An appraisal of genetic stability in human mesenchymal stem cells

Pedro H. Oliveira[1, 2], Joana S. Boura[1, 2], Manuel M. Abecasis[3], Cláudia Lobato da Silva[1, 2], Joaquim M. S. Cabral[1, 2]
[1] Department of Bioengineering, Instituto Superior Técnico (IST)
[2] IBB – Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering (IST)
[3] IPOFG - Instituto Português de Oncologia Francisco Gentil
Lisboa, Portugal
pcoliveira@ist.utl.pt

***Abstract*—Human bone-marrow mesenchymal stem cells (BM MSCs) and adipose derived stem cells (ADSCs) were evaluated for evidence of senescence during exposure to hypoxic conditions and protracted *ex vivo* expansion. Under hypoxia we have observed microsatellite instability and down-regulation of genes involved in DNA repair. Simultaneously, a decrease in ATP content and in the number of mitochondrial genomes was observed. Irrespective of O_2 tension or cell passage, we observed several heteroplasmic point mutations, some of which have been reported in a wide range of tumors. These results alert for a careful analysis of cell integrity during the development and application of regenerative therapies.**

***Index Terms*—mutation; genomic DNA; mitochondrial DNA; senescence; cancer**

I. CONTEXT

Despite the heralded potential of stem cell-based therapies, the occurrence of genetic changes in stem cells constitutes a serious roadblock to regenerative medicine. Several lines of evidence have shown that recurrent events of genetic instability as well as other manifestations of senescence accumulate during the *ex-vivo* expansion of mesenchymal stem cells (MSCs) [1, 2], embryonic stem cells (ESCs) [3], neural stem cells (NSCs) [4], and others. Therefore, from the clinical point of view, there is a pressing need to tightly control the authenticity and quality of cells to be used for therapeutic applications, as well as to improve or develop appropriate assays to monitor the occurrence of genetic aberrations. Stem/progenitor cells such as MSCs have elicited great hopes due to their capacity to differentiate into multiple lineages and ability to self-renew *ex-vivo*. Some doubts remain though, regarding the best conditions to expand MSCs in a robust and reproducible way, while assuring their purity, safety and effectiveness for clinical applications. In this context, it is important to achieve the ideal balance between cell growth and quality, in terms of preventing culture-induced genetic or epigenetic changes. Although conflicting results have been reported, some examples of parameters able to have an impact on cell quality include donor age, type of medium used in the culture process, O_2 tension and consecutive passaging [5]. In this work we address the effects of expanding MSC's for prolonged periods of time under normoxia (Nx) or hypoxia (Hx), on the genetic stability of both nuclear and mitochondrial DNA.

II. GOALS

A. *Effect of O_2 tension and consecutive passaging on the genetic stability of stem cells*

We are currently engaged in the search and characterization of mutations in both genomic DNA (gDNA) and mitochondrial DNA (mtDNA) of human bone marrow mesenchymal stem cells (BM MSCs) and adipose derived stem cells (ADSCs). Particularly, we are evaluating the effect of hypoxia vs normoxia as well as of consecutive passaging on the expression of genes involved in mismatch repair (MMR), homologous recombination (HR) and non-homologous end-joining (NHEJ), as well as on telomere length and occurrence of microsatellite instability (MSI). Furthermore, we are looking at signs of mitochondrial senescence, namely changes in mutation load, ATP content, membrane potential and number of mtDNA genomes.

B. *To address the regional preference of certain genetic aberrations in human mtDNA*

Mitochondria play well-recognized roles in the generation of cellular energy and as mediators of events such as apoptosis. Although there is emerging evidence relating mtDNA genetic rearrangements with complex processes of cell aging and neoplasia [6], there is a lack of information concerning hotspot mapping, namely DNA repeats or other fragile sites. We thus pretend within this goal, to correlate common deletion/amplification breakpoints involved in genetic disorders and cancer, with the presence of certain recombination-prone sequences and/or other DNA physical constraints.

III. TEAM AND INSTITUTIONS

This work is within the frame of the scientific activities currently being developed at the Department of Bioengineering (DBENG, IST) and Institute for Biotechnology and Bioengineering (IBB). In particular, it is being conducted at the Stem Cell BioEngineering Laboratory headed by Prof. Joaquim Sampaio Cabral who has been focused for several years in the

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development of highly controlled culture systems for the *ex vivo* expansion of stem cells. Prof. Joaquim Cabral is also the Head of DBENG and Director of IBB.

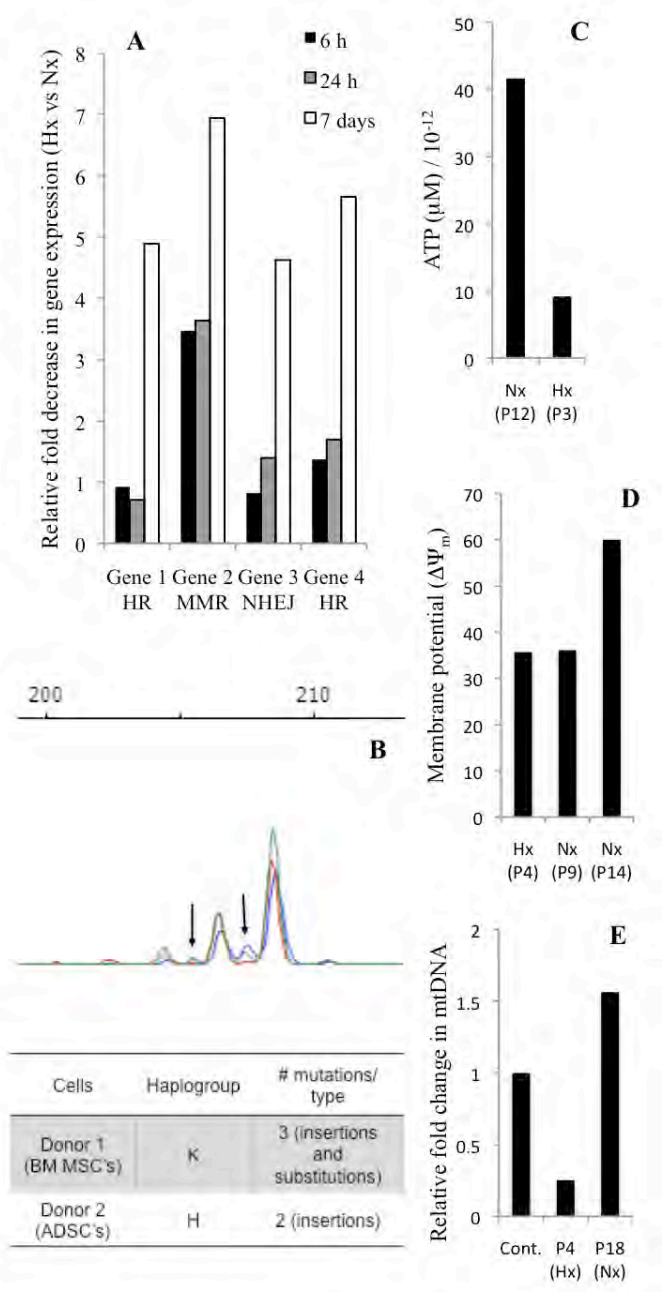

Figure 1. Cell expansion under Hx has led to a general decrease in the expression of genes involved in homologous recombination, mismatch repair, and non-homologous end joining (**A**). The latter was also accompanied by microsatellite instability events. Shown in **B** is an example of such instability detected in BM MSC's after 7 days under Hx (red), and for extensively passaged cells under Nx (P10-blue, P18-green). Arrows indicate absent fragments. Hx also led to a decrease (4.5-fold) in ATP concentration (**C**) and in the number of mitochondrial genomes, while a slight increase (1.6-fold) was observed for extensively passaged cells in Nx in comparison with a control sample (**E**). Changes in membrane potential were only observed in extensively passaged cells under Nx (**D**). Heteroplasmic point mutations were also detected in the mtDNA genome of ADSC's and BM MSC's irrespective of passage or O2 tension (see Table). All were located within the D-loop region.

IV. Implementation

Both BM MSC's and ADSC's were from healthy donors and were expanded in DMEM+10% FBS at 37ºC and 5% CO_2. Hypoxia (Hx), was created by culturing cells in a chamber flushed with premixed O_2, CO_2 and N_2 at varying ratios. Exhausted medium was changed twice a week. Fold variation in gene expression and in the number of mtDNA genomes was determined by RT-PCR using the $\Delta\Delta C_T$ method. Genotyping of several microsatellite markers was performed by analysis of fluorescently labeled PCR products in an ABI 3730 *xl* DNA analyzer (Applied Biosystems) in line with the manufacture's instructions. Intracellular ATP was measured by bioluminescence using the ATP determination Kit (Invitrogen). Membrane potential was determined by flow cytometry using the MitoProbe JC-1 assay kit (Invitrogen). Mutational analysis of the mtDNA was performed by PCR amplification of the entire mitochondrial genome followed by DNA sequencing. Haplogroup assignment was performed using tools such as PhyloTree and HaploGrep. Identification and classification of repeats in the mtDNA was performed using Repseek and Tandem Repeats Finder. An extensive list of deletion breakpoints was obtained from the Mitomap database (www.mitomap.org/).

V. Results

Our results show that Hx induced an immediate and concerted down-regulation of genes involved in DNA repair and damage response pathways (Fig. 1A), accompanied by genomic instability in selected microsatellite markers (Fig. 1B). Short-term exposure to hypoxic conditions led to a simultaneous decrease in mitochondrial ATP content (Fig. 1C) and in the number of mitochondrial genomes (Fig. 1E). Moreover, several heteroplasmic point mutations were consistently detected in cells exposed to both hypoxic and normoxic conditions (see Table), particularly within the hypervariable D-loop region. The latter include insertions and substitutions, which have been detected in a wide range of tumors. Short-term exposure to Hx did not lead to changes in mitochondrial membrane potential ($\Delta\Psi_m$) in comparison with Nx (Fig. 1D). Only extensively passaged cells have shown an increase in $\Delta\Psi_m$ (Fig. 1D) Simultaneously, we have been conducting several *in silico* analysis on the human mtDNA, with the purpose of explaining the regional preference behind several deletion-breakpoints described in the literature. We found that physical distortion induced by particular repetitive DNA tracts are related with certain mutational events, but are not the sole explanation. Other instability-prone motifs have also been detected next to deletion breakpoints, and were found to play a key role in promoting recombination (data not shown).

VI. Conclusions

Altogether, these results alert for the need to monitor genetic integrity during the *ex-vivo* cultivation of

stem/progenitor cells towards the development and application of stem cell-based regenerative therapies.

VII. Planned Developments

Currently we are looking at further evidence of senescence and loss of cell quality, including analysis of telomere instability and detection of large aberrations in the mitochondrial genome. In a near future, we aim to evaluate alternative O_2 tensions and extend this analysis to other types of stem cells, namely induced pluripotent stem cells (iPSCs). The use of chip-based platforms is also planned.

Acknowledgments

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