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Research paper

Non-targeting siRNA-mediated responses are associated with apoptosis in chemotherapy-induced senescent skin cancer cells

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ABSTRACT

It is widely accepted that siRNA transfection can promote some off-target effects in the genome; however, little is known about how the cells can respond to the presence of non-viral dsRNA. In the present study, non-targeting control siRNA (NTC-siRNA) was used to evaluate its effects on the activity of pathogen and host-derived nucleic acid-associated signaling pathways such as cGAS-STING, RIG-I, MDA5 and NF-κB in A431 skin cancer cells and BJ fibroblasts. NTC-siRNA treatment promoted cytotoxicity in cancer cells. Furthermore, NTC-siRNA-treated doxorubicin-induced senescent cancer cells were more prone to apoptotic cell death compared to untreated doxorubicin-induced senescent cancer cells. NTC-siRNA stimulated the levels of NF-κB, APOBECs, ALY, LRP8 and phosphorylated STING that suggested the involvement of selected components of nucleic acid sensing pathways in NTC-siRNA-mediated cell death response in skin cancer cells. NTC-siRNA-mediated apoptosis in cancer cells was not associated with IFN-β-based pro-inflammatory response and TRDMT1-based adaptive response. In contrast, in NTC-siRNA-treated fibroblasts, an increase in the levels of RIG-I and IFN-β was not accompanied by affected cell viability. We propose that the use of NTC-siRNA in genetic engineering may provoke a number of unexpected effects that should be carefully monitored. In our experimental settings, NTC-siRNA promoted the elimination of doxorubicin-induced senescent cancer cells that may have implications in skin cancer therapies.

1. Introduction

Post-transcriptional gene silencing (RNA interference, RNAi), based on double-stranded (ds)RNA-mediated (small interfering RNA (siRNA) and microRNA) degradation of sequence-specific mRNA, is a fundamental mechanism regulating gene expression associated with biological processes such as cell growth and cell death, development and differentiation [1,2].

Antisense pairing-related gene silencing by exogenous dsRNAs, a common genetic engineering approach to manipulate gene expression and study gene function, can also have therapeutic implications [2,3]. However, the use of RNAi-based drugs can be limited due to off-target and immunostimulatory effects of siRNA [4–8]. Gene silencing by siRNA transfection can affect the levels of genes that are functionally unrelated to the target gene [4]. For example, non-targeting siRNA can modulate the protein levels of cell cycle regulators, namely p53 and p21, thus affecting cell cycle progression [4]. siRNA can also stimulate the expression of an endoplasmic reticulum (ER)-resident protein, non-selenocysteine containing phospholipid hydroperoxide glutathione peroxidase (NPGPx) as a response to non-targeting siRNA-induced stress [7]. NPGPx binding to exoribonuclease XRN2 promoted XRN2-mediated elimination of non-targeting siRNA, and NPGPx depletion resulted in the accumulation of non-targeting siRNA and apoptotic cell death [7]. siRNA transfection can be also associated with non-specific induction of inflammatory cytokines and type I interferon [6]. RNA can be recognized by selected immunoreceptors such as the members of the Toll-like receptor (TLR) family (TLR3, TLR7, TLR8) and cytosolic RNA-binding proteins such as the dsRNA-dependent protein kinase (PKR) and the helicases retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) that can induce type I interferon (IFN-α and IFN-β)-based antiviral innate defense response [6,9,10]. Short harpin RNA (shRNA) introduced into the cells by the means of a lentiviral vector can also induce RIG-I-mediated IFN activation [11]. Surprisingly, siRNA can also promote DNA-mediated interferon lambda-1 response (IFN-λ1/IL-29) *via* a crosstalk between siRNA sensor, RIG-I and DNA sensor, IFI16 signaling pathway [8]. Indeed, although different receptors are involved in the RNA and DNA sensing, the downstream signaling components can be physically and functionally

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interconnected [12]. For example, a canonical DNA sensing pathway, namely the cyclic GMP-AMP synthase (cGAS) and stimulator of interferon genes (STING) (the cGAS-STING pathway) can take part in the direct response to RNA viral infection [13]. Thus, siRNA-mediated cellular responses and their consequences should be studied in the context of both RNA and DNA sensing pathways.

The aim of the present study was to evaluate non-targeting control siRNA (NTC-siRNA)-based effects in terms of the modulation of cell viability and nucleic acid sensing pathways using two cellular models *in vitro*, namely A431 skin cancer cells and BJ normal human fibroblasts. NTC-siRNA transfection limited cell viability of cancer cells that was mediated by changes in selected components of nucleic acid sensing pathways. NTC-siRNA transfection also sensitized doxorubicin-induced senescent cancer cells to apoptotic cell death that may have therapeutic implications. We postulate that NTC-siRNA-related effects should be considered while conducting and interpreting siRNA-mediated gene silencing experiments and results, respectively.

2. Materials and methods

2.1. Cell lines and culture conditions

Human foreskin fibroblasts BJ (ATCC® CRL-2522™, ATCC, Manassas, VA, USA) and human squamous carcinoma A431 cells were used (85090402, ECACC, Public Health England, Porton Down, Salisbury, UK). Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM medium with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B, Corning, Tewksbury, MA, USA) at 37 °C in the presence of 5% CO_2. Cells were routinely passaged using trypsin/EDTA solution. Proliferatively active fibroblasts were only used as described previously, namely at population doubling levels (PDLs) between 30 and 40 [14].

2.2. Non-targeting control siRNA transfection

To test the effects of non-targeting control siRNA (NTC-siRNA), control siRNA (fluorescein conjugate)-A (sc-36869, Santa Cruz Biotechnology, Dallas, TX, USA) and siGENOME non-targeting siRNA Pool #1 (D-001206-13, Dharmacon™, Horizon Discovery Ltd., Lafayette, CO, USA) were used. The protocol of siRNA transfection was used according to suppliers' recommendations. Briefly, 30 and 60 nM NTC-siRNA, Lipofectamine™ 3000 Transfection Reagent (L3000001, Thermo Fisher Scientific, Waltham, MA, USA) and two time points, namely 24 and 48 h, were considered. The uptake of control siRNA fluorescein conjugate was routinely monitored using fluorescence microscopy.

2.3. MTT test

The effects of NTC-siRNA-mediated changes in metabolic activity were tested using MTT assay. Briefly, A431 cells were transfected with 30 and 60 nM NTC-siRNA (sc-36869, Santa Cruz Biotechnology, Dallas, TX, USA) for 24 and 48 h using lipofection, and standard protocol of MTT test was applied [15]. Metabolic activity at control growth conditions (untreated control) was considered as 100%. Furthermore, to select doxorubicin (DOX, 44583, Merck KGaA, Darmstadt, Germany) concentration for further analysis (DOX-induced senescence model), A431 cells were treated with 35 nM, 50 nM and 1 μM DOX for 24 and 48 h, and MTT test was then assayed.

2.4. Apoptosis

The pro-apoptotic activity of 60 nM NTC-siRNA (sc-36869, Santa Cruz Biotechnology, Dallas, TX, USA) was analyzed after 24 and 48 h post-transfection in A431 cells, namely NTC-siRNA-induced apoptosis was evaluated using a Muse® Cell Analyzer and Muse® Annexin V and Dead Cell Assay Kit according to manufacturer's instructions (Luminex Corporation, Austin, TX, USA). For evaluation of phosphatidylserine externalization, cells were stained with Annexin V and 7-AAD (7-aminoactinomycin D), and flow cytometry approach was considered to revealed four cell subpopulations, namely (1) Annexin V (−) and 7-AAD (−) (live cells), (2) Annexin V (+) and 7-AAD (−) (early apoptotic cells), (3) Annexin V (+) and 7-AAD (+) (late apoptotic cells/necrotic cells), and (4) Annexin V (−) and 7-AAD (+) (necrotic cells) (%). The pro-apoptotic activity of 60 nM NTC-siRNA (sc-36869, Santa Cruz Biotechnology, Dallas, TX, USA) was also analyzed in DOX-induced senescent skin cancer cells and hydrogen peroxide-stimulated senescent fibroblasts. To induce chemotherapy-promoted senescence program, A431 cells were treated with 35 nM DOX for 48 h, the drug was removed, and cells were then cultured up to 7 days with medium change every 48 h. To induce oxidative stress-mediated senescence in normal cells, proliferatively active fibroblasts were stimulated twice with 100 μM hydrogen peroxide for 2 h at 24 h interval (HP, 95321, Merck KGaA, Darmstadt, Germany) and then cultured up to 7 days with medium change every 48 h. Cells were then subjected to standard siRNA transfection protocol (two NTC-siRNAs were considered, namely sc-36869 (Santa Cruz Biotechnology, Dallas, TX, USA) and D-001206-13 (Dharmacon™, Horizon Discovery Ltd., Lafayette, CO, USA), and 48 h post-transfection, NTC-siRNA-mediated apoptosis was assayed as described above (phosphatidylserine externalization). Furthermore, for NTC-siRNA-treated DOX-induced senescent cancer cells, caspase activation assay was also considered, namely Muse® Caspase-3/7 Kit (Luminex Corporation, Austin, TX, USA) and flow cytometry analysis. Four cell subpopulations were distinguished, namely (1) Caspase-3/7 activity (−) and 7-AAD (−) (live cells), (2) Caspase-3/7 activity (+) and 7-AAD (−) (early apoptotic cells), (3) Caspase-3/7 activity (+) and 7-AAD (+) (late apoptotic cells/necrotic cells), and (4) Caspase-3/7 activity (−) and 7-AAD (+) (necrotic cells) (%). The effects of lipofectamine action were also analyzed.

2.5. Immunofluorescence

A431 skin cancer cells were treated with NTC-siRNA from Santa Cruz Biotechnology (sc-36869, Dallas, TX, USA), whereas BJ fibroblasts were treated with NTC-siRNA from Dharmacon™ (D-001206-13, Horizon Discovery Ltd., Lafayette, CO, USA). The effects of 60 nM NTC-siRNA-mediated changes on selected components of nucleic acid sensing pathways were studied 24 h post-transfection. Briefly, BJ and A431 cells were fixed and immunostained as described elsewhere [16]. The following primary and secondary antibodies were used, namely anti-LRP8 (PA5-109269, 1:50), anti-ALY (PA5-96489, 1:50), anti-APOBEC3A (PA5-99584, 1:100), anti-APOBEC3G (PA5-89318, 1:50), anti-STING (MA5-26030, 1:100), anti-phospho-STING (Ser366) (PA5-105674, 1:100), anti-RIG-I (700366, 1:100), anti-MDA5 (700360, 1:100), anti–NF–κB (PA5-16545, 1:50), anti-DNMT2/TRDMT1 (sc-365001, 1:100), anti–IFN–β (ab176343, 1:200), secondary antibodies conjugated to Texas Red (1:1000, T-2767) or Texas Red-X (1:1000, T-6390) (Thermo Fisher Scientific, Waltham, MA, USA, Abcam, Cambridge, UK, and Santa Cruz Biotechnology, Dallas, TX, USA). Digital cell images were acquired using a laser-based confocal imaging and HCA system IN Cell Analyzer 6500 HS (Cytiva, Marlborough, MA, USA). Quantitative analysis was performed using IN Carta software (Cytiva, Marlborough, MA, USA). The levels of analyzed proteins are presented as relative fluorescence units (RFU). When applicable, immuno-specific foci were automatically scored.

2.6. qRT-PCR

A431 skin cancer cells were treated with NTC-siRNA from Santa Cruz Biotechnology (sc-36869, Dallas, TX, USA). NTC-siRNA-mediated changes in gene expression of selected genes (*CDKN2D*, *MYC*, *TRDMT1*) were investigated 24 h post-transfection. Briefly, RNA was

isolated using GenElute™ Mammalian Total RNA Miniprep Kit (Merck KGaA, Darmstadt, Germany) and cDNA was synthesized using 2 μg of RNA and Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The expression of *CDKN2D*, *MYC* and *TRDMT1* genes was evaluated using Applied Biosystems StepOnePlus™ Real-Time PCR System and dedicated probes, namely HS00176481 (*CDKN2D*), HS00153408 (*MYC*) and HS00189402 (*TRDMT1*), respectively, according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). *GAPDH* gene (HS03929097) was used as a housekeeping gene. Gene expression data were normalized to control (CTR).

2.7. Statistical analysis

Results are presented as the mean ± SD from at least three independent experiments. If applicable, box and whisker plots were also considered. Differences between control conditions and treated samples (MTT assay, apoptosis) were evaluated using one-way ANOVA and Dunnett's multiple comparison test. Differences between control conditions and treated samples (uptake, protein levels, gene expression, senolysis) were assessed using Student's *t*-test. Statistical significance was revealed using GraphPad Prism 5. *P*-values of less than 0.05 were considered significant.

3. Results and discussion

3.1. Non-targeting control siRNA (NTC-siRNA) promotes apoptosis in skin cancer cells

We have previously observed that non-targeting control siRNA (NTC-siRNA) may promote IFN-β-mediated pro-inflammatory response in normal human fibroblasts (WI-38 and BJ cells) as a side effect of siRNA transfection [14]. Of course, the concentration of NTC-siRNA was used according to manufacturer's protocol and cell viability was not affected [14]. Thus, this pro-inflammatory response was not due to excessive amount of NTC-siRNA. We did not evaluate detailed molecular mechanisms underlying NTC-siRNA-associated adverse effects [14]. As mRNA levels of *DNMT2/TRDMT1* (RNA methyltransferase) were found to be elevated upon transfection with NTC-siRNA, we postulated that DNMT2/TRDMT1 may be involved in exogenous RNA-mediated responses in human fibroblasts [14]. However, little is known if NTC-siRNA-based side effects are cell type specific or more common responses. This issue deserves further elucidation as siRNA transfection approach is a standard and widely used procedure in genetic engineering and siRNA-based side effects may affect obtained results and data interpretation.

In the present study, we have used human skin cancer cells, namely A431 cells as a cellular model to investigate NTC-siRNA-mediated changes in the activity of nucleic acid sensing pathways and accompanying effects on cell viability. As a comparison, BJ normal human fibroblasts were used to analyze if NTC-siRNA-associated effects on nucleic acid sensing pathways may be considered as a common phenomenon. Furthermore, for experiments involving BJ cells another NTC-siRNA was used that was purchased for alternative company (see Materials and Methods for details).

Firstly, we have used a commercially available FITC conjugate of NTC-siRNA at two recommended concentrations of 30 and 60 nM (concentrations ranging from 20 to 80 nM are recommended to be used) and evaluated NTC-siRNA-mediated effects on metabolic activity (MTT assay) upon 24 and 48 h post-transfection in A431 cells (Fig. 1A).

No significant effects were observed when 30 nM NTC-siRNA was used (Fig. 1A). In contrast, 60 nM NTC-siRNA transfection for 24 and 48 h decreased metabolic activity of 10 and 27% compared to untreated control, respectively (Fig. 1A). No effects of lipofection reagent (lipofectamine) were observed (Fig. 1A). FITC conjugate of NTC-siRNA was taken up by A431 cells as judged by increased fluorescence signals upon

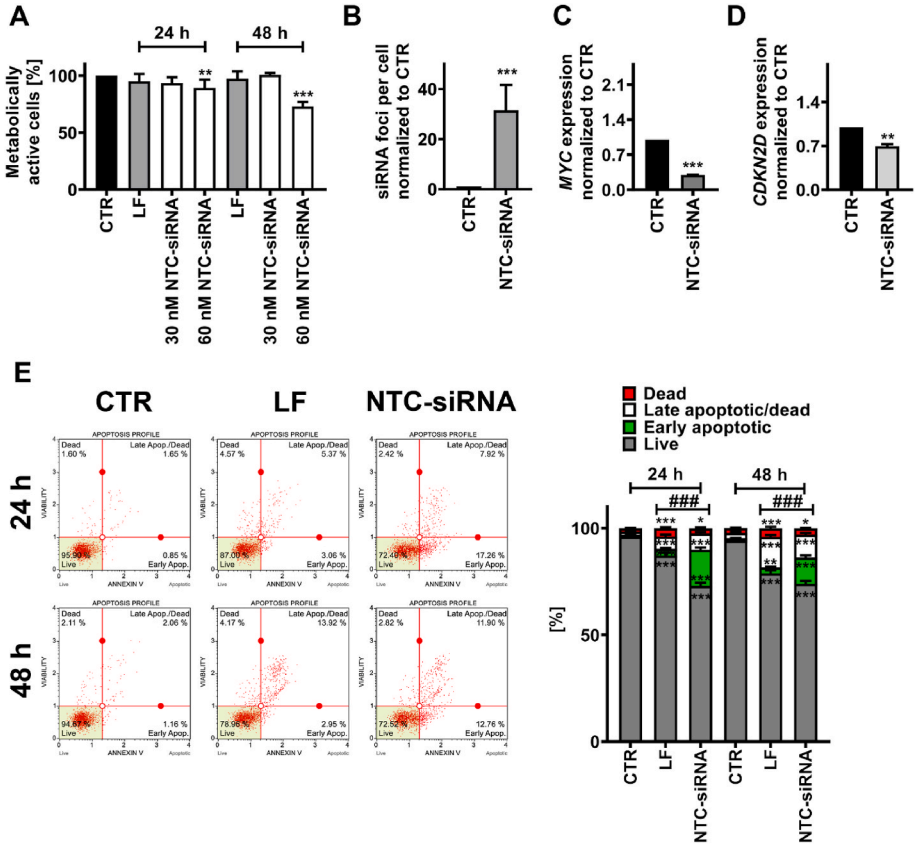

Fig. 1. NTC-siRNA-mediated effects on proliferation and cytotoxicity parameters in A431 skin cancer cells. (A) Metabolic activity was assayed using MTT test. Two concentrations and time points were considered. The effect of lipofection (LF) reagent is also shown. Metabolic activity at control untreated conditions is assumed as 100%. (B) NTC-siRNA uptake was revealed using FITC conjugate of NTC-siRNA and imaging flow cytometry. (C, D) NTC-siRNA-mediated expression of proliferation-related genes, namely *MYC* (C) and *CDKN2D* (D) was analyzed using qRT-PCR and dedicated probes. (E) NTC-siRNA-induced apoptosis was assayed using flow cytometry and Annexin V staining. Representative dot plots are shown. (A–E) Bars indicate SD, n = 3. (A, E) $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ compared to control (CTR) (ANOVA and Dunnett's a posteriori test), $^{\#\#\#}p < 0.001$ compared to lipofection reagent (LF) treatment (Student's *t*-test). (B, C, D) $^{***}p < 0.001$, $^{**}p < 0.01$ compared to control (CTR) (Student's *t*-test). CTR, control; LF, lipofection reagent stimulation; NTC-siRNA, transfection with non-targeting control siRNA (NTC-siRNA, sc-36869, Santa Cruz Biotechnology).

siRNA transfection (Fig. 1B). The levels of two regulators of cell proliferation were then analyzed in A431 cells, namely *MYC* (oncogene) and *CDKN2D* (p19) (Fig. 1C and D). NTC-siRNA-mediated decrease in the pools of *MYC* and *CDKN2D* was noticed that may indicate that NTC-siRNA may modulate cell proliferation in human skin cancer cells. It was previously reported that dedicated siRNA-based gene silencing may result in changes in non-targeted gene expression [4]. Elevated protein expression of two cell cycle regulators, namely p53 and p21 was observed in human cervical cancer cells HeLa, Ca Ski and SiHa cells, and breast cancer cells MCF-7 cells as a side effect (off-target effect) of siRNA transfection [4]. The authors postulated that it would be difficult to identify the interactions underlying the siRNA effects as p53 and p21 can be activated as a response to a number of stress stimuli such as DNA damage inducing conditions, oxidative stress, excessive mitogenic stimuli, and interferon activation [4].

We have then studied if NTC-siRNA-mediated changes in metabolic activity (Fig. 1A) may reflect NTC-siRNA-associated cytotoxicity in A431 cells (Fig. 1E). Indeed, NTC-siRNA transfection promoted apoptotic cell death in skin cancer cells (Fig. 1E). Some cytotoxic effects of lipofection reagent were also observed upon 48 h post-transfection in skin cancer cells (Fig. 1E). We have previously observed that siRNA transfection protocol had limited effects on cell viability of human normal fibroblasts, namely WI-38 and BJ cells [14]. NTC-siRNA promoted very slight, but significant 3% increase in the levels of early apoptotic cells upon NTC-siRNA transfection in BJ cells compared to untreated control, whereas NTC-siRNA did not affect cell viability of WI-38 cells [14]. NTC-siRNA also had no effect on the levels of a marker of cell proliferation, namely Ki67 in WI-38 and BJ cells [14]. NTC-siRNA also did not modulate the cell cycle progression in WI-38 cells, however, NTC-siRNA promoted the G_0/G_1 cell cycle arrest in BJ cells as 6% increase in the levels of BJ cells at the G_0/G_1 cell phase was noticed compared to untreated cells [14]. Thus, this may suggest that NTC-siRNA-related effects on cell viability and proliferation may reflect different genetic backgrounds of the same cell type used, namely two different fibroblast cell lines, and these observations may be also diverse in normal and cancer cells, namely fibroblasts and skin cancer cells (this

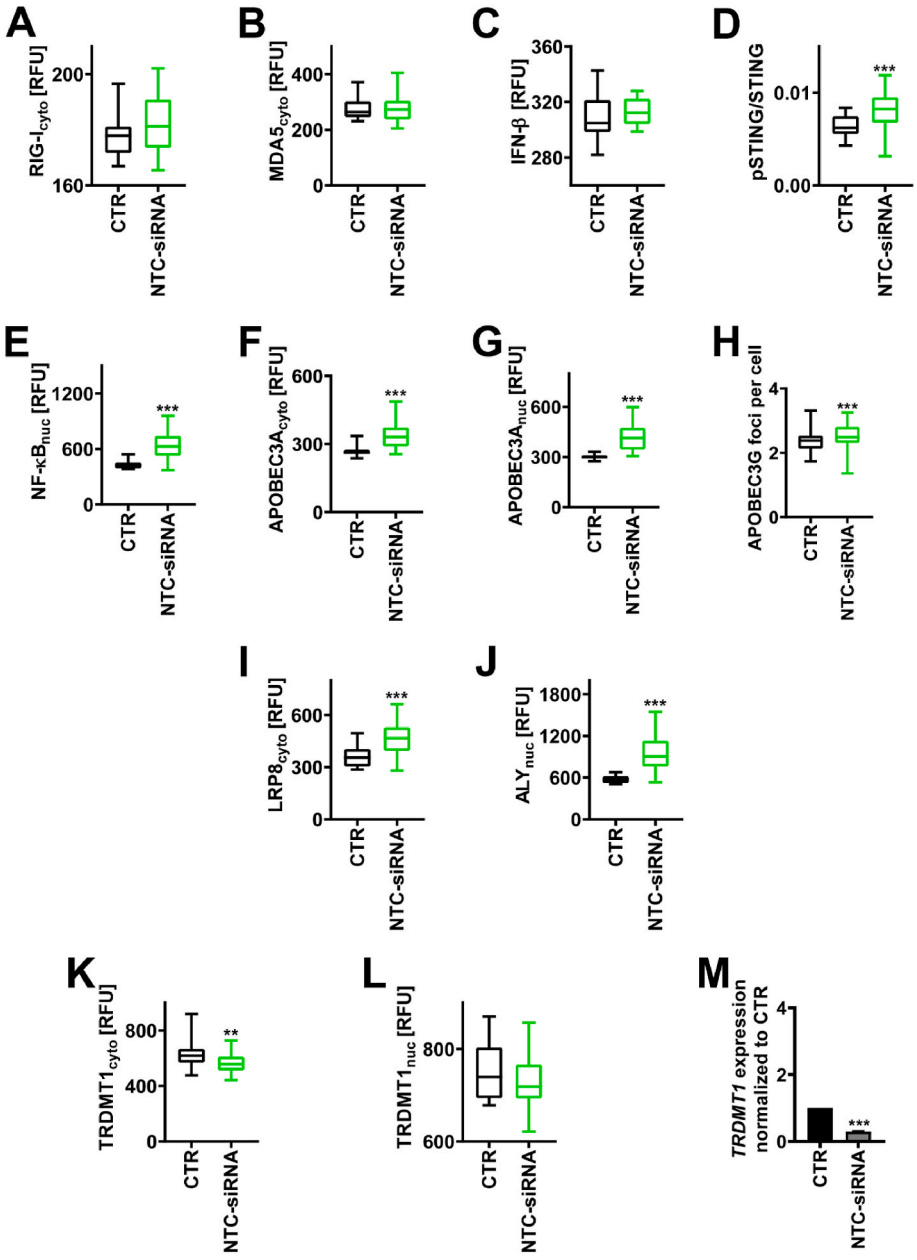

Fig. 2. NTC-siRNA-mediated activation of nucleic acid sensing pathways in A431 skin cancer cells. Cells were transfected with non-targeting control siRNA (sc-36869, Santa Cruz Biotechnology), fixed and selected components of nucleic acid sensing pathways and related responses were then analyzed using immunostaining and imaging flow cytometry. If applicable, qRT-PCR was also used to analyze gene expression at mRNA levels (M). Box and whisker plots are shown (A–L) or bars indicate SD (M), n = 3. ***$p < 0.001$, **$p < 0.01$ compared to control (CTR) (Student's *t*-test). Protein levels are presented as relative fluorescence units (RFU) (A, B, C, E, F, G, I, J, K, L). Phosphorylated signals of STING are presented as a ratio of phosphorylated STING to unmodified STING (D). APOBEC3G is presented as a number of foci per cell (H). *DNMT2/TRDMT1* expression was normalized to control (CTR) (M). CTR, control; NTC-siRNA, transfection with non-targeting control siRNA (NTC-siRNA).

study and [14]).

We have then analyzed selected signaling pathways that may affect cell viability and modulate adaptive responses upon NTC-siRNA transfection in human cancer cells (Fig. 2) and for comparison, in human normal fibroblasts (Fig. 3).

3.2. NTC-siRNA-mediated effects on nucleic acid sensing pathways and related proinflammatory response in skin cancer cells and normal fibroblasts

Mammalian cells can respond to the presence of viral and non-viral (extracellular and endogenous) RNA and DNA by activating several pathways that engage innate immune effectors and the induction of type-I interferon (IFN-I), and modulate a number of cell-intrinsic and cell-extrinsic processes, such cell proliferation and senescence, DNA replication and DNA damage repair, apoptosis and autophagy, cell stemness, angiogenesis, cell metabolism, antiviral and anticancer immunity, but their biological outcomes can be considered complex and heterogenous [17–19]. Pattern recognition receptors (PRRs) can sense foreign pathogen associated molecular patterns (PAMPs) or host-derived danger associated molecular patterns (DAMPs) such as DNA (the cGAS-STING pathway) or RNA (Rig-I-like receptor (RLR) pathway) [17,18,20]. Three RLRs (cytosolic RNA sensors) can be distinguished, namely RIG-I, MDA5, and laboratory of genetics and physiology 2 (LPG2) [18]. RIG-I and MDA5 can sense 5-triphosphate containing RNA (5′ppp) or long dsRNA, respectively [18]. However, a crosstalk between DNA and RNA sensing pathways is also suggested [12,13]. Thus, we decided then to analyze NTC-siRNA-mediated changes in both nucleic acid sensing pathways in A431 cells (Fig. 2) and BJ cells (Fig. 3) using NTC-siRNA from two different suppliers. NTC-siRNA did not affect the levels of RIG-I and MDA5 in A431 cells (Fig. 2A and B). In contrast, cytosolic levels of RIG-I, but not MDA5, were elevated in BJ cells upon NTC-siRNA stimulation (Fig. 3A and B). NTC-siRNA also did not stimulate IFN-β production in A431 cells (Fig. 2C) that suggest that NTC-siRNA-induced apoptosis (Fig. 1E) is not mediated by increased secretion of IFN-β in skin cancer cells. The data on siRNA-mediated changes in IFN-β secretion can be considered contradictory [4,14]. No interferon response was observed upon siRNA stimulation in human cervical cancer cells [4]. In contrast, NTC-siRNA transfection resulted in elevated levels of IFN-β in two different human fibroblast cell lines (WI-38 and BJ cells) as judged by Western blot-based results [14]. An increase in the levels of IFN-β was also confirmed in NTC-siRNA-treated BJ cells using imaging cytometry (Fig. 3C). Thus, one can conclude that perhaps siRNA-based stimulation of IFN-β production can be considered as a cell type specific phenomenon and its involvement in the regulation of cell viability requires further studies as siRNA-mediated increase in the levels of IFN-β (Fig. 3C) was not associated with elevated cytotoxicity in BJ cells [14]. Chemically synthesized siRNA also enhanced DNA-mediated IFN-λ1 induction in HeLa cells [8]. Type III IFN signaling was promoted through crosstalk between RNA sensor RIG-I and DNA

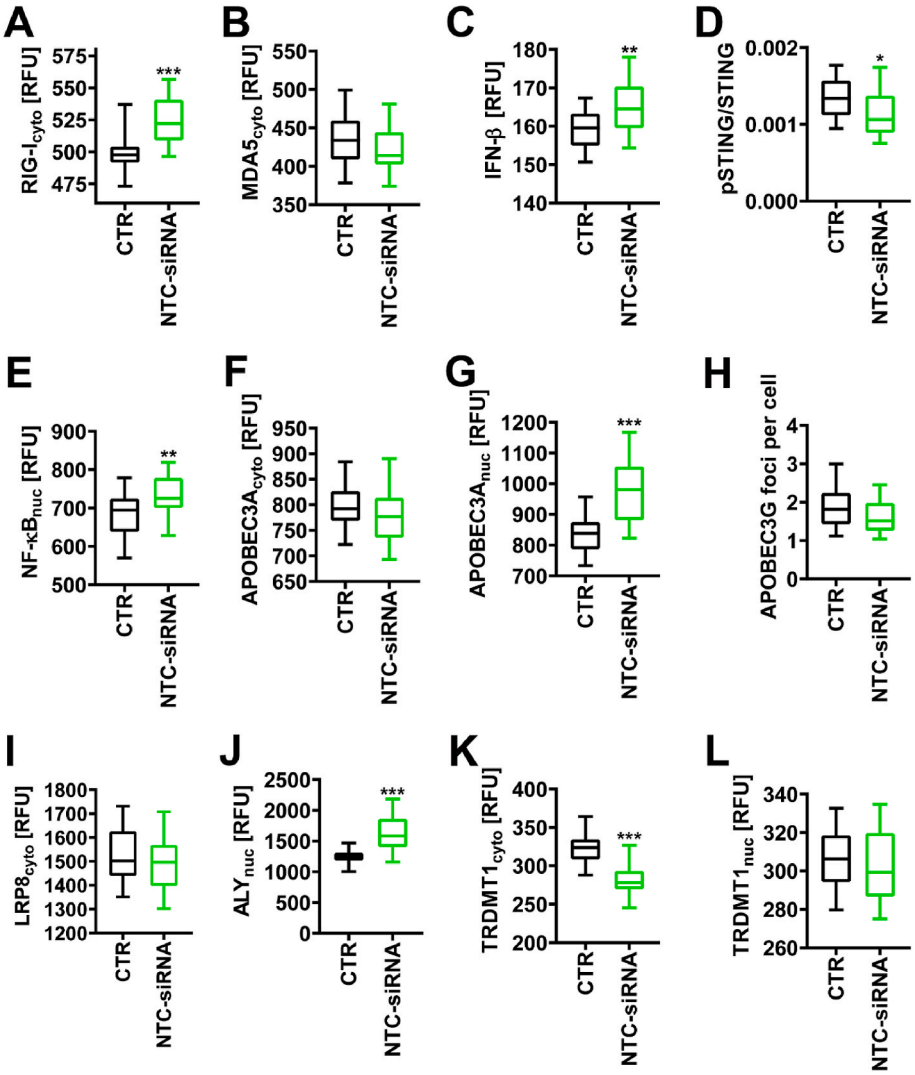

Fig. 3. NTC-siRNA-mediated activation of nucleic acid sensing pathways in BJ fibroblasts. Cells were transfected with non-targeting control siRNA (D-001206-13, Dharmacon™, Horizon Discovery Ltd.), fixed and selected components of nucleic acid sensing pathways and related responses were then analyzed using immunostaining and imaging flow cytometry. Box and whisker plots are shown (A–L), n = 3. ***$p <$ 0.001, **$p <$ 0.01, *$p <$ 0.05 compared to control (CTR) (Student's *t*-test). Protein levels are presented as relative fluorescence units (RFU) (A, B, C, E, F, G, I, J, K, L). Phosphorylated signals of STING are presented as a ratio of phosphorylated STING to unmodified STING (D). APOBEC3G is presented as a number of foci per cell (H). CTR, control; NTC-siRNA, transfection with non-targeting control siRNA (NTC-siRNA).

sensor [8]. The activation of downstream STING and TANK-binding kinase 1 (TBK1) was required for IFN-λ1 induction [8]. Thus, the STING-TBK1-IRF3 pathway can be also involved in siRNA-based proinflammatory response [8]. In our experimental conditions, NTC-siRNA transfection resulted in elevated levels of phosphorylated form of STING that was accompanied by NF-κB activation in A431 cells (Fig. 2D and E). In contrast, in NTC-siRNA-treated BJ cells, there was no activation of STING (Fig. 3D), but elevated pools of nuclear NF-κB were noticed (Fig. 3E) that indicate that STING activation is not required for NTC-siRNA-induced NF-κB-based inflammation in normal fibroblasts (Fig. 3D and E). Cytosolic RNA:DNA hybrids, a novel class of intracellular PAMP molecules, were also characterized as activators of the cGAS-STING pathway [21]. A number of crosstalks between RIG-I and cGAS-STING pathways was described [12]. For example, DNA can induce upregulation of *RIG-I* mRNA, whereas RNA viruses can promote upregulation of *STING* mRNA, assembly of the RIG-I/MAVS/STING complex, coactivation of RIG-I and STING pathways, thus potentiating IFN response [12]. However, in our experimental setting, STING activation was not accompanied by increased production of IFN-β in A431 cells (Fig. 2C and D) and STING activation was not observed in NTC-siRNA-treated BJ cells with elevated levels of IFN-β (Fig. 3C and D). Instead, STING activation was accompanied by NF-κB activation in NTC-siRNA-treated A431 cells (Fig. 2E). It was previously reported that STING can trigger proinflammatory cytokine response (TNF, IL-1β, IL-6) by NF-κB activation [22,23]. TBK1 and IKKε kinases can act redundantly to mediate STING-induced NF-κB responses for antiviral and antitumor immunity [24]. Increased phosphorylated signals of STING and NF-κB activation were also accompanied by limited cell viability (apoptosis induction) in skin cancer cells subjected to NTC-siRNA (Fig. 1E). Interestingly, DNA demethylating anticancer drugs (e.g., 5-aza-2-deoxycytidine) can promote their antiproliferative effects by the formation of dsRNAs and activation of the MDA5/MAVS/IRF7 pathway [25]. Thus, DNA methylation inhibitors stimulated cancer cells to behave as virus-infected cells, and promote an MDA5/MAVS/IRF7-dependent ‘‘viral mimicry’’ state [25]. However, more recently, it was documented that cGAS-STING can also drive the IL-6-dependent survival of chromosomally instable cancers [26]. Chromosomal instability (CIN) promoted the cGAS-STING pathway and the non-canonical NF-κB pathway-dependent IL-6-STAT3-based signaling, and inactivation of cGAS-STING signaling impaired the survival of triple-negative breast cancer cells [26]. Thus, the role of cGAS-STING pathway in cancer cell survival can be considered complex.

APOBEC protein family (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) consists of 11 members in humans, namely activation-induced cytidine deaminase (AID), APOBEC1 (A1), APOBEC2 (A2), APOBEC3 (A3; with seven subfamily members - A3A, A3B, A3C, A3D, A3F, A3G, A3H), and APOBEC4 (A4) [27,28]. APOBECs are able to deaminate cytosine residues to uracil in ssDNA and RNA that may result in somatic mutations, DNA breaks, RNA modifications, or DNA demethylation [27,28]. Besides the role of APOBECs in virus restriction as a part of the innate antiviral immunity [29], APOBEC-mediated mutagenesis is widespread in human cancers that could be correlated with cancer development and poor prognosis [30–33]. Viral infection and genotoxic stress can induce the expression of APOBEC3A and pro-inflammatory genes through two distinct pathways [34]. APOBEC3A expression can be induced upon the stimulation of the transcription factor STAT2 by RIG-I, mitochondrial antiviral-signaling protein (MAVS), interferon regulatory factor 3 (IRF3), and IFN-dependent immune response [34]. Furthermore, APOBEC3A expression can be stimulated by an IFN-independent manner, namely genotoxic stress can promote the levels of APOBEC3A and other pro-inflammatory genes through the activation of the NF-κB pathway [34]. As APOBECs can operate in various cellular compartments [27], we decided then to analyze the cytosolic and nuclear pools of APOBEC3A upon siRNA stimulation (Fig. 2F and G, and Fig. 3F and G). Indeed, siRNA promoted the levels of APOBEC3A in both cellular compartments in A431 cells (Fig. 2F and G) and nuclear levels of APOBEC3A in BJ cells (Fig. 3G). Stimulation with siRNA also induced the levels of APOBEC3G in A431 cells, but not in BJ cells (Figs. 2H and 3H). Increased expression of APOBEC3A was not accompanied by increased secretion of IFN-β, but STING and NF-κB activation was noticed in A431 cells (Fig. 2). In contrast, in siRNA-stimulated BJ cells, elevated levels of nuclear APOBEC3A were accompanied by increased production of IFN-β and NF-κB activation (Fig. 3). More recently, it was reported that APOBEC3A can bind to IFN-stimulated response elements (ISRE) that inhibited phosphorylated STAT1 binding and suppressed IFN-stimulated gene 15 (ISG15) induction in response to IFN-I treatment [35]. Thus, in selected experimental settings, APOBEC3A may contribute to a negative feedback loop during IFN signaling [35]. APOBEC3A, upregulated across cancer types, can also drive CIN and metastasis in a STING-dependent manner and independently of the canonical deaminase functions [36]. Thus, siRNA-mediated upregulation of APOBEC3A in cancer cells (this study) may have pleiotropic effects.

Low-density lipoprotein receptor-related protein 8 (LRP8, also known as apolipoprotein E receptor 2, ApoER2) may have diverse roles during viral infections [37,38]. LRP8 promoted antiviral effects during respiratory syncytial virus (RSV) infection [37]. LRP8/ApoER2 can be also a receptor for multiple alphaviruses [38]. Stimulation with siRNA also promoted the levels of LRP8/ApoER2 in skin cancer cells (Fig. 2I), but not in fibroblasts (Fig. 3I). This may be a part of response to extracellular RNA (siRNA transfection) in cancer cells. However, increased levels of LRP8 can also promote oncogenic effects in triple-negative breast cancer (TNBC) cells [39]. Inhibition of LRP8 can significantly suppress breast cancer stem cells (BCSCs) and tumorigenesis in TNBC *via* Wnt/β-catenin signaling attenuation [39]. Thus, the stimulation of LRP8/ApoER2 levels in cancer cells should be carefully monitored as this may promote some adverse effects.

As ALY/REF, an mRNA export adaptor and m^5C-binding protein, functions in promoting mRNA export, including viral mRNA export, and thus modulating viral infections [40–42], we decided then to evaluate the levels of ALY upon NTC-siRNA stimulation (Figs. 2J and 3J). Indeed, nuclear pools of ALY were increased after siRNA transfection (Figs. 2J and 3J), thus ALY may be involved in the response to extracellular siRNA in both normal and cancer cells.

DNMT2/TRDMT1 methyltransferase can promote the stability of RNA and survival of human immunodeficiency virus 1 (HIV-1) by RNA cytosine methylation [43]. We have also observed that IFN-β-mediated response to the presence of NTC-siRNA was accompanied by elevated mRNA levels of *DNMT2/TRDMT1*, but not protein levels, in human WI-38 and BJ fibroblasts [14]. More recently, it was documented that Trdmt1 may play a protective role in LPS-induced inflammation by regulating the TLR4-NF-κB/MAPK-TNF-α pathway in rats [44]. As upon viral infection, DNMT2 can re-localize from the nucleus to the stress granules (SGs) [43], we have then analyzed the cytoplasmic and nuclear fractions of DNMT2/TRDMT1 in skin cancer cells and normal fibroblasts (Fig. 2K and L, and Fig. 3K and L, respectively). However, the levels of cytosolic DNMT2/TRDMT1 were decreased, while the levels of nuclear DNMT2/TRDMT1 were unaffected upon NTC-siRNA stimulation in A431 and BJ cells (Fig. 2K and L, and Fig. 3K and L, respectively). *DNMT2/TRDMT1* gene expression was also decreased after siRNA transfection in A431 cells (Fig. 2M). This may suggest that DNMT2/TRDMT1-based response to extracellular siRNA may be cell type specific and limited to the regulation at the transcriptional level (this study and [14]).

3.3. NTC-siRNA promotes senolytic effect during chemotherapy-induced senescence in skin cancer cells, but not during oxidative stress-induced senescence in normal fibroblasts

As NTC-siRNA was found to be cytotoxic to skin cancer cells when used at recommended concentration (Fig. 1E), we decided then to evaluated if NTC-siRNA may promote the elimination of chemotherapy-

induced senescent skin cancer cells (Fig. 4).

We have considered a well-established chemotherapy-induced model of senescence, namely doxorubicin treatment [45]. Indeed, NTC-siRNA stimulation in doxorubicin-induced senescent skin cancer cells promoted apoptotic cell death as judged by two biomarkers of apoptosis, namely phosphatidylserine externalization (Fig. 4A) and caspase 3/7 activity (Fig. 4B). Thus, one can conclude that NTC-siRNA can exert senolytic effects in chemotherapy-induced senescent skin cancer cells (Fig. 4). We were also interested if senolytic effect of NTC-siRNA can be also observed in normal cells subjected to stress-induced senescence. Thus, we have stimulated BJ fibroblasts with hydrogen peroxide, an oxidative stress stimulus as previously described [14] and analyzed NTC-siRNA-mediated apoptosis in senescent fibroblasts (Fig. 5).

We have considered two different siRNA controls that were purchased from two different suppliers (Fig. 5A and B, respectively). NTC-siRNA had no senolytic potential in stress-induced senescent fibroblasts (Fig. 5). Perhaps senolytic effects of NTC-siRNA may be cell type specific and limited to cancer cells and/or pro-senescent factor, here doxorubicin (Figs. 4 and 5), and also may be modulated by ploidy status and gene mutations. Senolysis, a selective elimination of senescent normal and cancer cells, may be achieved by two experimental setups, namely genetic engineering (genetic approach) and the use of senolytic drugs (pharmacological approach) that may in turn promote healthspan and lifespan in animal models and humans [46–48]. Perhaps NTC-siRNA can be considered as a novel senolytic agent in cancer cells (this study). This surprising observation may have potential therapeutic implications. However, further studies are needed to confirm such assumptions.

In conclusion, we have shown that NTC-siRNA may activate nucleic acid sensing pathways, promote NF-κB activity and limit cell viability of skin cancer cells as a side effect. The activation of selected components of nucleic acid sensing pathways was also documented in NTC-siRNA-treated normal human fibroblasts. However, we have previously reported that NTC-siRNA transfection was not accompanied by cytotoxic effects in WI-38 and BJ fibroblasts [14]. NTC-siRNA stimulation may also result in selective eradication of chemotherapy-induced senescent skin cancer cells, thus having beneficial effects (this study). Similar senolytic effects of siRNA were not observed in stress-induced senescent BJ fibroblasts. More studies are needed to reveal detailed molecular mechanisms underlying the effects of NTC-siRNA on normal and cancer cells.

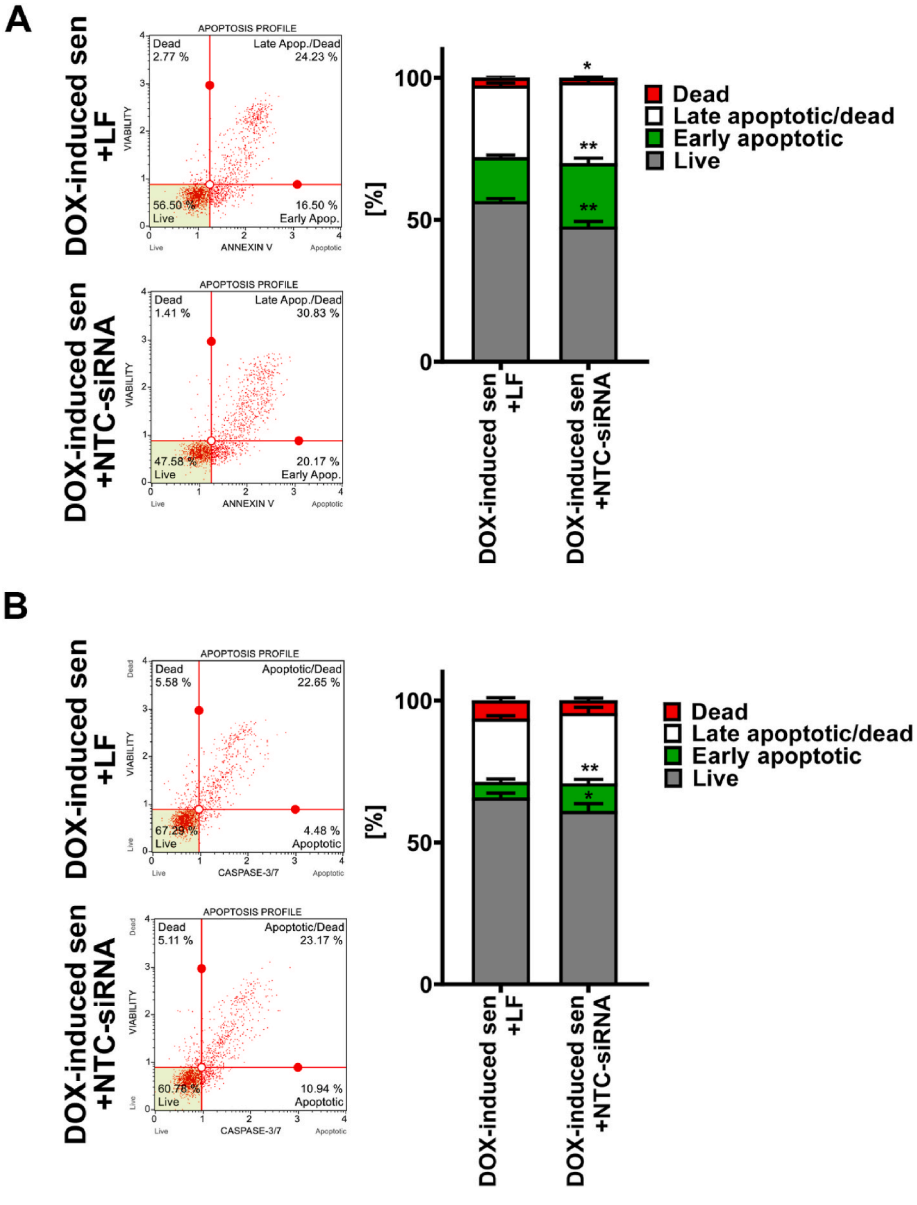

Fig. 4. NTC-siRNA promotes senolysis in doxorubicin-induced senescent skin cancer cells. A431 cells were treated with 35 nM doxorubicin to induce chemotherapy-mediated senescence program. 7 days after drug removal, senescent cells were transfected with NTC-siRNA (sc-36869, Santa Cruz Biotechnology) and two biomarkers of apoptotic cell death were then investigated, namely phosphatidylserine externalization (A) and caspase 3/7 activity (B). Apoptosis-mediated senolysis was documented using a Muse® Cell Analyzer and Muse® Annexin V and Dead Cell Assay Kit (A) or Muse® Caspase-3/7 Kit (B). Bars indicate SD, n = 3, $^{**}p < 0.01$, $^{*}p < 0.05$ compared to lipofection reagent (LF) treatment (Student's *t*-test). Representative dot plots are also presented. DOX-induced sen + LF, doxorubicin-induced senescent skin cancer cells treated with lipofection reagent (LF); DOX-induced sen + NTC-siRNA, doxorubicin-induced senescent skin cancer cells transfected with non-targeting control siRNA (NTC-siRNA).

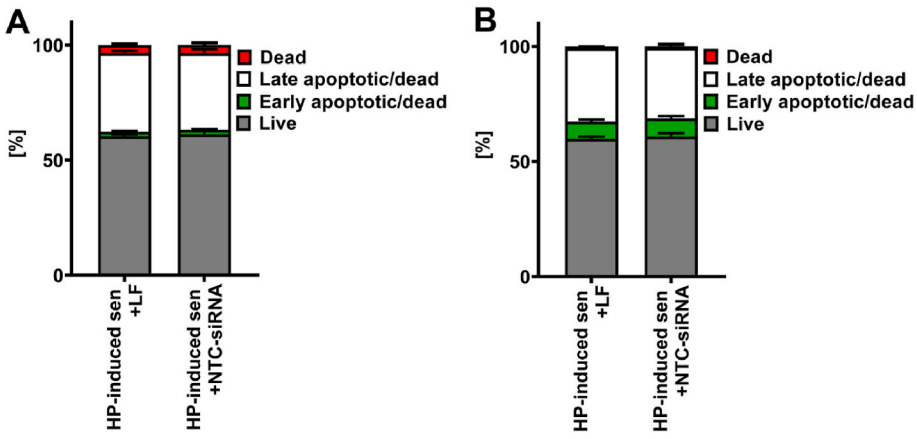

Fig. 5. The effect of NTC-siRNA on apoptosis induction in hydrogen peroxide-induced senescent BJ fibroblasts. BJ cells were treated with 100 μM hydrogen peroxide to induce oxidative stress-mediated senescence program. 7 days after oxidant removal, senescent fibroblasts were transfected with NTC-siRNA (A, sc-36869, Santa Cruz Biotechnology and B, D-001206-13, Dharmacon™, Horizon Discovery Ltd.) and a biomarker of apoptotic cell death was then assayed, namely phosphatidylserine externalization. Apoptosis was analyzed using a Muse® Cell Analyzer and Muse® Annexin V and Dead Cell Assay Kit. Bars indicate SD, n = 3. HP-induced sen + LF, hydrogen peroxide-induced senescent BJ fibroblasts treated with lipofection reagent (LF); HP-induced sen + NTC-siRNA, hydrogen peroxide-induced senescent BJ fibroblasts transfected with non-targeting control siRNA (NTC-siRNA).

Author contributions

Conceptualization, A.L., M.W.; methodology, M.W., A.L.; investigation, G.B., D.B., A.L. and M.W.; writing - original draft preparation, A.L.; writing - review and editing, M.W.; visualization, G.B., A.L.; supervision, A.L., M.W.; funding acquisition, A.L., M.W. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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