Brg1 chromatin remodeling factor is involved in cell growth arrest, apoptosis and senescence of rat mesenchymal stem cells

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Summary

Self-renewal, proliferation and differentiation properties of stem cells are controlled by key transcription factors. However, their activity is modulated by chromatin remodeling factors that operate at the highest hierarchical level. Studies on these factors can be especially important to dissect molecular pathways governing the biology of stem cells. SWI/SNF complexes are adenosine triphosphate (ATP)-dependent chromatin remodeling enzymes that have been shown to be required for cell cycle control, apoptosis and cell differentiation in several biological systems. The aim of our research was to investigate the role of these complexes in the biology of mesenchymal stem cells (MSCs). To this end, in MSCs we caused a forced expression of the ATPase subunit of SWI/SNF (Brg1 – also known as Smarca4) by adenoviral transduction. Forced Brg1 expression induced a significant cell cycle arrest of MSCs in culture. This was associated with a huge increase in apoptosis that reached a peak 3 days after transduction. In addition, we observed signs of senescence in cells having ectopic Brg1 expression. At the molecular level these phenomena were associated with activation of Rb- and p53-related pathways. Inhibition of either p53 or Rb with E1A mutated proteins allowed us to hypothesize that both Rb and p53 are dispensable for Brg1-induced senescence, whereas only p53 seems to play a role in triggering programmed cell death. We also looked at the effects of forced Brg1 expression on canonical MSC differentiation in adipocytes, chondrocytes and osteocytes. Brg1 did not induce cell differentiation per se; however, this protein could contribute, at least in part, to the adipocyte differentiation process.

In conclusion, our results suggest that whereas some ATP-dependent chromatin remodeling factors, such as ISWI complexes, promote stem cell self-renewal and conservation of an uncommitted state, others cause an escape from ‘stemness’ and induction of differentiation along with senescence and cell death phenomena.

Key words: Marrow stromal stem cells, Chromatin remodeling, Differentiation, Apoptosis, Senescence, Cell cycle

Introduction

Stem cells within normal tissues are defined by common characteristics: self-renewal to maintain the stem cell pool over time; regulation of stem cell number through a strict balance between cell proliferation, cell differentiation and cell death; ability to give rise to a broad range of differentiated cells (Gage, 2000; Morrison et al., 1997; Temple, 2001).

Chromatin state is fundamental for gene expression. In fact, euchromatin is permissive for transcription, whereas heterochromatin is repressive. Chromatin remodeling factors can modify the balance between euchromatin and heterochromatin by acting as main regulators of gene expression. Self-renewal, proliferation and differentiation properties of stem cells are controlled by key transcription factors. However, their activity is modulated by chromatin remodeling factors that operate at the highest hierarchical level. Studies on these factors can be especially important to dissect molecular pathways governing the biology of stem cells (Buszczak and Spradling, 2006; Edlund and Jessell, 1999; Rasmussen, 2003).

Eukaryotic cells contain at least five classes of chromatin remodeling complexes: SWI/SNF, ISWI, CHD(Mi-2), INO80 and SWR1. These complexes have a catalytic ATPase subunit that is fundamental to allow or impair the access to nucleosomal DNA by altering the structure, composition and positioning of nucleosomes (Saha et al., 2006).

The different classes of chromatin remodeling factors contribute to distinct functions in the regulation of cell biology. Some remodeling factors can promote self-renewal and inhibit stem cell commitment and differentiation, such as those that contain the RING finger protein Bmi1. In fact, it has recently been shown that Bmi1 protein is necessary for self-renewal of adult hematopoietic stem cells, as well as adult peripheral and central nervous stem cells. It has also been demonstrated that Bmi1 has only a minimal or no role in stem cell commitment and differentiation. Other remodeling complexes, however,
could be involved in stem cell differentiation and/or senescence, such as the SWI/SNF remodeling factors (Buszczak and Spradling, 2006; Park et al., 2004; Rasmussen, 2003).

The mammalian SWI/SNF family includes several members that share most of the same subunits and are distinguished by the ATPase enzyme, either the Brg1 (also known as Smarca4) or the Brm protein, and/or the presence of tissue-specific isoforms. Complexes containing Brg1 have been shown to be required for cell cycle control, apoptosis and cell differentiation in several biological systems (Bultman et al., 2000; de la Serna et al., 2006; Dunaief et al., 1994; Hendricks et al., 2004; Martens and Winston, 2003; Murphy et al., 1999; Reisman et al., 2002). Homozygous knockout mice for the Brg1 gene die at the embryonic stage, whereas heterozygotes are prone to tumors (Bultman et al., 2000; de la Serna et al., 2006; Saha et al., 2006). Brg1 protein can interact with different proteins involved in the regulation of transcription, such as factors involved in myeloid, erythroid, lymphocyte, muscle, neural and adipocyte commitment and/or differentiation (Bultman et al., 2000; de la Serna et al., 2006; Dunaief et al., 1994; Hendricks et al., 2004; Martens and Winston, 2003; Murphy et al., 1999; Reisman et al., 2002). Several lines of evidence suggest that Brg1 plays a role in negative regulation of cell cycle progression by binding to the retinoblastoma 1 (Rb) protein and the tumor suppressor protein p53 (Hendricks et al., 2004; Kang et al., 2004; Lee et al., 2002; Strobeck et al., 2000).

It appears that SWI/SNF complexes containing Brg1 could counteract the effect of chromatin remodeling complexes associated with the Bmi1 protein of the Polycomb group. For this reason we aimed to investigate the role of Brg1 in the biology of mesenchymal stem cells (MSCs). In addition to hematopoietic stem cells, bone marrow contains cells called marrow stromal stem cells, which meet the criteria for stem cells of non-hematopoietic tissues. These stem cells are currently referred to as either MSCs, because of their ability to differentiate into mesenchymal cells (such as bone and cartilage cells, adipocytes), or marrow stromal cells, because they appear to stem from the complex array of supporting structures found in marrow (Bianco and Gehron Robey, 2000; Colter et al., 2000; Prockop, 1997). MSCs have been shown to possess great somatic plasticity because they can differentiate into non-mesenchymal lineages. In fact, it has been demonstrated that MSCs are capable of differentiating into neurons and astrocytes in vitro and in vivo. MSCs are of interest because they are easily isolated from a small aspirate of bone marrow and can be expanded through as many as 50 population doublings in approximately 10 weeks (Prockop, 1997). For these reasons, the cells are currently being tested for their potential use in cell and gene therapy for several human diseases.

Knockout mice for Brg1 and the expression of the dominant-negative form of Brg1 in vitro contributed significantly to dissecting the role of Brg1 in cell biology. However, complementary studies, such as those based on forced expression of Brg1, could provide further details on the Brg1 molecular signature. To this end, we transduced MSCs with adenoviruses expressing Brg1 and analyzed the effects on cell proliferation, apoptosis, senescence and differentiation.

**Results**

**Brg1 endogenous expression increases during replicative senescence of MSCs**

After several passages in vitro, MSCs reduce their proliferative potential and progressively lose their typical multilineage potential. These are typical signs of senescence (Colter et al., 2000; Colter et al., 2001; Sethe et al., 2006). Under our experimental conditions, MSCs isolated from rat bone marrow reached confluence 7-10 days after initial plating and were able to differentiate into adipocytes, osteocytes and chondrocytes (data not shown). Starting from this point (day 1), cells were trypsinized and plated at low confluence for propagation. When cells reached 70% culture-confluence they were trypsinized again for further propagation. We followed the expression level of endogenous Brg1 mRNA in cultures of MSCs during a 30-day time period. We observed a progressive increase in Brg1 expression until 15 days, when it reached a plateau value. At this time point, the Brg1 level was 20 times higher than that at day 1 (P<0.01). Protein expression followed the same expression profile (data not shown).

The increase of Brg1 expression during cell cycle exit and senescence of MSCs could suggest that its expression is functionally related to cell senescence or, alternatively, increase in Brg1 expression could be considered only a signature of expression that characterizes cell cycle exit and senescence of MSCs. In order to get further insight into the role of Brg1 in the biology of MSCs we analyzed the effect of forced Brg1 expression on cultured MSCs.

**Setup of Brg1 adenoviral transduction**

The overexpression of a protein in a cell could produce biased data, because high and prolonged production of ectopic proteins could induce non-physiological effects. Transduction experiments were performed by making certain to avoid overwhelming the cell machinery with non-physiologically high and prolonged production of ectopic proteins. The transduction protocol we used induced a slight increase of Brg1 protein (+60±8%) in cells treated with Ad-CMV-Brg1 compared with controls 3 days after transduction.

**Brg1-promoted cell cycle arrest**

Forced Brg1 expression induced a significant cell cycle arrest of MSCs in culture as determined by FACS analyses. Twenty-four hours after Brg1 transduction, the percentage of G1/G0 cells was 87% compared with 52% in control cultures. The decrease in G2/M cells was more impressive: 0.21% versus 10%. Also, 3 days after transduction, cells overexpressing Brg1 were locked in G1/G0 phase (Fig. 1).

Molecular analyses showed that mRNA expression of cyclin A and cyclin E, whose activity determines G1/S progression, was significantly decreased in cells overexpressing Brg1 compared with controls (Fig. 2).

Brg1 acts through interaction with p53 and Rb (Dunaief et al., 1994; Kang et al., 2004; Lee et al., 2002; Strobeck et al., 2000). For this reason, we analyzed in depth their biochemical pathways. Forced Brg1 expression did not change p53 mRNA level; instead, it increased protein levels 3 days after transduction (Figs 2, 3 and Table 1).

The mRNA levels of Rb and its related gene Rb2/p130 increased 1 day after viral transduction, but declined at 3 days
of treatment. By contrast, the corresponding proteins were upregulated 3 days after transduction, suggesting that increased Rb and Rb2/p130 mRNAs were translated and shortly after degraded (Figs 2, 3 and Table 1).

Cyclin/cyclin dependent kinase (CDK) complexes promote cell cycle progression and are negatively regulated by several CDK inhibitors, such as p21cip and p27kip1, which are related to p53 and Rb pathways (Galderisi et al., 2003; Giaccia and Kastan, 1998; Park et al., 1999). Brg1 ectopic expression induced an increase of p21cip at both the mRNA and protein level (Figs 2, 3 and Table 1). The effect on p27 expression was different: we detected an upregulation of mRNA (+160±22%) and a downregulation of protein level (Fig. 3 and Table 1). These data suggest that only p21 contributes to Brg1-induced cell cycle arrest.

Brg1 triggers programmed cell death
FACS analysis showed that cell cycle arrest was associated with a huge increase in apoptosis that reached a peak 3 days after transduction: 23% of dead cells in Brg1-transduced cells versus 0.2% in controls (Fig. 1). These data were confirmed by a TUNEL assay, which showed an increased percentage of apoptotic cells in cultures treated with Ad-CMV-Brg1 compared with controls (data not shown).

At the molecular level, 3 days after transduction, we observed an increase in both the Bax/Bcl-2 ratio (4.75-fold) and the BclXs/BclXl ratio (1.9-fold) in cells expressing ectopic Brg1 as detected by reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 4). This could suggest that apoptosis was triggered through canonical pathways. In addition, we detected an upregulation of Bad (+292±20%) and Bak (+680±60%) mRNA levels (Fig. 4); these are well-known pro-apoptotic genes (Adams and Cory, 1998; Reed, 1997).

Brg1 induces senescence
We observed signs of senescence in cells overexpressing Brg1, as detected by in situ acid-beta-galactosidase staining 3 days after viral transduction (Fig. 5 and Table 2). To expand upon this finding, we measured telomerase activity by a primer extension assay in which telomerase reverse transcriptase (TERT) synthesizes telomeric repeats onto oligonucleotide primers [telomeric repeat amplification protocol (TRAP)] (Adams and Cory, 1998; Kim et al., 1994; Kim and Wu, 1997; Reed, 1997). Telomerase-positive extracts were obtained from MSCs, as expected for stem cell samples. However, the telomerase activity was reduced in cells treated with Ad-CMV-Brg1 compared with control cells (Fig. 6). These data were in agreement with a reduced expression of TERT mRNA (–40±7%) in cells showing an upregulation of Brg1 protein.

**Table 1. Protein expression in MSCs transduced with Ad-CMV and Ad-CMV-Brg1**

|                     | Ad-CMV-Brg1 | Ad-CMV |
|---------------------|-------------|--------|
| p53                 | 0.51±0.02   | 0.26±0.03 |
| pRb                 | 0.4±0.02    | 0.12±0.03 |
| pRb2/p130           | 0.21±0.05   | 0.11±0.02 |
| p21                 | 1.65±0.1    | 1.0±0.05  |
| p27                 | 0.08±0.01   | 0.16±0.02 |

Fig. 1. FACS analysis of MSCs transduced with Ad-CMV and Ad-CMV-Brg1. Analyses were performed 1 and 3 days after transduction. Pre G1 indicates sub-diploid cells that are undergoing programmed cell death.

Fig. 2. Semiquantitative RT-PCR analysis of mRNA expression of the indicated genes of MSCs transduced with Ad-CMV and Ad-CMV-Brg1. Analyses were performed 3 days after transduction. mRNA levels were normalized with respect to HPRT, chosen as an internal control. Each experiment was repeated at least three times.

Fig. 3. Western blot of protein expression in MSCs transduced with Ad-CMV and Ad-CMV-Brg1. Analyses were performed 3 days after transduction. Protein levels were normalized with respect to β-tubulin, chosen as an internal control. Each experiment was repeated at least three times. Variations in protein expression are given as arbitrary units.
Brg1 could contribute to adipogenesis

We also looked at the effects of Brg1 overexpression on canonical MSC differentiation in adipocytes, chondrocytes and osteocytes. Cell cycle exit represents a key step to trigger differentiation of cells and induction of a novel program of gene expression leading to cell differentiation (Edlund and Jessell, 1999; Galderisi et al., 2003; Morrison et al., 1997). We wanted to verify whether Brg1-mediated cell cycle exit could directly trigger biochemical pathways leading to cell commitment and differentiation. To this end, cells were transduced with Ad-CMV-Brg1 and with a control virus and were grown for 14 days in culture media without the addition of hormones and cytokines that promote cell differentiation.

Brg1 did not induce cell differentiation per se, as determined by cytochemical staining (data not shown). However, Brg1 could contribute, at least in part, to adipocyte lineage commitment, because we observed the activation of several adipocyte differentiation markers: PPAR-\(\gamma\) (+162\(\pm\)18\%) and lipoprotein lipase (LPL) (+159\(\pm\)19\%) (Fig. 7). This hypothesis was further strengthened by the observation that, in media promoting adipocyte cell differentiation of MSCs, Brg1 overexpression accelerated the acquisition of mature phenotypes associated with a huge increase in differentiation markers: PPAR-\(\gamma\) (+216\(\pm\)25\%) and LPL (+976\(\pm\)79\%) (Fig. 7).

Pathways involved in apoptosis and senescence

Brg1 could rely upon Rb and p53 to trigger its biological effects. It has been demonstrated that Brg1 interacts both with Rb and p53 in several biological systems (Dunai et al., 1994; Hendricks et al., 2004; Lee et al., 2002). Also in MSCs, Brg1 could interact both with Rb and p53, as suggested by data obtained in communoprecipitation experiments (Fig. 8). It is well known that apoptosis and senescence entail the activation of several biochemical pathways; among these, p53- and Rb-mediated pathways play a key role (Artandi and Attardi, 2005; Campisi, 2001; Narita et al., 2003; Pelicci, 2004).

Forced Brg1 expression in MSCs induced both apoptosis and replicative senescence, and we decided to further investigate the role played by Rb and p53 in these phenomena. We worked with E1A adenoviral protein, which interacts and inhibits both Rb and p53. We used adenovirus carriers expressing normal and mutated E1A proteins: Ad-CMV-E1A, Ad-CMV-E1A(YH47-928) and Ad-CMV-E1A(RG2). The mutated E1A(YH47-928) encodes for a protein that interacts and inhibits p53 and not Rb, whereas E1A(RG2) has an opposite effect: it blocks Rb activity and not p53 (Dornan et al., 2003; Moran, 1993; Wang et al., 1995; Wang et al., 1993).

We performed double transduction experiments on MSCs. In cells transduced with Ad-CMV-Brg1 or with a control virus, we inhibited p53 with Ad-CMV-E1A(YH47-928), Rb with Ad-CMV-E1A(RG2) or both with Ad-CMV-E1A.

Table 2. Acid-beta-galactosidase assay

| Senescent cells (%) |  
|---------------------|---|
| Ad-CMV-Brg1         | 16\(\pm\)1.1\% |
| Ad-CMV              | 11\(\pm\)0.9\% |
| Ad-E1AYH47 (p53 \(\pm\))\* | 17\(\pm\)1.3\% |
| Ad-E1ARG2 (Rb \(\pm\))\† | 12\(\pm\)1.0\% |

*Adenovirus carrying mutated E1A isoforms that inhibit p53.
†Adenovirus carrying mutated E1A isoforms that inhibit Rb.

Fig. 4. Semiquantitative RT-PCR analysis of mRNA expression of the indicated genes of MSCs transduced with Ad-CMV and Ad-CMV-Brg1. Analyses were performed 3 days after transduction. Each experiment was repeated at least three times.

Fig. 5. Senescence-associated \(\beta\)-galactosidase assay performed on MSCs transduced with the recombinant adenoviruses indicated in Table 2. Assays were performed 3 days after transduction. Ad-YH47 and Ad-RG2 indicate adenoviruses carrying mutated E1A isoforms that inhibit p53 (p53 \(\pm\)) and Rb (Rb \(\pm\)), respectively. The picture shows several senescent cells as detected with \(\beta\)-galactosidase assay. White asterisk, a typical flat large senescent cell.

Fig. 6. Polyacrylamide gel electrophoresis of TRAP assay products obtained from protein extracts of MSCs transduced with Ad-CMV and Ad-CMV-Brg1. Analyses were performed 3 days after transduction. The picture shows the PCR-amplified products of telomerase activity. Three different reactions for each transduction experiment are shown. Black arrow, TRAP internal control.
Both Rb and p53 appeared to play a role in Brg1-mediated senescence. In situ acid-beta-galactosidase staining showed that the inhibition of p53 with Ad-CMV-E1A(YH47-928) and of Rb with Ad-CMV-E1A(RG2) reduced the percentage of senescent cells in cultures treated with Ad-CMV-Brg1 compared with controls (Fig. 5 and Table 2). However, Rb-related pathways seem to play a major role in senescence compared with the p53 pathways. Also, the TRAP assay and TERT mRNA levels confirmed these data (data not shown). It has been demonstrated that distinct heterochromatin structures accumulate during senescence and could represent a hallmark of this process. In particular, Narita et al. observed Rb-mediated heterochromatin formation during cellular senescence and demonstrated that DNA from senescent cells was more resistant to limited micrococcal nuclease digestion compared with normal cells (Narita et al., 2003). Cells transduced with Ad-CMV-Brg1 showed an increased resistance to nuclease digestion compared with cells treated with a control virus. Inhibition of p53 and Rb promoted nuclease digestion of DNA from cells overexpressing Brg1, suggesting that both pathways contribute to Brg1-induced senescence (Fig. 9A,B).

Induction of apoptosis by forced Brg1 expression appeared to be primarily associated with the p53 pathway. In fact, inhibition of p53 greatly reduced the expression of the pro-apoptotic gene Bax (−270±19%) in cells treated with Ad-CMV-Brg1 compared with controls. Data from the TUNEL assay were in agreement with this hypothesis (Table 3).

**Discussion**

The access of transcription factors to promoters of genes is regulated through the chromatin status by epigenetic means. The accessibility of promoters appears to be a crucial feature in stem cell biology. The self-renewal and uncommitted state of a stem cell are associated with the expression of genes such as those encoding the transcription factors OCT4, NANOG and SOX2 (Buszczak and Spradling, 2006). By contrast, lineage-specific transcription factors are linked to loss of stemness, triggering of differentiation and acquisition of specific cell phenotypes. Adenosine triphosphate (ATP)-dependent chromatin remodeling factors can alter histone-DNA contacts and induce chromatin structural changes that can either promote or inhibit gene expression. ATP-dependent remodeling factors are grouped into several classes that reveal

**Fig. 7.** Semiquantitative RT-PCR analysis of mRNA expression of the indicated genes of MSCs transduced with Ad-CMV and Ad-CMV-Brg1. Analyses were performed 3 days after transduction. mRNA levels were normalized with respect to HPRT, chosen as an internal control. Each experiment was repeated at least three times. The picture shows experiments performed on MSCs grown in proliferating medium (control medium) and adipocyte differentiation medium (inducing medium).

![Image](2908_JCS_120_16)
cell type-specific and gene-specific roles for these multiprotein complexes (de la Serna et al., 2006; Kingston and Narlikar, 1999; Kornberg and Lorch, 1999). Several studies have identified chromatin remodelers that have a role in maintaining stem cell self-renewal and uncommitted state, such as those containing the Bmi1 protein (Park et al., 2004). Few data have addressed the role of chromatin remodeling in stem cell senescence and apoptosis.

ATP-dependent remodeling complexes containing Brg1 have been shown to be required for cell cycle control, apoptosis and cell differentiation in several biological systems. Specifically, Brg1 plays a role in negative regulation of cell cycle progression by binding to the retinoblastoma protein and the tumor suppressor protein p53 (Dunaief et al., 1994; Kang et al., 2004; Lee et al., 2002; Strobeck et al., 2000). Both Rb and p53 are master genes in the control of cell cycle exit that can lead either to cell differentiation or to senescence. Moreover, both genes have a role in controlling programmed cell death (Artandi and Attardi, 2005; Campisi, 2001; Narita et al., 2003; Pelicci, 2004). For these reasons, we evaluated whether forced expression of Brg1 in MSCs can cause a loss of proliferative potential along with induction of replicative senescence and/or induction of cell differentiation. We also evaluated the apoptosis process following increased Brg1 expression.

Forced expression of Brg1 in MSCs induced an arrest in the G1/G0 phase of the cell cycle. This is in agreement with other reports showing that this gene induces growth arrest and could be considered a putative tumor suppressor gene (Dunaief et al., 1994; Hendricks et al., 2004; Murphy et al., 1999; Strobeck et al., 2000). Growth arrest is associated with increased expression of p53 and Rb, which are known effectors of Brg1 biological activity. In addition, genes related to p53 and Rb are known to be implicated in the absence of SWI/SNF function. However, the activity of this complex was later required for progression of adipogenesis (Salma et al., 2004). In fact, they demonstrated that histone hyperacetylation, binding of co-activators, RNA polymerase II and general transcription factors initially occurred at inducible PPAR-gamma-2, an adipocyte-specific transcription factor, in the absence of SWI/SNF function. However, the activity of this complex was later required for progression of adipogenesis.

In conclusion, our research showed that Brg1 could play a key role in multiple aspects of MSC biology. Remodeling of chromatin through forced expression of Brg1 appears to negatively affect stem cell self-renewal properties, inducing senescence and apoptosis. This further highlights the importance of chromatin remodelers for stem cell identity. Whereas some complexes, such as those associated with Bmi1 protein, prevent senescence and are required for maintenance of ‘stemness’, other remodeling complexes can reduce replicative senescence and cell death. A careful balance between the activity of these players is fundamental for the homeostasis of the stem cell compartment.

### Materials and Methods

#### Animals and MSC cultures

In accordance with protocols devised by Prockop and co-workers (Colter et al., 2000; Colter et al., 2001; Javazon et al., 2001), MSCs were harvested from the bone marrow of the femurs and tibias of 4- to 12-month-old rats by inserting a 21-gauge needle into the shaft of the bone and flushing it with α-modified Eagle’s medium (αMEM) containing 10% fetal bovine serum (FBS), 2 ng/ml basic fibroblast growth factor (bFGF), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (proliferation medium). Cells from one rat were plated onto two 100 mm dishes. After 24 hours, non-adherent cells were discarded, and adherent cells representing MSCs along with committed progenitors were washed twice with phosphate-buffered saline (PBS). Cells were then incubated for 7-10 days in proliferating...
medium to reach confluence and extensively propagated for further experiments. All cell culture reagents were obtained from Invitrogen Italia (Milan, Italy) unless otherwise stated.

**MISC differentiation**

Adipocyte differentiation: cells were grown for 14 days in αMEM containing 10% FBS, 0.1% indomethacin, 1 μM hydrocortisone, 1 μg/ml insulin and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). Adipocytes were detected by standard Oil Red O staining.

Osteocyte differentiation: cells were grown for 14 days in αMEM containing 10% FBS, 100 nM dexamethasone, 0.2 mM ascorbic acid and 10 nM β-glycerophosphate. Osteocytes were detected with Alizarin Red staining.

Chondrocyte differentiation: cells were grown for 14 days in αMEM containing 10% FBS, 100 mM dexamethasone, 0.3 mM acetic acid, 1× insulin-transferrin-selenium (ITS) (Sigma-Aldrich, Milan, Italy) and 5 ng/ml TGF-β1. Chondrocytes were detected with toluidine blue staining.

**Adenoviral production and transduction**

The Ad-CMV- and Ad-CMV-βgal viruses were generated as previously described (Claudio et al., 1999). The Ad-CMV-Brg1 was kindly provided by E. Lee (DNAX Research, Palo Alto, CA). The Ad-CMV-E1A, Ad-CMV-E1A(Y7H-928) and the Ad-CMV-E1A(RG2) were kindly provided by E. Moran (Temple University, Philadelphia, PA).

To obtain enough viruses for MSC transduction, recombinant adenoviruses were expanded by infecting 293 cells and then purified.

Cell cultures were transduced at different multiplicities of infection (MOI) with Ad-CMV-Brg1, Ad-CMV and Ad-CMV-βgal and cytotoxic effects were determined by trypan blue exclusion and CytoTox 96 cell death assay (Promega, Madison, WI). β-galactosidase activity was evaluated by an in situ detection β-galactosidase staining kit (Roche, Mannheim, Germany) to determine the percentage of transduced cells. Typically, we found that approximately 80% of MSCs were positive for β-galactosidase activity when transduced with 200 MOI during a 48-hour incubation period.

**Cell cycle analysis**

For each assay 3×10^6 cells were collected and resuspended in 500 μl of a hypotonic buffer (0.1% Triton X-100, 0.1% sodium citrate and 50 μM propidium iodoide/RNase A). Cells were incubated in the dark for 30 minutes and then analyzed. Samples were acquired on a FACSCalibur flow cytometer using the Cell Quest software (Becton Dickinson, Franklin Lakes, NJ) and analyzed in accordance with standard procedure using the Cell Quest software and the ModFitLT software version 3 (Becton Dickinson).

**TUNEL assay and determination of apoptotic index**

The cells for the TUNEL assay (in situ cell death detection kit from Roche) were grown on coverslips. Cells were fixed for 15 minutes using 4% paraformaldehyde and the TUNEL reaction was performed according to the manufacturer’s instructions. The apoptotic index was calculated by the number of positive TUNEL cells out of at least 500 cells in different microscopic fields.

**RNA extraction and RT-PCR and real-time PCR**

Total RNA was extracted from cell cultures using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s protocol. The mRNA levels of the genes analyzed were measured by RT-PCR amplification, as previously reported (Galdersi et al., 1999).

Sequences for rat mRNAs from the GenBank (DNASTAR, Madison, WI) were used to design primer pairs for RT-PCR reactions (OLIGO 4.05 software; National Biosciences, Plymouth, MN). Primer sequences are available on request. Appropriate regions of HPRT cDNA were used as controls. PCR cycles were adjusted to have linear amplification for all the targets. Each RT-PCR reaction was repeated at least three times. A semi-quantitative analysis of mRNA levels was performed by the GEL DOC UV system (Bio-Rad, Hercules, CA).

When minimal differences in gene expression were detected, experiments were repeated using the real-time PCR approach. The real-time PCR assays were run on an Opticon 4 machine (MJ Research, Waltham, MA). Reactions were performed according to the manufacturer’s instructions by using the SYBR Green PCR master mix. Primer sequences are available on request.

**Western blotting and coimmunoprecipitation**

Cells were lysed in a buffer containing 0.1% Triton for 30 minutes at 4°C. The lysates were then centrifuged for 10 minutes at 10,000 g at 4°C. After centrifugation, 10-40 μg of each sample was loaded, electrophoresed in a polyacrylamide gel and electrotoblotted onto a nitrocellulose membrane. Primary antibodies to detect Brg1, pRb, p53, p21, p27 and tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibody to detect p53/p27/p130 was obtained from BD Biosciences (Bedford, MA). All the antibodies were used according to the manufacturers’ instructions.

Immunoreactive signals were detected with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz) and reacted with Supersignal WestPico or WestPico (Pierce, Rockford, IL).

For immunoprecipitation, cells were lysed in RIPA buffer (TBS+1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing protease inhibitors. Lysates were precleared for 30 minutes with mouse immunoglobulin G (IgG) and protein G plus protein A-agarose (Santa Cruz). Two micrograms of Rb- or p53-specific antibodies (BD Biosciences) or control IgG and protein G plus protein A-agarose (40 μl) were added to the precleared lysates and gently rotated overnight at 4°C. The immunoprecipitates were washed four times with lysis buffer and then were resuspended in a 2X Laemmli sample; western blots were performed as described above. Rb and p53 immunoprecipitates were used to detect Brg1.

**Senescence-associated β-galactosidase staining**

Cultured cells were washed twice with PBS and then fixed for 10 minutes with 2% formaldehyde/0.2% glutaraldehyde at room temperature. Cells were then washed twice with PBS and 1 to 2 ml of senescence-associated β-galactosidase staining solution per 35 mm dish was added. Cells were incubated at 37°C for several hours until blue staining was clearly detectable under a light microscope. The percentage of senescent cells was calculated by the number of β-galactosidase-positive cells out of at least 500 cells in different microscopic fields. Just before using the senescence-associated β-galactosidase staining solution, we added 20 ml of citric acid/sodium phosphate (pH 6) in a volume of 100 ml of 150 mM NaCl, 2 mM MgCl₂, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml X-Gal.)

**TRAP assay**

The TRAP assay was performed according to Kim and co-workers (Kim et al., 1994; Kim and Wu, 1997). Briefly, 5×10^6 cells were lysed in 10 mM Tris-HCl, pH 7.5, 2.5 mM MgCl₂, 1 mM EGTA, 0.5% CHAPS, 10% glycerol, 5 mM β-mercaptoethanol and 1 mM AEBSF for 30 minutes at 4°C. The lysates were then centrifuged for 10 minutes at 10,000 g at 4°C. After centrifugation, protein concentration was determined by the Bradford assay. The reaction mixture for the TRAP assay was done as follows: 1X Taq DNA polymerase buffer (Promega, Milan, Italy), 1.5 mM MgCl₂, 50 μM dNTPs, primer M2(TS) (5'-AACTCG-GTCGACGAGT'T3'), and TRAP internal control (5'-AATCCGTGGCG-CAGGTTAAAGCGCGGAGAGGGAAT3'). This mixture was incubated for 30 minutes at 25°C; we then added primer ACX (5'-GCGCGCCTTACCACT-ACC3'), primer NT (5'-ATCCGCTCTCGGCTT3') and 2.5 U Taq DNA polymerase (Promega). The reaction was diluted for 5 minutes at 94°C, then amplified for 30-35 cycles (94°C for 15 seconds, 60°C for 15 seconds, 72°C for 15 seconds). Primers ACM and M2(TS) amplify the telomerase products, whereas primers M2(TS) and NT amplify the TRAP internal control. PCR products were resolved on 20% polyacrylamide gels stained with Gelstar (Cambrex Bio Science, Copenhagen, Denmark).

**Micrococcal nuclease assay**

Cells were permeabilized with 0.1% L-lyso-phosphatidylcholine (Sigma-Aldrich) in 150 mM sucrose, 80 mM KCl, 35 mM HEPES, pH 7.4, 5 mM K2HPO4, 5 mM MgCl₂ and 0.5 mM CaCl₂ for 90 seconds, followed by digestion with 2 U/ml micrococcal nuclease (Sigma-Aldrich) in 20 mM sucrose, 50 mM Tris-HCl, pH 7.5, 50 mM NaCl and 2 mM CaCl₂ at room temperature for various times. Digested DNA was resolved by electrophoresis on a 1% agarose gel.

**Statistical analysis**

Statistical significance was evaluated using analysis of variance (ANOVA) followed by Student’s t-tests and Bonferroni’s tests.

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