphate nanoparticle group (NP) compared to the group in osteogenic medium (O+) * $P<0.05$, ** $P<0.01$; NP group compared to the group in non-osteogenic medium (O-): # $P<0.05$, ## $P<0.01$.

4. DISCUSSION

Effective gene delivery into mammalian somatic cells *in vitro* and *in vivo* is an essential step in gene therapy. One of the factors that limits the transfection efficiency, is the rapid degradation of DNA within the endosomal and lysosomal compartments after endocytosis. In this study, we have demonstrated gene transfection efficiency and odonto-stimulatory effect in STRO-1-selected rat DPSCs through the pEGFP-BMP2 loaded calcium phosphate nanoparticles.

Calcium ions are known to form ionic complexes with the helical phosphates of DNA, and these complexes have easy transportability across the cell membrane *via* ion-channel mediated endocytosis. However, this easy and inexpensive method can not lead to satisfactory transfection efficiency in cells, mainly due to low endosomal escape and no protection from nuclease degradation.²¹ Previous studies have reported the method of pDNA encapsulated in calcium phosphate nanoparticles as gene carriers, and this amelioration has enhanced the transfection efficiency.^{9,10} Literature shows that 5.88µg of pDNA per mg calciumphosphate can be nearly completely loaded in the nanoparticles.⁹ Using the same ratio, we also acquired particles with a size of less than 100 nm. It has been confirmed that particles greater than 200 nm in diameter are not efficiently taken up by endocytosis and thus only *via* phagocytosis.²² One previous study showed that the surface charge of DNA-loaded calcium phosphate nanoparticles was positive in neutral aqueous buffer,⁹ which can result in unmovable nanoparticles in electrophoresis towards the anode. Furthermore, DNA encapsulated calcium phosphate particles was protected for the external DNase environment, which was favorable for the survive of DNA after endocytosis.

The endosomal escape as well as nuclear uptake is the final obstacle for of DNA delivery.⁸ It has been demonstrated that calcium ions play an important role in endosomal escape, cytosolic stability and enhanced nuclear uptake of DNA through nuclear pore complexes.²³

The inorganic nanoparticles are necessary as DNA carriers for entry into the cell. In our case, the DNA doped nanoparticles are non crystalline, in contrast to void calcium phosphate nanoparticles.^{9,10} In the present study, we observed successful gene expression from nanoparticle-mediated cells and the gene products were bioactive. As a control, the LipofectamineTM reagent showed similar transfection efficiency. However, LipofectamineTM-mediated cells produced higher hBMP2 levels at day 2 but less at day 8 and 10, compared to nanoparticle-mediated group. This difference can be the result of different methods for endosomal escape. After endocytosis, lipid-DNA compounds internalize into the endosome and initiate the destabilization of the endosomal membrane for escaping.^{8,24} However, the dissolution of inorganic nanoparticles in the acidic endosomal compartment (pH 5.0-5.5) results in an osmotic disbalance and the consequent disruption of the endosome so that DNA can escape into the cytosol.⁹ Further, compared to the nanoparticle group, the cells transfected with LipofectamineTM showed a lower cell number from day 1 to 8, which can be due to the known cytotoxicity of cationic liposomes.²⁵ Altogether, LipofectamineTM was advantageous for the transfection efficiency at the beginning of transfection (i.e. at day 2), however after that the nanoparticle was superior.

Titanium fiber mesh is a highly biocompatible material, and its non-degradable nature is not necessarily disadvantageous for hard tissue applications. Previous studies already demonstrated that titanium fiber mesh, which has been used for bone regeneration,²⁶ supported the attachment, growth, and differentiation of dental pulp cells *in vivo* and *in vitro*.^{19,27} In the current study, the non-osteogenic DPSCs culture demonstrated a faster proliferation, due to the inverse relationship between proliferation and differentiation, i.e. when cells are more proliferative then the differentiation of the cells is declined, and vice versa.²⁸ It is known that BMP-2 in high concentrations can stimulate the early differentiation of cells and the formation of extracellular matrix, while not influencing proliferation.²⁹

ALP is a prerequisite for the differentiation of dental pulp cells. Compared to previous study,¹⁹ we observed a prolonged period of ALP activity. Of course a 3-D scaffold provides a larger surface area for cell attachment compared to the smooth substrate. This is favorable for the spreading and interaction of cells, and thus results in a longer increase of their ALP activity.¹⁹ Moreover, in our study STRO-1-selected rat DPSCs were used, which were proved to show an earlier up-regulation of ALP activity, compared to non-selected rat dental pulp cells on a similar scaffold,¹⁹ which is due to the more homogeneous cells from STRO-1 selection.¹⁸ The analysis of the ALP activity between the two groups that were cultured in the osteogenic medium revealed that the secreted BMP-2 from the nanoparticle-transfected cells exerts its effect and accelerates the odontogenic differentiation and matrix mineralization of DPSCs. Previous studies have revealed that BMP-2 functions in a dose-dependent manner. High concentrations (i.e. 100 ng/ml) of BMP-2 demonstrated the obvious induction of an osteoblastic phenotype.^{29,30} Our study shows that the transfected cells mediated with nanoparticles produce less than 100 ng/ml of hBMP-2, however the transfected cells can continuously secrete the *Bmp2* gene product into the medium, which may account for similar bio-functionality to the higher concentrations necessary with BMP-2 as a protein supplement in a single application.³¹ Furthermore, calcium content measurement demonstrated that the transfected cells led to more mineralization, and SEM observation showed many cells were fully covered by mineralized deposition. Although a previous study has shown that calcium phosphate nanocomposite by themselves can induce cell differentiation and bone formation,³² we considered that gene products were mostly responsible for cell differentiation, especially bearing in mind the low dose calcium phosphate particles used presently. Ideally an empty nanoparticle could have been used as negative control. However this was not possible as the production of non-DNA loaded particles would have resulted in very different properties regarding morphology, composition, and crystallinity.

The real-time PCR further confirmed the results mentioned above. It is known that the expression of mRNA is always seen prior to the actual protein expression. The mRNA expression of ALP also continued to increase, and reached a peak at day 16 for cells in osteogenic culture, while the transfected cells demonstrated a more sensitive reaction. Collagen type I is the predominant collagen in dentin and bone matrix and the foundation of the extracellular matrix on which future mineralization takes place. Higher levels of collagen type I mRNA were found in cells from non-osteogenic culture, which agreed with the cell growth profiles found through DNA analysis and SEM. OC, a non-collagenous protein, is also present in dentin and bone matrix. The mRNA expression of OC showed similar profiles to a previous study.¹⁹ However, the two groups in osteogenic culture only demonstrated differences at day 8. This may be due to the BMP-2, which stimulates the early differentiation of cells, while OC is known as a late phase marker for terminal cell differentiation and matrix mineralization.³³ DSPP is the major part of non-collagenous proteins in natural dentin, and plays a crucial role during dentin mineralization.³⁴ DMP-1 is another specific protein in dentin, and is a candidate gene for dentinogenesis imperfecta.³⁵ Expression of these mRNAs can prove that these cells exhibit odontoblast phenotype.

5. CONCLUSIONS

Based on our results, we concluded that rat STRO-1 selected dental pulp stem cells can be successfully transfected with pEGFP-BMP2 loaded calcium phosphate nanoparticles. A relatively high transfection efficacy was achieved, analogous to that of LipofectamineTM. In some aspects, this nanoparticle vector was superior to the commercial transfection reagent. The secreted BMP-2 reached a functional level and accelerated the odonto-differentiation action. Consequently, calcium phosphate nanoparticles can be considered a valid candidate as non-viral gene vector. Meanwhile, the established combination of a more homogeneous cell

population with gene transfection can be a strategy to improve the efficacy of DPSCs for hard tissue engineering.

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Chapter 8

General Conclusions and Future Perspectives

1. SUMMARY AND ADDRESS TO THE AIMS

Adult stem cells are residing within differentiated tissues or organs, can renew themselves, and differentiate into specialized cells. Adult stem cells can be isolated from an individual, and thereafter be used for autogenous tissue regeneration, which will eliminate the possibility of immune rejection. Furthermore, unlike embryonic stem cells, the use of adult stem cells does not arouse ethical debate. Hence, adult stem cells provide enormous promise for tissue engineering applications.

Recent studies proved the existence of stem cells in postnatal dental pulp tissue. These dental pulp stem cells (DPSCs) demonstrated self-renewal capability, and could differentiate towards odontoblasts and form a dentin-like structure. However, as a potential candidate for dental tissue engineering, the behavior of DPSCs was quite variable, even for cells derived from the same source. These results suggest a hierarchy of progenitors in adult dental pulp, including a minor population of self-renewing, highly proliferative, multipotent stem cells, among a larger compartment of perhaps more committed progenitors. Hence, for successful dental tissue engineering strategies, stem cell technology needs to be optimized. Besides cell selection, also the addition of growth factors could be an effective strategy.

Therefore, the aim of this thesis was to isolate this putative DPSC subpopulation from postnatal rat dental pulp, and investigate its differentiation potential under both *in vitro* and *in vivo* environments. Furthermore, bone morphogenetic protein (BMP)-2 gene transfected DPSCs were investigated on differentiation characteristics *in vitro* and *in vivo*. We addressed to the following questions.

1.1 Can a more homogeneous cell subpopulation be sorted from rat dental pulp cells using STRO-1 selection?

In Chapter two, the STRO-1 antigen, defining a mesenchymal stem cell or progenitor subpopulation, was used for separating rat dental pulp cells with fluorescence-activated cell sorting (FACS). Subsequently, the STRO-1 positive cells were tested for their ability to differentiate towards an odontoblast-like phenotype. Three cell populations (STRO-1 positive, STRO-1 negative, and non-sorted cells) were cultured in odontogenic medium containing dexamethasone and β -glycerophosphate. Cultures were analyzed by light- and scanning electron microscopy (SEM), and assessed for proliferation, ALP activity, and calcium content. Results showed that the STRO-1 positive cell population was able to differentiate into the odontoblast phenotype, similar to the non-sorted population. The negative cells however showed a fibroblast-like phenotype. SEM and real-time PCR confirmed such results. In conclusion, the STRO-1 selection proved applicable for rat-derived material, to obtain a cell population which is more homogenous. This positive cell fraction was capable of differentiating into the odontogenic pathway, whereas the negative fraction was not. However, the effect was not always advantageous, when compared to non-sorted cells.

1.2 What is the multilineage potential of STRO-1⁺ rat dental pulp stem cells *in vitro*?

In chapter three, two cell populations (STRO-1⁺ and non-sorted cells) were cultured under conditions promoting neurogenic, adipogenic, myogenic, and chondrogenic differentiation. Results of light microscopy, histochemistry, and immunohistochemistry showed that STRO-1⁺ cells were capable to advance into all four differentiation pathways under the influence of inductive media. Quantitative PCR and statistical analysis on specific differentiation markers confirmed that there were significant up-regulations in STRO-1⁺ cells compared to the other populations during induction culture. On the basis of our results, we concluded that 1) rat STRO-1⁺ dental pulp stem cells were capable of differentiating towards multilineage cell types including neural cells, adipocytes, myocytes, and chondrocytes; 2) the STRO-1⁺

population had a more defined multilineage potential compared to non-sorted cells, probably because of its more homologous nature.

1.3 Do STRO-1 selected dental pulp stem cells have the ability for hard tissue formation *in vivo*, when seeded in porous HA/TCP ceramic scaffolds?

In Chapter four, STRO-1-selected rat dental pulp-derived stem cells were cultured with three different media: “BMP-plus” medium containing dexamethasone and 100ng/ml of rhBMP-2, “odontogenic” medium containing dexamethasone, and “control” medium without supplements. The cell-scaffold complexes were cultured in these media for 1, 4, or 8 days before implantation. Histological analysis demonstrated that the cultures with BMP plus and 4 days of culture gave the highest percentage of hard tissue formation per implant ($36 \pm 9\%$ of pore area). Real-time PCR confirmed these results. In conclusion, STRO-1-selected dental pulp stem cells show effectively hard tissue formation *in vivo*, and a short *in vitro* culture period and addition of BMP-2 can enhance this effect.

1.4 What is the influence of adenoviral-mediated Bmp2 transfection on dental pulp stem cells *in vitro*?

In Chapter five, STRO-1-selected rat dental pulp-derived stem cells were transfected with adenoviral-mediated human bone morphogenetic protein-2 gene (AdBMP-2). Subsequently, the cells were evaluated for their odontogenic differentiation ability in medium, not containing dexamethasone or other stimuli. Cultures were investigated by light microscopy, SEM, and evaluated for cell proliferation, ALP activity and calcium content. Real-time PCR was performed for gene expression of *Alp*, *OC*, collagen type I, *Bsp*, *Dspp*, and *Dmp1*. Finally, an oligo-microarray was used to profile the expression of odontogenesis-related genes. Results of ALP, calcium content, and real-time PCR showed that only *Bmp2*-

transfected cells had the ability to differentiate into the odontoblast phenotype, and to produce a calcified extracellular matrix. SEM and oligo-microarray confirmed these results. In contrast, the non-transfected cells represented a more undifferentiated cell phenotype. Based on our results, we concluded that the adenovirus can transfect STRO-1 selected cells with a high efficacy. After *Bmp2* gene transfection, these cells had the ability to differentiate into odontoblast phenotype, even without the addition of odontogenic supplements to the medium.

1.5 How is the *in vivo* behavior of adenoviral-mediated *Bmp2* transfected dental pulp stem cells?

In Chapter six, the feasibility of hard tissue formation from rat dental pulp-derived stem cells transfected with the *Bmp2* gene was studied, in an *in vivo* mouse subcutaneous implantation model. STRO-1 selected dental pulp stem cells were transfected with adenoviral-mediated human bone morphogenetic protein-2 gene (AdBMP-2) and seeded onto a porous ceramic scaffold material. The cell-scaffold complexes were cultured in medium not containing dexamethasone or other stimuli for 4 days before implantation. Samples were retrieved after 1, 4 and 12 weeks and evaluated by histology and real-time PCR. The results indicated that only the cultures with *Bmp2* gene transfection showed obvious hard tissue generation, and 12 weeks of implantation gave the highest percentage of hard tissue formation per implant ($33 \pm 7.3\%$ of pore area). Real-time PCR confirmed these results. In conclusion, *Bmp2*-transfected dental pulp stem cells effectively show hard tissue formation *in vivo*.

1.6 Can calcium phosphate nanoparticles be applied as a non-viral vector for *Bmp2* transfection into dental pulp stem cells?

In Chapter seven, nanoparticles of calcium phosphate encapsulating plasmid DNA (pEGFP-BMP2) were prepared. Then, STRO-1-selected rat dental pulp stem cells were

transfected using these nanoparticles. The transfection efficiency and bioactivity of the secreted BMP2 were examined. Thereafter, the transfected cells were cultured on a fibrous titanium mesh. The cultures were investigated by SEM, and evaluated for cell proliferation, ALP activity and calcium content. Finally, real-time PCR was performed for odontogenesis-related gene expression. The results showed that the size of the DNA-loaded particles was around 100 nm in diameter. Nanoparticles could protect the DNA encapsulated inside from external DNase, and release the loaded DNA in low acidic environment (pH 3.0). *In vitro*, nanoparticle transfection showed a high efficiency analogous to that of LipofectamineTM 2000. In addition, we found that nanoparticle transfection accelerated or promoted the odontogenic differentiation of rat dental pulp stem cells, when cultured in the three dimensional scaffolds. Based on our results, pDNA-loaded calcium phosphate nanoparticles appears to be an effective non-viral vector for gene delivery, and functioned well for odontogenic differentiation through *Bmp2* transfection.

2. CLOSING REMARKS AND FUTURE PERSPECTIVES

In this thesis, the *in vitro* and *in vivo* differentiation potential of STRO-1-selected stem cells derived from rat dental pulp was evaluated. All studies focused on two main directions: the ability for differentiation towards odontoblast-like cells, and the practical potential for hard tissue regeneration. As mentioned in Chapter one, the behavior of DPSCs can vary considerably, even for cells from the same source. Only a minor population of DPSC strains, derived from single colonies, can generate ectopic dentin *in vivo*. STRO-1 is the most extensively used antibody that appears to recognize stage-and/or lineage-specific stromal antigens. The monoclonal antibody STRO-1 reacts with an unidentified cell surface antigen expressed by a minor subpopulation of bone marrow stromal cells. Hence, we isolated a STRO-1 positive population from dental pulp by fluorescence-activated cell sorting. These

cells showed multilineage differentiation potential, and generally were more homologous than non-sorted cells. These STRO-1 positive DPSCs also formed abundant hard dentin-like tissue after subcutaneous implantation on a ceramic carrier, and thus exhibited great potential for therapeutic application.

Besides cell selection, a second strategy described in this thesis was the transfer of the *Bmp2* gene into DPSCs. Our studies showed that STRO-1-selected rat dental pulp stem cells can be genetically modified with the adenoviral vector containing the hBMP-2 gene. *Bmp2*-transfected DPSCs underwent more effective induction toward the odontoblast phenotype, and formed abundant hard tissue after subcutaneous implantation on a ceramic carrier, even without supplements like dexamethasone. The combination of homogeneously STRO-1 selected DPSCs with *Bmp* transfection shows potential to devise new therapies in the future.

However, the histological results of the current study, revealed only a bone-like hard tissue formation, with the absence of typical dentin-like features. Possible explanations are that, although the inductive supplements used in dental tissue engineering research are always described as “odontogenic”, neither dexamethasone nor BMP-2 are strictly to be defined as “odontogenic” factors. Such formulations originated as an osteogenic supplement to promote osteoblast differentiation from bone marrow. Furthermore, other possibilities for the absence of dentin formation can lie in the micro-morphology and/or chemical characteristics of HA/TCP ceramic. To actually progress to potential therapeutic applications for dentin regeneration, the development of more suitable stimulating factors, or scaffolds seems essential. Improved scaffold designs in composition as well as morphology can facilitate the formation of more natural tissue constructs.

Finally, we also explored the possibility of using plasmid DNA loaded calcium phosphate nanoparticles for gene transfection applications. The *in vitro* results showed that this vector can mediate *Bmp2* transfection into DPSCs in a highly efficient way, and this accelerated the

odontogenic differentiation of DPSCs. However, the *in vivo* response of this transfection lacked hard tissue regeneration, although some odontogenic genes were demonstrated to be upregulated. The results of this preliminary study are evidently not in accordance with the *in vitro* study. We assume that, unlike adenovirus, calcium phosphate nanoparticles mediate extrinsic gene expression for a shorter period. Thus, the nanoparticle-mediated gene transfection scheme needs to be optimized for application *in vivo*, which will be done in follow-up studies.

The outcomes of these studies are promising, but still far from applicable. Several problems need to be considered before clinical applications can be achieved.

Chapter 8

Samenvatting in het Nederlands

Summary in Dutch

1. SAMENVATTING EN EVALUATIE VAN DE DOELSTELLINGEN

Adulte stamcellen zijn aanwezig in de volledig gedifferentieerde weefsels en organen, kunnen zichzelf vernieuwen, en differentiëren tot gespecialiseerde celtypen. Adulte stamcellen kunnen geïsoleerd worden vanuit elk individu, en vervolgens gebruikt worden voor autogene weefsel regeneratie technieken, zonder kans op immunologische afstoting. Verder roept het gebruik van adulte stamcellen geen ethische tweestrijd op, in tegenstelling tot het gebruik van embryonale stamcellen. Adulte stamcellen zijn dan ook veelbelovend voor toepassing in het veld van de weefselregeneratie.

Recente studies hebben bewezen dat stamcellen ook aanwezig zijn in het postnatale dentale pulpa weefsel. Deze dentale pulp stamcellen (DPSCs) hebben het vermogen tot zelfvernieuwing, kunnen differentiëren tot odontoblasten, en vormen een dentine-achtige structuur. Belemmeringen voor het gebruik van DPSCs voor dentale weefselregeneratie zijn echter dat het gedrag zeer variabel kan zijn, zelfs voor cellen die vanuit een zelfde bron gewonnen zijn. Zulke resultaten geven duidelijk aan dat er een zekere hiërarchie van voorlopercellen bestaat. In de dentale pulpa is een kleine populatie van zelfvernieuwende, snel groeiende, multi-potente stamcellen aanwezig, samen met een grotere hoeveelheid van waarschijnlijk meer vastgelegde voorlopercellen. Voor succesvolle tandheelkundige weefselregeneratie strategieën lijkt het daarom van belang om stamceltechniek verder te optimaliseren. Naast celselectie, kan ook het toevoegen van groeifactoren deel uitmaken van zo'n effectieve strategie.

Het onderliggende doel van dit proefschrift was dan ook de genoemde DPSC subpopulatie te isoleren uit de postnatale dentale pulpa van ratten, en vervolgens het differentiatie potentiaal van deze populatie te bestuderen, zowel *in vitro* als *in vivo*. Vervolgens werden DPSCs bestudeerd na transfectie met het gen voor bone morphogenetic protein (BMP)-2. De volgende onderzoeksvragen werden gesteld.

1.1 Kan een meer homogene subpopulatie van dentale pulpa cellen van ratten verkregen worden door middel van STRO-1 selectie?

In hoofdstuk twee werd het STRO-1 antigeen gebruikt om rat dentale pulpa cellen te scheiden met behulp van de fluorescence-activated cell sorting (FACS) techniek. STRO-1 komt tot expressie bij een subpopulatie van mesenchymale stam- of voorloper cellen. Vervolgens werden de STRO-1 positieve cellen getest op het vermogen tot differentiatie naar een odontoblast-achtig fenotype. Drie celpopulaties (STRO-1 positief, STRO-1 negatief, en niet-geselecteerde cellen) werden opgekweekt in odontogeen medium met toevoeging van dexamethason en β -glycerolfosfaat. De celkweeken werden geanalyseerd met behulp van licht- en scanning elektronen microscopie (SEM), en verder beoordeeld op proliferatie, ALP activiteit, en hoeveelheid calcium. De resultaten toonden aan dat de STRO-1 positieve celpopulatie kon differentiëren tot het odontoblast fenotype, op een vergelijkbare wijze als de niet-gesorteerde populatie. De STRO-1 negatieve cellen vertoonden echter een fibroblast-achtig fenotype. SEM en real-time PCR experimenten bevestigden deze resultaten. In conclusie kan gezegd worden dat STRO-1 selectie bruikbaar is om een homogene celpopulatie te verkrijgen, zelfs bij materiaal verkregen uit ratten. De positieve cel fractie was in staat te differentiëren tot odontoblast, in tegenstelling tot de negatief geselecteerde fractie. Echter, de celselectie had niet altijd positief resultaat in vergelijking tot niet gesorteerde cellen.

1.2 Kunnen STRO-1⁺ ratten dentale pulpa stamcellen in celweek differentiëren tot meerdere fenotypen?

In hoofdstuk drie werden twee celpopulaties (STRO-1⁺ en niet-gesorteerde cellen) gekweekt onder condities die de differentiatie bevorderen naar een neurogene, adipogene,

myogene, of chondrogene ontwikkeling. Resultaten van licht microscopie, histochemie, en immunohistochemie lieten zien dat STRO-1⁺ cellen in staat waren tot ontwikkeling in alle vier differentiatie richtingen in de inductieve kweekmedia. Kwantitatieve PCR en statistische analyse van specifieke differentiatie markers bevestigde dat STRO-1⁺ cellen significant beter differentieerden ten opzichte van de andere celpopulatie. Op basis van deze resultaten kan geconcludeerd worden dat 1) STRO-1⁺ dentale pulpa stamcellen uit de rat in staat zijn in meerdere richtingen te differentiëren inclusief neurale cellen, adipocyten, myocyten, en chondrocyten; 2) de STRO-1⁺ populatie een beter differentiatie potentiaal heeft in vergelijking met niet-gesorteerde cellen, waarschijnlijk vanwege de meer homologe aard.

1.3 Kunnen STRO-1 geselecteerde dentale pulpa stamcellen, aangebracht op een poreus HA/TCP keramisch dragermateriaal, *in vivo* hard weefsel vormen ?

In hoofdstuk vier werden STRO-1-geselecteerde dentale pulpa stamcellen gekweekt in drie verschillende media: “BMP-plus” medium met dexamethason en 100ng/ml rhBMP-2, “odontogeen” medium met dexamethason, en “controle” medium zonder toevoegingen. De cellen werden gezaaid op een dragermateriaal, vervolgens 1, 4, of 8 dagen in deze media gekweekt, en daarna geïmplant. Histologische analyse liet zien dat de kweek in aanwezigheid van BMP, en met 4 dagen kweektijd, uiteindelijk leidde tot het hoogste percentage hard weefsel per implantaat ($36 \pm 9\%$ van de porie grootte). Real-time PCR bevestigde dit resultaat. In conclusie kan gezegd worden dat STRO-1-geselecteerde dentale pulpa stamcellen *in vivo* effectief hard weefsel kunnen vormen, waarbij korte *in vitro* kweektijden en toevoeging van BMP-2 de hard weefsel vorming bevorderen.

1.4 Wat is de invloed van adenovirus-gemedieerde Bmp2 transfectie op dentale pulpa stamcellen *in vitro*?

In hoofdstuk vijf werden STRO-1-geselecteerde dentale pulpa stamcellen met behulp van een adenovirus getransfecteerd met het gen voor humaan bone morphogenetic protein-2 (AdBMP-2). Vervolgens werden de cellen geanalyseerd op hun odontogene differentiatie capaciteit, in medium zonder dexamethason of andere toevoegingen. De celkweken werden onderzocht met behulp van licht microscopie, SEM, en beoordeeld op celproliferatie, ALP activiteit en hoeveelheid calcium. Real-time PCR werd gebruikt om de gen expressie van *Alp*, *OC*, collagen type I, *Bsp*, *Dspp*, en *Dmp1* te beoordelen. Tenslotte werd een oligo-microarray gebruikt om een profiel te maken van de expressie van odontogenese-gerelateerde genen. De resultaten van de ALP, calcium, en real-time PCR experimenten lieten zien dat alleen de *Bmp2*-getransfecteerde cellen in staat waren om te differentiëren tot een odontoblast-achtig fenotype, en om een gecalcificeerde extracellulaire matrix te vormen. De SEM en oligo-microarray experimenten bevestigden deze resultaten. De niet-getransfecteerde cellen lieten een meer ongedifferentieerd fenotype zien. Op basis van de resultaten kan geconcludeerd worden dat adenovirussen STRO-1 geselecteerde cellen met een hoge efficiëntie kunnen transfecteren. Na transfectie met het *Bmp2* gen, waren de cellen in staat om te differentiëren tot een odontoblast-achtig fenotype, zelfs zonder toevoeging van odontogene supplementen aan het kweekmedium.

1.5 Hoe gedragen dentale pulpa stamcellen zich *in vivo*, na adenovirale transfectie met *Bmp-2* ?

In hoofdstuk 6 werd hard weefsel vorming door dentale pulpa stamcellen onderzocht na transfectie met het *Bmp2* gen, in een subcutaan implantatie model in de muis. STRO-1-geselecteerde dentale pulpa stamcellen werden getransfecteerd met een adenovirus met het humane bone morphogenetic protein-2 gen (AdBMP-2) en daarna uitgezaaid op een poreus keramisch dragermateriaal. De cel-dragermateriaal complexen werden opgekweekt in medium

zonder toevoeging van dexamethason of andere stimulerende factoren, gedurende 4 dagen. De samples 1, 4 en 12 weken na implantatie uitgehaald, en beoordeeld met histologie en real-time PCR. De resultaten lieten alleen bij de cellen met *Bmp2* gen transfectie duidelijk de vorming van hard weefsel zien, waarbij 12 weken implanteren tot het hoogste percentage hard weefsel leidde ($33 \pm 7.3\%$ van de porie grootte). Real-time PCR bevestigde deze resultaten. In conclusie kunnen we zeggen dat *Bmp2*- getransfecteerde dentale pulpa stamcellen effectief hard weefsel kunnen vormen *in vivo*.

1.6 Kunnen calcium fosfaat nanodeeltjes gebruikt worden als een niet-virale vector voor *Bmp2* transfectie in dentale pulpa stamcellen?

In hoofdstuk 7 werden nanodeeltjes van calcium fosfaat gemaakt, met daarin plasmide DNA (pEGFP-BMP2). Vervolgens werden STRO-1-geselecteerde dentale pulpa stamcellen getransfecteerd met deze nanodeeltjes. Eerst werd de effectiviteit van de transfectie, en de bioactiviteit van het afgegeven BMP2 bestudeerd. Daarna werden de getransfecteerde cellen gekweekt op titaan vezelgaas. De kweken werden onderzocht met behulp van SEM, en beoordeeld op celproliferatie, ALP activiteit en hoeveelheid calcium. Tenslotte werd real-time PCR gebruikt om de expressie van odontogenese-gerelateerde genen te onderzoeken. De resultaten lieten zien dat de deeltjes geladen met DNA ongeveer 100 nm in diameter waren. De nanodeeltjes waren in staat om het gebonden DNA te beschermen tegen extern toegevoegd DNase, en konden het DNA vrijgeven in een zure omgeving (pH 3.0). *In vitro* was transfectie met de nanodeeltjes net zo effectief als met LipofectamineTM 2000. Daarnaast werd aangetoond dat transfectie met de nanodeeltjes leidde tot een versnelde en verhoogde odontogene differentiatie van de dentale pulpa stamcellen op het dragermateriaal. Gebaseerd op deze resultaten lijken pDNA-geladen calcium fosfaat nanodeeltjes een effectieve niet-

virale vector, die voldeed om *Bmp2* transfectie en odontogene differentiatie te bewerkstelligen.

2. AFSLUITENDE OPMERKINGEN EN TOEKOMSTPERSPECTIEF

In dit proefschrift werd *in vitro* en *in vivo* het differentiatie potentieel geanalyseerd van STRO-1-geselecteerde stamcellen verkregen uit de dentale pulpa van de rat. Het onderzoek kan verdeeld worden in twee hoofdrichtingen: nl de differentiatie tot odontoblast-achtige cellen, en de praktische toepassing voor het regenereren van hard weefsel. Zoals gezegd in hoofdstuk 1 is het gedrag van DPSCs erg variabel, zelfs voor cellen die uit een zelfde bron verkregen zijn. Slechts een klein deel van DPSC lijnen, afgeleid uit losse kolonies, kan *in vivo* ectopisch dentine vormen. STRO-1 is het meest gebruikte antilichaam dat stadium-en/of lijnspecifieke stromale antigenen lijkt te herkennen. Het monoclonale antilichaam STRO-1 reageert met een nog onbekend antigen op het celoppervlak dat tot expressie komt bij een kleine subpopulatie van stromale cellen in het beenmerg. Daarom werd een STRO-1 positieve populatie geselecteerd uit dentale pulpa met behulp van fluorescence-activated cell sorting. De verkregen cellen hadden het vermogen om naar meerdere fenotypen te differentiëren, en waren in het algemeen eenvormiger dan niet gesorteerde cellen. Deze STRO-1 positieve DPSCs vormden na subcutane implantatie op een keramisch dragermateriaal een ruime hoeveelheid hard dentine-achtig weefsel, en lijken dus in potentie zeer geschikt voor therapeutische toepassingen.

Naast celselectie, wordt er in het proefschrift nog een tweede strategie beschreven, namelijk het inbrengen van het gen voor *Bmp2* in de DPSCs. Deze studies lieten zien dat STRO-1-geselecteerde dentale pulpa stamcellen genetisch gemodificeerd kunnen worden met een adenovirale vector die het hBMP-2 gen bevatte. *Bmp2*-getransfecteerde DPSCs namen effectiever het odontoblast fenotype aan, en vormden ook na subcutane implantatie op

een keramisch dragermateriaal een ruime hoeveelheid hard weefsel, zelfs wanneer geen supplementen zoals dexamethason gebruikt werden. De combinatie van de homogene STRO-1 geselecteerde DPSCs samen met *Bmp* transfectie lijkt veelbelovend om in de toekomst nieuwe therapieën te ontwikkelen.

De histologische resultaten van alle studies geven echter wel aan dat er een bot-achtig hard weefsel wordt gevormd, waarin typische dentine-achtige structuren ontbreken. Een mogelijke verklaring hiervoor lijkt te vinden in de supplementen die normaliter gebruikt worden voor tandheelkundige weefsel regeneratie. Deze worden weliswaar “odontogeen” genoemd, maar strikt gesproken zijn dexamethason noch BMP-2 “odontogene” factoren. Deze factoren zijn eigenlijk in eerste instantie beschreven als osteogene supplementen om osteoblast differentiatie uit beenmerg te bevorderen. Een andere verklaring voor de afwezigheid van dentine vorming zou de micro-morfologie en/of de chemische karakteristiek van het gebruikte HA/TCP keramiek kunnen zijn. Om daadwerkelijk tot therapeutische toepassingen voor dentine regeneratie te komen, lijkt het essentieel om beter geschikte stimulerende factoren, of dragermaterialen te ontwikkelen. Dragermaterialen met een meer geschikte samenstelling en morfologie zouden de vorming van meer natuurlijk gelijkende weefsel constructen kunnen bevorderen.

Tenslotte is ook de mogelijkheid onderzocht om calcium fosfaat nanodeeltjes geladen met plasmide DNA te gebruiken voor gen transfectie. De resultaten van het *in vitro* experiment lieten zien dat deze vector op een zeer effectieve manier *Bmp2* transfectie in DPSCs kan mediëren, en dat dit de odontogene differentiatie van de DPSCs versnelde. Echter, *in vivo* leidde deze transfectie methode nog niet tot hard weefsel regeneratie, alhoewel een aantal odontogene genen duidelijk hoger tot expressie kwamen. De resultaten van deze preliminaire studie zijn duidelijk nog niet in overeenstemming met de *in vitro* studie. We nemen aan dat de calcium fosfaat nanodeeltjes een extrinsieke gen expressie geven gedurende een kortere

periode, in vergelijking met het adenovirus. Daarom zal de transfectie methode met de nanodeeltjes eerst verder geoptimaliseerd moeten worden voor *in vivo* gebruik. Hiervoor lopen op dit moment al vervollexperimenten.

De uitkomsten van alle studies lijken veel belovend, maar zijn nog niet toepasbaar. De hierboven genoemde problemen vereisen verdere studie voordat klinische toepassingen bewerkstelligd kunnen worden.

Chapter 8

结论及展望 (in Chinese)

1. 结论

成体干细胞是存在于已分化组织或器官中的干细胞。它们既能自我更新，又可分化成具有特殊功能的细胞。现在越来越多的证据显示，成体干细胞不仅可以分化出其来源组织或器官的细胞类型，而且还可以跨谱系分化，分化产生与其胚层来源不同的细胞类型。从个体组织或器官中分离培养的成体干细胞可以用于自体组织再生。自身来源的成体干细胞不会引起免疫排斥反应，而且避免了应用胚胎干细胞所带来的伦理学争论。因此，成体干细胞在未来组织工程领域的应用前景非常乐观。

近来的研究从成体牙髓中分离出了具有自我更新及分化潜能的细胞群，并命名为牙髓干细胞 (dental pulp stem cells, DPSCs)。牙髓干细胞能够分化为成牙本质细胞，形成牙髓牙本质样复合体结构。此外，牙髓干细胞也具有多向分化潜能，可以转向分化 (trans-differentiation) 为脂肪细胞、神经细胞、肌细胞及软骨细胞。同时，牙髓干细胞能够长期冻存且仍然保持干细胞特性。牙髓成为又一潜在的成体干细胞储库，牙髓干细胞显示了在牙体组织修复及牙组织工程的应用前景。然而，研究表明不同克隆来源的牙髓干细胞的生物学行为差异很大，包括增殖能力及体内外分化能力。这一现象提示上述牙髓干细胞群落间存在异质性 (hierarchy)，仅仅少数细胞亚群为具有高度增殖更新及多向分化潜能的干细胞，其余大多数群落只是具有一定增殖能力的已经处于定向分化途径上的前体细胞。这一生物学性状势必影响牙髓干细胞在组织工程的应用前景及效率。因此，为了成功实施牙组织工程策略，需要优化牙髓干细胞的分离策略。除此之外，生长因子的引入也不失为有效的手段。

因此，本论文的目的在于探讨一种可靠的方法，从成体牙髓中分离具有干细胞生物学活性的 DPSCs 亚群；同时，研究其体内外分化潜能。此外，骨形成蛋白 2 (BMP2) 基因被转染引入上述细胞中，观察其体内外分化特点。我们主要致力于以下问题：

1.1 能否通过 STRO-1 筛选从大鼠牙髓细胞中获得生物学性状更具同源性的细胞亚群？

本论文的第二章主要目的是利用流式分选方法从大鼠牙髓中获得的 STRO-1⁺ 细胞，并分析此细胞在体外培养时的分化能力及优势。牙髓细胞源自 Wistar 大鼠切牙牙髓，与 STRO-1 单克隆抗体进行免疫反应后，使用流式细胞分选 (FACS) STRO-1⁺ 细胞并扩增培养。三组细胞 (STRO-1⁺/STRO-1⁻未分选的细胞) 在成牙本质诱导培养条件下研究其在体外的分化能力。通过测定细胞增殖率、碱性磷酸酶 (ALP) 活性及钙含量来观察细胞的生长模式。利用扫描电镜观察细胞基本形态及形成的矿化结节。并用实时定

量 PCR (real-time PCR) 检测成牙本质细胞分化相关基因的表达。诱导后的 STRO-1⁺ 细胞能够形成矿化结节, 并显著上调相关基因的表达, 显示出 STRO-1⁺ 细胞具有向成牙本质细胞表型分化的能力。然而, STRO-1⁻ 细胞更多的呈现出成纤维细胞样的表型特点, 没有表现出向成牙本质细胞分化的潜能。未分选的第三代牙髓细胞也有分化的表现, 甚至在诱导培养早期较之 STRO-1⁺ 细胞还体现一定的优势。

1.2 STRO-1⁺大鼠牙髓干细胞的体外分化能力如何?

本论文的第三章进一步使用成神经性、成脂肪性、成肌性及成软骨性诱导培养体系分析 STRO-1⁺ 细胞多向分化潜能, 并比较两组细胞 (STRO-1⁺/未分选的细胞) 分化潜能的差异。细胞形态、免疫学和组织化学染色结果表明, 在上述四种诱导环境下, STRO-1⁺ 细胞能够发生相应的形态学改变, 分泌或表达相应细胞分化标志物, 显示出向神经细胞、脂肪细胞、肌细胞及软骨细胞分化的能力。同时, real-time PCR 结果显示, 较之未分选的细胞, STRO-1⁺ 细胞更显著的上调分化相关基因的表达。基于上述结果, 我们得出结论: 1) STRO-1⁺ 大鼠牙髓干细胞具有多向分化的潜能; 2) 由于含有更多同源性的细胞, STRO-1⁺ 群落表现出更稳定和更确定的分化能力。

1.3 STRO-1 分选的牙髓干细胞体内硬组织形成能力如何?

本论文的第四章, STRO-1 分选的大鼠牙髓干细胞接种于 HA/TCP 支架, 生长于三种培养基中: 1) “BMP-plus” 培养基, 包含地塞米松及 100ng/ml rhBMP-2; 2) 成牙本质诱导培养基, 包含地塞米松; 3) 非诱导培养基。细胞一支架复合物于培养的第 1、4 或 8 天移植入免疫缺陷小鼠皮下。组织学分析显示, 经过 4 天的移植前培养, 能够在皮下获得最高的异位成骨率 ($36 \pm 9\%$)。Real-time PCR 检测从另一方面也验证上述结果。综上所述, STRO-1 分选的大鼠牙髓干细胞显示出有效的体内硬组织形成能力。而且, 较短的体外培养期及添加 BMP-2 能够增强细胞这种效能。

1.4 腺病毒介导 *Bmp2* 基因转染对牙髓干细胞体外分化有何影响?

本论文的第五章, 该部分研究的主要目的是使用腺病毒载体, 介导人 *Bmp2* 基因转染 STRO-1 分选的大鼠牙髓干细胞, 分析转染的功能性效果及对细胞成牙本质能力的影响。两组细胞 (转染/未转染) 通过细胞增殖率、碱性磷酸酶活性及钙含量的检测来观察生长状态的改变。利用扫描电镜观察细胞基本形态及形成的矿化结节。通过实时定

量 PCR 检测成牙本质细胞分化相关基因 (碱性磷酸酶、I 型胶原、骨钙素、骨唾蛋白、牙本质唾磷蛋白及牙本质基质蛋白 1) 的表达。另外, 使用特异性寡核苷酸微阵列技术检测两组细胞基因差异表达的情况。碱性磷酸酶活性、钙含量、扫描电镜及 PCR 结果显示, 在非诱导培养基环境中只有转染的细胞有向成牙本质细胞表型分化的能力, 而且能够形成矿化的细胞外基质。寡核苷酸微阵列分析同样也证实了上述的结论。在此培养环境中, 未转染的 STRO-1 分选的大鼠牙髓干细胞则仍然更多的呈现出未分化的状态。综上所述, 腺病毒载体能够高效的转染 STRO-1 分选的大鼠牙髓干细胞。通过 *Bmp2* 基因的转染, 即使在非诱导培养环境下细胞也获得了向成牙本质细胞分化的能力。基因转染的策略能够赋予干细胞更多的分化能力。

1.5 腺病毒介导 *Bmp2* 基因转染的牙髓干细胞在体内的反应性如何?

本论文的第六章, 腺病毒载体介导人 *Bmp2* 基因转染 STRO-1 分选的大鼠牙髓干细胞, 接种于 HA/TCP 支架。细胞一支架复合物体外培养 4 天后移植入免疫缺陷小鼠皮下。结果显示, 转染后的移植物能够在体内形成明显的骨样硬组织, 而且经过 12 周的移植, 能够在皮下获得最高的异位成骨率 ($33 \pm 7\%$)。Real-time PCR 检测也验证上述结果。综上所述, 通过 *Bmp2* 基因转染能够显著增强牙髓干细胞体内硬组织形成能力。

1.6 磷酸钙纳米微球能否作为非病毒载体介导 *Bmp2* 基因转染牙髓干细胞?

本论文的第七章, 该部分研究的主要目的是制备复合质粒 DNA 的磷酸钙纳米微球, 评估其相关物化特性。使用此纳米微球作为载体转染大鼠牙髓干细胞, 评估转染的效率及产物的生物学活性。此外, 研究转染的大鼠牙髓干细胞在钛纤维三维支架上的生长特点及分化表现。同时, 使用脂质体作为衡量转染效果的对照。结果显示, 制备的负载质粒 DNA 的磷酸钙微球能够达到纳米尺寸, 该复合物能够保护包裹的质粒 DNA 抵御外界的酶降解作用。纳米微球能够在酸性环境下 (pH 5.0) 逐步释放质粒 DNA。制备的纳米微球能够转染大鼠牙髓干细胞, 并达到同脂质体相近的效率($\approx 70\%$)。ELISA 检测到转染细胞能够分泌功能性水平 hBMP2。通过 W20-17 细胞评估, 所分泌的 hBMP2 具有生物学活性。体外三维培养环境下, 细胞在钛纤维上生长状态良好, 转染的大鼠牙髓干细胞周围显示更明显的矿化基质沉积, 成牙本质细胞分化相关基因的表达也得到更显著的上调。综上所述, 纳米尺寸的负载质粒 DNA 的磷酸钙微球能够成

功转染大鼠牙髓干细胞，此转染系统能够产生功能性的效果。钛纤维支架上，细胞能够良好的生长，转染后的细胞显示出更明显的向成牙本质细胞分化的能力。负载质粒 DNA 的磷酸钙纳米微球能够作为又一有效的非病毒载体，在今后的基因治疗中使用。

2. 结语及对未来的展望

目前认为，组织工程的目的是从形态结构和功能上修复或再生缺损的组织器官。合适的细胞资源、生物活性因子及理想的支架材料是组织工程三个基本组成部分。自体组织来源的成体干细胞具备自我更新及多向分化的能力，更避免了免疫排斥反应，是天然的用于修复来源组织的组织工程反应细胞。牙髓干细胞的发现为成体干细胞提供了一个新的来源，也为牙体组织工程提供潜在的最佳选择。

本研究成功的利用流式分选得到 STRO-1 阳性的大鼠牙髓细胞亚群，并研究了细胞在体内、外增殖及分化的表现。结果证实，STRO-1 阳性筛选得到的细胞具有明显的干细胞特性；而且，STRO-1 阳性表型细胞的聚集导致细胞同源性更为显著、整体未分化性增加以及对外界分化刺激更为敏感。此外，细胞显示出较强的体内硬组织形成能力。STRO-1 阳性分选优化了牙髓干细胞获得途径，提升了组织工程反应细胞的效能。

通过特定基因的转染能够产生长效的内源性生物活性因子，通过自分泌形成的细胞微环境进而对细胞生长分化和组织形成产生有效的影响。本研究使用携带 *Bmp2* 基因的腺病毒载体转染 STRO-1 阳性的大鼠牙髓干细胞，在未加其它诱导刺激的环境中能够有效促使细胞向成牙本质细胞方向分化。体内移植实验显示，转染增强了细胞的硬组织形成能力。STRO-1 分选结合基因转染能够进一步提高牙髓干细胞的分化潜能和实际应用效能。

作为新的非病毒基因载体，磷酸钙纳米微球技术被尝试用于牙髓干细胞的转染试验。结果显示，负载 *Bmp2* 基因的磷酸钙纳米微球能够有效地转染细胞，获得相似于商业转染试剂的效率，而且细胞毒性更低。进而，转染的细胞培养于钛纤维支架上。转染组表现出更显著的成牙本质细胞分化表型，提示其实际应用价值。

本研究主要探讨了体外培养的 STRO-1 阳性大鼠牙髓细胞的生物学表现，涉及了细胞、生物因子和支架三个方面的内容。合适的细胞、合适的因子以及合适的支架能够模拟出类似天然的组织生长环境，进而提高组织工程方案的成功率和应用效能。本研究结果提示了牙髓干细胞乐观的应用前景。然而，在真正应用于临床之前，仍有大量的工作需要完成。

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