Impact of long-term expansion on mesenchymal stromal cell differentiation potential

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START-MSC I and II

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Introduction

Mesenchymal stromal cells (MSCs) are promising candidates for novel cell therapeutic applications especially in hard and soft tissue regeneration. Due to their relatively low frequency it is required to expand these cells to obtain clinically relevant cell dose. Although the majority of data indicate that MSCs have a potent MSC safety system as they undergo replicative senescence, few data suggested that prolonged expansion, however, may pose the risk of accumulation of DNA damage and subsequent transformation. Thus we investigated whether long-term expansion affects MSC qualities and analysed MSC differentiation capacities by histochemistry, colorimetric assays and reverse transcription quantitative PCR (RT-qPCR).

Methods

Adipogenic and osteogenic differentiation was induced in lipoaspirate-derived (LA) MSCs of early, middle and late passage. Early passage corresponds to cumulative population doublings (CPD) of 5-10, middle of 10-25 and late of >30.

To detect the differentiation capacity on day 21, the following analyses were performed:

1. staining: von Kossa, to detect the calcium deposition during the osteogenesis and oil red O to stain the lipid droplets during adipogenesis.

2. colorimetric assays: Fluitest® Ca – CPC, *in vitro* test for the quantitative determination of calcium and Fluitest® TG, enzymatic *in vitro* test for the quantitative determination of triglycerides (TG).

3. Reverse transcription quantitative PCR (RT-qPCR): We used Roche Light Cycler 480 with the universal probe library system by Roche and followed MIQE guidelines in designing RT-qPCR experiments. RT-qPCR includes reference genes (RG) to normalise mRNA levels between different samples. For accurate RT-qPCR expression profiling we used geNorm software to determine the most stable RG from a set of RG. To define osteogenesis-associated marker expression, we selected osteopontin (OPON) and osteocalcin (OCAL) and calculated their expression relative to the reference genes TATA-binding protein (TBP), splicing factor arginine/serine-rich 4 (SFRS4) and beta-2-microglobulin (B2M). For adipogenesis, adiponectin (ADPQ), adipophilin (ADFP), peroxisome proliferator activated receptor gamma (PPARg) and perilipin were normalised to the reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-2-microglobulin (B2M) and splicing factor, arginine/serine-rich 4 (SFRS4).

RT-qPCR was used to measure the levels of 5 RGs, which have been reported in the literature to be invariant. The RGs were analysed in MSCs under adipogenic and osteogenic differentiation conditions as well as uninduced MSCs. mRNA of 12 samples was isolated, RNA quality was checked using the Agilent Bioanalyzer and cDNA was synthesised.
Expression stability (M) of all RGs was evaluated using the software-based method geNorm. In addition to gene stability measure M, the geNorm program calculates a normalization factor (NF). Crossing points of all genes of interest (GOI) were transformed to relative values (V_r), and divided by the sample specific NF (geometric mean of the three best reference genes) to achieve the relative gene expression. **Rel Exp GOI= (1) VrGOI = primer efficiency^(min CPmean – Cpmean)/NF.** Rescaled relative expression was calculated by the division of the lowest expression.

This calculation was compared to the classical 2^ΔΔ Cp method ignoring the primer specific efficiency and normalised to a single reference gene. Here the theoretical optimal efficiency of 2 was used to calculate the relative expression and also only a single RG was used:
2^(-ΔΔ Cp) = (1) d Cp uninduced = CpGOI –CpRG and Δ Cp induced = CpGOI –CpRG (2) ΔΔ Cp = ΔΔ Cpinduced –ΔΔ CPuninduced.

Results

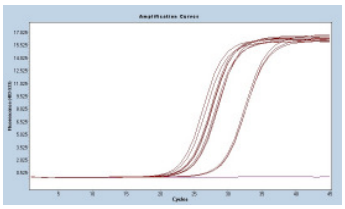

Fig. 1 Differentiation may significantly affect the expression of the reference gene GAPDH.

As function of the MSC culture heterogeneity and differentiation, marked differences in the Cp from 20 to 26 were obvious.

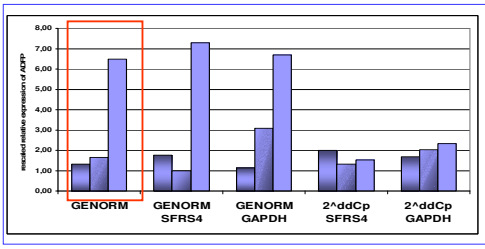

Fig. 2 Comparison of different calculation methods and RGs for relative gene expression

We depict the relative expression of adipophilin in 3 different samples normalised againts two different RGs (SFRS4 and GAPDH). Compared to the geNorm based calculation (red bordered) which includes both RGs, the expression of the same GOI calculated with other methods resulted in pronounced expression variations.

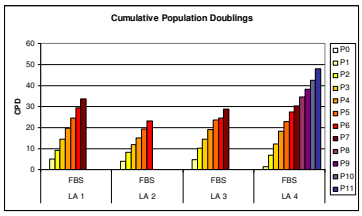

Fig.3 Cumulative population doublings of MSC cultivated until onset of replicative senescence.

Isolates from four different donors are depicted. It is obvious that isolate-specific differences exist affecting the life span of the MSCs.

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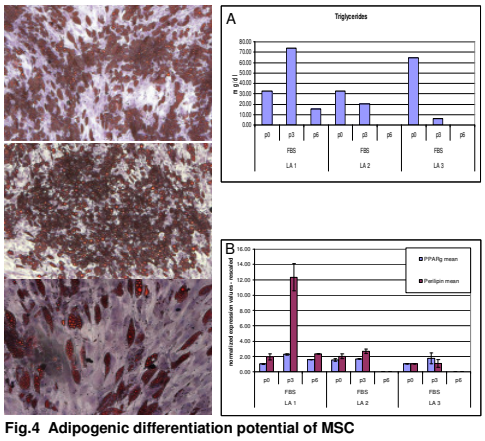

Fig.4 Adipogenic differentiation potential of MSC

The left panel depicts oil red O stained cells 21d after adipogenic induction in different passages. A- Quantification of triglyerides after induction as function of expansion culture. B- relative gene expression of PPARg and perilipin.

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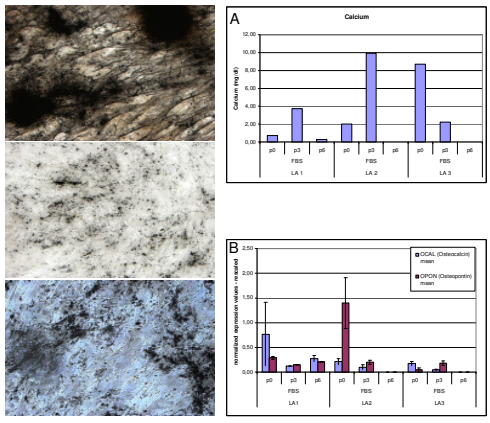

Fig.5 Osteogenic differentiation potential of MSC

The left panel depicts von Kossa stained cells 21d after osteogenic induction in different passages. A- Quantification of calcium after induction as function of expansion culture. B- relative gene expression of osteocalcin and osteopontin.

Discussion

Our results revealed that MSCs undergo replicative aging which rapidly affects adipogenic differentiation potential. Osteogenic potential appeared less influenced by the culture age of MSCs. Moreover our data underline the necessity to carefully assess appropriate and valid normalisation controls for RT-qPCR. This is important as we observed that reference genes may be regulated in a condition-specific manner that is not suitable for use in target gene normalization.

These data support the current agreement to use MSCs up to CPD levels of 10-15, at least if differentiated cells are intended to be clinically applied in hard or soft tissue augmentation. Accordingly, CPD better reflect culture-dependent aging rather than passages. Laboratories aiming optimised expansion need to compromise maintained MSC qualities and accelerated cell expansion.