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The Effect of Telomerase Template Antagonist GRN163L on Bone-Marrow-Derived Rat Mesenchymal Stem Cells is Reversible and Associated with Altered Expression of Cyclin d1, cdk4 and cdk6

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Abstract Telomerase activity is essential for the continued growth and survival of malignant cells, therefore inhibition of this activity presents an attractive target for anti-cancer therapy. The telomerase inhibitor GRN163L, was shown to inhibit the growth of cancer cells both in vitro and in vivo. Mesenchymal stem cells (MSCs) also show telomerase activity in maintaining their self-renewal; therefore the effects of telomerase inhibitors on MSCs may be an issue of concern. MSCs are multipotent cells and are important for the homeostasis of the organism. In this study, we sought to demonstrate in vitro effects of GRN163L on rat MSCs. When MSCs were treated with 1 μM GRN163L, their phenotype changed from spindle-shaped cells to rounded ones and detached from the plate surface, similar to cancer cells. Quantitative-RT-PCR and immunoblotting results revealed that GRN163L holds MSCs at the G1 state of the cell cycle, with a drastic decrease in mRNA and protein levels of cyclin D1 and its cdk counterparts, cdk4 and cdk6. This effect was not observed when MSCs were treated with a mismatch control oligonucleotide. One week after GRN163L was removed, mRNA and protein expressions of the genes, as well as the phenotype of MSCs returned to those of untreated cells. Therefore, we concluded that GRN163L does not interfere with the self-renewal and differentiation of MSCs under short term in vitro culture conditions. Our study provides additional support for treating cancers by administrating GRN163L without depleting the body's stem cell pools.

Keywords Mesenchymal stem cells · Telomerase · Cyclin D1 · cdk4 · cdk6 · GRN163L

SM Grazynov is an employee of Geron Corporation and performed many studies to show the use of telomerase inhibitors in different type cancers. He received equity interest, patent rights, or corporate affiliations, including consultantships, for GRN163L.

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Introduction

Telomerase is a ribonucleoprotein complex consisting of a reverse transcriptase catalytic subunit (hTERT) and an RNA moiety (hTR or hTERC). The template region of hTR is complementary to telomeric DNA and together with hTERT elongates telomeres by adding TTAGGG repeats to the end of the chromosome. This leads to stabilization of telomere lengths, prevention of t-loop and overhang loss, and continued cell division [1, 2]. Telomerase activity is reduced or absent in majority of normal tissues, which leads to a progressive shortening of the telomere with each cell division. In contrast, tumor cells and self-renewable tissues express telomerase to sustain their growth. The presence of this activity in many different kinds of human tumors, but

not in normal somatic cells, makes inhibition of telomerase activity an attractive tool on targeted cancer therapy [3–5].

Experimental studies based on anti-telomerase cancer therapies, such as immunotherapy, gene therapy, small molecule inhibitors and oligonucleotide-based therapeutics [3–5] have been making progress in recent years. A novel human telomerase RNA (hTR) antagonist GRN163L (Imetelstat) is a lipid-modified N3′→P5′ thio-phosphoramidate that is complementary to the template region of hTR, preventing it from binding to the telomeric repeats. The GRN163L sequence (5′-Palm-TAGGGTTAGACAA-3′) is apparently unique in the human transcriptome, and shows greatly enhanced stability as well as specific and high-affinity binding to telomerase. It has been shown that GRN163L causes telomerase inhibition and subsequent telomere shortening in many cell types, including in breast cancer, lung cancer, multiple myeloma and non-Hodgkin's lymphoma cell lines [6–8]. GRN163L has recently entered into Phase I/II clinical trials in patients with chronic lymphocytic leukaemia and some solid tumors such as in lung and breast cancer. Despite being a promising therapeutic agent, inhibition of telomerase activity may lead to side effects since germline cells, proliferating stem and progenitor cells also exhibit telomerase activity. Thus inhibition of telomerase could potentially cause a decline or defect in regenerative capacity and organ homeostasis [9].

Mesenchymal stem cells (MSCs) are multipotent cells capable of self-renewal and differentiating into multiple lineages such as osteocytes, adipocytes, chondrocytes, myoblasts, cardiomyocytes [10–12]. MSCs are also promising agents for cell-based therapies due to their ability to migrate in vivo to promote regeneration of damaged tissue, treat inflammation, and promote angiogenesis without inducing immune reaction in addition to the lack of ethical concerns [13, 14]. Additionally, their high self-renewal potential makes them strong candidates for stem cell gene therapy treating acquired and inherited disorders and restoring organ system functions, since tissue repair and regeneration is made possible by the presence of adult stem cells [15]. For the above reasons, the effects of telomerase-targeting therapies on MSCs must be well defined. The present study was undertaken to investigate the in vitro effects of GRN163L on the self-renewal and differentiation processes of MSCs.

Materials and Methods

Isolation and Culture of MSCs

MSCs were obtained from female, 9-week-old, 280–300 g Sprague–Dawley rats. After the rats were sacrificed by cervical dislocation, bone marrow a heterogeneous cell population was collected from the femurs and tibias by flushing with a 5 mL syringe containing 10% FBS (HyClone, Logan, USA) in DMEM (HyClone). The cells were cultured in plastic cell-culture dishes with MesenCult medium (StemCell Technologies, Vancouver, Canada) with a 20% supplement (StemCell Technologies) and a 1% penicillin–streptomycin solution (HyClone) in a 5% CO2 incubator at 37°C. The next day, the media of the tissue culture plates were changed and the nonadherent cells were removed. The media of the cells were changed every 3 days, after washing with sterile 1x PBS. Our experimental study protocol was approved by the Animal Ethics Committee of Bilkent University (BILHADYEK). All the animals received care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science of the USA, and this study protocol complied with Bilkent University's guidelines on the humane care and use of laboratory animals.

Experimental Groups

After 14 days of culture, the cells were trypsinized (HyClone) and transferred to culture dishes for RNA and protein isolation as well as for differentiation and telomerase activity assays. One day after the transfer, GRN163L (Geron Co., CA, USA) and its mismatch control oligo were added at a concentration of 1 μM to the cell plate. The media were changed every 3 days with the fresh GRN163L (hereafter stated as 163 L group) and mismatch control oligonucleotide (hereafter stated as mismatch group) together with control MSCs with no treatment (hereafter stated as control group). 163 L and mismatch cultures continued for 1 week. Finally, MSCs were left for recovery for 1 week after GRN163L was removed (hereafter stated as 163LR (recovery) group).

Total RNA Isolation and Reverse Transcription

MSCs were trypsinized and the total cellular RNA was isolated from the precipitate by using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, with additional DNase treatment. The cDNAs were synthesized from the total RNA samples with the DyNAmo cDNA synthesis kit (Finnzymes, Espoo, Finland) according to the manufacturer's protocol.

RT-PCR

cDNA amplification for *CD90, CD71, CD45, CD34, CD29* and *cyclophilin* were performed by using DyNAzyme II (Finnzymes). The primers are listed in Table 1. The initial

Table 1 Primer sequences for Q-RT-PCR and RT-PCR and product size

Gene	Sequence	Product size (bp)
TERT	(F) 5′-GCTACGCCATCCTGAAGGTC-3′ (R) 5′-GGTAGCAGAGCCAACGTGTG-3′	99
CYCLIN B1	(F) 5′-TCGATGTGGAGCAGCATACT-3′ (R) 5′-GTCCATTCACCGTTGTCAAG-3′	142
CDK4	(F) 5′-GAAGACGACTGGCCTCGAGA-3′ (R) 5′-ACTGCGCTCCAGATTCCTCC-3′	109
CDK6	(F) 5′-TTGTGACAGACATCGACGAG-3′ (R) 5′-GACAGGTGAGAATGCAGGTT-3′	151
CDK2	(F) 5′-TGACCAACTCTTCCGGATCT-3′ (R) 5′-ATAACAAGCTCCGTCCGTCT-3′	164
CDK1	(F) 5′-GTTGACATCTGGAGCATAGG-3′ (R) 5′-CTCTACTTCTGGCCACACTT-3′	144
CYCLIN A2	(F) 5′-CTGCCTTCCACTTAGCTCTC-3′ (R) 5′-GAGGTAGGTCTGGTGAAGGT-3′	125
RB	(F) 5′-TTGAAAGAAGAGGCACTCCC-3′ (R) 5′-CGCTACCTTAAATACCGCCT-3′	116
P53	(F) 5′-GTTCCGAGAGCTGAATGAGG-3′ (R) 5′-AGACTGGCCCTTCTTGGTCT-3′	109
Bmi-1	(F) 5′-ATGTGTGTCCTGTGTGGAGG-3′ (R) 5′-AGCCATTGGCAGCATCTGCT-3′	281
P21	(F) 5′-ATGTCCGATCCTGGTGATGTC-3′ (R) 5′-CGGCTCAACTGCTCACTGTC-3′	92
Cdc25a	(F) 5′-AGTGAAGAGAGCAGACCGAT-3′ (R) 5′-GGAGAGAGACTGGTGTGGAA-3′	126
P16INK4a	(F) 5′-CTTCACCAAACGCCCCGAACA-3′ (R) 5′-CGGGAGAGGGTGGTGGGGTC-3′	132
P19ARF	(F) 5′-GCAGAGCATGGGTCGCAGGTTC-3′ (R)5′-CGGGAGAGGGTGGTGGGGTC-3′	295
CYCLIN D1	(F) 5′-AATGCCAGAGGCGGATGAGA-3′ (R) 5′-GCTTGTGCGGTAGCAGGAGA-3′	189
CYCLIN E	(F) 5′-TTACTGATGGTGCTTGCTCC-3′ (R) 5′-GTCGTTGACGTAGGCCACTT-3′	136
Cyclophilin	(F) 5′-GGGAAGGTGAAAGAAGGCAT-3′ (R) 5′-GAGAGCAGAGATTACAGGGT-3′	211
CD 90	(F) 5′-CCAGTCATCAGCATCACTCT-3′ (R) 5′-AGCTTGTCTCTGATCACATT-3′	374
CD 34	(F) 5′-TGTCTGCTCCTTGAATCT-3′ (R) 5′-CCTGTGGGACTCCAACT-3′	281
CD 71	(F) 5′-ATGGTTCGTACAGCAGCAGA-3′ (R) 5′-CGAGCAGAATACAGCCATTG-3′	182
CD 29	(F) 5′-ACTTCAGACTTCCGCATTGG-3′ (R) 5′-GCTGCTGACCAACAAGTTCA-3′	190
CD 45	(F) 5′-ATGTTATTGGGAGGGTGCAA-3′ (R) 5′-AAAATGTAACGCGCTTCAGG-3′	175

denaturation step was at 95°C for 5 min, followed by 30 (for *CD90* and *CD34*), 35 (for *CD 71*), 26 (for *CD29* and *CD45*) and 23 (for *cyclophilin*) cycles of denaturation for 30 s for all genes at 94°C, annealing for 30 s at 55°C (for *CD90, CD34* and *cyclophilin*), 60 s at 66°C (for *CD71*) and 30 s at 60°C (for *CD29* and *CD45*), followed by extension for 30 s (for *CD90, CD34, CD29, CD45* and *cyclophilin*) and 45 s (for *CD71*) at 72°C. A final extension at 72°C for 5 min was applied to all the reactions.

Q-RT-PCR

The primers used for Q- RT-PCR are shown in Table 1. Before performing Q- RT-PCR reactions for experimental samples, the amplification efficiencies of all primers were calculated using a standard dilution series. Fold changes in the expression of the genes were estimated based on the comparative $Ct\left(2^{-\Delta\Delta Ct}\right)$ method, using the normal MSCs as calibrator. The Q- RT-PCR conditions for all

investigated genes have an initial denaturation 95°C, 10 min followed by 45 cycles (for all target genes) and 35 cycles (for housekeeping *cyclophilin*) of denaturation for 30 s at 94°C, annealing for 30 s at 60°C (for *TERT, cdk4, cyclin A* and *Bmi-1*), at 55°C (for *cyclin B1, cdk6, cdk2, cdk1, RB, p21* and *cyclophilin*), at 65°C (for *cyclin D1* and *p53*), at 59°C (for *cdc25A* and *cyclin E*), at 62°C (for $p16^{INK4a}$), at 68°C (for $p19^{ARF}$), followed by extension of 30 s at 72°C. Final extension at 72°C for 5 min was applied to all the reactions.

Protein Isolation and Quantification

MSCs were scraped from the cell culture plates in 1x PBS and the precipitate was treated with a lysis buffer containing 0.05 M Tris HCl, 1x protease inhibitor, 0.25 M NaCl and 1% (v/v) IGEPAL for 30 min on ice. Then the lysate was centrifugated for 20 min at 13,000 rpm at 4°C. Protein concentrations of supernatants were determined with Bradford protein assay as described [16].

Western Blotting

The proteins were separated on 8% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with blocking solution for 1 h at room temperature and was incubated in antibody solution at the concentration of 1:200 (for Bmi-1, TERT, Cdc25a, Cyclin A, Cdk4, Cyclin D1, Cyclin E), 1:1000 (for Cdk1, Cdk2, Cyclin B1, p53), 1:2000 (for Cdk6, α-Tubulin) for o/n at 4°C. Then horseradish peroxidase-linked secondary antibodies were applied for 1 h in blocking solution. Finally, Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, IL, USA) was applied to the membrane for 5 min and placed in an X-ray film cassette and developed. Anti-Bmi-1, TERT, Cdc25a, Cyclin A, Cdk4, Cyclin D1, and Cyclin E antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-Cdk1, Cdk2, Cyclin B1 and p53 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti- α-Tubulin antibody was purchased from Calbiochem (Darmstadt, Germany).

Estimation of Telomerase Activity Using Telomeric Repeat Amplification Protocol Assay (TRAP)

1×10^6 MSCs were collected by trypsinization and a telomerase assay was performed by using TRAP-eze Telomerase Detection Kit (Millipore, MA, USA). A gel-based assay was performed for visualization of the telomeric repeats after PCR, according to the manufacturer's protocol. Gels were stained with SYBR Green I (Sigma, MO, USA) at 1:10000 dilution in TAE and visualized with Vilber Lourmat ChemiCapture (Marne-La-Vallée Cedex, France).

Adipogenic Differentiation

An adipogenic induction medium was prepared by freshly adding 1 μM dexamethasone (Sigma), 10 μg/ml insulin (Sigma), 100 μM indomethacin (Sigma) and 0.5 mM IBMX to LG-DMEM (HyClone), containing 1x penicillin-streptomycin (HyClone) and 10% FBS (HyClone). The MSCs were cultured for 21 days, changing the medium every 2 days with freshly prepared medium. After 21 days the cells were fixed with 4% paraformaldehyde and stained with Oil Red O (Sigma).

Osteogenic Differentiation

An osteogenic induction medium was prepared by freshly adding 0.1 μM dexamethasone (Sigma), 0.2 mM ascorbic acid 2-phosphate (Sigma) and 10 mM glycerol-2-phosphate (Sigma) to LG-DMEM (HyClone), containing 1x penicillin-streptomycin (HyClone) and 10% FBS (HyClone). The MSCs were cultured for 21 days, changing the medium every 2 days for freshly prepared medium. After 21 days the cells were fixed with 70% ethanol and stained with Alizarin Red S (Sigma).

Statistical Analysis

All data are expressed as means ± SD. Data were analyzed by performing a paired *t*- test using Minitab Statistical Software ® (State College, Pennsylvania, USA). A value of $P< 0.05$ was considered to be statistically significant.

Results

We first investigated the phenotypic effect of GRN163L on MSCs (Fig. 1). Untreated control MSCs (Fig. 1a) and mismatch oligonucleotide treated MSCs (Fig. 1b) showed normal phenotypes with fibroblastic morphology and spindle-shaped cells. At the same time, when MSCs were plated in the presence of single dose of 1 μM GRN163L (163L), we observed phenotypic effects as early as 3 days after the treatment, such as the cells losing their fibroblastic shapes, rounding up, and appearing to detach from the plate surface (Fig. 1c). However, when the drug was removed and the cells were given time to recover (163LR), GRN163L-treated MSCs reattached to the plate surface and regained their normal phenotype within 1 week (Fig. 1d). By using RT-PCR, we showed that all of the experimental groups were positive for mesenchymal stem cell markers (*CD29, CD71* and *CD90*) and negative for

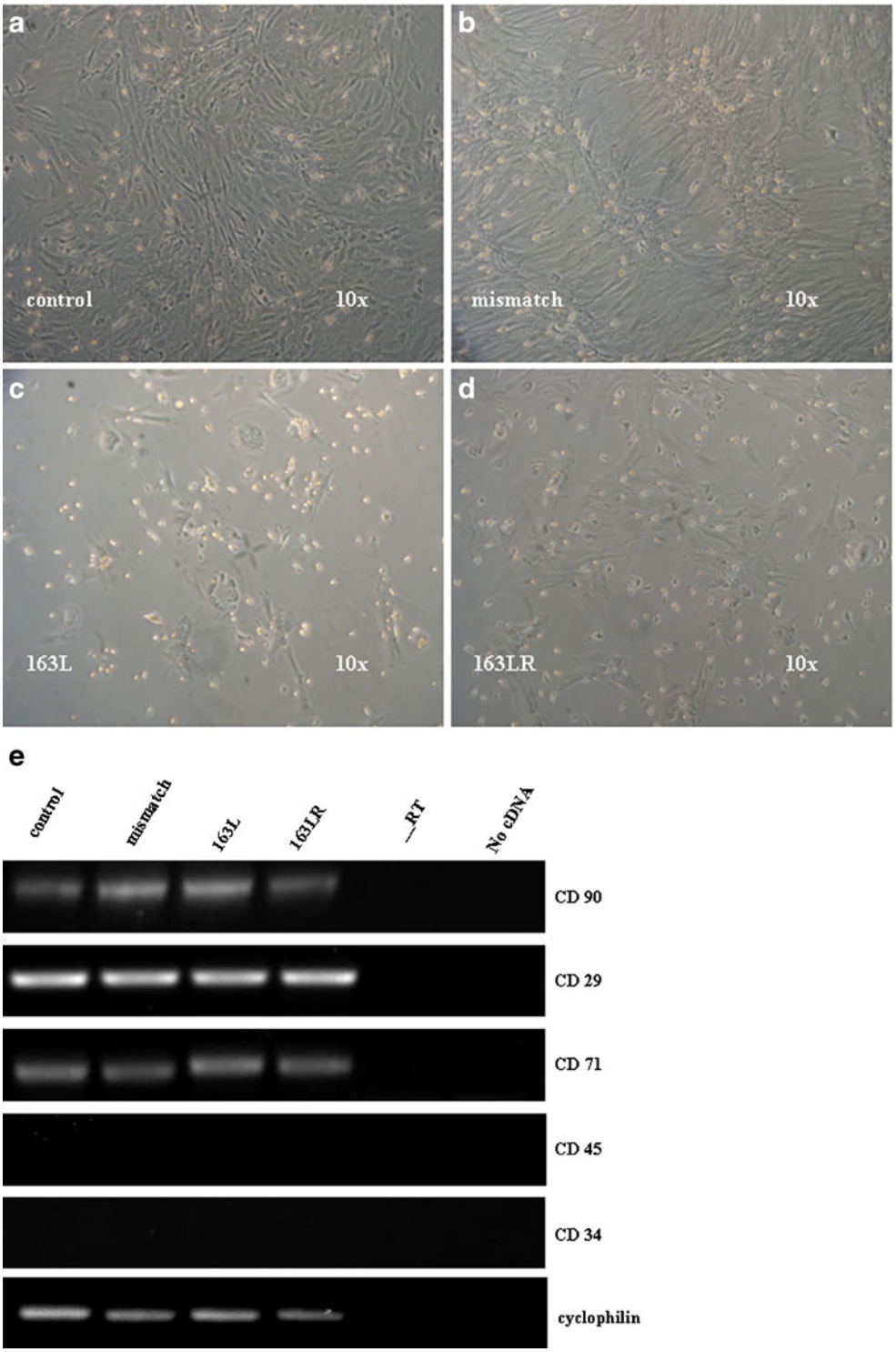

Fig. 1 The effect of GRN163L a Untreated control MSCs (*control*); **b** Mismatch oligonucleotide treated MSCs (*mismatch*); **c** 1 μM GRN163L treated MSCs (*163L*); **d** MSCs that were treated, and then left for 1 week without the ligand (*163LR*); **e** The expression of the markers of MSCs (CD90, CD29 and CD71) and hematopoietic cells (CD34, CD45)

hematopoietic cell markers (*CD34, CD45*) (Fig. 1e). We also examined telomerase activity of MSCs from control, 163L, mismatch and 163LR groups by the TRAP assay (Fig. 2). The telomerase activity of the MSCs in the 163L group was completely inhibited, whereas inhibition was not observed in the mismatched, 163LR or control MSCs. The effect of GRN163L on telomerase activity was reversible because the telomeric repeats were also observed in the 163LR group with a similar pattern as in the untreated control MSCs (Fig. 2a). No telomeric repeats were present when cell extracts were heat inactivated (Fig. 2b).

To illustrate if after the removal of the ligand whether MSCs regain their differentiation properties, we induced lipidogenic (Fig. 3) and osteogenic (Fig. 4) differentiation

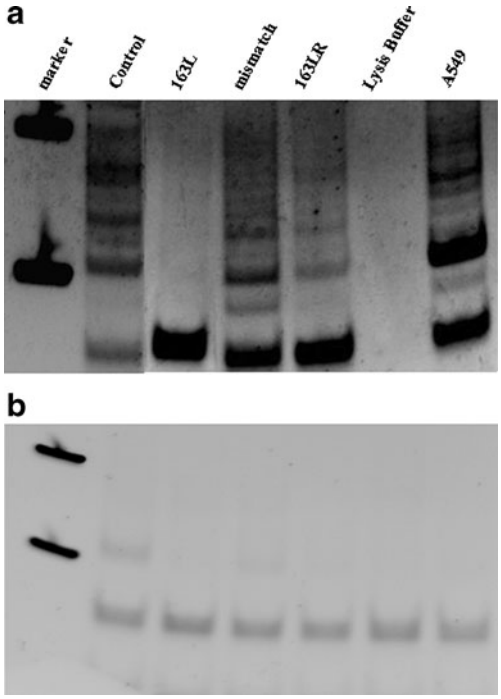

Fig. 2 TRAP assay of MSCs from control, mismatched, 163L and 163LR groups. A549 lung cancer cell line was used as a positive control to determine the telomerase inhibition by 163L. Cell extracts were prepared from **a** normal and **b** heat inactivated samples

in 163LR and compared them to that of control group. These results show that MSCs are able to differentiate into adipocytes (Fig. 3c) and osteocytes (Fig. 4c) after they recovered from the GRN163L treatment, similar to the MSCs from the control group (Figs. 3a and 4a). We did not observe differentiation of MSCs from control and 163LR groups into adipocytes or osteocytes when these cells were cultured in non-inducing media (Figs. 3b, d and 4b, d respectively).

As the MSCs cease growing in the presence of GRN163L and then regain their growth abilities when the drug was removed, the expression of cell cycle genes (*cyclin A, B, D* and *E* and their counterparts, *cdk 1, 2, 4* and *6)* were checked both at the mRNA with qRT-PCR (Fig. 5) and protein level with Western blotting (Fig. 6). In addition, *cdc25A, p16, p19, p21, p53, RB, TERT* and *Bmi-1* levels were also investigated. The expression of all the tested genes were found to decrease upon 163L treatment, compared to the control group at the mRNA level. Among those genes, the decrease was statistically significant in the expression of *cyclin D1, cdk6, cyclin E, cyclin B1, cdc25A, Bmi1, p21*and *p19* (Fig. 5; control vs 163L). Interestingly, the expression of all the genes increased in the 163LR group where the ligand was removed and the MSCs recovered. Among these genes the increase was significant in the expression of *cdc25A, p53, p21* and *p19* (Fig. 5; 163L vs 163LR). The effect of the ligand appears telomerase

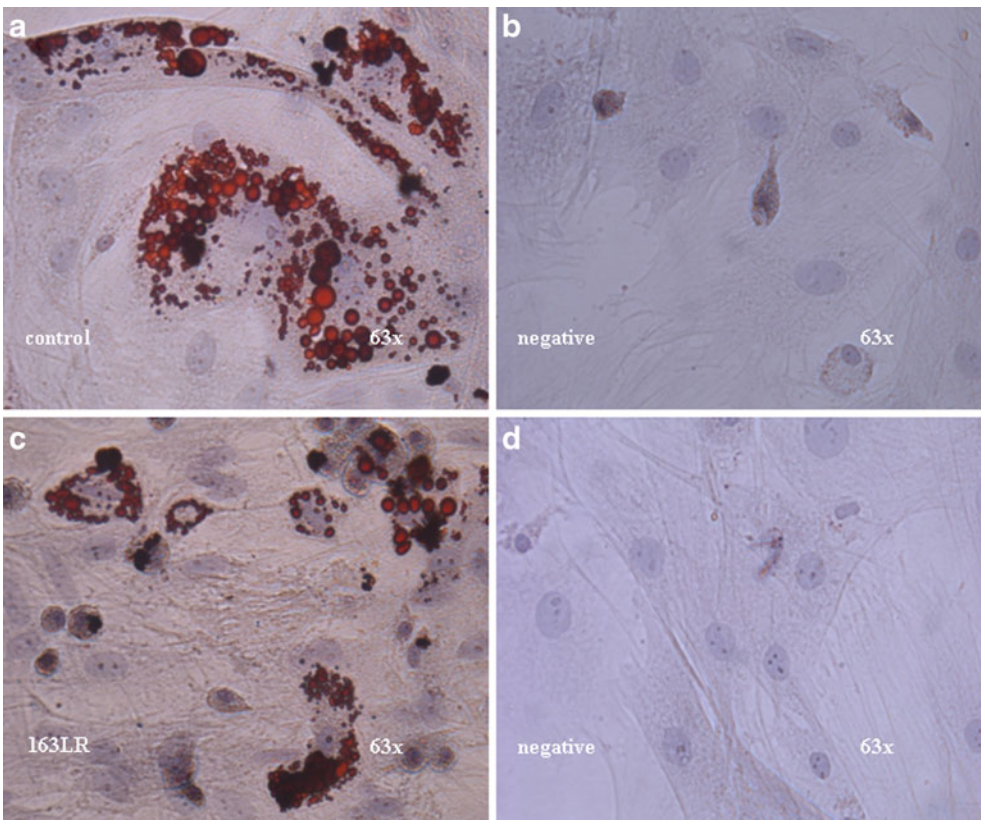

Fig. 3 Oil Red O staining to determine adipogenic differentiation of MSCs. **a** control and **c** 163LR group by using induction media. MSCs from **b** control group and **d** 163LR groups did not induce adipogenic differentiation when non-induction media was used

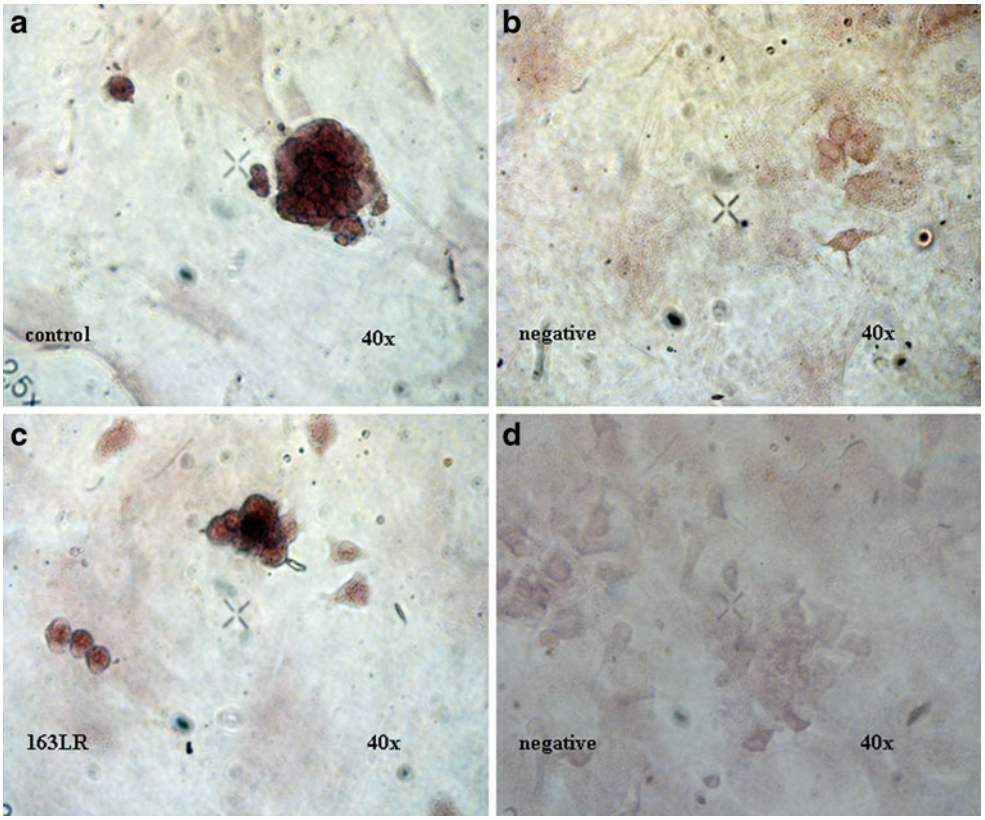

Fig. 4 Alizarin Red S staining to determine osteogenic differentiation of MSCs. **a** control and **c** 163LR group by using induction media. MSCs from **b** control group and **d** 163LR groups did not induce osteogenic differentiation when non-induction media was used

inhibition specific, since the expression of these genes in the mismatch group did not decrease (Fig. 5; 163L vs mismatch). The expression of all the genes, except *cyclin B1* and *RB*, which were higher than the control group (Fig. 5; control vs mismatch). A similar pattern of expression, that is, to decrease upon the 163L treatment and to increase 1 week after the removal of 163L was also observed in the expression of some of these genes at the protein level (Fig. 6). Our Western blot results showed that the expression of Cdk2, Cdk4, Cdk6, Cyclin D1, and Bmi-1 decreased in the 163L group compared to the control group (Fig. 6; control vs 163L) and increased 1 week after the removal of 163L (Fig. 6; 163L vs 163LR). In contrast, the expression of Cyclin E and Cdc25A were similar in control and 163L groups, however, they increased after 163L was removed (Fig. 6; 163L vs 163LR). Other proteins that were tested did not show any difference between the groups.

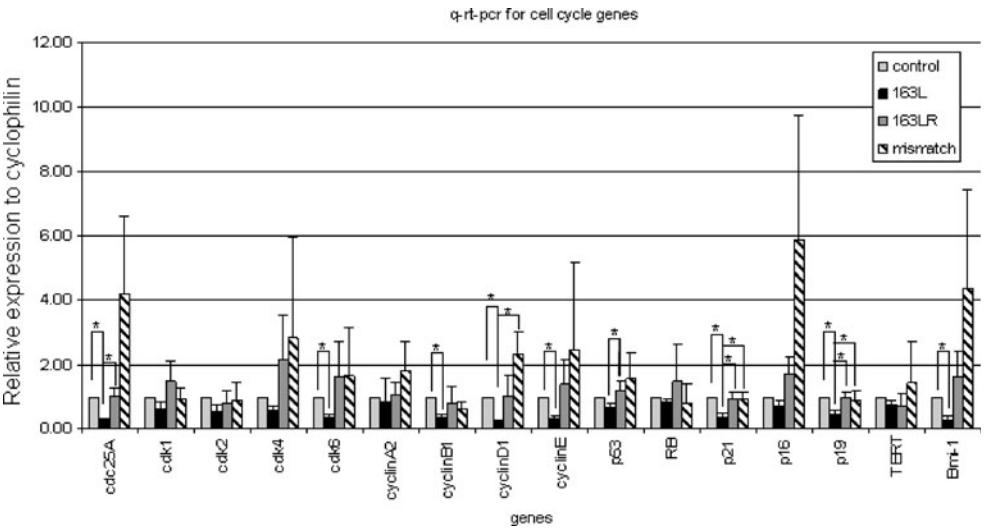

Fig. 5 mRNA expression of cell cycle genes in MSCs from control, mismatch, 163L and 163LR groups determined with qRT-PCR. * indicates $p \leq 0.05$

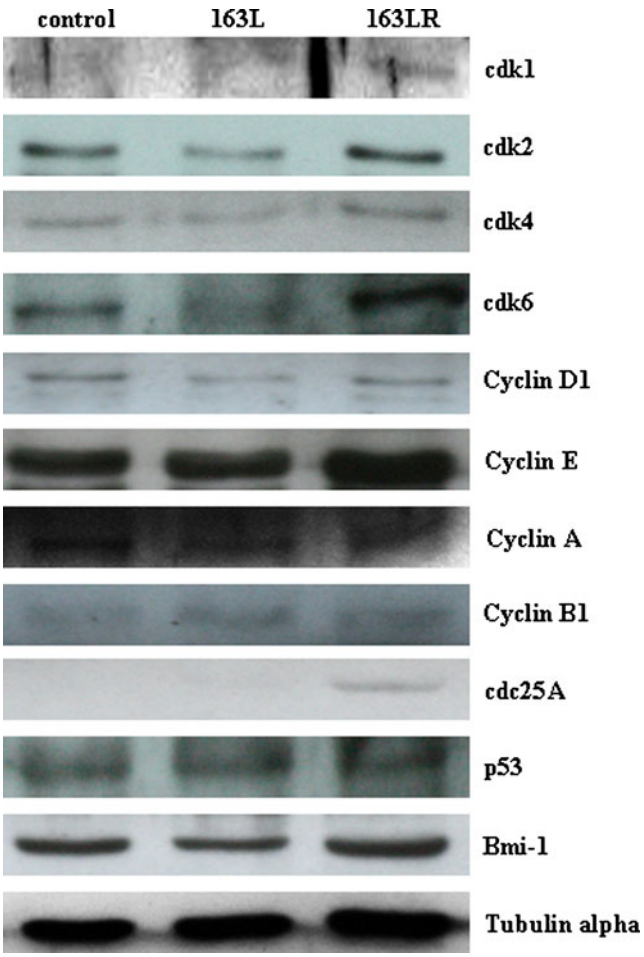

Fig. 6 Protein expression of cell cycle genes in MSCs from control, 163L and 163LR groups determined by Western blot

Discussion

The use of anti-telomerase agents as cancer therapeutic agents is still in an early stage for complete analyses of clinical outcomes, efficacy, tolerance and toxicity on normal tissue [17]. Potential effects of the inhibition of telomerase activity on stem cells are important because different stem cell compartments have different levels of telomerase activity [18]. Embryonic stem cells express high levels of telomerase activity [19] while several adult stem cell compartments, such as hematopoietic stem cells, epidermal stem cells, neural stem cells and mesenchymal stem cells also maintain telomerase activity but with low levels [9, 18, 20].

Among these stem cells, the effect of telomerase inhibitor GRN163L on mesenchymal stem cells is an issue of potential concern when applied to tumor tissue in vivo because MSCs emerge as an important player of cell-based therapies in a wide variety of pathologies, including cardiovascular disease and myocardial infarction (MI), brain and spinal cord injury, cartilage and bone injury, Crohn's disease and graft-versus-host disease (GVHD) during bone marrow transplantation [15, 21]. In vivo bone formation potential of telomerase-over expressing hMSCs was highly enhanced suggesting telomerase is required for both cell replication and differentiation [22]. Moreover, mouse MSCs with their telomerase activity knocked-down completely failed to differentiate into adipocytes or chondrocytes [23]. Thus, one could speculate that hMSCs may express at least a low level of telomerase activity to carry out the regenerative capacity as well as the differentiation potential [18]. Treatment with a telomerase inhibitor could have unwanted side effects on MSCs. While the potential side effects are unknown, they are expected to be mild because stem cell populations have longer telomeres than tumor cells, and as they divide rarely, their telomeres should shorten at a much slower rate than proliferating cancer cells [24].

The present study was undertaken to investigate the in vitro effects of GRN163L on the self-renewal and differentiation processes of MSCs. First, we evaluated the morphological changes of MSCs upon treatment of GRN163L. Previous studies have shown that rapid morphological changes occur within 24 h in cancer cells when plated in the presence of GRN163L and cells become rounded up, loosely attached to the surface, cease growing and die [25, 26]. These morphological changes were independent of telomerase inhibitory effect of GRN163L via hTR antagonism, but related to the lipidation of the compound, the nitrogen-phosphorous-sulfur–containing backbone chemistry (NPS linkage) and the presence of triple-G motif. It has also been suggested that antimetastatic effects of GRN163L may be related with the anti-cell adhesive effects of this novel cancer therapeutic agent [25]. In our study, similar to the data reported for cancer cell lines, we observed similar morphological changes when MSCs were plated in the presence of GRN163L, suggesting a similar mechanism of action of GRN163L both in MSCs and cancer cells. The cells' rounding effects were observed within 24 h after GRN163L treatment (Fig. 1c), whereas mismatch oligonucleotide-treated MSCs became tightly attached to the cell culture plate, similar to the untreated control cells (Fig. 1b and a, respectively). Moreover, "rounding effect" was not observed when GRN163L was withdrawn (163LR group) and they regained the original mesenchymal stem cell morphology as the unreated control group (Fig. 1d).

MSCs play an important role in the homeostasis of organisms therefore GRN163L would not be predicted to cause any alteration in the long term irreversible differentiation potential of MSCs. To address this issue, we treated MSCs with GRN163L, let them recover for 1 week and then evaluated their osteogenic and adipogenic differ-

entiations (Figs. 3 and 4, respectively). Our results showed that there was no noticeable difference in the potential of these MSCs to differentiate into adipocytes and osteocytes when compared with the untreated control cells. Observing of no measurable effects on the differentiation of MSCs upon GRN163L treatment is of particular clinical importance, mainly for older patients. Aging deteriorates the differentiation potential of MSCs [27, 28]; therefore to use GRN163L for anti-cancer treatment of the elderly would seem not further decrease the potential.

Several studies have examined telomerase activity in MSCs, providing somewhat differing results. As discussed in Sethe et al. 2006, some studies find no telomerase activity in MSCs, whereas others have detected telomerase activity [29]. Our results clearly showed that MSCs do have telomerase activity, and the activity is inhibited by GRN163L (Fig. 2). Importantly, telomerase activity recovers upon withdrawal of GRN163L (Fig. 2; 163LR group). The reason for this disparity could be explained by the presence of the heterogeneous group of MSCs (telomerase positive and negative) and it clearly warrants further research [30, 31]. The difference could also be related to the passage number of MSCs used. It has been recently shown that telomerase activity is present at 10 days but negative at 30 days [32]. The MSCs that were used in this study derived from the first passage and were physiologically more relevant.

As the "rounded" MSCs were not able to proliferate as much as the untreated cells, several cyclins and cdks that regulate cell cycle were analyzed to determine whether telomerase inhibition leads to cell cycle arrest. Our real-time PCR and Western blot data clearly showed that inhibiting telomerase activity with GRN163L does leads to cell cycle arrest at the G1 phase, and that importantly, these effects were reversible. Therefore, it is possible to conclude that the MSCs will regain their proliferation and self-renewal capacity following the cessation of anti-telomerase therapy. Additionally, the mismatch oligonucleotide had no effect on the proliferation status of these cells, indicating high specificity of this compound.

It is also important to note that the in vitro effects of GRN163L on MSCs may not necessarily reflect their possible in vivo effects. When the telomerase activity was knocked down, mouse MSCs failed to differentiate into adipocytes or chondrocytes [23]. On the other hand telomerase-over expressing hMSCs had been shown to have a high osteogenic differentiation potential [22]. Systemic effects of telomerase inhibition on bone marrow-derived MSCs might be different than in vitro reversible effects. Therefore, new studies are warranted to investigate the role of GRN163L on in vivo conditions.

In summary, we have investigated the potential effects of GRN163L on MSCs. Our results suggest that the inhibitory effect of GRN163L on mesenchymal stem cells is reversible in under short term in vitro cell culture conditions. We conclude that hTR antagonist GRN163L does not interfere with the self-renewal and differentiation of MSCs and can be used without apparent toxic side effects. Nevertheless, in clinical settings anti-telomerase cancer treatment should be stopped before telomere depletion in stem cells, so that telomerase activity and telomeres in reproductive and stem cells can be restored. Our study provides additional support for treating cancers by administrating GRN163L without depleting the body's stem cell pools.

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