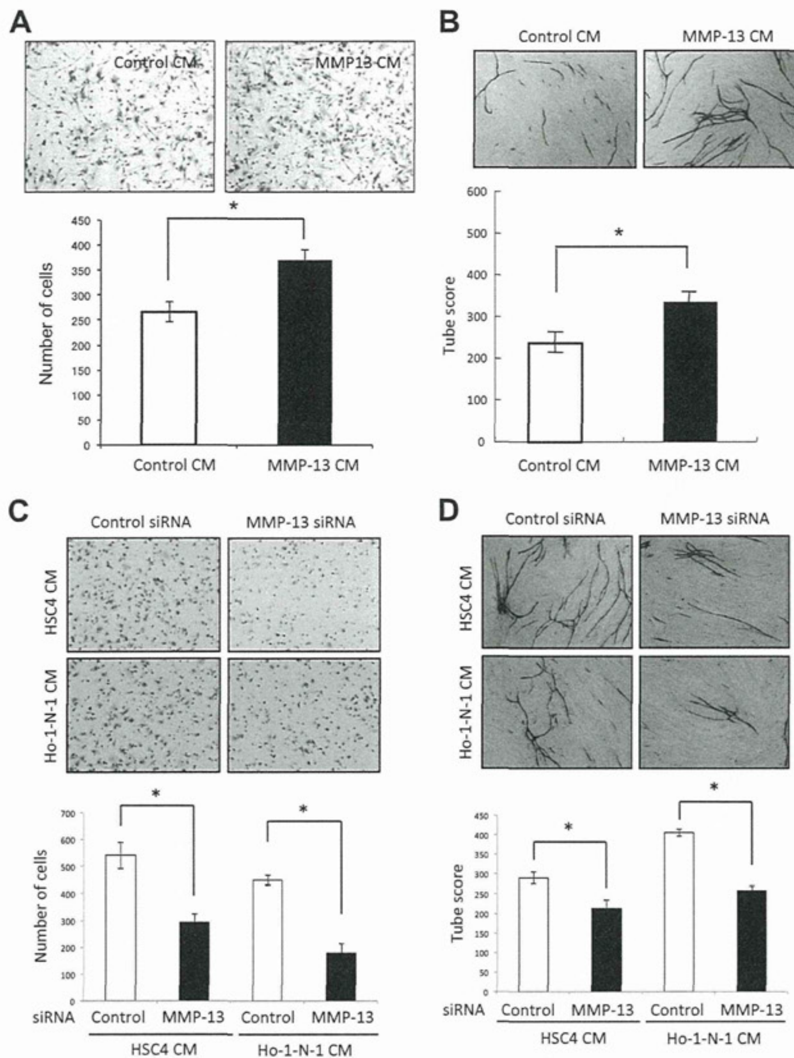

FIGURE 2. **Involvement of MMP-13 in migration and capillary tube formation of HUVECs.** *A*, migration activity by conditioned medium from MMP-13-overexpressing cells. Migration activity was measured by the use of a 24-well cell culture insert with 8-μm pores. The lower compartment contained 0.5 ml of conditioned medium from empty vector-transfected HSC3 cells (*control CM*) or MMP-13-overexpressing HSC3 cells (MMP-13 CM). After trypsinization, 5×10^4 of immortalized HUVECs (HuhT1) were resuspended in 100 μl of serum-free medium and placed in the upper compartment of the cell culture insert for 4 h. To examine the activity of migration, the cells that had penetrated onto the lower side of the filter were fixed with formalin and stained with hematoxylin. 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. *, significantly different from control at $p < 0.05$. *B*, *upper panel* shows the representative area of capillary tube formation by conditioned medium from empty vector-transfected HSC3 cells (control CM) or MMP-13-overexpressing HSC3 cells (MMP-13 CM) (×40). An angiogenesis assay kit was used according to the manufacturer's instructions with minor modifications. HUVECs were treated with mixture of conditioned medium and HuMedia-EG2 in a percentage of 1:1. The mixed media were changed every 3 days. After 12 days, the cells were fixed and stained with anti-human CD31 antibody as described under "Materials and Methods." The *lower graph* shows the average capillary tube score after conditioned medium treatment. 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. *, significantly different from control at $p < 0.05$. *C*, migration activity of HuhT1 cells by conditioned medium from control or MMP-13 siRNA-treated cells. MMP-13 siRNA were transfected into HSC4 and Ho-1-N-1 cells. Migration activity was measured as described in *A*. 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. *, significantly different from control at $p < 0.05$. *D*, upper panel shows the representative area of capillary tube formation by conditioned medium from control or MMP-13-depleted cells (×40). Capillary tube formation was examined as described in *B*. The *lower graph* shows the average capillary tube score after conditioned medium treatment. 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. *, significantly different from control at $p < 0.05$.

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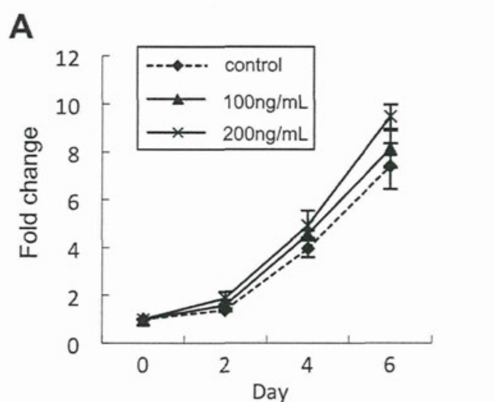

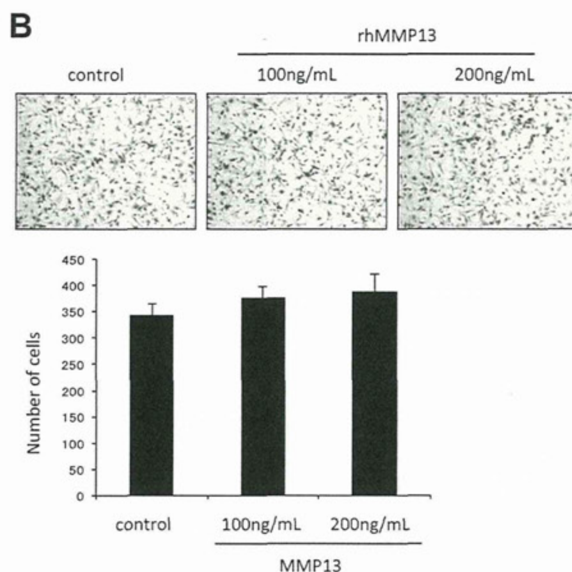

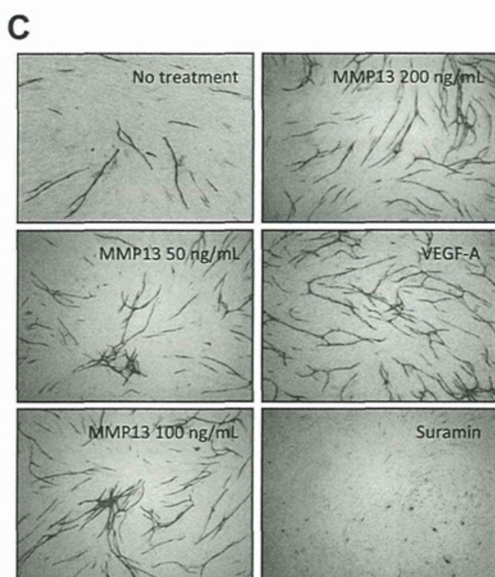

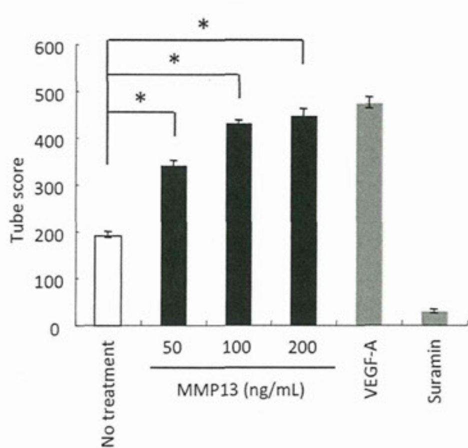

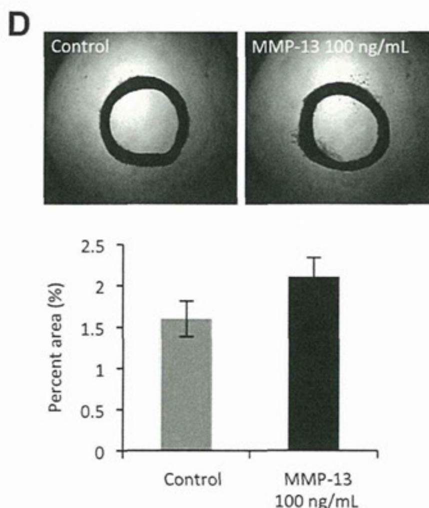

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methanol containing 0.3% H_2O_2 for 30 min. Antigen retrieval was done by the microwaving using a citrate phosphate buffer (pH 6.0), and then the sections were incubated with the primary antibody at 4 °C overnight. Immunohistochemical staining was 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

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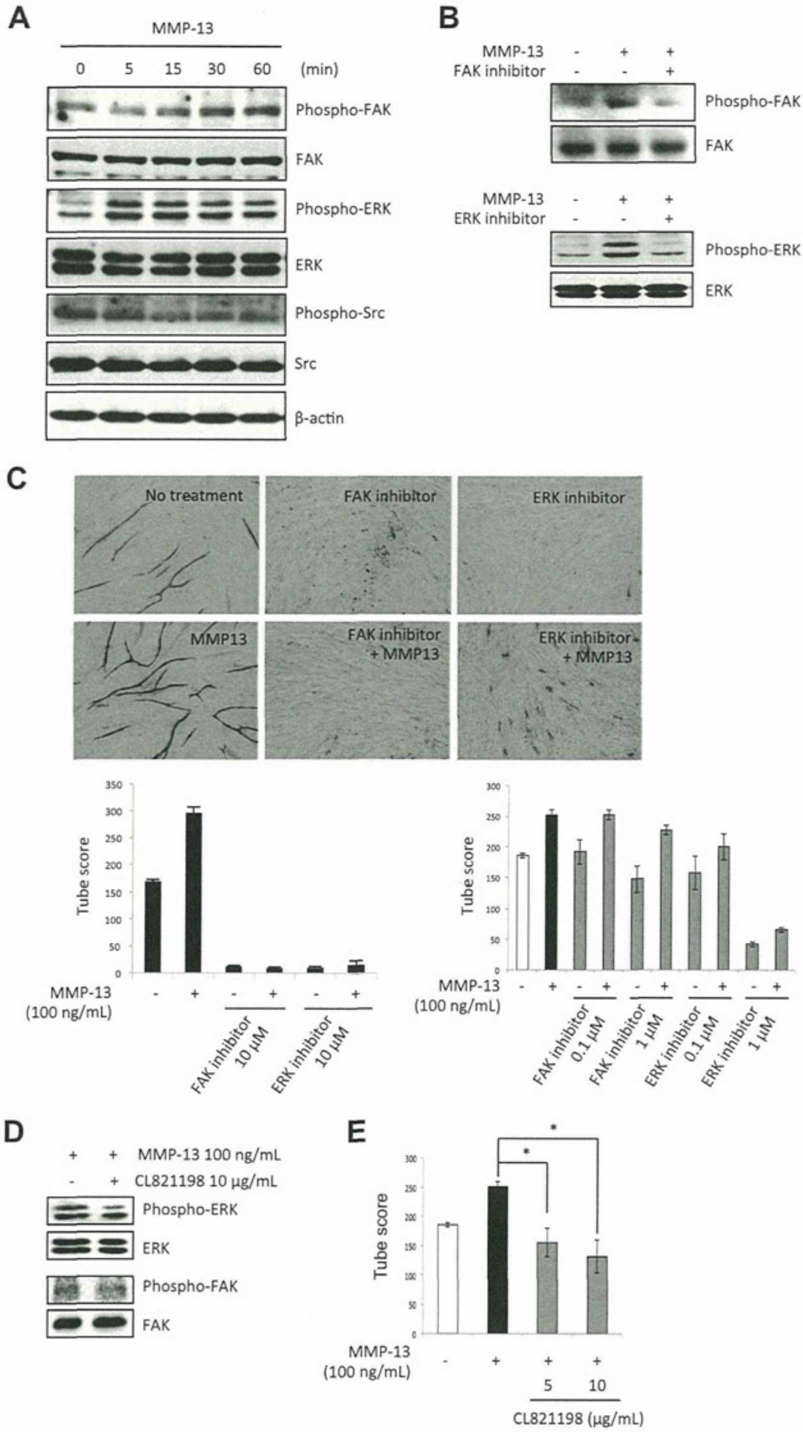
FIGURE 3. **MMP-13 promoted angiogenesis both *in vitro* and *in vivo*.** *A*, effect of MMP-13 on the proliferation of HuhT1 cells. Cells were plated on 24-well plates, and trypsinized cells were counted by Cell Counter at 0, 2, 4, and 6 days after adding recombinant MMP-13 protein (100 or 200 ng/ml). *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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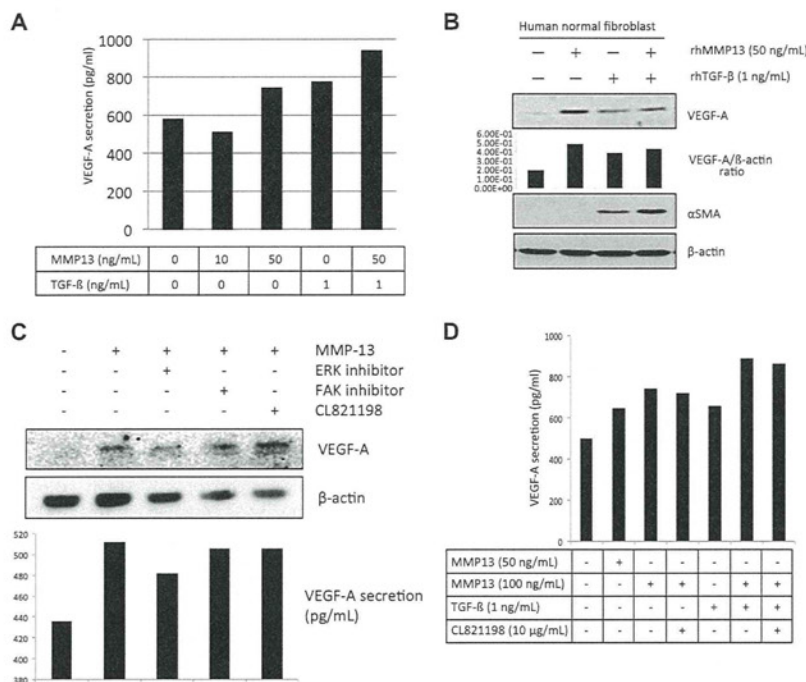

FIGURE 5. **VEGF-A secretion by MMP-13 treatment in fibroblasts.** *A*, fibroblasts were seeded on a culture dish. After incubation for 24 h, medium was changed to DMEM without FBS. After 24 h, MMP-13 (0, 10, and 50 ng/ml) and TGF-β (1 ng/ml) with or without MMP-13 (50 ng/ml) were treated for 24 h. The 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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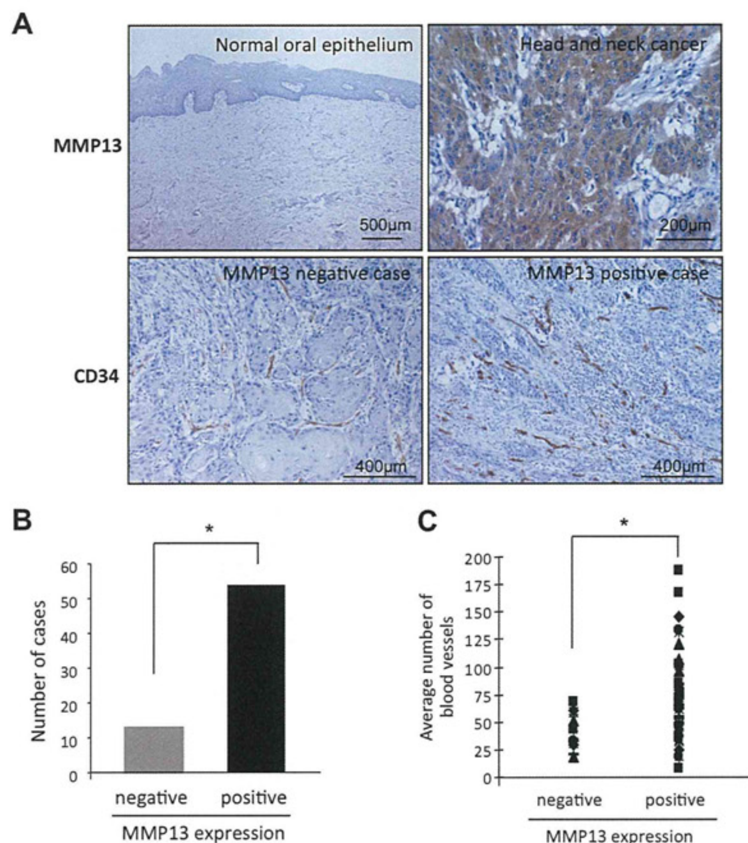

FIGURE 6. **MMP-13 expression is well correlated with the number of blood vessels in human cancer cases.** *A*, immunohistochemical staining of MMP-13 and CD34 in normal oral epithelium and HNSCC. Representative cases of MMP-13 expression in normal oral epithelium and HNSCC are shown. Representative 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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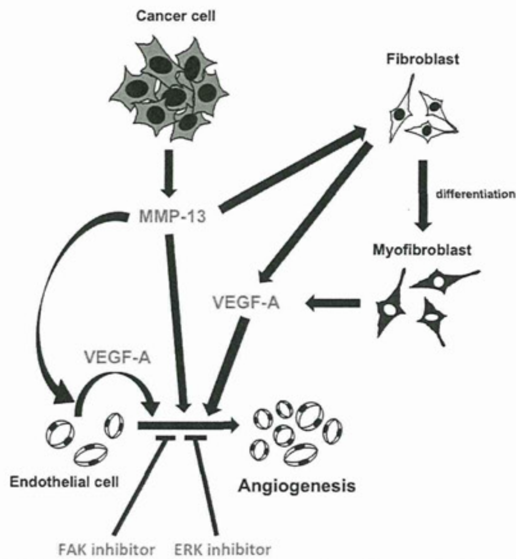

FIGURE 7. **A model of MMP-13-promoted angiogenesis.** MMP-13 is secreted from cancer cells. MMP-13 promotes angiogenesis through FAK and ERK signaling pathway. Moreover, MMP-13 enhances the secretion of VEGF-A from endothelial cells, fibroblasts or myofibroblasts. Secreted VEGF-A promotes angiogenesis.

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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REFERENCES

1. Fidler, I. J. (1990) Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes memorial award lecture. *Cancer Res.* **50,** 6130–6138
2. Howell, G. M., and Grandis, J. R. (2005) Molecular mediators of metastasis in head and neck squamous cell carcinoma. *Head Neck* **27,** 710–717
3. Folkman, J. (2000) Tumor angiogenesis in *Cancer Medicine* (Holland, J. F., ed) 5th Ed., pp. 132–152, B. C. Decker, Inc., Ontario, Canada
4. Hanahan, D., and Weinberg, R. A. (2000) The hallmarks of cancer. *Cell* **100,** 57–70
5. Bouck, N., Stellmach, V., and Hsu, S. C. (1996) How tumors become angiogenic. *Adv. Cancer Res.* **69,** 135–174
6. Fukumura, D., Xavier, R., Sugiura, T., Chen, Y., Park, E. C., Lu, N., Selig, M., Nielsen, G., Taksir, T., Jain, R. K., and Seed, B. (1998) Tumor induction of VEGF promoter activity in stromal cells. *Cell* **94,** 715–725
7. Egeblad, M., and Werb, Z. (2002) New functions for the matrix metalloproteinases in cancer progression. *Nat. Rev. Cancer* **2,** 161–174
8. Milner, J. M., and Cawston, T. E. (2005) Matrix metalloproteinase knockout studies and the potential use of matrix metalloproteinase inhibitors in the rheumatic diseases. *Curr. Drug Targets Inflamm. Allergy* **4,** 363–375
9. Werner, J. A., Rathcke, I. O., and Mandic, R. (2002) The role of matrix metalloproteinases in squamous cell carcinomas of the head and neck. *Clin. Exp. Metastasis* **19,** 275–282
10. Coussens, L. M., Fingleton, B., and Matrisian, L. M. (2002) Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* **295,** 2387–2392
11. Littlepage, L. E., Sternlicht, M. D., Rougier, N., Phillips, J., Gallo, E., Yu, Y., Williams, K., Brenot, A., Gordon, J. I., and Werb, Z. (2010) Matrix metalloproteinases contribute distinct roles in neuroendocrine prostate carcinogenesis, metastasis, and angiogenesis progression. *Cancer Res.* **70,** 2224–2234

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12. Bergers, G., Brekken, R., McMahon, G., Vu, T. H., Itoh, T., Tamaki, K., Tanzawa, K., Thorpe, P., Itohara S., Werb, Z., and Hanahan, D. (2000) Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat. Cell Biol.* **2,** 737–744
13. Lee, S., Jilani, S. M., Nikolova, G. V., Carpizo D., and Iruela-Arispe, M. L. (2005) Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. *J. Cell Biol.* **169,** 681–691
14. Ribatti, D. (2009) Endogenous inhibitors of angiogenesis: a historical review. *Leuk. Res.* **33,** 638–644
15. Kosaki, N., Takaishi, H., Kamekura, S., Kimura, T., Okada, Y., Minqi, L., Amizuka, N., Chung, U. I., Nakamura, K., Kawaguchi, H., Toyama, Y., and D'Armiento, J. (2007) Impaired bone fracture healing in matrix metalloproteinase-13 deficient mice. *Biochem. Biophys. Res. Commun.* **354,** 846–851
16. Zijlstra, A., Aimes, R. T., Zhu, D., Regazzoni, K., Kupriyanova, T., Seandel, M., Deryugina, E. I., and Quigley, J. P. (2004) Collagenolysis-dependent angiogenesis mediated by matrix metalloproteinase-13 (collagenase-3). *J. Biol. Chem.* **279,** 27633–27645
17. Freije, J. M., Díez-Itza, I., Balbín, M., Sánchez, L. M., Blasco, R., Tolivia, J., and López-Otín, C. (1994) Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. *J. Biol. Chem.* **269,** 16766–16773
18. Nielsen, B. S., Rank, F., López, J. M., Balbin, M., Vizoso, F., Lund, L. R., Danø, K., and López-Otín, C. (2001) Collagenase-3 expression in breast myofibroblasts as a molecular marker of transition of ductal carcinoma in situ lesions to invasive ductal carcinomas. *Cancer Res.* **61,** 7091–7100
19. Lederle, W., Hartenstein, B., Meides, A., Kunzelmann, H., Werb, Z., Angel, P., and Mueller, M. M. (2010) MMP13 as a stromal mediator in controlling persistent angiogenesis in skin carcinoma. *Carcinogenesis* **31,** 1175–1184
20. Anno, K., Hayashi, A., Takahashi, T., Mitsui, Y., Ide, T., and Tahara, H. (2007) Telomerase activation induces elongation of the telomeric single-stranded overhang, but does not prevent chromosome aberrations in human vascular endothelial cells. *Biochem. Biophys. Res. Commun.* **353,** 926–932
21. Schor, S. L., Schor, A. M., Rushton, G., and Smith, L. (1985) Adult, foetal and transformed fibroblasts display different migratory phenotypes on collagen gels: evidence for an isoformic transition during foetal development. *J. Cell Sci.* **73,** 221–234
22. Kudo, Y., Ogawa, I., Kitajima, S., Kitagawa, M., Kawai, H., Gaffney, P. M., Miyauchi, M., and Takata, T. (2006) Periostin promotes invasion and anchorage-independent growth in the metastatic process of head and neck cancer. *Cancer Res.* **66,** 6928–6935
23. Bishop, E. T., Bell, G. T., Bloor, S., Broom, I. J., Hendry, N. F., and Wheatley, D. N. (1999) An *in vitro* model of angiogenesis: basic features. *Angiogenesis* **3,** 335–344
24. Fox, S. B., Leek, R. D., Weekes, M. P., Whitehouse, R. M., Gatter, K. C., and Harris, A. L. (1995) Quantitation and prognostic value of breast cancer angiogenesis: comparison of microvessel density, Chalkley count, and computer image analysis. *J. Pathol.* **177,** 275–283
25. Bauer, K. S., Cude, K. J., Dixon, S. C., Kruger, E. A., and Figg, W. D. (2000) Carboxyamido-triazole inhibits angiogenesis by blocking the calcium-mediated nitric-oxide synthase-vascular endothelial growth factor pathway. *J. Pharmacol. Exp. Ther.* **292,** 31–37
26. Deraz, E. M., Kudo, Y., Yoshida, M., Obayashi, M., Tsunematsu, T., Tani, H., Siriwardena, S. B., Keikhaee, M. R., Kiekhaee, M. R., Qi, G., Iizuka, S., Ogawa, I., Campisi, G., Lo Muzio, L., Abiko, Y., Kikuchi, A., and Takata, T. (2011) MMP-10/stromelysin-2 promotes invasion of head and neck cancer. *PLoS One* **6,** e25438
27. Noma, K., Smalley, K. S., Lioni, M., Naomoto, Y., Tanaka, N., El-Deiry, W., King, A. J., Nakagawa, H., and Herlyn, M. (2008) The essential role of fibroblasts in esophageal squamous cell carcinoma-induced angiogenesis. *Gastroenterology* **134,** 1981–1993
28. Sappino, A. P., Skalli, O., Jackson, B., Schürch, W., and Gabbiani, G. (1988) Smooth-muscle differentiation in stromal cells of malignant and non-malignant breast tissues. *Int. J. Cancer* **41,** 707–712
29. Uitto, V. J., Airola, K., Vaalamo, M., Johansson, N., Putnins, E. E., Firth, J. D., Salonen, J., López-Otín, C., Saarialho-Kere, U., and Kähäri, V. M. (1998) Collagenase-3 (matrix metalloproteinase-13) expression is induced in oral mucosal epithelium during chronic inflammation. *Am. J. Pathol.* **152,** 1489–1499
30. Tardif, G., Reboul, P., Pelletier, J. P., and Martel-Pelletier, J. (2004) Ten years in the life of an enzyme: the story of the human MMP-13) collagenase-3. *Mod. Rheumatol.* **14,** 1489–1499
31. Ashworth, J. L., Murphy, G., Rock, M. J., Sherratt, M. J., Shapiro, S. D., Shuttleworth, C. A., and Kielty, C. M. (1999) Fibrillin degradation by matrix metalloproteinases: implications for connective tissue remodeling. *Biochem. J.* **340,** 171–181
32. Culhaci, N., Metin, K., Copcu, E., and Dikicioglu, E. (2004) Elevated expression in MMP-13 and TIMP-1 in head and neck squamous cell carcinomas may reflect increased tumor invasiveness. *BMC Cancer* **4,** 1–8
33. Johansson, N., Vaalamo, M., Grénman, S., Hietanen, S., Klemi, P., Saarialho-Kere, U., and Kähäri, V. M. (1999) Collagenase-3 (MMP-13) is expressed by tumor cells in invasive vulvar squamous cell carcinomas. *Am. J. Pathol.* **154,** 469–480
34. Pendás, A. M., Uría, J. A., Jiménez, M. G., Balbín, M., Freije, J. P., and López-Otín, C. (2000) An overview of collagenase-3 expression in malignant tumors and analysis of its potential value as a target in antitumor therapies. *Clin. Chim. Acta* **291,** 137–155
35. Krecicki, T., Fraczek, M., Jelen, M., Podhorska, M., Szkudlarek, T., Zatonski, T. (2003) Expression of collagenase-1 (MMP-1), collagenase-3 (MMP-13) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in laryngeal squamous cell carcinomas. *Eur. Arch Otorhinolaryngol.* **260,** 494–497
36. del Casar Lizcano, J. M., Vizoso Piñeiro, F., González Sánchez, L. O., Martin Suarez, A., Gava, A., Cuesta Fernandez, E., and Diez Santisteban, M. C. (2003) Expression and clinical significance of collagenase-3 (MMP-13) in gastric cancer. *Gastroenterol. Hepatol.* **26,** 1–7
37. Roeb, E., Arndt, M., Jansen, B., Schumpelick, V., and Matern, S. (2004) Simultaneous determination of matrix metalloproteinase (MMP)-7, MMP-1, -3, and -13 gene expression by multiplex PCR in colorectal carcinomas. *Int. J. Colorectal. Dis.* **19,** 518–524
38. Corte, M. D., Gonzalez, L. O., Corte, M. G., Quintela, I., Pidal, I., Bongera, M., and Vizoso, F. (2005) Collagenase-3 (MMP-13) expression in cutaneous malignant melanoma. *Int. J. Biol. Markers* **20,** 242–248
39. Luukkaa, M., Vihinen, P., Kronqvist, P., Vahlberg, T., Pyrhönen, S., Kähäri, V. M., and Grénman, R. (2006) Association between high collagenase-3 expression levels and poor prognosis in patients with head and neck cancer. *Head Neck* **28,** 225–234
40. De Wever, O., and Mareel, M. (2003) Role of tissue stroma in cancer cell invasion. *J. Pathol.* **200,** 429–447
41. Johansson, N., Airola, K., Grénman, R., Kariniemi, A. L., Saarialho-Kere, U., and Kähäri, V. M. (1997) Expression of collagenase-3 (matrix metalloproteinase-13) in squamous cell carcinomas of the head and neck. *Am. J. Pathol.* **151,** 499–508
42. Direkze, N. C., Hodivala-Dilke, K., Jeffery, R., Hunt, T., Poulsom, R., Oukrif, D., Alison, M. R., and Wright, N. A. (2004) Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts. *Cancer Res.* **64,** 8492–8495
43. Hattori, N., Mochizuki, S., Kishi, K., Nakajima, T., Takaishi, H., D'Armiento, J., and Okada, Y. (2009) MMP-13 plays a role in keratinocyte migration, angiogenesis, and contraction in mouse skin wound healing. *Am. J. Pathol.* **175,** 533–546
44. Zhao, X., and Guan, J. L. (2011) Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. *Adv. Drug Deliv. Rev.* **63,** 610–615
45. Ilić, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995) Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* **377,** 539–544
46. Gabarra-Niecko, V., Schaller, M. D., and Dunty, J. M. (2003) FAK regulates biological processes important for the pathogenesis of cancer. *Cancer Metastasis Rev.* **22,** 359–374
47. Baek, Y. Y., Cho, D. H., Choe, J., Lee, H., Jeoung, D., Ha, K. S., Won, M. H., Kwon, Y. G., and Kim, Y. M. (2011) Extracellular taurine induces angiogenesis by activating ERK-, Akt-, and FAK-dependent signal pathways. *Eur. J. Pharmacol.* **674,** 629–640
48. Bissell, M. J., and Radisky, D. (2001) Putting tumors in context. *Nat. Rev. Cancer* **1,** 46–54
49. Dvorak, H. F. (1986) Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N. Engl. J. Med.* **315,** 1650–1659

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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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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

show that multiple miRNAs such as miR-17-5p, miR-20a/b, miR-93, miR-106a/b, miR-146a-b, miR-372 and miR-373 bypass Ras-induced senescence by directly targeting p21 [27]. Therefore, one miRNA can regulate several genes to cooperate in senescence, whereas many miRNAs can target one gene to control senescence (Fig. 1).

Traditionally, cellular senescence has been thought to arise as a consequence of altered gene expression including senescence genes, senescence suppressor genes, oncogenes, tumor suppressor genes, stemness genes, epigenetic genes and others (Table. 1). Cellular senescence can be triggered by telomere shortening due to the end replication problems in human somatic cells. Telomerase, a ribonucleoprotein enzyme for telomere elongation, is inactivated in normal somatic cells, but most cancer cells and stem cells have telomerase activity due to the activation of human telomerase reverse transcriptase (hTERT) gene. Although little is known about hTERT regulation by miRNAs, it is reported that miR-138 potentially targets the hTERT gene, involved in the development of thyroid carcinoma [49]. miR-128a was identified to directly target Bmi-1 oncogene, increasing p16 expression to promote cellular senescence [50]. The p53-independent up-regulation of miR-34a provoked senescence through repression of v-myc myelocytomatosis viral oncogene homolog myc [51]. miR-372 and miR-373 prevent Ras-induced senescence through the down-regulation of tumor suppressor LATS2 [52]. miR-21 modulates apoptosis by regulating Ras [53] and phosphatase and tensin homolog (PTEN)-dependent activation of PI 3-kinase signaling [54]. The miR-302-367 cluster acts after Oct4, Sox2 and Nanog to maintain stemness of human embryonic stem cells [55]. Let-7b is involved in decline of neuronal stem cell self-renewal during aging by reducing HMGA2 levels in old but not in young mice [56]. In addition, miR-146a can inhibit cellular senescence by targeting IL-6/8 [57]. The proto-oncogene LRF, leukemia/lymphoma related factor, is post-transcriptionally regulated by miR-20a, inducing senescence in mouse embryonic fibroblasts through preventing p19ARF transcription [16]. miR-29 and miR-30 can mediate repression of B-Myb expression during Rb activation, resulting in cellular senescence [58]. Some receptors such as ER [59] and ERBB2/3 [60] are regulated by miR-22 and miR-125, respectively. Above all, these miRNAs modulate cellular senescence through targeting various genes on p53/pRB pathway and/or other signaling pathways.

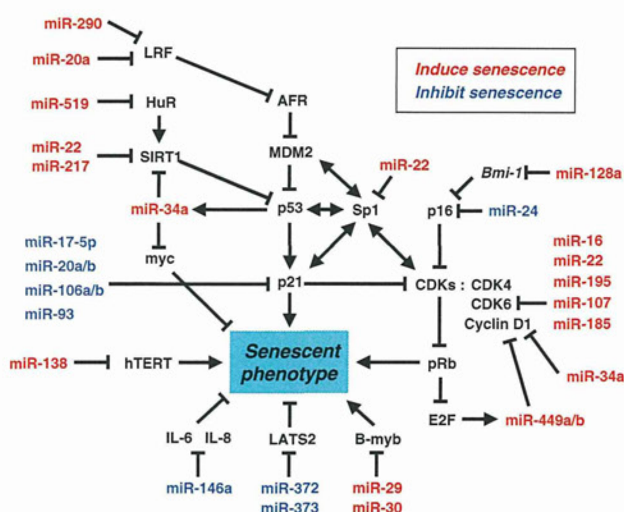

Fig. 1. miRNAs are involved in cellular senescence through p53/pRb pathway and other pathways. Cellular senescence is modulated by a variety of miRNAs as shown. These regulatory miRNAs induce senescence phenotypes (indicated in red) or delay the onset of senescence (indicated in blue).

Table 1
Genes associated with cellular senescence.

Genes	Functions	Examples	miRNAs	References
Senescence genes	Cell cycle regulators	p16	miR-24	[46]
		p21	miR-106a/b	[27]
Senescence suppressor genes	Regulators of telomere length	hTERT	miR-138	[49]
Oncogenes	Signal transduction molecules	Ras	miR-21	[53]
		Bmi-1	miR-128a	[50]
		myc	miR-34a	[51]
Tumor suppressor genes	Signal transduction molecules	p53	miR-34a	[44]
		LATS2	miR-372, 373	[52]
		PTEN	miR-21	[54]
Stemness genes	Self-renewal regulators	SOX2	miR302-367	[55]
		OCT3/4		
Epigenetic genes	Chromatin factors	HMGA2	Let-7b	[56]
	Histone modification	SIRT1	miR-22	[17]
Others	Inflammatory factors	IL-6, 8	miR146a	[57]
	Transcription factors	LRF	miR-20a	[16]
		Sp1	miR-22	[17]
		B-Myb	miR-29, 30	[58]
	Receptors	ER	miR-22	[59]
		ERBB2/3	miR-125a-5p	[60]

4. Involvement of secretory factors in cellular senescence

Senescent cells secrete not only exosomes but also other inflammatory cytokines and chemokines [61]. Recently, it has been reported that senescent cells assume a novel phenotype, the so-called senescence-associated secretory phenotype (SASP), which developed in normal fibroblasts, epithelial cells, and epithelial tumor cells after genotoxic stress in culture, and in epithelial tumor cells in vivo after treatment of prostate cancer patients with DNA damaging chemotherapy [62]. SASP is characterized by the secretion of myriad factors associated with inflammation and malignancy factors as well as the release of exosomes [21,43,62].

The secreted factors including matrix components, extracellular proteases, cytokines, and chemokines set the stage for crosstalk between senescent cells and their environment [63]. Of note, the effect of secreted factors is complex: they seem to reinforce the senescent phenotype in nearby senescent cells but at the same time can increase the proliferation of nearby neoplastic cells. For example, interleukin (IL)-6 contributes to the induction of senescence of nonmalignant cells in an autocrine manner [64], whereas its tumor promoting function by paracrine signaling has been proposed to directly or indirectly target the angiogenic compartment [65]. In cultured premalignant epithelial cells, SASPs induced an epithelial–mesenchyme transition and invasiveness, hallmarks of malignancy, by a paracrine mechanism that depended largely on the IL-6 and IL-8. Senescent cells contribute to induction of senescence in cells in its immediate vicinity mediated by secreted factors, which could constitute a danger signal that sensitizes normal neighboring cells to enter senescence, and block growth factors signaling directly by inhibition at the receptor level, thereby potentially contributing to the induction of senescence [66]. Tumor cells can misuse signaling of secreted factors, inducing senescence in fibroblasts. These senescent fibroblasts could in turn secrete factors that contribute to tumor growth in the later stage of tumorigenesis [67]. In addition, aging can lead to an increase in the number of senescent cells, thereby potentially stimulating expansion of premalignant cells [40]. Therefore, it is thought that cellular senescence suppresses tumorigenesis early in life, whereas it may promote cancer in aged organisms.

In addition to secretion of cytokines, senescent cells produce high levels of exosomes that are able to modulate the microenvironment during senescence. Exosomes are secreted by most cell types including dendritic cells, B cells, T cells, epithelial cells, and tumor cells, and interact with surrounding cells upon secreted. Exosomes can introduce

regulatory secreted factors into the interiors of the cells or receptors into the external membranes of the cells, providing intercellular communication both within and between cells [6]. Exosomes participate in the surface-membrane traffic and the horizontal transfer of protein and RNAs as well miRNAs among neighboring cells, which are necessary for the rapid phenotype adjustments in a variety of conditions. [68]. It is reported that senescence is associated with the release of exosomes, demonstrating a significantly increased release of exosome-like microvesicles during senescence in normal human fibroblasts. Importantly, senescence-associated exosomes were also observed to release from human senescent prostate cancer cells, suggesting senescence-associated exosomes can transfer cargos with both immunoregulatory potential and genetic information, between cells during cellular senescence [19]. EGFR is repressed at the chromatin level during senescence and is shown to be potentially regulated during cellular senescence [69]. Exosome-like microvesicles released by EGFR-bearing tumor cells are incorporated by neighboring endothelial cells, resulting in activation of the MAPK and Akt pathways and the autocrine production of VEGF. Their transfer between EGFR-bearing glioma cells can accelerate the growth of tumor cells that take up the vesicles. Thus, the effects of exosomal release in senescence could be wide ranging and influence the microenvironment [70].

The secretory activity and the release of exosomes in senescent cells are regulated by p53 tumor suppressor (Fig. 2) [71]. In cells undergoing senescence, p53 acts as a transcription factor and up-regulates a variety of secreted factors such as insulin-like growth factor-binding protein 3 (IGFBP-3). IGFBP3, secreted by some senescent cells, is known to be up-regulated in senescent fibroblasts and endothelial cells, leading to senescence [71,72]. In addition, it is reported that p53 up-regulates tumor suppressor-activated pathway-6 transcript (TSAP6), which is an intracellular factor that stimulates exosome release into the extracellular medium [71,73]. Chmp4 that recruits Alix protein to the endosome to function in MVB vesicle formation is also induced by p53 activation [74]. The p53 protein can also activate caveolin-1, the main component of the caveolae plasma membranes, and facilitate the endocytosis, accompanied with endosomal clearance of membrane receptors from cell surface [74,75] . In addition, overexpression of caveolin-1 induced premature senescence in human fibroblasts [76], whereas knockout of caveolin-1 by shRNA blocks the induction of bleomycin-induced senescence [77]. Maspin, a mammary serine protease inhibitor, is also up-regulated by p53 in response to stress, and plays a role in the exosome secretion [71]. At present, it is well known that both secreted factors and exosomes are secreted from senescent cells, and involved in a variety of biological function, but it is still unclear whether they coordinately influence each other for the biological effects of age-associated diseases.

5. Biological processes mediated by miRNAs and exosomes during senescence

Recent findings have revealed the complexities of the senescence phenotypes, demonstrating that senescent cells differ from proliferating cells in three way: (1) they arrest cell growth and cannot be stimulated to reenter the cell cycle by known physiological stimuli; (2) they become resistant to apoptotic cell death; (3) they acquire altered differentiated functions [21,78]. All of these changes might result from cooperative changes in multiple systems with the resultant changes to pathways regulating different cellular processes.

The ability of miRNAs to regulate a variety of target genes allows them to induce changes in multiple biological processes, implicating that miRNAs may provide a significant link between senescence and other biological processes. Using the systems biology tool Metacore from Genego, pathway analysis of senescence-associated miRNA targets demonstrates the common signaling pathways resulting in senescence [79]. Dhahbi et al. used DAVID and Gene Ontology to functionally annotate miRNA-regulated genes, revealing the biological processes targeted by miRNA expression changes during senescence [80]. Among them, the most highly represented and enriched term is regulation of cell proliferation, which is consistence with a pivotal role of miRNAs in cell proliferation regulation since inhibition of proliferation is the hallmark of senescence [21]. Senescence-associated miRNAs, miR-22 and miR-34a

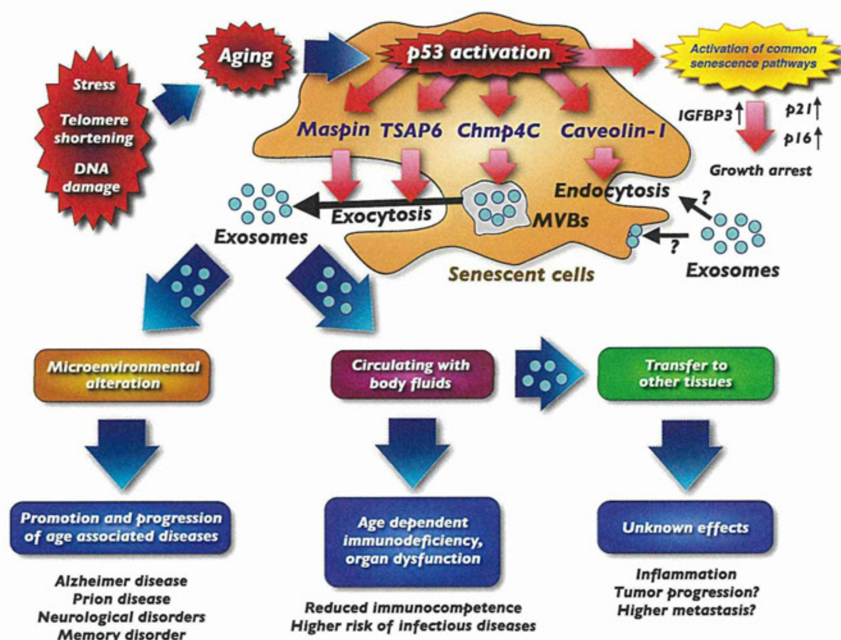

Fig. 2. The secretory activity and the release of exosomes are regulated by p53 tumor suppressor in senescent cells. Senescence-inducing factors such as oxidative stress, telomere shortening and DNA damage induce p53 activation followed by the increase in p21, p16 or IGFBP3 on the senescence pathways. The activation of p53 also induces up-regulation of Maspin, TSAP6, Chmp4C and Caveolin-1, which can enhance the endosome production, the exosome production and release from the plasma membrane. Higher production of exosomes which cargo miRNA and proteins from senescent cells may induce the alteration of microenvironment in age-associated diseases, cause immunodeficiency and organ dysfunctions in infectious diseases, and result in other unknown effects such as inflammation.

inhibit cell proliferation by targeting common senescence pathways [17,18]. Other miRNAs also contribute to regulate cell proliferation through common signaling pathways of senescence (Fig. 1). Notably, miRNAs regulate actin cytoskeleton structure changes that contribute to the enlarged and flattened cell morphology characteristic of senescence phenotype. Our previous study demonstrates that miR-22 induces senescent-like morphology in SiHa cells, accompanied with cytoskeleton remodeling such as actin fiber formation [17]. miRNAs control key molecules in the multiple processes governing the cell cycle during senescence induction, which allows these miRNAs to affect the cell cycle regulatory process as whole rather than at just one checkpoint or stage [79]. Because the capability of cells to adhere to extracellular matrix is linked to cytoskeleton remodeling and cell shape, the changes in cell adhesion regulation may contribute to the morphological changes induced in senescence. Senescent cells are known to acquire resistance to apoptotic stimuli, whereas miRNAs may sway the cellular decision to commit to senescence instead of apoptosis. Alteration of protein metabolism and modification is responsible for the increase in cell volume and mass resulting in the enlarged and flattened morphology. So far, various studies support that miRNAs could facilitate the complex cellular changes required to establish the senescent phenotype, associated with inhibition of cell proliferation, cellular metabolic processes, negative regulation of apoptosis, stimulation of cell adhesion and cytoskeleton remodeling. Here, we summarize the effects of several miRNAs, either up or down in senescence, on biological processes changed during cellular senescence (Table. 2).

Exosomes are secreted into surrounding body fluids, targeting cells to transfer exosomal molecules including proteins, mRNAs, and miRNAs that can aid cell proliferation, immune response and other cellular process. For example, exosomes released by prematurely senescent prostate cancer cells were enriched in B7-H3 protein [19], which is a member of the B7 family of proteins that are capable of modulating CD4 T-cell responses and antitumoral immunity [81]. Secreted factors IL-6/8 from senescent fibroblasts in SASP are involved in the induction of a classic epithelial–mesenchyme transitions and invasiveness in human breast cancer cell lines [62]. Previous studies have found miRNAs circulating in the peripheral blood and have identified exosomes as mediators of cell–cell communication. It is reported that macrophage-secreted exosomes would deliver oncogenic miRNAs to breast cancer cells, contributing to the regulation of the invasiveness of breast cancer cells [82]. Therefore, exosome formation, cargo content, and delivery to surrounding cells are thought to be of immense biological interest in cellular processes. Little is known about the role of exosomes in vivo aging; however, these in vitro experimental data give the biological importance of exosomes in aging.

6. Potential of exosomes and miRNAs in aging and cancer

Aging is a well-recognized risk factor in the development of many diseases such as neurodegenerative disorders, cardiovascular disease, diabetes and cancer, which are the primary cause of death and disability in the elderly population. The process of aging impacts on a wide range of functions within the human body, displaying age-related changes occur in cellular, molecular and physiological functionality of tissues and organs. Nowadays, it is a well-known fact that some age-related molecular changes (e.g. DNA methylation, telomere shortening) can be detected in several types of colorectal cancers [83]. The understanding connection of aging process to age-related diseases is of great clinical importance regarding prevention and modern therapeutic strategies.

Exosomes are believed to play a role in various physiological and pathological conditions. Senescence-associated exosomes could transfer many regulatory factors including proteins and miRNAs, which contribute to the aging process in an autocrine, paracrine and even endocrine fashion. Although the relationship between miRNA and aging is not fully understood, the accumulating evidence suggests that exosomes act as significant communicator in human body. Exosomes have been implicated in the spread of neurodegenerative diseases including age-related macular degeneration (AMD) and Alzheimer's disease (AD). Age-related macular degeneration (AMD) is a major cause of loss of central vision in the elderly. Wang et al. demonstrated that increased autophagy and the release of intracellular proteins via exosomes by the aged retinal pigment epithelium may contribute to the formation of drusen that is a risk factor for developing AMD [84]. Alzheimer's disease (AD) is an irreversible age-related degenerative disorder, characterized by amyloid plaques, neurofibrillary tangles, inflammation, and neuronal damage in the brain. Accumulation of β-amyloid peptides (Aβ) in the senile plaques is a hallmark of the progression of disease. Interestingly, Aβ peptides formed by the β cleavage of amyloid precursor protein (APP) by two unknown proteases, β-secretase and γ-secretase, are secreted from the cells in association with exosomes [85]. Similar observation was found in prion diseases, which are fatal and infectious neurodegenerative disorders characterized by the accumulation of an abnormally folded form

Table 2
Analysis of biological processes changed during cellular senescence.

	Up-regulated miRNAs			Down-regulated miRNAs		
Biological processes	Let-7	miR-22	miR-34a	miR-17	miR-21	miR-155
Cell proliferation	Inhibited	Inhibited	Inhibited	Promoted	Promoted	Promoted
Apoptosis	Inhibited	Inhibited	Induced	Inhibited	Induced	Inhibited
Cell cycle arrest	G1/S arrest G2/M arrest	G1/S arrest	G1/S arrest G2/M arrest	Inhibitor of miR-17 induces G0/G1 arrest	Antisense targeting miR-17 induces G1 arrest	Antisense targeting miR-155 induces G0/G1 arrest
Cell differentiation	Involved	Involved	Involved	Involved	Involved	Involved
Metabolic processes	Involved	Inhibited	Inhibited	Involved	Involved	ND
Protein metabolism and modification	Involved	Involved	Involved	Involved	Involved	Involved
Cell adhesion	ND	Involved	decreased	decreased	Involved	decreased
Cytoskeleton remodeling	ND	Increased	ND	ND	Involved	ND
Cell migration and cell motility	Inhibited	Inhibited	Inhibited	decreased	Involved	Inhibited

Note: all the data are summarized from published articles. ND means not determined.

of glycosylphosphatidylinositol-linked prion protein (PrP), scrapie PrP [86–88]. These results suggest that exosomes might affect the microenvironment of these age-related diseases.

It is found that miRNAs can be stably detected in circulating plasma and serum, since miRNAs are packaged by exosomes to be protected from RNA degradation [13]. Ago2 and HDL that are associated with miRNAs are also stably found in serum, but their biological significance is still being discussed [89–91]. While miRNA profiling between cells and exosomes seems to be different, similar miRNA profiling was observed in exosomes containing miRNAs and Ago2 binding miRNAs. Therefore, the functional regulation of exosome secretion to deliver specific miRNAs may exist during aging and in human diseases. Recent studies have indicated that circulating exosomal miRNAs can serve as surrogates for the tumor cell-associated counterparts, extending their diagnostic potential to asymptomatic individuals [92]. Considering the high relevance between exosomes and miRNAs in many aspects, we suppose that circulating miRNAs will have a potential role in aging and age-related diseases.

Loss of immune function is typical symptom in human aging. Among human body fluids, one of major body fluid is blood, and a potential major in vivo source of exosomes is the B cell. Little is known about the function and the possible targets of B cell-derived exosomes. Exosomes derived from B cells in both human and mice elicit immune response by inducing antigen-specific MHC class II-restricted T-cell responses [93]. The frequency and number of bone marrow common lymphocyte progenitors (CLPs) are considerably reduced by 7 months of age. Furthermore, while about 90% of CLPs numbers is reduced in 20 month old mice, age-dependent reduction of the frequency and number of pro-B cell is observed. Due to recombination of impaired V-DJ heavy chain gene, the transition of pro-B cell into pre-B cells also decreases with aging. It is possible that these alterations may reflect on profiling and secretion of exosomes from these cells.

Studies on tumor derived exosomes reveal that exosomes may help establish an oncogenic niche systemically via delivery of protein, mRNA, and miRNAs that can aid tumor growth and metastasis. The presence of HLA-G in exosomes secreted by melanoma cells might imply a role in creating a tumor tolerance associated response [94]. Utilizing VEGF and cytokines cargo, exosomes enhance recruitment of hematopoietic and endothelial precursor cells to enhance neo-angiogenesis in the tumor [95]. Additionally, exosomes are known to trigger apoptotic reduction in anti-tumor immune cells through FasL and TNF-pathways and influence planar cell polarity and the extracellular matrix to allow tumor cell mobilization [96].

In summary, exosomes allow for reciprocal communication between different tissues, as shown in Fig. 3. Exosome production and secretion are altered during in vivo aging and in cancer, which has biological significance in metabolism, glycolysis, renal function, immunocompetence, tumor progression and brain memory function. Secretory factors are circulating in the tissues through the blood, so they may impact on the phenotypes by coordinate alteration of miRNAs, exosome proteins, lipids and cytokines. Furthermore, it is possible that individual difference in the production of secretory factors during aging may reflect the cancer risk as well as the cure rate. To date, limited information is available for understanding the mechanism and function of age-associated exosomes. We suppose that alteration of circulating miRNA, RNA, lipids and proteins via exosomes may coordinately influence many biological processes in human aging and cancer.

There is an increasing interest in using miRNAs as biomarker in the diagnosis of diseases and also as a promising biomedicine in the therapy of human diseases. Recent study shows that the sensitivity of miRNA amplification from human biologic fluids such as serum and saliva can be improved by exosome isolation [97], indicating exosomal miRNAs might become a potential starting point for earlier

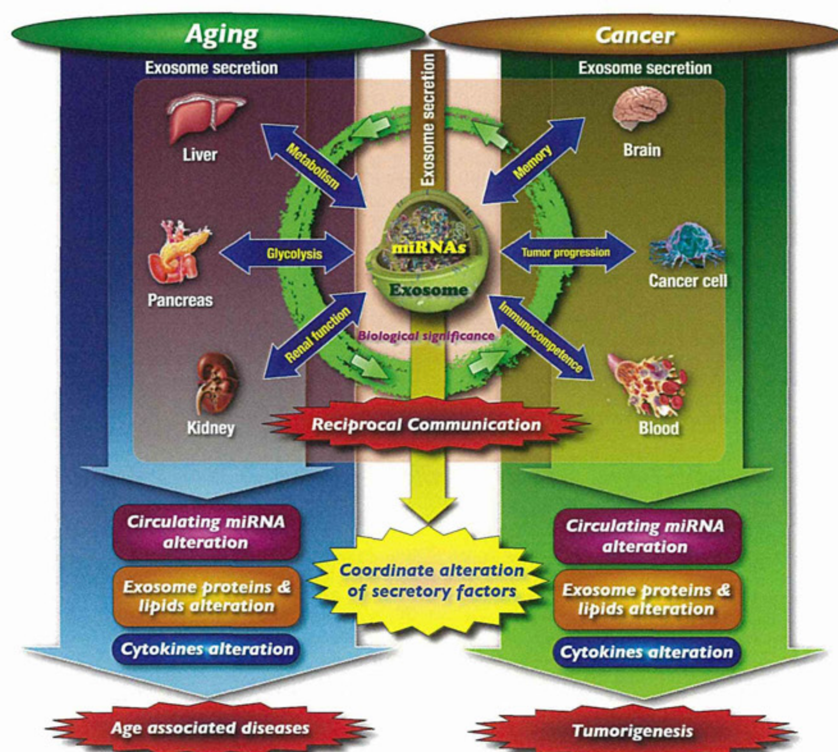

Fig. 3. Exosomes that allow for reciprocal communication between different tissues are critical in coordinating regulation of cellular function through miRNAs and proteins transfer. Epigenomic alterations during aging or in cancer may increase exosome secretion, accompanied with alteration in expression of circulating miRNAs, exosome proteins and cytokines, which are involved in cell-to-cell communication via exosomes. Therefore, exosomes play an important role in age-associated diseases and tumorigenesis.

biomarker studies to reduce the probability of false negative results in clinic diagnosis of human diseases.

Nowadays, therapeutic potential of RNA drug to the clinic has been realized. With the development of nonimmunogenic delivery technologies, it is reported that exosomes can be used as nanocarriers to mediate siRNA delivery to the brain in mice, demonstrating efficient knockdown of BACE1, a therapeutic target in Alzheimer's disease [98]. In conclusion, exosomes provide a novel biomarker discovery and therapeutic platform for delivery of a variety of therapeutic modalities with great promise.

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References

[1] C. Thery, L. Zitvogel, S. Amigorena, Exosomes: composition, biogenesis and function, Nat. Rev. Immunol. 2 (2002) 569–579.
[2] S. Mathivanan, H. Ji, R.J. Simpson, Exosomes: extracellular organelles important in intercellular communication, J. Proteomics 73 (2010) 1907–1920.
[3] J. Ratajczak, M. Wysoczynski, F. Hayek, A. Janowska-Wieczorek, M.Z. Ratajczak, Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication, Leukemia 20 (2006) 1487–1495.
[4] R.J. Simpson, J.W. Lim, R.L. Moritz, S. Mathivanan, Exosomes: proteomic insights and diagnostic potential, Expert Rev. Proteomics 6 (2009) 267–283.
[5] M. Record, C. Subra, S. Silvente-Poirot, M. Poirot, Exosomes as intercellular signalosomes and pharmacological effectors, Biochem. Pharmacol. 81 (2011) 1171–1182.
[6] C. Thery, M. Ostrowski, E. Segura, Membrane vesicles as conveyors of immune responses, Nat. Rev. Immunol. 9 (2009) 581–593.
[7] K. Al-Nedawi, B. Meehan, J. Rak, Microvesicles: messengers and mediators of tumor progression, Cell Cycle 8 (2009) 2014–2018.
[8] W. Stoorvogel, M.J. Kleijmeer, H.J. Geuze, G. Raposo, The biogenesis and functions of exosomes, Traffic 3 (2002) 321–330.
[9] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.
[10] N. Bushati, S.M. Cohen, microRNA functions, Annu. Rev. Cell Dev. Biol. 23 (2007) 175–205.
[11] N. Kosaka, H. Iguchi, T. Ochiya, Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis, Cancer Sci. 101 (2010) 2087–2092.
[12] X. Chen, Y. Ba, L. Ma, X. Cai, Y. Yin, K. Wang, J. Guo, Y. Zhang, J. Chen, X. Guo, Q. Li, X. Li, W. Wang, J. Wang, X. Jiang, Y. Xiang, C. Xu, P. Zheng, J. Zhang, R. Li, H. Zhang, X. Shang, T. Gong, G. Ning, K. Zen, C.Y. Zhang, Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases, Cell Res. 18 (2008) 997–1006.
[13] H. Valadi, K. Ekstrom, A. Bossios, M. Sjostrand, J.J. Lee, J.O. Lotvall, Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells, Nat. Cell Biol. 9 (2007) 654–659.
[14] K. Ohshima, K. Inoue, A. Fujiwara, K. Hatakeyama, K. Kanto, Y. Watanabe, K. Muramatsu, Y. Fukuda, S. Ogura, K. Yamaguchi, T. Mochizuki, Let-7 microRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line, PLoS One 5 (2010) e13247.
[15] L. Pitto, M. Rizzo, M. Simili, D. Colligiani, M. Evangelista, A. Mercatanti, L. Mariani, F. Cremisi, G. Rainaldi, MiR-290 acts as a physiological effector of senescence in mouse embryo fibroblasts, Physiol. Genomics 39 (2009) 210–218.
[16] L. Poliseno, L. Pitto, M. Simili, L. Mariani, L. Riccardi, A. Ciucci, M. Rizzo, M. Evangelista, A. Mercatanti, P.P. Pandolfi, G. Rainaldi, The proto-oncogene LRF is under post-transcriptional control of MiR-20a: implications for senescence, PLoS One 3 (2008) e2542.
[17] D. Xu, F. Takeshita, Y. Hino, S. Fukunaga, Y. Kudo, A. Tamaki, J. Matsunaga, R.U. Takahashi, T. Takata, A. Shimamoto, T. Ochiya, H. Tahara, miR-22 represses cancer progression by inducing cellular senescence, J. Cell Biol. 193 (2011) 409–424.
[18] H. Tazawa, N. Tsuchiya, M. Izumiya, H. Nakagama, Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 15472–15477.
[19] B.D. Lehmann, M.S. Paine, A.M. Brooks, J.A. McCubrey, R.H. Renegar, R. Wang, D.M. Terrian, Senescence-associated exosome release from human prostate cancer cells, Cancer Res. 68 (2008) 7864–7871.
[20] L. Hayflick, The limited in vitro lifetime of human diploid cell strains, Exp. Cell Res. 37 (1965) 614–636.
[21] J. Campisi, F. d'Adda di Fagagna, Cellular senescence: when bad things happen to good cells, Nat. Rev. Mol. Cell Biol. 8 (2007) 729–740.
[22] G.P. Dimri, X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E.E. Medrano, M. Linskens, I. Rubelj, O. Pereira-Smith, et al., A biomarker that identifies senescent human cells in culture and in aging skin in vivo, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 9363–9367.
[23] J. Campisi, Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors, Cell 120 (2005) 513–522.
[24] H. Vaziri, S.K. Dessain, E. Ng Eaton, S.I. Imai, R.A. Frye, T.K. Pandita, L. Guarente, R.A. Weinberg, hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase, Cell 107 (2001) 149–159.
[25] C.J. Sherr, F. McCormick, The RB and p53 pathways in cancer, Cancer Cell 2 (2002) 103–112.
[26] J.P. Brown, W. Wei, J.M. Sedivy, Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts, Science 277 (1997) 831–834.
[27] V. Borgdorff, M.E. Lleonart, C.L. Bishop, D. Fessart, A.H. Bergin, M.G. Overhoff, D.H. Beach, Multiple microRNAs rescue from Ras-induced senescence by inhibiting p21(Waf1/Cip1), Oncogene 29 (2010) 2262–2271.
[28] C.L. Olsen, B. Gardie, P. Yaswen, M.R. Stampfer, Raf-1-induced growth arrest in human mammary epithelial cells is p16-independent and is overcome in immortal cells during conversion, Oncogene 21 (2002) 6328–6339.
[29] C.J. Sherr, J.M. Roberts, CDK inhibitors: positive and negative regulators of G1-phase progression, Genes Dev. 13 (1999) 1501–1512.
[30] M. Ruas, F. Gregory, R. Jones, R. Poolman, M. Starborg, J. Rowe, S. Brookes, G. Peters, CDK4 and CDK6 delay senescence by kinase-dependent and p16INK4a-independent mechanisms, Mol. Cell. Biol. 27 (2007) 4273–4282.
[31] G.H. Stein, L.F. Drullinger, A. Soulard, V. Dulic, Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts, Mol. Cell. Biol. 19 (1999) 2109–2117.
[32] E. Simboeck, J.D. Ribeiro, S. Teichmann, L. Di Croce, Epigenetics and senescence: learning from the INK4-ARF locus, Biochem. Pharmacol. 82 (2011) 1361–1370.
[33] N. Dietrich, A.P. Bracken, E. Trinh, C.K. Schjerling, H. Koseki, J. Rappsilber, K. Helin, K.H. Hansen, Bypass of senescence by the polycomb group protein CBX8 through direct binding to the INK4A-ARF locus, EMBO J. 26 (2007) 1637–1648.
[34] M.F. Robert, S. Morin, N. Beaulieu, F. Gauthier, I.C. Chute, A. Barsalou, A.R. MacLeod, DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells, Nat. Genet. 33 (2003) 61–65.
[35] J.Y. Fang, L. Yang, H.Y. Zhu, Y.X. Chen, J. Lu, R. Lu, Z.H. Cheng, S.D. Xiao, 5-Aza-2'-deoxycytidine induces demethylation and up-regulates transcription of p16INK4A gene in human gastric cancer cell lines, Chin. Med. J. (Engl) 117 (2004) 99–103.
[36] T.S. Lin, H. Lee, R.A. Chen, M.L. Ho, C.Y. Lin, Y.H. Chen, Y.Y. Tsai, M.C. Chou, Y.W. Cheng, An association of DNMT3b protein expression with P16INK4a promoter hypermethylation in non-smoking female lung cancer with human papillomavirus infection, Cancer Lett. 226 (2005) 77–84.
[37] J.H. Tong, D.C. Ng, S.L. Chau, K.K. So, P.P. Leung, T.L. Lee, R.W. Lung, M.W. Chan, A.W. Chan, K.W. Lo, K.F. To, Putative tumour-suppressor gene DAB2 is frequently down regulated by promoter hypermethylation in nasopharyngeal carcinoma, BMC Cancer 10 (2010) 253.
[38] M. Wagner, G. Brosch, W. Zwerschke, E. Seto, P. Loidl, P. Jansen-Durr, Histone deacetylases in replicative senescence: evidence for a senescence-specific form of HDAC-2, FEBS Lett. 499 (2001) 101–106.
[39] J.C. Jeyapalan, J.M. Sedivy, Cellular senescence and organismal aging, Mech. Ageing Dev. 129 (2008) 467–474.
[40] A. Krtolica, S. Parrinello, S. Lockett, P.Y. Desprez, J. Campisi, Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 12072–12077.
[41] J. Campisi, Cellular senescence as a tumor-suppressor mechanism, Trends Cell Biol. 11 (2001) S27–S31.
[42] N. Ohtani, D.J. Mann, E. Hara, Cellular senescence: its role in tumor suppression and aging, Cancer Sci. 100 (2009) 792–797.
[43] J. Campisi, Aging and cancer cell biology, 2008, Aging Cell 7 (2008) 281–284.
[44] M. Yamakuchi, C.J. Lowenstein, MiR-34, SIRT1 and p53: the feedback loop, Cell Cycle 8 (2009) 712–715.
[45] F. Sun, H. Fu, Q. Liu, Y. Tie, J. Zhu, R. Xing, Z. Sun, X. Zheng, Downregulation of CCND1 and CDK6 by miR-34a induces cell cycle arrest, FEBS Lett. 582 (2008) 1564–1568.
[46] A. Lal, H.H. Kim, K. Abdelmohsen, Y. Kuwano, R. Pullmann Jr., S. Srikantan, R. Subrahmanyam, J.L. Martindale, X. Yang, F. Ahmed, F. Navarro, D. Dykxhoorn, J. Lieberman, M. Gorospe, p16(INK4a) translation suppressed by miR-24, PLoS One 3 (2008) e1864.
[47] Y. Takahashi, A.R. Forrest, E. Maeno, T. Hashimoto, C.O. Daub, J. Yasuda, MiR-107 and MiR-185 can induce cell cycle arrest in human non small cell lung cancer cell lines, PLoS One 4 (2009) e6677.
[48] Q. Liu, H. Fu, F. Sun, H. Zhang, Y. Tie, J. Zhu, R. Xing, Z. Sun, X. Zheng, miR-16 family induces cell cycle arrest by regulating multiple cell cycle genes, Nucleic Acids Res. 36 (2008) 5391–5404.
[49] S. Mitomo, C. Maesawa, S. Ogasawara, T. Iwaya, M. Shibazaki, A. Yashima-Abo, K. Kotani, H. Oikawa, E. Sakurai, N. Izutsu, K. Kato, H. Komatsu, K. Ikeda, G. Wakabayashi, T. Masuda, Downregulation of miR-138 is associated with overexpression of human telomerase reverse transcriptase protein in human anaplastic thyroid carcinoma cell lines, Cancer Sci. 99 (2008) 280–286.
[50] S. Venkataraman, I. Alimova, R. Fan, P. Harris, N. Foreman, R. Vibhakar, MicroRNA 128a increases intracellular ROS level by targeting Bmi-1 and inhibits medulloblastoma cancer cell growth by promoting senescence, PLoS One 5 (2010) e10748.

[51] N.R. Christoffersen, R. Shalgi, L.B. Frankel, E. Leucci, M. Lees, M. Klausen, Y. Pilpel, F.C. Nielsen, M. Oren, A.H. Lund, p53-independent upregulation of miR-34a during oncogene-induced senescence represses MYC, Cell Death Differ. 17 (2010) 236–245.
[52] P.M. Voorhoeve, C. le Sage, M. Schrier, A.J. Gillis, H. Stoop, R. Nagel, Y.P. Liu, J. van Duijse, J. Drost, A. Griekspoor, E. Zlotorynski, N. Yabuta, G. De Vita, H. Nojima, L.H. Looijenga, R. Agami, A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors, Cell 124 (2006) 1169–1181.
[53] J. Ren, D. Zhu, M. Liu, Y. Sun, L. Tian, Downregulation of miR-21 modulates Ras expression to promote apoptosis and suppress invasion of Laryngeal squamous cell carcinoma, Eur. J. Cancer 46 (2010) 3409–3416.
[54] F. Meng, R. Henson, M. Lang, H. Wehbe, S. Maheshwari, J.T. Mendell, J. Jiang, T.D. Schmittgen, T. Patel, Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines, Gastroenterology 130 (2006) 2113–2129.
[55] A. Barroso-del Jesus, G. Lucena-Aguilar, P. Menendez, The miR-302-367 cluster as a potential stemness regulator in ESCs, Cell Cycle 8 (2009) 394–398.
[56] J. Nishino, I. Kim, K. Chada, S.J. Morrison, Hmga2 promotes neural stem cell self-renewal in young but not old mice by reducing p16Ink4a and p19Arf Expression, Cell 135 (2008) 227–239.
[57] D. Bhaumik, G.K. Scott, S. Schokrpur, C.K. Patil, A.V. Orjalo, F. Rodier, G.J. Lithgow, J. Campisi, MicroRNAs miR-146a/b negatively modulate the senescence-associated inflammatory mediators IL-6 and IL-8, Aging (Albany NY) 1 (2009) 402–411.
[58] I. Martinez, D. Cazalla, L.L. Almstead, J.A. Steitz, D. DiMaio, miR-29 and miR-30 regulate B-Myb expression during cellular senescence, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 522–527.
[59] D.P. Pandey, D. Picard, miR-22 inhibits estrogen signaling by directly targeting the estrogen receptor alpha mRNA, Mol. Cell. Biol. 29 (2009) 3783–3790.
[60] G.K. Scott, A. Goga, D. Bhaumik, C.E. Berger, C.S. Sullivan, C.C. Benz, Coordinate suppression of ERBB2 and ERBB3 by enforced expression of micro-RNA miR-125a or miR-125b, J. Biol. Chem. 282 (2007) 1479–1486.
[61] A.R. Davalos, J.P. Coppe, J. Campisi, P.Y. Desprez, Senescent cells as a source of inflammatory factors for tumor progression, Cancer Metastasis Rev. 29 (2010) 273–283.
[62] J.P. Coppe, C.K. Patil, F. Rodier, Y. Sun, D.P. Munoz, J. Goldstein, P.S. Nelson, P.Y. Desprez, J. Campisi, Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor, PLoS Biol. 6 (2008) 2853–2868.
[63] T. Kuilman, D.S. Peeper, Senescence-messaging secretome: SMS-ing cellular stress, Nat. Rev. Cancer 9 (2009) 81–94.
[64] T. Kuilman, C. Michaloglou, L.C. Vredeveld, S. Douma, R. van Doorn, C.J. Desmet, L.A. Aarden, W.J. Mooi, D.S. Peeper, Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network, Cell 133 (2008) 1019–1031.
[65] B. Ancrile, K.H. Lim, C.M. Counter, Oncogenic Ras-induced secretion of IL6 is required for tumorigenesis, Genes Dev. 21 (2007) 1714–1719.
[66] S.S. Huang, S.M. Leal, C.L. Chen, I.H. Liu, J.S. Huang, Identification of insulin receptor substrate proteins as key molecules for the TbetaR-V/LRP-1-mediated growth inhibitory signaling cascade in epithelial and myeloid cells, FASEB J. 18 (2004) 1719–1721.
[67] G. Yang, D.G. Rosen, Z. Zhang, R.C. Bast Jr., G.B. Mills, J.A. Colacino, I. Mercado-Uribe, J. Liu, The chemokine growth-regulated oncogene 1 (Gro-1) links RAS signaling to the senescence of stromal fibroblasts and ovarian tumorigenesis, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 16472–16477.
[68] E. Cocucci, G. Racchetti, J. Meldolesi, Shedding microvesicles: artefacts no more, Trends Cell Biol. 19 (2009) 43–51.
[69] M. Narita, S. Nunez, E. Heard, A.W. Lin, S.A. Hearn, D.L. Spector, G.J. Hannon, S.W. Lowe, Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence, Cell 113 (2003) 703–716.
[70] K. Al-Nedawi, B. Meehan, R.S. Kerbel, A.C. Allison, J. Rak, Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 3794–3799.
[71] X. Yu, S.L. Harris, A.J. Levine, The regulation of exosome secretion: a novel function of the p53 protein, Cancer Res. 66 (2006) 4795–4801.
[72] I. Kirman, N. Poltoratskaia, P. Sylla, R.L. Whelan, Insulin-like growth factor-binding protein 3 inhibits growth of experimental colocarcinoma, Surgery 136 (2004) 205–209.
[73] A. Lespagnol, D. Duflaut, C. Beekman, L. Blanc, G. Fiucci, J.C. Marine, M. Vidal, R. Amson, A. Telerman, Exosome secretion, including the DNA damage-induced p53-dependent secretory pathway, is severely compromised in TSAP6/Steap3-null mice, Cell Death Differ. 15 (2008) 1723–1733.
[74] X. Yu, T. Riley, A.J. Levine, The regulation of the endosomal compartment by p53 the tumor suppressor gene, FEBS J. 276 (2009) 2201–2212.
[75] Z. Feng, p53 regulation of the IGF-1/AKT/mTOR pathways and the endosomal compartment, Cold Spring Harb. Perspect. Biol. 2 (2010) a001057.
[76] D. Volonte, K. Zhang, M.P. Lisanti, F. Galbiati, Expression of caveolin-1 induces premature cellular senescence in primary cultures of murine fibroblasts, Mol. Biol. Cell 13 (2002) 2502–2517.
[77] A. Linge, K. Weinhold, R. Blasche, M. Kasper, K. Barth, Downregulation of caveolin-1 affects bleomycin-induced growth arrest and cellular senescence in A549 cells, Int. J. Biochem. Cell Biol. 39 (2007) 1964–1974.
[78] J. Campisi, Cancer, aging and cellular senescence, In Vivo 14 (2000) 183–188.
[79] K. Lafferty-Whyte, C.J. Cairney, N.B. Jamieson, K.A. Oien, W.N. Keith, Pathway analysis of senescence-associated miRNA targets reveals common processes to different senescence induction mechanisms, Biochim. Biophys. Acta 1792 (2009) 341–352.
[80] J.M. Dhahbi, H. Atamna, D. Boffelli, W. Magis, S.R. Spindler, D.I. Martin, Deep sequencing reveals novel microRNAs and regulation of microRNA expression during cell senescence, PLoS One 6 (2011) e20509.
[81] W.K. Suh, B.U. Gajewska, H. Okada, M.A. Gronski, E.M. Bertram, W. Dawicki, G.S. Duncan, J. Bukczynski, S. Plyte, A. Elia, A. Wakeham, A. Itie, S. Chung, J. Da Costa, S. Arya, T. Horan, P. Campbell, K. Gaida, P.S. Ohashi, T.H. Watts, S.K. Yoshinaga, M.R. Bray, M. Jordana, T.W. Mak, The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses, Nat. Immunol. 4 (2003) 899–906.
[82] M. Yang, J. Chen, F. Su, B. Yu, L. Lin, Y. Liu, J.D. Huang, E. Song, Microvesicles secreted by macrophages shuttle invasion-potentiating microRNAs into breast cancer cells, Mol. Cancer 10 (2011) 117.
[83] K. Leiszter, O. Galamb, F. Sipos, K. Toth, G. Valcz, V.A. Patai, J. Molnar, B. Molnar, Z. Tulassay, Age-related microscopic and molecular changes of the human colon, and their role in the development of colorectal cancer in elderly people, Orv. Hetil. 151 (2010) 885–892.
[84] A.L. Wang, T.J. Lukas, M. Yuan, N. Du, M.O. Tso, A.H. Neufeld, Autophagy and exosomes in the aged retinal pigment epithelium: possible relevance to drusen formation and age-related macular degeneration, PLoS One 4 (2009) e4160.
[85] L. Rajendran, M. Honsho, T.R. Zahn, P. Keller, K.D. Geiger, P. Verkade, K. Simons, Alzheimer's disease beta-amyloid peptides are released in association with exosomes, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 11172–11177.
[86] B. Fevrier, D. Vilette, H. Laude, G. Raposo, Exosomes: a bubble ride for prions? Traffic 6 (2005) 10–17.
[87] I. Porto-Carreiro, B. Fevrier, S. Paquet, D. Vilette, G. Raposo, Prions and exosomes: from PrPc trafficking to PrPsc propagation, Blood Cells Mol. Dis. 35 (2005) 143–148.
[88] S. Alais, S. Simoes, D. Baas, S. Lehmann, G. Raposo, J.L. Darlix, P. Leblanc, Mouse neuroblastoma cells release prion infectivity associated with exosomal vesicles, Biology of the cell/ under the auspices of the European Cell Biology Organization 100 (2008) 603–615.
[89] A. Turchinovich, L. Weiz, A. Langheinz, B. Burwinkel, Characterization of extracellular circulating microRNA, Nucleic Acids Res. 39 (2011) 7223–7233.
[90] J.D. Arroyo, J.R. Chevillet, E.M. Kroh, I.K. Ruf, C.C. Pritchard, D.F. Gibson, P.S. Mitchell, C.F. Bennett, E.L. Pogosova-Agadjanyan, D.L. Stirewalt, J.F. Tait, M. Tewari, Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 5003–5008.
[91] K.C. Vickers, B.T. Palmisano, B.M. Shoucri, R.D. Shamburek, A.T. Remaley, MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins, Nat. Cell Biol. 13 (2011) 423–433.
[92] D.D. Taylor, W. Zacharias, C. Gercel-Taylor, Exosome isolation for proteomic analyses and RNA profiling, Methods Mol. Biol. 728 (2011) 235–246.
[93] M. Ostrowski, N.B. Carmo, S. Krumeich, I. Fanget, G. Raposo, A. Savina, C.F. Moita, K. Schauer, A.N. Hume, R.P. Freitas, B. Goud, P. Benaroch, N. Hacohen, M. Fukuda, C. Desnos, M.C. Seabra, F. Darchen, S. Amigorena, L.F. Moita, C. Thery, Rab27a and Rab27b control different steps of the exosome secretion pathway, Nat. Cell Biol. 12 (2010) 19–30.
[94] B. Riteau, F. Faure, C. Menier, S. Viel, E.D. Carosella, S. Amigorena, N. Rouas-Freiss, Exosomes bearing HLA-G are released by melanoma cells, Hum. Immunol. 64 (2003) 1064–1072.
[95] S. Gesierich, I. Berezovskiy, E. Ryschich, M. Zoller, Systemic induction of the angiogenesis switch by the tetraspanin D6.1A/CO-029, Cancer Res. 66 (2006) 7083–7094.
[96] J.W. Kim, E. Wieckowski, D.D. Taylor, T.E. Reichert, S. Watkins, T.L. Whiteside, Fas ligand-positive membranous vesicles isolated from sera of patients with oral cancer induce apoptosis of activated T lymphocytes, Clin. Cancer Res. 11 (2005) 1010–1020.
[97] A. Gallo, M. Tandon, I. Alevizos, G.G. Illei, The majority of microRNAs detectable in serum and saliva is concentrated in exosomes, PLoS One 7 (2012) e30679.
[98] L. Alvarez-Erviti, Y. Seow, H. Yin, C. Betts, S. Lakhal, M.J. Wood, Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes, Nat. Biotechnol. 29 (2011) 341–345.

Mutations in *UVSSA* cause UV-sensitive syndrome and destabilize ERCC6 in transcription-coupled DNA repair

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UV-sensitive syndrome (UV^S^S) is an autosomal recessive disorder characterized by photosensitivity and deficiency in transcription-coupled repair (TCR), a subpathway of nucleotide-excision repair that rapidly removes transcription-blocking DNA damage[1]. Cockayne syndrome is a related disorder with defective TCR and consists of two complementation groups, Cockayne syndrome (CS)-A and CS-B, which are caused by mutations in *ERCC8 (CSA)* and *ERCC6 (CSB)*, respectively[2]. UV^{S}S comprises three groups, UV^{S}S/CS-A, UV^{S}S/CS-B and UV^{S}S-A, caused by mutations in *ERCC8*, *ERCC6* and an unidentified gene, respectively[3–6]. Here, we report the cloning of the gene mutated in UV^{S}S-A by microcell-mediated chromosome transfer. The predicted human gene *UVSSA* (formerly known as *KIAA1530*)[7] corrects defective TCR in UV^{S}S-A cells. We identify three nonsense and frameshift *UVSSA* mutations in individuals with UV^{S}S-A, indicating that *UVSSA* is the causative gene for this syndrome. The UVSSA protein forms a complex with USP7 (ref. 8), stabilizes ERCC6 and restores the hypophosphorylated form of RNA polymerase II after UV irradiation.

To clone the gene responsible for UV^{S}S-A, mouse chromosomes were randomly transferred to Kps3 cells (UV^{S}S-A) by microcell-mediated chromosome transfers (MMCTs)[9] using mouse A9 cells as the donor. Kps3 cells fused with A9 microcells were UV irradiated periodically over 6 weeks. We obtained four independent UV-resistant cellular clones (15A-7, KAB1-14, KAGA2-6 and KAGB2-4) that exhibited normal levels of UV sensitivity and recovery of RNA synthesis after UV irradiation (UV-RRS) (**Supplementary Fig. 1**). To identify the mouse genomic DNA that was integrated in each clone, a comparative genomic hybridization (CGH) array analysis was performed. An intact mouse chromosome 5 was found in KAB1-14, whereas chromosome 5 with segments of chromosomes 12 and 17 was found in KAGA2-6. Only a few fragments of chromosome 5 were found in the 15A-7 and KAGB2-4 clones (**Fig. 1a**). These results indicated that the causative gene was located within the common 600-kb region of chromosome 5 that was integrated in the 15A-7 and KAGB2-4 clones (**Fig. 1b**). There are 11 known genes in that region (**Fig. 1c**). Six BAC clones[10] encompassing the 600-kb region were introduced into Kps3 cells (**Fig. 1c**). Kps3 clones transfected with BAC 476L03 acquired normal levels of UV resistance, whereas transfection with BAC 154C21 failed to complement UV-sensitive cells (**Fig. 1d**). These results indicate that *4933407H18Rik* is a complementing gene for UV^{S}S-A. The *4933407H18Rik* gene is the mouse homolog of a predicted human gene, *KIAA1530*, which has subsequently been renamed *UVSSA* (encoding UV-stimulated scaffold protein A) in light of this finding, with support from the Human Gene Nomenclature Committee (HGNC).

Sequencing of *UVSSA* cDNA in UV^{S}S-A cells revealed that Kps3 (Japanese)[4] and XP24KO (Japanese)[11] cells had a homozygous c.367A>T transversion, resulting in premature termination at amino acid 123 (p.Lys123*). TA-24 (Iranian)[3] cells had a homozygous deletion, c.87delG, that caused a frameshift at position 29, leading to premature termination at amino acid 39 (p.Ile31Phefs*9) (**Fig. 2a,b**). In addition, the expression of cDNA encoding Flag-HA-UVSSA successfully conferred normal levels of UV-RRS and UV resistance to Kps3 cells (**Fig. 2c,d**). Taken together, these results indicate that *UVSSA* is the causative gene for UV^{S}S-A.

The *UVSSA* gene is located on human chromosome 4 at p16.3 and is 40.7 kb long. The transcript of the gene is 4,336 nt long and is composed of 14 exons, encoding a protein that consists of 709 amino acids with a deduced molecular weight of 81 kDa[7,12]. Analysis of the amino-acid sequence of UVSSA revealed that there is a Vps27, Hrs and STAM (VHS) domain and a structurally similar epsin NH_2-terminal homology (ENTH) domain in the N-terminal region and a well-conserved domain of unknown function (DUF) 2043 domain in the C-terminal region (**Fig. 2a**). It was reported that the VHS/ENTH domains of SCAF8 and NRD1 interact with the C-terminal domain of RNA polymerase II (RNA Pol II)[13,14], suggesting that UVSSA also interacts with RNA Pol II. Homologs of UVSSA have been identified in *Arabidopsis thaliana*, rice and *Caenorhabditis elegans*, but there are no reports of their function.

To elucidate the function of UVSSA in TCR, we first examined its interaction with other TCR factors (**Fig. 3**). The UVSSA protein complex was affinity purified from the soluble or chromatin

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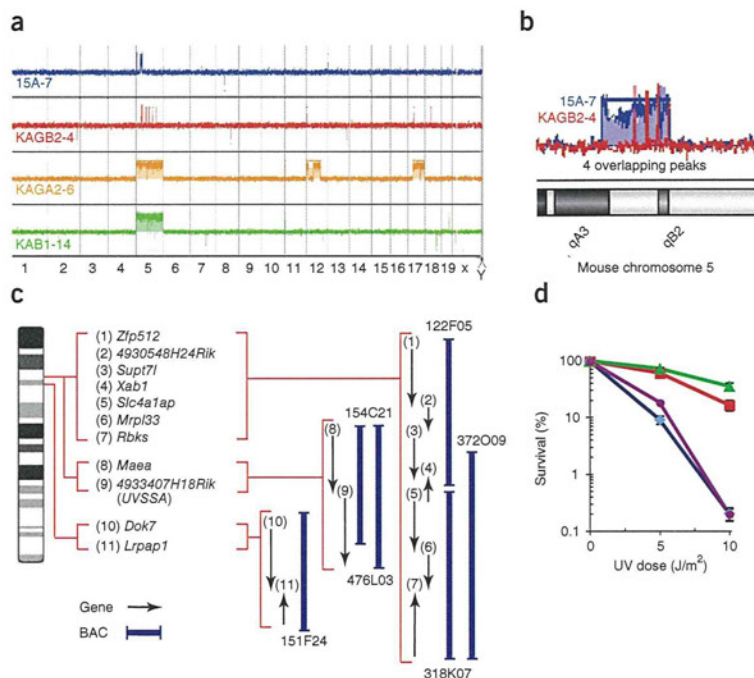

Figure 1 CGH array analysis and identification of BAC clones encompassing mouse genomic DNA integrated in the 15A-7 and KAGB2-4 clones. (**a**) CGH array analysis for identification of mouse genomic DNA integrated into the Kps3 clones containing mouse A9 chromosome segments. Kps3 cells were used as a control. (**b**) Enlarged view of the mouse chromosome 5 region integrated into the 15A-7 and KAGB2-4 clones. (**c**) Schematic of BAC clones encompassing the common 600-kb region of mouse genomic DNA integrated in clones 15A-7 and KAGB2-4. Genes included in the region are listed in positional order with a corresponding number. Chromosomal regions in red brackets correspond to peaks 2 to 4, from the left side, in **b**; there are no genes in peak 1. Arrows indicate the direction of the genes. (**d**) UV survival of Kps3 cells transfected with BAC 476L03 (green triangles) or BAC 154C21 (purple circles), compared to normal human WI38VA13 cells (red squares) and parental Kps3 cells (blue rhombuses). Error bars, s.d. calculated from three independent experiments.

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fraction of extracts derived from Kps3 cells expressing Flag- and HA-tagged wild-type UVSSA. In the soluble fraction, ERCC8 was coimmunoprecipitated with UVSSA, irrespective of UV irradiation, but not with RNA Pol II or ERCC6 (**Fig. 3a**). In the chromatin fraction, ERCC6, ERCC8 and RNA Pol II were coimmunoprecipitated with UVSSA after UV irradiation (**Fig. 3b**).

Next, we searched for new TCR proteins that interact with UVSSA. The wild-type UVSSA protein fused with Flag and HA tags was stably expressed in HEK293 cells. Whole-cell extract was used for affinity purification of the UVSSA complex with antibodies to Flag and HA. Mass spectrometric analysis of the UVSSA complex identified USP7 as an interacting protein (**Supplementary Fig. 2**). USP7 is a ubiquitin-specific protease that recognizes and removes ubiquitin from proteins. Numerous proteins have been identified as potential substrates of USP7, including Mdm2, p53, claspin, Chfr and histone H2B[8,15]. To determine whether USP7 is involved in TCR, wild-type cells were treated with *USP7* small interfering RNA (siRNA) (**Fig. 3c**). *USP7* siRNA–treated cells showed decreased UV-RRS and UV survival, implicating USP7 in TCR (**Fig. 3d,e**). In the *USP7* siRNA–treated cells, not only USP7 protein levels, but also those of UVSSA, were decreased (**Figs. 3c** and **4a**). The amount of *UVSSA* mRNA was not affected in *USP7* siRNA–treated cells (**Supplementary Fig. 3**). Conversely, knockdown of *UVSSA* did not result in decreased USP7 protein levels (**Fig. 4a**), probably because of a much greater quantity of USP7 relative to UVSSA in the cells. The amount of ERCC8 protein was not affected in *USP7* siRNA–treated cells (**Fig. 4b**). These results indicate that UVSSA forms a stable complex with USP7 and transiently binds ERCC8. Also, the UVSSA-USP7 complex was coimmunoprecipitated with ERCC8, irrespective of UV irradiation in the soluble fraction, and it was coimmunoprecipitated with ERCC6, ERCC8 and RNA Pol II in the UV-damaged chromatin fraction (**Fig. 3a,b**). Coimmnunoprecipitation was also detected in the cells expressing a normal level of *UVSSA* (**Supplementary Fig. 4**). These results indicate that the UVSSA-USP7 complex and ERCC8 are recruited to the sites of RNA Pol II and ERCC6 in the UV-damaged chromatin fraction. In the accompanying paper by Schwertman *et al.*, it was shown that both ERCC6 and UVSSA were recruited to the spot locally irradiated with 254-nm UV-C light, although the recruitment of ERCC6 and UVSSA was much weaker than that of the global genome-repair protein XPC[16]. These results suggest that only a small portion of cellular UVSSA is involved in TCR.

It is known that ERCC6 has an essential role in recruiting TCR factors to the stalled RNA Pol II complex[17,18]. To examine whether the binding of UVSSA, USP7 and ERCC8 to RNA Pol II is dependent on ERCC6, the Flag- and HA-tagged UVSSA protein was expressed in CS1AN (CS-B) cells, and immunoprecipitation of the cell extracts was performed using antibodies to Flag and HA. USP7 and ERCC8 were coimmunoprecipitated with UVSSA in the chromatin fraction, irrespective of UV irradiation, but RNA Pol II was not, even after UV irradiation (**Fig. 3f**). These results suggest that UVSSA, USP7 and ERCC8 are loaded onto RNA Pol II in an ERCC6-dependent manner.

Next, to examine whether the binding of UVSSA and USP7 to ERCC6 and RNA Pol II is dependent on ERCC8, the Flag- and HA-tagged UVSSA protein was expressed in CS3BE (CS-A) cells, and immunoprecipitation of the cell extracts was performed using antibodies to Flag and HA. USP7 was coimmunoprecipitated with UVSSA in the chromatin fraction, irrespective of UV irradiation, but RNA Pol II and ERCC6 were not coimmunoprecipitated, even after UV irradiation (**Fig. 3g**). These results suggest that ERCC8 is required for recruitment of the UVSSA-USP7 complex to ERCC6 and RNA Pol II in the UV-irradiated chromatin fraction. In the accompanying paper by Schwertman *et al.*, it was shown that GFP-UVSSA accumulated at sites of UV damage caused by a 266-nm UV-C laser in CS-A and CS-B cells, and a UV-independent interaction between hyperphosphorylated RNA Pol II (RNA Pol IIo) and UVSSA was detected in CS-B cells by chromatin immunoprecipitation (ChIP) using cross-linking reagent[16]. We assume that ERCC6 is required for stable integration of UVSSA, USP7, ERCC8 and RNA Pol II into a functional TCR complex.

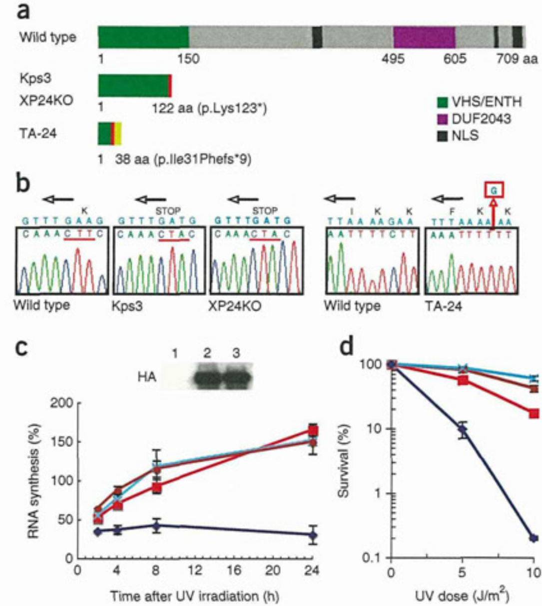

Figure 2 Mutations within the *UVSSA* gene in three subjects with UV^sS-A. (**a**) Schematic of the UVSSA protein and of amino-acid changes in Kps3, XP24KO and TA-24 cells. Amino-acid changes are shown in red. (**b**) Sequence chromatograms showing mutations in Kps3, XP24KO and TA-24 cells. The homozygous c.367A>T transversion is shown in Kps3 and XP24KO cells, and the homozygous c.87delG mutation is indicated in TA-24 cells. Translated amino acids are labeled above the cDNA sequence. Arrows represent the direction in which the sequence is read. (**c**) UV-RRS of Kps3 clones stably expressing *UVSSA* cDNA. Upper, protein blot of the Kps3 clones stably expressing Flag- and HA-tagged UVSSA using antibody to HA: lane 1, parental Kps3; lane 2, UVSSA-corrected Kps3 clone 1; lane 3, UVSSA-corrected Kps3 clone 2. Lower, UV-RRS of clones 1 (brown circles) and 2 (aqua crosses) and of WI38VA13 (red squares) and parental Kps3 cells (blue rhombuses). Error bars, s.d. calculated from three independent experiments. (**d**) UV survival of the Kps3 clones stably expressing Flag- and HA-tagged UVSSA. Symbols for each cell line are the same as in **c**. Error bars, s.d. calculated from three independent experiments.

We have found that the ERCC8 protein is translocated to the nuclear matrix after UV irradiation in an ERCC6- and transcription factor IIH (TFIIH)-dependent manner and that it colocalizes with Pol IIo[17,19]. Using a cell-free system[19], we found that UVSSA enhances UV-induced translocation of ERCC8 to the nuclear matrix (**Supplementary Fig. 5**).

It is known that inhibition of transcription after UV irradiation not only results from a blockage of transcription elongation by DNA damage on the transcribed strand but also can be caused by a reduction in the levels of the hypophosphorylated form of RNA polymerase II (RNA Pol IIa), which is required for the initiation of transcription[20]. The restoration of RNA Pol IIa levels and transcription after UV irradiation are deficient in CS-A and CS-B cells[20]. Consistent with this, we found that RNA Pol IIa disappeared after UV irradiation (10 J/m^2) in both the parental CS3BE (CS-A) cells and in ERCC8-corrected CS3BE cells. Approximately 16 h after UV irradiation, RNA Pol IIa reappeared in the ERCC8-corrected CS3BE cells but not in the parental CS3BE cells (**Supplementary Fig. 6**). We then examined the phosphorylation status of RNA Pol II in whole-cell lysates from Kps3 cells and UVSSA-corrected Kps3 cells after 10 J/m^2 of UV irradiation (**Fig. 4c**). RNA Pol IIa disappeared in both the parental and UVSSA-corrected Kps3 cells. However, it reappeared 8 h after UV irradiation in the UVSSA-corrected Kps3 cells but not in the parental Kps3 cells, indicating that UVSSA is required, in addition to ERCC6 and ERCC8, for the reappearance of RNA Pol IIa after UV irradiation.

Moreover, the amount of ERCC6 protein decreased in the non-irradiated parental Kps3 cells compared to the amount in the UVSSA-corrected Kps3 cells, and a greater decrease was detected after UV irradiation in the parental Kps3 cells, whereas there was little decrease in the UVSSA-corrected Kps3 cells (**Fig. 4c**). These results indicate

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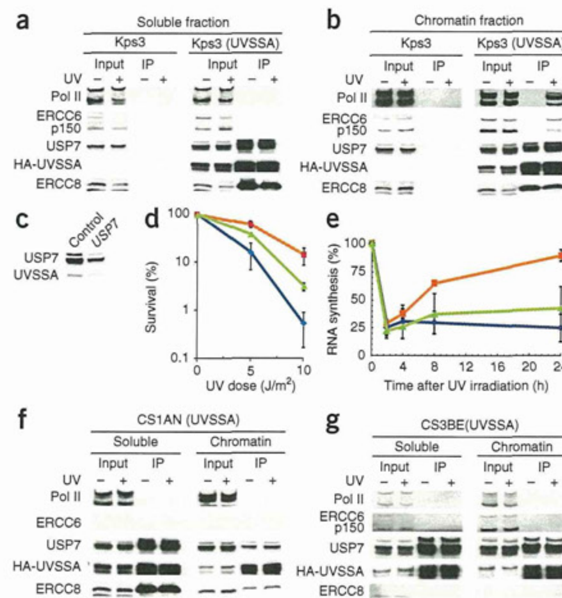

Figure 3 Interaction of UVSSA with RNA Pol II, ERCC6, ERCC8 and USP7. (**a**,**b**) Parental Kps3 cells and Flag- and HA-tagged wild-type UVSSA-corrected Kps3 cells were either irradiated with 20 J/m^2 of UV or were not irradiated and were incubated for 30 min. The UVSSA protein complex (IP) was then affinity purified from the soluble (**a**) or chromatin (**b**) fraction of each cell extract using anti-FLAG M2 agarose and anti-HA agarose and was subjected to protein blotting with antibodies to HA (UVSSA), RNA Pol II, ERCC6, ERCC8 and USP7. p150 corresponds to the CPFP protein (fusion protein consisting of N-terminal ERCC6$^{1-465}$ and the piggyback transposon)[32]. (**c**) Protein blot analysis of USP7 and UVSSA in UVSSA-corrected Kps3 cells and in isogenic cells transfected with *USP7* siRNA. The upper band of USP7 may be a modified form. (**d**) UV survival of parental Kps3 cells (blue rhombuses), UVSSA-corrected Kps3 cells (orange squares) and UVSSA-corrected Kps3 cells transfected with *USP7* siRNA (green triangles). Error bars, s.d. calculated from three independent experiments. (**e**) RNA synthesis after 10 J/m^2 of UV irradiation in the three cell lines in **d**. The symbols for each cell line are the same as in **d**. (**f**) Interaction of UVSSA with RNA Pol II, ERCC8 and USP7 in CS-B cells. Flag- and HA-tagged UVSSA protein was expressed in CS1AN (CS-B) cells, and immunoprecipitation of the cell extracts was performed using antibodies to Flag and HA. (**g**) Interaction of UVSSA with RNA Pol II, ERCC6 and USP7 in CS-A cells. Flag- and HA-tagged UVSSA protein was expressed in CS3BE (CS-A) cells, and immunoprecipitation of the cell extracts was performed using antibodies to Flag and HA. Error bars, s.d. calculated from three independent experiments.