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Predicting the therapeutic efficacy of MSC in bone tissue engineering using the molecular marker CADM1

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ABSTRACT

Mesenchymal stromal cells (hMSCs) are advancing into the clinic but the therapeutic efficacy of hMSCs faces the problem of donor variability. In bone tissue engineering, no reliable markers have been identified which are able to predict the bone-forming capacity of hMSCs prior to implantation. To this end, we isolated hMSCs from 62 donors and characterized systematically their *in vitro* lineage differentiation capacity, gene expression signature and *in vivo* capacity for ectopic bone formation. Our data confirms the large variability of *in vitro* differentiation capacity which did not correlate with *in vivo* ectopic bone formation. Using DNA microarray analysis of early passage hMSCs we identified a diagnostic bone-forming classifier. In fact, a single gene, *CADM1*, strongly correlated with the bone-forming capacity of hMSCs and could be used as a reliable *in vitro* diagnostic marker. Furthermore, data mining of genes expressed correlating with *in vivo* bone formation represented involvement in neurogenic processes and Wnt signaling. We will apply our data set to predict therapeutic efficacy of hMSCs and to gain novel insight in the process of bone regeneration. Our bio-informatics driven approach may be used in other fields of cell therapy to establish diagnostic markers for clinical efficacy.

1. Introduction

Many human diseases are caused by failure of tissue function, with well-known examples such as diabetes, damage inflicted by myocardial infarcts and degeneration of the hip joint. The disciplines of tissue engineering and cell therapy aim at restoring worn-out or diseased tissues for which the patient's own body represents a source of autologous cells [1]. For instance, a much used source of autologous cells in the field of bone tissue engineering is human multipotent mesenchymal stromal cells (hMSCs), also referred to as mesenchymal stem cells [2]. Because hMSCs can be easily isolated from bone marrow aspirates and expanded *in vitro*, they are used for various cell-based therapeutic applications [3]. hMSCs are multipotent cells which are able to differentiate, depending on the stimulus, into several lineages including the osteogenic, chondrogenic and adipogenic lineage *in vitro* [4]. Osteogenic differentiation of hMSCs is characterized by expression of alkaline phosphatase (ALP) and the formation of a mineralized extracellular matrix (ECM) containing hydroxyapatite. Molecules such as dexamethasone (dex), 3′-5′-cyclic adenosine monophosphate (cAMP), 1,25-dihydroxyvitaminD (vitD3) and bone morphogenetic protein 2 (BMP-2) are used to drive osteogenic differentiation of hMSCs *in vitro* [5–7]. For bone tissue engineering, we and others have demonstrated ectopic bone formation by seeding hMSCs onto porous calcium phosphate scaffolds and subsequent subcutaneous implantation into immune-deficient mice [8,9]. Although proof of principle exists for bone tissue engineering in animal models [10], clinical application is hampered by large donor variation in the ability of hMSCs to deposit bone tissue *in vivo* [11,12]. Unfortunately, bone tissue engineering efficacy is not correlated to known clinical or molecular labels. This is partly due to the fact that the field of hMSC biology lacks an elaborate classification system of CD markers to define stem cells, progenitor

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cells and differentiated cells as it is known for the hematopoietic stem cell system. Cell surface markers such as Stro-1 and the nerve growth factor receptor have been used to prospectively isolate clonogenic hMSCs from a crude bone marrow aspirate [13,14], but the resultant population of cells is still heterogenic in its biological performance. CD146 defines an hMSC subpopulation with the ability to organize a hematopoietic niche *in vivo* but its expression on hMSCs does not correlate to bone formation per se [15]. Therefore, it would be beneficial to define diagnostic markers in culture expanded hMSCs which can predict their *in vivo* performance. The markers could be used to select patients eligible for clinical trials but also provide biological tools to interfere with the osteogenic potential of hMSCs.

The use of diagnostic markers for tissue engineering outcome is successfully used in the field of cartilage regeneration, where Dell'Accio et al. identified a set of molecular markers predictive for *in vivo* cartilage formation of adult human articular chondrocytes [16]. The genes were identified based on their known involvement in the chondrogenic process and similarly, we and others have tried to correlate the expression of genes involved in the osteogenic process in hMSCs to their potency to form bone *in vivo*. Although correlations were found between collagen type I and osteoprotegerin [17] or ALP expression [18] and bone formation, the data sets used were too small to firmly establish a link between gene expression and bone formation and no new insight in the process was obtained. To this end, larger data sets are required for which genome-wide gene expression profiling can be applied. Recently, Larsen et al. identified a molecular phenotype for hMSCs with *in vivo* bone-forming capacity by comparing low versus high bone-forming hMSC-TERT cell populations [19]. Also, in the same group Burns et al. described a correlation between *in vivo* bone formation and *in vitro* expression of matrix proteins determined by analysis of the same cell populations in three-dimensional hydroxyapatite-tricalcium phosphate osteospheroid cultures [20]. However, in both cases donor-to-donor variation is not taken into account since both cell populations originate from the same donor, and thus the molecular signature of bone-forming hMSCs remains incomplete.

We have previously reported on a microarray based approach to distinguish metastasizing from non-metastasizing breast tumors starting from RNA isolated from a breast tumor biopsy [21]. In this study, we have used a similar strategy to find *in vitro* diagnostic markers which are able to predict the *in vivo* bone-forming capacity of hMSCs. We developed a bank of hMSCs from 62 different donors, performed various *in vitro* differentiation assays and analyzed the *in vivo* bone formation for each donor. In addition, we determined the gene expression profile of the hMSCs from the different donors and correlated it with the *in vivo* bone-forming capacity (outlined in Fig. 1). In this manuscript, we present a molecular signature of bone-forming hMSCs.

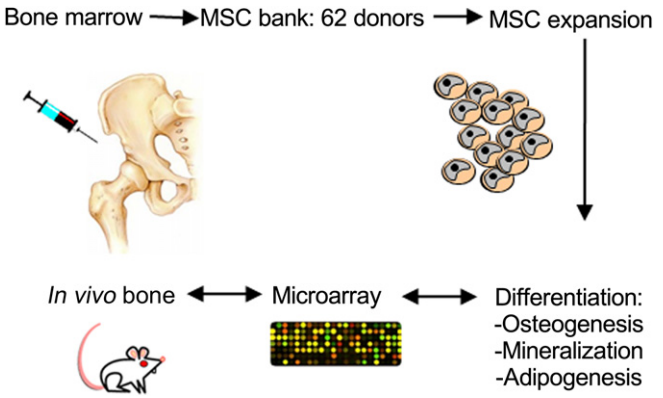

Fig. 1. Study outline. Bone marrow aspirates were obtained from 62 donors, hMSCs were isolated and expanded, subsequently the *in vivo* bone formation, microarray expression profile and differentiation capacity of the cells were determined.

2. Materials and methods

2.1. Isolation and culture of hMSCs

Bone marrow aspirates (5–20 mL) were obtained from donors with written informed consent, and hMSCs were isolated and proliferated as described previously [22]. Briefly, aspirates were resuspended using a 20-gauge needle, plated at a density of 500,000 cells/cm^2 and cultured in hMSC proliferation medium containing α-MEM (Gibco), 10% heat-inactivated fetal bovine serum (Biowhittaker), 0.2 mM ascorbic acid (Sigma), 2 mM L-glutamine (Gibco), 100 U/mL penicillin with 100 mg/mL streptomycin (Gibco) and 1 ng/mL basic fibroblast growth factor (Instruchemie, Delfzijl, The Netherlands). The serum batch was selected based on proliferation rate and osteogenic differentiation potential. Cells were grown in a humid atmosphere with 5% CO_2. After plating of the bone marrow aspirate, the cells obtained from the first trypsinization were considered as PD (population doubling) 0. Relative population doublings refers to the number of population doublings that cells had undergone, relative to PD 0. Basic medium was composed of proliferation medium without basic fibroblast growth factor, osteogenic medium was composed of basic medium supplemented with 10^{-8} M dex (Sigma) and mineralization medium was composed of basic medium supplemented with 10^{-8} M dex and 0.01 M β–glycerophosphate (Sigma). After expansion, cells of the same batch were split into the different media for differentiation assays.

2.2. In vivo bone formation

To evaluate the bone-forming capacity of hMSCs, cells were seeded onto porous biphasic calcium phosphate (BCP) ceramic granules of approximately 2–3 mm, prepared and sintered at 1150 °C as described previously [23]. In total, 200,000 cells per three particles were seeded, in osteogenic medium. This seeding density is on the lower hand to avoid an outcome of 100% bone-forming donors. After one week of culturing, tissue-engineered constructs were implanted subcutaneously in immune-deficient mice (Hsd-cbp:NMRI-nu, Harlan, $n = 6$ for each donor). The mice were anesthetized by intramuscular injection of 0.05 mL of 0.5 mg/mL of anesthetic (1.75 mL of 100 μg/mL ketamine, 1.5 mL of 20 mg/mL xylazine and 0.5 mL of 0.5 mg/mL atropine). Four subcutaneous pockets were made dorsally and each pocket was implanted with three particles. Animals were housed at the Central Laboratory Animal Institute (Utrecht University, Utrecht, The Netherlands), and experiments were approved by the local animal care and use committee. After six weeks, the mice were sacrificed using CO_2 and samples were explanted, fixed in 1.5% glutaraldehyde (Merck) in 0.14 M cacodylic acid (Fluka) buffer (pH 7.3), dehydrated and embedded in methyl methacrylate (LTI) for sectioning. Sections were processed on a histological diamond saw (Leica SP1600). Sections were etched with an HCl/ethanol mixture and sequentially stained to visualize bone, with 1% methylene blue (Sigma) and 0.03% basic fuchsin (Sigma), which stained cells blue and bone pink. Histomorphometry was performed by making low-magnification images from three sections per sample, with a standard selection procedure for each donor. In short, one section in the middle of the scaffold was chosen and the other two sections (left and right side) imaged had the same spacing from the middle section. Scaffold and bone were pseudo colored, and image analysis was performed with KS400 software (Zeiss Vision). A custom-made program (University of Utrecht) was used to measure percentage of bone area compared to scaffold area.

2.3. Mineralization

To determine the mineralization capacity and calcium deposition, hMSCs were seeded in T25 flasks at 5000 cells/cm^2. Cells were cultured in mineralization medium for three weeks, in triplicate. The total calcium deposition was analyzed by using a Calcium Assay Kit (Quantichrom, BioAssay Systems) according to manufacturer's protocol. Briefly, 0.5 N HCl was used to release calcium and the calcium content was measured at 620 nm and expressed as mg/dl.

2.4. Adipogenesis

Adipogenic differentiation capacity of hMSCs was determined as described previously [24]. In short, after three weeks of culture in adipogenic medium, lipid formation was visualized by staining with Oil red O and staining was quantified by extraction of color and measuring absorbance at 540 nm.

2.5. Chondrogenesis

Cells were grown in pellet culture for 21 days with 250,000 cells/pellet in serum-free chondrogenic medium containing TGFβ3 [25]. Chondrogenic medium was supplemented with 250 ng/mL human BMP6 (Biovision) [26]. Pellets were fixed and stained with Alcian Blue (Sigma).