BioResearch

Monitoring of Human Mesenchymal Stem Cell Differentiation with Histo- or Biochemical Assays and miRNA Expression Analysis

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1. Abstract

Human mesenchymal stem cells (hMSC) are of tremendous interest for the field of regenerative medicine and cell therapy due to their tissue regenerative properties and potential to modulate the immune system. An inherent characteristic of hMSC is their ability to differentiate into chondrocytes, osteoblasts and adipocytes. The potency of hMSC is commonly tested by determining this so-called trilineage propensity of the cells by histo- or biochemical methods.

MicroRNAs (miRNAs) are short non-coding RNA molecules that function as post-transcriptional regulators. They bind to complementary sequences on target mRNA resulting in translational repression or target degradation. As translation modulators miRNAs might be early indicators of cell differentiation.

In order to determine whether miRNA analysis could be an alternative tool for monitoring hMSC trilineage propensity, hMSC were differentiated into the chondrocytes, osteoblasts and adipocytes lineages. The miRNA expression profile of differentiated hMSC was determined with microarray analysis. Differentially expressed miRNAs were identified by comparison to undifferentiated hMSC and human dermal fibroblasts. Results were compared to standard histo- or biochemical assays.

2. Material and Methods

Adult Normal Human Dermal Fibroblasts (NHDF-Ad, Lonza CC-2511) from 8 independent donors were expanded for 14 days in FGM™-2 Growth Medium (Lonza, CC-3132). hMSC (Lonza PT-2501) from 6 independent donors were expanded for 7 – 14 days in MSCGM™ Growth Medium (Lonza, PT-3001). Cells were passaged twice a week. Differentiation protocols and assays are described in Figures 2-4.

RNA was extracted on the indicated days using the miRCURY™ RNA Isolation Kit – Cell & Plant (Exiqon, #300110) according to manufacturer's protocol. Samples were analyzed by Sistemic Ltd, on the Agilent miRNA platform using Agilent's SurePrint G3 Human v16 microRNA 8x60K microarray slides; miRBase version 16.0. Each slide contains 8 individual arrays, each array represents 1,349 microRNAs (1,205 Human; 144 viral). 100 ng of Cy3-labeled total RNA were used as input for each microarray experiment. The analysis of microarray data was carried out using SistemQC™ data analysis suit.

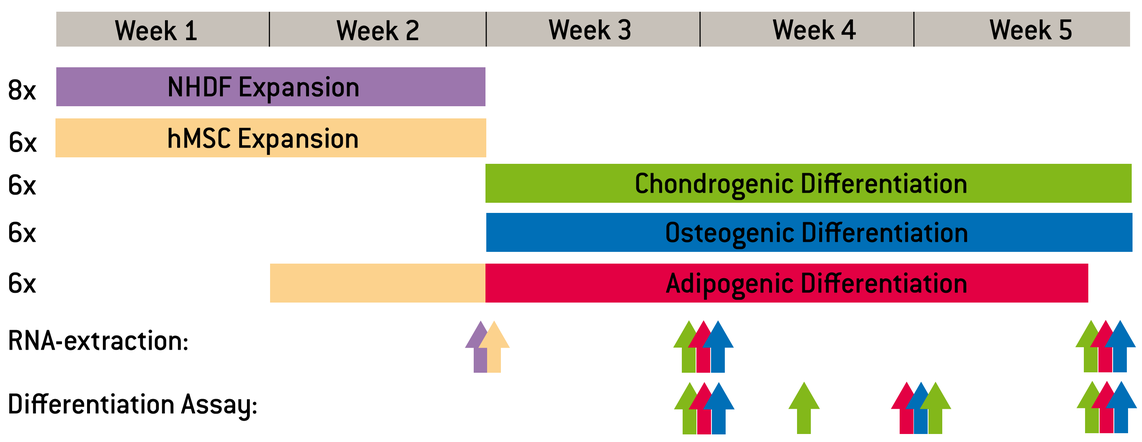

Figure 1. **Experimental set-up.** For details see Material and Methods and Figure 2-4.

4. Discussion

- All hMSC lots used in this study were able to differentiate into the chondrocytes, osteoblasts and adipocytes lineages as measured by standard bio- or histochemical assays. The final extent of differentiation varied between different lots and allowed us to correlate the differentiation potential measured by standard assays to the miRNA expression profile. Additionally, for osteogenic differentiation, the temporal initiation of differentiation was highly variable between the different lots.

- Differentially expressed miRNAs can be detected in day 7 differentiated hMSC and can therefore be early predictors of differentiation.

  - hsa-miRNA-101-3p was highly up-regulated in day 7 adipogenic differentiated hMSC relative to undifferentiated and chondro/osteo lineages. Use of hsa-miRNA-101-3p expression as an early predictive indicator of hMSC adipogenic potency is suggested by a strong correlation with differentiation day 18 AdipoRed™ signal.

  - Increased expression of several miRNAs during osteogenesis was detected on day 7 despite the absence of increased OsteoImage™ signal. Therefore, miRNA analysis with e.g. microarray analysis or qPCR could be an appropriate tool for monitoring changes in cell identity (e.g. cell differentiation or de-differentiation) at early stages. This might be of particular interest for cell types or differentiation pathways where easy-to-use, robust and cost-effective assays like the AdipoRed™ Assay or OsteoImage™ Mineralization Assay are not available.

- In order to replace classical histo- or biochemical assays by miRNA analysis, appropriate qPCR protocols for each miRNA have to be established. The selected miRNAs used for monitoring cell differentiation should be independent from e.g. media composition, cell density or passage number. Validation of miRNA expression as a prognostic biomarker for hMSC differentiation would require a specific miRNA pattern that is not only necessary, but sufficient for a particular differentiation process.

3. Results

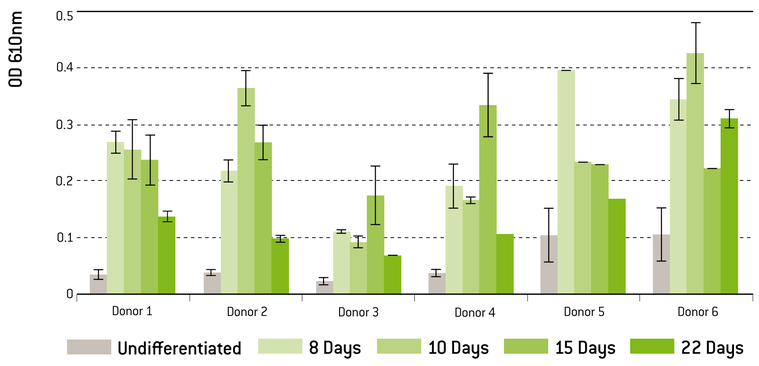

Figure 2. **Chondrogenic Differentiation** hMSC were differentiated with the hMSC Differentiation BulletKit™ – chondrogenic (Lonza, PT-3003) in the presence or absence (undifferentiated control) of 10ng/ml TGF-ß3 (Lonza, PT-4124). Chondrogenic differentiation was monitored on the indicated days using Alcian blue 8GX staining (Sigma, A3157). Absorption was measured with the FLUOstar Omega (BMG Labtech) at 610 nm (data is expressed as mean ± SD for 3 replicates). All lots underwent chondrogenic differentiation as measured by glycosaminoglycan production compared to undifferentiated controls. Lot-to-lot dependent variability was observed.

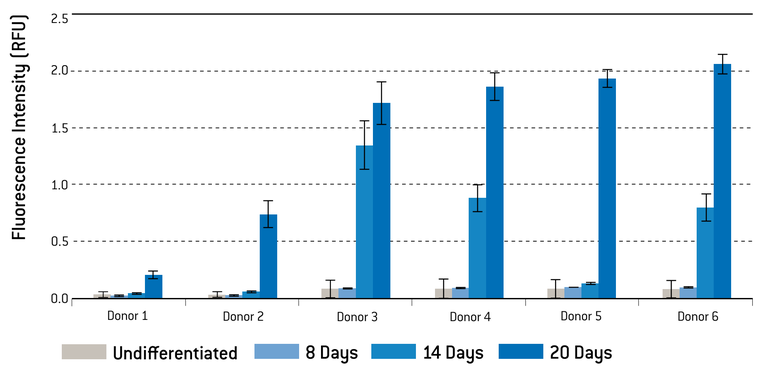

Figure 3. **Osteogenic Differentiation** hMSC were differentiated with the hMSC differentiation BulletKit™ – osteogenic (Lonza, PT-3002) according to manufacturer's instructions with exception of the seeding density (1,85E+03 cells/cm²). Osteogenic differentiation was monitored on the indicated days with the OsteoImage™ Mineralization Assay (Lonza, PA-1503). Fluorescence intensity was measured with the Fluoroskan Ascent (Labsystems; excitation 485 nm/emission: 520 nm; 293 data points/well; data is expressed as mean ± SD for 1 – 3 replicates). All donors underwent osteogenic differentiation as measured by hydroxyapatite deposition of cells. Lot-to-lot dependent variability was observed with respect to total amounts of deposited hydroxyapatite per well as well as the time required for detectable hydroxyapatite deposition.

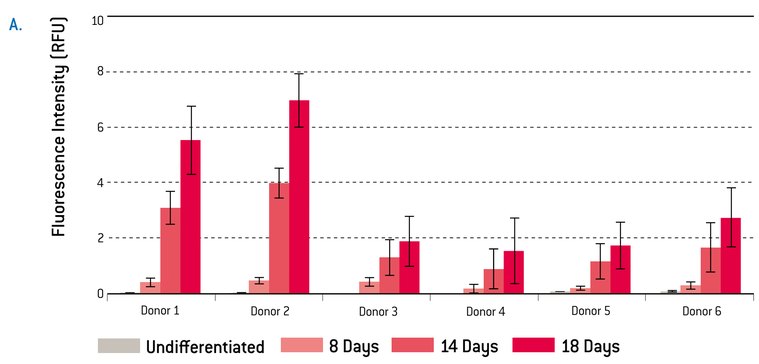

Figure 4. **Adipogenic Differentiation** hMSC were differentiated with the hMSC Differentiation BulletKit™ – adipogenic (Lonza, PT-3004) according to manufacturer's instructions. Cell differentiation was monitored on the indicated days with the AdipoRed™ Assay (Lonza, PT-7009) containing the hydrophilic stain Nile Red. A) Fluorescence intensity was measured with the Fluoroskan Ascent (Labsystems; excitation 485nm/emission: 538nm; 293 data points/well; data is expressed as mean ± SD for 1 – 3 replicates). B) Exemplary fluorescence microscopy overlay. Nile Red stained intracellular triglyceride droplets are shown in red. Hoechst 33342 stained nuclei are shown in blue. Scale bar: 200 µm. All hMSC lots underwent adipogenic differentiation as measured by the accumulation of intracellular lipid droplets within the cells. The percentage of differentiated cells and/or the total lipid content per cell varied between different lots.

A.

	# of DE miRNA
Chrondrogenic	5
Osteogenic	15
Adipogenic	6

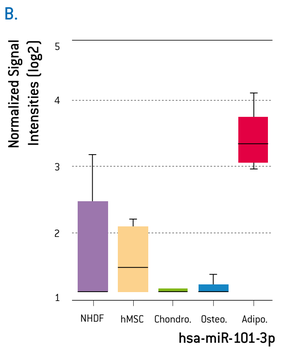

Figure 5. **SistemQC™ Microarray Analysis Identifies Putative Differentially Expressed miRNAs** in 7 days differentiated hMSC A) Number of miRNAs that were significantly differentially expressed (DE) in all 6 hMSC lots on day 7 of chondrogenic, osteogenic and adipogenic differentiation in comparison to undifferentiated hMSC and other differentiation lineages (≥ 1.5 fold change compared to other sample types; pFDR < 0.05 (A,C); pFDR < 0.1 (0); FDR = False Discovery Rate: pFDR = multiple test inflation adjusted p-values generated from t-tests using the Benjamini-Hochberg method[1]) Statistically significant DE of individual miRNAs was in the 2 – 8-fold range (see also Figure 5B). Interestingly, for osteogenic differentiation, 5 significantly DE miRNAs could be observed on day 7 of differentiation. At that time point, no differentiation could be detected with the biochemical assay OsteoImage™. (see also Figure 3). B) Normalized expression of hsa-miRNA-101-3p in NHDF, undifferentiated and 7-day differentiated hMSC. Hsa-miRNA-101-3p is significantly up-regulated in adipogenic differentiation compared to undifferentiated as well as day 7 chondro- and osteo differentiated hMSC. The black horizontal line within each box represents the median. The boxes range from the 25th percentile to the 75th percentile (covering 50% of the data) and the whiskers cover 95% of the data.

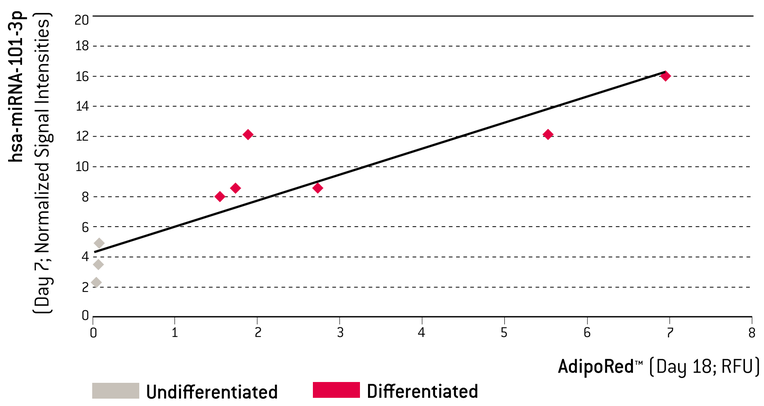

Figure 6. **Hsa-miR-101-3p Expression May Be An Early Marker of hMSC Adipogenesis.** Hsa-miRNA-101-3p expression in adipogenic differentiated hMSC on day 7 measured by microarray analysis shows positive correlation with the final differentiation success measured by the standard AdipoRed™ Assay on day 18 ($R^2 = 0,8108$; each data point represents one donor). Low expression of hsa-miRNA-101-3p is observed in undifferentiated hMSC. Interestingly, a role for miR-101-3p up-regulation in adipogenesis has been reported[2]. This miRNA has been shown to be significantly up-regulated in a comparison of pre-adipocytes to adipocytes and may therefore represent a positive marker of early adipogenesis.

References

1. Benjamini Y and Hochberg Y (1995). J Royal Stat Soc B, **57**:289–300
2. Ortega et al. (2010) PLoS One, **5**(2):e9022

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