Aptamer-based capture molecules as a novel tool to isolate target cells and promote cell adhesion

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Table of Content

Abbreviations

aPTT	Activated partial thromboplastin time
BSA	Bovine serum albumin
CFU-F	Colony forming units-fibroblast
CPB	Cardiopulmonary bypass
DNA	Deoxyribonucleic acid
ES	Embryonic stem cells
FACS	Fluorescence activated cell sorting
FCS	Fetal Calf Serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
MAB	Monoclonal antibody
MAPS	Multipotent adult progenitor cells
MHC	Major histocompatibility complex
MI	Myocardial infarction
MSC	Mesenchymal stem cell
PBS	Phosphate-buffered saline
PE	R-Phycoerythrin
PDGF	Platelet-derived growth factor
PLA	Poly-lactic-acid
PLGA	Poly--lactide co-glycolic-acid
RNAi	RNA interference
ssDNA	Single-stranded DNA
SELEX	Systematic evolution of ligands by exponential enrichment
siRNA	Small interfering RNA
STRO-1	Stromal cell surface marker antibody
tRNA	Transfer ribonucleic acid
VEGF	Vascular endothelial growth factor

Assertion

Ich erkläre hiermit, dass ich die der biologischen Fakultät der Universität Tübingen zur Promotion eingereichte Arbeit mit dem Titel: ‘’Aptamer-based capture molecules as a novel tool to isolate target cells and promote cell adhesion’’ selbständig ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Angaben wahr sind und dass ich nichts verschwiegen habe. Mir ist bekannt, dass die falsche Abgabe einer Versicherung an Eides statt mit einer Freiheitsstrafe bis zu drei Jahren oder mit einer Geldstrafe bestraft wird.

Bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskriptes habe ich Unterstützungsleistungen von folgenden Personen erhalten:

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Die vorgelegte Dissertation wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt. Mit der Arbeit wurde weder ein akademischer Grad erworben noch eine staatliche Prüfung absolviert.

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Abstract

Aptamers have been introduced to analytical applications, target validation, and drug discovery processes and, recently, applied directly as therapeutic agents. Aptamers can be generated by a method called SELEX (Systematic Evolution of Ligands by Exponential Enrichment). This is quite remarkable for such a young technology, which is only created in the early 1990s. Given their small size, ease of synthesis, low cost, and high specificity, aptamers provide versatile tools for validation of intracellular and extracellular targets. With a number of additional aptamers expected to enter into clinical trials over the next years, aptamers appear to make a significant contribution to the treatment of acute and chronic diseases. MSCs are one of the stem cell populations that are being introduced in the clinic for treatment of several degenerative diseases. Mesenchymal stem cells have several advantages including the differentiation potential and the stability of their phenotype *in vitro*. The use of these cells in therapy showed also promising results in phase I clinical trials. It is hoped that using stem cells in the clinic will bring major advances in the therapy of several chronic and degenerative diseases. But, due to the lack of specific phoenotype, the isolation of pure MSCs is an obstacle on the application ways. The traditional method of the isolation is based on their selective adherence to plastic surfaces; other methods are based on the characteristics of MSC, using antibodies against MSCs or positive depletion of other cells, but the specificity of the antibodies are still under research. Thus, the cell populations obtained by current methods are essentially heterogeneous.We first used osteoblasts to show the proof of principle and then applied this principle on pig mesenchymal stem cells (MSC). We targeted pigMSCs and generated aptamers which can bind them with high affinity and specificity. Using this aptamers we set up a new method to isolate MSCs from bone marrow, which is quick and efficient. Then we used the fresh isolated

MSCs to analyse the phenotype and found some interesting changes which may contribute to the subpopulations of MSCs. This study shows for the first time that immobilized aptamers can work as capture molecules on biomaterials to fish out the target cells from a biological mixture. This new application of aptamers will bring novel aspects of MSCs to the field of regenerative medicine and tissure engineering.

Introduction

1. Aptamers

1.1 Concept of aptamers

Aptamers are nucleic acid molecules that bind to molecular targets, including proteins, with high affinity and specificity. Aptamers are typically from 15 to 50 nucleotides in length and can be composed of DNA, RNA, peptide(PNA) or nucleotides with a chemically modified sugar backbone (i.e., 2′-fluoro, 2′-O-methyl, phosphorothioate). The secondary structure of aptamers consists primarily of short helical arms and single-stranded loops which are the effective part to bind the target via van der Waals, hydrogen bonding, and electrostatic interactions. Aptamers can recognize their targets such as most small molecules, peptides, or protein targets, with KD values ranging from 10 pM to 10 nM for proteins with great specificity (for antibodies, the KD value is from pM to uM). For instance, an aptamer to bFGF (FGF-2) binds with up to 20,000-fold greater affinity to bFGF than it does to its closely related fibroblast growth factor (FGF) -1, -4, -5, -6, and -7 homologues.[1] Aptamers can also distinguish between closely related members of a protein family, or between different functional or conformational states of the same protein.[2]

1.2 SELEX

Aptamers can be generated by a method called SELEX (Systematic Evolution of Ligands by Exponential enrichment). [3] This method starts with a large amount of random sequence single-stranded oligonucleotides library, RNA or DNA. This library pool normally has up to 10^{14}-10^{15} variants. The complexity of the library provides a source for a variety of the targets. The further method involves an iterative process of binding, partitioning, and amplifying novel

nucleic acids. The applications of this technology extend from basic research reagents to the identification of novel diagnostic and therapeutic agents. Examples of these applications are described along with a discussion of underlying principles and future developments expected to the utility of SELEX. Meanwhile the development of SELEX has driven from the bench top to fully automated systems.[4-6]

1.3 Advantage of aptamers

When aptamers are considered as binding molecules, they are inevitably compared to antibodies, which are still the general reagents in the field of diagnostics. Monoclonal antibodies (MABs) became widespread in the 1970s [7] and have since then made a great contribution to many diagnostic applications. Soon after the discovery of aptamers, they started to demonstrate their potential and versatility in diagnostic assay formats where they substituted for monoclonal antibodies (MABs).[8] Recently, Somalogic Inc. (Boulder, USA) reported the development of an aptamer chip that can assess approximately 50 different analytes in patient samples.[9] These new data underline the potential of aptamers as powerful diagnostic reagents in sophisticated assay formats. Aptamer-based assays reach very low detection limits and can be performed in solution by simple one-tube reactions without the need for washes and separations. These “mix and measure” assays benefit from the fact that aptamers share certain properties with both MABs and nucleic acids: aptamers combine specific recognition of their corresponding target’s 3-D shape with the broad diversity of nucleic acids in terms of enzymatic and synthetic chemistry. [10, 11]

In addition, aptamers have a number of advantages that make them very promising in analytical and diagnostic applications. The selection of aptamers starts from a big ssDNA library which normally includes $10^{14\text{-}15}$ different sequences. During the selection procedure, a counter-SELEX can be made to

get rid of the unspecific binding of other targets to guarantee the specificity of aptamers to the designed target. Another big advantage is that there is no need of animals for their production. Thus it shows low costs and high efficiency. Most of antibody production starts in biological systems by inducing an immune response to the target, but the immune response can fail when the target molecule, i.e. protein, has a structure similar to endogenous proteins or when the antigen consists of toxic compounds. On the contrary, aptamers are isolated by in vitro methods that are independent from animals: an in vitro combinatorial library can be generated against any target. In addition, generation of antibodies in vivo means that the animal immune system selects the sites on the target protein to which the antibodies bind. The in vivo parameters restrict the identification of antibodies that can recognize targets only under physiological conditions limiting the extension to which the antibodies can be functionalized and applied. Moreover, the aptamer selection process can be manipulated to obtain aptamers that bind to a specific region of the target and with specific binding properties in different binding conditions. After selection, aptamers are produced by chemical synthesis and purified to a very high degree by eliminating the batch-to-batch variation found when using antibodies. Additionally, by chemical synthesis, modifications in the aptamer can be introduced enhancing the stability, affinity and specificity of the molecules. Finally, because of their simple structure, sensor layers based on aptamers can be regenerated more easily than antibody-based layers, are more resistant to denaturation and have a much longer life. Moreover, the selection process itself, with the amplification steps, gives some advantages to aptamers in respect to other “non-natural” receptors, such as oligonucleic acids, peptides, which cannot be amplified during their selection procedure.

Due to all these characteristics, aptamers have been used in numerous investigations, as therapeutic [12] or diagnostic tools[13] and for the development of new drugs. Moreover, aptamers have been recently used in analytical chemistry applications, as immobilized ligands or in homogeneous assays.

1.4 Examples for the application of aptamers

Vascular Endothelial Growth Factor (VEGF)

Angiogenesis is critical in numerous physiological and pathological states. VEGF is the best-characterized factor involved in benign and neoplastic angiogenesis and represents a promising target in anticancer and other antiangiogenic therapies. [14]

Pharmacokinetic studies of the 2-fluoropyrimidine and 2-O-methylpurine aptamer to VEGF, renamed NX-1838, were conducted in rhesus monkeys. This aptamer was conjugated to a 40-kDa PEG moiety, and following intravenous administration, showed a terminal half-life (t1/2) of 9.3 h and a clearance rate of 6.2 ml/h. Subcutaneous administration resulted in almost 80% absorption into the plasma compartment, reaching a peak concentration of 4.9 μg/ml in 8–12 h.[15] Preclinical and clinical studies of NX-1838, renamed Macugen, have been conducted by Eyetech Pharmaceuticals, Inc., for the treatment of exudative age-related macular degeneration and diabetic macular edema.[16-17] The antiangiogenic activity of the VEGF aptamer has been evaluated in rat corneal angiogenesis and mouse retinopathy of prematurity (ROP) models. These studies revealed significant inhibition of VEGF-mediated neovascularization, with the aptamer reducing 80% of retinal neovascularization in the ROP model.[16] A phase IA safety study based on these exciting preclinical results showed no significant risks with a single intravitreal injection of the drug. Moreover, 80% of treated patients exhibited stable or improved vision three months after treatment, and 27% demonstrated a three-line or greater improvement on the Early Treatment for Diabetic Retinopathy Study (ETDRS).[16] A phase II study reported no serious side effects resulting from multiple intravitreal injections of Macugen with or without photodynamic therapy; 87.5% of the treated patients exhibited stabilized or improved vision three months after treatment and 25% demonstrated a three-line or greater improvement in vision on ETDRS. Furthermore, a

three-line gain after three months was seen in 60% of patients who received a combination of Macugen and photodynamic therapy. [17] A phase III study is under way to further evaluate the efficacy of Macugen. [18] These results are the first to show that in vitro selected aptamers can be clinically efficacious drugs in humans.

Thrombin

Thrombin is a key regulatory enzyme in the coagulation cascade. It is a serine protease produced from prothrombin by the action of factor Xa. Thrombin, in turn, converts fibrinogen into fibrin, which is the building block of the fibrin matrix of blood clots.[19] A single-stranded DNA-aptamer was isolated that prolonged clotting time from 25 s to 169 s in purified fibrinogen and from 25 s to 43 s in human plasma. [20]In cynomolgus monkeys, the prothrombin time (PT) increased by 1.7-fold 10 min after infusion of the aptamer and returned to baseline 10 min after the infusion was terminated.[21] In a regional anticoagulation model via sheep hemofiltration, PT increased to 40–45 s (baseline, 21.7 s) while the systemic PT remained close to baseline.[21] In a canine cardiopulmonary bypass (CPB) model, the aptamer-treated group exhibited increases in PT, activated partial thromboplastin time (aPTT), and activated clotting time that subsequently returned to baseline after aptamer infusion ceased. [22]Pharmacokinetic studies revealed the elimination half-life of the drug was ～1.9 min pre- and post-CPB, but while on CPB for 60 minutes the half-life increased to 7.7 min. Currently this DNA aptamer is being evaluated in preclinical studies by Archemix Corporation in preparation for human clinical trials. In an RNA selection against thrombin, 2-fluoro-modified RNA molecules were exposed to a mixture of human and porcine α-thrombin in round 1 and then toggled between human and porcine thrombin in alternate rounds of selection. An aptamer named Toggle-25 (Tog-25) was identified that bound to human thrombin with a Kd of 2.8 ± 0.7 nM and to porcine thrombin

with a Kd of 83 ± 3 pM. The aptamer increased the thrombin time from 11.6 ± 0.2 s to 22.6 ± 1.4 s in human plasma and from 15.7 ± 0.7 s to 61.9 ± 1.2 s in porcine plasma. The aptamer inhibited thrombin-dependent platelet activation in a dose dependent manner in both human and porcine platelets.[23]

Prostate-Specific Membrane Antigen

Prostate cancer cells over-express a well-characterized surface antigen, prostatespecific membrane antigen (PSMA). [24]An RNAselection was conducted against the extracellular component of PSMA; called sPSM.[25] SELEX identified two aptamers that inhibited xPSM with a Ki of 2.1 and 11.9 nM, respectively. Aptamer xPSM-10 was truncated (xPSM-10-3) and fluorescently end-labeled to evaluate its ability to bind PSMA-expressing cancer cells. The aptamer bound to LNCaP cells but not PC-3, showing its specificity for PSMA and its potential in therapeutic development for this target.

Immunoglobulin E

Immunoglobulin E (IgE) plays an important role in protecting mammals from parasites. [26]Overproduction of IgE due to exposure to environmental antigens, however, can result in diseases such as allergies, atopic dermatitis, and allergic asthma (94). A 2-NH2 pyrimidine-modified RNA selection and a DNA selection were performed against human IgE and yielded aptamers that bind human IgE with high affinities (RNA, Kd = 30 nM; DNA, Kd = 9 nM).[27] Unfortunately, both of these aptamers had 300- to 1000-fold greater binding to human IgE than to rat or mouse orthologs, which limited preclinical testing of the aptamers. The aptamers inhibited IgE binding to its receptor Fc ε RI, resulting in a Ki = 21 nM for the RNA aptamer and a Ki = 6 nMfor the DNA aptamer. The aptamers also prevented IgE-mediated cellular degranulation in serum of patients with a grass allergy. In patients exposed to grass extract, the IC50s for the RNA and DNA aptamers were 2–6 μ M, and when triggered by anti-IgE antibodies, the IC50s were200–300 nM.

2. Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells (MSCs) are clonogenic, non-haematopoietic stem cells present in bone marrow and are able to differentiate into multiple mesoderm-type cell lineages e.g. osteoblasts, chondrocytes, myocytes, endothelial-cells and also non- mesoderm-type lineages e.g. neuronal-like cells.

2.1 Genesis of MSCs

The existence of MSCs in bone marrow has been suspected for many years. However, Freidenstein et al.[28] were the first to develop in vitro culture methods for their isolation and for testing their differentiation potential in 1991. They termed this group of cells bone marrow fibroblasts. These cells were named by later investigators: bone marrow stromal cells, mesenchymal stem cells or skeletal stem cells. The stem cell characteristics of mesenchymal stem cells are based on their ability to differentiate into multiple cell types including osteoblasts, chondrocytes, myocytes, endothelial cells and even neuronal-like cells.

2.2 Isolation methods of MSCs

No prospective markers exclusively defining MSCs are available at the moment. Thus, cell populations obtained by the current methods are essentially heterogenous. Currently, there are several methods for isolation of mesenchymal stem cells from bone marrow. Traditionally, they are isolated based on their selective adherence to plastic surfaces.[29-31] One disadvantage of this method is the unavoidable haematopoietic cell contamination and the cellular heterogeneity of cultures. Other investigators tried different methods to isolated homogenous cell populations. Simmons et al.[32] developed a monoclonal antibody: Stro-1 that has been used to isolate a pure population of

cells with mesenchymal stem cells characteristics.[32,33] Reyes et al.[34] isolated a pluripotent mesenchymal stem cells population (termed Multipotent Adult Progenitor Cells: MAPC) from CD45a/GlycoproteinA- depleted bone marrow-derived mononuclear cell fraction that selectively adhered to laminin-coated plates under low serum conditions. And, marrow-isolated adult multilineage inducible (MIAMI) cells were isolated from the whole bone marrow cells by selective adhesion to fibronectin-coated plates in presence of reduced serum conditions and under low oxygen tension. Interestingly, side by side comparison of these different cell populations did show major differences.[35] Recently, CD271 (LNGFR, low affinity nerve growth factor receptor) has also been shown to be the best known marker for the enrichment of nonhematopoietic stem cells from bone marrow aspirates,[36,37] termed marrow stromal cells (MSCs): colony forming unit - fibroblast (CFU-F) activity was found to occur only in isolated CD271+ cells, but not CD271– cells.[38] MSCs selected for CD271 expression were shown to have a 10- to 1000-fold higher proliferative capacity in comparison to MSCs isolated by plastic adherence.[39]Newer studies have also demonstrated the possibility of isolating MSC-like cells from the “stroma” of a number of organs including synovial membranes [40] and deciduous teeth. [41] MSCs were detected circulating also in peripheral blood [42] and in umbilical cord blood. [43]

2.3 Characterization of MSCs

2.3.1 Phenotype

Much progress has been made to characterize the cell surface antigenic profile of human bone marrow-derived MSC populations using fluorescence activated cell sorting (FACS) and magnetic bead sorting techniques. To date, however, a single marker that definitively delineates the *in vivo* MSCs has yet to be identified, due to the lack of consensus from diverse documentations of the MSC phenotype.[44-46] However, analyses using a combination of monoclonal

antibodies raised against surface markers of *in vitro*-derived MSCs (e.g., STRO-1, SH2, SH3, SH4)[44,47] have shown some promise toward immuno-phenotyping these cells. On the other hand, the fact that MSCs share common features with endothelial, epithelial and muscle cells [46] and present a highly variable profile of cell surface antigens [48-51] makes it a daunting task to identify a universal single marker for MSCs. Differences exist among the reported studies in the surface marker characteristics that may be explained by variations in culture methods and/or differentiation stage of he cells. Despite this controversy of what defines a 'mesenchymal stem cell', there is general agreement that MSCs lack typical hematopoietic antigens, namely, negative for CD45, CD34, CD14 , CD31, and CD133 and positive for CD105, CD166, CD54, CD55, CD13 and CD44. [51]

2.3.2 Subpopulations of MSCs

Prockop et al[52, 53] have used the low-density plating methods of Friedenstein and isolated and studied the population of rapidly dividing cells from human bone marrow, termed recycling stem (RS) cells, as a subpopulation of MSCs. RS cells were termed RS-1 for the small agranular, rapidly dividing cells, and RS-2 for small, granular cells, whereas the more typical fibroblastic MSCs were considered mature MSCs in the cultures. RS cells divided very quick when plated at low density and may yield 10^9 cells in 6 weeks.[54] Most recently, Prockop et al identified the Wnt inhibitor dickkopf-1 as an effector molecule for hMSC proliferation in vitro.[55] Another exciting findings came from Verfaillie et al, who described marrow progenitor cells or multipotent adult progenitor cells (MAPCs).[50, 56-58] The cultured cells have many of the attributes of MSCs, but they are reported to expand indefinitely and have lineage potentials that include extended ectodermal and endodermal cell types, making them more like ES cells. Additionally, they lack major histocompatibility complex (MHC) class I and class II on their surface, so presumably, they may be used

allogeneically, although the lack of MHC I molecules may make them vulnerable to elimination by natural killer cells. Much of the published data are from cells of mouse origin, and it will be important to verify that the human MAPCs are as potent in these respects as the mouse MAPCs.[59, 60]

2.3.3 Multilineage differentiation potential of MSCs

The multilineage differentiation potential of MSCs populations has been extensively studied in vitro since their first discovery in 1960s.[61] These studies demonstrate that populations of bone marrow derived MSCs from human, canine, rabbit, rat, porcine and mouse have the capacity to develop into terminally differentiated mesenchymal phenotypes both in vitro and in vivo, including bone, [62] cartilage, [63] tendon, [64, 65] muscle, [66,67] adipose tissue, [68,69] and hematopoietic-supporting stroma. [69] The ability of MSCs to differentiate into a variety of connective tissue cell types has rendered them an ideal candidate cell source for clinical tissue regeneration strategies, including the augmentation and local repair and regeneration of bone, [70] cartilage [71] and tendon.[64] Individual colonies derived from single MSC population which derived from single MSC precursors have also been reported to be heterogeneous in terms of their multilineage differentiation potential. For instance, Pittenger *et al.*[45] reported that only one-third of the initial adherent bone marrow-derived MSC clones are pluripotent (osteo/chondro/adipo). Furthermore, non-immortalized cell clones examined by Muraglia *et al.*[72] demonstrated that 30% of the *in vitro* derived MSCs clones exhibited a tri-lineage (osteo/chondro/adipo) differentiation potential, while the remainder displayed a bi-lineage (osteo/chondro) or uni-lineage potential (osteo). These observations are consistent with other *in vitro* studies using conditionally immortalized clones.[73-75] Additionally, Kuznetsov *et al.*[76] demonstrated that only 58.8% of the single colony-derived clones had the ability to form bone within hydroxyapatite-tricalcium phosphate ceramic scaffolds after

implantation in immunodeficient mice. Similar results were reported by using purer populations of MSCs maintained *in vitro*.[77] Taken together; these results suggest that clonally-derived MSCs are heterogeneous with respect to their developmental potential.

2.3.4 Self-renewal potential of MSCs

The self-renewal potential of MSCs is the ability to generate identical copies of themselves through mitotic division over extended time periods (even the entire lifetime of an organism). The absolute self-renewal potential of MSCs remains an open question, in large part due to the different methods employed to derive populations of MSCs and the varying approaches used to evaluate their self-renewal capacity. As a population, bone marrow derived MSCs have been demonstrated to have a significant but highly variable self-renewal potential during *in vitro* serial propagation.[78] Continuous labeling of fresh bone marrow cell harvests with tritiated thymidine reveals that CFU-Fs are not cycling *in vivo*, [79]and their entry into cell cycle and subsequent development into colonies depends on serum growth factors. [80] Cell seeding density also plays a role in the expansion capacity of MSCs. For example, Colter *et al* demonstrated that higher expansion profiles of MSCs can be attained when plated at low density (1.5-3 cells/cm2) but not at high density (12 cells/cm2), which resulted a dramatic increase in the fold expansion of total cells (2,000-fold vs. 60-fold expansion, respectively). This work and other similarly reported work [81] strongly suggest that MSCs and isolated MSCs clones are heterogeneous with respect to their self- renewal capacity.

3. Applications of MSCs

Adult MSCs are ideal candidates for regenerative medicine in cell and gene therapy applications, because of their multipotentiality and capacity for

extensive self-renewal. Four areas for potential clinical use of mesenchymal stem cells have been explored: (1) local implantation for localized diseases, (2) systemic transplantation, (3) combining stem cell therapy with gene therapy, (4) use in tissue engineering protocols.[82] Locally injected expanded autologus mesenchymal stem cells for treatment of large bone defects in patients with defective fracture healing has been reported successful.[83] Systemic transplantation of allogenic normal mesenchymal stem cells has been tried in children with severe osteogenesis imperfecta. Homing of mesenchymal stem cells in bone as well as the production of normal collagen by the transplanted mesenchymal stem cells has been demonstrated. [84] However, the contribution of the transplanted mesenchymal stem cells to the clinical improvement observed in these patients is not clear. And one of the challenges is to improve the engraftment efficiency of mesenchymal stem cells to bone marrow and bone, but what is encouraging is that neither autologous nor allogeneic MSCs induce any immuno-reactivity in the host, [85-88] thus rendering MSCs an ideal carrier to deliver genes into the tissues of interest for gene therapy applications. Genetic modification of stem cells is an attractive target for gene therapy because of their higher proliferative capacity and long-term survival compared with other somatic cells. Mesenchymal stem cells have been demonstrated to be able to express exogenous proteins (e.g. factor VIII and IL-3) for an extended period of time and to maintain this ability after transplantation in vitro. [89] Several approaches have been examined and used to introduce exogenous DNA into MSCs to render them useful in tissue regeneration therapies. Viral transduction, particularly using adenovirus mediated gene transfer, can generate stable cell clones with high efficiency and low cell mortality, thus making it a popular option in gene therapy. For example, MSCs infected with an adenovirus vector containing dominant-negative mutant collagen type I gene have been used successfully to repair the bone in individuals with the brittle bone disorder, osteogenesis imperfecta etc.[90] However, the safety concerns associated with viral transduction have prompted researchers to look

for alternative non-viral gene delivery approaches.

Tissue engineering provides alternative ways for obtaining tissues and organs needed for transplantation due to lack of sufficient number of organ donors and limitations attributable to immunological rejection and mismatch of physical dimensions. Tissue engineering may allow obtaining the patient's own cells, seeding them on bio-degradable scaffolds that allow formation of a particular tissue. These tissues can be used to repair tissue defects due to disease or trauma. Also, tissue engineering may also allow ex vivo engineering of tissue by the means of 3-dimensional bio-scaffolds seeded with mature cell or stem cells and cultivated in bioreactors that lead to the formation of tissues or organs like liver, hearts, cartilage or kidney.[91] Mesenchymal stem cells are good cell types for use in tissue engineering protocols because the relative ease for establishing the cells in vitro cultures and their good proliferation and differentiation potential.[92-93] Several scaffolds are currently available and may be classified as either biologically derived polymers isolated from extracellular matrix, plants and sea-weed, e.g. collagen type I or fibronectin, alginate from brown algae or synthetic e.g. hydroxyapatite, tri-calcium phosphate ceramics, polylactide and polyglycolide and a combination of these in the form of poly DL-lactic-co-glycolic acid. There are several animal experiments that show the success of using this approach e.g. for treatment of large bone defects in animal models[94] and it is expected that transplantation of tissues based on these methods to human beings will be achieved in the next years.

3.1 Bone tissue engineering

The use of natural and synthetic biomaterials as carriers for MSC delivery has shown increasing promise for orthopaedic therapeutic applications, especially bone formation. Recent advances in the field of biomaterials have led to a

transition from nonporous, biologically inert materials to more porous, osteoconductive biomaterials, and, in particular, the use of cell-matrix composites. [95] The parameters that need to be considered in the selection of a suitable delivery vehicle include physicochemical properties, such as surface area, porosity, local acidification, material chemistry, dimensional architecture, mechanical integrity, degradation characteristics, natural versus synthetic, and potential for drug delivery; and biological properties, such as the ability of the scaffold to support cellular attachment, proliferation, differentiation, matrix deposition, angiogenesis, prevention of dedifferentiation, and enrichment with a suitable quantity of cells. A number of delivery vehicles have been successfully used in cell-matrix composites in vivo, such as porous ceramics of hydroxyapatite and β-tricalcium phosphate loaded with autologous MSCs. [96] These constructs were capable of healing criticalsized segmental bone defects not capable of being healed by resident cells or by the addition of the osteoconductive device alone. A recent *in vitro* study comparing the biodegradable polymers poly-L-lactide (PLA) and poly-Llactide- co-glycolide (PLGA) on the basis of adherence and proliferation of seeded trabecular-bone-derived osteoprogenitor cells showed that PLGA was the better substrate for the attachment and subsequent osteogenic differentiation of these progenitor cells. [97]

3.2 Cartilage tissue engineering

The damage of the articular cartilage by trauma or degenerative joint diseases such as primary osteoarthritis often causes the disablility of joint. Articular cartilage functions to provide uncompromised movement by minimizing friction between joints and allows load bearing through distribution of and resistance to compressive forces, but possesses very limited potential for healing. Current treatment methods for restoration of function due to articular cartilage

damage, other than total joint arthroplasty, include autografting, allografting, periosteal and perichondrial grafting, stimulation of intrinsic regeneration by intentionally drilling full-thickness defects, pharmacological intervention, and autologous cell transplantation. A potential resolution of this disease state is the regeneration of cartilage tissue using autologous MSCs, thereby avoiding any donor-site morbidity as is seen with current repair methods, but requiring an understanding of the mechanisms responsible for the generation, maintenance, and particularly the regeneration of cartilage tissues.

MSC-based repair of full-thickness articular cartilage defects has been attempted in animal models, using various carrier matrices. [98-102] Natural polymers such as collagen have shown promise in early applications. Using autologous MSCs dispersed in a collagen-type-I gel, Wakitani *et al.* [98] succeeded in repairing full-thickness defects on the weight-bearing surface of medial femoral condyles. The regenerating cartilage was subsequently replaced by bone in a proximal-to-distal fashion until the underlying subchondral bone was completely repaired without disruption of the overlying cartilage. Use of synthetic polymers in such applications has also been promising, in particular the α-hydroxyesters PLA and PGA and their copolymer, PLGA.

3.3 Soft tissues

Tendon

In addition to the well-established bone, cartilage, and adipose lineages, the induction of MSC differentiation into other connective tissues, such as muscle, tendons, and ligaments is also being investigated. For tenogenesis, key factors include culture conditions, growth factors, and physical stimulation, such as mechanical loading. For a tissue-engineering approach, marrow-derived MSCs have been used for Achilles tendon repair. MSCs

seeded onto a collagen-type-I construct incorporated into healing tendons that subsequently exhibited greater load-related MSC-loaded scaffolds had better alignment of cells and collagen fibers and were more similar to the native tendon than unloaded controls. [103] Much of the improvement seen with MSC-loaded constructs was seen at a biochemical level and in maximum stress, modulus, and strain energy density, rather than a histological level, and without much improvement in the microstructure of the tissue itself. [104] Another factor in this process is the initial seeding density of the cells, showing a plateau of density-dependent effect at approximately 4 million cells per milliliter. [105] One important issue concerning cell-based tendon tissue engineering is the mechanical loading and subsequent activation of the forming tissue. While no specific studies addressing this in MSCs are available, information gathered from tendon/ligament fibroblasts strongly suggests that tensile strength and stretch loading are essential for the proper formation and alignment of the tendon or ligament structure. [106]

Adipose tissue

In vitro adipogenic induction requires specific medium supplementations, including dexamethasone and 3- isobutyl-1-methylxanthine. Indomethacin, a nonsteroidal anti-inflammatory drug, binds to and activates the transcription factor peroxisome proliferator-activated receptor gamma (PPAR-γ), which is crucial for adipogenesis. [107] Several groups have also shown the ability of MSCs to interconvert between the adipogenic and osteogenic lineages. The concept of interconvertibility is appealing because *in vivo* the bone marrow progressively adopts a more 'fatty' or adipose-like, versus hematopoietic, structure as a function of age. It has been proposed that the stromal elements of the marrow, perhaps containing MSCs, can differentiate into either the osteogenic or the adipogenic lineage, depending upon microenvironmental cues.[108, 109]

Muscle

Marrow MSCs have been induced into the myogenic lineage both *in vivo* and *in vitro*. While skeletal muscle itself contains stem cells known to be active in regeneration, these cells are distinct from MSCs and the subject is reviewed elsewhere. [110] Examination of the myogenic differentiation of MSCs is currently being applied to cardiac muscle as well as skeletal muscle. In particular, regeneration of cardiomyocytes is the goal of many groups, on the basis of previous experiments showing the induction of murine marrow stem cells into the cardiomyocyte phenotype.[111,112] Some groups have examined the treatment of myocardial infarction by application of autologous MSCs in the pig model, and these studies show engraftment, differentiation, and improved function in animals treated with autologous marrow MSCs. [113] In recent human study, the intracoronary application of autologous bone-marrow cells after myocardial infarction leads to significant improvements of function in comparison with a group given standard therapy. Not only was the infarct region itself much smaller in these patients, but also the level of function of the heart was vastly improved over those receiving only the standard therapeutic interventions. [114] While the exact mechanisms responsible for such phenotypic conversion remain unknown, these findings hold much promise for the future of tissue engineering and regeneration. [115]

3.4 MSCs for Cardiomyoplasty

Interest in MSCs, in general, and as a cardiac therapy, in particular, has increased exponentially during the past 5 years. Compared with other cells types considered for cardiomyoplasty, MSCs appear to possess unique properties that may allow for convenient and highly effective cell therapy.

MSCs can be used allogeneically, delivered systemically, and differentiate into a cardiomyocyte-like phenotype when implanted in healthy myocardium.[116,117]

Furthermore, MSCs can be readily transduced by a variety of vectors and maintain transgene expression after in vivo differentiation.[117,118] Transgene expression by MSCs may be used ultimately to augment cell engraftment or the extent of differentiation. Recent data from Mangi et al[119] support this possibility. This group used a retroviral vector to overexpress the prosurvival gene Akt in MSCs before implantation in infarcted rat myocardium. Akt protein overexpression was reported to greatly enhance MSC survival and prevent pathologic remodeling after infarction, with impressive improvement in cardiac output. The first use of bone marrow cells for cardiomyoplasty was reported in 1999 by the laboratories of Weisel and Lee at the University of Toronto.[120] In this study, autologous bone marrow cells were implanted in the left ventricle (LV) of rats by direct injection 3 weeks after cryoinjury. Transplanted marrow cells could be identified in all animals 8 weeks after injury and were found to express muscle-specific proteins not present before implantation. Furthermore, improved systolic and diastolic functions were reported in animals that received cells pretreated with the DNA-demethylating agent 5-azacytidine, which has been reported to enhance myogenic differentiation of pluripotent cells. It should be noted that bone marrow, as opposed to a purified MSC population, was used in this study. Development of a causative relationship between the observed functional improvements and a particular cell population is problematic because of the fact that in addition to being a major repository for MSCs, whole marrow contains a number of cell types. As opposed to the muscle precursor cells, allo-MSCs have the ability to be used immediately after acute injury. The ability to treat MI patients with allo-MSCs in an emergent setting at the time of coronary reperfusion may constitute a distinct clinical advantage over autologous cellular cardiomyoplasty. Furthermore, MSCs appear to have the ability to home to the site of myocardial injury when administered intravenously after acute infarction.

One of the most interesting characteristics of MSCs is their ability to home to

sites of tissue damage or inflammation.[121] Although the cytotactic factors responsible for injury-specific MSC migration and its physiologic consequences have yet to be fully elucidated, it is postulated that MSCs migrate to participate in wound repair. This extraordinary ability of MSCs to home to sites of acute tissue injury has been demonstrated in the settings of bone fracture[121-122] and cerebral ischemia[123] as well as the infarcted heart.[124, 125] Saito et al[124] were the first to demonstrate that MSCs administered intravenously engraft within regions of MI. In this study, mouse MSCs were transduced with a LacZ reporter gene and injected intravenously to rats (in the absence of immunosuppression). In healthy (noninjured) animals, intravenous MSCs preferentially engraft in the marrow cavity. However, when these rats were subjected to a cycle of ischemia/reperfusion, significant numbers of labeled xenogeneic MSCs could be identified in the circulation and, subsequently, the infarcted region of the heart. The majority of engrafting MSCs were found to be positive for cardiomyocyte- specific proteins, whereas a distinct subpopulation was determined to participate in angiogenesis.[125]

One of the problems inherent to the study of cellular cardiomyoplasty, particularly with intravenous administration, is the difficulty in tracking the movement of implanted cells. Recently, Barbash et al[126] used camera imaging of 99mTc-labeled MSCs to determine their distribution after intravenous or intraventricular injection in a rat MI model. They reported that 4 hours after intravenous injection, a significant number of MSCs were trapped in the lungs, with lesser numbers in the heart and other organs. Cardiac engraftment was augmented significantly (and pulmonary plugging attenuated) with intraventricular administration. MRI of iron-labeled cells is another technique that has been used recently to track MSC distribution in vivo.[127, 128] Hill et al[128] demonstrated that iron labeling does not alter MSC multipotentiality and that imaging in a standard 1.5-T magnet provides excellent special resolution in the beating heart. Furthermore, the iron label can be conjugated to a fluorescent

particle to aid in histologic MSC identification. MSC localization in vivo with either MRI or imaging is of tremendous value to those attempting to demonstrate the biodistribution or persistence of MSC cardiomyoplasty. However, the inability of the techniques to accurately quantify the number of MSCs is troubling to those seeking to optimize the therapeutic use of MSCs. When attempting to calculate dose/response relationships in such studies, it is the number of cells engrafting that is of concern, as opposed to the number administered. Accurate cell engraftment quantification has been elusive regardless of cell type examined. Some investigators have attempted to generate indexes of engraftment by simply counting the number of labeled cells in a representative sampling of high-powered fields. This methodology is hindered by the possibility of counting multiple cell layers or fractions of cells and, therefore, is not exact. We have attempted to quantify MSC engraftment using RT-PCR amplification of transgene expression. Although theoretically sound, in our experience, PCR-based quantification of transduced MSCs lacks the sensitivity required for accurate tissue quantification when the number of engrafted cells is low. A method found to provide a more accurate quantification of MSC engraftment is radiolabeling of MSCs with tritiated thymidine ([3H]-thymidine). After implantation, the degree of radioactivity in a tissue can be correlated to MSC engraftment in a linear fashion. Although PCR techniques would allow for detection of 50 000 cells in a rat heart, tritium labeling has a 10-fold lower threshold of detection (5000 cells), greatly improving accuracy of measurements as well as our ability to detect modest numbers of cells in the heart. Using these types of techniques has allowed investigators to quantify the degree of MSC engraftment in the heart after delivery. Although the exact numbers vary, it can be said that in all cases, the extent of engraftment is currently modest. Some experiments have indicated that 3% of MSCs administered by direct injection persist after 2 weeks. Administration of higher numbers of cells results in only modest augmentation of long-term engraftment.

4. Our objective

Adult mesenchymals stem cells (aMSCs) are a stem cell population present in bone marrow which can be isolated and expanded in culture and characterized in vitro and in vivo. The potential of aMSC to replicate undifferentiated and to mature into different various mesenchymal tissue cells suggests that it is an attractive source for tissue engineering. However, due to the lack of specific phenotype of MSC, to separate MSC from bone marrow is a barrier for its application. Though many research groups look for new antibodies to isolate MSC, for example, w8b2 and 57D2[129], the binding specificity of these antibodies is still under investigation. We tried to find other ways than antibodies to separate MSC from bone marrow. Aptamers may come out to realize this task. Aptamers are ssDNA or ssRNA which can fold into second structure to bind to targets with high affinity and specificity. With its advantage such as easy synthesis, a small molecule etc, the application of aptamers has been greatly investigated. In our studies, we want to make new use of aptamers as capture molecules to MSCs. The term capture molecule means aptamers could play the role of a specific antibody to MSCs, to separate MSCs from bone marrow; on the other hand, they could be used as a coating material to enhance the MSCs adhesion and enrichment. To prove the principle, we first used osteoblast cell line from sarcoma as a target to generate aptamers and test our design. We started from a ssDNA library which includes about 10^{14} random sequences. 10^5 osteoblasts were used for each round of selection. After 10 rounds selection, the binding sequences were cloned and sequenced. The binding affinity of individual aptamer was identified by FACS. The aptamer with the best affinity against osteoblasts was synthesized and coated on solid surfaces to attach osteoblasts out of a solution. The adhesion looks quite good and further electronic scanning microscope shows that the binding between the aptamer coated surfaces with osteoblasts is much stronger than the binding between the non-coated surfaces with osteoblasts.

When this principle was fully approved, we used MSCs from adult pigs to perform the experiment. Targeting porcine aMSC, an aptamer that binds to the porcine aMSC with high affinity and specificity was identified. To identify the binding ability of the aptamers generated, we first used the FACS assay to test it. Then we used the streptavidin magnetic microbeads to bind with biotin labeled aptamers and then use this beads-aptamer to separate MSC out of bone marrow blood. This new application of aptamers can facilitate aMSC isolation and enrichment greatly. With the aptamer, aMSCs could be separated by FACS sorting or by magnetic beads (MACS, magnetic associated cell sorting), thereby enhancing the rate of aMSC-derived cells after in vitro differentiation for various applications in regenerative medicine. Additionally, the analysis of freshly isolated aMSC by aptamers from bone marrow reveals novel insights to the aMSC subpopulations and their antigenic profile in their natural environment. The potential clinical applications will not only reveal unknown aspects of isolation and characteristics of MSC but also provide a new tool for the fascinating field of tissue engineering and regenerative medicine.

Results Part 1:

Aptamer-based capture molecules as a novel coating strategy to promote cell adhesion.

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Ketai Guo, Hans P. Wendel, Lutz Scheideler, Gerhard Ziemer, AM Scheule

The SELEX-technology for the generation of aptamers against osteosarcoma cells was exclusively developed by me. All experiments were planed, performed and analyzed by me. The publication was written by me. The idea of this study was from Hans P. Wendel and he supervised my work and corrected the manuscript. L. Scheideler introduced to me the culture of the osteosarcoma cells. G. Ziemer and AM. Scheule were intensively involved in the planning of the study.

Aptamer-based capture molecules as a novel coating strategy to promote cell adhesion

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Abstract

To improve cell seeding efficiency and cytocompatibility, we designed a new coating material for scaffolds. We used aptamers, highly specific cell binding nucleic acids generated by combinatorial chemistry with an in vitro selection called systematic evolution of exponential enrichment (SELEX). Aptamers do have high binding affinity and selectivity to their target. In our study, human osteoblasts from osteosarcoma were used as a target to get the aptamer. Single aptamer mediated cell sorting assay showed the binding affinity with osteoblasts. Additionally, the immobilized aptamers on tissue culture plates could capture osteoblasts directly and quickly from the cell solution. This model proves that applying aptamer on scaffolds can greatly enhance the adhesion of seeding cells.

Key words: aptamer, osteoblast, scaffold, tissue engineering

Introduction

Current scaffolds, made by conventional scaffold fabrication techniques, are generally made of synthetic polymers. The cells can not recognize such surfaces well, resulting in an impaired seeding efficiency and an ability to grow orderly in such scaffolds. Recent research activities focus on material selectivity and the fabrication methods of scaffolds as well as the functionalizing of the surfaces to make them more compatible for the seeding of cells. Especially, coating surfaces with PEG or RGD peptides have shown better cell adhesion [1-3]. Surfaces conjugated with growth factors like BMP-2 can also increase the cell attachment [4-6]. Here we introduce a new coating strategy for scaffolds, using aptamers as cell capture molecules.

Aptamers consist of ssDNA or RNA obtained from a random oligonucleotide library by Systematic Evolution of Ligands by Exponential Enrichment (SELEX) [7, 8]. SELEX is a technique used to screen large combinatorial libraries of oligonulceotides by an iterative process of in vitro selection and amplification.

The combinatorial libraries based on replicable oligos offer the convenience of amplification, and also make the screening process fast and easily. In the selection procedure, the library is incubated with a target of interest which is often fixed on a solid vector such as nitrocellulose membrane or columns. The population of unbound sequences is washed away and the binding sequences are isolated and amplified to obtain a smaller enrichment library for the next selection round. The enrichment efficiency could be detected by carrying on binding assays like FACS. Once binding saturation is achieved after several rounds of selection, the selected oligos are cloned and sequenced to obtain the single sequence, which is called the aptamer. The length of an aptamer is uauslly 50-80 base pair. The relative small molecular weight makes it suitable to apply to the molecular reorganization. Aptamers could fold into a three-dimensional structure based on their nucleic acid sequence to bind with the target. The dissociation constants (Kd) between the aptamer and its target is often in the pico molar or even nano molar range which shows a highly specific interaction [9, 10]. With the advantage of non-immunogenic; rapid kidney clearance and easy plasticity, aptamers are being used on both diagnostic and therapeutic applications [11-15]. Aptamers that have high affinity and specificity for cells and tissues have also been produced, demonstrating that complex targets, including purified protein, amino acid, and cells are compatible with the SELEX process. [16] In our approach we investigated a new application of aptamers, using them as a probe to capture the target cells. To test the "proof of theory", we used SAOS-2 osteoblasts from human osteosarcoma as a target of SELEX to generate the DNA aptamers.

MATERIALS AND METHODS

Cells and culture

SAOS-2 osteoblasts were cultured in McCoy’s medium supplemented with 10% FCS, 1%Penicillin/Streptomycin, and 1% L-Glutamine. (Invitrogen, Karlsruhe, Germany) as previously described [17].

DNA library and primers

The DNA oligonucleotide library contains a 40-base central random sequence flanked by primer sites on either side. (5'-GAATTCAGTCGGACAGCG-N40-GATGGACGAATATCGTCTCCC-3') The FITC-labeled forward primer (5'-C_{12}-FITC-GAATTCAGTCGGACAGCG -3') and biotin-labeled reverse primer (5'-Bio -GGGAGACGATATTCGTCCATC-3') were used in PCR to get the double-labeled DNA and to separate the single strand DNA by streptavidin dynabeads (Dynal, Denmark). The library and all primers were synthesized by Operon Technologies (Cologne, Germany).

SELEX procedure

Selecting DNA aptamer with a high affinity to osteoblast was performed as follows. ssDNA pools were denatured by being heated at 80 °C for 10 min in a selection buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM KCl, 100 mM NaCl, 1 mM MgCl2, and 0.1% NaN3 and then renatured at 0 °C for 10 min. To reduce background binding, a 5-fold molar excess of yeast tRNA (Invitrogen) and bovine serum albumin (BSA. Sigma-Aldrich, Munich) were added. The osteoblasts were incubated with the ssDNA library at 37 °C for 30 min in the selection buffer. Partitioning of bound and unbound ssDNA sequences was done by centrifugation. After centrifugation and having been washed three times with a 1ml selection buffer (with 0.2% BSA), cell-bound aptamers were amplified by PCR (Master Mix from Promega, Mannheim). FITC- and biotin-labeled primers were used in PCR amplification (25 cycles of 1 min at 94 °C, 1 min at 48 °C, and 1 min at 72 °C, followed by 10 min at 72 °C). M280 streptavidin magnetic beads were added to the PCR product to separate the ssDNA which was used as the library for the next selection round. [18]

Cloning and sequencing

Aptamers obtained from the tenth round of selection were PCR-amplified using

unmodified primers and cloned into Escherichia coli using the TA cloning kit (Invitrogen). Plasmids of individual clones were isolated by the plasmid extraction kit (Qiagen Germany, Duesseldorf), and inserts were amplified by PCR and sent to be sequenced (Applied Biosystems). Individual FITC-aptamers were prepared to perform the binding affinity identification [19].

FACS assay of aptamer binding affinity

200 pmol FITC-labeled aptamer was incubated with 10^5 osteoblasts at 37 °C for 30min, washed three times and analyzed by the FACS detection. The same amounts of osteoblasts were used as a control. The aptamers which had the best binding affinity were chosen to continue with further identification. The second structure of the aptamer was analyzed by DNASYS software.

Capture ability assay of aptamer

12-well cell culture plates (Greiner Germany, Frickenhausen) were coated with streptavidin overnight at 4°C, and washed with PBS-T (0.05% Tween-20) several times. Biotinylated-aptamer and biotinylated-library were added to different wells and incubated at 30°C for 4 hours. The plate was washed with PBS-T and incubated with osteoblasts solution at 37°C for 30 min with gentle shaking. Then the cell solution ($1 * 10^5$)was decanted and the plate was washed several times to discard the non-adhered cells. Cell attachment was observed under an inverse microscope (Zeiss Axiovert 135, Zeiss, Germany) with 200 fold magnification and the adherent cell number from three individual groups were calculated per eyeshot and the deviations were analyzed by t-test.

Plasma stability

Fresh human plasma was prepared by 3000g centrifugation of the whole blood for 20 min. 8nmol aptamer was incubated at 37°C in a final volume of 0.5 ml heparinized human plasma. Samples of 50 µl were taken out and collected

after 0, 0.5, 1, 1.5,2, 3, 4, and 6 h. Full-length and digested oligonucleotides were separated on a 2% agarose gel and photographed.

Results

Selection of ssDNA Molecules bound to osteoblasts

Human sarcoma osteoblasts were used as the target for in vitro selection of aptamers from a random pool of 10^{15} DNA molecules. The starting library was composed of 79-mer single-stranded DNA molecules containing randomized 40-nucleotide inserts. To monitor the enrichment of specific cell-binding aptamers during selection, SELEX pools of the second and following rounds were analyzed by flow cytometry after the incubation with osteoblasts (Fig. 1). In each round of selection, the concentration of competitor DNA was increased to further selection to create a small but high-affinity and high-specificity aptamer pool. Analysis of fluorescent-labeled pools in successive cycles of selection showed a shift of second round histogram toward higher fluorescent intensity. After 7 rounds of selection, the fluorescence of the osteoblast-selected round-8 pool showed no further increase, the pool was then cloned and sequenced.

Sequences from 36 clones were obtained, and their inserts were analyzed and sorted into putative families through the alignment of consensus motifs. Motifs were identified by inspection with the aid of computer-assisted search engines (data not shown). On the basis of the demonstrable binding to osteoblast, one aptamer, O-7, was chosen for further characterization. The fluorescence of O-7 binding to osteoblast was shown in Figure 2, and the second structure of O-7 was shown in Figure 3.

Capture ability assay of aptamer

In this assay, we immobilized the biotinylated aptamerO7 on the streptavidin coated plate and then added osteoblasts to see if they could bind. Compared to the plate without aptamer coating, the plate with aptamer coating attached

more cells in a short time. The result shows that the aptamer could bind well with the target when fixed on a solid surface (see Figure 4). There is a significant deviation between the sample groups (83±1.5) and control groups (3.667±1.88) ($p<0.01$).

Plasma stability

For clinic or therapeutic applications, aptamers must resist rapid degradation by exo- and endonucleases. Human plasma predominantly contains a high 3'-exonuclease activity [20]. In human blood plasma, the natural aptamer has an estimated life of at least 6 h as determined by agarose gel analysis (Figure 5). This is enough time for the cells to grow on the scaffold following their first attachment. This result showed that the aptamer O7 could resist the enzyme digestion and did not need extra modification.

Discussion

Since the SELEX method was invented in the 1990s, research interests have greatly increased in this area. Numerous nucleic acid ligands, also termed aptamers, have been developed using this technology. The targets vary from protein, antigen, virus to cell, irons etc [21] [22]. The affinity and specificity of aptamers to a given target make it possible to isolate virtually any target; from adjusting their bioavailability to their clinical utility. However, to our knowledge, no research has been reported on the use of aptamers in scaffold as a coating material. In cell-based tissue engineering, scaffolds play important roles in guiding tissue morphogenesis. The matrix stimulates cells to influence differentiation and the cells actively remodel the matrix via local proteolysis activity. The matrix can signal cells by binding adhesion and growth factors, and they also respond to the remodeling influence of cell-associated proteases [23]. When used as scaffolds, common polymers, such as poly (lactic acid) and related polyesters do not accommodate the sensitivity to cell-associated proteases and binding to cell surface adhesion receptors. Here, we present a

new molecular strategy for the first time. We make use of the high affinity between aptamers and its target cells. By immobilizing the aptamers on a solid surface to attach suspended cells, we proved the principle that aptamers work well when immobilized on the scaffold. This approach may be useful to improve the adherence of the seeding cells. For example, local presentation or delivery of progenitor cells on the scaffold can speed up the repair or enhance the tissue function after trauma by proliferation and differentiation. The immobilized aptamers on the scaffold can increase the attachment of cells to the surface significantly, so the seeding efficiency will be largely increased and the time for the whole tissue regeneration may be shortened. This innovative application of aptamers will bring new improvements to the clinical use of scaffolds and advanced chances for the field of tissue engineering and regenerative medicine.

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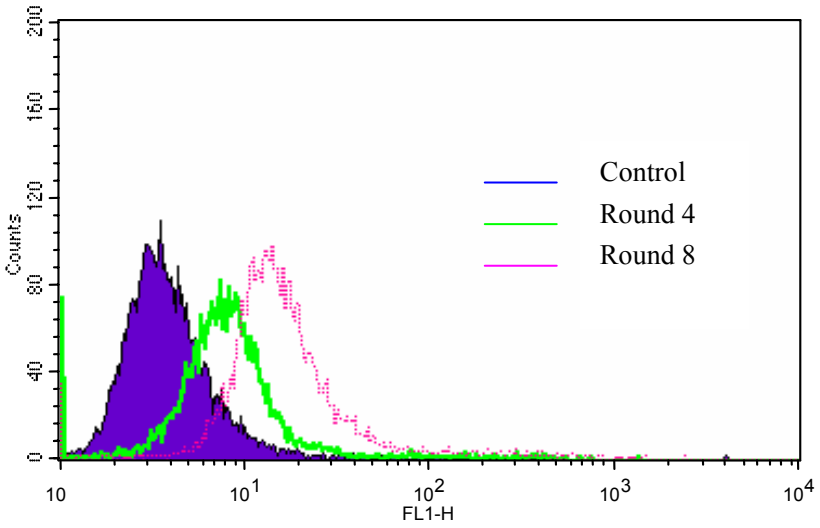

Figure 1 Fluorescence screening of selection rounds.
The blue curve is the control, the green curve is the round 4 and the red curve is the round 8, the fluorescence is increased gradually.

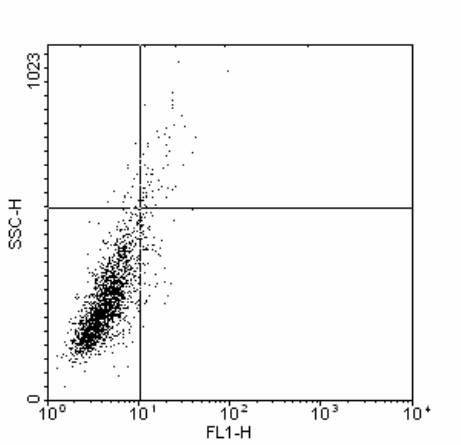

Figure 2a

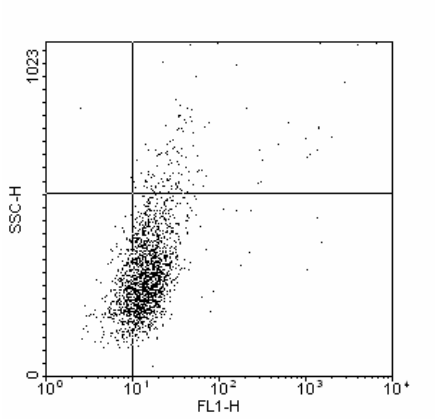

Figure 2b

Figure 2: Fluorescence identification of cell binding affinity assay with FITC-aptamer O-7. A is the control (without aptamer, only cells); b is the cells incubated with FITC-aptamers. The binding ratio is about 70%.

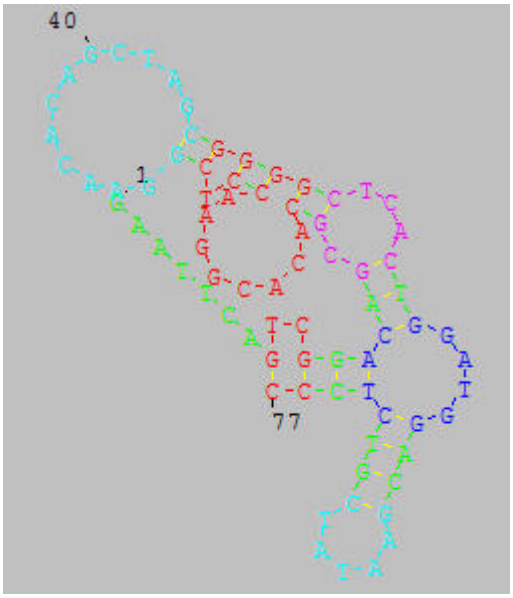

Figure 3 second structure of aptamer O-7

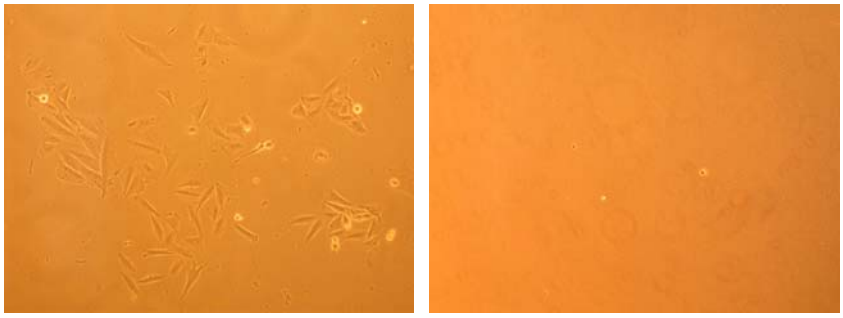

Figure 4: capture ability assays of aptamer

The left picture is the culture plate coated with aptamer, where a lot of cells were captured and started growing; the right is the culture plate coated with the library, where fewer cells were attached.

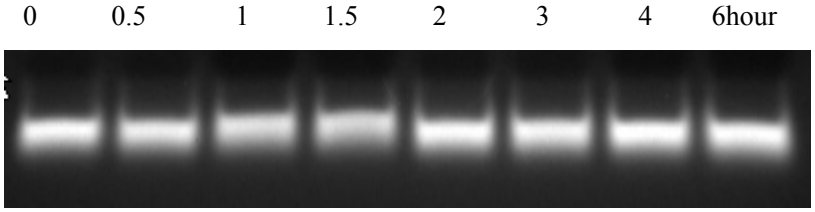

Figure 5 plasma stability assay of aptamer
Analysis of the stability of aptamerO7 in human blood plasma was observed by agarose gel electrophoresis. Samples were taken out on different time point from 0 hour to 6 hours. The result shows that the aptamer O7 can resist the degradation at least 6 hours.

Results Part 2:

The effect of electrochemical functionalization of Ti-alloy surfaces by aptamer-based capture molecules on cell adhesion.

Biomaterials. accepted

Ke-Tai Guo, Dieter Scharnweber, Bernd Schwenzer, Gerhard Ziemer, Hans P. Wendel

The SELEX-technology for the generation of aptamers against osteosarcoma cells was exclusively developed by me. All experiments were planed, performed and analyzed by me, with the exception of the part “Immobilization procedure of aptamers on Ti-alloy“ which was performed by D. Scharnweber and B. Schwenzer. The publication was written by me. The idea was from Hans P. Wendel and he supervised my work and corrected the manuscript. G Ziemer was intensively involved in the planning of the study. This manuscript is submitted to Biomaterials.

Electrochemical functionalization of Ti-alloy surfaces by aptamer-based capture molecules accelerates cell adhesion

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Abstract

To improve cell seeding efficiency and cytocompatibility, we designed a new coating material for scaffolds. We used aptamers, highly specific cell binding nucleic acids generated by combinatorial chemistry with an in vitro selection called systematic evolution of exponential enrichment (SELEX). In this study, we functionalized Ti-alloy surfaces to enhance cell adhesion. By coating the material with a cell specific aptamer, working as a capture molecule, we could improve the attachment of cells effectively and avoid the limitations of the currently available materials. Aptamers, immobilized by partial electrochemical entrapment in oxide layers on Ti-alloy surfaces were able to capture cells out of a flowing suspension rapidly. This model proves that surface immobilized aptamers can greatly enhance the attachment of seeded cells. This technology opens new perspectives towards clinical application of stem cell and tissue engineering strategies.

Key word: aptamer, Ti-alloy

Introduction

Nowadays, new ideas arise rapidly to optimize and develop the surface properties of medical implants or even tissue engineered scaffolds for culturing cells for various therapeutic applications. Different metallic as well as biodegradable and non-degradable polymers with specified surface modifications are used as implants or scaffolds. Interactions between cells and artificial surfaces which occur for instance at the bone-implant interphase are the direct result of the biochemical environment that is provided by the biomaterial surface. The surface chemistry depends both on the properties of the bulk material and the chemistry of the surrounding environment. This is due to the fact that almost all implanted materials rapidly become coated with e.g. plasma proteins and lipids and these adsorbed biomolecules are the principal mediators of the cellular acceptability to most materials. The net effect involves an interaction between a given surface and the biomolecules available in the respective environment and their surface adsorption. As proteins adsorb, they usually undergo a change in conformation, which may include denaturation or unfolding. This, in turn, may either hide or expose sites within the protein that interact with cell surface receptors [1, 2]. Current implants and scaffolds used in tissue engineering can be classified in three main groups: first metallic materials with excellent mechanical properties; second synthetic polymers made by conventional scaffold fabrication techniques; third the natural biomaterials that possess elaborate optimized

space-filling, three-dimensional architectures [3, 4]. Recent research activities focus on material selectivity and fabrication methods of implants as well as on functionalizing of surfaces to make them more compatible for cell seeding. Especially, coating of surfaces with PEG or RGD peptides has shown better cell adhesion [5-7]. Surfaces conjugated with growth factors like BMP-2 can also increase the cell attachment [8, 9]. Many factors affect the application of implants including bulk material, three-dimensional architecture and porosity, surface chemistry, mechanical properties, initial scaffold environment (osmolarity and pH) and late material environment (degradation characteristics). The first and important step concerning the application of an implant is to attach the seeded cells, but most of the materials face the difficulty, that the desired cells cannot recognize the surface well, and therefore cannot grow orderly. To solve this problem, we tried to modify the surface of an implant material to facilitate the cell attachment. In the following exposition we present a new coating material out of high specific DNA-aptamers to enhance the rapid adhesion of seeded cells on Ti-alloy surfaces.

2 Materials and Method

2.1 Cells and culture

SAOS-2 osteoblasts were cultured in McCoy's medium supplemented with 10% FCS, 1%Penicillin/Streptomycin, and 1% L-Glutamine. (Invitrogen, Karlsruhe, Germany) as previously described [10].

2.2. DNA library and primers

The DNA oligonucleotide library contains a 40-base central random sequence flanked by primer sites on either side. (5'-GAATTCAGTCGGACAGCG-N40-GATGGACGAATATCGTCTCCC-3') The FITC-labeled forward primer (5'-C_{12}-FITC-GAATTCAGTCGGACAGCG -3') and biotin-labeled reverse primer (5'-Bio -GGGAGACGATATTCGTCCATC-3') were used in PCR to get the double-labeled DNA and to separate the single strand DNA by streptavidin dynabeads (Dynal, Denmark). The library and all primers were synthesized by Operon Technologies (Cologne, Germany).

2.3. SELEX procedure

Selecting DNA aptamer with a high affinity to osteoblast was performed as follows. ssDNA pools were denatured by being heated at 80 °C for 10 min in a selection buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM KCl, 100 mM NaCl, 1 mM MgCl2, and 0.1% NaN3 and then renatured at 0 °C for 10 min. To reduce background binding, a 5-fold molar excess of yeast tRNA (Invitrogen) and bovine serum albumin (BSA. Sigma-Aldrich, Munich) were added. The osteoblasts were incubated with the ssDNA library at 37 °C for 30 min in the selection buffer. Partitioning of bound and unbound ssDNA sequences was done by centrifugation. After centrifugation and having been washed three times with a 1ml selection buffer (with 0.2% BSA), cell-bound aptamers were

amplified by PCR (Master Mix from Promega, Mannheim). FITC- and biotin-labeled primers were used in PCR amplification (25 cycles of 1 min at 94 °C, 1 min at 48 °C, and 1 min at 72 °C, followed by 10 min at 72 °C). M280 streptavidin magnetic beads were added to the PCR product to separate the ssDNA which was used as the library for the next selection round. [11] Aptamers obtained from the tenth round of selection were PCR-amplified using unmodified primers and cloned into Escherichia coli using the TA cloning kit (Invitrogen). Plasmids of individual clones were isolated by the plasmid extraction kit (Qiagen Germany, Duesseldorf), and inserts were amplified by PCR and sent to be sequenced (Applied Biosystems). Individual FITC-aptamers were prepared to perform the binding affinity identification [12].

2.4 Immobilization procedure of aptamers on Ti-alloy

Ti-alloy discs (Ø16mm x 2 mm) of Ti6Al7Nb (Synthes Inc.) were ground down to a size of P600 (26μm) and etched for 2 min in a mixture of 0.4 M HF and 1 M HNO3 at room temperature. The immobilization was carried out in a conical cell made from acrylic glass. After filling the cell with 800 μl 0.04 M acetate buffer (pH = 4.0) containing the 5'-phosphorylated aptamer in concentration of 400 nM it was incubated for 15 minutes. Subsequently, for stable fixation the anodic polarization was carried out in potentiostatic mode by a potential of 8 V_{SCE} for 100 seconds using a Voltalab 4.0 combined with a high voltage booster HVB100 (Radiometer Analytical, Copenhagen) after adding 3 ml with

0.04 M acetate buffer (pH = 4.0) to the electrochemical cell. Following electrochemical treatment the cell including the sample was washed three times for two minutes with 3 ml of 40 mM acetate buffer three times for two minutes with distilled water and afterwards the samples were dried in air.

2.5 Binding affinity of aptamer to osteoblasts

2.5.1 FACS assay of aptamer binding affinity

200 pmol FITC-labeled aptamer was incubated with 10^5 osteoblasts at 37 °C for 30min, washed three times and analyzed by the FACS detection. The same amounts of osteoblasts were used as a control. The aptamers which had the best binding affinity were chosen to continue with further identification.

2.5.2 Aptamer mediated cell sorting with magnetic beads

The biotinylated aptamer was incubated with osteoblasts for 30 minutes and anti-biotin microbeads were added. The same amount of osteoblasts without aptamers was also incubated with anti-biotin microbeads, acting as a negative control. The mixture was washed three times and percolated through a magnetic column. The magnetic beads which were bound to the aptamer were collected and put into cell culture medium.

2.6 *The attachment of osteoblasts on Ti-alloy coated with aptamer*

The aptamer was immobilized on a Ti-alloy surface by partial oriented

electrochemical entrapment in the oxide layer [13]. For that the 5'-phosphorylated aptamer and 5'-phosphorylated-library was adsorbed on the Ti-alloy surface followed by a potentiostatic polarisation to 8 VSCE resulting in a regiospecific entrapment (5'-end) of the aptamer with a depth of about 8 nm. The aptamer coated Ti-alloy (Ti6AI7Nb) was incubated with osteoblasts and human embryonic kidney cell (293 cells) at 37°C for 45minutes with a gentle vibration to imitate the circulation inside the bone. The fibronectin coated Ti-alloy (Ti6AI7Nb); the oligo library (scrambled sequence) coated Ti-alloy and non-coated Ti-alloy was incubated with osteoblasts and HEK 293 cells respectively as controls. After several times of washing the Ti-alloy surface was stained with fluorescindiacetate and examined under the microscope (Zeiss, Germany). To observe the binding between aptamers and osteoblasts, after the incubation of osteoblasts to the aptamer-coated Ti-alloy surfaces, the Ti-alloys were fixed in 2.5% glutaraldehyde, dehydrated through a graded series of ethanol, vacuum dried, mounted onto aluminium stubs and sputter coated with gold-palladium. Samples were examined under a scanning electron microscope (stereoscan 250, Cambridge Instruments limited, UK).

2.7 Plasma stability

To valid the biological stability for potential applications of aptamers on scaffolds inside the bone, we carried out an aptamer plasma stability experiment. Fresh human plasma was prepared by 3000g centrifugation of the

whole blood for 20 min. 8nmol aptamer was incubated at 37°C in a final volume of 0.5 ml heparinized human plasma. Samples of 50 µl were taken out and collected from 0 to 24 h. Full-length and digested oligonucleotides were separated on a 2% agarose gel and photographed.

2.8 Aptamer mediated protein purification

An affinity purification procedure was performed to identify the aptamer target on osteoblasts. Cell surface extracts were incubated with biotinylated oligonucleotides immobilized on streptavidin magnetic beads. The aptamer-bead complexes were washed extensively and the bound proteins were eluted and then resolved electrophoretically before coomassie staining.

3 Results

3.1 Selection of ssDNA Molecules bound to osteoblasts

Human sarcoma osteoblasts were used as the target for in vitro selection of aptamers from a random pool of 10^{15} DNA molecules. In each round of selection, the concentration of competitor DNA was increased to further selection to create a small but high-affinity and high-specificity aptamer pool. After 7 rounds of selection, the fluorescence of the osteoblast-selected round-8 pool showed no further increase, the pool was then cloned and sequenced. Sequences from 36 clones were obtained, and their inserts were analyzed and sorted into putative families through the alignment of consensus

motifs. Motifs were identified by inspection with the aid of computer-assisted search engines (data not shown). On the basis of the demonstrable binding to osteoblast, one aptamer, O-7, was chosen for further characterization. The sequence of O-7 was 5'-GAATTCAGTCGGACAGCGCACACGGAACCTCGGAACACAGCTAGCGG GGCTCACTGGATGGACGAATATCGTCTCCC-3'

3.2 Binding ability of aptamer to osteoblasts

The flow cytometry assay shows aptamer O7 can bind with osteoblasts (Figure1). The cell culture showed no cell attachment within the negative control (Figure 2-B), whereas those osteoblasts incubated with surface immobilized aptamers on the microbeads recorded high cell number and growth (Figure 2-A). This result showed that the generated aptamer can capture osteoblasts out of a cell solution.

3.3 *The attachment of osteoblasts on Ti-alloy coated with aptamer*

The results (Figure 3) showed that aptamer coated Ti-alloy attached significant more osteoblasts compared to library coated Ti-alloy. The fibronectin coated Ti-alloy attached some osteoblasts while the control surface without coating bonded almost no cells. For the HKE 293 cells, the fibronectin-coated Ti-alloy surface binds more cells than the aptamer-coated, library-coated and non-coated Ti-alloy surface. After culturing for 24 and 48 hours, the

osteoblasts grow well on the aptamer-coated Ti-alloy surface, see Figure 4. This model shows, that surfaces functionalized with specific aptamers are able to significantly and specifically increase the adhesion of osteoblasts. Figure 5-A showed that Ti-alloy surfaces with aptamer coating attach cells stronger and tighter than natural Ti-alloy (Figure 5-B).

3.4 Plasma stability

For clinic or therapeutic applications, aptamers must resist rapid degradation by exo- and endonucleases. Human plasma predominantly contains a high 3'-exonuclease activity [20].The electrophoresis results showed that the aptamer could resist plasma degradation for 48 hours (Figure 6). This time is enough for the cells to grow on the scaffold following their first attachment. This result showed that the aptamer O7 could resist the enzyme digestion and did not need extra modification.

3.5 Aptamer mediated protein purification

The aptamer O-7 (Figure 7) showed a specific interaction with a high-molecular weight polypeptide corresponding to 200kDa. The band was cut off, trypsinized and further analysed. The sequence of the protein was blasted in the data and showed, that the gene MYH-9 coded a special type of a non muscle myosin.

Discussion

SELEX is a combinatorial technique to screen ultra large libraries consisting of 10^{15} different oligonucleotides by an iterative process of in vitro selection and amplification. [14, 15] The combinatorial libraries based on replicable nucleotides offer the convenience of amplification and make the screening process fast and easy. In the selection procedure, the library is incubated with a target of interest, which is often fixed on a solid vector such as nitrocellulose membranes or columns. The population of unbound sequences is washed away; the binding sequences are isolated and amplified to obtain an enrichment library for the next round of selection. The enrichment efficiency can be detected by continual binding assays like in flow cytometry. When the binding saturation is achieved after several rounds of selection, the very few selected oligonucleotides which remained from the start library will be cloned and sequenced to obtain the single sequences with high affinity to their target, which were called aptamers. The length of an aptamer is mostly 50-80 bp. The relative small molecular structure makes it suitable for application on the molecular reorganization. Aptamers can fold into a three-dimensional structure based on their nucleic acid sequence to bind with the target. The dissociation constants (Kd) between the aptamer and its target are frequently in the picomolar or even nanomolar ranges showing a high specific interaction. As a

benefit from the advantage of being non-immunogenic and showing rapid kidney clearance and easy plasticity, aptamers can be used as both diagnostics and therapeutics.

Cytocompatible surfaces play an important role in cell-based tissue engineering. Ideally, they should provide a surface to promote the attachment, migration, proliferation and desired differentiation of progenitor cells. To improve the compatibility of materials for seeded cells, chemical modifications either of the surface or the bulk materials that endow implants with biological functions as well as regulating attachment has been developed [16]. One method to improve cell adhesion and migration onto a material is to tether cell-binding peptides which mimic and induce cell-extracellular matrix (ECM) protein interactions to the biomaterial surface by physical or chemical modification methods [17]. Many investigations focused on the interactions of bone-forming cells with various integrin-binding domains like the arginine-glycine-aspartate (RGD) oligopeptide [18-20]. Most of the methods were complex and extremely laborious. In our research we successfully applied a new aptamer-based capture molecule in order to modify the surface of a scaffold. In principle, nucleic acids with aptamer function have considerable advantages over proteins and peptides as bioactive molecules immobilized on surface. Aptamers can be generated easily and the coating technique on Ti-alloy can be performed quick and easy and reproducible. Using the described method of fast and stable electrochemical entrapment [21]

the nucleic acids could be immobilized in a manner that substantially retains their accessibility and biological functionality as an aptamer. Compared to proteins, the resulting surface stated with nucleic acid-mediated specific biochemical information shows very low immunogenic potential. Furthermore, by using several different aptamers for entrapment into the oxide layer a modular system for tailoring biochemical surface properties should be available. If necessary for using under physiological conditions, the stability of nucleic acids against nucleases may be enhanced by modification of individual nucleotide residues. Moreover, it was shown that it is possible to sterilize the coated scaffolds using gamma radiation without loosing the binding affinity of aptamers [13].

This physical entrapment of cells will not change the character of seeded cells as shown in Fig.1d. It enhances cell adhesion without any side effects. Although the concept of using aptamers as therapeutic agents exists for more than 10 years and meanwhile the first aptamer-based drug has been approved by the FDA [22], there is no report with regard to the application in the field of tissue engineering. For the first time, this study showed a new and successful principle to apply aptamers on Ti-alloy surfaces to promote cell adhesion. This concept may have the potential to boost the clinical application of cell-based tissue engineered implants. Further research will focus on using aptamers for stem cell immobilization on surfaces of real scaffolds to facilitate clinical applications like bone repair or local stem cell homing.

Conclusion

This study showed the prove of principle for the application of surface-coated aptamers to work as capture molecules for cell adhesion. We generated aptamers with high affinity to osteoblasts which were able to rapidly attach osteoblasts from cell solutions, when coated on Ti-alloy surfaces. This technology opens new perspectives towards clinical application of stem cell and tissue engineering strategies.

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Legends to figures

Figure 1

Fluorescence identification of cell binding affinity assay with FITC-aptamer O-7.

The red curve is the control (without aptamer, only cells); the black curve represents the cells incubated with FITC-aptamer O-7

Figure 2

Binding assay of aptamers.

Cell sorting. Osteoblasts were incubated with biotinylated aptamers and pulled down together with anti-biotin microbeads (Figure 2-A). They growed well in culture; while the non-labeled microbeads did not bind any cells; they were washed through the magnetic filter and no cells were held back on the magnetic columns, resulting in very less cells on the culture flask (Figure 2-B).

Figure 3

Fluorescence cell staining of osteoblasts and HEK 293 cells captured by aptamer coated; uncoated, fibronectin-coated and library-coated Ti-alloy surfaces (A-H). Each experiment was made with osteosarcoma osteoblasts incubating on the Ti-alloy discs for 45 minutes (100 x).

(A) Osteoblasts on aptamer-coated Ti-alloy

(B) Osteoblasts on library- coated Ti-alloy

(C) Osteoblasts on fibronectin- coated Ti-alloy

(D) Osteoblasts on non- coated Ti-alloy

(E) 293 cells on aptamer- coated Ti-alloy

(F) 293 cells on fibronectin - coated Ti-alloy

(G) 293 cells on library- coated Ti-alloy

(H) 293 cells on non- coated Ti-alloy

Figure 4

The growth of Osteoblasts on aptamer- coated Ti-alloy after culturing for 24 hours (Figure A, 400 x) and 48 hours (Figure C, 400 x), non- coated Ti-alloy surfaces incubated with osteoblasts for 24 hours (Figure B) and 48 hours (Figure D, 400 x)

Figure 5

Scanning electron micrograph of cell-seeded Ti-alloy discs.

Figure 4-B shows the binding of cells on the non-coated Ti-alloy, Figure 4-A is the cell binding on the aptamer coated Ti-alloy surface, showing more strong and tight incorporation, reflecting the cell-friendly surface properties (magnification: bar=10μm).

Figure 6

Plasma stability assay.

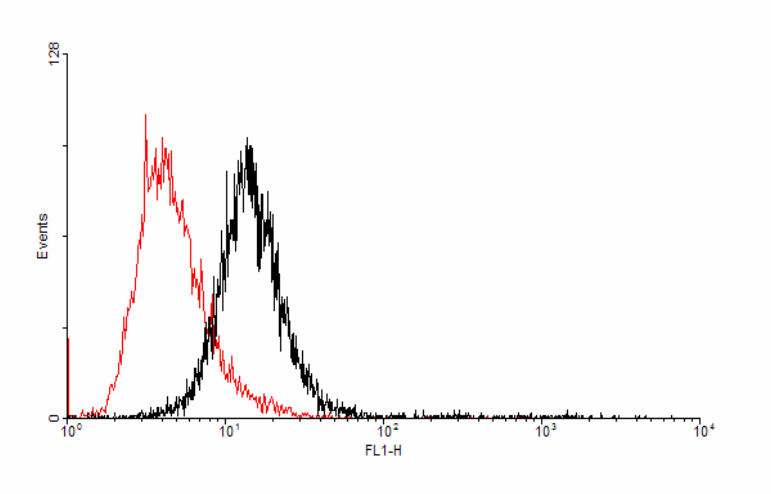
Analyses of the stability of aptamer O-7 in human blood plasma was shown by agarose gel electrophoresis. Samples were taken out on different time points from 0 to 48 hours. The results show, that the aptamer can resist degradation at least until 48 hours.

Figure 7

Aptamer mediated protein purification.

Lane 1 shows the whole cell lysis extraction, lane 2 is the purified protein by aptamer O-7, and the arrow is the target protein on the membrane.

Figures



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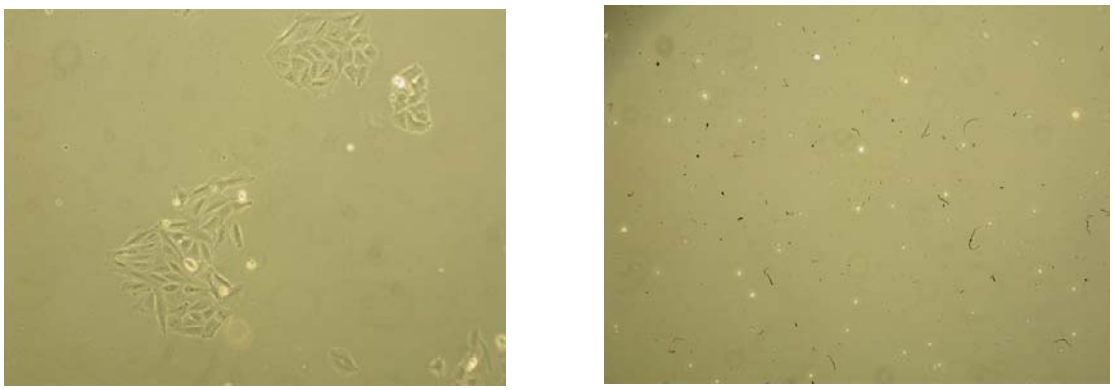
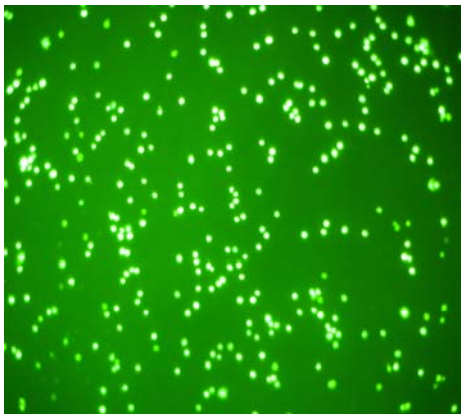


A B

Figure 2



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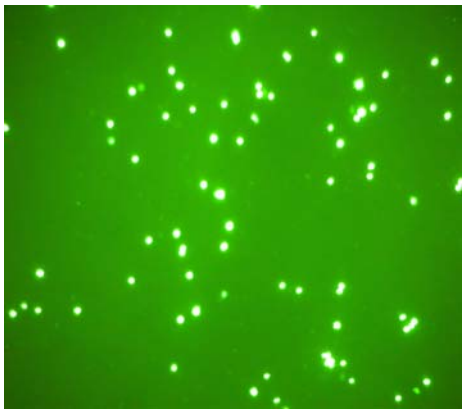

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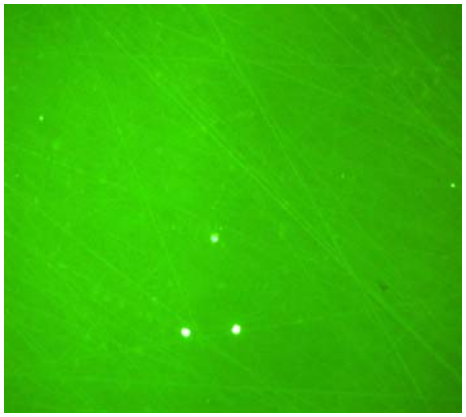

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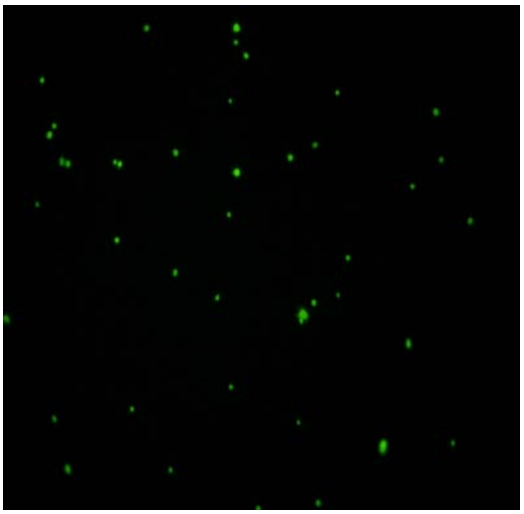

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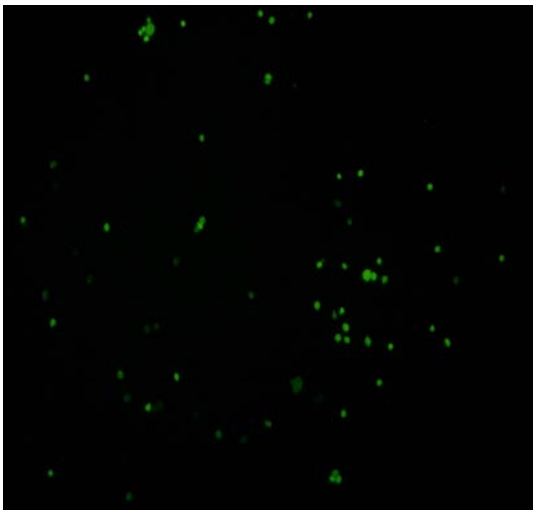

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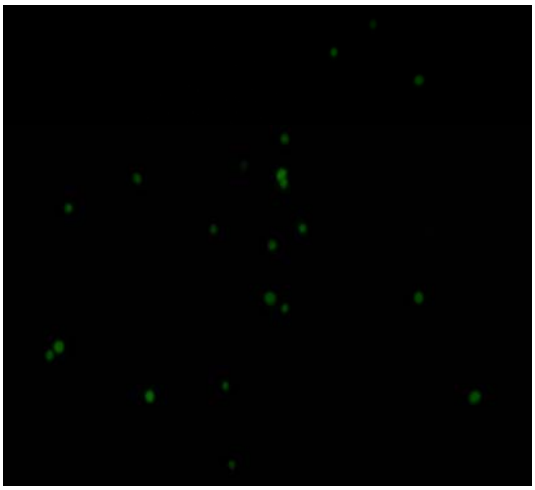

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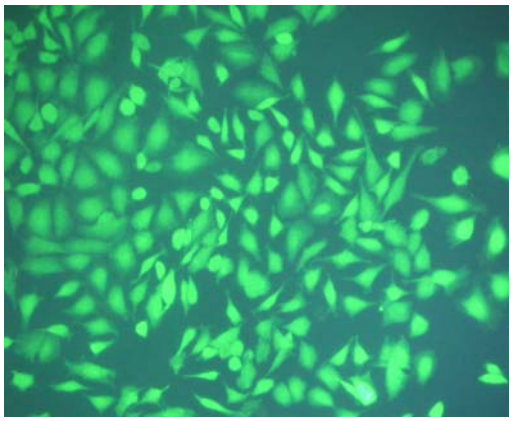
Figure 3



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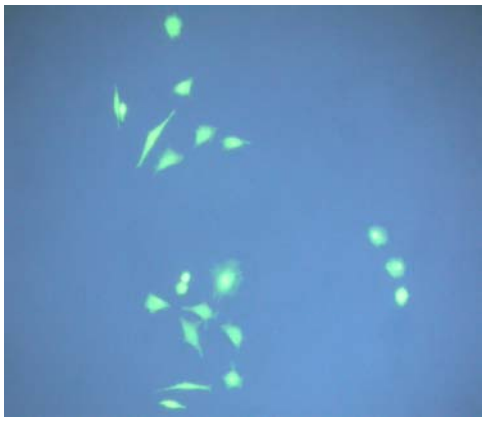

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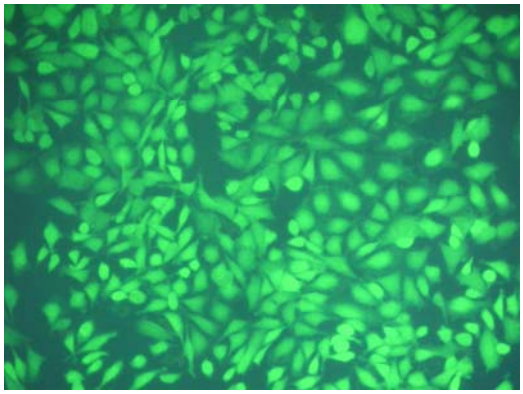

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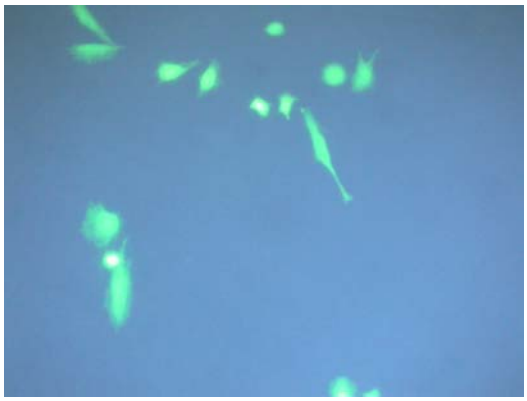

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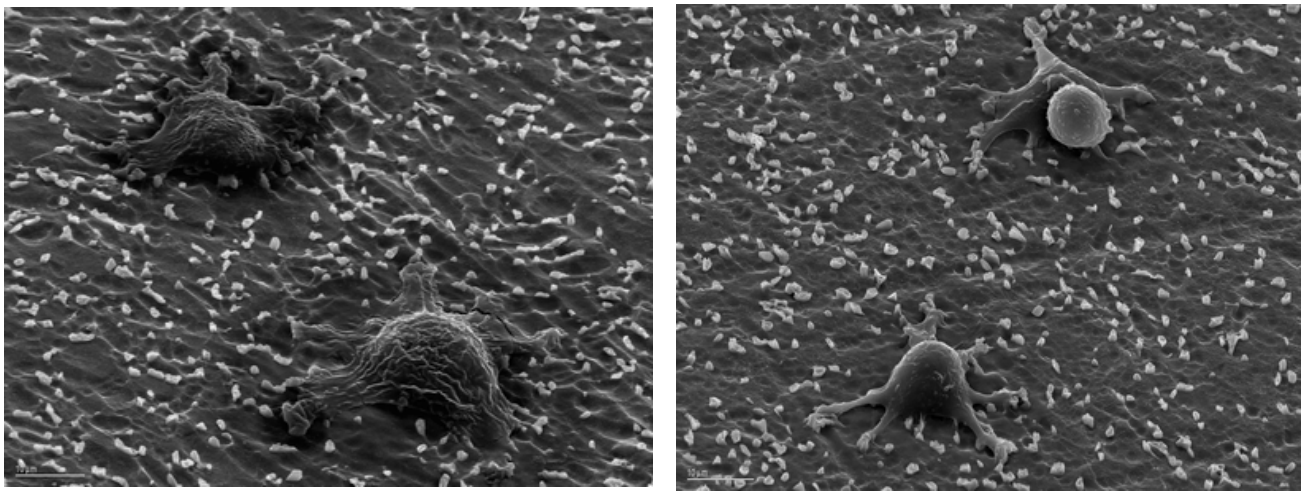
Figure 4



A B

Figure 5

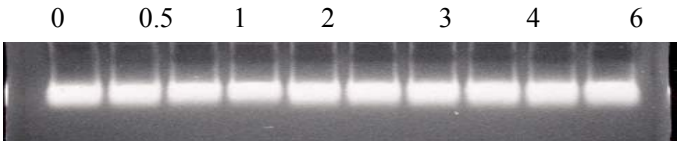


Figure 6

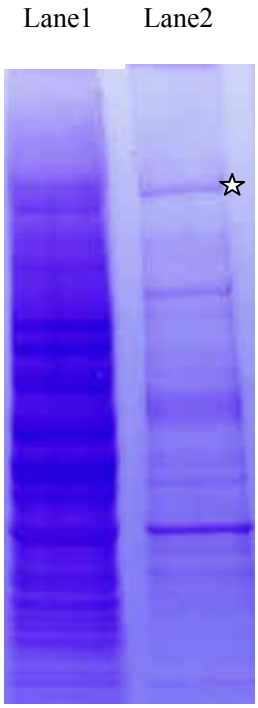


Figure 7

Results Part 3:

A new technique for the isolation and surface immobilization of mesenchymal stem cells from whole bone marrow using high specific DNA-aptamers.

Stem Cells. In press 2006

Ke-Tai Guo, Richard Schäfer, Angela Paul, Annika Gerber, Gerhard Ziemer, Hans P. Wendel

The SELEX-technology for the generation of aptamers against pig mesenchymal stem cells was exclusively developed by me. All experiments were planed, performed and analyzed by me, with the exception of the figure 1 a (C-F), figure 1b, figure 2(a-c), figure 6 which were performed by R. Schäfer and A. Gerber. Figure 4 were performed by me and R. Schäfer together. A. Paul helped me with the culture of the mesenchymal stem cells. The publication was written by me. The idea was from Hans P. Wendel. He supervised my work and corrected the manuscript. G. Ziemer was intensively involved in the planning of the study.

A new technique for the isolation and surface immobilization of mesenchymal stem cells from whole bone marrow using high-specific DNA-aptamers

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ABSTRACT

Adult mesenchymal stem cells (aMSCs) are a stem cell population present in bone marrow which can be isolated and expanded in culture and characterized. Due to the lack of specific surface markers, it is difficult to separate the MSCs from bone marrow directly. Here we present a novel method using high-specific nucleic acids called aptamers.

Porcine MSCs were used as a target to generate aptamers by combinatorial chemistry out of a huge random library with in vitro technology called systematic evolution of exponential enrichment (SELEX). After cloning and sequencing, the binding affinity was detected using a cell sorting assay with streptavidin-coated magnetic microbeads. We also used 12-well plates immobilized with aptamers to fish out MSCs from the cell solution, and a FITC-labeled aptamer to sort MSCs from bone marrow using high-speed FACS. The cells showed high potency to differentiate into osteogenic, as well as into adipogenic lineages with typical morphological characteristics. Surface marker staining showed that the attached cells were $CD29^{+}$, $CD44^{+}$, $CD45^{-}$, $CD90^{+}$, SLA class I^{+}, SLA DQ^{-} and SLA DR^{-}.

Compared to existing methods, this study established a novel, rapid, and efficient method for direct isolation of aMSCs from porcine bone marrow by using an aptamer as a probe to fish out the aMSCs. This new application of aptamers can facilitate aMSCs isolation and enrichment greatly, thereby enhancing the rate of aMSC-derived cells after in vitro differentiation for various applications in the emerging field of tissue engineering and regenerative medicine.

INTRODUCTION

Adult Mesenchymal stem cells (aMSCs) are commonly regarded as multipotent $CD29^+$, $CD44^+$, $CD90^+$, $CD11b^-$,$CD34^-$,$CD45^-$ progenitor cells with broad differentiation potential into mesodermal and ectodermal, as well as entodermal cells and tissues respectively [1,2]. Previous studies have shown that when aMSCs are induced in vitro they are capable of differentiating into osteoblasts, chondrocytes, and adipocytes[3, 4]. Remarkably, less than 0.05 % of the bone marrow cells show aMSC-specific properties. aMSCs have many advantages for application in bone tissue engineering. The isolation of aMSCs from bone marrow is easy due to their adherence to tissue-culture plastic, they have potential proliferation and differentiation ability, and it is easy to culture and store them in vitro [5]. Current aMSCs isolation strategies depend on adherence to plastic surfaces. First, the isolated mononuclear cells from bone marrow were cultured until cell confluence is formed. Then, the desired differentiation potential of the adherent cells must be identified to confirm the right cell source. This procedure is very time consuming and requires at least two additional weeks. Therefore, a quick and efficient method to isolate aMSCs directly from bone marrow is urgently needed for therapeutic applications, as well as for tissue engineering applications. To overcome this problem, antibody-based separation techniques have been developed, for example by using LNGFR (Low-affinity nerve growth factor receptor, also called CD271) [7]. However, the biological impacts of the antibody binding to aMSCs are unknown, and this technique cannot be used to fix the living cells onto scaffolds for the regeneration of structural and functional tissues.

In this study, we present a new class of capture molecules called aptamers which can be used to isolate aMSCs from whole bone marrow directly and easily. Further, the analysis of freshly aptamer-isolated aMSCs from bone marrow will reveal novel insights into the aMSCs subpopulations and their antigenic profile in their natural environment.

Aptamers are single-stranded DNA or RNA molecules which can fold into a

three-dimensional structure to bind to a variety of targets, including proteins, peptides, enzymes, antibodies and various cell surface receptors [8-10]. Aptamers can be up to 100 bases long and can be generated from combinatorial libraries through an in vitro selection process called systematic evolution of ligands by exponential enrichment (SELEX) [11]. Aptamers have significant advantages compared to other targeting agents. They bind to their target with high affinity and selectivity without immunogenic or toxic effects, and show quick circulation clearance. Moreover, they can be easily synthesized in vitro. Aptamers are used in both diagnostic and therapeutic fields to place antibodies, as specific inhibitors[12] or as biosensors to detect a desired target [10]. These favorable properties of aptamers might also be applicable in the emerging field of regenerative medicine for isolation and selective binding of aMSCs.

MATERIALS AND METHODS

aMSCs isolation and culture

Fresh bone marrow was extracted from porcine femur under sterile conditions. The animals (pigs [German Land Race], 50 kg, male) were kept and treated according to the animal welfare regulations of the University of Tuebingen. Porcine aMSCs were isolated according to the modification methods previously described[13]. Briefly, mononuclear cells (MNCs) were isolated from bone marrow aspirate by centrifugation over a Ficoll histopaque layer (30 min, 400g, density 1.077). After centrifugation, the cells were cultivated under standard culture conditions with low-glucose Dulbecco's modified Eagle's medium (DMEM; Gibcol) supplemented with 10% fetal calf serum, penicillin (50U/ml), and streptomycin (50ug/ml). The medium was changed after the first 24 hours and then twice a week. When cells reached 80% confluence they were detached using 0.25% Trypsin-EDTA and re-plated in preparation for SELEX and differentiation potential assessments.

For the specificity tests (FACS with Aptamer), at and human aMSCs were isolated and characterized as above. The animals (Sprague Dawley rats) were kept and treated according to the animal welfare instructions of the University of Tuebingen. The human bone marrow was taken in the course of orthopedic operations, which was approved by the local Ethics Committee of the University of Tuebingen according to the Declaration of Helsinki. The murine P19 cells were purchased from ATCC (Manassas, VA, USA).

aMSCs characteristics

The potential of aMSCs to differentiate into adipogenic and osteogenic lineages was assayed as follows. For osteogenic differentiation, aMSCs were cultured in an osteogenic culture medium which includes 0.2 mM L-ascorbic acid 2-phosphate magnesium salt n-hydrate and 0.01 μM dexamethasone (Dex) (Sigma-Aldrich Co.), 10mM β-Glycerophosphate were added to the medium. After 21 days, the subcultured cell layers were washed with PBS, fixed with 4% paraformaldehyde, and stained according to the alkaline

phosphatase staining kit (Sigma kit #85). Five weeks after culturing, the deposition of mineralized bone matrix was identified using Von Kossa staining. Cell layers were fixed with 4% paraformaldehyde, incubated with 2% silver nitrate solution (w/v) for 10 min in the dark, washed thoroughly with deionized water, and then exposed to UV light for 15 min [13].

For adipogenic differentiation, aMSCs were stimulated with growth medium supplemented with 0.5 μM hydrocortisone, 0.5mM 3-isobutyl-1-methylxanthine and 60 μM indomethacin (Sigma-Aldrich) for three weeks. During these three weeks, the medium was changed twice a week. Cells were then rinsed twice with phosphate-buffered saline (PBS), fixed with 10% formalin for 10 min, washed with distilled water, rinsed in 60% isopropanol and covered with a 0.3% oil red O solution (Sigma-Aldrich) in 60% isopropanol for 10 min. Cultures were then briefly rinsed in 60% isopropanol and thoroughly in distilled water and then left to dry at room temperature [14]. The cultured MSCs were performed the surface marker identification by FITC-labeled monoclonal antibodies to CD29, CD44, CD45, CD90, SLA-class I, SLA DQ, and SLA DR (Becton Dickinson, Germany, Heidelberg). For the isotype controls, nonspecific mouse IgG was substituted for the primary antibody.

Selection of aptamer binding to aMSCs

DNA library and primers

The DNA oligonucleotide library contains a 40-base central random sequence flanked by primer sites on either side (5'-GAATTCAGTCGGACAGCG-N40-GATGGACGAATATCGTCTCCC-3'). The size of the library is about 10^{15}. The FITC-labeled forward primer (5'-C_{12}-FITC-GAATTCAGTCGGACAGCG -3') and biotin-labeled reverse primer (5'-Bio -GGGAGACGATATTCGTCCATC-3') were used in PCR to get the double-labeled DNA and separate the single stranded DNA by streptavidin-coated magnetic beads (M-280 Dynabeads, Dynal, Hamburg, Germany). The library and all primers were synthesized by Operon

Technologies (Cologne, Germany).

SELEX procedure The selection of the DNA aptamers of porcine aMSCs was performed as follows. 4nmol ssDNA pools were denatured by heating at +80 °C for 10 min in a selection buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM KCl, 100 mM NaCl, 1 mM $MgCl_2$, and 0.1% NaN3 and then renatured at 0 °C for 10 min. To reduce background binding, a 5-fold molar excess of yeast tRNA (Invitrogen, Karlsruhe, Germany) and bovine serum albumin (BSA, Sigma-Aldrich, Munich, Germany) were added. The mesenchymal stem cells (Passage 2, 10^6 cells for the first round and 10^5 cells for subsequent rounds) were incubated with the ssDNA at 37 °C for 30 min in the selection buffer. Partitioning of bound and unbound ssDNA sequences was done by centrifugation. After centrifugation and being washed three times with 1 ml selection buffer (with 0.2% BSA), cell-bound ssDNA were amplified by PCR (Master Mix from Promega, Mannheim, Germany). FITC- and biotin-labeled primers were used in PCR amplification (25 cycles of 1 min at 94 °C, 1 min at 48 °C, and 1 min at 72 °C, followed by 10 min at 72 °C). For flow cytometric analysis, FITC labeled ssDNA was prepared as described above. Aptamers obtained from the tenth round of selection were PCR-amplified using unmodified primers and cloned into Escherichia coli using the TA cloning kit (Invitrogen). Plasmids of individual clones were isolated by the plasmid extraction kit (Qiagen, Duesseldorf, Germany), and inserts were amplified by PCR and sequenced with the ABI PRISM® 377 DNA Sequencer (Applied Biosystems, Darmstadt Germany). Individual FITC-aptamers were prepared to perform the binding affinity tests [15]. As a rule, the SELEX procedure takes one to several months.

Aptamer binding to MSCs

FACS assay of aptamer binding affinity to aMSCs. 200 pmol FITC-labeled aptamer was incubated with 10^5 aMSCs at 37 °C for 30min, washed three

times and analyzed using flow cytometry (BD, Heidelberg, Germany). The same amounts of murine P19 cells, rat aMSCs and human aMSCs incubated with aptamer separately were used as a control. The aptamer which had the best binding affinity was chosen to continue the further identification process. The secondary structure of the aptamer was analyzed using DNASYS software (version 2.5; Hitachi Software Engineering Co).

***Aptamer binding to aMSCs*:** Biotinylated aptamer G-8 was synthesized by OPERON and incubated with 10^5 aMSCs for 30 min at 37°C, washed three times and incubated with anti-biotin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15min at 0°C. The same number of aMSCs without aptamer were incubated with anti-biotin microbeads and acted as a negative control. The mixture was washed three times and filtered through a magnetic column. Then the column was removed from the magnet holder and the beads were put into cell culture medium.

Aptamer binding to aMSCs in whole bone marrow.

Flowcytometry: 10ml fresh bone marrow was lysed with ammonium chloride and incubated with FITC-labeled aptamer (200pmol) for 30 min at 37°C. After being washed three times, the cells were analyzed using FACS. The same amount of peripheral blood was treated identically to act as a control.

Capture experiment: 20ml fresh bone marrow was lysed with ammonium chloride and resuspended using PBS (2%FBS, 1mM EDTA). FcR blocking antibody and 1nmol aptamer were added to the bone marrow solution for 30min at room temperature. EasySep biotin selection cocktail (cellsystems, St. Katharinen, Germany) was added to the solution and incubated for 15min. EasySep magnetic nanoparticles were added and the mixture was incubated for 10min. Then the mixture was put into the magnet and set aside for 5min. The supernatant was poured out and the magnetically labeled cells were washed twice with buffer and further cultured.

Aptamer-mediated aMSCs adhesion on a solid surface. A 12-well cell culture plate (Greiner, Nuertingen, Germany) was coated with streptavidin and incubated overnight at 4°C, and then washed with PBS-T (0.05% Tween-20) three times. The biotinylated-aptamer and the biotinylated-library (control) (1nmol) were added to different wells and incubated at 30°C for 4 hours. The plate was washed with PBS-T and incubated with aMSCs at 37°C for 30 min with gentle shaking. The medium was then removed from the plate and the non-adherent cells were discarded. Cell attachment was observed under an inverse microscope (Zeiss Axiovert 135, Zeiss, Oberkochen, Germany).

FITC-aptamer mediated aMSCs sorting. 20ml bone marrow was lysed to remove the red blood cells. 4nmol FITC-labeled aptamer G-8 was then incubated with the bone marrow for 30 min under 37°C, followed by three washing steps. Then the bone marrow cells were analyzed using flowcytometry. The FITC positive cells were sorted and collected for further analyses.

Characterization of the sorted aMSCs

Phenotype identification of the sorted aMSCs

20ml of whole bone marrow from an adult pig was lysed to remove the red blood cells. The FITC-labeled aptamer G-8 was added and incubated for 30 min at 37°C. After being washed three times, the cells were analyzed under sterile conditions using high speed FACS (FACS-Sort; Becton Dickinson, Heidelberg, Germany) and the FITC-positive cells were sorted and collected in PBS. Some of the sorted cells were analyzed the second time using the PE-labeled CD4, CD8, CD29, CD44, CD45 and CD90; the rest of the sorted cells were cultured for two weeks and then analyzed using PE-labeled antibodies CD29, CD44, CD45 and CD90 (Becton Dickinson, Heidelberg, Germany).

Differentiation of the sorted aMSCs

The sorted aMSCs were cultured in osteogenic and adipogenic culture mediums. The alkaline phosphatase staining and oil red staining were performed as noted.

Comparison of efficiency of aMSCs isolation between conventional plastic adherence and aptamer-based aMSC-isolation

To evaluate the efficiency of aMSCs isolation, the adipogenic and osteogenic differentiation potential and the quantities of isolated cells were compared. Mononuclear cells were isolated from fresh porcine whole bone marrow via density gradient, and plated at a density of 500 cells/well. After 24 hours, medium was changed to remove non adherent cells. Then, adipogenic, osteogenic, or normal medium was added. Aptamer-sorted aMSCs were plated at the same density (500 cells/well). After 24 hours, medium was changed and adipogenic, osteogenic, or normal medium was added. After 5 weeks, when the Aptamer-sorted cells reached confluency, the adipogenic and osteogenic staining procedures were started.

Plasma stability

Fresh human plasma was prepared by centrifugation of 3000g whole blood for 20 min. 8nmol of the aptamer G-8 and a random ssDNA (GGGAGCTCAGCCTAAACGCTCAAGGATCGTTCGCAACGGTTCGACGCA GTTCGTTCGACATGAGGCCCGGATC) were incubated separately at 37°C in a final volume of 0.5 ml of freshly prepared heparinized human plasma. Samples of 50 µl were removed after 0.5, 1, 2, 4, 6, and 24h. Reactions were terminated by adding 5µl loading buffer and subsequently storing the mixture on ice. Full-length and digested oligonucleotides were separated on a 2% agarose gel and photodocumented. [16]

RESULTS

aMSCs isolation and characteristics

Porcine aMSCs were successfully isolated from bone marrow via gradient centrifugation, expanded in monolayer culture and evaluated for osteogenic differentiation potential. Spindle bipolar to polygonal fibroblastic cells were observed 4 days after the first seeding. The cells reached confluence after 12 days. On initial inspection (first passage), the cells showed a uniform monolayer. The aMSCs cultured in osteogenic medium showed ALP positive and Von Kossa positive (calcium mineral precipitation) after 8 days and 28 days, respectively. The aMSCs cultured in adipogenic differentiation medium showed positive for red oil staining, while all the controls were negative (Figure 1a). Surface marker staining showed that the attached cells were $CD29^{+}$, $CD44^{+}$, $CD45^{-}$, $CD90^{+}$, SLA class I^{+}, SLA $DQ^{-}$and SLA DR^{-} (Figure 1b).

Selection of aptamers with high affinity to aMSCs

aMSCs derived from porcine bone marrow were used as the target for in vitro selection of aptamers from a random pool of DNA molecules. The starting library consisted of 79-mer single-stranded DNA molecules containing randomized 40-nucleotide inserts. This library was applied to a number of cultured cells in the same passage, which minimized nonspecific interaction. To monitor the enrichment of specific cell-binding aptamers during selection, SELEX pools of the second and subsequent rounds were analyzed using flow cytometry after incubation with aMSCs. In each round of the selection, the concentration of competitor DNA was increased to further selection toward a high-affinity and high-specificity aptamer pool. Analysis of the histograms of the fluorescent-labeled pools in successive cycles of selection showed a shift beginning with the second round toward higher fluorescent intensity. After 10 rounds of selection, the fluorescence of the aMSC-selected round-11 pool showed no further increase. This pool was then cloned and sequenced.

Sequences from 36 clones were obtained, and their inserts were analyzed and

sorted into putative families using the alignment of consensus motifs. Motifs were identified by inspection with the aid of computer-assisted search engines (data not shown). On the basis of the demonstrable binding to aMSCs, one aptamer, G-8, was chosen for further characterization.

Binding of aptamers to aMSCs

Flow cytometric tests: Fluorescence of G-8 binding to aMSCs is shown in Figure 2a-c, which illustrates that the aptamer binds only to aMSCs, and not to murine P19 cells or humanMSC.

Fishing experiment: aMSCs which bound with the biotinalyted aptamer could be sorted and congregated using anti-biotin microbeads. When filtered through a magnetic column, aMSCs could be fixed by the biotinalyted aptamer. After two weeks in culture, as shown in Figure 3a, the anti-biotin microbeads alone were not able to fish out aMSCs, which means there were no cells growing in the culture flask (3a-A, negative control). The anti-biotin microbeads with biotinalyted-aptamer fixed on their surfaces can bind with aMSCs, and growing cells could therefore be detected (3a-B). This result shows that the aptamer is able to fish out aMSCs from the cell solution.

Binding of aptamers to aMSCs in whole bone marrow

Flow cytometric assay: the aptamer G-8 showed almost no binding to peripheral blood cells compared to the whole bone marrow. (Figure 1b)

Capture experiment: With the Easysep biotin selection kit, aMSCs from whole bone marrow could be fished out using streptavidin magnetic beads which coat with biotinylated aptamer and grow well in culture flasks (Figure 3b).

Aptamer-mediated aMSCs adhesion on solid surfaces

The biotinylated aptamer was immobilized onto a streptavidin coated plate. The aMSCs solution was added to the plate and incubated for the 30 minutes with gentle shaking. Compared to the plate without coated aptamer,

significantly ($p<0.001$) more cells (216.7 ± 13.3 cells) attached to the aptamer coated plate in a short time than to the uncoated plate (34.3 ± 5.1 cells). The results showed that the aptamer could trap MSCs from agitated medium (Figure 3c).

Characterization of the sorted aMSCs

Phenotypic identification of the sorted aMSCs

Mononuclear cells from bone marrow were collected with the FITC-labeled aptamer G-8 using high speed flow associated cell sorting (FACS) (1% yield) and analyzed using PE-labeled antibodies. The results showed 2 subpopulations of sorted cells. The first subpopulation (R1), containing small granular cells, was $CD4^-$ (82.2%), $CD8^-$ (80.5%), $CD29^-$(70.7%), $CD44^+$(90.9%), $CD45^+$(86.4%) and $CD90^-$(77.6%). The second subpopulation (R2), containing small and densely granular cells, was $CD4^-$(98.9%), $CD8^-$(98.9%), $CD29^-$(83.7%), $CD44^+$ (87.7%), $CD45^+$ (99.2%) and $CD90^+$ (91.8%). The sorted cells were cultured for 14 days (passage 0) and also stained using PE-labeled antibodies. The results were CD29+ (98.0 %), CD44+ (99.6 %), CD90+ (99.5%) and CD45-(87.6 %), which are consistent with previously described markers of aMSCs in culture (Figure 4). In contrast to the freshly sorted cells, no distinct subpopulation could be detected, and the cultured cells upregulated CD29 and lost the CD45 antigen.

Differentiation of the sorted aMSCs

Adipogenic and osteogenic differentiation of the aptamer-sorted porcine aMSCs passage 0 showed that the sorted aMSCs have a high potential to differentiate into adipocytes and osteoblasts (Figure 5).

Efficiency of aMSCs isolation

No cell growth could be detected in the wells in which monoculear cells from whole bone marrow were seeded (initially plated: 500 cells/well; conventional 24h plastic adherence procedure for isolation of aMSC, Fig. 5a, A; Fig. 5b, C),

whereas Aptamer-sorted cells grew well and showed adipogenic (Fig. 5a, B) and osteogenic (Fig. 5b, B) differentiation (initially plated: 500 cells/well; medium change after 24h).

Plasma stability

For clinical or therapeutical applications, aptamers should be resistant against rapid degradation by exo- and endonucleases. Human plasma predominantly contains high 3'-exonuclease activity [17]. In human blood plasma, the random sequence cannot resist the degradation of plasma (Figure 5-A). The unmodified aptamer G-8 can resist the degradation of nucleases for 24 hours, which was detected using agarose gel analysis (Figure 5-B) and does not need extra modification to improve its stability.

DISCUSSION

This study established a new, rapid, and efficient method for direct isolation of aMSCs from porcine bone marrow. Using an aptamer as a probe to fish out the aMSCs, this method is quick and efficient compared to the existing methods. Due to the lack of a special surface marker, the traditional method of isolating aMSCs from bone marrow mainly depends on the plastic adherence characteristics of MSCs, or on negative selection, or removing other cells which expose surface markers.[18, 19] However, the first (plastic adherence) method is time consuming. Identification of the functional differentiation of aMSCs takes between 2 and 4 weeks. The observation that not a single cell could be cultured with the conventional plastic adherence method after seeding 500 mononuclear cells from fresh bone marrow confirms the known low frequency of real aMSCs in bone marrow.

In contrast, seeding 500 aptamer-isolated cells leads to confluent cultures of aMSCs with adipogenic and osteogenic differerentiation potential. Our experiments show that the MSCs indicate an antigen shift from isolation to culture. In addition to previous reports of CD45 loss in culture [20], we found an upregulation of CD29 during culture. The R1 population lacks the phenotype CD90, were CD29 negative (here R1 lacked the antigen CD90) and CD45 positive. Previous reports on aMSCs subpopulations described the recycling stem cells (RS2), which are also CD90 negative [21]. Furthermore, using CD29 or CD90 magnetic beads to select CD29 or CD90 positive MSCs, or using CD45 negative selection to isolate and purify MSCs leads to the loss of significant amounts of MSCs. Therefore, the negative isolation method is expensive and inefficient.

Our method shows the advantage of isolating very young MSCs, which have strong potential to differentiate into osteoblasts and adipose. Additionally, aptamer synthesis is easy and aptamers can resist enzymatic digestion for several hours. aMSCs are considered a readily accepted source of stem cells due to their efficacy in multiple types of cellular therapeutic strategies, like

bone tissue regeneration[22]. The field of developing oligonucleotides as molecules with a utility beyond genetics has grown rapidly in the last decade. The application of in vitro selection methods has allowed nucleic acid aptamers to be generated which have the ability to bind targets with high selectivity, specificity and non-immunogenicity. A great advantage of this technology is that a variety of sequences are capable of binding to a variety of ligands, including small molecules, inorganics, proteins and cellular targets[23-25]. Great progress has been made toward clinical applications of aptamers [26-31]. Here we have pioneered the use of aptamers in the isolation of aMSCs from bone marrow using an aptamer with high specificity for porcine aMSCs. Moreover, using the scaffold-immobilized aptamer as a probe to fish out circulating cells is a new application for material and tissue engineering research. The cell adhesion experiments show that immobilized aptamers can enhance aMSCs attachment in the very short term. The aMSCs differentiation assay indicates the long term compatibility between an aptamer and aMSCs without affecting stem cell specific plasticity. Compared to the established method of aMSCs isolation based on plastic adherence, the aptamer-based isolation of aMSCs shows a much higher efficiency combined with high specificity without affecting stem cell plasticity. We have done experiments on aptamer-protein precipitation using lysed MSCs and looked at the target of the aptamer using proteomics tools. However, we could not find any known protein sequence, perhaps due to the limited porcine protein library. We anticipate that target protein characterization will work much better using aptamers against human MSCs. As our long term goal is to create a scaffold coated with aptamers, we chose an aptamer coated surface to mimic the target condition. This new aptamer against aMSCs can improve the current scaffold design by modifying the surface of the scaffold, increasing environmentally mediated stem cell plasticity and making the cells respond well to the microenvironmental conditions. Furthermore, this new technique could be useful for the identification and isolation of aMSCs and their various desired

subpopulations.

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FIGURE LEGENDS

Figure 1

(a) Characterization and identification of aptamer-positive high-speed FACS-sorted aMSCs from bone marrow. The cells showed high potency to differentiate into osteogenic, as well as into adipogenic lineages with typical morphological characteristics. Figures A and B show osteogenic differentiation and Von Kossa staining (100x) (A is control), figures C and D show cells stained with alkaline phosphatase+ Hematoxylin (200 x) (C is control). Cells in figures E and F were differentiated adipogenic and stained with Red oil + Hematoxylin (400 x) (E is control)

(b) Epitope identification of the cultured aMSCs. The adult porcine aMSCs were $CD29^{+}$, $CD44^{+}$, $CD90^{+}$, SLAclass I^{+}, SLA class II DQ^{-}, SLA class II DR^{-} (the red curve is the isotype control).

Figure 2

Affinity of aptamer G-8 to aMSCs from different species tested using flowcytometry, which shows that aptamers bind to aMSCs, not to murine P19 cells or human MSCs.

(a) The green curve represents the porcine aMSCs incubated with FITC-G-8, the red curve represents the murine P19 cells incubated with FITC-G-8.

(b) The green curve represents the porcine aMSCs incubated with FITC-G-8, the red curve represents the rat aMSCs incubated with FITC-G-8.

(c) The green curve represents the porcine aMSCs incubated with FITC-G-8, the red curve represents the human aMSCs incubated with FITC-G-8.

(d) As negative controls, the aptamer G-8 shows no affinity to peripheral blood. Figure d1 shows the binding of the aptamer to whole bone marrow, d2 shows the binding of the aptamer to peripheral blood (red curve represents the aptamer incubated with cells; the green curve represents the cell control).

Figure 3

(a) Aptamer-based cell sorting.

The cells which bind to the biotinylated aptamer can be pulled down together with anti-biotin microbeads (3a-B) and grow well in the culture flask, whereas the non-coated microbeads did not bind to the cells. The cells were washed through the magnetic filter and no cells were held on the magnetic columns, resulting in fewer cells in the culture flask (3a-A) (x100).

(b) Captured aMSCs from bone marrow.

The left picture is the control, only beads incubated with whole bone marrow, where there were only very few cells growing on the culture flask. The right picture shows the whole bone marrow incubated with the aptamer (fixed on the magnetic microbeads); there are more cells congregated and growing (x100).

(c) Surface binding of aMSCs on aptamer-coated plates.

After 30 minutes' incubation of aMSCs, the aptamer-coated culture plate captured many aMSCs (216.7 ± 13.3 cells per eyeshot) (3c-B); the culture plate coated with the library captured only very few aMSCs. (34.3 ± 5.1 cells per eyeshot) (3c-A) (x100)

Figure 4

Phenotypic identification of aptamer-sorted aMSCs from porcine bone marrow either directly after the sorting process or after 2 weeks in culture.

(a) The subpopulation R1 of the sorted aMSCs was stained with PE-labeled antibodies immediately after sorting. The results showed they were $CD4^-$, $CD8^-$, $CD29^-$, $CD44^+$, $CD90^-$.

The subpopulation R2 of the sorted aMSCs was stained with PE-labeled antibodies immediately after sorting. The results showed $CD4^-$, $CD8^-$, $CD29^-$, $CD44^+$, $CD90^+$. The red curves show the isotype controls.

After 2 weeks in culture, the sorted aMSCs were stained with PE-labeled

antibodies. The cells were $CD29^+$, $CD44^+$, $CD45^-$, and $CD90^+$, the red curves show the isotype controls.

Figure 5

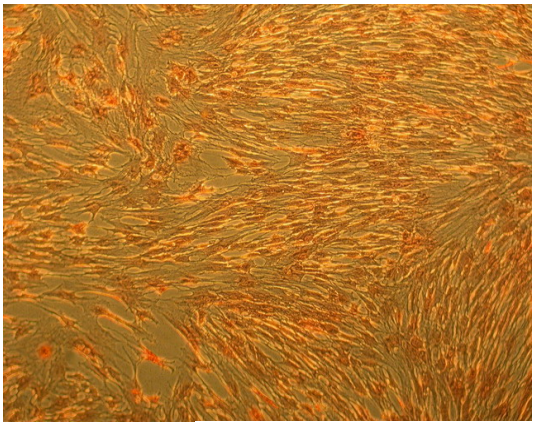
To proof the plasma stability against fast enzymatic degradation, the aptamer G-8 was incubated in human blood plasma for up to 24 hours at 37°C, and visualized using agarose gel electrophoresis. A random ssDNA sequence was chosen as a control. Figure 5A shows that random ssDNA sequences cannot resist the plasma digestion for 30 minutes (positive control); Figure 5B shows that aptamers can resist degradation for at least 24 hours.

Figure 6

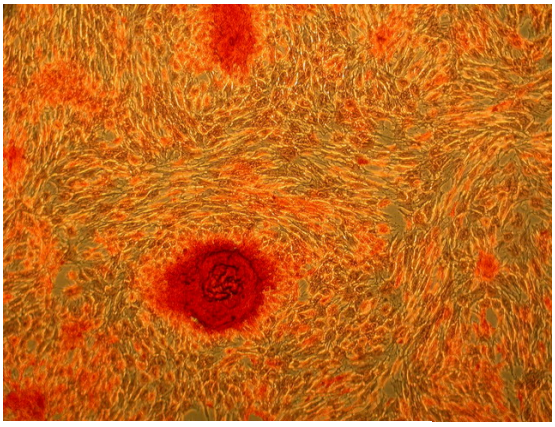
Adipogenic (Fig. 6a) and osteogenic (Fig. 6b) differentiation of the aptamer sorted porcine aMSCs (passage 0) versus plastic adherence procedure for isolation of aMSCs (passage 0). Mononuclear cells were isolated from fresh whole bone marrow according to density gradient and plated at a density of 500 cells/well (A+C). After 24 hours, the medium was changed to remove non-adherent cells. Then adipogenic, osteogenic, or normal medium was added. Aptamer-sorted aMSCs were plated at a density of 500 cells/well (B+D). After 24 hours, the medium was changed and adipogenic, osteogenic, or normal medium was added. The staining procedure was started after 5 weeks, once the Aptamer-sorted cells reached confluence: **Fig. 6a** (adipogenic differentiation): **A**: whole bone marrow – 24h adherence, adipogenic medium; **B**: Aptamer-sorted aMSCs — 24h adherence, adipogenic medium; **C**: whole bone marrow – 24h adherence, control (normal medium); **D**: Aptamer-sorted aMSCs — 24h adherence, control (normal medium). Staining with oil red O, haematoxylin counterstaining. **Fig. 6b** (osteogenic differentiation): **A**: whole bone marrow – 24h adherence, osteogenic medium; **B**: Aptamer-sorted aMSCs — 24h adherence, osteogenic medium; **C**: whole bone marrow – 24h adherence, control (normal medium); **D**: Aptamer-sorted aMSCs — 24h

adherence, control (normal medium). Staining for alkaline phosphatase, haematoxylin counterstaining. No cell growth could be detected in wells A and C (plastic adherence procedure for isolation of aMSCs), whereas aptamer-sorted cells (B and D) grew well and showed adipogenic (Fig. 6a, B) and osteogenic (Fig. 6b, B) differentiation.

Figures



A



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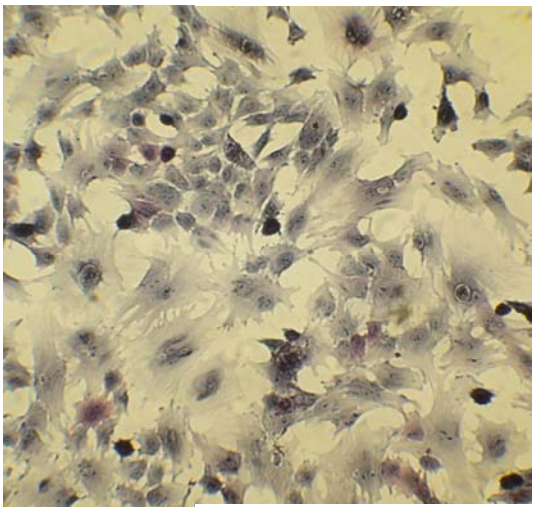

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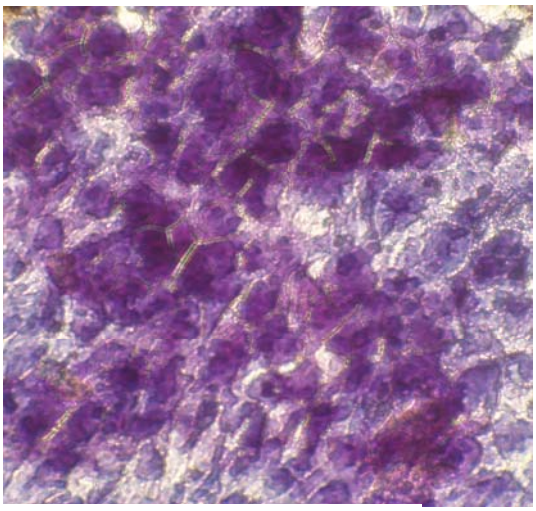

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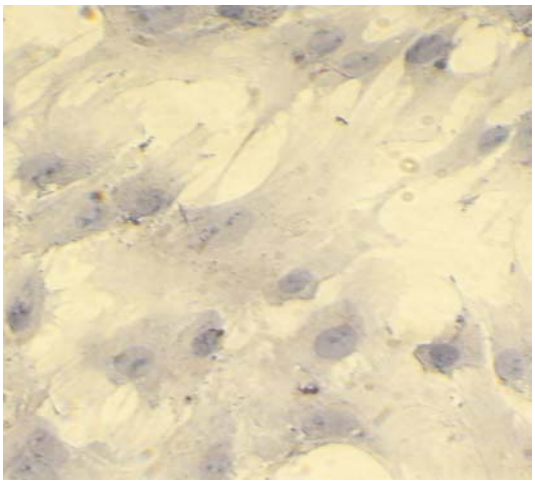

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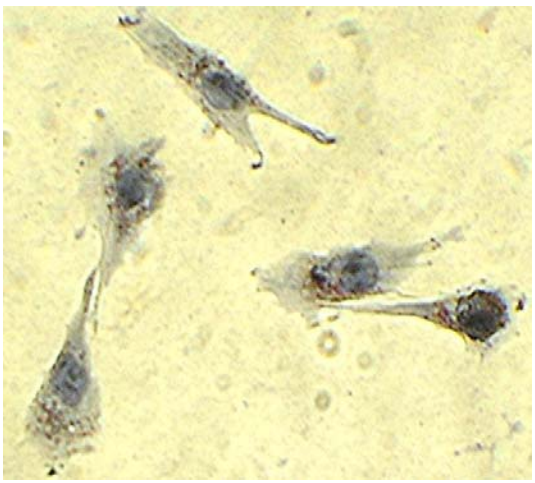
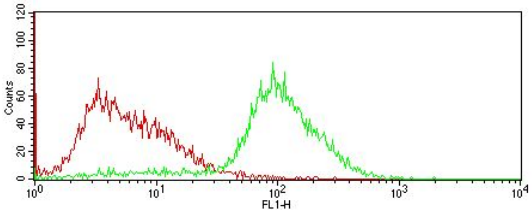
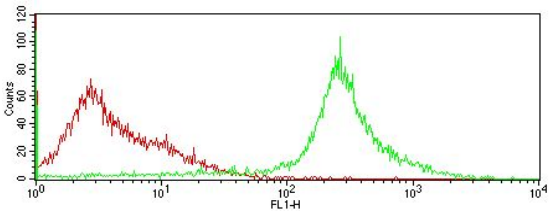


F

(a)



CD29



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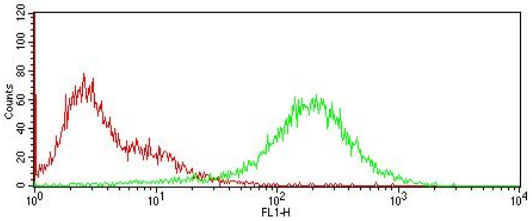
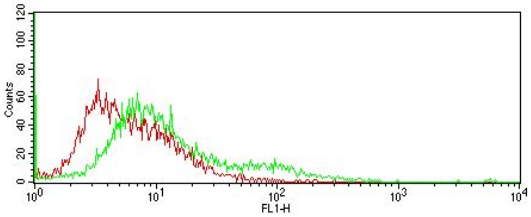


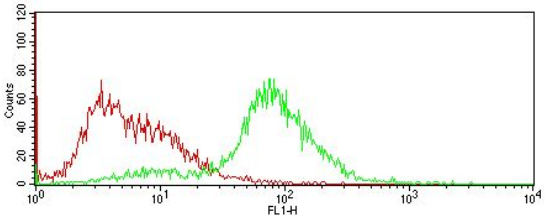
CD90

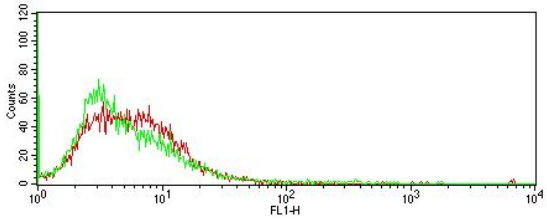


CD45

SLA class I

SLA class II (DQ)





SLA class II (DR)

(b)

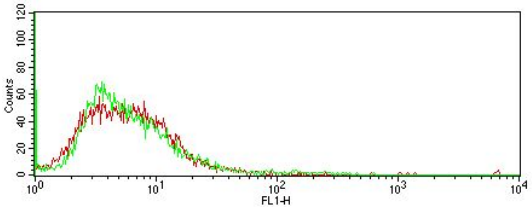
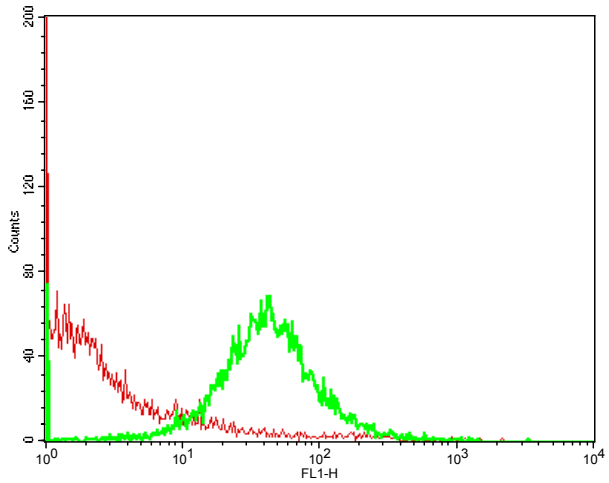


Figure 1



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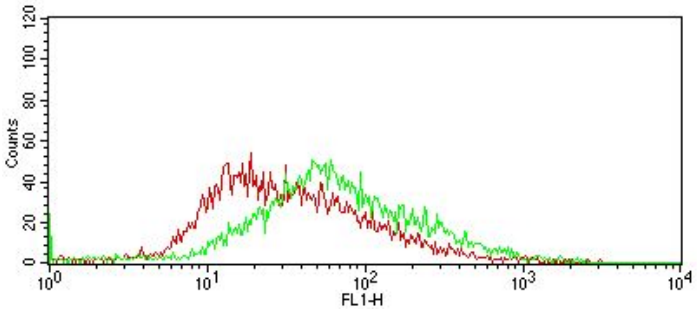

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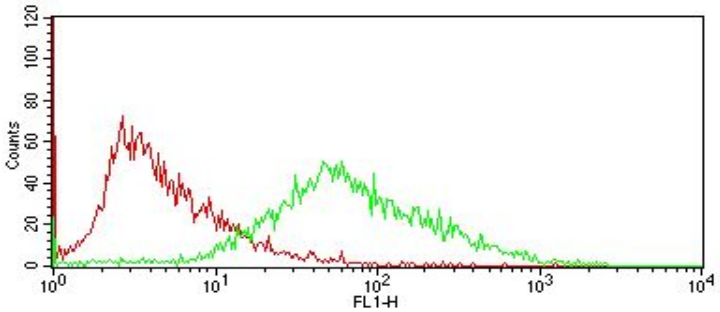

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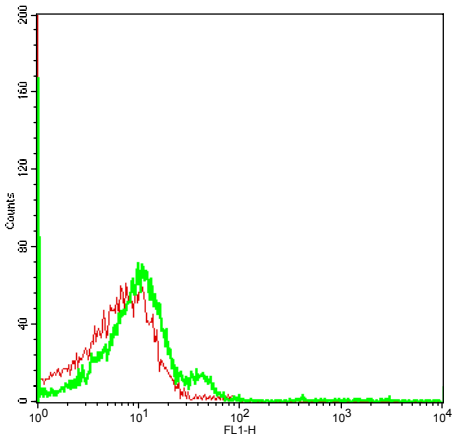
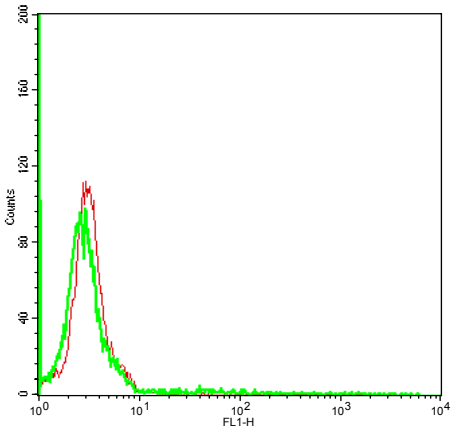
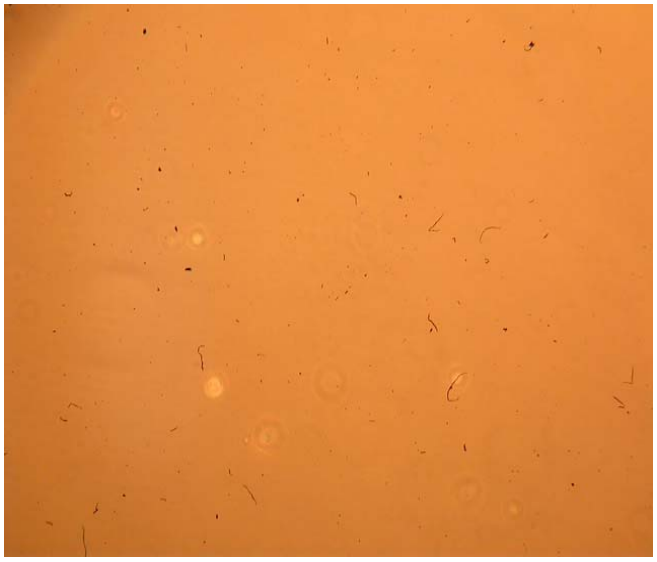


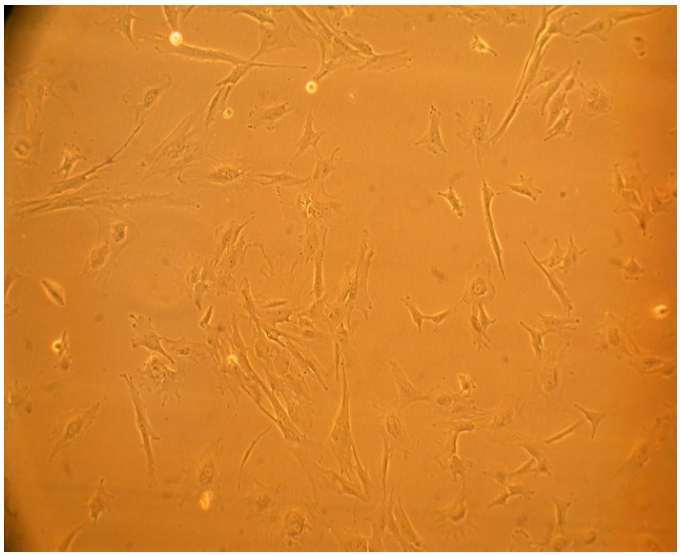


d1 d2

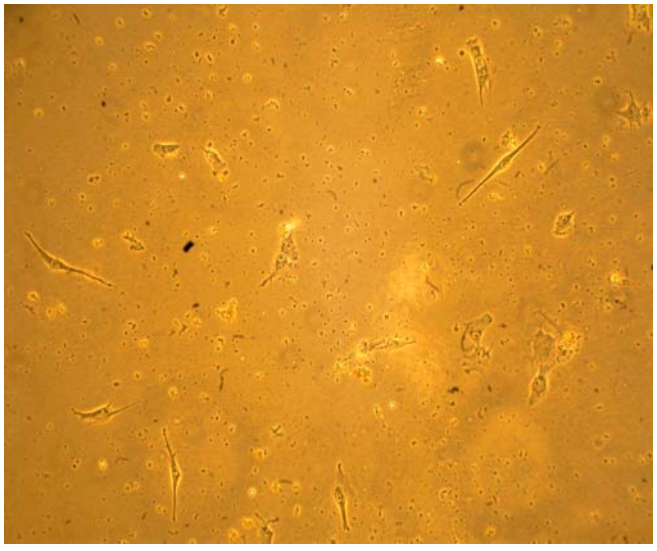
Figure 2



(a)—A



(a)—B



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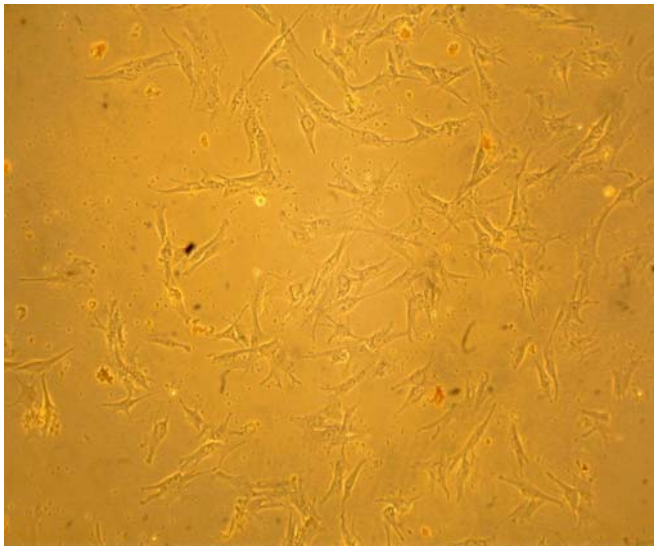
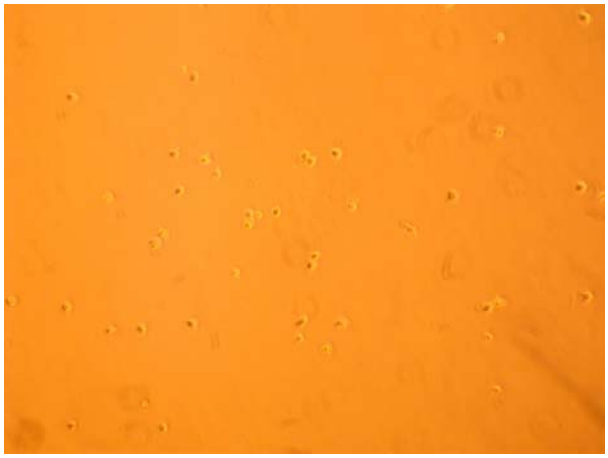
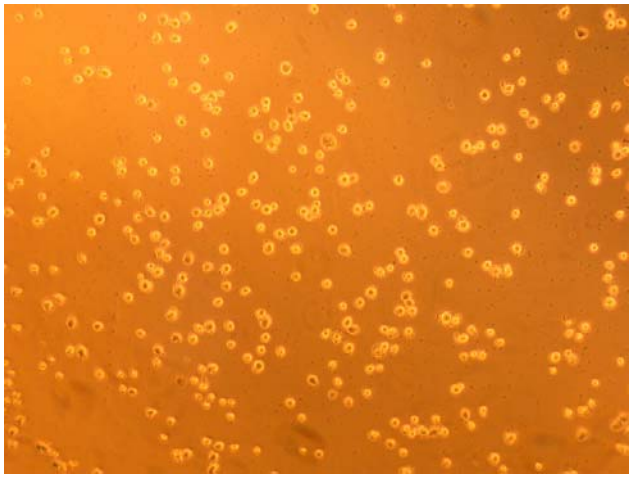
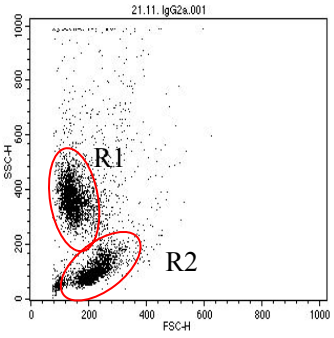






(c)-A (c)-B

Figure 3



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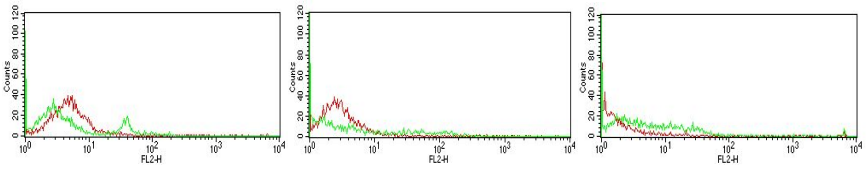
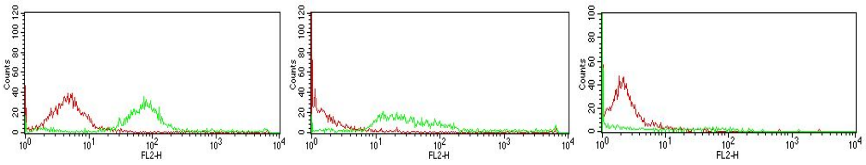
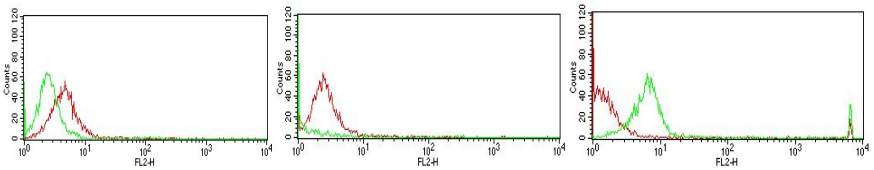


CD4 CD8 CD29

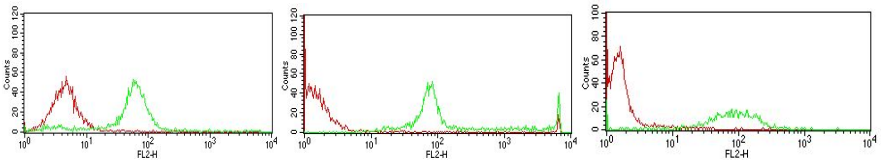


CD44 CD45 CD90

R2:

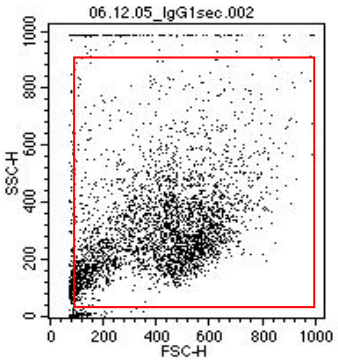


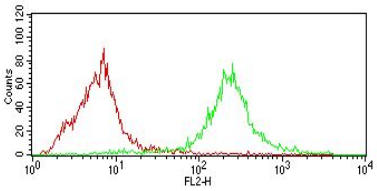
CD4 CD8 CD29



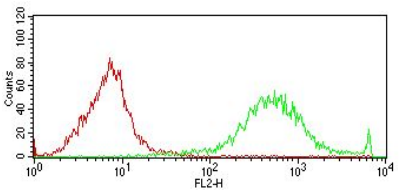
CD44 CD45 CD90

(a)

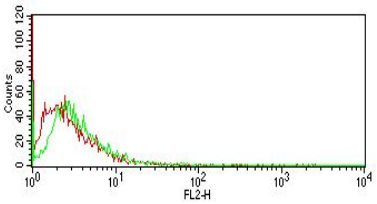




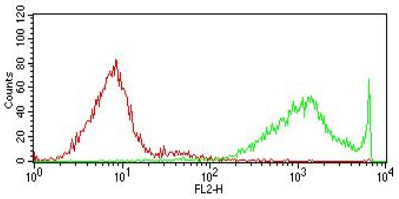
CD29



CD44



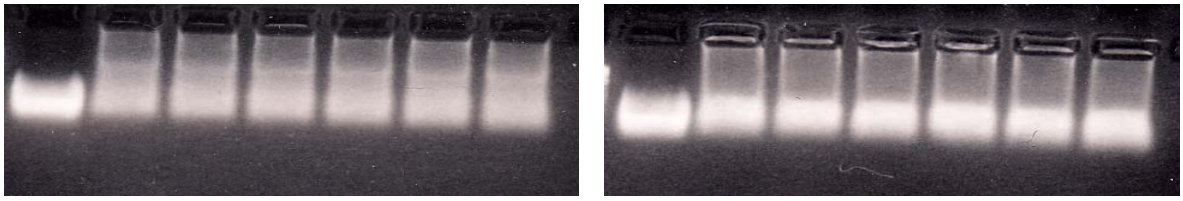
CD45



CD90

(b)

Figure 4



A B

Figure 5

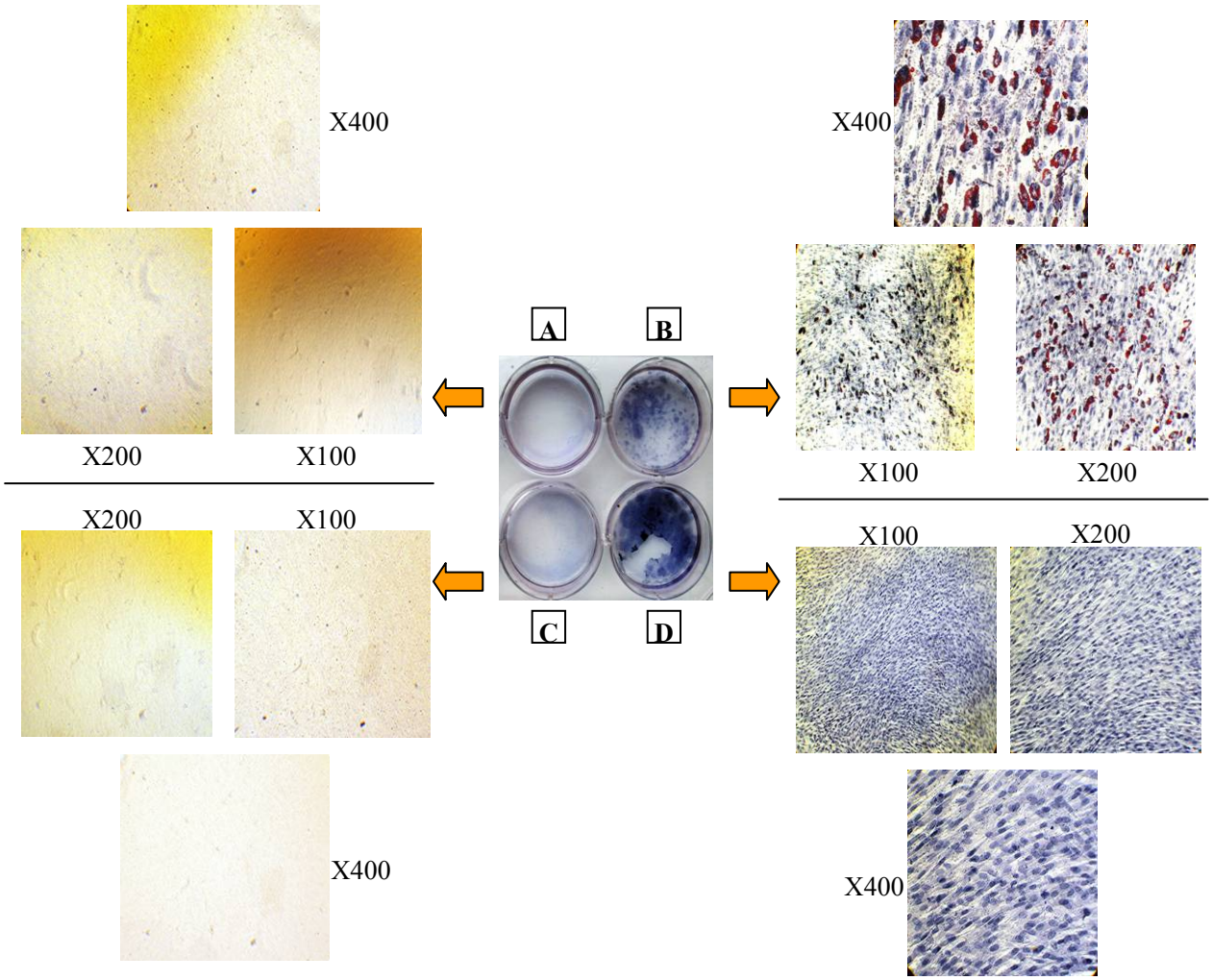


Figure 6a

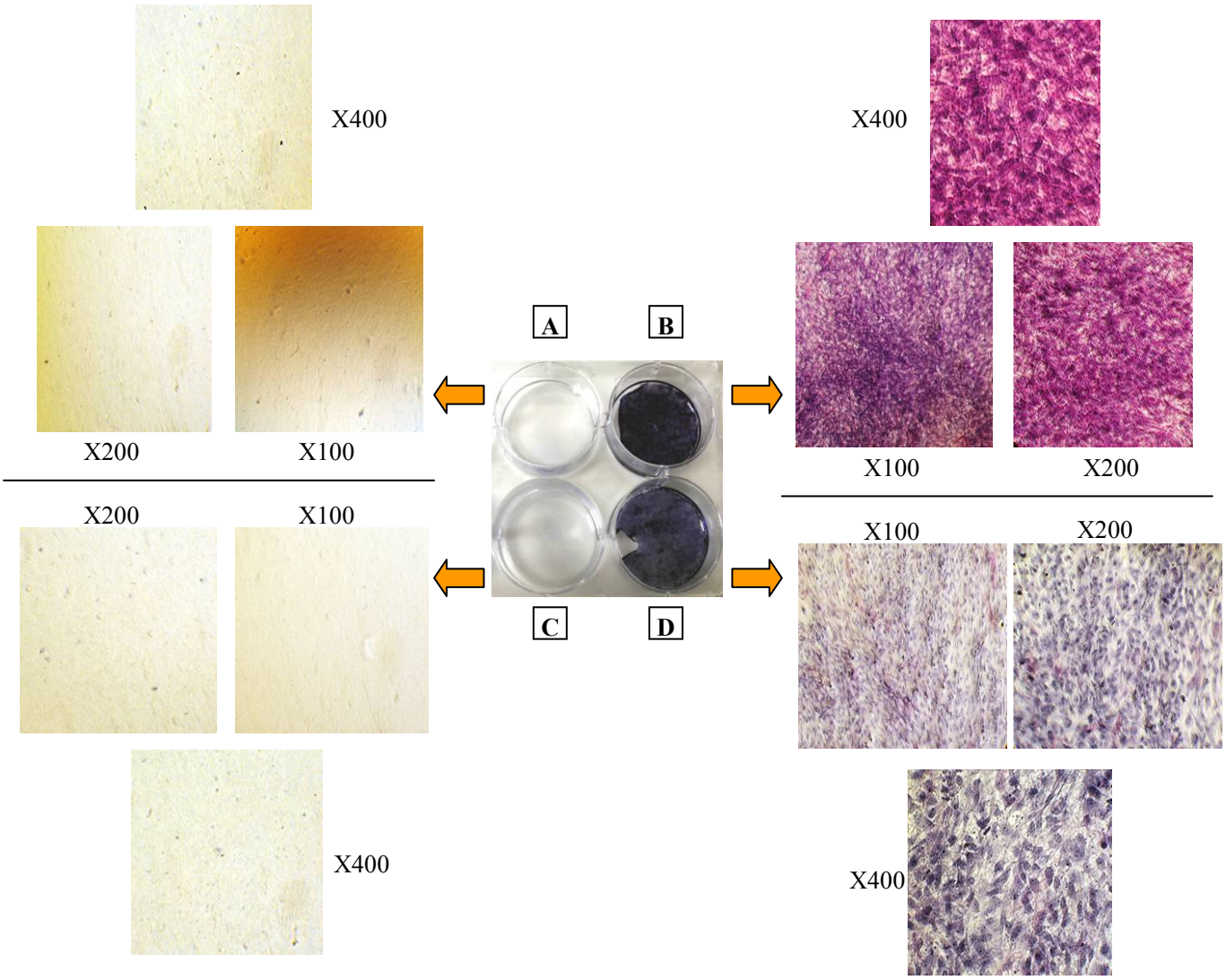


Figure 6b

Figure 6

Results Part 4:

The immunostimulatory activity of CpG oligonucleotides on microglial N9 cells is affected by a polyguanosine motif.

Journal of Neuroimmunology 2005, 161:68–77

Zhiren Zhang, Ketai Guo, Hermann J. Schluesener

All the experiments of real time-PCR in the manuscript of “The immunostimulatory activity of CpG oligonucleotides on microglial N9 cells is affected by a polyguanosine motif” were performed by me. All experiments were planed, performed and analyzed by Zhiren Zhang. The publication was written by Zhiren Zhang. The idea of the study was from H Schluesener’ group.

The immunostimulatory activity of CpG oligonucleotides on microglial N9 cells is affected by a polyguanosine motif

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Abstract

Oligonucleotides (ODN) with hexameric motifs containing central unmethylated CpG dinucleotides are immunostimulatory. Also ODN with continuous guanosines (polyG motif) show a wide range of immunological activity. Depending on the position, the chemical property of the ODN backbone and the cell type, polyG motifs have either an enhancing or a suppressing effect on the immunostimulatory activity of the CpG-ODN. Microglial cells are central components of the innate immune system of the brain and are activated by CpG-ODN in vitro and in vivo. Here we present the analysis of the immunomodulatory effects of CpG-ODN carrying a polyG motif on the microglial cell line N9.

Our data show that N9 cells express Toll-like receptor 9 (TLR9) and are activated by CpG-ODN, which leads to expression of interleukin-12p40 (IL12p40), tumor necrosis factor-a (TNF-α) and inducible nitric oxide synthase (iNOS). A 3'-end polyG motif inhibits phosphothioate (PS) CpG-ODN immunostimulatory activity but enhances the immunostimulatory activity of phosphodiester (PE) CpG-ODN. Correspondingly, a 3'-end polyG motif improves the cellular uptake of PE CpG-ODN but does not change their cellular distribution pattern. Furthermore, PE CpG-ODN with a 3'-end polyG motif interact with a much higher number of cellular proteins than PE CpG-ODN. These data indicate that the 3'-end polyG motif could enhance the immunostimulatory activity of PE CpG-ODN in microglial N9 cells through increasing interaction with cellular proteins. Therefore PE CpG-ODN containing a 3'-end polyG motif resulting in increased immunostimulatory activity might be promising alternate analogues for studies in the central nervous system.

Keywords: Microglia; CpG oligonucleotides; Polyguanosine motif

1. Introduction

DNA has complex effects on the immune system. Unmethylated CpG dinucleotides within specific flanking bases (referred to as CpG motifs) are immunostimulatory and are at a much higher frequency present in bacterial than in vertebrate DNA. Bacterial DNA or synthetic ODN with CpG motifs are recognized as danger signal by the innate immune cells via TLR9 to trigger a Th1-polarized immune response. CpG-ODN can trigger B cells, plasmacytoid dendritic cells, monocytes, macrophages and natural killer cells to proliferate, mature, up-regulate several costimulatory molecules and to secrete a variety of Th1-promoting cytokines, chemokines and immunoglobulins. Based on their strong immunostimulatory activity, a number of CpG-ODN are at various stages of preclinical and clinical evaluation as adjuvant or therapeutic agents for cancer and allergic diseases (Krieg, 2002; Rothenfusser et al., 2003; Dalpke et al., 2001; Hemmi et al., 2000).

However the use of phosphodiester (PE) CpG-ODN is limited because of their rapid degradation in serum and inside cells, which results in weaker activity (Krieg et al., 1995). To overcome this problem, ODN with a phosphothioate (PS) backbone have been used to increase resistance to cellular nucleases and thus to prolong their action. However, PS ODN have non-specific effects. In vitro PS ODN cause transcription factor Sp1 activation, platelet aggregation, inhibition of cell proliferation and migration, perturbation of cell-surface receptor binding and induction of changes in cell morphology (Perez et al., 1994; Wang et al., 1996; Rockwell et al., 1997; Anselmet et al., 2002). In clinical studies, PS ODN caused thrombocytopenia, fatigue, fever, rashes, leukopenia and complement activation (Yuen and Sikic, 2000). In addition, PS ODN might be neurotoxic at therapeutic concentrations higher than 1 μM (Agrawal, 1991), causing paralysis and necrosis of the spinal cord (Wojcik et al., 1996). Moreover, PS CpG-ODN might exacerbate inflammatory tissue damage, cause autoimmune disease or increase sensitivity to toxic shock

(Pisetsky, 1997; Deng et al., 1999; Segal et al., 2000; Cowdery et al., 1996; Sparwasser et al., 1997).

Single-stranded guanosine-rich ODN, which have the potency to form quadruplex structures that result in increased nuclease resistance and cellular uptake, are another type of ODN motifs that have a wide range of immunomodulatory activity (Dapic et al., 2003). In vitro, PS deoxyguanosine oligomers inhibit the production of interferon-γ by murine splenocytes upon induction by ConA or bacterial DNA (Halpern and Pisetsky, 1995). PE or PS deoxyguanosine oligomers can inhibit murine macrophage and dendritic cell activation (Zhu et al., 2002a,b). PS guanosine-rich ODN can directly lead to proliferation of macrophage progenitors from murine bone marrow cells (Lang et al., 1999). In addition, PS polyguanosines ODN display the ability to costimulate $CD8^+$ T cells (Lipford et al., 2000). They also block the downstream functions of interferon-γ, and the binding of interferon-g to its receptor (Balasubramanian et al., 1998; Lee et al., 1996). Furthermore, polyG motifs exert suppressive or stimulating effects on the immunostimulatory activity of CpG-ODN according to the cell type, the modification of the ODN backbone and their location within the ODN. The addition of a polyG motif to the 5' or 3'-end of the PE CpG-ODN improves their ability to activate NK cells (Kimura et al., 1994; Ballas et al., 1996) and to induce interferon-α production by dendritic cells (Krug et al., 2001). In PS CpG-ODN, a polyG motif can block their ability to activate NF-κB in B cells and in PE CpG-ODN it can reduce the level of B cell activation (Lenert et al., 2001). In macrophages and dendritic cells, only polyG located at the 3'-end of PE CpG-ODN can improve their immunostimulatory activity (Dalpke et al., 2002a; Lee et al., 2000).

Microglia, cells derived from the mononuclear-phagocyte lineage, are the brain's ubiquitous but normally inconspicuous immune effector cells. They constitute the brain's autochthonous source of macrophages and thus are part of its intrinsic immune system. Microglia respond rapidly to subtle, acute and

chronic pathological stimuli and are prominently involved in many diseases. Microglial cells express TLR9 and can be activated by CpG-ODN in vitro and in vivo (Dalpke et al., 2002b; Takeshita et al., 2001; Schluesener et al., 2001; Carpentier et al., 2000). Up to now the effects of polyG motif on CpG-ODN have not been studied in microglia. Here we describe the immunomodulatory effects of polyG motif on the immunostimulatory activity of CpG-ODN in microglial cells.

2. Materials and methods

2.1. Synthetic ODN

All ODN used in this study were synthesized by MWG-Biotech AG, Ebersberg, Germany. PS ODN, PE ODN, FITC labelled ODN, biotin labelled ODN and unlabelled ODN were used. Sequences of ODN are as follows: 1826: TCCATGACGTTCCTGACGTT, 1826GC: TCCATGAGCTTCCTGAGCTT, 1826-G6: TCCATGACGTTCCTGACGTTGGGGGG, 1826GC-G6: TCCATGAGCTTCCTGAGCTTGGGGGG, G6-1826: GGGGGGTCCATGACGTTCCTGACGTT, G6-1826GC: GGGGGGTCCATGAGCTTCCTGAGCTT.

2.2. Cell cultures

Murine N9 microglial cells were cultured in RPMI-1640 with 10% heat inactivated fetal calf serum (FCS) with penicillin and streptomycin at 100 U/mL (Gibco, Grand Island, NY) at 37°C in 5% CO_2.

2.3. RNA preparation and cDNA synthesis

Total RNA from cultured cells was prepared using the RNeasy Mini Kit

(QIAGEN GmbH, Hilden, Germany) according to the manufacture's instruction. 1 µg RNA was reverse transcribed into cDNA using randomised primers.

2.4. RT-PCR

The expression of TLR9 and β-actin by N9 cells was determined using reverse transcription PCR with 1 µL cDNA. The following primers were used: β-actin (sense, CCC TGT GCT GCT CAC CGA; antisense, ACA GTG TGG GTG ACC CCG TC), TLR9 (sense, GGG CCC ATT GTG ATG AAC C; antisense, GCT GCC ACA CTT CAC ACC AT). Cycle conditions were 94°C 3 min, then 30 cycles of: 94°C for 40 s, 55°C for 30 s and 72°C for 1 min. PCR products were analyzed on a 2% agarose gel using ethidium bromide staining.

2.5. Detecting cytokine induction by real-time RT-PCR

10^6 Cells/well were plated in 12-well cell culture plates and cultured for 24 h. Afterwards cells were stimulated with 1.5 µM of different unlabelled ODN and incubated for 12 h. Subsequently mRNA expression of IL12p40, iNOS and TNFα was quantified by real-time PCR in comparison with unstimulated cells using SYBR-Green as detection reagent and β-actin as reference standard. Following primers were used: IL12p40 (sense, CAG AAG CTA ACC CAT CTC CTG GTT TG; antisense, CCG GAG TAA TTT GGT GCT CCA CAC), TNF-α (sense,AAAATTCGAGTGACAAGCCTG TAG; antisense, CCC TTG AAG AGA ACC TGG GAG TAG) and iNOS (sense, CAG CTG GGC TGT ACA AAC CTT; antisense, CAT TGG AAG TGA AGC GTT TCG).

2.6. ODN uptake analysis

5 x 10^5 cells/well were seeded in 12-well cell culture plates and cultured for 24 h. Afterwards cells were washed with FCS-free RPMI 1640 and incubated with

FCS-free RPMI 1640 at 37°C for 1 h to remove residual FCS. After incubation, medium was replaced by serum-free RPMI 1640 containing FITC labelled ODN at the indicated concentrations. Cells were cultured in the dark as long as indicated. After washing with PBA buffer (phosphate-buffered saline (PBS), 2% BSA, and 0.1% sodium azide) and PBS cells were detached from the plate with trypsin-EDTA. Cells were collected by centrifugation at 1000 rpm for 5 min and cell fluorescence intensity was quantified by flow cytometry. For the inhibition experiments, cells were preincubated with fucoidan (0.14 mg/mL) for 15 min at 37°C and then FITC labelled ODN were added for further incubation. The following steps were the same as described above.

2.7. ODN cellular distribution analysis using fluorescence microscopy

10^5 Cells in 0.4 mL RPMI1640 were seeded onto 6-well chamber slides (Lab-Tek Chamber Slide, Nalgene Nunc International Corp. Naperville, USA) and cultured for 24 h. After washing and incubating with FCS-free medium, cells were washed once with PBS and then incubated with FITC labelled ODN (0.5 μM) in FCS-free RPMI 1640 for 1 h at 37°C. Subsequently cells were fixed with 2% paraformaldehyde (PFA) for 15 min at room temperature and mounted in a mounting medium containing DAPI (VECTASHIELD Mounting Medium with DAPI, Vector Laboratories, Inc., Burlingame, USA). The cellular distribution of ODN was observed using fluorescence microscopy.

2.8. CpG-ODN mediated protein purification

N9 cells cultured in flasks were rinsed twice with ice-cold PBS and collected with a scraper. Cells were resuspended in solubilization buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100) and sonicated. Afterwards a protease inhibitor cocktail (Sigma, Munich, Germany) was added and cell lysates were incubated for 2 h on ice. Lysates were cleared by centrifugation at 14,000 g at 4°C for 5

min. An aliquot of the supernatants was used for protein purification.

One milligram (100 µL) of magnetic streptavidin microbeads (Dynal Biotech ASA, Oslo, Norway) was coated with 200 pmol of biotin labelled ODN by incubation in 1 mL selection buffer for 30 min at room temperature. The lysates were incubated with the ODN coated magnetic microbeads in the presence of a 100-fold excess of tRNA (20 nmol) as an unspecific competitor in selection buffer (total volume 1.5 mL, 0°C, 30 min). The protein-ODN-magnetic bead-complex was recovered in a magnet stand and washed five times (first wash: 1 mL of selection buffer with 150 mM NaCl; second through fifth wash: 200 µL of selection buffer with 100 mM NaCl with 2 nmol tRNA). Proteins were removed from CpG-ODN coated beads by heating in the loading buffer and analyzed by 10% polyacrylamide gel electrophoresis and detected by staining with Coomassie Blue (Blank et al., 2001).

3. Results

3.1. Microglial N9 cells express TLR9 and are activated by CpG-ODN

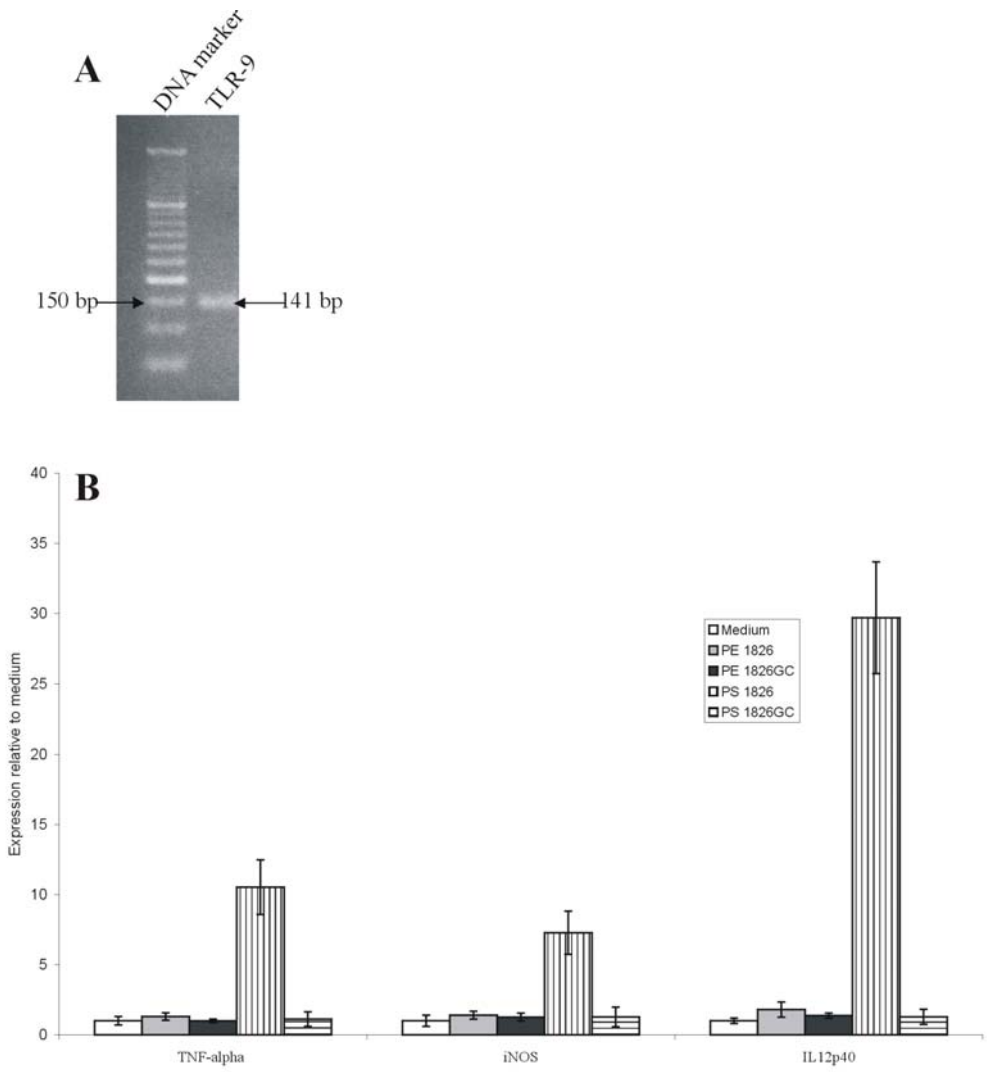

Fig. 1. Microglial N9 cells express TLR9 and produce TNF-α, iNOS and IL12p40 upon stimulation by CpG-ODN. (A) RT-PCR analysis shows that microglial N9 cells express TLR9. (B) N9 cells were stimulated for 12 h by various ODN (1.5 μM) and then expression of TNF-α, iNOS and IL12p40 was measured using real-time PCR. Data are expressed relative to incubation with medium alone and each bar represents the mean and SEM of triplicates.

To investigate the influence of a polyG motif on the immunostimulatory activity of CpG-ODN in microglial N9 cells, we first tested the expression of TLR9 in N9 cells using RT-PCR. As shown in Fig. 1A, microglial N9 cells expressed TLR9 at the mRNA level. Subsequently, a previously reported CpG-ODN (1826) was applied to stimulate N9 cells and to examine the biological activity of TLR9. PS 1826 strongly induced the expression of proinflammatory cytokines, TNF-α and IL12p40, and anti-pathogen effector molecule, iNOS in N9 cells (Fig. 1B). This induction is CpG motif specific, since PS 1826GC did

not have any immunostimulatory activity. PE 1826, however, only marginally induced N9 cells to express TNF-α, IL12p40 and iNOS, which correspond to previously reported data.

3.2. The extension of CpG-ODN by polyG motifs affects immunostimulatory activity on N9 cells

Subsequently the effects of polyG motifs on the immunostimulatory activity of PE and PS 1826 were probed in microglial N9 cells. As shown in Fig. 2, PE 1826-G6, which contained a 3'-end polyG motif, markedly enhanced TNF-α, IL12p40 and iNOS induction compared with PE 1826. The conversion of CpG dinucleotides to GpC dinucleotides (PE 1826-G6/PE 1826GC-G6) completely abrogated induction of TNF-α, IL12p40 and iNOS. This indicates that a polyG motif alone could not induce TNF-α, IL12p40 and iNOS expression. The introduction of a 5'-end polyG motif (PE G6-1826) only slightly enhanced TNF-α, IL12p40 and iNOS induction compared with PE 1826, which suggests that the location of the polyG motif is important for the immunomodulatory activity to PE CpG-ODN. These data indicate that the addition of a polyG motif at the 3'-end of PE CpG-ODN increases immunostimulatory activity in N9 cells. Furthermore, the effects of a polyG motif on PS CpG-ODN were analyzed. Unexpectedly, extension of the 3'- or 5'-end (PS 1826-G6 or PS G6-1826) with polyG motif inhibited TNF-α, IL12p40 and iNOS induction compared with PS 1826, suggesting that the conjugation of a polyG motif at the 3' or 5' terminus inhibit the immunostimulatory potential of PS CpG-ODN (Fig. 2).

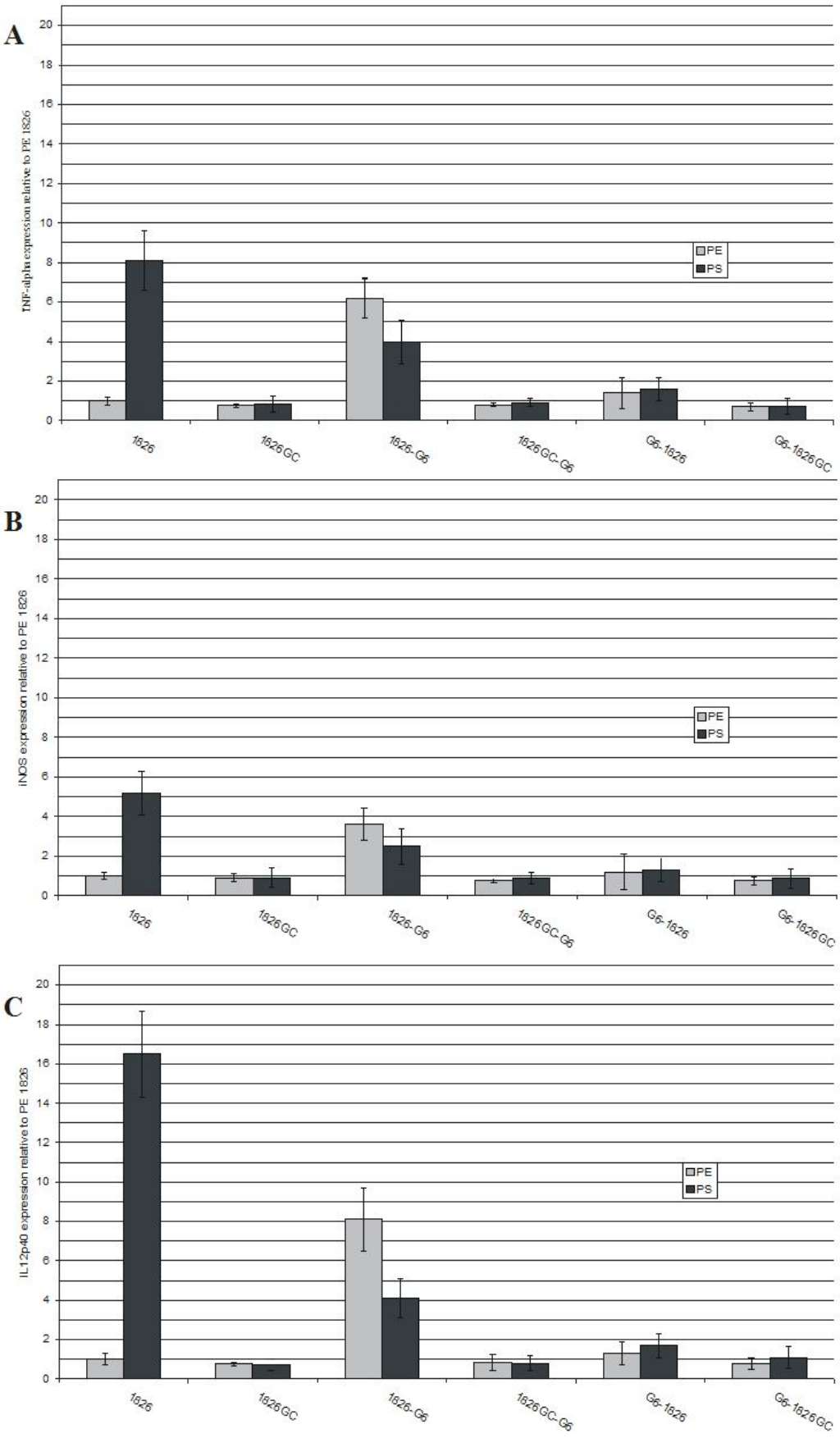
A
TNF-alpha expression relative to PE 1826
PE
PS
1826
1826 GC
1826-G6
1826 GC-G6
G6-1826
G6-1826 GC
B
iNOS expression relative to PE 1826
PE
PS
1826
1826 GC
1826-G6
1826 GC-G6
G6-1826
G6-1826 GC
C
IL12p40 expression relative to PE 1826
PE
PS
1826
1826 GC
1826-G6
1826 GC-G6
G6-1826
G6-1826 GC

Fig. 2. Influences of a polyG motif on the immunostimulatory activity of CpG-ODN in microglial N9 cells. N9 cells were incubated with various ODN (1.5 μM) for 12 h. Induction of TNF-α (A), iNOS (B) and IL12p40 (C) was measured using real-time PCR. Data are expressed relative to reference PE 1826 and each bar represents the mean and SEM of triplicates.

3.3. The extension of CpG-ODN with a polyG motif enhances CpG-ODN uptake but without affection cellular distribution

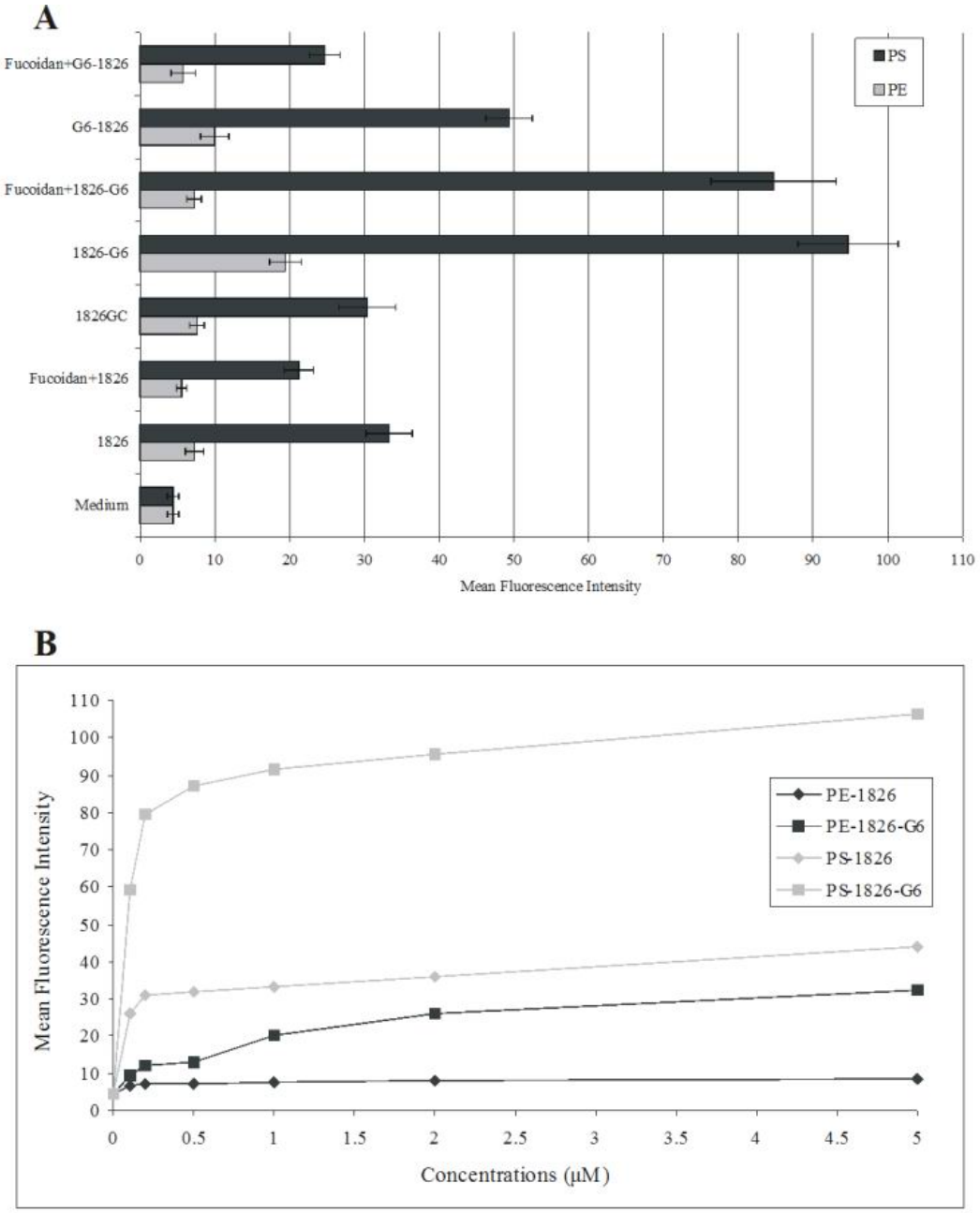

Fig. 3. Influences of a polyG motif on CpG-ODN uptake in microglial N9 cells. (A) N9 cells were incubated with various FITC labelled ODNs (1 μM) for 1 h at 37°C in serum-free RPMI 1640. Thereafter, cells were washed and mean fluorescence intensity was measured by flow cytometry. For inhibition, fucoidan (1.4 mg/mL) was added 15 min prior to addition of FITC-ODN. Each bar represents the mean and SEM of triplicates. (B) Dose–response curve for some of the ODN of panel (A) are given in detail.

Uptake is an essential prerequisite for the action of CpG-ODN.To study the mechanism of the effects of a polyG motif on CpG-ODN, a panel of PE or PS CpG-ODN, with or without polyG motif, was FITC labelled and their cellular uptake into N9 cells was analyzed. Cells were incubated with FITC labelled CpG-ODN (1 μM) for 1 h. Thereafter, cells were detached from the plate with trypsin-EDTA and dislodged cells were analyzed by flow cytometry.

As shown in Fig. 3A, uptake of the PS ODN was much higher than that of their PE counterparts with the same sequences (1826, 1826-G6 and G6-1826). This indicates that the PS phosphothioate modification highly increases cellular uptake. Moreover, uptake was independent of CpG motif (PE/PS 1826 compared with PE/PS 1826GC). PolyG motif either located at the 3' or the 5' terminus increased PE/PS 1826 uptake. Furthermore, cellular uptake of PE/PS ODN, with or without polyG motif, decreased when cells were co-incubated with fucoidan, a known ligand of scavenger receptor A. In addition, CpG-ODN uptake was dose-dependent (Fig. 3B).

After cell entry, CpG-ODN move into early endosomes and are subsequently transported to a lysosomal compartment. TLR9, which is localized to the endoplasmic reticulum, is recruited to the CpG-ODN containing compartment. The interaction of CpG-ODN with TLR9 activates a signal pathway and induces proinflammatory gene expression (Latz et al., 2004). Therefore we investigated whether the polyG motif could change the cellular distribution of CpG-ODN.

Fluorescence microscopy was used to study the intracellular distribution of FITC labelled CpG-ODN (nuclei were counterstained with DAPI). As shown in Fig. 4, PE and PS 1826 mainly localized in the cytoplasm and showed a spotted distribution after entry. PE/PS 1826-G6 as well as G6-1826 showed a similar spotted cytoplasmic distribution. Thus, in the present experimental setting polyG motifs did not change the cellular distribution of CpG-ODN.

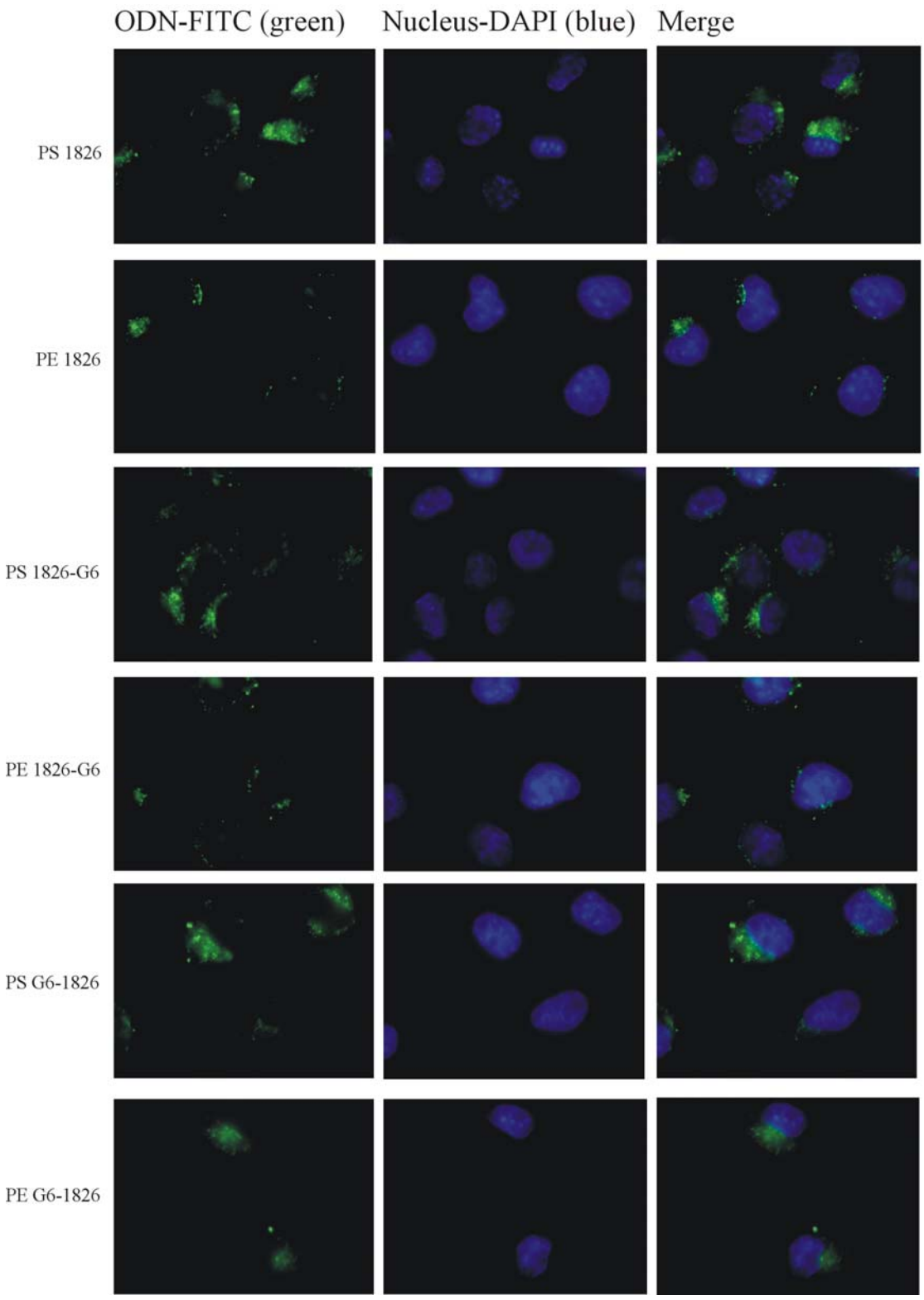

Fig. 4. PolyG motifs do not change the cellular distribution of CpG-ODN in microglial N9 cells. N9 cells were cultured in 6-wells chamber slides for 24 h and then incubated with 0.5 μM of various FITC labelled ODN for 1 h at 37°C. Subsequently cells were washed and fixed with PFA (2%) at room temperature for 15 min. Mounting medium with DAPI was used to show the cell nucleus. After covering with glass slides, cells were observed using a fluorescence microscope.

3.4. The extension of CpG-ODN with a 3'-end polyG motif enhances the number of cellular binding proteins

The affinity of ODN to cell membrane proteins positively correlates with their cellular uptake efficiency; therefore we tested whether a 3'-end polyG motif could increase the number of cellular binding proteins. PE 1826 and PE 1826-G6 were biotin labelled and were used to capture cellular proteins from microglial N9 cells. The captured proteins were analyzed by SDS-PAGE. As shown in Fig. 5, PE 1826-G6 interacted with more proteins than PE 1826.

Fig. 5. CpG-ODN containing a polyG motif bind more cellular proteins.
Biotin labelled ODN were bound via streptavidin to magnetic microbeads.

4. Discussion

DNA has multiple and complex effects on the immune system. One of the most well-studied types of DNA is the CpG-ODN, which interacts with TLR9 and induces a Th1- polarized immune response. Another type is the singlestranded

guanosine-rich ODN, which forms a quadruplex structure under physiologic conditions that result in nuclease resistance and increase cellular uptake. Due to these advantages, CpG-ODN containing a polyG motif have been tested in several types of immune cells.

Microglia are the brain's immune effector cells. Several studies have shown that primary microglia as well as microglial cell line, such as BV-2, express TLR9 and can be stimulated by CpG-ODN to express different cytokines (Dalpke et al., 2002b; Takeshita et al., 2001; Bsibsi et al., 2002). These observations are corresponding with our result that microglial N9 cells express TLR9 and can be activated by CpG-ODN (Fig. 1).

In this study we investigated the effects of a polyG motif on the immunostimulatory activity of CpG-ODN in microglial N9 cells. In general, CpG-ODN containing a polyG motif showed immunomodulatory activity in microglial N9 cells but only polyG motifs located at the 3'-end of PE CpG-ODN increased the immunostimulatory activity. PolyG motifs located at the 3'-end of PS CpG-ODN or 5'-end of PE or PS CpG-ODN, however, suppressed the immunostimulatory activity in microglial N9 cells. PolyG alone has no immunostimulatory activity in microglial N9 cells either. Other groups have tested the immunomodulatory effects of PolyG motif on CpG-ODN in several other immune cells. Using macrophage cell line, RAW 264.7 and primary dendrite cells, Dalpke et al. and Lee et al. respectively prove only 3'-end polyG motifs increase immunostimulatory activity of PE CpG-ODN (Dalpke et al., 2002a; Lee et al., 2000).

The interaction of CpG-ODN with TLR9, which is localized to the endoplasmic reticulum, triggers a signal pathway and finally induces immunostimulatory gene expression (Latz et al., 2004). Therefore cellular uptake of CpG-ODN is considered to be the rate-limiting step for CpG-ODN activity. CpG-ODN that were linked to latex, magnetic or gold beads could not be taken up and lost their stimulatory activity (Manzel and Macfarlane, 1999; Sester et al., 2000).

Being polyanionic chemicals, ODN are unlikely to enter cells by simple diffusion. Receptor-mediated endocytosis is considered to be the major mechanism of ODN uptake in most cells (Juliano and Yoo, 2000). Several putative DNA binding/transport proteins have been isolated from cell membranes and Mac-1, a scavenger receptor and a cell membrane nucleic acid channel has been found to potentially mediate endocytosis of nucleic acids (Benimetskaya et al., 1997; Biessen et al., 1998; Hanss et al., 1998).

We observed that addition of a polyG motif at the 3'- or 5'-end enhanced cellular uptake of PE or PS CpG-ODN by N9 cells. But 3'-end polyG had greater efficiency in enhancing cellular uptake than 5'-end polyG. Uptake could be decreased by fucoidan, a ligand of scavenger receptor. Single-stranded guanosine-rich ODN, which have the ability to form quadruplex structures, were reported to be effective ligands of scavenger receptor A, one of the membrane receptors involved in ODN uptake (Kaur et al., 2003). Antisense ODN tethered to polyG sequences at their 3'-end were recognized by scavenger receptors on macrophages and were taken up more efficiently than those ODN that lacked the polyG sequences (Prasad et al., 1999). Furthermore, quadruplex structure formation of the polyG motif in CpG-ODN correlates with its ability to induce IL12 (Lee et al., 2000). The reports corroborating with our observation indicated that polyG motifs might enhance cellular uptake of ODN through quadruplex structure formation and the resulting interaction with the scavenger receptor. Furthermore, PE 1826-G6 binds a higher number of cellular proteins than PE 1826 (Fig. 5), this suggests that the polyG motif might also interact with other proteins to increase uptake. The location of the polyG motif within the ODN affects its ability to increase ODN uptake, 3'-end location proved to be better than 5'-end in our experiment. This might be due to quadruplex structure formation being dependent on the position of the polyG motif.

After cell entry CpG-ODN move into the early endosomes and are

subsequently transported to a lysosomal compartment, where TLR9 and the adaptor molecule MyD88 are recruited and initiate a signal cascade. Thus the cellular distribution of CpG-ODN is important for its activity. We compared cellular distribution of CpG-ODN and CpG-ODN with a polyG motif. There was no significant difference between different CpG-ODN, indicating that the polyG motif does not affect the cellular location of the CpG-ODN. The molecular recognition events between CpG-ODN and TLR9 are still not fully understood but a free 5'-end is important for CpG-ODN activity. Kandimalla et al. reported that the incorporation of ligands in the 5'-flanking sequences potentiates the immunostimulatory activity of CpG-ODN, while the incorporation of the same ligands in the 3'-flanking sequences has minimal effect on the immunostimulatory activity (Kandimalla et al., 2002). In addition, Klinman et al. observed that the immunostimulatory activity of CpG-ODN could be blocked by a 5'-end suppressing motif (Klinman et al., 2003). In our study, CpG-ODN with a 5'-end polyG motif were shown to be much less efficient at inducing TNF-α, iNOS and IL12p40 compared with CpG-ODN containing a 3'-end polyG motif. This can partly be explained by lower uptake, but might also partly be due to inhibitory effects of the 5'-end polyG motif.

Surprisingly, a 3'-end polyG motif increased PS CpG-ODN uptake but inhibited its immunostimulatory activity, which is totally different from that of PE CpG-ODN. The same inhibitory effects of 3'-end polyG motifs on PS CpG-ODN were also observed in macrophages and dendrite cells (Lee et al., 2000; Dalpke et al., 2002a,b). The reason for these contradictory observations is unknown but Dalpke et al. suggested a two-step model of CpG signalling that could explain the effects. In a first step CpG-ODN are taken up into an endosome. This step is CpG motif independent and enhanced by the polyG motif. In a second step CpG-ODN are transported to a lysosomal compartment where the CpG motifs are exposed with the help of certain enzymes and can then be recognized by TLR9. For this step, it is assumed that the PE polyG

motif can be easily degraded and thereby allow exposure of the CpG motif, however, the PS polyG motif is difficult to degrade and thus the CpG motif is not presented.

CpG-ODN are strong Th1-polarized stimuli and thus potential therapeutic candidates. Complete or partial modification of the CpG-ODN backbone with phosphothioate is widely used to increase uptake and nuclease resistance. However PS ODN may cause certain kinds of unwanted effects and PS CpG-ODN might exacerbate inflammatory tissue damage, autoimmune disease or increase sensitivity to toxic shock, which limits the applications of PS CpG-ODN. But PE CpG-ODN are easily digested by nucleases and therefore show low bioavailability, which is an obstacle for PE CpG-ODN application. Guanosine-rich ODN, which form a quadruplex structure that result in nuclease resistance and increase cellular uptake, might help to overcome this obstacle. Here we show that a 3'-end polyG motif enhances both the cellular uptake of PE CpG-ODN and their immunostimulatory activity in microglial N9 cells. Therefore these hybrid PE CpG-ODN might be promising alternative analogues for application of CpG-ODN in the central nervous system.

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Discussion

Adult-derived MSCs, initially thought to be limited in potential to mesenchymal tissues, have been shown to be capable of greater plasticity and transdifferentiation than previously expected. [130-138] Even MSCs display a finite life span in *in vitro* culture and approach senescence much more rapidly than ESCs, current techniques for the long-term culture expansion and maintenance of the undifferentiated phenotype of MSCs already allow them to be grown in sufficient number for clinical application.[139,140] Interestingly, another multipotent adult progenitor cell, capable of differentiating at the single-cell level into cells of visceral mesoderm, neuroectoderm, and endoderm *in vitro* (specifically, cells of the hematopoietic lineage), as well as epithelium of the liver, lung, and gut, was recently copurified along with the MSC from rodent bone marrow. [141] Although the existence of such multipotent adult progenitor cells needs to be confirmed in humans, adult MSCs are likely to offer the same therapeutic potential without evoking the ethical, moral, and legal issues associated with the use of ESCs. In conclusion, MSCs derived from adult tissue present an exciting progenitor cell source for applications of tissue engineering and regenerative medicine. Modalities may include direct injection or implantation and/or *ex vivo* tissue engineering, in combination with biocompatible/biomimetic biomaterials and/or natural or recombinantly derived biologics. MSCs may also be considered for gene therapy applications for the delivery of genes or gene products. Another intriguing prospect for the future is the use of MSCs to create ‘off-the-shelf’ tissue banks. To fully harness the potential of these cells, future studies should be directed to ascertain their cellular and molecular characteristics for optimal identification, isolation, and expansion, and to understand the natural, endogenous role(s) of MSCs in normal and abnormal tissue functions.

To talk about the application of MSC, another type of stem cell has to be

refered, which is called embryonic stem cell. ESCs are derived from the inner cell mass of the embryonic blastocyst. These cells can be maintained indefinitely in vitro without loss of differentiation potential, and when reimplanted into a host embryo, they give rise to progenies that differentiate into all tissues. However, the research is always complicated by the current complexities of ethical issues. Controversies surrounding the legal and moral status of human embryos and the use of ES cells encompass fundamental issues such as contraception, abortion, the definition of human life, and the rights and legal status of an embryo. Despite such challenging considerations, it is instructive to explore the fundamental biological differences between MSCs and ES cells, especially for applications of regenerative medicine. The transient life span of ES cells in vivo is in sharp contrast to that of MSCs, which reside much later into adult life. The seemingly unlimited potential of human ES cells to self-renew and differentiate into a large variety of tissues was first characterized by Thomson *et al.* [142] For example; use of allogeneic cells could involve the potential risks of immunorejection and heterotopic tissue formation (teratomagenesis). These problems could be circumvented using autologous cells created by 'somatic-cell nuclear transfer', but will eventually evoke ethical and legal issues similar to those surrounding reproductive cloning. [143]

MSC targeting by aptamers: Aptamers are a promising class of compounds, both for target validation and therapy. Since aptamers were introduced 15 years ago, the application is under extremely exploration. Given their small size, ease of synthesis, and low cost, aptamers provide versatile tools for validation of intracellular and extracellular targets. In the case of extracellular targets, such as VEGF, thrombin, and PDGF etc, aptamer-based validation affords a direct path to therapeutic development. Therapeutic aptamer can be readily stabilized or shielded from renal filtration by chemical or compositional modification for evaluation in in vivo preclinical discovery programs. But the limitation of aptamers that also applies to antibody fragments or peptides seems to be that, unlike small molecules, they cannot enter animal cell culture

cells unaided. Most researchers in industry therefore focus on extracellular aptamer targets, because of the much easier access of the aptamer drugs to either target molecules. In vivo delivery of aptamers to intracellular targets has mostly been addressed up to now by incorporating them into liposome vesicles. In clinical trials of another functional therapeutic class of RNA molecules, ribozymes, the drugs seem however to be able to penetrate into patient cells without additional transfection or delivery reagents. The clinical formulations required to date seem only to be simple saline solutions. The same could be true for aptamer therapeutics, but this still needs to be examined. Alternatively, aptamers as 'intrameric' entities intramers are being more and more successfully explored in cell culture systems and animal models by using gene therapeutic approaches and vector delivery systems. Perhaps the most exciting perspective for aptamers lies in drug discovery. Due to their unique binding characteristics, the way aptamer-based lead substances potentially engage their target molecules is different from other types of ligands. This can lead to new therapeutic approaches with unique mechanisms of action. The rapid generation of aptamer ligands by automated selection and the unique options to fully control the experimental setting in the selection process open up further opportunities to link the lead substance’s binding to specific pathophysiological parameters. Moreover, aptamers can potentially provide a smart link between early target validation, diagnostic application, drug development, and therapeutic intervention. The speculation that, analogous to nature where nucleic acids carry out sophisticated tasks beyond information storage (e.g., ribosomal RNA catalyzing peptide-bond formation), customized transfer “nucleic acid biotools” will find a natural place in research for various tasks. The numerous ways in which aptamers can be employed are still evolving. Finally, in specific situations, aptamers themselves may indeed be the best therapeutic solution. One of the advantages of aptamers versus small-molecule drugs is their exquisite specificity. Aptamers are designed and developed for high affinity to only a certain target molecule or only a particular

isoform of a target protein and can distinguish between different conformational states of the same protein. [144,145] Their high specificity is reflected in their many different possible modes of target recognition. Much work to elucidate the molecular structure of aptamer and ligand interactions has had a great impact on our understanding of aptamer function. [146] Most aptamers are very adaptive and flexible when unbound. Different aptamers show totally different binding motifs, which range from stacking of flat ligand moieties between bases to binding of target moieties or amino acids deeply inside the groove of twisted double-helix parts of the aptamer.

However, the future role of aptamers as pharmaceuticals or tools in pharmaceutical development remains to be seen, given the potential of other new methods currently under evaluation, such as RNAi, peptide, or antisense technologies. RNAi has become a widely used tool for the suppression of gene activity in invertebrates, plants, and, with the advent of small interfering RNA (siRNA) techniques, in mammalian cells. [147-149] siRNAs can be introduced via direct transfection or by expression from various plasmids either transiently or stably. [149-151] The siRNA molecules bind to a protein complex, called the RNA-induced silencing complex. This complex contains a helicase activity that unwinds the two strands of RNA molecules, allowing the antisense strand to bind to the targeted RNA molecule [151] and an endonuclease activity that hydrolyzes the target RNA at the site where the antisense strand is bound. Recently, nucleic acid-derived aptamers have been used to regulate intracellular protein activity. [152] Aptamer 'decoys', short RNAs containing the RNA-binding site for HIV-1 RNA-binding proteins, such as Rev and Tat, have been developed that inhibit HIV-1 replication when over-expressed. [153-154] Aptamers specific to non-RNA-binding proteins, as well as RNA-binding proteins, can be generated. These non-decoy aptamers can bind with high specificity and affinity [155] and can knock-out intracellular protein activity. [156] A good example for combine these two methods come out recently. An anti-NFB aptamer, a-p50 [157] which can inhibit the DNA-binding activity of the p50

subunit of the NFB(NF-kappa B) transcription factor in yeast, [158] and a kind of siRNA which can also knock down the activity of NFB were used alone and combined separately. The result was that both aptamers and siRNAs when used alone knock-down the activity of NFB to a similar extent and interestingly, when used in combination the two methods work better than either method alone, leading to essentially complete (90%) knock-down of NFB activity.[159]

Aptamers as capture molecules for cells isolation is a new application, here we selected intact MSCs as the target and using aptamers as a coating material to enhance cell enrichment, and this is totally new application. We combined the aptamer technology with tissue engineering and contribute this application to regenerative medicine. The function part of aptamers is from the secondary structure, complementary base pairing defines aptamer secondary structure, consisting primarily of short helical arms and single-stranded loops. Stable tertiary structure, resulting from combinations of these secondary structures, allows aptamers to bind to targets via van der Waals, hydrogen bonding, and electrostatic interactions. Due to the importance of mesenchymal stem cells and lack of special phenotype, we want to get the aptamer to be used as a special antibody, with two functions: to bind MSCs and to fish out MSCs from the bone marrow: when coated on scaffolds as a material, and the scaffold is plated with MSCs, the coating material can enhance the attachment. In our first pre-experiment, we use osteoblasts as the seeding cells, and the results turned out good. Due to the difficulties to get human MSCs and due to the fact that the animal experiments must be made before clinical applications we selected porcine MSCs as the target cells. We found some interesting results in our experiment: the MSCs captured by aptamers showed CD45 positive when freshly isolated, but after culture this phenotype is quickly lost. This result is in accordance to the reportage before.[160] There are some groups reporting about antigenic shifts and differences between subpopulations of aMSC (e.g. RS-2 cells CD90-, mature MSC CD90+). The freshly aptamer-isolated cells showed surface characteristics which had been

described previously (CD45+, CD44+) and others which to our knowledge had not yet been described before (CD29-, CD90- (population R1)). It has to be emphasized that most of the cells which were described in the literature were cultured cells, whereas with the aptamer-technique we could isolate and analyze MSC nearly in the condition of their natural environment, the bone marrow. The method is quite quick and without damage to the cells. After 2 weeks in culture the aptamer-isolated cells showed very homogeneously the previously described surface epitopes (CD29+, CD44+, CD90+, SLA class I+, CD45-, SLA class II-). MSCs cannot be identified and sufficiently characterized only by analyzing their epitope profile. To confirm the character of stem cells, additionally the differentiation potential has to be evaluated. The aptamer-isolated cells showed adipogenic and osteogenic differentiation potential defining them as real MSCs. And to the purity of isolated MSCs, we could not exclude having co-isolated hematopoietic stem cells, but, this possibility does not be very likely due to the above mentioned arguments.

And to compare the advantage of the traditional separating method and our aptamer based method, we seeded 500 cells with the conventional method (ficoll-24h plastic adherence), not a single cell or colony could be detected after 5 weeks in culture which is due to the low frequency of aMSC in the adult bone marrow. In contrast, 500 aptamer-isolated cells proliferated and grew until confluence and could be differentiated. This shows that our method is an efficient technology.

More important is that this technique can easily be adjusted for generation of aptamers against human MSCs or other haemopoietic sources. Recently We have started further experiments with human MSCs. Our future goal is to make more applications of aptamers in the field of tissue engineering and regenerative medicine.

Conclusion

Within this study, we aim to ascertain the application of aptamers as capture molecules to separate cells from mixed solutions like whole blood or bone marrow. Furthermore we immobilized aptamers on solid substrates to enhance the cell attachment to artificial implant materials. To test the proof of principle for this application, we first used an immortal cell line as target cells to select the aptamers. When the aptamers against these osteoblasts were selected out, we immobilized the aptamer on a Ti-alloy surface by electrochemical methods and incubated it with a osteoblast solution. The aptamer coated surfaces bound more cells than the non-coated surfaces. The pictures from scanning electronic microscope showed that the binding of the osteoblasts to the aptamer coated surfaces was much stronger than the binding of the non-coated surfaces to the osteoblasts. When this principle was successful proved, we targeted adult porcine mesenchymal stem cells (aMSCs) and generated a specific aptamer against them. To test the binding ability of this aptamer, first we used FITC-labeled aptamers to bind with aMSCs and analysed by FACS assay. Then we fixed biotin-labeled aptamers on streptavidin magnetic beads and incubated the mixture with aMSCs, then filtered through a magnet to collect the cells which were bound on the beads. Both results showed the high binding affinity of the aptamer to aMSC. Then we applied aptamers to separate MSCs from bone marrow blood. By FACS sorting, the MSC could be sorted out by FITC-aptamer. The further phenotype analysis showed that the isolated MSCs indicated an antigen shift from isolation to culture. Besides the previously report of CD45 loss in culture, we found that there was an upregulation of CD29 during culture. We speculated that the isolated MSCs represented a type of very young populations which will reveal novel insights to the MSC subpopulations and their antigenic profile in their natural environment. In addition, the isolated MSCs had strong potential

to differentiate into osteoblasts and adipocytes compared to the MSCs isolated by the traditional method which is based on their plastic adherence. The potential clinical applications will not only reveal unknown aspects of isolation and characterisation of MSCs, but also provide a new tool for the fascinating field of regenerative medicine and tissue engineering.

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List of Publications

Full articles.

1. Zhang Z, **Guo K**, Schluesener HJ.

The immunostimulatory activity of CpG oligonucleotides on microglial N9 cells is affected by a polyguanosine motif. *J Neuroimmunol. 2005 Apr;161(1-2):68-7*.

2. **Guo K**, Wendel HP, Scheideler L, Ziemer G, Scheule AM.

Aptamer-based capture molecules as a novel coating strategy to promote cell adhesion. *J Cell Mol Med. 2005 Jul-Sep; 9(3):731-6.*

3. Zhang Z, Weinschenk T, **Guo K**, Schluesener HJ.

siRNA binding proteins of microglial cells: PKR is an unanticipated ligand. *J Cell Bio*chem. *J Cell Biochem*. 2006 Apr 15; 97(6):1217-29.

4. **Guo KT**, Schaefer R, Paul A, Gerber A, Ziemer G, Wendel HP.

A new technique for the isolation and surface immobilization of mesenchymal stem cells from whole bone marrow using high specific DNA-aptamers. *Stem Cells.in press 2006*.

5. **Guo KT**, Scharnweber D, Schwenzer B, Ziemer G, Wendel HP.

Electrochemical functionalization of Ti-alloy surfaces by aptamer-based capture molecules accelerates cell adhesion. *Biomaterials. accepted*.

6. Zhang Z, Schittenhelm J, **Guo K**, Bühring HJ, Trautmann K, Meyermann R, Schluesener HJ.

Upregulation of frizzled 9 (FZD9) in astrocytomas. *Neuropathology and applied neurobiology. Accepted.*

7. Guo LH, **Guo KT**, Wendel HP, Schluesener HJ.
Combinations of TLR and NOD2 ligands stimulate rat microglial P2X4R expression. *Biochemical and Biophysical Research Communications 2006.Accepted.*

Posters and Abstracts

1. Selection of aptamers to porcine mesenchymal stem cells

4th annual Meeting of European Tissue Engineering Society, Aug.31st –Sep. 3rd, Munich, Germany

2. Selection of aptamers to porcine mesenchymal stem cells

8th annual meeting of Tissue Engineering Society Internation. Oct 22nd-25th, Shaihai, P. R. China

3. A new technique for the isolation and surface immobilization of mesenchymal stem cells from whole bone marrow using high specific DNA-aptamers.

2006 Regenerate World Congress on Tissue Engineering and Regenerative Medicine. Apr 25th-27th, Pittsburgh, USA.

Award

Travel award 2006 Regenerate World Congress on Tissue Engineering and Regenerative Medicine. Apr 25th-27th, Pittsburgh, USA.

Assertion of Publication

1. Aptamer-based capture molecules as a novel coating strategy to promote cell adhesion.

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Journal of Cellular and Molecular Medicine. 2005 Jul-Sep; 9(3):731-6.

The SELEX-technology for the generation of aptamers against osteosarcoma cells was exclusively developed by me. All experiments were planed, performed and analyzed by me. The publication was written by me. The idea of this study was from Hans P. Wendel and he supervised my work and corrected the manuscript. L. Scheideler introduced to me the culture of the osteosarcoma cells. G. Ziemer and AM. Scheule were intensively involved in the planning of the study.

2. Electrochemical functionalization of Ti-alloy surfaces by aptamer-based capture molecules accelerates cell adhesion

Ketai Guo[1], Dieter Scharnweber[2], Bernd Schwenzer[3], Gerhard Ziemer[1], Hans P. Wendel[1] *

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2 Max Bergmann Center of Biomaterials, Technical University, Dresden, Germany
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The SELEX-technology for the generation of aptamers against osteosarcoma cells was exclusively developed by me. All experiments were planed, performed and analyzed by me, with the exception of the part “Immobilization procedure of aptamers on Ti-alloy“ which was performed by D. Scharnweber and B. Schwenzer. The publication was written by me. The idea was from Hans P. Wendel and he supervised my work and corrected the manuscript. G Ziemer was intensively involved in the planning of the study. This manuscript is

submitted to Biomaterials.

3. A new technique for the isolation and surface immobilization of mesenchymal stem cells from whole bone marrow using high-specific DNA-aptamers.

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Stem Cells. In press 2006

The SELEX-technology for the generation of aptamers against pig mesenchymal stem cells was exclusively developed by me. All experiments were planed, performed and analyzed by me, with the exception of the figure 1 a (C-F), figure 1b, figure 2(a-c), figure 6 which were performed by R. Schäfer and A. Gerber. Figure 4 were performed by me and R. Schäfer together. A. Paul helped me with the culture of the mesenchymal stem cells. The publication was written by me. The idea was from Hans P. Wendel. He supervised my work and corrected the manuscript. G. Ziemer was intensively involved in the planning of the study.

4. The immunostimulatory activity of CpG oligonucleotides on microglial N9 cells is affected by a polyguanosine motif

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All the experiments of real time-PCR in the manuscript of “The immunostimulatory activity of CpG oligonucleotides on microglial N9 cells is affected by a polyguanosine motif” were performed by me. All experiments were planed, performed and analyzed by Zhiren Zhang. The publication was written by Zhiren Zhang. The idea of the study was from H Schluesener’ group.

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I want to thank my loving family: my parents, my brother and my husband, their love are always my power resource to go ahead. I love them forever!

Curriculum Vitae

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