Molecular Therapy
Nucleic Acids
Letter to the Editor

MicroRNA-146a-5p Limits Elevated TGF-β Signal during Cell Senescence

MicroRNAs (miRNAs) are small non-coding RNAs implicated in post-transcriptional suppression of target genes in a sequence-specific manner and are thereby able to modulate cellular processes. miR-146a seems to play multiple roles in terms of regulating different phenotypes by targeting a wide range of different genes in various cellular contexts. Notably, miR-146a-5p targets Smad4[1] and tumor necrosis factor receptor-associated factor 6 (TRAF6),[2] which both serve as important mediators in the transforming growth factor-β (TGF-β) pathway. TGF-β is a potent anti-tumor cytokine because of its function as a strong inhibitor of the growth of epithelial cells and its regulation of a wide array of cellular processes, such as cell growth, differentiation, and apoptosis;[3] therefore, its misregulation can result in tumor development. However, the role of miR-146a-5p in regulation of stress-induced senescence of normal human keratinocytes has not been fully addressed yet.

Serial subculture of primary normal human oral keratinocytes (NHOKs) induces stress-induced senescence and provides an excellent in vitro model for stress-induced senescence.[4,5] To determine whether stress-induced senescence results from continued serial subculture of primary NHOKs, the protein level of $p16^{INK4A}$ and senescence-associated β-galactosidase (SA-β-gal) activity, biomarkers of senescence, were determined during serial subculture of primary NHOKs. The level of cellular $p16^{INK4A}$ gradually increased in the serially subcultured keratinocytes with increasing population doubling (PD) levels (Figure 1A). Moreover, although senescent cells were detected in exponentially proliferating NHOKs at PD 13.6, the percentage of SA-β-gal positive cells was very low; in contrast, the majority of cells at PD 16.9 displayed characteristics of stress-induced senescence, showing SA-β-gal-positive staining and growth arrest (Figure 1B). These indicate that the serial subculture of primary NHOKs to the post-mitotic stage of proliferation induces stress-induced senescence.

In preliminary screening with a miRNA microarray, we found that miR-146a-5p expression was increased in NHOKs at later passages compared with those at earlier passages (data not shown). To clarify the expression pattern of miR-146a-5p in stress-induced senescence, we first examined the levels of miR-146a-5p expression by a TaqMan miRNA assay during stress-induced senescence of primary NHOKs. The expression level of miR-146a-5p was gradually enhanced with increasing PD levels (Figure 1C). Furthermore, the protein level of the p65 subunit of nuclear factor kappa B (NF-κB), which transactivates miR-146a-5p,[6] was also steadily enhanced and reached a plateau just before cell cycle arrest (Figure 1A). Because TGF-β1 treatment of exponentially proliferating NHOKs significantly inhibited cell proliferation (Figure 1D) and concomitantly promoted apoptosis (Figure 1E), we next determined whether TGF-β1 was involved in stress-induced senescence of primary NHOKs. The level of TGF-β1 protein gradually increased with increasing PD levels and peaked in senescent cells during stress-induced senescence of primary NHOKs (Figure 1F); its expression pattern seemed to be identical to that of miR-146a-5p (Figure 1C). Therefore, we assessed whether TGF-β1 can regulate the expression of NF-κB and miR-146a-5p in normal human keratinocytes. TGF-β1 induced the expression of NF-κB (Figure 1G) and also promoted translocation of NF-κB into the nucleus within 1 hr of TGF-β1 treatment in a small portion of exponentially proliferating NHOKs (Figure 1H). Moreover, TGF-β1 treatment significantly enhanced miR-146a-5p expression (Figure 1I). Although TGF-β1 induced NF-κB expression in our experiment, it is uncertain whether the up-regulation of NF-κB during stress-induced senescence of NHOKs is due to TGF-β1 because of a slight difference in chronological expression patterns. However, considering the similar chronological expression patterns of TGF-β1 and miR-146a-5p and the nuclear translocation of NF-κB induced by TGF-β1, TGF-β1 seems to be the rate-limiting factor of miR-146a-5p upregulation during stress-induced senescence of primary NHOKs. Taken together, our data indicate that miR-146a-5p expression is associated with stress-induced senescence of primary NHOKs that is, in part, linked to the elevated levels of TGF-β1.

Next, to test the role of miR-146a-5p in keratinocyte senescence, exponentially proliferating NHOKs were transfected with miR-146a-5p mimic or negative control. Exogenous miR-146a-5p expression, which was verified by a TaqMan miRNA assay (data not shown), led to significantly increased cell proliferation (Figure 1J) and inhibited apoptosis of NHOKs (Figure 1K). Furthermore, the protein level of $p16^{INK4A}$ was decreased in miR-146a-5p mimic-transfected cells (Figure 1L); however, there is no significant difference in SA-β-gal activities between negative control- and miR-146a-5p mimic-transfected NHOKs (Figure 1M). These data suggest that the introduction of exogenous miR-146a-5p may play a role in inhibiting keratinocyte senescence in exponentially proliferating NHOKs, which expressed small amounts of TGF-β1 compared to senescent cells.

To determine whether canonical Smad signaling is involved in keratinocyte senescence, we investigated the expression of Smad proteins in both exponentially proliferating NHOKs and senescent cells. The expression levels of Smad signaling cascades that represent the canonical Smad signaling, including phospho-Smad2-Ser465/467, Smad2/3, and Smad4 proteins, were decreased in the senescent NHOKs compared to those of exponentially proliferating NHOKs (Figure 1N). This observation suggests that the canonical Smad signaling may be turned off in senescent keratinocytes.

miR-146a-5p has been implicated in the modulation of TGF-β signaling by targeting Smad4 and TRAF6;[1,2] therefore, we next examined whether miR-146a-5p could abrogate TGF-β signaling in NHOKs.

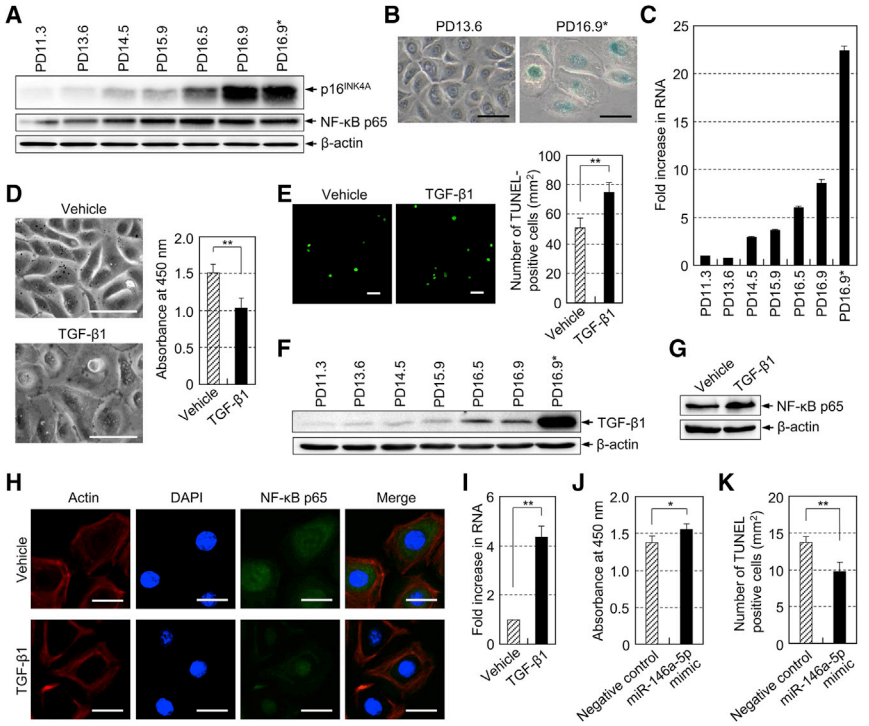

Figure 1. miR-146a-5p Is a Self-Limiting Factor of TGF-β Signaling that Is Upregulated during Stress-Induced Senescence of Primary NHOKs

(A) Immunoblot analyses of $p16^{INK4A}$ and NF-κB during serial subculture of primary NHOKs. Primary NHOKs were serially subcultured once they were 70% confluent until they reached the post-mitotic stage of proliferation (PD16.9), at which time the culture was maintained for 12 days without further passaging (PD16.9*). (B) Phase-contrast micrographs of SA-β-gal staining of the exponentially growing NHOKs (PD 13.6) and senescent NHOKs (PD 16.9*). Scale bars, 100 μm. (C) The miR-146a-5p expression profile during serial subculture of primary NHOKs. The assay conditions were the same as in (A). (D) and (E) show the effects of TGF-β1 on proliferation (D) and apoptosis (E) of NHOKs. Exponentially proliferating NHOKs were treated with vehicle or 20 ng/mL TGF-β1 for 2 days. Scale bars, 100 μm (D) and 50 μm (E). **$p < 0.01$. (F) Immunoblot analysis of TGF-β1 during serial subculture of primary NHOKs. The assay conditions were the same as in (A). (G) Immunoblot analysis of NF-κB in NHOKs treated with TGF-β1. The assay conditions were the same as in (D) and (E). (H) Translocation of NF-κB into the nucleus in NHOKs in response to TGF-β1. Exponentially proliferating NHOKs were treated with vehicle or 20 ng/mL TGF-β1 for 1 hr, and translocation of NF-κB from the cytoplasm into the nucleus was characterized by confocal microscopy. Scale bars, 20 μm. (I) Effect of TGF-β1 on miR-146a-5p expression. The assay conditions were the same as in (D) and (E). **$p < 0.01$. (J) and (K) show the effects of exogenous miR-146a-5p expression on the proliferation and apoptosis of primary NHOKs. Exponentially proliferating NHOKs were transfected with miR-146a-5p mimic (20 nM) or negative control (20 nM) for 2 days. Cell proliferation (J) and apoptotic cells (K) were determined in miR-146a-5p mimic-transfected cells by cell viability assay and TUNEL assay, respectively. (L) Immunoblot analyses of Smad proteins and $p16^{INK4A}$ in miR-146a-5p mimic-transfected NHOKs. The assay conditions were the same as in (J) and (K). (M) shows phase-contrast micrographs and incidence of senescent cells detected by SA-β-gal staining in the miR-146a-5p mimic-transfected NHOKs. The assay conditions were the same as in (J) and (K). Scale bars, 100 μm. (N) shows immunoblot analyses of $p16^{INK4A}$ and Smad proteins in both exponentially proliferating NHOKs (PD13.6) and senescent cells (PD16.9*). (O) and (P) show the effects of TGF-β1 on cell proliferation (O) and protein levels of miR-146a-5p target genes, Smad proteins, $p15^{INK4B}$, and $p16^{INK4A}$ (P) in miR-146a-5p mimic-transfected NHOKs. Exponentially proliferating NHOKs transfected with a miR-146a-5p mimic (20 nM) or the negative control (20 nM) for 2 days were further treated with 20 ng/mL TGF-β1 for 2 days. **$p < 0.01$. (Q)–(S) show the effects of TGF-β1 on cell proliferation (Q), apoptosis (R), and the protein level of p53 (S) in TRAF6-specific siRNA-transfected NHOKs. Exponentially proliferating NHOKs transfected with TRAF6-specific siRNA (40 nM) or control siRNA (40 nM) for 2 days were further treated with 20 ng/mL TGF-β1 for 2 days. Scale bars, 50 μm. **$p < 0.01$.

Pretreatment with a miR-146a-5p mimic partly reversed the anti-proliferative effect of TGF-β1 in exponentially proliferating NHOKs (Figure 1O). In addition, the expression of TRAF6, which is implicated in non-canonical signaling of TGF-β, was notably reduced after the introduction of exogenous miR-146a-5p (Figure 1P). However, the expression levels of phospho-Smad2-Ser465/467, phospho-Smad3-Ser423/425, Smad2/3, and Smad4 proteins were not affected (Figures 1L and 1P), suggesting that the canonical Smad signaling is not affected by the miRNA. A considerable body of evidence indicates that Smad4 is a valid target gene of miR-146a-5p;[1,7] however, exogenous miR-146a-5p expression did not affect the expression levels of Smad4 in NHOKs. TGF-β1 induced the expression of $p15^{INK4B}$, which is known to be induced by TGF-β/Smad signaling pathway,[8] and its expression was also retained despite miR-146a-5p pretreatment (Figure 1P), further supporting that the canonical Smad signaling is not affected by the miRNA. As expected, TGF-β1 induced the expression of $p16^{INK4a}$; however, introduction of exogenous miR-146a-5p inhibited $p16^{INK4a}$ expression, and its expression was retained despite TGF-β1 treatment (Figure 1P). To further test whether TRAF6 suppression is implicated in the abrogation of TGF-β signaling by miR-146a-5p, exponentially proliferating NHOKs were transfected with TRAF6-specific or negative control small interfering RNA (siRNA) and subsequently treated with TGF-β1. TRAF6 knockdown (Figure 1Q, upper panel) notably reversed the cellular responses to TGF-β1 treatment in these cells (Figures 1Q, lower panel, and 1R) and also markedly reduced the expression level of p53 protein (Figure 1S). Taken together, our data indicate that miR-146a-5p can function as a self-limiting factor of the TGF-β1-induced reduction of proliferation and apoptosis by directly targeting TRAF6.

Nevertheless, the importance of miR-146a-5p upregulation during stress-induced senescence was an enigma. A plausible explanation is that miR-146a-5p may serve to restrain excessive TGF-β signaling in order to maintain tissue homeostasis

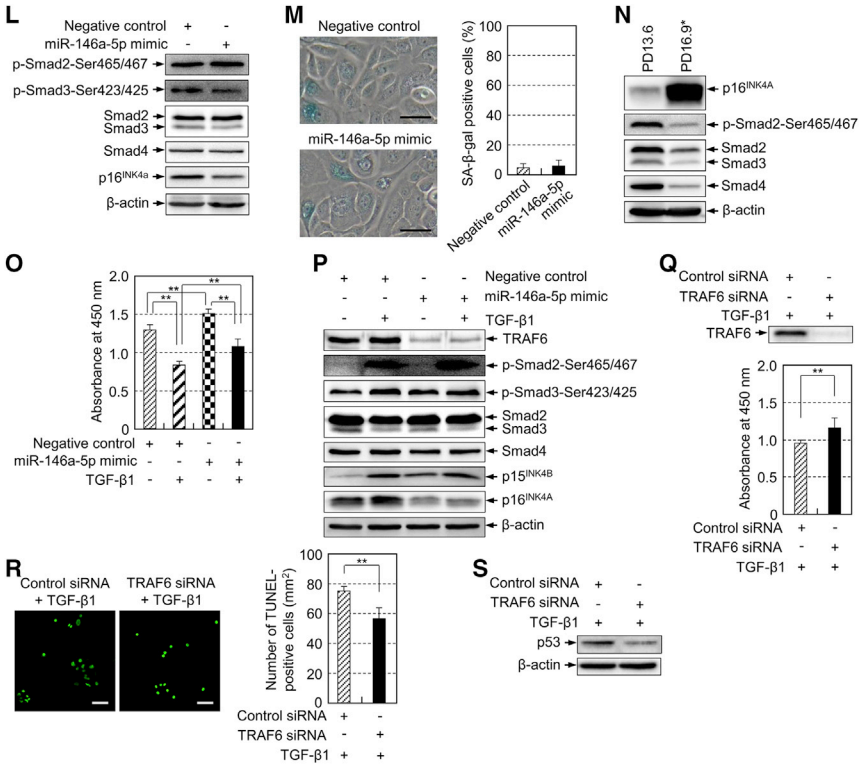

Figure 1. (*continued*).

during stress-induced senescence in primary NHOKs. Consistent with this, miR-146a-5p rescues cells from oncogenic Ras-induced senescence.[9] In addition, miR-146a regulates toll-like receptor 2-induced inflammatory responses in keratinocytes in a negative feedback manner by targeting interleukin-1 receptor-associated kinase 1 and TRAF6.[10]

Not surprisingly, miR-146a-5p is implicated in multiple epithelial lesions. miR-146a-5p is highly expressed in both progressive premalignant leukoplakia and oral squamous cell carcinoma compared with normal mucosa and non-progressive leukoplakia.[11] Theoretically, once cells achieve resistance against senescence, miR-146a-5p can further drive transformation of keratinocytes by conferring resistance against cell static signals, such as TGF-β. Epithelial lesions characterized by dysregulation of immune cells, such as oral lichen planus, are also linked with dysregulation of miR-146a-5p.[12] Altogether, thorough comprehension of the miRNA may result in a valuable armament in clinical applications against such diseases in the future. In summary, our findings demonstrate that miR-146a-5p is overexpressed as a consequence of stress-induced senescence of primary NHOKs and mediates a negative regulatory circuit within TGF-β signaling by targeting TRAF6.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Seung-Ki Min,[1,3] Sung Youn Jung,[2] Hyun Ki Kang,[2] Seung Bin Jo,[2] Myung-Jin Kim,[1] and Byung-Moo Min[2]

[1]Department of Oral and Maxillofacial Surgery, Seoul National University School of Dentistry, Seoul 03080, Republic of Korea; [2]Department of Oral Biochemistry and Program in Cancer and Developmental Biology, Dental Research Institute, Seoul National University School of Dentistry, Seoul 03080, Republic of Korea

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[3]Present address: Oral Oncology Clinic, Research Institute and Hospital, National Cancer Center, Goyang-si, Gyeonggi-Do 10408, Republic of Korea

Correspondence: Byung-Moo Min, Department of Oral Biochemistry and Program in Cancer and Developmental Biology, Seoul National University School of Dentistry, 101 Daehak-Ro, Jongno-Gu, Seoul 03080, Republic of Korea.
E-mail: bmmin@snu.ac.kr

Correspondence: Myung-Jin Kim, Department of Oral and Maxillofacial Surgery, Seoul National University School of Dentistry, 101 Daehak-Ro, Jongno-Gu, Seoul 03080, Republic of Korea.
E-mail: myungkim@snu.ac.kr

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