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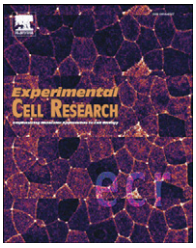

Research Article

p53 regulates the proliferation, differentiation and spontaneous transformation of mesenchymal stem cells

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ABSTRACT

Mesenchymal stem cells (MSC) have been extensively studied and gained wide popularity due to their therapeutic potential. Spontaneous transformation of MSC, from both human and murine origin, has been reported in many studies. MSC transformation depends on the culture conditions, the origin of the cells and the time on culture; however, the precise biological characteristics involved in this process have not been fully defined yet. In this study, we investigated the role of p53 in the biology and transformation of murine bone marrow (BM)-derived MSC. We demonstrate that the MSC derived from p53KO mice showed an augmented proliferation rate, a shorter doubling time and also morphologic and phenotypic changes, as compared to MSC derived from wild-type animals. Furthermore, the MSC devoid of p53 had an increased number of cells able to generate colonies. In addition, not only proliferation but also MSC differentiation is controlled by p53 since its absence modifies the speed of the process. Moreover, genomic instability, changes in the expression of c-myc and anchorage independent growth were also observed in p53KO MSC. In addition, the absence of p53 implicates the spontaneous transformation of MSC in long-term cultures. Our results reveal that p53 plays a central role in the biology of MSC.

Introduction

Adult stem cells reside in specific niches in a quiescent state during most of the host lifetime maintaining the tissue homeostasis [1,2]. In addition to hematopoietic stem cells, bone marrow contains mesenchymal stem cells (MSC), also known as bone marrow fibroblasts, skeletal stem cells or marrow stromal cells [3,4]. MSC were originally described by Friedenstein and colleagues [5] as a plastic-adherent clonal cell population capable of differentiating into multiple mesoderm-type cells (e.g., osteoblast, adipocytes and chondrocytes) [3,6,7]. Due to the lack of specific markers and their heterogeneity, MSC are phenotypically characterized throughout a panel of cell surface molecules [8]. Recently, MSC have been used on several cell therapy approaches [3,9,10], although basic aspects of their biology and mechanism of action are still to be determined. This way, cell expansion *ex vivo*, which represents a selection for rapidly dividing cells, may increase the risk of spontaneous transformation in both humans [11,12] and mice MSC [13,14]. Therefore, knowledge of the pathways directly implicated in the cellular transformation and the control of the culture conditions of MSC are needed to improve the use of these cells for therapeutic applications.

Proliferation, self-renewal and genomic stability are tightly controlled in embryonic and adult stem cells [15]. The p53 tumor suppressor gene is inactivated in most human cancers and has functionally been involved in tumor development, cell cycle

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Abbreviations: MSC, Mesenchymal Stem Cells; BM, Bone Marrow; hMSC, human Mesenchymal Stem Cells; p53KO, p53 Knock-Out; WT, Wild Type; PI, Propidium Iodide; s.c, subcutaneously; CFSE, Carboxyfluorescein Diacetate Succinimidyl Ester; RT-qPCR, Reverse Transcription quantitative PCR; FCS, Foetal Calf Serum; TC, Tumor Cells; NT, Non Tumor detected

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control, apoptosis and genomic stability [16,17]. Mutations in p53 have been implicated in age-related mesenchymal stem cell transformation [18] and in the self-renewal and differentiation of neural stem cells [19,20]. Additionally, a direct role of p53 in the differentiation process of MSC has been recently proposed [21] and its misfunction or absence has been related with the osteogenesis and osteosarcoma development [22,23].

In this work, we analyzed the role of p53 in bone marrow-derived MSC by comparing MSC cultures obtained from p53 knockout (KO) and p53 wild-type (WT) mice. We observed that the lack of p53 promotes a higher proliferation rate on MSC, which acquire the typical MSC surface phenotype earlier than WT MSC. Besides, p53KO MSC showed an increment in the number of precursors able to form colonies. Moreover, the time required by these cells to differentiate, to adipocytes or osteocytes, is shorter than in WT MSC. On the other hand, we have also shown that the absence of p53 increases genomic instability and c-myc expression and leads to the spontaneous transformation of long-term MSC cultures. Consequently, the tumor suppressor protein p53 plays a pivotal role in the biology of MSC, regulating their proliferation, differentiation and tumorigenesis.

Materials and methods

Mice

C57Bl/6 mice were obtained from IFFA-Credo (France), and C57Bl/6 p53KO mice were obtained from the CNIO (Spanish National Cancer Research Centre, Madrid) animal facility. Animals were housed under standard conditions in our animal facility (CIB-CSIC). Genotyping was performed by PCR using the following primers: Neo (186/186-1), 5′-gaagcgggaagggactggctgcta-3′ and 5′-cgggagcggcgataccgtaaagc-3′; p53KO (IMRs), 5′-cttgggtggaga-ggctattc-3′ and 5′-aggtgagatgacaggagatc-3′; and p53WT (OM2/OM3), 5′-tatactcagagccggcct-3′and 5′-acagcgtggtggtacctta-3′. All animal manipulations and experimental methods described in this manuscript have been approved by the CSIC Committee for Animal Experimentation. The number of animals employed and their suffering was in all circumstances minimized. All experiments were performed in compliance with the European Union and Spanish laws on experimentation and animal care (Council Directive 86/609/EEC).

Mesenchymal stem cell isolation, culture and characterization

Bone marrow was collected from 6- to 8-week-old mice by flushing femurs. Cells were cultured and expanded in MesenCult® (Stemcell Technologies, Inc.) medium supplemented with 2 mM L-glutamine (GibcoBRL) and 50 U/ml penicillin/streptomycin (GibcoBRL) at 37 °C in a humidified atmosphere with 95% air and 5% CO_2 and allowed to attach for 4 days. Non-adherent cells were removed at that point by changing the culture medium. Initially, cells were passed at dilutions ranging from 1:2 to 1:5 when they reached confluency using 2 mM EDTA/PBS to detach the cells. After 10–15 passages, the cells were passed at dilutions ranging from 1:10 to 1:20 due to their increased proliferation rate. For proliferation assays, MSC from >80 days were counted and seeded at 4×10^3 cells/cm^2.

Flow cytometry

Surface expression of CD45 (eBioscience), CD29 or Sca-1 (BD Pharmingen) was assessed by flow cytometry analysis to characterize the culture; 10^4 cells were routinely analyzed and subsequent fluorescent secondary antibodies were used in each case. Cell cycle analyses were performed using propidium iodide (PI) labeling. Cells were washed with PBS and fixed with 70% ethanol–PBS at 4 °C for at least 4 h. Following incubation at 37 °C for 20 min with 0.1 mg/ml RNase (Roche), 20 μg/ml of PI (Sigma) was added to each sample. All assays were analyzed in an EPICS XL flow cytometer and the Expo 32 ACS software® (Coulter Electronics).

Colony-forming unit–fibroblast (CFU-F) assay

Whole bone marrow or MSC (>80 days in culture) were counted and seeded at different densities in culture dishes and the medium was replaced every 3–4 days. After 15 days in culture, colonies were stained with 0.2% (w/v) violet crystal (Sigma) solution in 20% (v/v) methanol.

Cell viability and CFSE assays

Cell viability was analyzed with the CellTiter 96™ Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. Briefly, 5×10^3 cells per well were seeded in flat-bottomed 96-well plates, and kinetic analysis was performed with an automatic microplate spectrophotometer (Multiskan, Biochromatic; Labsystem) at 492 nm.

CFSE (Carboxy*f*luorescein Diacetate *S*uccinimidyl *E*ster) assay was performed, incubating 2×10^6 cells with 2.5 mM CFSE (Molecular Probes) for 5 min at room temperature (RT). Reaction was stopped with 10% FCS during 1 min and the cells were washed and seeded in 60-mm culture dishes. After different time intervals, cells were recovered, washed in phosphate-buffered saline (PBS) and analyzed by flow cytometry as described.

Differentiation assays

MSC WT and p53KO were seeded at 4×10^3 cells/cm^2 in six-well plates. Cells were grown in α-MEM medium supplemented with L-glutamine and penicillin/streptomycin, during at least 7 days after which they were growing in different conditional media.

To induce adipogenic differentiation, the MSC were cultured for 15 days in differentiation medium: 10% FCS in α-MEM supplemented with 4.5 g/l glucose, 1 μM dexamethasone (Sigma) and 5 μg/ml insulin (Sigma). Adipocytes were easily discerned from undifferentiated cells by contrast microscopy. They were fixed in 4% paraformaldehyde in PBS for 1 h at 4 °C and stained with Oil Red O for 15 min and washed with distilled water.

Osteogenic differentiation was induced by culturing the MSC for 15 days in differentiation medium: 15% FCS in α-MEM supplemented with 50 μg/ml 2-phosphate-L-ascorbic (Sigma), 10 mM β-glycerol phosphate (Sigma) and 10 nM dexamethasone (Sigma). Mineralized deposits were visualized by Alizarin Red staining for 15 min at RT. Cells were fixed in cold ethanol (70%) for 1 h at 4 °C. The images were visualized using a Nikon i30 microscope.