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Efficacy of TRAIL treatment against HPV16 infected cervical cancer cells undergoing senescence following siRNA knockdown of E6/E7 genes

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ABSTRACT

In this study we investigated E6 and E7 oncogenes from the Human Papilloma Virus as targets for siRNA knockdown in order to boost the efficacy of the anti-cancer drug 'tumor necrosis factor-related apoptosis inducing ligand' (TRAIL). SiHa cells were treated with TRAIL following transfection with E6/E7 siRNA and the expression of death receptors DR4 and DR5, cell viability, apoptosis, senescence and cell cycle analysis were undertaken using flow cytometry, MTT viability assay and cellular β-galactosidase activity assays. E6/E7 siRNA resulted in significant upregulation of death receptors DR4 and DR5 but did not result in an enhanced sensitivity to TRAIL. Our results indicate that E6/E7-siRNA induces senescence rather than apoptosis in SiHa cells. The occurrence of senescence in drug resistant cervical cancer cells such as the SiHa cell line by E6/E7 siRNA, among other factors, may prevent TRAIL induced activation of extrinsic and intrinsic pathways that lead to apoptotic cell death. Our findings are significant for combinatorial strategies for cancer therapy since the induction of senescence can preclude apoptosis rendering cells to be recalcitrant to TRAIL treatment.

1. Introduction

Every year, about half a million cases of cervical cancer are diagnosed worldwide [1]. According to the CDC, in the United States alone nearly 12,000 women were diagnosed with cervical cancer in 2006 and almost 4000 died [2]. In many developing countries, where 80% of new cases arise, it is the leading female malignancy and a common cause of death for middle-aged women [3,4]. Through epidemiological studies, it has been discovered that the sexually transmitted Human Papilloma Virus (HPV) is the etiological agent for cervical cancer [5,6]. HPV is a double-stranded DNA virus that infects human epithelial tissue resulting in warts, or in extreme cases, cancer. In a worldwide study, it was discovered that HPV DNA was detected in 93% of cervical tumors [7]. HPVs are divided into low-risk and high-risk categories according to their ability to transform epithelial cells and induce cancer. Examples of low-risk HPVs are HPV 6 and 11 which are responsible for 90% of genital warts whereas the high-risk subtypes such as HPV 16 and 18 are responsible for a combined 70% of cervical cancers [8]. HPV 16 is by far the most prevalent HPV that causes cervical cancer, being associated with about 50% of the cases.

The ability of HPV to transform normal epithelial tissue and the causative process have been well studied [9]. The HPV genome encodes for six early expressed genes (E1, E2, E4, E5, E6 and E7) which are associated with viral replication, and two late expressed genes (L1 and L2) for viral capsid proteins. Malignancy occurs through the pleiotropic transforming effects exerted by both E6 and E7 viral proteins. The *de novo* expressed E6 oncoprotein forms a complex with p53 and the ubiquitin-ligase enzyme which leads to the ubiquitination of p53 and its subsequent proteosomal degradation [10]. The removal of p53 allows cells to replicate indefinitely through the impairment of the of cells' ability to initiate apoptosis since p53 is no longer able to detect DNA damage and regulate the G_1/S cell cycle checkpoint. The E7 oncoprotein sequesters hypophosphorylated Retinoblastoma (pRb) which is a negative regulator of the transcription regulator E2F and promotes its eventual proteosomal degradation. As pRb is lost, E2F is free to release transcription factors that promote cell cycle progression and the eventual clonal expansion of transformed cells [1,11]. Expression of either E6 or E7 alone does not guarantee carcinogenesis; however, when they work in unison the chances that a cell will become oncogenic is drastically increased [11].

Among the newer strategies under investigation for targeted treatment of cancer, blocking viral oncogene expression using RNA interference (RNAi) offers the promise of high specificity combined with limited undesired side effects. RNAi is a gene silencing mechanism in which double stranded RNA molecules, known as

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short interfering RNA (siRNA), can block translation of gene specific mRNA through complementary base pairing [12]. For cervical cancer therapy, inhibition of E6 and E7 expression by siRNA in HPV transformed cells can potentially restore p53 and pRb function leading to restoration of the cell cycle checkpoints. Unfortunately, reports of the induction of apoptosis in E6/E7 siRNA treated cervical cells are inconsistent. RNA interference of E6 and E7 expression has shown some success in cultured cervical cancer cells with a gain of function observed for both p53 and pRb and subsequently, apoptosis [13]. However, in other studies, the siRNA-mediated gene silencing of E6 or E7 did not induce apoptosis, but rather exhibited only reduced cell proliferation [14]. Other cervical cancer cell lines such as the HPV 18 infected HeLa cells, when transfected with E6/E7 siRNA, undergo cellular senescence [13].

The targeted induction of apoptosis in cancer cells can be achieved using tumor necrosis factor-related apoptosis inducing ligand (TRAIL). TRAIL is a membrane bound and soluble ligand from the tumor necrosis factor family that has been the focus of basic as well as clinical research for cancer therapy due to its ability to promote apoptosis in cancerous cells while leaving healthy cells relatively unaffected [15,16]. TRAIL acts by binding to its own set of cellular receptors and is capable of inducing apoptosis through both an extrinsic and intrinsic mechanism [17]. There are two functional death receptors (DR), DR4 and DR5, that become active when TRAIL binds the receptors resulting in caspase-dependent apoptosis [18,19]. TRAIL can also activates the intrinsic pathway of apoptosis after binding the death receptors by promoting the Bcl2 family dependent release of cytochrome C from the mitochondria, formation of the apoptosome and subsequent caspase cascade, complementing p53 apoptotic activity [20,21]. The discovery of the presence of DR4 and DR5 in cervical neoplasia specimens suggests that therapy using TRAIL may be efficacious [22]. The *in vitro* effects of TRAIL on cultured cervical cancer cells have been mixed; while HeLa and CaSki cells show varying degrees of cytotoxicity, SiHa cells are not responsive [16,23].

In this study, we explore the strategy of a synergistic approach for the treatment of non-responsive SiHa cervical cancer cells involving induction of apoptosis specifically using a combination of siRNA and TRAIL. Our initial hypothesis was that the siRNA knockdown of E6 and/or E7 would prevent proteosomal degradation of p53 in SiHa cells and lead to the transactivation of the death receptor expression on the cell surface, subsequently sensitizing these cells to TRAIL and leading to induction of apoptosis. Contrary to our premise, we report here that the knockdown of E6/E7 genes in SiHa cervical cancer cells by siRNA leads to senescence rather than apoptosis and does not enhance sensitivity to TRAIL. To understand the mechanisms of this phenomenon, we compared our results to the effects of MG132, a proteosome inhibitor which can recover p53, increase DR5 expression, and lead to sensitization of SiHa cells to TRAIL [23]. Our results indicate that during combinatorial approaches for cancer therapy, the induction of senescence may preclude apoptosis, thus rendering cells recalcitrant to TRAIL treatment.

2. Materials and methods

2.1. Transfection of SiHa Cells

The human cervical cell line SiHa was obtained from the American Type Culture Collection (Manassas, VA) and cultured according the supplier's instructions. Transfections were performed with 10 nM of E6/E7 siRNA using HiPerfect Transfection Reagent (Qiagen, Valencia, CA). The guide strand sequence for E6 siRNA was 5′-AGACAUACAUCGACCGGUCCCA-3′ (base pair position 397, Naito et al. [24,25]). The guide strand sequence for E7 siRNA was 5′-CAUUCGUACUUUGGAAGACCUGUUA-3′ (base pair position 225) and its design is based on Stealth technology (Invitrogen, Carlsbad, CA). Two rounds of transfections were performed for each experiment; 1 and 4 days after plating the cells (See Table 1, Supplementary material). A medium-content GC (MGC) scrambled siRNA (Invitrogen) was used as the negative transfection control throughout the experiments. Western Blot Analysis of p53 levels was undertaken as mentioned in the Supplementary material section.

2.2. Flow cytometry analysis

Cells were analyzed for the expression of death receptors DR4 and DR5 by flow cytometry following transfection with E6/E7 or treatment with 10 μM MG132 according to Hougardy et al. [23]. For the analysis of apoptosis, cells were treated with siRNA, MG132 and TRAIL (see below) and stained with Annexin V (eBiosciences, San Diego, CA) and propidium iodide (Roche, Basel, Switzerland). For the analysis of cell cycle, siRNA, MG132 and TRAIL treated cells were fixed and stained with propidium iodide (Roche). The methods used are described in detail in the Supplementary material.

2.3. TRAIL treatment and analysis of cell viability by MTT assay

SiHa cells were plated 2500 cells per well in a 96 well cell culture plate in triplicates and transfected. Twenty-four hours after the second transfection, 1 μg/ml human recombinant TRAIL (Calbiochem/EMD Biochemicals, Gibbstown, NJ) was added to the wells and incubated overnight. For the MG132 treatment, cells were pretreated with 10 μM MG132 for 2 h and then TRAIL was added. Following a 24 h incubation, viability of the cells was analyzed with an MTT cell proliferation kit (Trevigen, Gaithersburg, MD).

2.4. Senescence activated β-galactosidase staining

Cells were stained for β-galactosidase activity using the Senescent Cells Histochemical Staining Kit (Sigma–Aldrich, St. Louis, MO). Cells were transfected as mentioned before and analyzed for β-galactosidase activity on the sixth day. Brightfield images (10×) were scored based on percentages of stained/unstained cells.

3. Results and discussion

3.1. Expression of death receptors in response to knockdown of E6 and E7 genes

Transfection of SiHa cells with 10 nM of E6 and E7 siRNA results in a marginal decrease in cell viability over a 4 day period, as measured by MTT assay, in comparison to controls which were transfected with control siRNA or incubated with the transfection reagent alone. A recovery of cell viability is observed for E6/E7 siRNA treated cells from day five onwards (data not shown). Previous studies have shown that multiple siRNA transfections over a week are necessary to obtain a robust suppression of E6/E7 gene expression in SiHA cells [26]. Based on these findings and our results, a second transfection was carried out on day 3 to provide a sustained knockdown of E6 and E7 proteins. Expression of cell surface DR4 and DR5 proteins were analyzed by flow cytometry 1 day after the second transfection. A significant increase in the levels of death receptors was observed for cells transfected with E6/E7 siRNA or treated with the proteosomal inhibitor MG132 as compared to control siRNA transfected cells (Fig. 1A). The percentage shifts in the geometric mean of the flow cytometry profiles indicate that

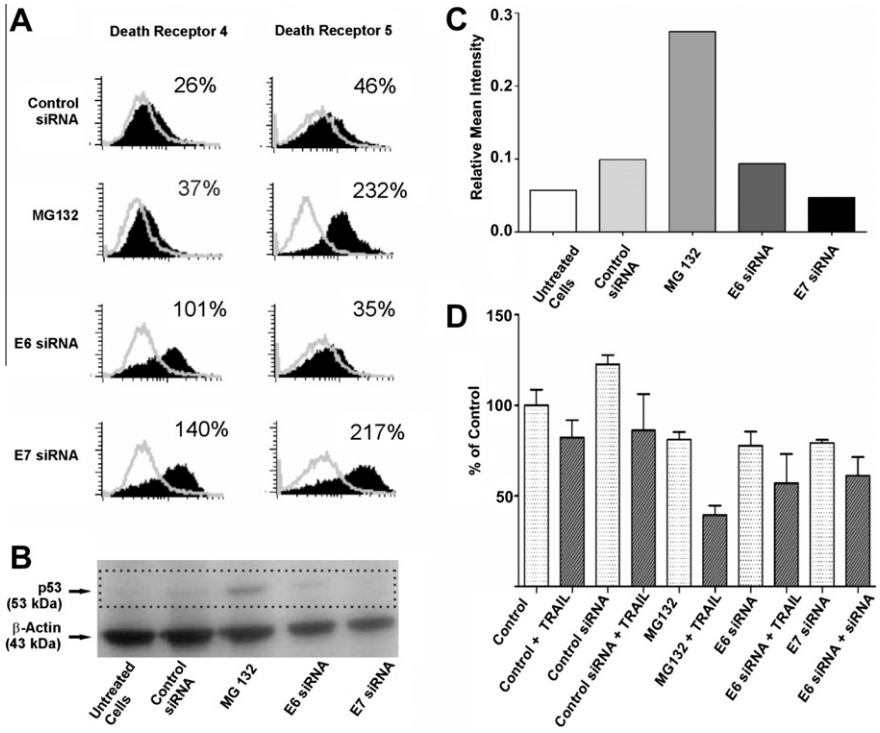

Fig. 1. (A) Flow cytometry analysis of the expression of death receptors 4 and 5 on the cell surface of SiHa cells following treatment with E6/E7 siRNA or MG132. The gray line is the untreated control and the black shaded area is the experimental sample. Percentage shift in the geometric mean of the treated cells as compared to the sample is indicated. (B) Western blot analysis of p53 recovery in SiHa cells following transfection with E6/E7 siRNA or treatment with MG132. (C) Bar graph representing the β-actin-normalized levels of p53 from the Western blot. (D) Response of SiHa cells to a combination of TRAIL following E6/E7 siRNA or MG132 treatment. Viability of cells following various treatments was analyzed by MTT assay and represented as a percentage of untreated cells. Error bars represent standard error of the mean ($N = 3$).

DR4 expression was upregulated by 101% and 140% in response to E6 and E7 siRNA transfections, respectively. E7 siRNA treatment resulted in a 217% increase in DR5 expression while E6 siRNA caused a marginal increase (35%) (Fig. 1A). The effects of MG132 were similar to previous reports [23] with a higher increase in DR5 (232%) as compared to DR4 levels (37%).

p53 has been implicated in the upregulation of cell surface death receptors upon MG132 treatment in cervical cancer SiHa cells. MG132 can restore p53, which may contribute to upregulation of death receptor gene expression since p53 has been shown to be a transcription factor that regulates both DR4 and DR5 genes [27,28]. During Western blot analysis, we found a significant recovery of p53 protein levels in SiHa cells treated with MG132 while E6 and E7 siRNA did not result in a recovery (Fig. 1B). The recovery of p53 in SiHa cells, in response to E7 siRNA, appears inconsistent since Tang et al. [14] found a significant increase in p53 after transfection with 10 nM E7 siRNA. Nevertheless, the induction of death receptors does not depend on p53 levels because even though the greatest recovery of p53 was observed with MG132 (Fig. 1C), cells transfected with E6 or E7 siRNA exhibited a similar increase in both DR4 and DR5 (Fig. 1A). The lack of correlation between p53 levels and expression of death receptors indicates that death receptor expression is only partially regulated by p53 and that treatment with E6/E7 siRNA may also independently enhance expression.

3.2. Cellular responses to TRAIL following E6/E7 siRNA transfection

Untreated SiHa cells show very little expression of DR4 and DR5 (Fig. 1A) and have been reported to show no response to TRAIL [23]. In contrast, in response to TRAIL treatment we found a reduction of cell viability of up to 18% in untransfected cells and up to 36% in MGC control siRNA transfected cells (Fig. 1D). Since an upregulation of DR4 and DR5 was observed by E6/E7 siRNA, we tested if TRAIL would be effective in further reducing cell viability in SiHa cells after siRNA knockdown of these viral oncogenes. Theoretically, TRAIL should be able to stimulate the *de novo* expressed death receptors – following siRNA transfection – resulting in apoptosis. Surprisingly, we found that a combination of E6/E7 knockdown and subsequent addition of TRAIL does not enhance cell mortality any further than the cumulative effects of these two treatments that were observed individually (Fig. 1D). To understand the lack of enhanced sensitization to TRAIL and absence of robust apoptosis, flow cytometry analysis was undertaken after staining the cells with Annexin V and PI (Fig. 2A). In the MG132 + TRAIL treated cells a significant increase in staining with Annexin V alone or a combination of Annexin V/PI indicates that 60% of these cells are undergoing apoptosis in the early or late stage (Fig. 2B). However the addition of TRAIL to E6/E7 knockdown transfected cells did not change the level of staining for Annexin V, as compared to negative controls, indicating that apoptosis had not been induced in these cells (Fig. 2A). However a much higher staining was observed for PI alone indicating that cells are more permeable to this dye due to generalized membrane disruption possibly as a result of necrosis or senescence (Fig. 2A and B). These cells also show altered morphology and reduction in cell proliferation as compared to controls. These effects that E6 and E7 siRNA have on SiHa cells appear to be consistent with autophagic cell death associated with senescent cells. Similar changes have been observed in other senescent cells such as high passage keratinocytes [29].

3.3. E6/E7 siRNA induced senescence in SiHa cells

To further confirm the induction of senescence instead of apoptosis in SiHa cells in response to E6/E7 siRNA, the cell cycle

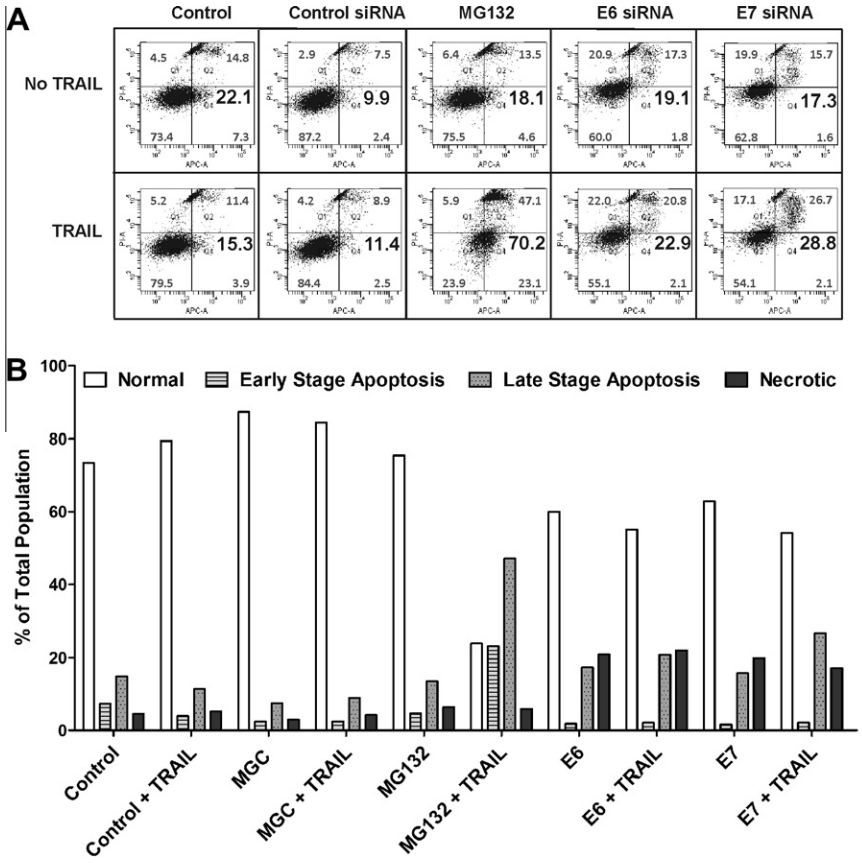

Fig. 2. Analysis of apoptosis by flow cytometry of siRNA/MG132/TRAIL treated SiHa cells. (A) Propidium iodide staining is found on the *y*-axis and Annexin V staining is on the *x*-axis. The numbers in the far corners of each quadrant represent the percentage of the population in that quadrant. The larger number on the center-right represents the percentage of apoptotic cells indicated by combined Annexin V-PI staining in quadrants 2 and 4. (B) Bar graph representation of flow cytometry analysis of apoptosis from Fig. 2A. Percentage of cells in each of the quadrants from Fig. 2A representing normal, early stage apoptosis, late stage apoptosis and necrotic cells – based on the pattern of staining for Annexin V and propidium iodide – are indicated.

progression and endogenous β-galactosidase activity were investigated. The cell cycle was analyzed by staining for DNA content with PI. Cells that show an arrest in cell cycle as a result of senescence show an increase in the population of the G_1 phase as compared to the G_2 phase. Flow cytometry analysis (Fig. 3A) of PI stained cells indicate that the E6 and E7 siRNA treated cells show a significant increase in the DNA content of the G_1 population compared to the controls. Untreated and control siRNA treated cells showed less than 70% of their cell population in the G_1 phase whereas the E6 and E7 siRNA treated cells showed an increase to

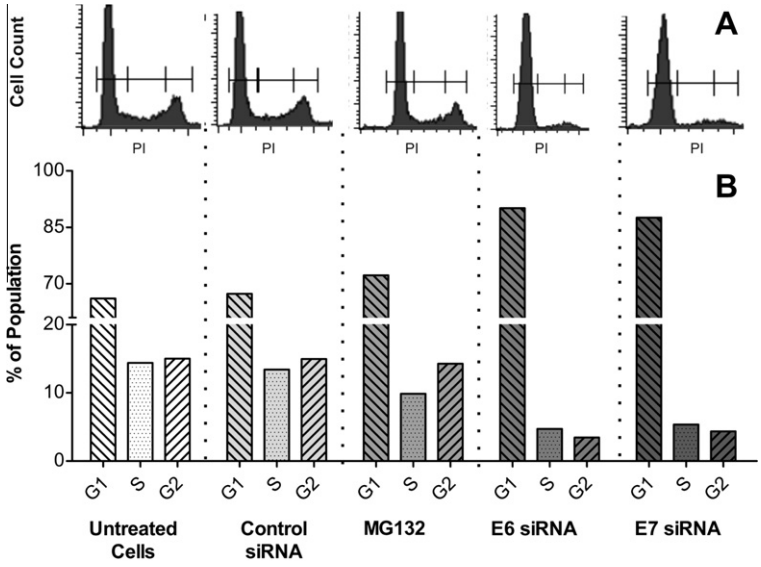

Fig. 3. Analysis of cell cycle progression in SiHa cells treated with siRNA/MG132. (A) Flow cytometry profiles of cells stained with propidium iodide for DNA content. (B) Bar graph representation of the percentage of cells in G_1, S and G_2 phases of the cell cycle.

90% and 88%, respectively. There was also a concomitant decrease of about 20–25% in the S- and G_2 cell populations for E6/E7 siRNA treated cells compared to the controls (Fig. 3B). These results clearly indicate a cell arrest at the G_1/S checkpoint for E6/E7 siRNA treated cells. The DNA content of the positive control, MG132, was similar to negative controls as well with only 74% of the cell population in G_1 phase inferring that there was minimal cell cycle arrest taking place (Fig. 3B).

The occurrence of senescence in E6/E7 siRNA treated cells was confirmed with β-galactosidase staining (Fig. 4). The activity of β-galactosidase inside cells at pH 6.0 is a known marker for senescence [30]. The results from this experiment confirm the strong induction of senescence in SiHa cells when they are incubated with E6/E7 siRNA. The baseline percentage of senescence in a population of untreated SiHa cells was 7% and doubled to 14% when treated with the control siRNA or the positive control, MG132 (Fig. 4). The percentage of senescent cells increases dramatically when E6 and E7 siRNA is used, increasing to 62% and 82%, respectively (Fig. 4). The morphology of these cells is also indicative of senescence. While apoptotic cells become smaller, rounded and exhibit "blebbing", senescent cells have large, flattened cytoplasm's that are usually more obtuse in shape than normal cells [31].

3.4. Senescence may preclude TRAIL induced apoptosis

One of the effects of E6/E7 siRNA treatment and lack of sensitization of SiHa cells to TRAIL may be a result of senescence associated autophagy. Unlike apoptosis, cells undergoing autophagy exhibit packaging of cellular organelles in double membranes that then bind to lysozymes which contain hydrolitic enzymes and an acidic pH that can metabolize the organelle [32]. Cells undergoing autophagy are unlikely to respond to TRAIL for two reasons. Firstly, the packaging of mitochondria during autophagy sequesters them from the rest of the cell and isolates cytochrome C which is critical for the induction of intrinsic apoptosis. Secondly, disruption of the cell membrane during autophagy may affect the structure and function of the death receptors leading to the inhibition of TRAIL induced extrinsic apoptosis. Further experiments to investigate these changes are necessary to confirm senescence associated autophagy in SiHa cells. In addition, several other factors have the potential to contribute to the refractory response of SiHa cells to TRAIL induced apoptosis. Hougardy et al. [22] have shown that SiHa cells have low levels of the pro-apoptotic protein and caspase-8 target, bid, and that after TRAIL binding, there is minimal pro-caspase 8 activation compared to HeLa cells. SiHa cells are more likely to express higher levels of cFLIP, which prevents the activation of caspase 8 and consequent caspase cascade since a correlation between elevated cFLIP levels and disruption of the E2 gene in HPV16 infected cervical cancer cells such as SiHa has been noted [33]. In comparison to six other cancerous cervical epithelial cell lines, SiHa expressed the highest level of NFκB activity and was the most resistant to the tested cancer drugs [34]. NFκB can provide survival signals to the cell by inducing the expression of cFLIP, and other inhibitors of apoptosis such as Bcl-XL and XIAP [19].

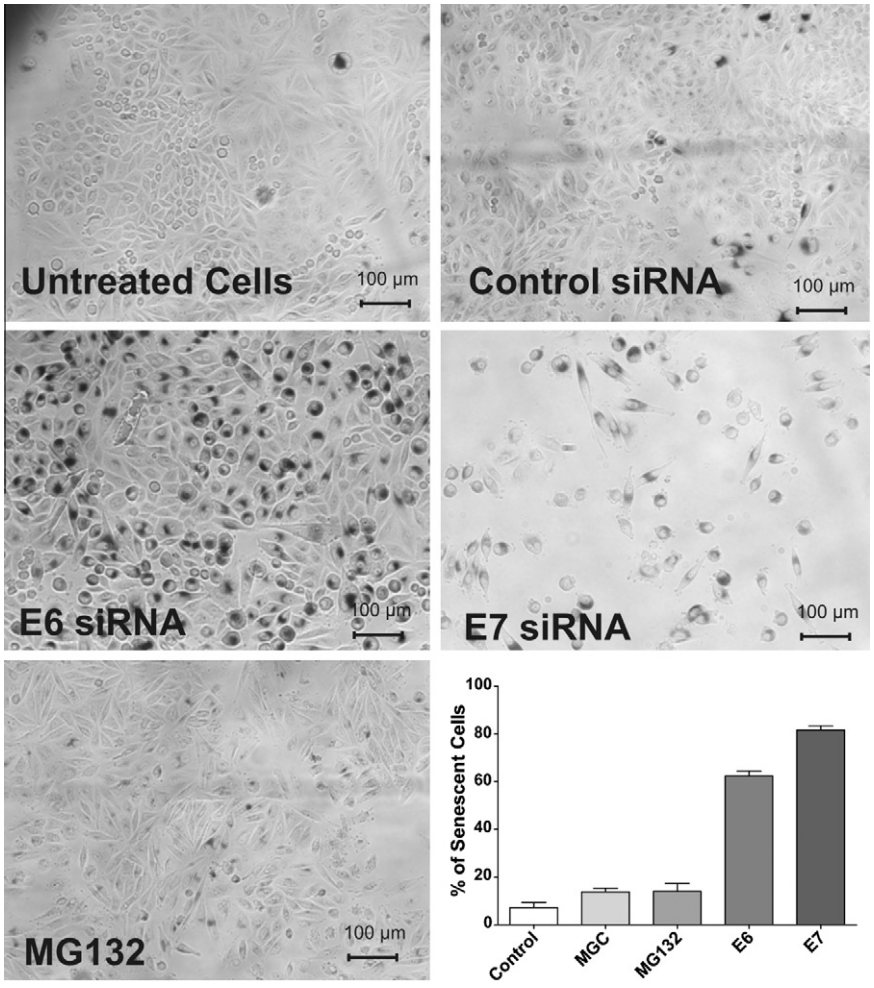

Fig. 4. Senescence in SiHa cells treated with siRNA/MG132 indicated by staining for β-galactosidase (pH 6.0) activity. Bar graph on the lower-right shows the percentage of the total population of cells that was positive for senescent staining. Error bars are standard error of the mean (N = five randomly chosen images).

These factors may work in concert with senescence and eventual autophagic cell death to prevent TRAIL induced apoptosis in SiHa cells following the knockdown of E6/E7 by siRNA. Molecular pathways involving these molecules are interesting targets for further investigation. In conclusion, sensitization to TRAIL is still possible as seen in combination with MG132. However, future combinatorial treatment strategies for cervical cancer involving TRAIL need to take into account the inhibition of apoptosis due to the induction of senescence.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.12.056.

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