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Identification of suitable reference genes in bone marrow stromal cells from osteoarthritic donors

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Abstract Bone marrow stromal cells (BMSCs) are key cellular components for musculoskeletal tissue engineering strategies. Furthermore, recent data suggest that BMSCs are involved in the development of Osteoarthritis (OA) being a frequently occurring degenerative joint disease. Reliable reference genes for the molecular evaluation of BMSCs derived from donors exhibiting OA as a primary co-morbidity have not been reported on yet. Hence, the aim of the study was to identify reference genes suitable for comparative gene expression analyses using OA-BMSCs.

Passage 1 bone marrow derived BMSCs were isolated from n = 13 patients with advanced stage idiopathic hip osteoarthritis and n = 15 age-matched healthy donors. The expression of 31 putative reference genes was analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using a commercially available TaqMan® assay. Calculating the coefficient of variation (CV), mRNA expression stability was determined and afterwards validated using geNorm and NormFinder algorithms.

Importin 8 (IPO8), TATA box binding protein (TBP), and cancer susceptibility candidate 3 (CASC3) were identified as the most stable reference genes. Notably, commonly used reference genes, e.g. beta-actin (ACTB) and beta-2-microglobulin (B2M) were among the most unstable genes.

For normalization of gene expression data of OA-BMSCs the combined use of IPO8, TBP, and CASC3 gene is recommended.

Introduction

Osteoarthritis (OA) is one of the most frequent musculoskeletal disorders and represents the main indication for total joint arthroplasty (Pereira et al., 2011; Pivec et al., 2012). Notably, in the western countries 10% of individuals above the age of 60 years suffer from osteoarthritis of the hip joint (Sun et al., 1997). Due to the increasingly aging western populations OA represents one of the most important socio-medical and economic musculoskeletal diseases (Woolf and Pfleger, 2003). OA is defined as a premature destructive joint degeneration accompanied by pain and reduced function. The disease is characterized by alterations in both the cartilaginous (e.g. increased matrix-metalloproteinase-mediated enzymatic breakdown of extracellular matrix components) as well as by structural alterations in the adjacent subchondral bone (Bijlsma et al., 2011). Cellular and subcellular mechanisms are in the focus of ongoing research concerning OA etiology (Müller-Hilke, 2007). Genetic factors are discussed as reason for OA besides apoptosis, mechanical load, and oxidative stress (Aigner

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et al., 2007; Loughlin, 2011). To date, therapeutic options are predominantly symptom-orientated and include the use of anti-inflammatory drugs, changes in life-style, autologous chondrocyte transplantation, as well as joint axis realigning and joint-replacing surgery.

Bone marrow stromal cells (BMSCs) provide an excellent source of progenitor cells because of their proliferation and differentiation capacity along a number of connective tissue lineages, e.g. bone, cartilage, adipose, and muscular tissue (Pittenger et al., 1999; Vater et al., 2011). BMSCs can be easily isolated from bone marrow aspirates and are key components for innovative cell-based therapies to treat musculoskeletal diseases (Loughlin, 2011). It can be assumed that a large proportion of patients who would potentially benefit from cell-based strategies suffer from osteoarthritis as the underlying disease or comorbidity.

Previously OA research primarily focused on morphological and metabolic alterations of chondrocytes and extracellular matrix (ECM) based on the rationale that the osteoarthritic joint is characterized by an imbalance between anabolic and catabolic processes at the cellular and extracellular level resulting in ECM degradation-mediated chondrolysis (Aigner et al., 2007).

Interestingly, researchers recently demonstrated alterations in the proliferation and differentiation capacity of BMSCs from osteoarthritic as compared to healthy donors supposing a key role of BMSCs in the pathogenesis of OA. Murphy et al. observed that OA-BMSCs showed a significantly reduced proliferation capacity as well as a reduction of the in vitro adipogenic and chondrogenic activities (Murphy et al., 2002). Lamas et al. demonstrated in bone marrow-derived OA-BMSCs isolated from the femoral channel a down regulation of the collagen gene set, particularly collagen, type X, alpha 1 (COL10A1), as well as several Wnt/-catenin pathway related genes (Lamas et al., 2010).

Currently, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) can be regarded as the most accurate and specific technique to assess gene expression. Because of its reproducibility, large dynamic range, ease of use, capability of high throughput, high accuracy, and affordability qRT-PCR is the most frequently applied method for gene expression analyses (D'haene et al., 2010). However, the accuracy of gene expression assessment is influenced by several factors, e.g. type and number of cells, quality and handling of mRNA (Bustin, 2002; Bustin and Nolan, 2004), type of detection chemistry (Bustin and Nolan, 2004), and cDNA synthesis conditions (Lekanne Deprez et al., 2002). Thus normalization of qRT-PCR data is an inevitable step to ensure accounting for all inter-sample variables mentioned above. Therefore, genes which are equally and constantly expressed independent of any treatment or disease should be used as internal controls. Target gene expression is related to the expression of these reference or "housekeeping" genes. The most popular and commonly used reference genes are glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin (ACTB). However, these genes are not constantly expressed in all types of physiological and diseased cells and tissues (Zhu et al., 2001; Suzuki et al., 2000). Although the combined use of several reference genes is recommended (Vandesompele et al., 2002), the vast majority of gene expression investigations are performed using only one reference gene. A proper selection of suitable reference genes needs to be performed for each tissue, cell type, and disease before using them in gene expression studies. At present, no standard method for the selection of reference genes exists. For the optimal choice of reference genes a number of statistical algorithms, such as NormFinder (Andersen et al., 2004), Global Pattern Recognition (Akilesh et al., 2003), equivalence test (Haller et al., 2004), Bestkeeper© (Pfaffl et al., 2004), and geNorm (Vandesompele et al., 2002) were introduced with all these algorithms being based on the gene expression stability.

The aim of this study was the identification of suitable reference genes for accurate and reliable quantification of gene expression using comparative qRT-PCR analyses of BMSCs from osteoarthritic versus healthy donors.

Methods

Cell isolation and cultivation

Bone marrow stromal cells were isolated from pelvic bone marrow aspirates of n = 13 (67 ± 6 years) patients with advanced stage idiopathic hip osteoarthritis and n = 15 age-matched (61 ± 4 years) healthy donors by density gradient-centrifugation. The fraction of mononuclear cells (MNCs) containing the BMSCs was transferred to cell culture flasks. BMSCs were separated by polystyrene adherence, cultured in DMEM up to 90% confluence, and harvested in passage 1. The study was approved by the local institutional review board (approval no. EK203082008).

Quantitative reverse transcription PCR analysis (qRT-PCR)

Total RNA isolation of passage 1 BMSCs with 3–5 × 10^6 cells per sample was carried out by AROS Applied Biotechnology A/S, Aarhus, Denmark. cDNA synthesis was performed using 1 μg total RNA with oligo-dT and SuperScript™ First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A total of 28 biological samples (n = 13 OA group and n = 15 control group) were analyzed at once with the number of donors in each group denoting biological replicates. Thus biological inter-individual differences in gene expression were registered.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using commercially available TaqMan® Express Human Endogenous Control Fast 96-Well Plates (Applied Biosystems), spotted with 32 lyophilized TaqMan® Gene Expression Assays in triplets. Table 1 provides a summary of all genes investigated in this study. Instead of analyzing 18s RNA expression, the corresponding wells were used for no template controls thereby leaving a total of n = 31 potential reference genes to be evaluated.

Ten microliter qPCR reaction volume containing 0.6 μl aqua dest (no template control) or 0.6 μl template cDNA corresponding to 29 ng of RNA, 4.4 μl sterile distilled water, and 5 μl TaqMan® Fast Universal PCR Master Mix (2×, Applied Biosystems) were added to each well. The amplification was performed using 40 cycles at 95 °C for 15 s and 60 °C for 60 s as recommended by the manufacturer. The quantification of gene expression was determined using the 7500 Software v2.0.5 (Applied Biosystems). The obtained cycle threshold (Ct) values were exported in Microsoft Excel for further analysis.