Changes in microRNA expression induced by three-dimensional aggregation of mesenchymal stem cells

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Summary
Recent studies showed that culture of mesenchymal stem cells (MSC) as three-dimensional (3D) aggregates enhanced their therapeutic potential. The aim of this study was to determine the changes associated to MSC aggregation, with particular focus on multipotency and microRNA expression patterns. To this aim, MSC isolated from adult mouse bone marrow were aggregated by incubation in hanging drops for 72h. The aggregates were cultured on gelatin-coated plates to generate explants, which were either analysed for microRNA expression pattern (using TaqMan MicroRNA Assays and miRWalk and Panther databases) or dissociated and re-cultured for multipotency assessment. The results showed no difference between the differentiation capacity of explanted and normal cultured MSC. However, microRNA expression pattern revealed the induction of three microRNAs (miR-380-5p, 434-5p and 302c) as well as an overall increased microRNA expression level (1.6 fold versus control). In silico analysis of these microRNAs suggested the activation of several cellular processes involved in engraftment, such as biological adhesion, response to stimulus and prevention of ECM proteins degradation, as well as inhibition of apoptosis in explanted MSC versus control. By concluding, 3D aggregation of MSC did not affect their multipotency, yet induced changes which might impact the resistance and behaviour of cells post-transplantation.

Introduction
Stem cell transplantation appears as a suitable therapy for various diseases by providing cells able to differentiate and replace the dead tissue. Mesenchymal stem cells (MSC) stand out as the main candidates for cellular transplantation therapy for several reasons: (i) they are easily accessible in large amounts (the best sources would be either bone marrow or adipose tissue), (ii) they have no immunogenicity, (Martens et al., 2006), (iii) they are able to differentiate into various cell types, such as adipocytes, osteoblasts, condrocytes, muscle cells and neurons (Jackson et al., 2007; Gimble et al., 2008), (iv) they secrete factors with anti-apoptotic and pro-angiogenic paracrine activity and (v) they have no associated ethical controversies as compared to their embryonic counterparts. However, the promises related to these cells exceed the current experimental success, as clinical studies indicate only mild and transient beneficial effects. It seems that the main reason for this limitation is the low retention and survival of the transplanted cells (Assunção-Silva et al., 2015). Recently, three-dimensional (3D) culture of MSC has been described as a way to increase their therapeutic properties. The explanation might reside in the modifications of the intercellular...
contacts that give rise to more complex organizations resembling in vivo settings. Furthermore, MSC spheroids assembled by incubation in hanging drops, a technique originally developed for embryonic stem cell differentiation, increased the secretion of anti-inflammatory molecules in comparison to MSC cultured in classical 2D system (Bartosh et al., 2010). This study focuses on the changes induced in MSC after aggregation into 3D spheroids, with particular focus on multipotency and microRNA expression pattern, as important contributors in multiple biological processes. Here we report that 3D aggregation of MSC led to important variations in microRNAs expression pattern that might improve their capacity to survive and engraft in the host tissue after transplantation.

Materials and methods

Isolation of mouse mesenchymal stem cells. All experiments were approved by the Institutional Ethical Committee. Animals were housed and used in accordance to the Guide for the Care and Use of Laboratory Animals (Washington DC, National Academies Press, 1996). MSC were obtained from bilateral tibias and femurs of 8-week-old C57bl/6 mice as previously reported (Rosca & Burlacu, 2010). Briefly, the medullar channels were flushed by using a syringe containing complete culture medium (low glucose DMDM supplemented with 10% MSC-qualified fetal bovine serum and 1% antibiotics, all from Life Technologies). The cell suspension was seeded on gelatin (0.1% in PBS)-coated Nunclon dishes at a final density of 4x10⁶ cells/ml and incubated at 37°C until confluency. MSC were first detected at fifth passage as fibroblast-like colonies. These colonies were further multiplied by trypsinization (using 0.25% trypsin supplemented with 0.5 mM EDTA) and seeding at 5000 cells/cm² on gelatin-coated plates every 5 days. Characterization of MSC was done by flow cytometry and by following the differentiation potential into adipocytes and osteoblasts, according to the guidelines of the International Society for Cellular Transplantation Society (Dominici et al., 2005).

Flow cytometry. Cells were detached with accutase for 5 minutes at 37°C, centrifuged and resuspended in PBS with 2% fetal bovine serum (PBS-FBS). One hundred thousand cells /200 μl PBS-FBS were incubated for 30 minutes on ice with anti-c-kit-PE, anti-Sca-1-PE or the specific isotype-PE (all from R&D Systems). At the end of the incubation period, cells were washed in PBS-FBS and analyzed in a Gallios™ Flow Cytometer (Beckman Coulter, Inc). A minimum of 10.000 events were considered for each sample and acquired data was analyzed using Summit v4.3 software (Cytomation, Inc).

Assessment of multipotency. MSC differentiation capacity was tested by incubation of confluent monolayers for two weeks in adipogenic and osteogenic specific differentiation conditions. The differentiation cocktails consisted in complete medium supplemented with 10-6M dexamethasone, 100 μM indomethacin and 1% ITS (for induction of adipogenic differentiation) and with 10-7 M dexametasone, 10 mM β-glycerophosphate and 0.2 mM ascorbic acid (for osteogenic differentiation). Differentiation was demonstrated by specific histological stainings. Thus, lipid droplets accumulated in the cytoplasm during the adipogenic differentiation were highlighted by Oil Red O staining, while the calcium deposits from the extracellular matrix of osteoblasts were stained with silver nitrate by Von Kossa method.

Spheroid assembling and explant formation. 3D aggregates were obtained by using a technique originally developed for embryonic stem cell differentiation (Banerjee & Bhonde, 2006; Wang & Yang, 2008). MSC were enzymatically detached Trypsin-EDTA, centrifuged and resuspended at a density of 1.25 x 10⁶ cells/ml. Twenty microliters containing 25.000 cells (Bartosh et al., 2010) were placed on the internal face of a culture plate lid, which was subsequently turned over the dish to generate hanging drops. The dish
was filled with 4 ml of PBS in order to prevent the evaporation of the small drops. After 72 hours of incubation, the spheroids were collected and placed on 0.1% gelatin-coated dishes to allow the development of explants. Four days after plating, explants were harvested for microRNA expression analysis or trypsinized and re-cultured in 6-well plates for evaluation of the differentiation potential.

**microRNA PCR array.**
Identification of changes in microRNA expression induced by 3D aggregation was performed by Real Time PCR. Thus, total RNA was extracted from control and 4-day explant MSC by using TRIzol reagent and mature forms of miR were reverse transcribed into cDNA starting from 1 μg total RNA, using TaqMan® MicroRNA Reverse Transcription Kit and Megaplex™ RT primers (Life Technologies), which allowed the discrimination between the mature form and precursors of microRNA. Next, 335 mouse microRNAs were further quantified in cDNA on a ViiA™ 7 Real-Time PCR System (Applied Biosystems), by using TaqMan microfluidic cards (TaqMan® Rodent MicroRNA Array A) and TaqMan® Universal PCR Master Mix (all from Life Technologies). Data analysis was performed with Expression Suite Software V1.0.3 (Applied Biosystems) by using the comparative CT method (2−ΔΔCT method). Small nuclear U6 RNA was employed for internal normalization.

In silico functional analysis.
MicroRNAs with induced, down-regulated or up-regulated expression were selected for in silico analysis. Putative targets for each selected microRNA were predicted by miRWalk on-line database (Dweep et al., 2011) and those with statistical significance (p< 0.001) were selected and further analysed with Panther on-line database (Mi & Thomas, 2009; Mi et al., 2013). Briefly, these targets were initially automatically grouped according to the biological processes and metabolic pathways in which they are involved. The influence of microRNAs on particular cellular processes, was expressed as the average target number (ATN) for each process, calculated by dividing the total number of targets of microRNAs in a category (induced, down- or up-regulated) involved in a certain cellular process to the number of microRNAs in that category. For example, the three induced microRNAs (380-5p, 434-5p and 302c) had 7 targets (1, 1, and 5 respectively) related to the apoptotic process; ATN of the induced microRNAs involved in apoptotic process is 2.33 (7:3=2.33).

**Results and discussions**
Characterization of MSC. The morphological aspect of a fibroblast-like colony observer at fifth passage is illustrated in Figure 1a. These cells were further multiplied and evaluated for the presence of the mesenchymal stem cell marker Sca-1 and hematopoietic stem cells marker c-kit by flow cytometry. The results confirmed the absence of c-kit marker (0.65+/−0.5%), and the presence of Sca-1 on the majority of cells (76.2+/−9.5%), as shown in Figure 1b. The ability to differentiate into adipocytes and osteoblasts was evaluated by incubation of cells under specific culture conditions. As shown in Figure 1c, two-week exposure to the adipogenic conditions resulted in massive accumulation of cytoplasmic lipid droplets, which were positively stained with Oil Red O. Similarly, von Kossa staining indicated extensive calcium accumulation in the extracellular matrix (Figure 1d), after two weeks of incubation of cells in the presence of the osteogenic cocktail. These results indicate a mesenchymal stem cell-specific phenotype and multilineage differentiation potential, which warranted these cells as genuine MSC.

Preservation of MSC multipotency after 3D aggregation. 3D MSC spheroids obtained by hanging drop assay were allowed to adhere on gelatin-coated plates to develop explants (Figure 2a). Analysis of explants revealed that cells in the close proximity of aggregates contained multiple vesicles inside the cytoplasm that attenuated as the cells proliferated and moved away (see Figure 2b).
The presence of these vesicles suggest a transient increased secretory capacity of the aggregate-connected cells at earlier times of their emerging from the aggregates, in relation to the cells they later produced by proliferation. A possible explanation for the absence of secretory vesicles in aggregate-distant cells may be that the aggregate-arising cells release the vesicles into the extracellular medium during initial rounds of proliferation. In order to test whether MSC preserve their capacity to differentiate into multiple lineages after enduring the process of 3D aggregation, trypsin-dissociated explants were let to adhere on gelatin-coated dishes to generate confluent cultures. These cultures were subsequently incubated under adipogenic and osteogenic conditions and their differentiation capacity was comparatively evaluated against control MSC (maintained in 2D culture). The Oil Red O staining of the lipid droplets (Figure 2c) and von Kossa staining of the calcification nodules (Figure 2d) confirmed that spheroid-derived MSC retained their capacity to differentiate towards the adipogenic and osteogenic lineages, as similar extent to control cells.

Evaluation of microRNA expression level in MSC explant and control cells
Real Time PCR performed on TaqMan microfluidic cards revealed that the microRNA expression pattern was considerably modified in MSC explants in comparison to initial MSC and the average level of expression was approximately 1.5 folds higher than in control MSC (Figure 3a). Moreover, 3 microRNAs were induced in MSC explants (miR-380-5p, 302c and 434-5p) while they were undetectable in control MSC (Figure 3b). Of a total of 170 microRNAs with different expression level between MSC explants and control cells, 49 microRNAs showed at least two-fold change (Figure 3c). Of these, 23 microRNAs had down-regulated expression (miR-34a, -503, -29b, -450a-5p, -21, -409-3p, -362-3p, -197, -101a, -301a, -9, -34c, -500, -301b, -322, -98, -203, -7a, -872, -669a, -423-5p, -148b, -467a) and 26 microRNAs had up-regulated expression (miR-128a, -685, -223, -667, -342-5p, -200c, -331-5p, -181a, -671-3p, -680, -687, -328, -30d, -194, -107, -23b, -574-3p, -
In silico functional analysis

In order to gain further insights onto the biological implications of the microRNAs with modified expression in explanted MSC versus control cells, the three induced microRNAs (miR-380-5p, -434-5p and -302c), as well as the microRNAs with four-times either down-regulated (miR-34a, -503, -29b and -450a-5p) or up-regulated (miR-128a, -685, -223, -667, -342-5p, -200c, -331-5p, -181a, -671-3p and -680) expression were selected for in silico functional analysis (Figure 4a). Targets of the selected microRNAs were predicted by using miRWalk on-line platform and those with statistical significance (p<0.01) were further analysed using Panther on-line database. Of the total processes these microRNAs may be involved in, those particularly relevant for survival and engraftment of cells after transplantation were considered for detailed analysis, namely apoptotic pathway, biological adhesion (including cell junctions and extracellular matrix (ECM) components) and response to stimulus.

The influence of microRNAs on each particular process or protein class was predicted based on ATN value (calculated as described above). By comparing ATN values, it appeared that apoptotic process was suppressed in MSC explants versus normal cultured cells (5.8 for up-regulated microRNA, versus 3.75 down-regulated microRNAs). On contrary, biological adhesion and ECM components had ATN values higher for down-regulated microRNAs.
(7.75 and 6.5 respectively), in comparison to up-regulated microRNAs (5 and 4.7, respectively), suggesting the activation of these targets in MSC explants. In the same direction, MSC explants were more responsive to stimulus, as demonstrated by ATN value for down-regulated versus up-regulated microRNAs (22.25 versus 20, respectively).

Fig. 3. Changes in microRNA expression in MSC induced by 3D aggregation. a) Variation of the expression level of microRNAs in explant MSC in comparison to control MSC, as obtained by miRNA PCR array. Note that the average fold increase of general microRNA expression in MSC explants was approximately 1.6 times versus control. Among them, several microRNAs were highly up-regulated and others were down-regulated; b) microRNAs with induced expression in MSC explants versus control MSC; c) List of microRNAs with down-regulated and up-regulated expressions in MSC explants in comparison to control MSC. Only microRNAs with minimum 2 fold variation in gene expression were listed.

Taken together, our data suggest that MSC explants may provide improved benefits for cell transplant therapies, due to their superior engraftment properties, lesser susceptibility to apoptosis, and augmented responsiveness to stimulus.

Discussion
Culturing MSC is an absolute requirement in order to obtain sufficient amounts of cells for clinical needs in regenerative therapy. However, the traditional 2D culture system imposes the cells to adhere to synthetic substrates and this minimizes the intercellular interaction and negatively impacts the natural biological properties of MSC (Saleh et al., 2012). Recently, 3D culture system came out as a method to increase the physiological relevance of various cell types, due to the natural cell-cell interactions within the aggregate, similarly to the in vivo settings (Antoni et al., 2015). For example, breast cancer cells grown in 3D system exhibit an increased resistance to chemotherapeutic drugs or radiation in comparison to cells grown in traditional system and better mimic the pathological in vivo condition (Anastasov et al., 2015). 3D aggregation was shown to enhance the therapeutic effects of adipose
MSC in a rat model of ischemia-reperfusion kidney injury (Xiaozhi et al., in press). MicroRNAs are well known mediators of important biological processes such as growth, apoptosis and differentiation (Friedman & Jones, 2009). In this study, we focused on the changes induced in microRNA gene expression by 3D aggregation of MSC, pursuing their influence on multipotency, cell death and capacity to interact with the surrounding environment. First, we showed that MSC multipotent capacity was not affected by 3D culture, which means that cells maintained their stemness after aggregation. This is an important attribute for cellular therapy, considering that these cells are expected to generate various cell types after transplantation. Thus, MSC explants emerged from spheroids generated by 72h-aggregation in hanging-drop assay were able to differentiate towards the adipogenic and osteogenic lineages, similarly to the 2D MSC culture.

![Table]

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<th>Category</th>
<th>380-5p</th>
<th>434-5p</th>
<th>302r</th>
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<th>296</th>
<th>450a-5p</th>
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<th>685</th>
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<th>432-5p</th>
<th>290i</th>
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However, transient 3D culture as spheroids had a dramatic effect on microRNA expression in MSC. Several modifications, such as down-regulation, up-regulation or induction of expression have been identified by microRNA PCR array in MSC after aggregation in comparison to normal cultured cells. In order to predict the influence of these changes on the cellular physiology, in silico functional studies have been performed, by using on-line available platforms, such as miRWalk (used for target prediction) and Panther (used for prediction of the biological functions of the identified microRNA targets). Data analysis suggested that MSC explants were more resistant to apoptosis then control.
MSC thanks to the up-regulation of several microRNAs predicted to decay multiple targets implicated in cell death process. Two of these targets were caspase-2 (targeted by miR-302c, which was induced in explants and not present in control cells) and caspase-9 (target of miR-342-5p (data not shown), whose expression was 8.6 times increased in comparison to control MSC. It is well known that caspase-9 is an important component of the apoptosome, the initiator of the caspase cascade, which indicates that its degradation impairs the initiation of the apoptotic process. Caspase-2 has been implicated in regulation of cell death induced by metabolic imbalance, DNA damage and endoplasmic reticulum stress, among others (Fava et al., 2012). Moreover, ATN value of the down-regulated and up-regulated microRNAs suggested that biological adhesion and response to stimulus were promoted in MSC explants in comparison to the 2D cultured cells. Likewise, ECM components could be protected from degradation.

All these data suggests that 3D aggregation improves the therapeutic properties of MSC, leading to better survival and engraftment in the host tissue after transplantation.

Conclusions

This study showed that 3D aggregation of MSC did not affect their multipotency, yet induced changes which might impact the resistance and behaviour of cells post-transplantation. Based on our results, we postulate that a 3D culture step of MSC before transplantation could assure an increased retention and a better survival rate in comparison to the transplantation of 2D cultured MSC.

By concluding,

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