

Antisenescence effect of REAC biomodulation to counteract the evolution of myelodysplastic syndrome

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Short title: REAC biomodulation to prevent acute myeloid leukemia

Summary

About 30 percent of patients diagnosed with myelodysplastic syndromes (MDS) progress to acute myeloid leukemia (AML). The senescence of bone marrow-derived mesenchymal stem cells (BMSCs) seems to be one of the determining factors in inducing this drift. Research is continuously looking for new methodologies and technologies that can use bioelectric signals to act on senescence and cell differentiation towards the phenotype of interest. The Radio Electric Asymmetric Conveyer (REAC) technology, aimed at reorganizing the endogenous bioelectric activity, has already shown to be able to determine direct cell reprogramming effects and counteract the senescence mechanisms in stem cells. Aim of the present study was to prove if the anti-senescence results previously obtained in different kind of stem cells with the REAC Tissue optimization – regenerative (TO-RGN) treatment, could also be observed in BMSCs, evaluating cell viability, telomerase activity, p19ARF, P21, P53, and hTERT gene expression. The results show that the REAC TO-RGN treatment may be a useful tool to counteract the BMSCs senescence which can be the basis of AML drift. Nevertheless, further clinical studies on humans are needed to confirm this hypothesis.

Keywords: REAC; myelodysplastic syndromes, acute myeloid leukemia, senescence, stem cells, cellular mechanism.

Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of hematopoietic stem cell disorders and hematological malignancies. About 30 percent of patients who are diagnosed with MDS progress to acute myeloid leukemia (AML)[1]. MDS are characterized by dysplastic morphology in the bone marrow (BM) in association with the transformation of hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs), leading to unsuccessful hematopoiesis.

Most of mature blood cell compartments need to be continuously replenished by HPCs and HSCs. Evidently, in MDS, cell senescence has a primary role in BMSCs 'loss of self-renewal capability, loss of function, and BM suppression.[2-4]

The underlying etiopathogenesis of MDS recognizes several causes, including genetic abnormalities in myeloid progenitors and epigenetic factors affecting bone marrow (BM)[5,6]. Concurrently, the transition to AML is also conditioned by epigenetic causes[7,8] that can modify endogenous voltage potentials, endogenous bioelectric fields (EBF), and cell polarity behavior, thus playing a decisive role in MDS and its transformation into AML[9].

In turn, epigenetic and transcriptional cascades are conditioned by endogenous bioelectric phenomena that can determine radical changes in cellular properties and behavior, such as proliferation, differentiation, migration, morphology, and apoptosis[10,11], which can be the basis of tumor drifts[12].

Moreover, some studies investigating endogenous bioelectric phenomena have unveiled important advances in our understanding of leukemia, with potential therapeutic implications[13].

Aging, also interpreted as the summation of epigenetic modifications, is another important risk factor in the development of these diseases, as most patients with MDS are over 50 years old (median age range of 65 to 70 years)[14].

Tissue optimization-regenerative (TO-RGN) treatments are specific treatment protocols with radio electric asymmetric conveyer (REAC) technology. The mechanism of action on which the REAC technology is based to create a non-uniform radio electric field, which can interact with particles even if not electrically charged, to optimize their endogenous bioelectric characteristics. Adequate manipulation of endogenous bioelectric activity can epigenetically reprogram cell fate. The REAC-RGN protocol was designed for this purpose. TO-RGN, which aims to reorganize endogenous bioelectric activity, has been shown to be capable of determining direct cell reprogramming effects[15-17], thus counteracting senescence in stem cells[18,19] by modulating both dependent and independent telomerase mechanisms[20].

The ability of these specific REAC treatments to counteract aging in stem cells is one of the assumptions that motivate this study. Onco-hematological diseases (OHD) represent a group of clonal diseases whose pathogenesis is fueled by the presence of acquired genetic defects that compromise the functionality of hematopoietic medullary precursors, such as hematopoietic stem cells (HSCs) and BMSCs. One of the factors that mainly condition the regenerative capability of BMSCs is linked to the effects of senescence on self-renewal processes and differentiation potential. Senescence is a complex process involving a continuous decline in cell proliferation, changes in cell morphology and differentiation, and abnormalities in signaling pathways [4]. In addition, replicative senescence is often associated with altered expression of key molecules involved in the interaction between hematopoietic stem and progenitor cells and the BM microenvironment [2,21]. An aged microenvironment may also be responsible for the occurrence of somatic mutations and the onset of acute myeloid leukemia (AML) [22]. Based on these findings, in the present study, we aimed to assess how a specific REAC treatment that is capable of reorganizing endogenous bioelectric activity can interfere with the senescent phenotype of BMSCs. This could lead to the identification of its preventive effects in the transformation of MDS into AML by inducing the most functional epigenetic response toward the exposome and counteracting the aging process. For this reason, BMSCs obtained from low-risk MDS patients were selected to evaluate the effect of REAC TO-RGN treatment on the expression of the following senescence-associated genes: 1) p19ARF, which regulates self-renewal and cellular senescence programs; 2) p21, the cyclin-dependent kinase inhibitor 1; 3) p53, which regulates cell cycle progression and apoptosis; and 4) telomerase reverse transcriptase (hTERT), which is the catalytic subunit of the enzyme telomerase that is responsible for telomere elongation and integrity. The activity of this enzyme was also assessed to confirm the gene expression analysis results.

Methods

Ethics statement

This study was conducted according to the guidelines of the Declaration of Helsinki of 1975, revised in 2013 and approved by the Institutional Review Board of the Centre for developmental biology and reprogramming (CEDEBIOR) of University of Sassari, Italy. Protocol code N.01/2021, date of approval 18 January 2021. Informed consent was obtained from all subjects involved in the study.

Radio electric asymmetric conveyer (REAC) technology

The REAC technology was designed to carry out neurobiological stimulation treatments through the manipulation of EBF. To achieve this effect, it is necessary that a particular type of electrode, called asymmetric conveyer probe (ACP) connected with the REAC device, is put in contact with the cell culture or specific areas of the body. This manipulation is possible because this technology considers the bioelectric asymmetry at the base of the ionic flows that generates the EBF. REAC technology can modulate the current flows at both cellular and body levels by acting on EBF and exerting a therapeutic effect. Unlike other technologies that impose their electrical, magnetic, or electromagnetic stimulus on cell cultures or tissues to be treated, and consequently the effect depends on their stimulation strength, REAC technology generates a very weak radio electric field, which at the origin is lower than 2 milliwatts. This radio electric field, once emitted, is dispersed in the environment undergoing a dispersion that decreases with the square of the distance from the emitter. The effect of this dispersion is extremely important to further decrease the strength of the REAC radio electric field so that it does not impose itself on the endogenous bioelectrical characteristics of cell cultures but generates in them a gradient such as to modulate the activation of ionic flows. The treatment used in this study was TO-RGN. The REAC device used in this study was BENE Mod. 110 (ASMED S.r.l., Florence, Italy).

Cell isolation and culturing

Bone marrow-derived mesenchymal stem cells (BMSCs) were isolated from bone marrow of 10 low-risk MDS onco-hematological adult female and male patients (age=50± 15 years) collected during tests carried out for clinical reasons. The samples were immediately processed using Ficoll-Paque PLUS density gradient media (GE Healthcare Life Sciences, United Kingdom) according to the manufacturer's protocol. The cells obtained were immediately counted and seeded in a tissue culture dish measuring 100 mm at a concentration of 8×10^5 - 1×10^6 cells/plate in a medium composed of Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, USA), 10% fetal bovine serum (FBS, Thermo Fisher Scientific, USA), 200 mM L-glutamine (Euroclone, Milano, Italy) and 200 U/mL penicillin-0.1 mg/mL streptomycin (Euroclone, Milano, Italy). The REAC-RGN treatment is a pre-programmed treatment. In this case cells were cultured for 24, 48, or 72h in the absence or presence of REAC- RGN treatment.

Trypan Blue exclusion test of cell viability

The dye exclusion test was used to evaluate the number of viable cells cultured in the presence or absence of REAC treatment. The living cells possess intact cell membranes that excluded Trypan Blue dye, while the dead cells appear with a blue cytoplasm. The cell suspension was mixed with 0.4% dye solution

(Thermo Fisher Scientific, USA) in a proportion of 1:1 and then counted using a LUNA-II™ Automated Cell Counter to obtain information about the cell viability and cell size distributions.

Gene expression analysis of real-time PCR

RNA extraction was performed using ChargeSwitch total RNA cell kits (Thermo Fisher Scientific, USA) at 0, 24, 48, and 72h for the cells cultured with electromagnetic fields and the untreated control. Approximately 1 µg of total isolated mRNA was reverse transcribed into cDNA using the Superscript Vilo cDNA synthesis kit (Thermo Fisher Scientific, USA), for a final volume of 20 µL, according to the manufacturer's protocol. Quantitative polymerase chain reaction was performed in triplicate according to the protocol specified in the Platinum® Quantitative PCR SuperMix-UDG Kit (Thermo Fisher Scientific, USA) using a CFX-96 Thermal Cycler (Bio-Rad) (Applied Biosystems), as previously described [19]. The standard qRT-PCR conditions were 50 °C for 2 min, 95 °C for 2 min, and then cycled at 95 °C for 15 s, 55–59 °C for 30 s and 60 °C for 1 min for 40 cycles. The total volume of each reaction was 25 µL, composed of 2X SuperMix with SYBR Green I, 0.1 µM of each primer, and 3 µL cDNA generated from 1 µg of the total RNA template. The target Ct values were normalized on hGAPDH, considered as a reference gene, while the mRNA levels of the BMSCs cultured in the presence or absence of REAC were expressed as fold of change ($2^{-\Delta\Delta Ct}$) relative to the mRNA levels observed in the BMSCs at time 0 before starting treatment. Each experiment included a distilled water control. The qRT-PCR analysis was performed for the following genes: telomerase reverse transcriptase (TERT), RNA silencing suppressor p19 ARF (p19ARF), cyclin-dependent kinase inhibitor 1 (p21), tumor suppressor protein p53 (p53), octamer-binding transcription factor 4 (Oct-4); Sex determining region Y-box 2 (Sox2) and homeobox protein Nanog (NANOG). All primers were highly specific and designed with Primer3[15] and were obtained from Invitrogen (Primer: hGAPDH, Forward: GAGTCAACGGAATTTGGTCGT, Reverse: GACAAGCTTCCCGTTCTCAG; Primer: TERT, Forward: GACGTGGAAGATGAGCGTG, Reverse: GACGACGTACACACTCATC; Primer: p19ARF, Forward: GCCTTCGGCTGACTGGCTGG, Reverse: TCGTCCTCCAGAGTCGCCCG; Primer: p21, Forward: CAAAGGCCGCTCTACATCTT, Reverse: AGGAACCTCTCATTCAACCCGA; Primer: p53, Forward: TGGCCTTGAAACCACCTTTT, Reverse: AACTACCAACCCACCAGCCAA; Primer: Oct-4, Forward: GAGGAGTCCCAGGCAATCAA, Reverse: CATCGGCCTGTGTATATCCC; Primer: Sox2, Forward: CCGTTCATGTAGGTCTCGGAGCTG, Reverse: CAACGGCAGCTACAGCTAGATGC; Primer: NANOG, Forward: CATGAGTGTGGATCCAGCT, Reverse: CCTGAATAAGCAGATCCAT).

Telomerase activity detection

To evaluate the telomerase activity, the TRAPeze® Kit RT Telomerase Detection Kit (Millipore, MA, USA) was used. It directly measures the fluorescence emission of telomerase activity through real-time PCR. The BMSCs were seeded in a TC dish measuring 100 mm and treated or not treated with REAC for 24, 48, and 72h. Then, they were pelleted for the performance of the assay in accordance with the manufacturer's instructions. All samples underwent the procedure in triplicate using a CFX-96 Thermal Cycler (Bio-Rad). Briefly, the cells were lysed with a CHAPS lysis buffer included in the kit; the protein concentrations were determined using Nanodrop (Thermo Fisher Scientific, USA). The master mix was composed of 5X TRAPeze® RT reaction mix, Taq polymerase (5 units/ μ L), nuclease free water, and samples, for a final volume of 20 μ L. The PCR amplification conditions were 30 °C for 30 min, 95 °C for 2 min, and then cycled at 94° C for 15 s, 59° C for 60 s, and 45° C for 10 s for 45 cycles. Each sample included a negative and a positive control. The telomerase activity of each sample was normalized to the Ct of the standard curve generated from the control reaction mix included in the kit.

Senescence-Associated β -Galactosidase Staining

Cell senescence was evaluated in BMSCs treated or not treated with REAC for 72h by Senescence-associated (SA) β -Galactosidase Staining Kit (Cell Signaling Technology, Euroclone, Milan Italy). After treatment, cells were fixed and processed according to the manufacturer's instructions. Detection of SA- β -Galactosidase activity, visible by blue staining, was performed by inverted light microscope (10X magnification in bright field). The number of positively blue-stained cells was calculated as the percentage of total number of cells with an image software analysis (ImageJ, version 1.8.0, National Institutes of Health, Bethesda, MD, USA) (mean \pm SD; n=6) (* p \leq 0.05; ** p \leq 0.01).

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Science Version 13 (SPSS Inc., Chicago, IL, USA). For this study, assuming a p value $<$ 0.05 as statistically significant, Wilcoxon signed-rank test was used to evaluate the congruity of the observed set, while Kruskal-Wallis rank sum was used to assess the values found in the different observation topics. The experiments were performed two times with three technical replicates for each sample (n=10).

Results

Cell viability

After plating (1×10^6 cells/plate), the cells were cultured in the absence or presence of REAC TO-RGN treatment for 24, 48, and 72h. Then, the BMSCs were collected and counted using an automatic cell counter. The percentage of vital cells was calculated as the number of positive cells divided by the total number of counted cells. The REAC TO-RGN treatment significantly increased the BMSC viability and proliferation at each time point compared with the untreated control cells (Figure 1 and Table 1).

Telomerase activity

The cells were cultured for 24, 48, or 72h in the absence or presence of REAC. The mRNA levels of TERT from the control (blue bars) or REAC-treated (yellow bars) cells were normalized to GAPDH and plotted as fold changes relative to the mRNA expression at time 0, defined as 1 (gray bar). All the REAC-treated cells after 24, 48 and 72h were significantly different from the untreated control cells. The REAC TO-RGN treatment modulates hTERT gene expression by determining its upregulation (Figure 2, Panel A).

The TRAPeze-RT assay was performed in the untreated control cells and in the cells exposed to REAC TO-RGN after 24, 48, and 72h in culture. The telomerase activity of the REAC-treated cells was compared with that of the untreated control cells. Telomerase activity was calculated by comparing the average Ct values from the sample wells against the standard curve generated by the TSR8 control template. The REAC TO-RGN treated-cells exhibited a significant increase in telomerase activity compared with the untreated control cells for each time of observation (Figure 2, Panel B).

P19ARF, P21, P53 gene expression

The cells were cultured for 24, 48, or 72h in the absence or presence of REAC TO-RGN treatment. As showed in Figure 3, the mRNA levels of p19ARF (Panel A), p21 (Panel B), and p53 (Panel C) from the control (blue bars) or REAC-treated (yellow bars) cells were normalized to GAPDH and plotted as fold changes relative to the mRNA expression at time 0, defined as 1 (gray bar). All of the REAC TO-RGN-treated cells at each time point were significantly different from each untreated control cell for P19ARF and for p21. REAC TO-RGN-treated cells at were significantly different from each untreated control cell at 48 and 72 hours for p53. The REAC TO-RGN treatment modulates p19ARF, p21, p53 gene expression by determining their downregulation.

Expression of stemness-related genes

The cells were cultured for 24, 48, or 72h in the absence or presence of REAC. As showed in Figure 4, the mRNA levels of Oct-4 (Panel A), Sox2 (Panel B), and NANOG (Panel C) from the control (blue bars) or

REAC-treated (yellow bars) cells were normalized to GAPDH and plotted as fold changes relative to the mRNA expression at time 0, defined as 1 (gray bar). All the REAC-treated cells after 48 and 72h were significantly different from the untreated control cells. The REAC TO-RGN treatment modulates stemness-related gene expression by determining its upregulation.

β-galactosidase activity

Figure 5 shows the results from β-galactosidase staining assay in BMSCs treated or not with REAC for 72h. Results have revealed that REAC significantly counteract the senescence process, as compared to control untreated BMSCs (Panel A). The number of blue positive senescent cells was significantly reduced after REAC treatment (Panel B). Data are expressed as mean ±SD (n=6) (**p ≤ 0,001).

Discussion

OHD represents a group of clonal diseases whose pathogenesis is characterized by the presence of acquired genetic defects that compromise the functionality of the hematopoietic precursors[23]. In recent years, the demonstration that the BM microenvironment plays a substantial role in supporting or inhibiting the evolution of tumor clones has attracted extensive interest[24-26]. The growing knowledge on the mechanisms that mediate the hematopoietic function of the medullary precursors has made it possible to elaborate on the concept of hematopoietic niche[27], which is designated as the most relevant functional unit in this context. BMSCs represent a subfamily of stem cells with lineage-oriented features in the connective sense[28]. In addition to their tissue regeneration capability that is common to other stem cell subtypes, they present two other functional attributes, namely, the ability to support hematopoiesis[29-31] and specific immunomodulation properties[32].

The regenerative capability of BMSCs is not only linked to the original tissue, the medullary microenvironment in which they are found, and the age of the donor, but also to the effects that senescence exerts on the self-renewal processes and on the differentiation potential[33,34].

Alongside conventional methodologies represented by chemical stimuli, innovative strategies, methods, and devices are sought and applied that would allow the control of differentiation and of the various physiological processes through the transmission of the signals necessary to direct the differentiation potential toward the phenotype of interest[35].

Bioelectric signaling plays an important role in the induction and maintenance of differentiation processes as it is a functional regulator of the differentiation process in various cells and tissues[12,36,37]. To attain maximum effectiveness in inducing cell proliferation, differentiation, migration, morphology, apoptosis, and anti-senescence, it is crucial that bioelectric signals are not imposed from the outside but are endogenously induced. The REAC technology was designed to obtain this type of endogenous induction in order to recover and optimize the endogenous bioelectric potentials and the functions that depend on them[15-20,38-46].

The objective of the present study was to determine whether the previous anti-senescence results obtained on different stem cell lines could also be observed in BMSCs. For this reason, we evaluated the effects of REAC TO-RGN treatment on cell viability and on the expression of the senescence-associated genes p19ARF, p21, and p53. Moreover, the expression of hTERT, which is the product of the telomerase gene, was analyzed, together with the telomerase activity. The expression of the stemness-related genes and β -galactosidase activity were also evaluated.

The results show that the REAC TO-RGN treatment can induce a significant increase in cell viability, as showed by increased number of live cells, as compared to control untreated cells, after trypan blue exclusion test. It also acts on the upregulation of hTERT gene expression in telomerase activity. BMSCs have the capability to maintain some basal level of telomerase activity that increases upon differentiation and proliferation. Nevertheless, other authors have described a negative correlation between hTERT activity, MDS[47], and AML prognosis[48].

It is important to consider that in this study, the upregulation of telomerase activity agrees with the observed increase in cell viability; it is also essential in preserving chromosome integrity in newborn cells. On the other hand, the REAC TO-RGN treatment was able to contemporarily downregulate the expression of the genes responsible for the establishment of a senescent phenotype, namely, p19ARF, p21, and p53, and increase the expression of stemness-related genes, Oct-4, Sox2 and NANOG. Based on the gene expression profiling of circulating BMSCs, we demonstrate that REAC TO-RGN endogenous bioelectric manipulation can improve the molecular pattern of cell senescence, ultimately inducing a higher telomerase activity.

The perspectives opened by this study can be easily translated to the clinic, because the specific therapeutic treatments of REAC neurobiological stimulation have already been in use for years to promote reparative and regenerative effects, with already standardized methods of administration.

Conclusions

These results provide insights into the possibility of reversing or counteracting the functional impairments observed in low-risk MDS patients and open the chance to reactivate a compromised hematopoiesis. Future translational studies are warranted to determine if the REAC TO-RGN treatment is a useful tool to counteract the senescence mechanism that gives rise to AML. Moreover, it may constitute a valuable tool for traditional therapy, such as in regular blood transfusions for MDS patients.

Data Availability Statement: The data used to support the finding of this study are included within the article.

Conflicts of Interest: SR and VF are the authors of REAC patent. The other authors declare no conflict of interest.

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Tables

Table 1. Cell concentration in control and REAC TO-RGN-treated cells (mean \pm SE; n = 6).

	T0	24h CTRL	24h REAC	48h CTRL	48h REAC	72h CTRL	72h REAC
Total cell concentration	6.20 \pm 2.9 x 10 ⁶	2.47 \pm 0.42 x 10 ⁶	2.74 \pm 0.7 x 10 ⁶	3.46 \pm 0.38 x 10 ⁶	3.23 \pm 0.43 x 10 ⁶	2.63 \pm 0.47 x 10 ⁶	2.07 \pm 0.54 x 10 ⁶
Live cell concentration	4.27 \pm 1.8 x 10 ⁶	2.17 \pm 0.36 x 10 ⁶	2.53 \pm 0.6 x 10 ⁶	2.69 \pm 0.38 x 10 ⁶	2.74 \pm 0.46 x 10 ⁶	2.06 \pm 0.38 x 10 ⁶	1.89 \pm 0.49 x 10 ⁶
Dead cell concentration	1.94 \pm 1.1 x 10 ⁶	3.04 \pm 0.10 x 10 ⁵	2.03 \pm 0.8 x 10 ⁵	7.61 \pm 0.21 x 10 ⁵	4.90 \pm 0.22 x 10 ⁵	4.74 \pm 0.19 x 10 ⁵	1.85 \pm 0.062 x 10 ⁵
Viability	68.87%	87.85%	93.13%	77.74%	84.82%	78.32%	91.30%

Figures

Figure 1. Increase of viability in REAC TO-RGN-treated cells. After plating (1×10^6 cells/plate), the cells were cultured in the absence or presence of REAC TO-RGN treatment for 24, 48, and 72h. Then, BMSCs were collected and counted using an automatic cell counter. The percentage of vital cells was calculated as the number of positive cells divided by the total number of counted cells (mean \pm SE; n = 6) (* p \leq 0.05).

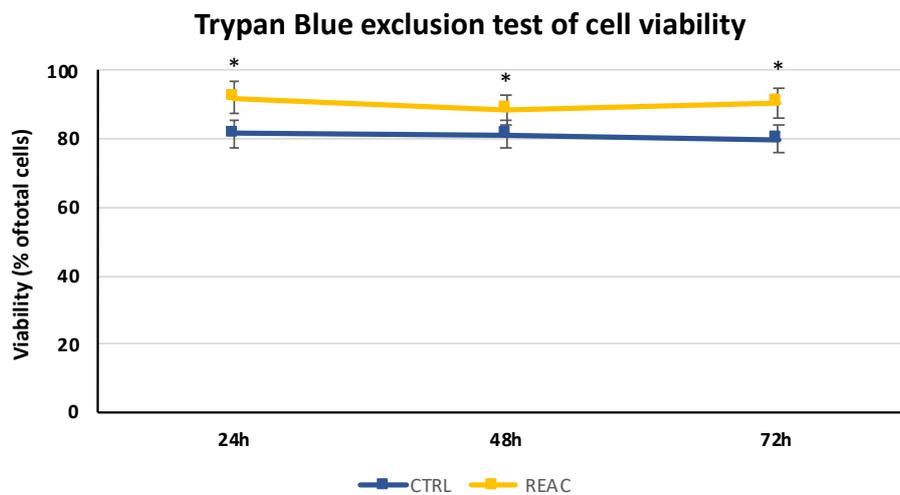


Figure 2. A) Effect of REAC TO-RGN in increasing the level of expression of TERT in the REAC TO-RGN-treated cells compared with the untreated control cells. For each timepoint, the mRNA level of the cells exposed to REAC TO-RGN treatment was significantly upregulated compared with the untreated controls (mean \pm SE; n=6) (* $p \leq 0.05$) **B)** REAC TO-RGN treatment increases telomerase activity. The REAC TO-RGN-treated cells showed an increase in telomerase activity compared with the untreated control cells for each time of observation (mean \pm SE; n = 6; * $p \leq 0.05$; ** $p \leq 0.001$).

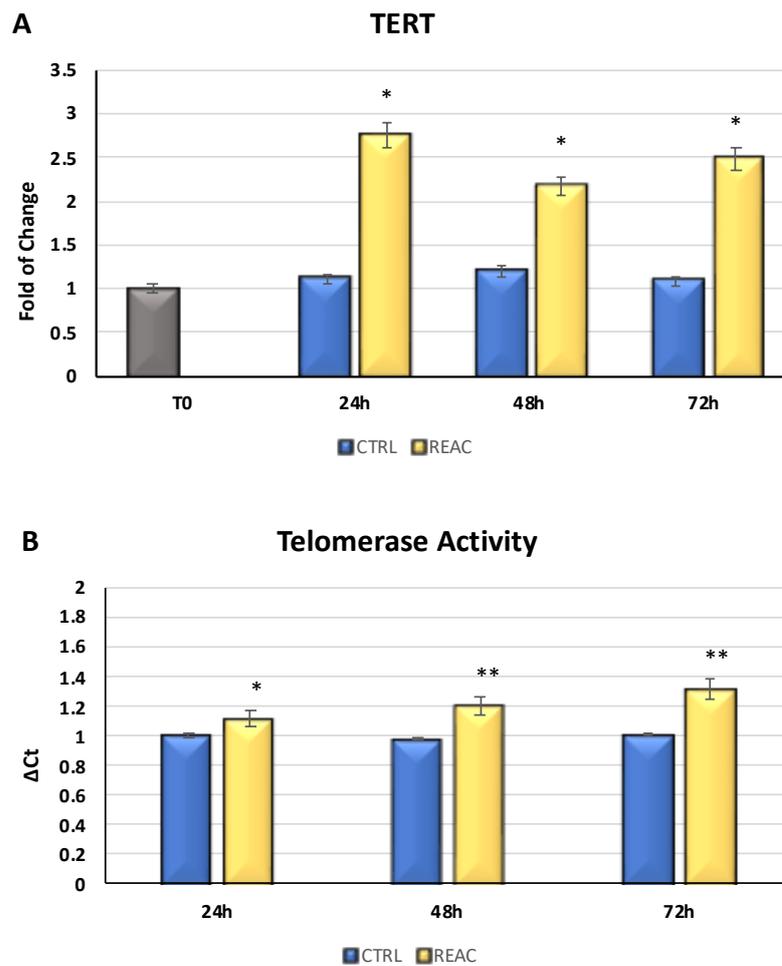


Figure 3. Gene expression of p19ARF (Panel A), p21 (Panel B), and p53 (Panel C) in BMSCs that were treated or not treated with REAC TO-RGN for 24, 48, or 72h. The expression of each gene increased for each time point in the untreated control cells, while it was significantly downregulated in the REAC TO-RGN-treated cells (mean \pm SD; n=6) (* $p \leq 0.05$; ** $p \leq 0.01$).

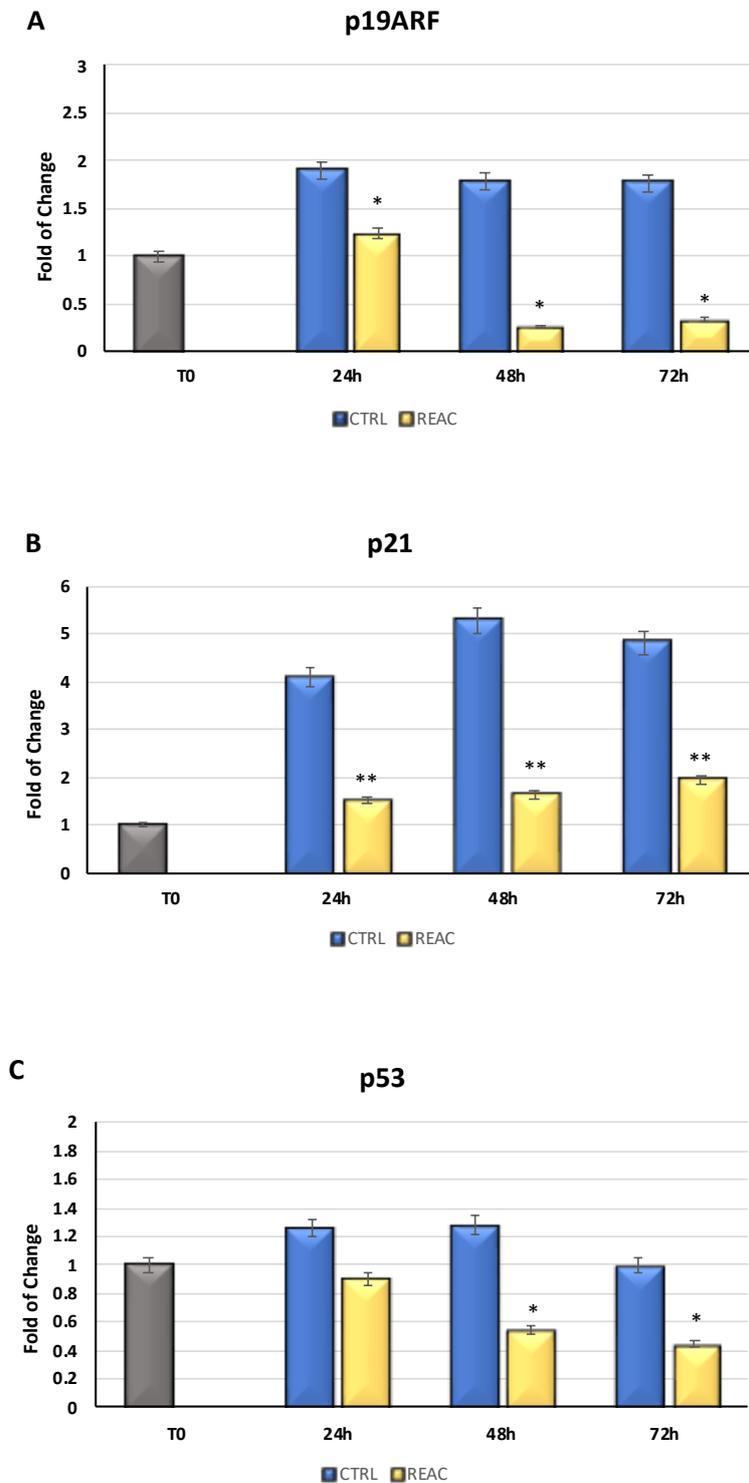


Figure 4. Effect of REAC TO-RGN in increasing the level of expression of Oct-4 (Panel A), Sox2 (Panel B), and NANOG (Panel C) in the REAC TO-RGN-treated cells compared with the untreated control cells. For each timepoint, the mRNA level of the cells exposed to REAC TO-RGN treatment was significantly upregulated compared with the untreated controls (mean \pm SD; n=6) (* $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.001$).

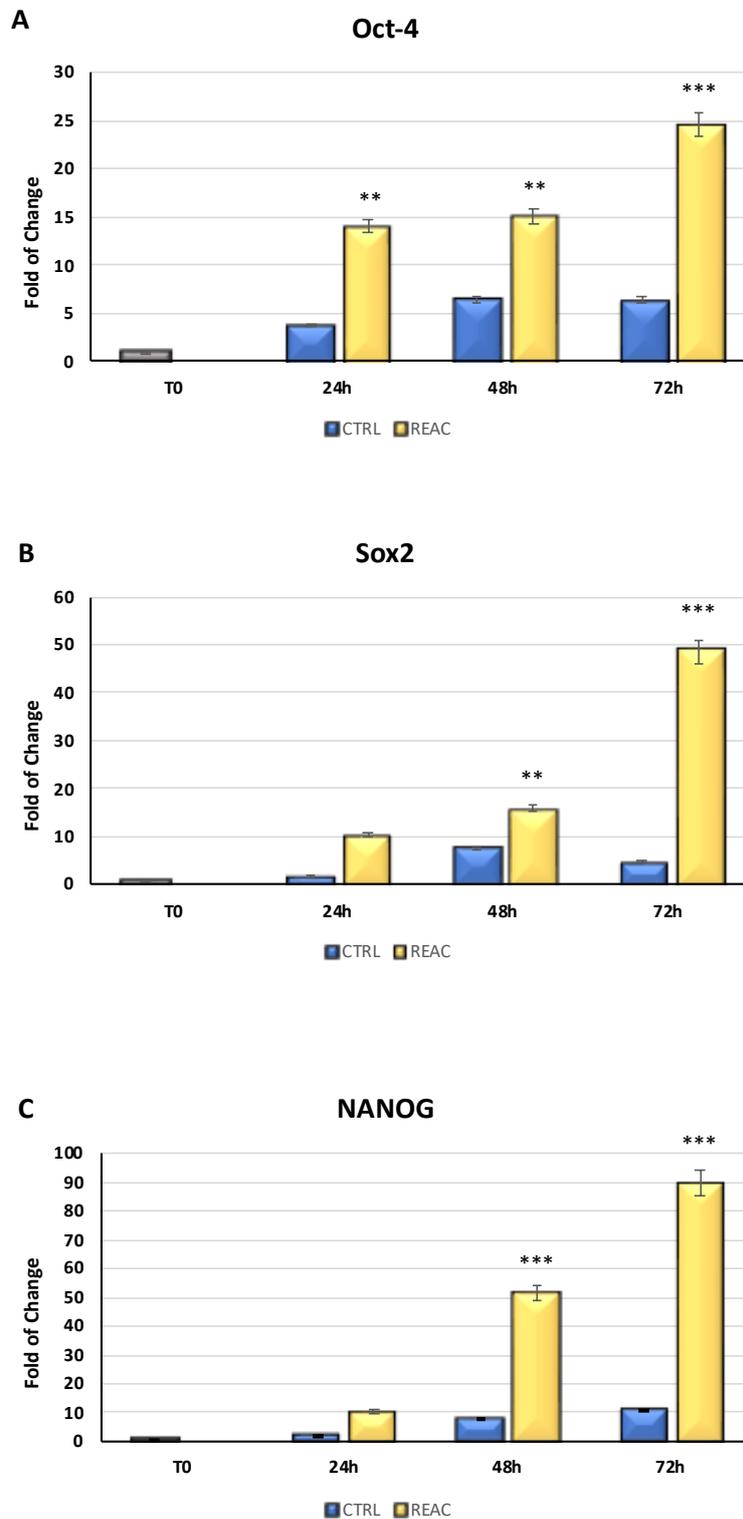


Figure 5. A) Senescence-associated β -galactosidase activity. β -galactosidase was evaluated in BMSCs treated or not with REAC for 72h. Blue arrows indicate the blue-positive cells. Scale bar=100 μ m. **B)** The number of blue positive cells was calculated using ImageJ. Data are expressed as mean \pm SD (n=6) (**p \leq 0,001).

