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An Effective Method for Adenoviral-Mediated Delivery of Small Interfering RNA Into Mesenchymal Stem Cells

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Abstract Mesenchymal stem cells (MSCs) promise as a main actor of cell-based therapeutic strategies, due to their intrinsic ability to differentiate along different mesenchymal cell lineages, able to repair the diseased or injured tissue in which they are localized. The application of MSCs in therapies requires an in depth knowledge of their biology and of the molecular mechanisms leading to MSC multilineage differentiation. The knockdown of target genes through small interfering RNA (siRNA) carried by adenoviruses (Ad) represents a valid tool for the study of the role of specific molecules in cell biology. Unfortunately, MSCs are poorly transfected by conventional Ad serotype 5 (Ad5) vectors. We set up a method to obtain a very efficient transduction of rat MSCs with low doses of unmodified Ad5, carrying the siRNA targeted against the mRNA coding for Rb2/p130 (Ad-siRNA-Rb2), which plays a fundamental role in cell differentiation. This method allowed a 95% transduction rate of Ad-siRNA in MSC, along with a siRNA-mediated 85% decrease of Rb2/p130 mRNA and a 70% decrease of Rb2/p130 protein 48 h after transduction with 50 multiplicities of infection (MOIs) of Ad5. The effect on Rb2/p130 protein persisted 15 days after transduction. Finally, Ad-siRNA did not compromise the viability of transduced MSCs neither induced any cell cycle modification. The effective Ad-siRNA-Rb2 we constructed, together with the efficient method of delivery in MSCs we set up, will allow an in depth analysis of the role of Rb2/p130 in MSC biology and multilineage differentiation. J. Cell. Biochem. 100: 293–302, 2007.

Key words: small interfering RNA; adenoviral vector; Coxsackie B and adenovirus receptor; serotype 5; mesenchymal stem cells; Rb2/p130

Mesenchymal stem cells (MSCs) have been first identified in adult bone marrow [Friedenstein et al., 1976]. Subsequent studies demonstrated that MSCs are widely distributed in vivo since they have been isolated also from other tissues, including lung, adipose tissue, skeletal muscle, trabecular bone, synovium, and the human umbilical cord perivascular cells derived from the Wharton's jelly [Tuan et al., 2003; Noth et al., 2005; Sarugaser et al., 2005]. MSCs can be distinguished from hematopoietic stem cells (HSCs) on the basis of their cell surface antigens and can be separated from HSCs by their propensity to adhere to cell culture plastics. Studies demonstrated that MSCs extensively proliferate in vitro while preserving a normal karyotype and telomerase activity on several passages [Pittenger et al., 1999].

Bone marrow-derived MSCs can potentially differentiate along different mesenchymal lineages, including those forming bone, cartilage, fat, ligament/tendon, muscle, neurons, astrocytes, and bone marrow stroma that supports hematopoiesis [Gao et al., 2001]. MSC potentiality has been first described by Prockop in a study showing that transplanted marrow cells engraft non-hematopoietic connective tissues such as spleen and liver [Prockop, 1997, 1998].

Many studies have been focused so far on MSCs as they promise as a main actor of

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cell-based therapeutic strategies, due to their intrinsic ability to differentiate into functional cell types able to repair the diseased or injured tissue in which they are localized. This trend to adopt the local identity may be correlated to local cytokines and matrix factors, as well as to adequate contact with host cells.

Furthermore, this source of stem cells can be directly obtained from individual patients through a relatively simple procedure, a small aspirate of bone marrow, followed by MSC quick expansion in vitro, thus eliminating the immune rejection of allogenic tissues.

The application of MSCs in therapeutic strategies requires an in depth knowledge of their biology and of the molecular mechanisms leading to MSC multilineage differentiation.

The up- or downregulation of specific target genes in MSCs through a variety of molecular tools can provide useful information about the molecules involved in MSC cell fate decisions. RNA interference (RNAi) based on small interfering RNA (siRNA) is an anti-mRNA strategy that prevents its translation into protein [Fire et al., 1998]. The RNAi mechanism has been described for the first time in *Caenorhabditis elegans* more than 10 years ago [Fire et al., 1991] as a biological reaction to double-stranded RNA (dsRNA), which interferes with the expression of specific genes containing a complementary region to the delivered dsRNA. Since then, this mechanism has been detected in many eukaryotics, thus revealing that this peculiar kind of inhibition of gene expression has been highly preserved during evolution [Shi, 2003]. RNAi is a natural mechanism of protection against virus infection and transposon jumping, and, as happened in the past for antisense (AS) oligonucleotides (ODNs), it has been exploited by researchers to inhibit the expression of specific target genes. The RNAi is induced by long dsRNA, which are processed into 21–23 bp dsRNA molecules by the RNase III-like enzyme DICER. These siRNA are then incorporated into the RNA-induced silencing complex (RISC), a protein–RNA complex which identifies and induces the degradation of cognate mRNA, thus preventing its translation. A detailed description of the components of the RNA silencing machinery has been recently reported by Meister and Tuschl [2004]. Short hairpin RNA (shRNA), containing a stem and loop structure, can also be used to interfere with specific gene expression, presenting some advantages in comparison with siRNA, since shRNA can be expressed continuously to establish stable gene silencing, while siRNA elicit only a transient silencing response [Paddison et al., 2004].

The RNAi mechanism, induced both in vitro and in vivo through chemically synthesized siRNA or produced by DICER from exogenous, vector-delivered long dsRNA, is a rapid and efficient tool to modulate target gene expression in mammalian cells. In fact, the RNAi revealed to be less expensive and time consuming than other methods, such as gene knock-out. As a matter of fact, siRNA are currently used in a number of protocols for inhibition of target mRNA in functional studies, as well as in clinical applications [Forte et al., 2005; Li et al., 2005].

One of the most widely used tools to introduce exogenous DNA in cells are viral vectors. Among these, adenoviruses (Ads) are often used for cell transduction. In particular, most of the Ad vectors that are used to transduce MSCs are classified as Ad serotype 5 (Ad5). Unfortunately, MSCs express low levels of the Coxsackie B and adenovirus receptor (CAR), a 46 kDa membrane protein which represents the primary attachment receptor for species C Ad [Studeny et al., 2002; Hung et al., 2004]. Consequently, MSCs are poorly transduced with conventional Ad5. This inconvenience has been overcome with very high multiplicities of infection (MOIs) of Ad5 [Conget and Minguell, 2000] or with Ad5 vectors containing modification of their fiber proteins, able to render the Ad independent of CAR [Tsuda et al., 2003; Knaan-Shanzer et al., 2005].

The aim of our investigation was to develop an effective method to transduce rat MSCs with low doses of unmodified Ad5, since this vector is frequently applied in the laboratory practice. In particular, we used an Ad5 expressing the siRNA targeted against the endogenous Rb2/p130 gene (Ad-siRNA-Rb2), known to play a fundamental role in differentiation, apoptosis, and negative regulation of cell cycle in several systems, including MSCs [Jori et al., 2004, 2005]. Our results indicate a maximal 95% transduction rate of Ad-siRNA, along with a maximal 85% decrease of Rb2/p130 mRNA, and a 70% decrease of Rb2/p130 protein in MSCs transduced with 50 MOIs of Ad-siRNA-Rb2 in comparison to MSCs transduced with equal MOIs of control Ad-siRNA-Luc. The effect of

siRNA on Rb2/p130 protein persisted 15 days after transduction. Finally, Ad-siRNA did not compromise the viability of transduced MSCs neither induced any cell cycle modification.

METHODS

Animals and MSC Cultures

Mesenchymal stem cells have been harvested from the bone marrow of the femurs and tibias of adult Wistar Kyoto (WKY) rats. Animals were handled in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institute of Health (NIH publication No. 85-23, revised 1985). All protocols related to this research were approved by the Animal Care and Use Committee of the Second University of Naples. Rats were acclimatized and quarantined for at least 1 week before undergoing MSC harvesting, and were housed at constant temperature ($21 \pm 1^{\circ}C$) and relative humidity (60%) under a regular light/dark schedule (light 7.00 a.m. to 7.00 p.m.). Food and water were freely available.

Wistar Kyoto rats have been anesthetized with an intraperitoneal injection of ketamine hydrochloride (2 mg/100 g) and MSCs have been harvested from the bone marrow by inserting a 21-gauge needle into the shaft of the bone and flushing it with complete α-modified Eagle's medium (αMEM) containing 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin. Cells from one rat have been plated into two 100 mm dishes. After 24 h, non-adherent cells have been discarded, and adherent cells, representing the marrow stromal stem cells, have been washed twice with PBS. The cells have been then incubated for 5–7 days to reach confluence. Finally, MSCs have been extensively propagated for further experiments. All cell culture reagents have been obtained from Invitrogen Corporation (Carlsbad, CA).

siRNA Design and Synthesis

We designed three hairpin siRNA targeted against rat Rb2/p130 mRNA (siRNA-Rb2) using the design algorithm developed by Cenix Bioscience GmbH on the basis of the rules and criteria described by Reynolds et al. [2004]. The only effective siRNA we selected resulted to target the following nucleotides within the rat Rb2/p130 mRNA sequence (GenBank accession number NM_031094): 5′GGGAAATGATCTCCATTGG3′.

The two complementary hairpin siRNA template ODNs have been synthesized (MWG Biotech) as 55-mer and included the 19-mer stem with two nucleotides overhang and the loop sequence 5′TTCAAGAGA3′. The 5′ ends of the two ODNs form the *Xho*I and *Spe*I restriction site overhangs that facilitate the efficient directional cloning into the Shuttle Vector (see below).

The siRNA negative control (siRNA-Luc) has been as published by Harborth et al. [2001], and targets the firefly (*Photinus pyralis*) luciferase gene (GenBank accession number X65324). The negative control siRNA-Luc exhibits no target mRNA in the rat transcriptome, as verified by multiple comparisons with GenBank sequences.

Adenoviral Production

The recombinant Ad-siRNA-Rb2 and the control Ad-siRNA-Luc have been produced using the pSilencer adeno 1.0-CMV System (Ambion, Austin, TX) according to manufacturer's instructions. Briefly, the two pairs of complementary 55-mer ODNs, related to the Rb2/p130 and to the luciferase mRNAs described above, have been annealed to form the siRNA template inserts. Successively, the siRNA template inserts have been ligated into the linearized Shuttle Vector 1.0-CMV supplied with the kit. One Shot TOP10 chemically competent *E. coli* cells (Invitrogen Corporation) have been transformed with the ligation products and plated on medium containing ampicillin to select for transformants. Clones have been screened for the siRNA inserts by digestion with *Xho*I and *Spe*I (Roche, Mannheim, Germany). Positive clones have been then expanded for a large-scale plasmid preparation. The inserts have been sequenced using the primers provided by the kit to verify that the hairpin siRNA templates had the correct sequences.

The Shuttle Vectors 1.0-CMV containing the siRNA templates and the Adenoviral LacZ Backbone provided with the kit have been linearized by digestion with *Pac*I (Roche). The two plasmids have then been transfected using calcium chloride into the 293 primary embryonic kidney cells (HEK-293), a packaging cell line which supplies the E1 function in *trans*. Homologous recombination has occurred in the region of overlap between the plasmids, thereby

producing the recombinant Ad-siRNA-Rb2 and Ad-siRNA-Luc.

HEK-293 cells have been harvested 12 days after transfection, when at least 60% of the cells have become infected and detached from the culture plate. To obtain enough viruses for subsequent MSC transduction, recombinant Ad-siRNA have been expanded by the infection of HEK-293 cells and then purified and titrated through the tissue culture infectious dose 50 ($TCID_{50}$) method, based on the development of cytopathic effect in cells using end-point dilutions to estimate the titer.

Adenoviral Transduction

Mesenchymal stem cells (5×10^4) have been seeded at equal density in six multiwell plates. Twenty-four hours later, MSCs have been transduced with Ad-siRNA-Rb2 or Ad-siRNA-Luc at different MOIs in serum-free αMEM. After 4 h, the medium has been supplemented with 2% FBS. Eighteen hours after transduction, the reduced medium containing the virus has been removed and replaced by the normal growth αMEM with 10% FBS. Forty-eight hours after transduction, MSCs have been harvested and submitted to RNA and/or protein extraction, to the detection of β-galactosidase (β-gal) activity or to the cell death assay.

Cell Death Assay

Mesenchymal stem cells (3×10^3) have been seeded at equal density in 96 multiwell plates and transduced with 5, 10, 20, 50, 100, 250, and 500 MOIs of control Ad-siRNA-Luc. Forty-eight hours after transduction, cell death has been determined by measuring the metabolic activity of cellular enzymes released in culture medium through the CytoTox 96 Cell Death Assay (Promega, WI) according to manufacturer's instructions.

Determination of Percentage of Transduced Cells

Mesenchymal stem cell cultures have been transduced with 5, 10, 20, 50, 100, 250, and 500 MOIs of control Ad-siRNA-Luc. The percentage of transduced cells has been then calculated by detecting the β-gal activity of the LacZ reporter gene contained in the Adenoviral LacZ Backbone used for recombinant Ad-siRNA production, by an "in situ" detection β-gal staining kit (Roche) according to manufacturer's instructions.

RNA Extraction and Semi-Quantitative Reverse Transcriptase-PCR (RT-PCR)

Total RNA has been extracted from MSC cultures using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. RNA has been then digested with DNase I (Ambion).

Two hundred nanograms of total RNA has been used for reverse transcription. First strand cDNA has been made with AMV reverse transcriptase (Promega).

The OLIGO 4.05 software (W. Rychlik copyright, 1992, National Biosciences, Inc., Plymouth, MN) has designed primer pairs for semi-quantitative PCR amplification on the basis of rat gene sequences reported in the Genbank database. All primers contain 50–60% G/C. The optimal annealing temperature for all the primer pairs is 55°C. We have used the following primers: GAPDH-472-5′GCATCCTGCACCAACTG3′; GAPDH-799-5′GCCTGCTTCACCTTCTT3′; HPRT-348-5′GACCAGTCAACGGGGGACATAAAAGTTA3′; HPRT-697-5′ACATCAACAGGACTCTTGTAGATTCAAC3′; Rb2-121-5′CAGAGGAAGAGGAGGAGGAT3′; Rb2-495-5′TCGGAAGTGTGGGGGTAAAT3′; Rb2-3191-5′CGAGTACAGTTATCTCAAAGTCACCCTA3′: Rb2-3532-5′ATGCATTAAGTCCTGCCAAAGAGTTCAT3′.

The localization of primer pairs used for semi-quantitative RT-PCR, as well as their position with respect to the siRNA target sequence, is shown in Figure 1.

Detailed descriptions of RT-PCR amplifications and agarose gel electrophoresis have been published elsewhere [Galderisi et al., 1999].

Densitometric analysis of RT-PCR product levels has been assessed by the Molecular Analyst software associated with Gel Doc 1000 (Bio-Rad Company, CA). The density values of RT-PCR products have been normalized with respect to endogenous glyceraldehyde-3-phosphate dehydrogenase (gapdh) and hypoxantine-guanine phosphoribosyltransferase (hprt) product levels. Each RT-PCR has been repeated at least three times. The number of cycles varied according to the expression level of the target gene. An appropriate number of cycles have been determined to ensure that PCR was taking place in the linear range, in order to guarantee a proportional relationship between input RNA and densitometric readout.

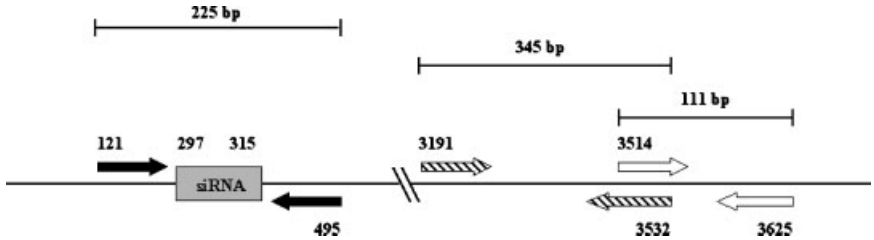

Fig. 1. Primer pairs and siRNA localization on Rb2/p130 cDNA sequence.

RT Real-Time PCR

The primer pairs for Real-Time PCR have been designed through the Primer Express software (Applied Biosystems). The optimal annealing temperature for all the primer pairs is 55°C. We have used the following primers: HPRT-592F-5′TTGGATATGCCCTTGACTATAATGAG3′; HPRT-592R-5′TTCAACTTGCCGCTGTCTTTT3′; Rb2-3514-5′TTGGCAGGACTTAATGCATGC3′: Rb2-3625-5′AGGCGTTTACTGCCACACTGA3′.

First strand cDNA has been made with an AMV reverse transcriptase (Promega). cDNA has been mixed with SYBR green (Sigma Aldrich, MO) and the Real-Time PCR has been performed by DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland) with an Opticon II PCR thermal cycler (Bio-Rad Company). Values of RT-PCR products have been normalized with respect to endogenous *hprt* product levels. As performed for semi-quantitative RT-PCR, each reaction has been repeated at least three times. After quantification, each reaction product has been also verified through agarose gel electrophoresis.

The localization of primer pairs used for RT Real-Time PCR, as well as their position with respect to the siRNA target sequence, is shown in Figure 1.

Western Blotting

Mesenchymal stem cells have been lysed in a buffer containing 50 mM Tris-HCl pH 7.4, 5 mM EDTA pH 8, 250 mM NaCl, 50 mM NaF, 0.1 mM Na_3VO_4 (all from Sigma-Aldrich), 0.1% Triton X-100 (Bio-Rad Company) for 30 min at 4°C. The lysates have been then centrifuged for 10 min at 10,000*g* at 4°C. After centrifugation, 20 μg of each sample has been loaded on 8% polyacrylamide gel and electroblotted onto a nitrocellulose membrane. Both the primary antibodies for detection of pRb2/p130 (BD Biosciences) and of alpha-tubulin (Sigma-Aldrich), which served as a control housekeeping gene, have been diluted 1:500 in 0.5% T-TBS containing 3% non-fat dry milk (Euroclone). All antibodies have been used according to manufacturers' instructions. Immunoreactive signals have been detected with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and reacted with ECL (Amersham Pharmacia Biotech, Uppsala, Sweden).

Flow Cytometry Analysis

For each assay 5×10^5 MSCs were collected and resuspended in 500 μl of a hypotonic buffer (0.1% Triton X-100, 0.1% sodium citrate and 50 μg/ml propidium iodide, RNAse A). Cells were incubated in the dark for 30 min and then analyzed. Samples were acquired on a FACSCalibur flow cytometer using the Cell Quest software (Becton Dickinson, NJ) and analyzed with standard procedure using the Cell Quest software and the ModFitLT software version 3 (Becton Dickinson).

Statistical Analysis

Statistical significance has been evaluated using ANOVA followed by Student's t and Bonferroni's tests. $P < 0.05$ has been considered significant.

RESULTS

Ad-siRNA Effectively Transduce MSCs Without Cytotoxic Effect

In order to identify the lowest MOIs of Ad-siRNA necessary to obtain a satisfying transduction rate, MSCs were transduced with 5, 10, 20, 50, 250, and 500 MOIs of Ad-siRNA, respectively. Figure 2 shows representative MSCs transduced with Ad-siRNA-Luc. It is worthwhile observing that β-gal activity is localized in MSC nucleus. Manufacturer's proprietary

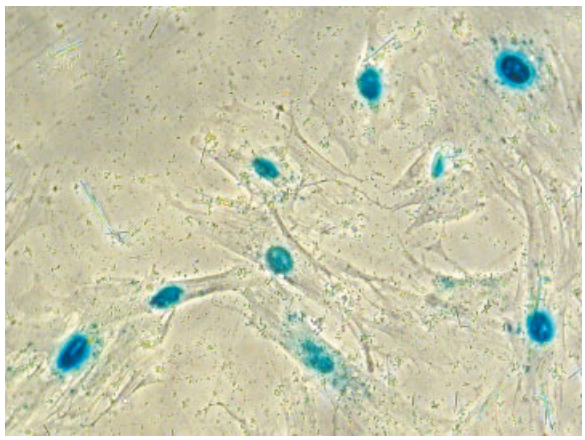

Fig. 2. Representative photomicrograph of MSCs transduced with Ad-siRNA-Luc and positive to β-gal assay. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

information does not allow to know if the gene coding for β-gal inserted in one of the two plasmids provided by the kit we used for Ad production contains a nuclear localization signal (NLS), as suggested by our observation.

We obtained a transduction rate of 80% with 20 MOIs of Ad-siRNA, of 93% with 50 MOIs of Ad-siRNA, and a maximal transduction rate of 95% using 100 MOIs of Ad-siRNA (Fig. 3). The cytotoxic effect, as determined by the CytoTox cell death assay, was maximal using 250 and 500 MOIs of Ad-siRNA for cell transduction, resulting in 20% and 24% of cell death, respectively (Fig. 3).

Since the CytoTox cell death assay is based on the measurement of the metabolic activity of cellular enzymes released in culture medium and consequently it does not allow to distinguish between cell apoptosis and necrosis, we further investigated by flow cytometry analysis the specific effect of Ad-siRNA on MSC apoptosis. MSCs were transduced with 100, 400, and 1,600 MOIs of control Ad-siRNA-Luc and submitted to flow cytometry assay 48 h later. No significant difference in the percentage of apoptotic cells, distinguishable by a discrete subdiploid DNA content, was detectable between control MSCs and Ad-siRNA-transduced MSCs (from a minimum of 0.1% to a maximum of 0.7% in all experiments).

Finally, no modification of cell cycle was detectable by flow cytometry analysis in MSCs transduced with up to 1,600 MOIs of Ad-siRNA-Luc, since the percentages of live cells in G_1 (from a minimum of 55% to a maximum of 58%), S (from a minimum of 41% to a maximum of 44%), and G_2/M (from zero to a maximum of 0.5%) phases of cell cycle were comparable to those obtained in control untransduced MSCs.

Ad-siRNA-Rb2 Reduces Rb2/p130 Expression

Mesenchymal stem cells transduced with 50 MOIs of Ad-siRNA were tested for Rb2/p130 knockdown through RT-PCR on total RNA extracted from MSCs 48 h after Ad-siRNA transduction and through Western Blot on protein lysates extracted from MSCs 48 h and 15 days after Ad-siRNA transduction.

Levels of Rb2 mRNA in MSCs treated with Ad-siRNA-Rb2 were compared to mRNA levels in MSCs treated with control Ad-siRNA-Luc through both semi-quantitative RT-PCR and RT Real-Time PCR.

Results of semi-quantitative RT-PCR were confirmed using two distinct pairs of primers, hybridizing to different regions of Rb2/p130 cDNA. In particular, the Rb2 primers 122–495

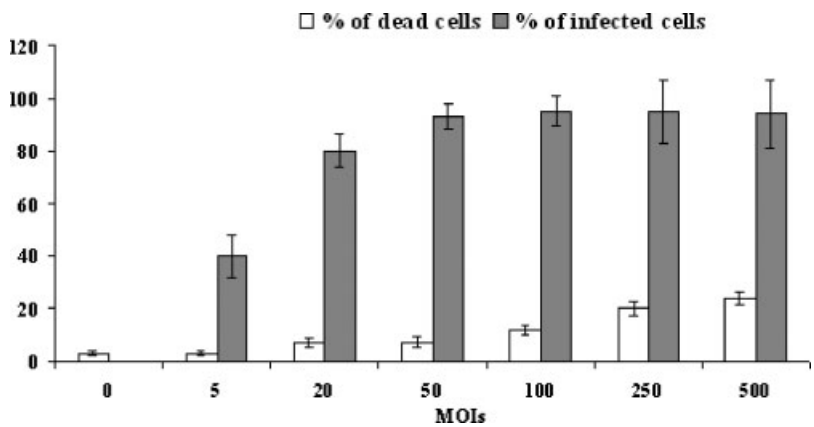

Fig. 3. Transduction rate and cytotoxic effect of Ad-siRNA-Luc on MSC.

were localized upper and lower with respect to the siRNA-Rb2 target sequence, while the Rb2 primers 3,191–3,532 were both localized lower with respect to the siRNA-Rb2 target sequence (Fig. 1). Furthermore, Rb2/p130 cDNA levels were normalized with respect to two different housekeeping genes (GAPDH and HPRT). The histogram in Figure 4A shows a maximal 85% decrease of Rb2/p130 mRNA detected by semi-quantitative RT-PCR using the Rb2/p130 primer pair 122–495, and a 63% decrease using the primer pair 3,191–3,532. The normalization of Rb2 cDNA levels with respect to GAPDH or HPRT resulted in similar values, indicating that MSC treatment with the Ad-siRNA did not affect the expression of these housekeeping genes.

An 85% decrease of Rb2/p130 mRNA was detected also by RT Real-Time PCR.

A comparable successful decrease of target mRNA was obtained in MSCs using conventional Ad5 vectors expressing siRNA against the Methyl-CpG-binding protein 2 (MeCP2) mRNA (data not shown).

The effect of Ad-siRNA-Rb2 was further verified by analyzing the protein levels of Rb2/p130 through Western Blotting. Results were normalized with respect to the protein expressed by the housekeeping gene alpha-tubulin. We detected a 70% decrease of Rb2/p130 protein 48 h after MSCs transduction with Ad-siRNA-Rb2 in comparison to MSC transduced with control Ad-siRNA-Luc. Such decrease of Rb2/p130 protein persisted 15 days after MSC transduction (Fig. 5).

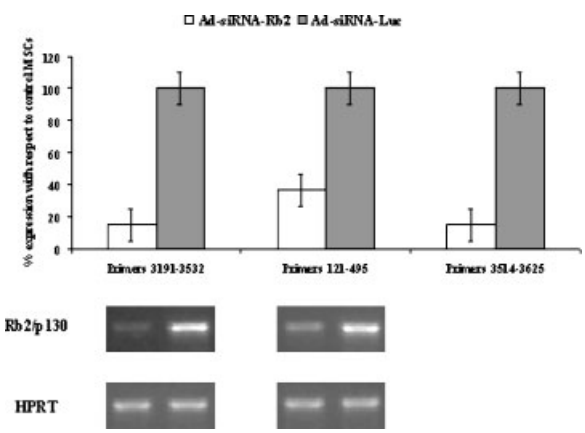

Fig. 4. Semi-quantitative RT-PCR and RT Real-Time PCR analysis of Rb2/p130 mRNA on total RNA extracted from MSCs harvested 48 h after Ad-siRNA transduction.

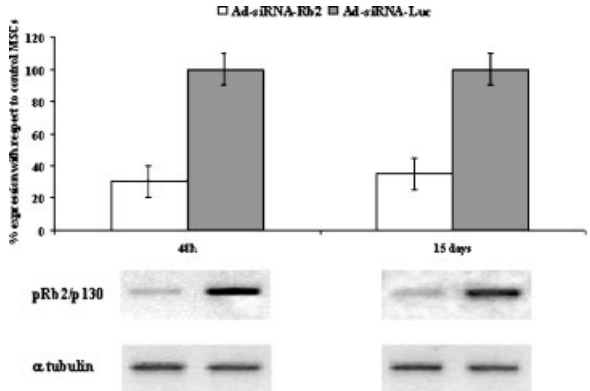

Fig. 5. Western Blot analysis of pRb2/p130 in MSCs harvested 48 h and 15 days after Ad-siRNA transduction.

DISCUSSION

Recombinant Ad continues to be one of the mostly used vectors for delivery of exogenous DNA aimed at the basic study of gene function and for gene therapy strategies. This is mainly due to the fact that they are relatively easy to construct and can be produced at high titers. Moreover, Ad vectors usually have high transduction efficiencies. Unfortunately, MSCs are poorly transfected by conventional Ad of serotype 5. Some groups reported a successful transduction of MSCs using high titers of conventional Ad5 vector (up to 2,000 MOIs) [Conget and Minguell, 2000; Olmsted-Davis et al., 2002; Toma et al., 2002]. Nevertheless, it should be considered that the transduction of cells with high quantities of replication-defective adenoviral recombinants is affected by a number of cell alterations, including cytotoxicity, both in vitro and in vivo. In particular, a global decrease of protein synthesis has been observed in many cell types by transduction with a number of different viral vectors, including Ad [Bello and Ginsberg, 1967; Beltz and Flint, 1979; Easton et al., 1998]. Moreover, cellular stress response following transduction with high titers of recombinant Ad has been detected by measuring the levels of phosphorylated c-Jun proteins in transduced neurons [Easton et al., 1998]. The same authors also revealed that the transduction of neurons with Ad vectors resulted in perturbations in cellular gene expression, for example, the induction of c-myb and cyclin D1 expression through the transcription factor E2F, which is in turn activated by the interaction with the adenoviral gene product E4orf6/7. Finally, high titers of

recombinant Ad were found to be directly cytotoxic on neurons because of the release of capsid proteins that occurs during uncoating of the virus [Caillaud et al., 1993].

This short overview clearly indicates that the application of Ad vectors in the laboratory practice, and in particular in the transduction of MSCs, is useful but requires the application of new strategies or the improvement of existing methods able to minimize undesirable cell alterations by using low MOIs of Ad vectors.

Some groups tested alternative non-viral methods of genetic modification of MSCs, such as electroporation [Peister et al., 2004] and liposomal-based transfection [Hoelters et al., 2005].

Other groups developed fiber-modified Ad vectors to increase the transduction efficiency of MSCs and of other cell types with a reduced expression of CAR. In this context, Mizuguchi et al. [2005] optimized the transduction to MSCs by three Ad vectors containing different modifications of the fiber knob, obtaining the transduction of all the cultured MSCs using 1,000 vector particles (VP)/cell of the modified Ad vectors. Similar results were obtained by Jacob et al. [2004], who evaluated the efficacy of an Ad vector containing an integrin-binding motif (Arg-Gly-Asp sequence) in the H1 loop of the fiber in various cancer cell lines and in MSCs. They showed that transduction of MSCs with 500–5,000 MOIs resulted in an appreciable expression of the green fluorescent protein (GFP) coded by the insert of the Ad vector. Knaan-Shanzer et al. [2005] used Ad5 vectors displaying fiber shaft and knob domains of Ad serotypes 50, 35, and 16. After transduction with 100 MOIs of modified Ad5, 80–100% of MSCs expressed GFP. Only 25% of MSCs expressed GFP when transduced with 100 MOIs of conventional Ad5.

We modified the standard protocol for cell transduction and obtained a very efficient transduction of rat MSCs with only 50 MOIs of conventional Ad5. Thus, our method revealed to be even more efficient than the above-mentioned investigations based on transduction with fiber-modified Ad, since they used from a minimum of 100 MOIs [Knaan-Shanzer et al., 2005] to a maximum of 5,000 MOIs of Ad vectors [Jacob et al., 2004] to obtain a satisfying transduction rate of MSCs.

Our results are in agreement with Hung et al. [2004] and with Conget and Minguell [2000], demonstrating that undifferentiated MSCs express CAR and consequently can be transduced by conventional Ad5 vectors. Nevertheless, we were able to effectively transduce 93% of MSCs using 50 MOIs of Ad, versus the 19% of transduction rate obtained by Conget and Minguell with 2,000 MOIs and the 90% of transduction rate reported by Hung et al. using 1,000 MOIs of Ad5.

It should be also mentioned that our method allowed the siRNA-mediated inhibition of Rb2/p130 gene expression without compromising the viability of the cells. In particular, 50 MOIs of Ad-siRNA-Rb2 resulted to be the optimal amount of virus, providing the highest level of cell transduction ($93 \pm 5\%$, Fig. 3) and of gene silencing (85% decrease of target mRNA and 70% decrease of protein, Figs. 4, 5) with the least cellular toxicity, as determined by CytoTox cell death assay ($7 \pm 2\%$, Fig. 3). We also aimed to dissect MSC necrosis from apoptosis by further analyzing transduced MSC by flow cytometry, since in this kind of analysis cell necrosis results in a smear on the left of the G_1 peak in the single-parameter DNA histogram corresponding to an heterogeneous subdiploid DNA content, while the hallmark of cell apoptosis is discrete peak on the left of the G_1 peak in the DNA histogram. Data revealed that MSC transduction with up to 1,600 MOIs of Ad-siRNA-Luc following our method did not induce any significant change in the percentage of apoptotic cells in comparison to control untransduced MSCs.

Flow cytometry analysis also revealed that MSC transduction with up to 1,600 MOIs of Ad-siRNA-Luc following our method did not induce any modification in cell cycle phases with respect to control MSCs. These data are in contrast with other studies [Wersto et al., 1998] observing in epithelial cell lines that the infection with Ad5 vectors caused a dose-dependent perturbation of normal cell cycle progression, with an increase in the number of cells arrested in G_2-M, associated with an inappropriate expression and increase in cyclins A, B1, and D, as well as in cyclin-dependent kinase $p34^{cdc2}$.

The differences in Rb2/p130 mRNA decrease we detected using two different primer pairs (85% vs. 63%) are probably related to the fact that the primer position with respect to the siRNA target sequence on mRNA can affect the RT-PCR detection of gene knockdown via RNAi.

Rb2/p130 has been selected as a target of siRNA as it plays a fundamental role in cell

differentiation. In more detail, this protein has been demonstrated to block the transcription of the E2F1 nuclear factor [Johnson, 1995], thus inhibiting the expression of a number of genes involved in DNA synthesis and cell cycle progression [Claudio et al., 1996; Stiegler et al., 1998]. The dramatic decrease of Rb2/p130 target mRNA observed in Ad-siRNA-Rb2 transduced MSCs is probably due to the high transduction efficiency we obtained and to the good design of siRNA sequence, which contributed to the almost complete degradation of target mRNA. In fact, the siRNA sequence was featured according to the eight criteria identified by the detailed analysis done by Reynolds et al. [2004]. It should also be mentioned that the hairpin siRNA template cloned in the shuttle vector was under the control of a modified Cytomegalovirus (CMV) promoter, recognized by a RNA polymerase II. This characteristic is alternative to many commonly used systems used for expressing siRNA in cells under the control of U6 or H1 RNA polymerase III. A recent study [Xia et al., 2002] demonstrated that the RNA polymerase II promoters are able to express very high levels of functional siRNA in cells. Furthermore, the CMV promoter has the advantage of being highly active in a broad range of cell types, without interfering with other transcription events.

The effective Ad-siRNA-Rb2 we constructed, together with the efficient method of delivery in MSCs we set up, will allow an in depth analysis of the role of Rb2/p130 in MSC biology and multilineage differentiation.

The gene or siRNA delivery in MSCs through the method we described could also further stimulate the use of MSCs as a tool for autologous stem cell-based therapies.

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