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SnoN/SKIL modulates proliferation through control of hsa-miR-720 transcription in esophageal cancer cells

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ABSTRACT

It is now evident that changes in microRNA are involved in cancer progression, but the mechanisms of transcriptional regulation of miRNAs remain unknown. Ski-related novel gene (SnoN/SKIL), a transcription co-factor, acts as a potential key regulator within a complex network of p53 transcriptional repressors. SnoN has pro- and anti-oncogenic functions in the regulation of cell proliferation, senescence, apoptosis, and differentiation. We characterized the roles of SnoN in miRNA transcriptional regulation and its effects on cell proliferation using esophageal squamous cell carcinoma (ESCC) cells. Silencing of SnoN altered a set of miRNA expression profiles in TE-1cells, and the expression levels of miR-720, miR-1274A, and miR-1274B were modulated by SnoN. The expression of these miRNAs resulted in changes to the target protein p63 and a disintegrin and metalloproteinase domain 9 (ADAM9). Furthermore, silencing of SnoN significantly upregulated cell proliferation in TE-1 cells, indicating a potential anti-oncogenic function. These results support our observation that cancer tissues have lower expression levels of SnoN, miR-720, and miR-1274A compared to adjacent normal tissues from ESCC patients. These data demonstrate a novel mechanism of miRNA regulation, leading to changes in cell proliferation.

1. Introduction

MicroRNAs (miRNAs) are endogenously expressed, short, non-coding RNAs 18–25 nucleotides in length that repress protein translation by binding to target messenger RNAs (mRNAs). miRNA expression depends on cell type and stage of differentiation [1]; aberrant expression is implicated in many diseases and dysregulation is linked to many types of cancer [2]. miRNAs can act as either oncogenes or tumor suppressors by their regulation of genes [3]. Interestingly, computational target prediction of specific miRNAs has demonstrated that they may have many putative targets, indicating their crucial roles in physiology and biology. Therefore, efforts to identify the precise target mRNAs of miRNAs are needed to understand and gain greater insight into miRNA biology. In cancer research, a number of studies have been conducted on miRNAs in an effort to elucidate disease mechanisms and improve upon current diagnostic and prognostic indicators [4]. However, the mechanisms that regulate miRNA transcription remain unknown. Recent studies have demonstrated that p53 regulates the transcription of several miRNAs [5–7]. In addition, transcription of two miR-200 clusters, one on chromosome 1 and the other on chromosome 12, are negatively regulated by the binding of zinc finger E-box binding homeobox 1 or 2 (ZEB1 or ZEB2) to the E-box of the miR promoter [8]. We recently found that K(lysine) acetyltransferase 3 (KAT3) histone acetyltransferase P300 and its cofactor PCAF (p300/CBP-associated factor) act as cofactors for ZEB1, forming a P300/PCAF/ZEB1 complex on the miR-200c/141 promoter; this binding results in lysine acetylation of ZEB1 and a release of ZEB1 suppression on miR-200c/141 transcription [9].

Ski-related novel gene (SnoN) is a 684 amino acid protein and a member of the Ski family of transcription regulators, defined by their homology with the viral transforming protein v-ski. The roles of SnoN and related Ski proteins in cancer biology are controversial. SnoN and related Ski proteins were originally known as negative regulators of transforming growth factor-beta (TGF-β) signaling. Many studies have focused on the roles of SnoN in tumorigenesis as a regulator of TGF-β signaling and have reported that SnoN level is elevated in many cancer cell lines and some tissues [10,11]. Amplification of chromosome 3q26, where SnoN/SKIL is coded, has been found in tumors in many areas of the body, such as the head and neck, esophagus, lung, ovary, cervix, and prostate, suggesting that SnoN/SKIL might have important roles

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in tumorigenesis and cancer progression [12]. In recent studies, however, SnoN has also been reported to be a tumor suppressor that regulates pathways beyond TGF-β signaling, in which it was shown that SnoN expression was downregulated in many cancers [13–15]. Taken together, SnoN has the potential to show both pro- and anti-oncogenic activity in cancers. SnoN might have various functions and may act as a transcriptional regulator through other pathways.

In the present study, we hypothesized that SnoN may regulate some miRNAs and contribute to tumorigenesis or cancer progression through a certain pathway via miRNAs. Using a PCR-based miRNA array on an esophageal cancer cell line indicated that a group of miRNAs was regulated by SnoN. Of these, the expression levels of miR-720, miR-1274A, and miR-1274B were clearly influenced by the level of SnoN expression. We further studied the downstream region of each miRNA, which suggested that miR-720 probably controlled cancer cell growth through its target protein, p63. These results demonstrate a new pathway for SnoN/miR-720/p63 in cancer cell biology, as well as a new transcriptional regulatory model for miRNA.

2. Materials and methods

2.1. Cancer cell lines and tissue samples

Five human cancer cell lines (TE-1, 4, 5, 8, and 10), established from esophageal squamous cell carcinoma (ESCC), were obtained from Cell Bank (RIKEN BioResource Center, Japan). These cells were cultured in RPMI-1640 (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Japan Bio Serum, Fukuyama, Japan) at 37 °C in a humidified atmosphere of 5% CO_2.

Tumor tissues and adjacent normal tissues were obtained from 19 ESCC patients who underwent surgery at Nippon Medical School Hospital (Tokyo, Japan). All specimens were snap frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted from frozen tissues using the RNeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's procedures. Written informed consent was obtained prior to the study.

2.2. miRNA expression profiling

PCR array assays were conducted using TaqMan Array Human MicroRNA A+B Cards Set v3.0 (Applied Biosystems, Foster City, CA). Total RNA (1000 ng) was reverse-transcribed using Megaplex RT Primers (Applied Biosystems) and was applied to each card. Real-time PCR was performed using the 7900HT Fast Real-Time System and the data were analyzed with the included software (SDS ver. 2.3 and RQ manager ver. 1.2) (Applied Biosystems). snRNA U6 was used as an internal control.

2.3. Small interfering RNA/plasmid transfection

Three SKIL-targeting siRNA (Silencer Select Pre-designed siRNA, ID: s12883, s12884, and s12885) and Silencer Select Negative Control #1 siRNA were obtained from Ambion (Austin, TX). Cells were cultured in RPMI-1640 with 10% FBS for 24 h to reach 80% confluence. The medium was changed to Opti-MEM I Reduced-Serum Medium (GIBCO/Invitrogen, Carlsbad, CA) before siRNAs were added. Cells were transfected with 5 nM siRNAs by Lipofectamine2000 (Invitrogen) and incubated for 72 h.

2.4. Western blot analysis

Cells were lysed in RIPA Buffer with protease inhibitors. Proteins were resolved on a 10% SDS-PAGE gel and electrotransferred to PVDF membranes. After blocking with 5% (w/v) milk, the membranes were incubated overnight at 4 °C with primary antibody and 1 h with appropriate HRP-conjugated secondary antibody. The following antibodies were used: SnoN Antibody (#4973) and Anti-rabbit IgG, HRP-linked antibody (#7074) (Cell Signaling Technology, Beverly, MA), anti-GAPDH (AM4300) (Ambion), and ECL Mouse IgG, HRP-linked whole antibody (GE Healthcare, Buckinghamshire, England).

2.5. Reverse transcription and real-time PCR

Total RNA was isolated with RNAiso Plus (TaKaRa Bio, Ohtsu, Japan) according to the manufacture's protocol. For quantification of miRNA or mRNA expression, TaqMan MicroRNA assays or TaqMan Gene Expression Assays were used (Applied Biosystems). The primers/probes were as follows: hsa-miR-720 (Assay ID: 002895), hsa-miR-1274A (ID: 002883), hsa-miR-1274B (ID: 002884), SKIL (ID: Hs00180524_m1), TP63 (ID: Hs00978343_m1), and ADAM9 (ID: Hs00177638_m1). PCR experiments were performed with the ABI 7300 Real-Time PCR System and conducted in triplicate. snRNA U6 for miRNA or GAPDH for mRNA were used as internal controls. The relative fold expression changes were calculated using the ΔΔCt method.

2.6. Target prediction

The predicted targets of hsa-miR-720, hsa-miR-1274A, and hsa-miR-1274B were determined using the TargetScan program (http://targetscan.org). After computational prediction, we selected p63 and ADAM9 out of the target candidates as putative targets for further analyses after careful review of published reports that dealt with individual miRNA [16,17].

2.7. Growth assay

For growth assays with total cell count, TE-1 cells were initially seeded at a density of 5.0×10^3 cells/well in 96-well plates with concurrent transfection of SKIL-targeting siRNA (ID: s12883). After 8–10 h, non-adherent cells were removed by washing with PBS. The numbers of remaining adherent cells were set as baseline (0 h). These cells were cultured under normal conditions. At the indicated times of harvesting, TE-1 cells were detached from the plates with TrypLE Express without Phenol Red (GIBCO/Invitrogen).

2.8. Statistical methods

Statistical analysis was performed with SPSS Ver. 20 (SPSS, Inc., Chicago, IL). A P value of <0.05 was considered statistically significant and all tests were two-tailed. All interval values are expressed as means ± SD. Statistically significant differences between two groups were calculated by the paired Student t-test. The Wilcoxon test was used to assess the difference in the miRNA levels between tumor tissues and adjacent normal tissues.

3. Results

3.1. Identification of the miR-720, miR-1274A, and miR-1274B which can be regulated by transcription co-factor SnoN/SKIL

To obtain a suitable study cell system to search for target miRNAs of SnoN/SKIL, we probed the expression levels of SnoN in several ESCC cell lines. Consistent with our previous observations, SnoN was expressed in all ESCC cell lines. TE-1 had the highest SnoN expression levels, however, the protein expression levels of SnoN are relatively low or nearly the same as A549, a lung cancer

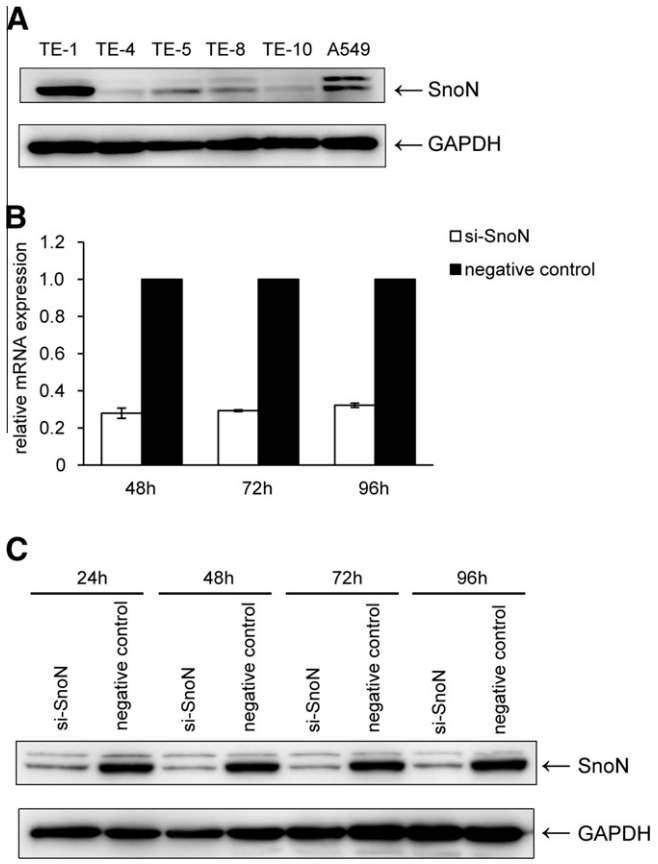

Fig. 1. SnoN expression in esophageal squamous carcinoma cell lines. (A) Western blot analysis was performed to examine protein expression levels of SnoN in six cell lines (TE-1, TE-4, TE-5, TE-8, TE-10, and A549). (B, C) Expression levels of SnoN in TE-1 24, 48, 72, and 96 h after siRNA transfection. The silencing efficiency of siRNAs was confirmed by real-time PCR (B) and Western blot analysis (C). GAPDH was used as an internal control.

cell line that is well characterized as having high SnoN expression [18] (Fig. 1A). Then we probed whether the miRNA profile in TE-1 cells could be altered in accordance with SnoN expression level. Small interfering RNA molecules, which target SnoN (s12883 denotes si-SnoN1, s12884 denotes si-SnoN2, and s12885 denotes si-SnoN3), were applied to the TE-1 cells. It was evident that these siRNA molecules were effectively silencing SnoN mRNA. The silencing effect was approximately 70% and was maintained at least 96 h after induction of siRNA molecules (Fig. 1B). Silencing effects of SnoN protein levels were also confirmed using Western blotting (Fig. 1C).

To identify miRNAs that could be controlled by SnoN, we obtained a differential display of 754 human miRNAs between control TE-1 and si-SnoN-treated TE-1. The most stably expressed snRNA U6 was chosen as an endogenous control. A total of 362 miRNAs that showed no expression in any RNAs were excluded from further analysis. To avoid false-positive transcripts, all amplification plots were manually checked for a regular sigmoid-shaped amplification curve and to be sure that each sample that was treated with si-SnoN moved in the same direction. A total of 274 miRNAs had less than 2-fold change in a sample or samples compared to control, and these were excluded. Of these, we chose 49 miRNAs (Fig. 2A), of which we ultimately selected three for further analyses. These miRNAs, miR-720, miR-1274A, and miR-1274B, had regular sigmoid-shaped amplification curves in all samples (data not shown) and were significantly decreased in TE-1 samples transfected with si-SnoNs compared to control, indicating that SnoN altered their transcription (Fig. 2B). To confirm the differences in expression between the samples, real-time PCR was performed for each miRNA (Fig. 2C).

3.2. Confirmation of target genes of SnoN/SKIL-associated miRNAs

Next we employed bioinformatics analysis to identify the target genes of the SnoN-associated miRNAs. The computational program TargetScan was used and it predicted hundreds of putative target genes. We screened and chose some of them based on their importance in cancer biology. We also examined published reports on the chosen target genes through PubMed and checked if those genes had an association with the miRNAs. Finally, we selected p63 and a disintegrin and metalloproteinase domain 9 (ADAM9) as target genes for miR-720 and miR-1274A, respectively. p63 is a member of the p53 gene family regulated by inhibitory apoptosis stimulating protein p53 [16]. Inhibitory apoptosis stimulating protein p53 is expressed predominantly in epithelial cells and binds selectively to p63, playing a role in epithelial development via miR-574-3p and miR-720. ADAM9 may play an essential role in the cleavage of major histocompatibility complex class I-related chain A in membranes. ADAM9 is over-expressed in human hepatocellular carcinoma tissues compared to normal liver tissues [19] and miR-1274a is upregulated in response to sorafenib and greatly reduces ADAM9 expression in hepatocellular carcinoma cells [17].

We examined the effects of SnoN expression level on the expression levels of p63 and ADAM9 in TE-1 cells. si-SnoN1 was selected and used for further analysis because of its effective silencing performance. As expected, mRNA levels of p63 and ADAM9 were elevated in TE-1 cells that were transfected with si-SnoN1 (Fig. 3).

3.3. Silencing of SnoN/SKIL is attributed to cell proliferation in TE-1 cells

As mentioned above, although the precise mechanisms are not known, SnoN has the potential to be a cancer suppressor [13]. To further test the effects of SnoN proteins on TE-1 cells, we tested for differences in proliferation using si-SnoN1. As expected, the silencing of SnoN resulted in significant increases in cell proliferation in TE-1 cells (72 h; $p = 0.013$, 96 h; $p = 0.014$: Fig. 4A), which suggests that SnoN is a negative regulator of cell proliferation in TE-1 cells.

3.4. Expression levels of SnoN and miR-720 in patient tissues

To identify the expression levels of SnoN and the three selected miRNAs, RT-PCR analysis was performed on tissue samples from 19 patients. In almost all samples, SnoN expression was lower in cancer tissues compared to non-cancer tissues. The mean expression level of SnoN was significantly lower in cancer tissues ($p = 0.001$) (Fig. 4B). In conjunction with SnoN, the mean expression level of each miRNA was significantly lower in the cancer tissue compared to adjacent noncancerous tissues. Therefore, the expression levels of SnoN and miRNAs were downregulated in cancer tissues, supporting our *in vitro* study (Fig. 4B,C).

4. Discussion

Dysregulation of miRNAs in esophageal cells may play a central role in pathological processes, including carcinogenesis and cancer progression [20,21]. One of the mechanisms of SnoN/SKIL function in cancer biology may be dependent on modulating p63 expression through miR-720. The ability of SnoN to modulate miRNA transcription is a novel discovery. The graphical abstract illustrates the probable pathway via which SnoN transcribes miRNAs and

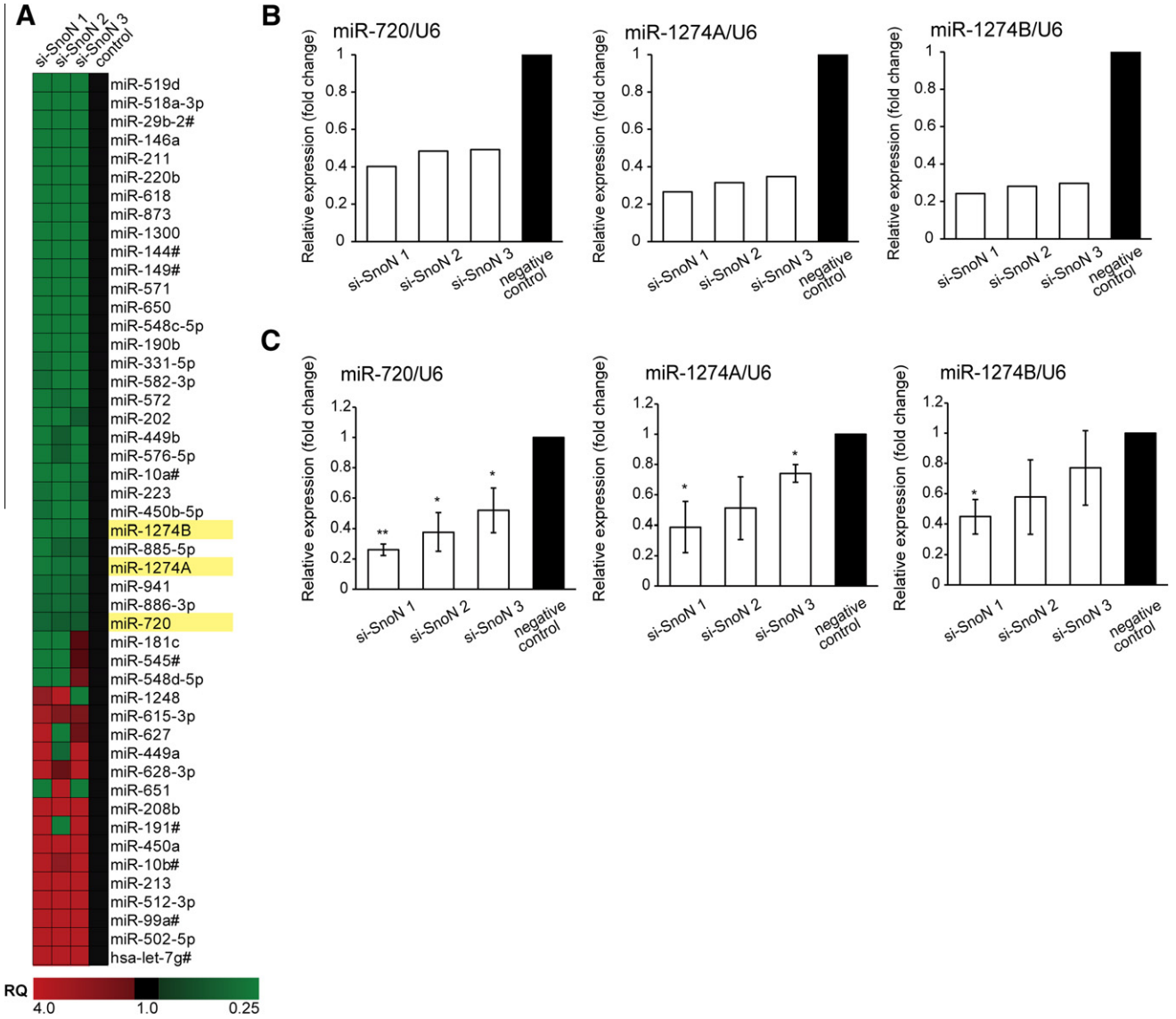

Fig. 2. Identification of miRNAs regulated by transcription co-factor SnoN/SKIL. (A) Comparison between control and SnoN silencing cells using an miRNA array. A heatmap of miRNA expression is shown, with miRNAs sorted according to their relative quantity (RQ) value. Samples are in columns, miRNAs are in rows. The miRNA expression level of the control cells was set as 1.0. (B) MicroRNA array analysis showed downregulation of miR-720, miR-1274A, and miR-1274B in TE-1 cells transfected with si-SnoNs. (C) Real-time PCR was performed on the RNA samples by individual miRNA. snRNA U6 was used as an internal control. There was a statistically significant difference as measured by a *t*-test ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$).

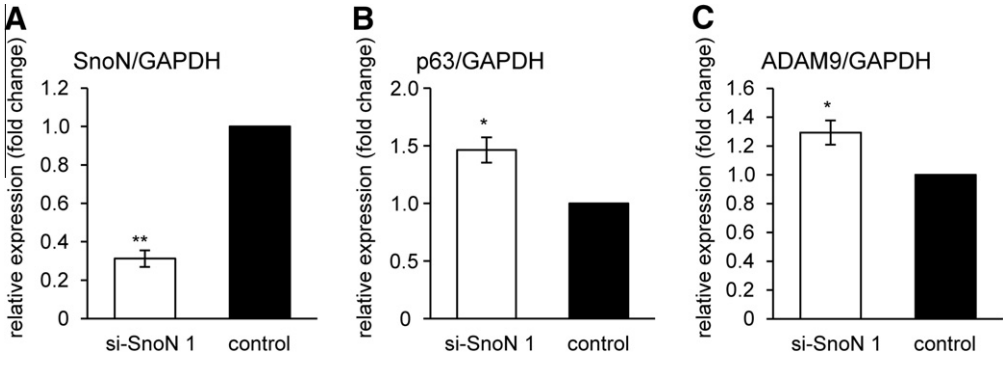

Fig. 3. Confirmation of the associations among SnoN, miRNAs, and target proteins. Real-time PCR analysis of p63 and ADAM9 in TE-1 cells. TE-1 cells were transfected with si-SnoN or with negative molecules. GAPDH was used as an internal control. There was a statistically significant difference as measured by a *t*-test ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$).

downregulates p63 and ADAM9, resulting in the increase in TE-1 esophageal cancer cells, and which supports previous reports that downregulation of SnoN/SKIL can also be a crucial contributor to cancer progression [13–15].

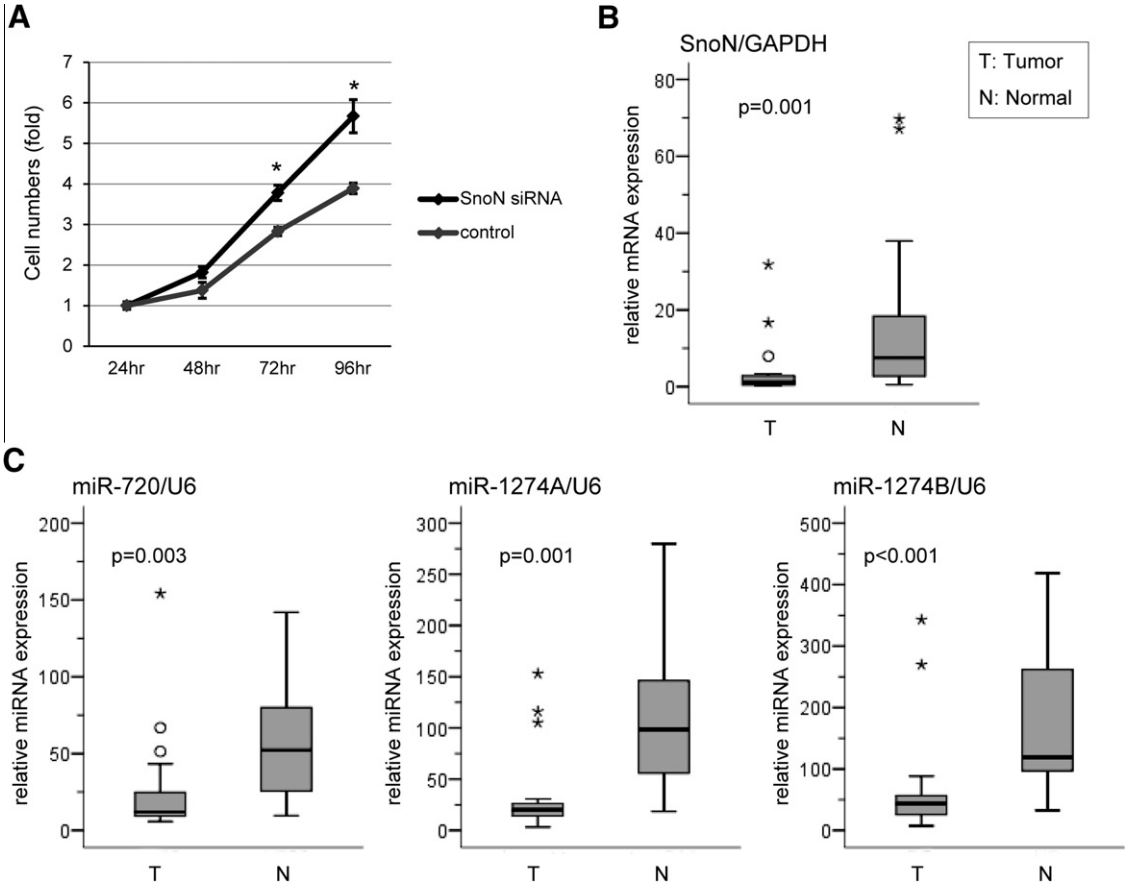

Fig. 4. (A) Growth curves of TE-1 cells transfected with si-SnoN, compared to controls. Representative data of the cell proliferation assay using total cell count are shown. TE-1 cells were transfected with si-SnoN or with negative molecules (controls), which had no silencing effect. Cell numbers were calculated at the indicated time points. The cell number at 0 h was set as the baseline. The assay was repeated twice (*$p < 0.05$). (B, C) SnoN and miRNA expression levels in patient tissues. (B) SnoN mRNA expression in patient samples was assessed by real-time PCR. GAPDH was used as an internal control. (C) Expression levels of miR-720, -1274A, and -1274B in patient samples were assessed by real-time PCR. Data are presented as ΔΔCt relative to snRNA U6. There was a statistically significant difference as measured by a Wilcoxon *t*-test (○: outlier, *extremum).

SnoN is an oncogene in the TGF-β pathway as well as a tumor suppressor gene that has dual effects on tumor development. In the TGF-β pathway, SnoN binds to Smad proteins, acting as a negative regulator of TGF-β signaling, leading to cell proliferation. While many cancer tissues show elevated SnoN levels, some cancer tissues show downregulation of SnoN expression. With regard to the tumor suppressive role of SnoN, Pan et al. [13] reported that SnoN regulates p53 through a PML-dependent mechanism. SnoN functions as a p53 regulator independent of the TGF-β pathway. Recent studies have revealed that SnoN plays an important role in the regulation of cell proliferation, senescence, apoptosis, and differentiation [15,22,23]. It might modulate multiple signaling pathways depending on various factors and its level of expression. In TE-1 cells, SnoN plays a potential anti-oncogenic role via the regulation of miR-720, miR-1274A, and miR-1274B. Further studies on the regulation of these miRNAs will likely reveal the mechanisms of SnoN function, including the identification of upstream regulators and downstream effectors in the regulation of tumorigenesis.

The mechanisms that control miRNA expression need to be elucidated. Recent studies have demonstrated that the transcription of several miRNAs including miR-34, miR-145, miR-107, miR-15/16, and miR-192/194/215 are regulated by p53 [5–7]. This transcription factor plays a central role in coordinating cellular responses to diverse stresses such as DNA damage. Therefore, these miRNAs contribute to tumor suppression by controlling cell cycle progression, epithelial–mesenchymal transition, stemness, cell survival, and angiogenesis in the p53 stress-response pathway. Conversely, the expression and activity of p53 may also be under the control of miRNAs. Expression levels of miR-200 family members and ZEB1/ZEB2 are closely and inversely associated. miR-200 downregulates the expression of the transcription factors ZEB1 and ZEB2 by binding to the 3' untranslated region of the mRNA and preventing translation. Our miRNA array demonstrated that 70% less expression of SnoN/SKIL altered a set of miRNA expression profiles. Out of those miRNAs, additional real-time PCR confirmation and bioinformatics analysis followed by a PubMed search extracted three miRNAs (miR-720, miR-1274A, and miR-1274B). In this regard, SnoN has the potential to control miRNA transcription. Further study, however, might recognize other candidate miRNAs that could be controlled by SnoN and uncover more precise mechanisms by which SnoN transcriptionally modulates miRNA expression.

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