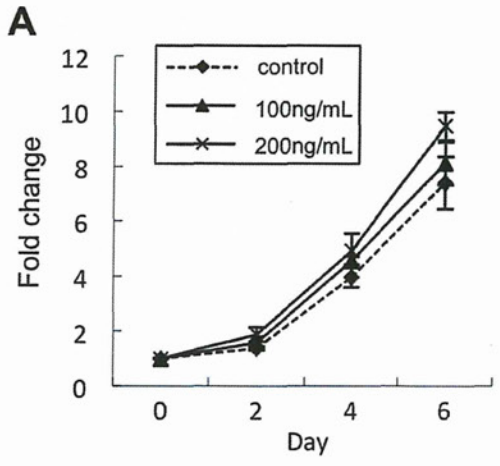

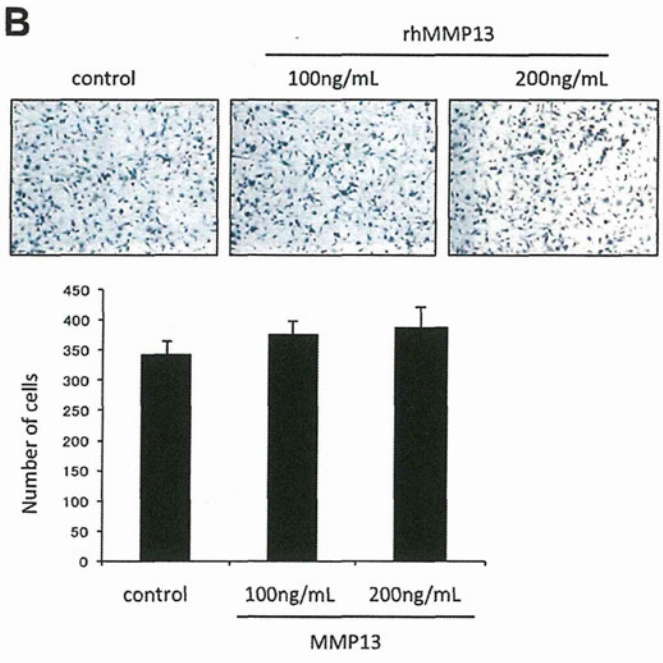

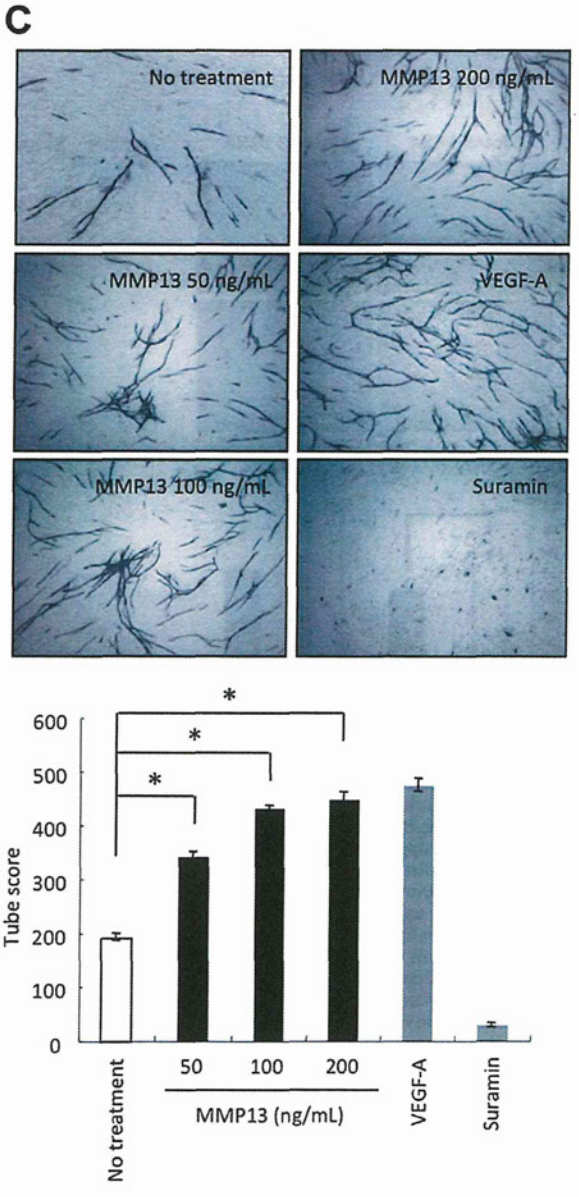

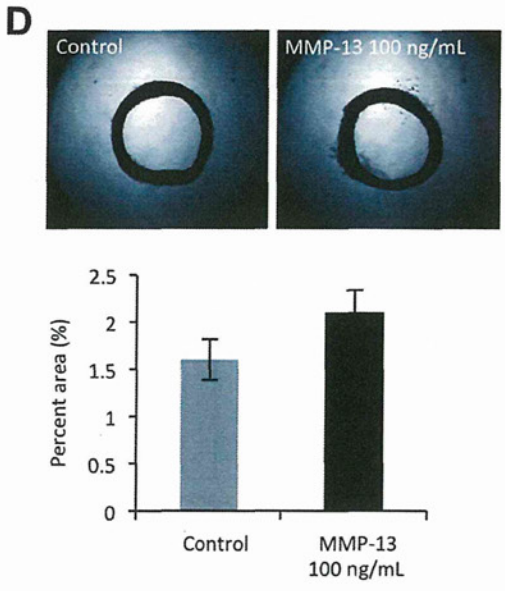

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methanol containing 0.3% H_2O_2 for 30 min. Antigen retrieval was done by the microwaving using a citrate phosphate buffer (pH 6.0), and then the sections were incubated with the primary antibody at 4 °C overnight. Immunohistochemical staining was carried out by a monoclonal anti-MMP-13 antibody (Fuji Company Industries, 1:80). For detection of the reaction after incubation with secondary antibodies, we used diaminobenzidine (DAKO, Glostrup, Denmark). The sections were counterstained by hematoxylin and dehydrated in ascending grades of ethanol, and finally, the slides were mounted. By considering the percentage of positive cells and the overall staining intensity, MMP-13 was considered positive if over 10% of the tumor cells showed strong or diffuse staining. If less than 10% of the cells showed weak or no staining, it was considered negative.

Assay for Blood Vessel Density—CD34 is an antigen present in hematopoietic progenitor cells and endothelial cells. Anti-CD34 antibody is a highly sensitive marker for endothelial cell differentiation and has also been studied as a marker for vascular tumors. To investigate the relation between angiogenesis and MMP-13, we stained all HNSCC cases with CD34 endothelial marker (Novocastra Laboratories Ltd., Newcastle, UK) by SABC method. To assess blood vessel density, we performed histomorphometric analysis. Three representative photomicrographs (areas where MMP-13 positivity were detected including invasive front) were taken from each case stained with CD34. First, we went through all the sections stained with MMP-13 and CD34 antibodies. The area was selected by the following criteria: (i) the expression of MMP-13, (ii) the included invasive tumor front, and (iii) the high number of blood vessels. Photographs of those tumors were taken in close proximity to MMP-13-expressing area including the invasive front. For MMP-13-negative cases, three areas from the invasive front were selected. Any positively stained endothelial cell or endothelial cell cluster, with or without a lumen, was considered as a single, countable blood vessel. Stromal area was quantitatively analyzed using digital image (Adobe Photoshop and Scion Image software). From each figure, total counts of blood vessels per stromal area were taken, and the average was calculated. The results were then compared with MMP-13 expression.

Statistical Analysis—A p value < 0.05 was required for assessing the significance. Correlation between variables was estimated using Fisher's exact test, and for correlation between MMP-13 expression and blood vessel density, a Welch test was used.

RESULTS

MMP-13 Promotes Angiogenesis—We previously identified periostin, interferon-induced transmembrane protein 1 (IFITM1), and Wnt-5b as cancer invasion-related factors by comparing the gene expression profiles between parent and highly invasive clone of a cancer cell line (22). MMP-13 was identified as a common up-regulated molecule by comparing the gene expression profiles between control cells and periostin-overexpressing cells, control cells and IFITM1-overexpressing cells, and control cells and Wnt-5b-overexpressing cells (Fig. 1*A*) (26). It is known that MMP-13 is highly expressed in various tumors and is related to tumor behavior and prognosis (18). To know the role of MMP-13 in cancer development, we generated MMP-13-overexpressing cancer cells. Expression of MMP-13 mRNA was examined in six head and neck cancer cell lines (Fig. 1*B*). Among six cell lines, HSC2 and HSC3 cells showed lower expression of MMP-13 mRNA. Expression level of MMP-13 in these cells was lower than that in other cancer cells. Therefore, we transfected a FLAG-MMP-13 plasmid into HSC3 cells. Then, we obtained four stable clones and one stable pool clone of MMP-13-overexpressing cells (Fig. 1*C*). All stable clones highly expressed ectopic MMP-13 (Fig. 1*C*). In further experiments, clone 1 was used. By using MMP-13-overexpressing cells, we examined the role of MMP-13 in cell growth and invasion. MMP-13 overexpression did not affect cell proliferation and slightly promoted the invasion of HNSCC cells (data not shown). We also confirmed that conditioned medium from MMP-13-overexpressing cells had a higher protease activity than that from control cells (Fig. 1*D*).

MMP-13 has recently been shown to play a critical role in the process of angiogenesis during the healing of fracture (15). Here, we examined the role of MMP-13 in angiogenesis. MMP-13 secretion was detected in conditioned medium from MMP-13-overexpressing-HSC3 cells by Western blot analysis (Fig. 1*E*). Expression level of ectopic MMP-13 in MMP-13-overexpressing HSC3 cells was similar to that of endogenous MMP-13 in Ho-1-N-1 or HSC4 cells (Fig. 1*F*). By using conditioned medium from MMP-13-overexpressing cells, we examined the migration of immortalized HUVECs. The HuhT1 cell line was previously established from HUVECs by transfection with human telomerase reverse transcriptase (20). Conditioned medium from MMP-13-overexpressing cells promoted migration of HuhT1 cells (Fig. 2*A*). Interestingly, conditioned medium from MMP-13-overexpressing cells significantly promoted capillary tube formation, in comparison with that from

FIGURE 3. **MMP-13 promoted angiogenesis both *in vitro* and *in vivo*.** *A*, effect of MMP-13 on the proliferation of HuhT1 cells. Cells were plated on 24-well plates, and trypsinized cells were counted by Cell Counter at 0, 2, 4, and 6 days after adding recombinant MMP-13 protein (100 or 200 ng/ml). *B*, migration activity by recombinant MMP-13 protein. Migration activity was measured as described in Fig. 1*E*. The *upper panel* shows the representative area of penetrated cells. The *lower graph* shows the average number of penetrated cells. The *bars* show the average values and S.D. of three independent experiments. *C*, *upper panel* shows the representative area of capillary tube formation by treatment with recombinant MMP-13 protein (50, 100, and 200 ng/ml) (×40). VEGF-A (2 μg/ml) was used as a positive control, and suramin (1 mM) was used as a negative control. Capillary tube formation was examined as described in Fig. 1*F*. The *lower graph* shows the average capillary tube score after treatment with recombinant MMP-13 protein. The capillary tube score was estimated with the Chalkley count method under a bright-field microscope. The values represent means of capillary tube score + S.D. based on three wells/data point in a single experiment. *, $p < 0.05$. *D*, *upper panel* shows representative case of culturing aortic explants in three-dimensional matrix gels with or without recombinant MMP-13 protein (100 ng/ml). Excited thoracic aorta (1-mm-long cross-sections) was placed on the Matrigel-coated wells and covered with an additional 50 μl of Matrigel. Afterward, Control was treated with EBM-2 medium only or EBM-2 medium containing recombinant MMP-13 protein. Each medium was added every other day. All assays were performed by using five aortic rings per sample. Aortic rings were photographed on day 15. The area of angiogenic sprouting was calculated using Image-Pro Plus software program (Media Cybernetics). The *lower graph* shows microvessel densities in square pixels.

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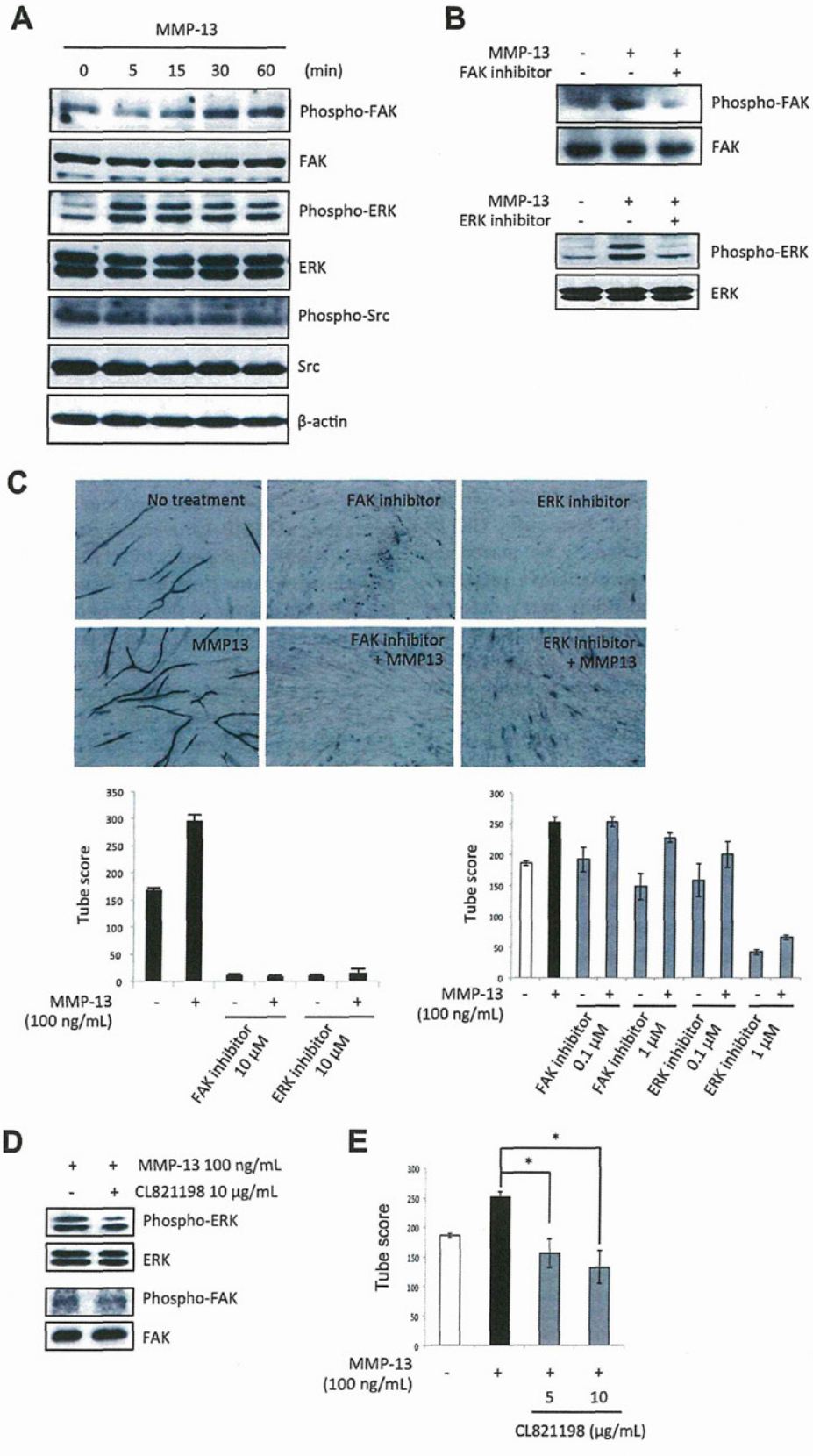

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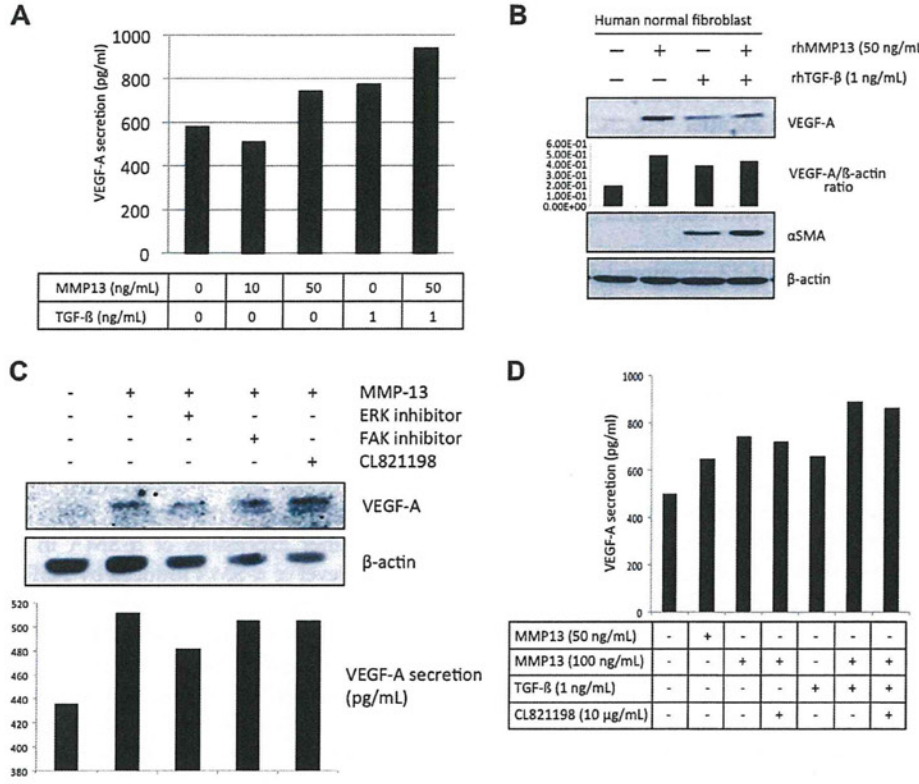

FIGURE 5. **VEGF-A secretion by MMP-13 treatment in fibroblasts.** *A*, fibroblasts were seeded on a culture dish. After incubation for 24 h, medium was changed to DMEM without FBS. After 24 h, MMP-13 (0, 10, and 50 ng/ml) and TGF-β (1 ng/ml) with or without MMP-13 (50 ng/ml) were treated for 24 h. The concentration of VEGF-A in the culture medium was quantified with commercial ELISA kits according to the manufacturer's instructions. *B*, after treatment with MMP-13 (0, 10, and 50 ng/ml) or TGF-β (1 ng/ml) with or without MMP-13 (50 ng/ml) for 24 h, fibroblasts were collected. Expressions of VEGF-A, α-SMA, and β-actin were examined by immunoblotting. The densitometric analysis of VEGF-A expression was performed. VGEF-A/β-actin ratio is shown. *C*, HuhT1 cells were seeded on a culture dish. After incubation for 24 h, medium was changed to HuMedia without FBS and growth factors. After 4 h, the recombinant MMP13 protein (100 ng/ml) with or without 10 μM of FAK inhibitor (FAK inhibitor 14), 10 μM of ERK inhibitor (U0126) or 10 μg/ml of CL-821198 were added and the cells were incubated for 1 h. Expression of VEGF-A and β-actin were examined by immunoblotting. The concentration of VEGF-A in the culture medium was quantified with commercial ELISA kits according to the manufacturer's instructions. *D*, fibroblasts were seeded on a culture dish. After incubation for 24 h, medium was changed to DMEM without FBS. After 24 h, MMP-13 (0, 50, and 100 ng/ml) and TGF-β (1 ng/ml) with or without MMP-13 (100 ng/ml) were treated for 24 h. Moreover, we treated CL-821198 (10 μg/ml). The concentration of VEGF-A in the culture medium was quantified with commercial ELISA kits according to the manufacturer's instructions.

empty vector-transfected HSC3 cells (Fig. 2*B*). Moreover, we examined MMP-13 knockdown in Ho-1-N-1 or HSC4 cells with MMP-13 expression. MMP-13 siRNA reduced MMP-13 expression and protease activity (Fig. 1, *D* and *F*). Conditioned medium from MMP-13-depleted cells suppressed migration and capillary tube formation (Fig. 2, *C* and *D*).

To exclude other factors in conditioned medium, we used recombinant MMP-13 protein for *in vitro* angiogenesis assay. We examined the effect of recombinant MMP-13 protein on cell growth and migration of HuhT1 cells. Treatment with recombinant MMP-13 protein did not significantly promote cell growth and migration of HuhT1 cells (Fig. 3, *A* and *B*). For

FIGURE 4. **MMP-13-promoted angiogenesis is mediated by FAK and ERK signaling pathway.** *A*, levels of total and phosphorylated forms of FAK, Src, and ERK after treatment of HuhT1 cells with MMP-13 (100 ng/ml) shown by Western blotting. β-Actin expression was used as a loading control. HuhT1 cells were seeded on a culture dish. After incubation for 24 h, medium was changed to HuMedia without FBS and growth factors. After 4 h, the recombinant MMP13 protein (100 ng/ml) was added and the cells were incubated for indicated times. *B*, phosphorylated forms of FAK (Tyr-576/577), Src (Tyr-416) and ERK (Thr-202/Tyr-204) in the presence of MMP-13 (100 ng/ml) after treatment with 10 μM FAK inhibitor (FAK inhibitor 14) or 10 μM ERK inhibitor (U0126). Expression of total FAK or ERK was used as a loading control. *C*, *upper panel* shows the representative area of capillary tube formation by FAK inhibitor (FAK inhibitor, 14 or 10 μM) or ERK inhibitor (U0126, 10 μM) with or without MMP-13 (100 ng/ml) (×40). The *lower left graph* shows the average tubule score after 10 μM FAK inhibitor (FAK inhibitor 14) or 10 μM of ERK inhibitor (U0126) with or without 100 ng/ml of recombinant MMP-13 protein. The *lower right graph* shows the average tubule score after FAK inhibitor (0.1 and 1 μM) or ERK inhibitor (0.1 and 1 μM) with or without 100 ng/ml of recombinant MMP-13 protein. The values represent means of capillary tube score + S.D. based on three wells/data point in a single experiment. *D*, to examine the effect of protease inhibition on FAK and ERK phosphorylation, CL-821198, which is a selective inhibitor of MMP-13 through the binding to the S1′ pocket of MMP-13 with its morpholine ring adjacent to the catalytic zinc atom, was used. HuhT1 cells were seeded on a culture dish. After incubation for 24 h, medium was changed to HuMedia without FBS and growth factors. After 4 h, CL-821198 (10 μg/ml) and/or recombinant MMP13 protein (100 ng/ml) were added, and the cells were incubated for 1 h. Levels of total and phosphorylated forms of FAK and ERK was examined by Western blotting. *E*, capillary tube formation was examined by using an angiogenesis assay kit. HUVECs were treated with the recombinant MMP-13 protein with or without CL-821198 (5 and 10 μg/ml), and the medium was changed every 3 days. After 12 days, the cells were fixed and stained with anti-human CD31 antibody. The graph shows the average capillary tube score after treatment with recombinant MMP-13 protein. The capillary tube score was estimated with the Chalkley count method under a bright-field microscope. The values represent means of capillary tube score + S.D. based on three wells/data point in a single experiment. *, $p < 0.05$.

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in vitro angiogenesis assay, we used VEGF-A as a positive control and suramin as a negative control. Similarly to conditioned medium from MMP-13-overexpressing cells, treatment with MMP-13 protein significantly promoted capillary tube formation in a concentration-dependent manner (Fig. 3*C*). Surprisingly, capillary tube score of MMP-13 treatment was similar to that of VEGF-A (Fig. 3*C*). However, MMP-13 did not significantly stimulate microvessel outgrowth from aorta comparing with control (Fig. 3*D*).

To clarify the mechanism of MMP-13-promoted angiogenesis, we examined the involvement of several intracellular signaling molecules such as FAK, Src, and ERK by Western blotting using phosphorylation specific antibodies in HuhT1 cells after adding recombinant MMP-13 protein. Increased phosphorylation of FAK and ERK was observed after adding MMP-13 protein (Fig. 4*A*). To demonstrate the involvement of FAK and ERK in MMP-13-promoted angiogenesis, we examined capillary tube formation after treatment with FAK inhibitor (FAK inhibitor 14) or ERK inhibitor (U0126) together with recombinant MMP-13 protein. We confirmed that treatment with FAK inhibitor 14 or U0126 suppressed FAK or ERK activity, respectively (Fig. 4*B*). Both inhibitors inhibited MMP-13-promoted capillary tube formation in a concentration-dependent manner (Fig. 4*C*). Both inhibitors also inhibited capillary tube formation without MMP-13 treatment in a concentration-dependent manner (Fig. 4*C*), suggesting that the FAK and ERK signaling pathway may be a conventional pathway of angiogenesis. Moreover, FAK inhibitor did not influence on ERK activity and ERK inhibitor did not influence on FAK activity in MMP-13-treated endothelial cells (data not shown), suggesting that the inhibitory effects exerted by either FAK or ERK inhibitor are separated. These findings suggest that MMP-13 may promote angiogenesis via a conventional pathway.

To know whether ERK or FAK activity induced by MMP-13 was caused by protease activity of MMP-13, we examined the effect of protease inhibition on FAK and ERK phosphorylation by using CL-821198, which is a selective inhibitor of MMP-13 through the binding to the S1′ pocket of MMP-13 with its morpholine ring adjacent to the catalytic zinc atom. CL-821198 treatment did not influence on ERK or FAK activity in HuhT1 cells (Fig. 4*D*). This finding indicates that ERK or FAK activity induced by MMP-13 is not caused by protease activity of MMP-13. We also examined the effect of CL-821198 on capillary tube formation. CL-821198 inhibited MMP-13-promoted tube formation. This finding suggests that MMP-13 activity may affect to capillary tube formation via an ERK- or FAK-independent manner (Fig. 4*E*).

MMP-13 Promotes VEGF-A Secretion in Fibroblasts and Endothelial Cells—A recent report shows that esophageal squamous cell carcinoma-derived TGF-β regulates angiogenesis through the release of VEGF from fibroblasts (27). We examined whether MMP-13 affected the release of VEGF from fibroblasts or endothelial cells as a similar function of TGF-β. Normal fibroblasts were obtained from gingival tissues using standard explant techniques (21). The level of VEGF-A secretion by fibroblasts was measured after being induced by MMP-13 with/without the presence of TGF-β. Interestingly, MMP-13 could promote the secretion of VEGF-A, especially in the presence of TGF-β (Fig. 5*A*). It is known that paracrine tumor-derived growth factors activate the cancer-associated fibroblasts, which undergo a myofibroblastic transdifferentiation defined by an elongated spindle shape, and the expression of contractile α-SMA and vimentin (28). Therefore, we examined the expression of α-SMA after TGF-β or MMP-13 treatment in fibroblasts. As previously reported, TGF-β induced α-SMA expression in fibroblasts (Fig. 5*B*). Although MMP-13 itself did not induce α-SMA expression in fibroblasts, both TGF-β and MMP-13 induced higher expression of α-SMA in comparison with the expression level of α-SMA induced by TGF-β (Fig. 5*B*).

TABLE 1
Correlation between MMP-13 expression and clinicopathologic findings in HNSCC

		MMP-13 expression		
	No. of cases	Low	High	p value
Non-neoplastic epithelium	30	30 (100%)	0 (0%)	$p < 0.001$
HNSCC	67	13 (19.4%)	54 (80.6%)	
Metastasis				
−	30	7 (23.3%)	23 (76.7%)	
+	37	6 (16.2%)	31 (83.8%)	

Moreover, we examined whether MMP-13-promoted VEGF-A secretion from the endothelial cell line HuhT1 or not. Interestingly, the expression and secretion levels of VEGF-A were increased by MMP-13 treatment (Fig. 5, *C* and *D*). Induction of VEGF-A in HuhT1 cells was partially dependent on ERK activity but not on FAK activity and MMP-13 protease activity (Fig. 5, *C* and *D*).

MMP-13 Is Highly Expressed in Human Cancer Tissues—To demonstrate *in vitro* and *in vivo* evidence of MMP-13-mediated angiogenesis, we examined the expression of MMP-13 and its relationship with tumor angiogenesis in clinical cancer cases. We examined the immunohistochemical expression of MMP-13 in 20 normal oral epithelium and 67 HNSCC tissues. Positive expression of MMP-13 was observed in 0 of 20 (0%) normal oral epithelium and 54 of 67 (81%) HNSCC cases (Fig. 5, *A* and *B*, and Table 1). We compared MMP13 expression with metastasis in HNSCC cases. MMP-13 expression was well correlated with metastasis (Table 1). Then, we compared MMP-13 expression with the number of blood vessels in HNSCC cases. The number of blood vessels was examined by staining using anti-CD34 antibody. CD34 is an antigen present in hematopoietic progenitor cells and endothelial cells. Anti-CD34 antibody is a highly sensitive marker for endothelial cell differentiation and has also been studied as a marker for vascular tumors. We observed an increased number of blood vessels at the invasive front of the MMP-13 positive tumor cases, compared with the MMP-13 negative cases ($p < 0.05$) (Fig. 6, *A* and *B*). The average number of blood vessel density assessed by histo-morphometric analysis was 41.4 ± 13.0 and 76.2 ± 26.6 in MMP-13 negative and positive cases, respectively ($p < 0.05$) (Fig. 6*C* and Table 1).

DISCUSSION

Angiogenesis, the formation of new blood vessels from preexisting ones, is a crucial step in tumor growth, progression, and metastasis. Regulation of angiogenesis *in vivo* is complex and is controlled by a variety of factors. Among them, VEGF is considered to play a dominant role. It has been well established that VEGF promotes a cancer progression by up-regulating

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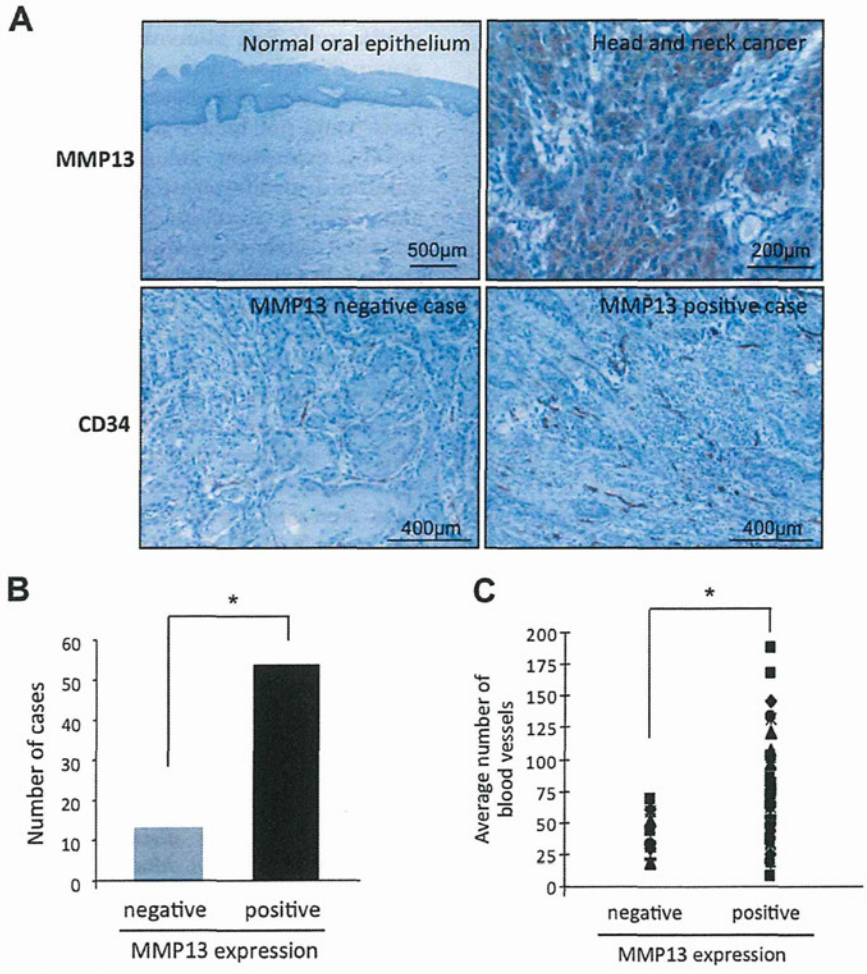

FIGURE 6. **MMP-13 expression is well correlated with the number of blood vessels in human cancer cases.** *A*, immunohistochemical staining of MMP-13 and CD34 in normal oral epithelium and HNSCC. Representative cases of MMP-13 expression in normal oral epithelium and HNSCC are shown. Representative cases of CD34 expression in HNSCC cases with or without MMP-13 expression are also shown. *B*, graph shows the number of cases with or without MMP-13 expression in 67 HNSCC cases. *, $p < 0.05$. *C*, graph shows the average number of blood vessels in HNSCC cases with or without MMP-13 expression. *, $p < 0.05$.

microvessel density (3). MMPs are zinc metalloenzymes with the ability to degrade the components of the ECM. Their action is crucial during the progression of cancer because they allow the remodeling of the surrounding healthy tissues and enable local invasion (8). MMP-13 is known as collagenase-3, which has the ability to degrade fibrillar collagen (29). However, it may also act as a potent gelatinase by degrading a wide variety of extracellular matrix components (30, 31). MMP-13 is overexpressed in a variety of tumors from such as head and neck, laryngeal, breast, chondrosarcoma, gastric, colorectal, vulvar carcinomas and cutaneous malignant lymphoma (17, 32–39). In most malignancies, MMP-13 has been correlated with tumor invasion, metastasis, and poor prognosis in patients (34, 35, 37, 39, 41). MMP-13 is predominantly expressed by tumor cells at the tumor invasive front and to some extent by stromal fibroblasts surrounding tumor cells (33, 41). Our immunohistochemical finding that MMP-13 expression is frequently observed, but no statistical correlation was observed in MMP-13 expression and metastasis in HNSCC (Table 1). As we used biopsy cases in this study, HNSCC cases with high expression of MMP-13 may have a potential to metastasize later. Indeed, MMP-13 expression was well correlated with number of blood vessels. Thus, it is well accepted that MMP-13 is involved in tumor progression.

In the present study, we demonstrate the novel role of MMP-13 in tumor angiogenesis. Although there is a study suggesting a role of MMP-13 in keratinocyte migration and angiogenesis during the healing of fracture (43), the role of MMP-13 in tumor angiogenesis has not been fully elucidated. It is known that some MMPs such as MMP-1, -2, -3, -7, -9, -14 and -16 are involved in tumor angiogenesis via the regulation of bioavailability of VEGF-A (11). Distinct from the function of other MMPs in angiogenesis, MMP-13 promoted angiogenesis through an increased number of blood vessels at the invasive front of the tumor and up-regulation of VEGF-A secretion from fibroblasts and endothelial cells (Fig. 7). We also found that MMP-13 promoted capillary tube formation was mediated by activation of FAK and ERK. FAK is a cytoplasmic tyrosine kinase that plays critical roles in integrin-mediated signal transductions and also participates in signaling by other cell surface receptors (44). Extensive studies in FAK knock-out mouse models indicated a critical role of FAK in angiogenesis during

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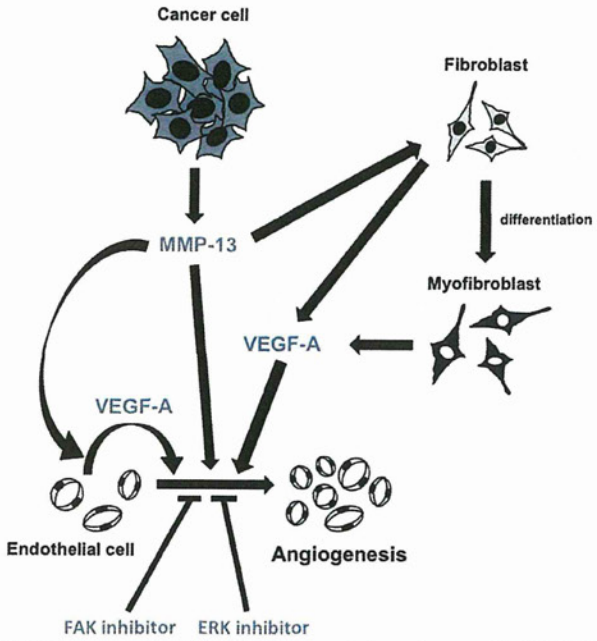

FIGURE 7. **A model of MMP-13-promoted angiogenesis.** MMP-13 is secreted from cancer cells. MMP-13 promotes angiogenesis through FAK and ERK signaling pathway. Moreover, MMP-13 enhances the secretion of VEGF-A from endothelial cells, fibroblasts or myofibroblasts. Secreted VEGF-A promotes angiogenesis.

embryonic development (45). Moreover, the increased expression of FAK in cancer cells has been suggested to play a role in the tumor angiogenic switch to promote aggressive tumor progression and metastasis (46). However, Baek *et al.* (47) found that the MEK/ERK pathway is involved in endothelial cell proliferation through up-regulation of positive cell cycle proteins and down-regulation of negative cell cycle proteins. Thus, activation of FAK and ERK is critically involved in angiogenesis. Indeed, treatment with FAK inhibitor or ERK inhibitor strongly inhibited the capillary tube formation of endothelial cells, suggesting that FAK and ERK signaling are essential in angiogenesis. Although MMP-13 has central roles in modulating extracellular matrix degradation through its direct matrix degrading capability as well as having a key involvement in the activation of other MMPs (7), FAK or ERK activity induced by MMP-13 was not mediated by matrix degrading capability (Fig. 4*D*). Our findings suggest that MMP-13-driven angiogenesis may be mediated by a conventional pathway via activation of FAK and ERK. However, the mechanism of ERK or FAK activation by MMP-13 is still unclear.

The tumor tissue consists of a dynamic mixture of tumor cells, fibroblasts, endothelial cells, and immune cells that all work together to drive tumor progression (48). Activated fibroblasts, also known as cancer-associated fibroblasts within the tumor microenvironment, is preceded by the chemoattraction and migration of precursor cells, which can either arise from the surrounding host fibroblasts or from circulating mesenchymal precursor cells (40, 42, 49). Cancer-associated fibroblasts are activated by paracrine tumor-derived growth factors, which undergo a myofibroblastic transdifferentiation (28). Noma *et al.* (27) showed that paracrine TGF-β from the esophageal cancer cells lead to activation of the fibroblasts and vascular network formation through the release of VEGF-A. Interestingly, MMP-13 induced the secretion of VEGF-A from fibroblasts in similar to TGF-β. Moreover, TGF-β induced α-SMA expression in fibroblasts, indicating that the phenotypic switch from fibroblast to myofibroblast may be caused by TGF-β. MMP-13 itself could not induce α-SMA expression, but it enhanced α-SMA expression induced by TGF-β. Although TGF-β induced a myofibroblastic transdifferentiation of fibroblasts and VEGF-A secretion from myofibroblasts, TGF-β itself inhibited capillary tube formation of endothelial cells (data not shown). Previous report shows MMP-13 increases the expression of VEGF and its receptor, VEGFR-2 (19). In this study, we could not detect VEGFR-2 expression in fibroblasts after treatment with MMP-13 and/or TGF-β by real-time PCR analysis (data not shown). In addition, VEGF-A induction by MMP-13 in fibroblasts and endothelial cells was not dependent on MMP-13 protease activity (Fig. 5, *C* and *D*). Although it is unclear how MMP-13 promotes VEGF-A secretion from fibroblasts and endothelial cells, it is interesting to examine the detailed role of MMP-13 in the tumor microenvironment.

In summary, our findings suggest that MMP-13 may directly and indirectly promote tumor angiogenesis. In various tumors, MMP-13 is correlated with tumor invasion, metastasis, and poor prognosis. Therefore, we believe that MMP-13 can be a potential target for therapeutic intervention to additionally obstruct tumor angiogenesis in cancer patients.

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The role of exosomes and microRNAs in senescence and aging ☆

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ABSTRACT

Senescence is viewed as a cellular counterpart to aging of tissues and organisms, characterized by an irreversible growth arrest and a combination of changes in cell morphology, function and behavior. microRNAs (miRNAs), the most studied small non-coding RNAs, play an important role in many biological processes by the regulation of gene expression. Recent evidence has shown that miRNAs are contained in exosomes that are tiny vesicles of endocytic origin and released by a variety of different cells as a means for cell-to-cell contact and information transfer. Exosomes and miRNAs have been found to participate in the complex networks of cellular senescence and contribute to aging. Here, we will give an overview on the involvement of secretory factors including exosomes and miRNA in the regulation of cellular senescence, demonstrating the potential role of exosomes and miRNAs in biological processes and signaling pathways of senescence and aging.

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Contents

1. Introduction

Exosomes are specialized membranous vesicles (40–100 nm in diameter) of endocytic origin. It was first described and observed by tracking the fate of recycling transferrin receptor during maturation of sheep reticulocytes. Exosomes are formed intracellularly via endocytic invagination and are generated by the outward budding at the limiting endosomal membrane of the multivesicular bodies (MVBs), sharing the biochemical characteristics with the internal vesicles of MVBs [1,2]. Exosomes are released into the extracellular environment from a variety of cells, such as, but not limited to, tumor cells, dendritic cells, lymphoid cells, mesothelial cells, epithelial cells, or cells from different tissues or organs. Thus, cells may communicate through membrane transfer by the secretion of exosomes [3]. Exosomes contain proteins, mRNAs, miRNAs, and signaling molecules that reflect the physiological state of their cells of origin and consequently provide a rich source of potential biomarker molecules [4,5]. Recently, exosomes have been recognized that they appear as a vectorized signaling system operating from inside a donor cell towards either the periphery, the cytosol, or possibly to the nucleus of target cells. Exosomes have been detected to date in various body fluids such as urine, serum, saliva and breast milk, function in intercellular communication, immune system modulation and tumor progression [6–8].

In the past few years, the importance of miRNAs, an abundant class of small non-coding RNAs, has rapidly emerged as important contributors to gene regulation. miRNAs form a particular class of 21- to 24-nucleotide RNAs that can regulate gene expression post-transcriptionally by affecting the translation and stability of target messenger RNAs (mRNAs). Due to the multiple target genes regulated by one miRNA, miRNAs resemble an

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additional layer of complexity to gene regulation and influence global cellular responses ranging from cell cycle regulation, cell proliferation, apoptosis, to development and differentiation [9,10]. They have been shown to have unique tissue-specific, developmental stage-specific or disease-specific patterns, which were reflected by miRNA expression profiles and specific miRNA functions.

In the most recent years, the relevance of exosomes and miRNAs in many fields has been recognized. Circulating miRNAs have been thought to be a new potential biomarker for cancer diagnosis and prognosis [11]. They are strikingly stable in blood plasma/serum and cell culture media, which might be associated with exosomes that contain miRNAs and therefore protect miRNAs against RNase activity [3,12]. Exosome-mediated miRNA transfer is an important mechanism of intercellular communication [13]. It is reported that let-7 miRNA family could be selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line, implying the involvement of exosomes in miRNA-regulated tumorigenesis [14].

In regard to senescence, some miRNAs have emerged as key regulators during cellular senescence [15–18]. Recently, it has been reported that senescence is associated with the release of exosomes, and senescence-associated exosomes can transfer cargos between cells to mediate cell–cell communication during cellular senescence [19]. In this review, we will focus on recent advances in understanding the diverse regulatory role of exosomes and miRNAs on senescence and aging.

2. Cellular senescence and signaling pathway of senescence

Cellular senescence is a permanent state of growth arrest, accompanied by altered cell physiology and behaviors. It was first described as a limit to the replicative life span of somatic cells. This type of senescence is called replicative senescence, which is triggered by telomere attrition after serial cultivation in vitro [20]. Cellular senescence is also caused prematurely by intrinsic- and/or extrinsic-stress factors including DNA damage, reactive oxygen species and activated oncogene expression [21], sharing similar apparent senescence phenotypes with replicative senescence. Senescent cells exhibit an enlarged and flattened morphology, distinctive nuclear structure of heterochromatin, induction of SA-β-galactosidase activity and altered gene expression [22,23].

Cellular senescence is mainly controlled by the p53–p21 and p16–pRB tumor suppression pathways. The p53 pathway is regulated at multiple points by proteins such as the E3 ubiquitin-protein ligase HDM2 (MDM2 in mice), which facilitates p53 degradation; alternate-reading-frame protein (ARF), which inhibits HDM2 activity; and Sirtuin 1 (SIRT1), which interacts with p53 and deacetylates the Lys382 residue of p53, thereby decreasing p53-mediated transcriptional activation [24,25]. p21 is a crucial transcriptional target of p53 and downstream mediator of p53-dependent senescence [26]. Moreover, p21 itself also mediates a transient DNA damage induced growth arrest and Ras-induced senescence [27]. Senescence signals that engage the p16–pRB pathway generally do so by inducing the expression of p16 that prevents pRB phosphorylation and inactivation. pRB halts cell proliferation by suppressing the activity of E2F, a transcription factor that stimulates the expression of genes that are required for cell cycle progression. E2F can also induce ARF expression, which engages the p53 pathway. So, there is reciprocal regulation between the p53–p21 and p16–pRB pathways. Senescence-inducing signals, including DNA damage, oncogenic stresses as well as oxidative stress, usually engage the p53 and/or the pRB pathways [21]. However, there are differences in how cells respond when one or the other pathway mediates a senescence response. In addition, RAF-induced senescence independent of both p53 and pRB has been reported in human cells [28].

Considering the importance of a family of protein kinases known as cyclin-dependent kinases (CDKs) and CDK inhibitors (CKIs) in controlling cell cycle progression, their activity is of special relevance to senescence. The activity of the CDK4–6/D kinases is followed by the subsequent activation of the CDK2/cyclin E and CDK2/cyclin A kinases, which in turn trigger DNA replication. CKIs fall into two families: the INK4 family and the Cip/Kip family. The INK4-type CKIs such as p16 specifically associate and inhibit the CDK4-6 kinases. The Cip/Kip proteins including p21, p27 and p57 associate to both CDK4-6/D and CDK2/E-A kinases with dramatically different effects, depending on the relative abundance of the different CDK complexes [29]. Overexpression of CDK4 and CDK6 extends the lifespan of human fibroblasts, suggesting that these factors are key regulator of cellular senescence [30]. It is reported that there were differential roles for p21 and p16 in the mechanisms of senescence in human fibroblasts [31]. Up-regulation of p16 may be essential for maintenance of the senescent cell cycle arrest, whereas p21 may be responsible for inactivation of both cyclin E- and cyclin D1-associated kinase activity at the early stage of senescence.

Recent studies about INK4-ARF locus link epigenetics to senescence pathway [32]. Transcriptional regulation of the INK4-ARF locus plays the pivotal role in senescence, placing chromatin regulation as a critical pathway in senescence. Epigenetic repression of INK4-ARF is controlled by a crosstalk of several chromatin modulators. The polycomb group (PcG) proteins are direct regulators of the INK4-ARF locus, which catalyze histone modifications that promote changes in chromatin structure. Overexpression of the PcG proteins CBX8 delays the onset of replicative senescence in human cells [33]. DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) are also epigenetic regulators of the INK4-ARF locus. Overexpressed DNMTs in cancer cells lead to aberrant hypermethylation, which impairs expression of tumor suppressor genes by methylation of the CpG islands within promoter regions [34]. The promoters of p16 and p21 are found to be hypermethylated and therefore repressed in various cancer cell lines [35,36], suggesting that DNA methylation may be involved in cellular senescence. Modulation of histone acetylation contributes to senescence phenotype, evidenced by HDAC levels decreased upon senescence in human fibroblasts, and HDAC1/2 could lead to a delay in the onset of senescence [37,38].

Cellular senescence is the biological consequence of aging, implicated in a variety of age-associated diseases. These diseases share fundamental and unappreciated pathology at the cellular and genetic levels through cellular senescence [39]. Senescence may promote carcinogenesis in surrounding tissues by secreting a wide variety of factors, which alter the microenvironment to enhance growth of neighboring tumor cells [40]. On the other hand, emerging evidence suggests that cellular senescence also acts as a barrier to tumor progression, because senescence inhibits aberrant cell proliferation of cancer cells, decreases cell motility, and prevents the oncogenic transformation of primary human cells [41,42]. Therefore, senescence is thought to act as a double-edged sword for tumorigenesis [43].

3. Contribution of miRNAs and miRNA-regulated genes to senescence

The miRNAs are important regulatory molecules of gene expression regarding senescence. Specific overexpression or down-regulation of certain miRNAs has been shown to play a role in senescence through potentially targeting genes on the p53–p21 and p16–pRB pathways. For example, miR-34a overexpression during senescence can induce cell cycle arrest by targeting CDK4/6, Cyclin D1, E2F and SIRT1 [18,44,45]. miR-22, up-regulated in senescent cells, can negatively regulate the CDK6 and SIRT1 to induce cellular senescence [17]. In contrast, miR-24, which was down-regulated during senescence, was found to modulate cellular senescence by targeting p16 [46]. Other miRNAs (miR-16, miR-195, miR-107 and miR-185) that also directly repress CDK6 by binding to its 3′-UTR induce cell cycle arrest [47,48]. Recent reports

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