Areca nut extract induced senescence in human oral fibroblasts enhances tumorigenesis of oral cancer cells

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Short title: Areca induces senescence and MMP-2 activity in oral fibroblast

Key words: areca, MMP-2 oral carcinoma, oral fibroblast, senescence

Abbreviations: ANE, areca nut extract; EGFR, epidermal growth factor receptor; IGFR, insulin like growth factor receptor; MMP, matrix metalloproteinase; NHOF, normal human oral fibroblast; asNHOF, ANE-induced senescent NHOF; pNHOF, presenescent NHOF; NHOK, normal human oral keratinocyte; OSCC oral squamous cell carcinoma; PA, plasminogen activator; PD, population doubling; PDL, population doubling level; ROS, reactive oxygen species; SA-ß-Gal, senescence-associated ß-galactosidase; TIMP, tissue inhibitor of metalloproteinase; X-gal, 5-bromo-4-chloro-3-indolyl-ß- D-galactosidase
Abstract

Areca use is tightly linked to oral mucosal and submucosal pathogenesis in Asians. This study investigated the effects of areca nut extract (ANE) on primary normal human oral fibroblasts (NHOFs) and the possible impacts on epithelial carcinogenesis. Chronic subtoxic ANE treatment (5 or 10 µg/ml) resulted in growth inhibition and the appearance of senescence-associated β-galactosidase in NHOFs. High or sustained p16 expression could underlie such a senescence process. A tremendous MMP-2 activation was elicited in the ANE-induced senescent (as)NHOFs. Oral cancer cells treated with asNHOF supernatant exhibited enhancement in proliferation, migration and anchorage-independent growth. The treatment also drastically activated IGFR and AKT as well as up-regulating cyclin D1 expression. Blockage of MMP-2 and AKT significantly attenuated the enhancement in anchorage-independent growth. Subcutaneous co-injection of cancer cells with asNHOFs into nude mice significantly enhanced tumorigenesis by SAS cells and also induced the creation of transient xenographic tumors with non-tumorigenic OECM-1 cells. Cultivated cells arising from the enhanced SAS tumors had significantly higher aggressiveness. Our results suggest that the senescence stress induced by ANE may aid oral cancer progression through up-regulating MMP-2 in asNHOFs. These findings seem to support an interactive role for areca-exposed stromal cells in the progression of oral cancers.
among a risk population.
Introduction

Around 200-400 million people in South and Southeastern Asia are engaged in chewing areca (betel), which is a combination of the major component, areca nut and other ingredients (1). Areca chewing is epidemiologically related to a higher risk of oral squamous cell carcinoma (OSCC), oral submucosal fibrosis (OSF) (2-4), other cancers and a range of systemic disorders (5, 6). Areca nuts contain arecoline, arecaidine, polyphenols and alkaloids (2). Areca nut extract (ANE) is cytotoxic and genotoxic to oral keratinocytes (7). It also induces reactive oxygen species (ROS) and COX-2 expression (8, 9). Our previous studies have shown that ANE elicits a rapid activation of MAPKs and NF-κB in oral keratinocytes (10-12). Furthermore, such NF-κB activation could be the basis of the COX-2 up-regulation that is associated with areca exposure (11).

Degradation of the extracellular matrix (ECM) represents a key element in the multistage processes of tumor invasion and metastasis; matrix metalloproteinases (MMPs) have been shown to have a central role among ECM-degrading enzymes (13). Studies have also indicated that MMPs are involved in early tumorigenesis and are able to modulate proliferation, apoptosis and angiogenesis (13). MMP-2 primarily hydrolyzes type IV collagen and laminin, which are key elements of the basement membrane and also play significant roles in the progression of cancers including
OSCC (14, 15). As well as laminin, it also degrades insulin-like growth factor (IGF)-binding proteins, which all are activated by IGFR and EGFR and modulate proliferation and apoptosis of epithelial cells (15, 16). Functional polymorphisms of the MMP-2 promoter are a risk factor for oral carcinogenesis in areca users (17). Under physiological condition, MMPs should be balanced against the tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activators (PAs). Areca ingredients seem to modulate MMPs towards pathogenesis since MMP-2 is increased in the saliva of areca chewers (18).

Normal cells gradually enter a state of replicative senescence during continuous culture. DNA-damage reagents, ROS and aberrant mitogenic signaling also are able to cause premature senescence in culture (19-21). Chronic subtoxic ANE treatment upsets the control and regulation of various cell cycle regulators and induce a senescence-associated phenotype in normal human oral keratinocytes (NHOKs) (12). Although senescence may be associated with the suppression of cancer growth (22), recent studies have shown that senescent dermal fibroblasts can assist the tumorigenesis of adjacent initiated epithelial cells (23, 24). OSF is a precancerous condition occurring exclusively in areca chewers. Although ANE-modulated ECM disruption plays an important role in the genesis of OSF (4), this study further demonstrates that the up-regulation of MMP-2 in NHOFs under long term low dose
ANE treatment is advantageous to the initiation and growth of xenographic OSCCs, which implies that there is also an indirect role for areca in epithelial oncogenesis.

**Materials and Methods**

**Cell culture and reagents.** NHOFs were cultivated following protocols previously established (25) as approved by an institutional review board. Calculation of population doublings (PDs) of NHOFs is described in details in supplements. SAS cells, a high grade tumorigenic OSCC cell and OECM-1 were cultivated following protocols previously established (11). OC3 cells, a low grade areca-associated OSCC cell which has a wild type p53 sequence and a non-tumorigenic phenotype, was cultivated under the conditions previously described (10). The preparation of ANE from ripe areca nuts was performed using the protocols previously described (25). Cells treated with ANE were washed and cultivated with serum-free medium for 24 h to collect supernatants. The use of active recombinant MMP-2, MMP-2 Inhibitor I, OA-Hy (26), a neutralizing MMP-2 antibody (27), an unrelated preimmune rabbit IgG and LY294002 (28) is described in supplements.

**Western blot analysis.** Whole cell lysate (50 µg) was separated by electrophoresis on a 10% denaturing polyacrylamide gel (12). The use of antibodies and the companies from which those antibodies were purchased will be provided upon
request.

**Zymography.** Supernatants were mixed with sodium dodecyl sulfate sample buffer without heating or reduction to preserve enzyme activity. The samples were electrophoresed in 10% polyacrylamide gels copolymerized with 0.1% gelatin according to documented methods (29). After electrophoresis, the gels were washed to remove the SDS, which re-natured the MMPs and this was followed by incubation with CaCl₂ and ZnCl₂. The gels were subsequently stained with 0.1% Coomassie blue and the cleared areas of gelatinolytic activity showed up on the blue background after destaining.

**Migration assay.** Cells were grown in serum-free media using the Transwell system (Corning, Acton, MA) with a porous transparent polyethylene terephthalate membrane having a pore diameter of 8 µm. After 12 h, the cells were fixed and stained. The cells attached on upper surface of the membrane were wiped off with cotton. The migrated cells were counted by microscopy.

**Anchorage-independent growth.** Cells were suspended in 1.3% methylcellulose (Methocel® MC, Sigma-Aldrich) in culture media at 0.8 or 3 x 10⁴ cells per well and plated on a layer of 0.9% agarose (Sigma-Aldrich) in culture media in six-well culture plates at 37 °C for 10 days. The number of total colonies and number of colonies with a diameter ≥ 100 µm (defined as large colonies) were counted over five fields per
well for a total of 15 fields in triplicate experiments.

Tumorigenesis. Various number of OSCC cells admixed with NHOFs or not were injected (0.05 ml) subcutaneously into the flank of 6- to 8-week-old female athymic nude mice. Tumor volumes were calculated using the formula \( V = 0.5 \times a \times b^2 \), where \( a \) and \( b \) are the long and short diameters of the tumors, respectively. After sacrifice, the tumors were bisected into two halves. One half of each tumor was subjected to fixation, processing and histopathological evaluation. The other portion of the tumors was subjected to cell culture.

Statistics. An unpaired t-test was used for statistical analysis. Results with \( p < 0.05 \) were considered to be statistically significant difference.

Results

ANE inhibited NHOF proliferation and induced senescence. NHOFs treated with a dosage \( \leq 10 \) µg/ml resulted in no apparent changes in the growth over 10 population doublings (PDs). Therefore, long-term treatment with 5 or 10 µg/ml ANE was imposed on the NHOFs to evaluate the effects of such long term exposure. In a total of 39 NHOFs that were treated with 10 µg/ml ANE, five showed no growth change for more than 20 PDs and were designated slow-response NHOFs (Fig. S14). 29 (74%) of the NHOFs exhibited a growth plateau at around 20 PDs and these were
designated normal-response NHOFs (Fig. S1B). Five NHOFs exhibited a sustained growth inhibition were designated rapid-response NHOFs (Fig. S1C). The treatment duration was divided into early, intermediate and late passage, representing treatments of < 10 PDs, 10-19 PDs and ≥ 20 PDs, respectively. Following an increase in PD, the ANE-treated NHOFs exhibited more prominent elongation and flattening in their cell morphology and the presence of abundant cytoplasmic vacuoles compared to the controls. In addition, there was a progressive increase in the number of SA-β-Gal labeled NHOFs (Fig. S1, right panel). Only normal response NHOFs were used during subsequent analysis. Prolonged treatment with 10 µg/mg ANE for one week longer than late passage resulted in the level of SA-β-Gal labeled cells being >70%. These cells were defined an ANE-induced senescent NHOFs (asNHOFs). NHOFs at the same passage without ANE treatment showed a level of SA-β-Gal labeled cells of <10% and these were defined as presenescent NHOFs (pNHOFs).

**ANE affected p16, Rb and p21 expression.** The status of p16, Rb, p53 and p21 during the senescence process of NHOFs was followed during ANE treatment. A pilot study showed either an absence or a barely detectable change in p53 expression between the controls and treated NHOFs; based on this, no further experiments were carried out to detect p53 changes. Figure S2A shows the results of a representative Western blot after 5 or 10 µg/ml ANE treatment at various passages. The asNHOFs
displayed similar molecular changes as the treated late passage cells. The expression of p16, Rb and p21 were normalized against GAPDH and are shown in Figure S2B. The columns represent the relative expression ratios of p16, Rb and p21 in treated cells relative to the controls at different passages. It seems that the early and sustained high p16 expression is consistent for entry into senescence in the ANE-treated NHOFs. The conspicuous down-regulation of p21 did not counteract this. Figure S2C and D illustrate the changes that occurred in two different NHOFs. In these NHOFs, there is prominent p16 up-regulation and p21 down-regulation, especially during intermediate or late passage following ANE treatment.

**ANE regulated MMP-2, TIMP-1 and u-PA mRNA expression.** A marked down-regulation of *KGF-1* and *TIMP-1* mRNA expression and a slight up-regulation of *MMP-2* and *u-PA* mRNA expression was noted in the NHOFs following 5 or 10 µg/ml ANE treatment at different passages (Fig. S3A). Analysis of three different NHOFs showed a remarkable and significant down-regulation of *KGF-1* and *TIMP-1* following treatment (Fig. S3B, up and down, respectively). A slight but significant up-regulation in *MMP-2* and *u-PA* mRNA expression, which was dose-dependent, was also noted (Fig. S3C, up and down, respectively). The notable down-regulation in *KGF-1* in this study agrees with a previous study (25) and this supports the validity of our analysis. No change was observed in the mRNA expression of *IL-6, MMP-3, -7,*
-9, -13 and -15 and $I_kB\alpha$ in the ANE-treated NHOFs.

**ANE up-regulated MMP-2 activity.** ANE-treated NHOFs and asNHOFs were switched to culture in a serum-free medium for 24 h without ANE treatment before harvesting the cell lysate and supernatant. Western blot analysis using the cell lysate indicated up-regulation of MMP-2 protein in the ANE-treated NHOFs (Fig. 1A). The MMP-2 protein became more abundant in ANE-treated NHOFs during late passage. In asNHOFs, the MMP-2 protein also became very abundant. The gelatin lysis activities of MMP-2 (72 kDa position as proform and 62 kDa position as activated form) in the supernatant of ANE-treated NHOF was much higher than that in the controls, particularly at late passage (Fig. 1B). In the asNHOFs, there is a very prominent increase in activated MMP-2.

**asNHOF supernatant enhanced the proliferation and migration of OSCC cells.** OC3 and SAS cells were treated with the concentrated supernatant from 4 x 10⁵ asNHOFs and pNHOFs. The MMP-2 activity of each asNHOF aliquot was equal to 0.15 µg MMP-2 as shown by zymography. Furthermore, 10 µM OA-Hy MMP-2 were used to block MMP-2 activity (15). asNHOF supernatant and 0.15 µg MMP-2 significantly increased the proliferation of OC3 cells. OA-Hy drastically inhibited the growth enhancement (Fig. 2A). asNHOF supernatant and MMP-2 also significantly increased the proliferation of SAS cells (Fig. 2B). With both OC3 and SAS cells,
asNHOF supernatant and MMP-2 significantly enhanced migration by more than 200%, but pNHOF supernatant did not do this (Fig. 2C, D). OA-Hy abolished the enhancement in migration.

**asNHOF supernatant activated IGFR and AKT and up-regulated cyclin D1.**

Western blotting analysis indicated a significant increase (>200%) in the phosphorylation of IGFR and FAK in SAS cells following treatment with asNHOF supernatant or MMP-2 for 15 min. The phosphorylation of AKT became significantly greater at 30 min following treatment (Fig. 3A). Activation of IGFR as well as a rather delayed AKT activation was the most prominent molecular events induced by asNHOF supernatant. An increase in cyclin D1 expression in OSCC cells was also noted following treatment with asNHOF supernatant or MMP-2 for 48 h, especially with OC3 cells (Fig. 3B).

**asNHOF supernatant enhanced anchorage-independent growth of OSCC cells.**

OSCC cells were treated with 0.03% DMSO (vehicle control), 0.15 μg MMP-2, the asNHOF supernatant or pNHOF supernatant. There was also a concomitant treatment with 10 μM OA-Hy, 4 μg IgG from a pre-immunized rabbit or 4 μg MMP-2 neutralizing antibody as recommended by original provider as part of the experimental controls. In addition, OSCC cells were pre-treated with non-toxic 2 or 10 μM LY294002 (28) followed by treatment with 0.1% DMSO, MMP-2 or asNHOF.
Figure 4 shows representative anchorage-independent growth results for SAS cells under the various treatments. Quantification of the total colonies is shown in Figure 5A and C. Both MMP-2 and asNHOF supernatant significantly enhanced anchorage-independent growth of the SAS cells and this could be abrogated significantly by concomitant OA-Hy, MMP-2 antibody and LY294002 treatment, indicating the possible involvement of MMP-2 and AKT activation in this anchorage-independent growth of the SAS cells. pNHOF supernatant did not cause a similar increase. Quantification of the large colonies is shown in Figure 5C and D. OA-HY, MMP-2 antibody and LY294004 were able to abrogate this induction of large colonies.

Figure S4 illustrates the results for OECM-1. There were only a few and smaller OECM-1 cell colonies that survived in the anchorage-independent growth environment (Fig. S4A). Both MMP-2 and asNHOF supernatant increased the colony number for OECM-1, which seemed to be MMP-2 dependent (Fig. S4B). What was intriguing is that, although asNHOF supernatant enhanced the number colonies by about 2.5-fold, most of the colonies were large (Fig. S4C), irregular (Fig. S4A) and very unlike the spheroid colonies formed by SAS cells. The anchorage-independent growth potential of OC3 cells was poor and was not augmented by MMP-2, asNHOF supernatant and pNHOF supernatant (not shown).
**asNHOF enhanced the tumorigenicity of OSCC cells.** A series of inoculate made up of $2.5 \times 10^5$ control SAS cells (SAS alone), the same amount of SAS cells mixed with $1 \times 10^6$ asNHOF (SAS + asNHOF), the same amount of SAS cells mixed with $1 \times 10^6$ pNHOF (SAS + pNHOF) and $1 \times 10^6$ asNHOF alone were injected subcutaneously to a number of nude mice. The SAS + asNHOF mice showed a significantly faster tumor induction and an increase in final tumor volume compared to the SAS alone or SAS + pNHOF groups at the end of 5+ weeks (Fig. 6A). No tumor mass was noted in mice injected with asNHOF. Histopathological examination revealed a massive growth of poorly-differentiated cells with prominent pleomorphism, aberrant mitosis, necrosis and conspicuous angiogenesis. Keratin pearl was minimal. A remarkable increase in the proportion of multinucleated giant tumor cells was found in the tumors from SAS cells admixed with NHOF compared to SAS alone cells (not shown).

OECM-1 was non-tumorigenic following subcutaneous injection of $5 \times 10^6$ cells over 8 weeks. However, co-injected with $1 \times 10^6$ asNHOF drastically induced the genesis of small xenographic OECM-1 tumors over a period of between 10 days and 30 days (Fig. 6B). It was intriguing that the tumors shrunk spontaneously after they reached about 200 mm³. Attempts to rescue the tumors by the injection of an
additional 1 x 10^6 asNHOFs into the area surrounding the regressing tumors were not successful.

**Discussion**

Areca chewing has been tightly linked to pathogenesis of epithelial and stromal cells in oral cavity (2, 4). A low concentration of ANE can mediate G_1 arrest of NHOK, which finally resulted in phenotypes related to premature senescence (12). In this study, the vast majority (74%) of NHOF exhibited conspicuous senescence-associated phenotypes including the arrest of cell growth, morphological changes, genesis of stress granules and remarkably high frequency of SA-β-Gal activity following a low dose ANE treatment for more than 20 PDs. These results together with our previous findings suggested the susceptibility of both oral epithelial and stromal cells to areca is linked to senescence-associated pathogenesis (30). An increase in p16, Rb and p21 expression in intermediate- or late-passage NHOFs relative to early-passage ones was found, which implies that there is a role for these proteins in replicative senescence. However, during the senescence process of NHOF modulated by ANE, p21 was substantially down-regulated, while p16 was persistently high or up-regulated. Specifically, p21 is able to activate arresting at various points in the cell cycle, while p16 acts specifically at G_1 phase. This implies that p16 activity at
$G_1$ could be a crucial effect underlying NHOF growth arrest. Although p16 and p21 each may play a distinctive role in the senescence process of fibroblasts (31), our data regarding an areca-induced senescence agrees with previous findings, where there was a demonstration of p16 up-regulation suggesting that p16 rather than p21 is a key protein in the terminal stages of growth arrest during the replicative senescence of fibroblasts (32). The findings also substantiated the existence of a p16-mediated irreversible senescence that is refractory to inactivation involving p21 (33).

Senescent fibroblasts affect the morphogenesis and carcinogenesis of the covering or adjacent epithelial cells by the generation of secreted or non-secreted factors (23, 24). Krtolica et al (34) demonstrated for the first time that senescent dermal fibroblasts promoted epithelial cell growth and tumorigenesis. A recent study has revealed that senescent fibroblast can secret vascular endothelial growth factor and this might promote tumorigenesis through an angiogenesis process (35). Dilley et al (21) have shown that a reduction in the gap junction of senescent dermal fibroblasts could be involved in the promotion of colony formation by initiated keratinocytes. In agreement with a previous study, we have found that there is a down-regulation of $KGF-1$ in NHOF following ANE treatment (25). Furthermore, an intensive screening has also stratified the overall increase in MMP-2 activity associated with asNHOFs and showed that it is due to a homeostatic disturbance in MMP-2 and the protein’s
regulators (4). SAS, OC3 and OECM-1 OSCC cells show little MMP-2 activity (not shown). On treatments with asNHOF supernatant, the proliferation and migration of SAS and OC3 were enhanced to different levels. Since a MMP-2 specific blocker reversed such enhancement together with the effects of the added MMP-2, this suggests that MMP-2 may be a crucial secreted molecule produced by asNHOFs that caused the enhancement. The rather prominent enhancement in migration suggests a novel concept whereby the senescent NHOF may affect adjacent epithelial cells by influencing their migration or invasion.

In SAS cells, the rather intensive activation of IGFR and AKT may be mechanistically involved in the phenotypic enhancement. Although MMP-2 can digest laminin and this releases a fragment that causes EGFR activation (15), no prominent EGFR activation was noted in this study. MMP-2 can cleave IGF binding proteins to release IGF, which results in IGFR activation (16). It is likely that by secreting MMP-2, the asNHOFs activate IGFR in the OSCC cells. The subsequent activation of the AKT cascade may finally lead to an increase in cyclin D1 expression and the enhancement of the tumor phenotype. It is also clear that blockage of MMP-2 and AKT impaired the anchorage-independent growth of SAS and OECM-1 cells that had been enhanced by the asNHOF supernatant. These findings support the hypothesis that asNHOFs enhance the transformation of OSCC cells, probably
through the AKT pathway. This is in agreement with previous findings where PI3K-AKT signaling was found to be crucial to the suppression of anoikis in transformed cells (36). Since asNHOF supernatant remarkably increase the fraction of large colonies compared to MMP-2 with SAS and OECM-1 cells, presumably the asNHOF supernatant also includes other factors in addition to MMP-2 that are able to further stimulate transformation. Proteomic analysis is being carried out at present to elucidate these novel factors.

The vast majority of areca chewers are also tobacco smokers, tobacco chewers and alcohol drinkers (1). Based on this, the carcinogenic effect of areca without tobacco has been queried even though areca has been classified as a class I carcinogen recently (3). Areca has been proved to be a promoter of oral chemical carcinogenesis in a rodent model (37). To evaluate the enhancing effects of asNHOFs on the growth of OSCC, both non-tumorigenic OC3 and OECM-1 cells and high grade tumorigenic SAS cells were used in a series of in vivo tumor induction experiments. The significant enhancing effect of asNHOFs on the xenographic SAS growth in nude mice supports the notion that the stromal cell changes elicited by ANE might support neoplastic growth of oral epithelium in an advantageous microenvironment. Since the cultivated cells from this tumor exhibited a significant higher mobility and anchorage-independent growth compared to the control cells, we can speculate that
such phenotypic changes become persistent in SAS cells. Anchorage independence is a measure of autonomous cell growth and an important hallmark of cancer. In agreement with the \textit{in vitro} studies involving the asNHOF supernatant, which was able to enhance the transformation of OECM-1 but not OC3, \textit{in vivo} experiments also revealed that co-injection of asNHOF was sufficient to induce transient tumorigenesis of OECM-1, but not of OC3. Krtolica et al (34) have shown that senescent dermal fibroblasts are able to stimulate premalignant epithelial cells to form tumors in mice. Whether the differences observed are due to variation in the origin of the fibroblasts, differences in the stress used to induce senescence, limitations in the proliferative enhancement that is provided by the asNHOFs or a lack of specific mutations in OC3 deserve further elucidation. It is likely that the recession of OECM-1 xenographic tumor was secondary to the mortality of the asNHOFs in the transplanted environment that originally sustained OECM-1. Data from OECM-1 possibly suggests that the asNHOFs act through an epigenetic mechanisms to affect OECM-1. The findings also suggest that factors produced by the asNHOFs may also be important to the initiation of tumor growth \textit{in vivo}. OC3 cells carry a wild type p53 but the p53 in OECM-1 is mutated (10). Chronic subtoxic ANE treatment results in the genesis of tetraploid OC3 (12). Wild type p53 is responsible for the tetraploidity checkpoint during the cell cycle (38). If p53 in OC3 can be knocked, this will impair the tetraploidity check
point and result in aneuploid OC3. This model, when applied to more advanced tumor phenotypes, might be useful as a way of addressing the interactive roles between ANE treated keratinocytes and fibroblast for epithelial oncogenesis.

In the present study we have identified the induction of senescence in oral fibroblast by areca nut extract treatment. The concomitant MMP-2 activation in these senescent fibroblasts may not only play a variety of roles in submucosal pathogenesis and impaired wound healing, but it also provides an advantageous environment for the degradation of the ECM and the activation of growth factors; these may contribute significantly to the progression of oral carcinomas. Evidence of this interactive role in oral cancer progression substantiates the view that areca per se might promote tumor formation by cancer cells. An anti-MMP-2 therapeutic regimen might prove to be invaluable as a way of intervening in OSCC progression.

Acknowledgements

This study was supported by an Excellent Research Grant V95ER2-011 from Veterans General Hospital, Taipei and Mackay-Yang-Ming Research Grant MMHY3-N-010-014.
References


Figure legends

Figure 1. MMP-2 protein expression and activity in NHOFs following ANE treatment. A, Western blot analysis of the cell lysate from a representative NHOF. The amount of cytosolic MMP-2 (72 kDa) gradually increased following the increase in culture passage and the increase in treatment dosage. asNHOF had the highest level of MMP-2 expression. B, Zymography of supernatant from the same NHOF as in A. The protein loadings were normalized against cell number. The zones of degradation detected at 72 kDa and 62 kDa represent pro-MMP-2 and active MMP-2, respectively. asNHOF produces a particularly large amount of active MMP-2. C and D, Quantitative analysis of pro-MMP-2 and active MMP-2, respectively, in three distinctive NHOFs. Values are expressed as means ± S.D. from three distinctive NHOFs. CTL, control; *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Figure 2. asNHOF supernatant affects the proliferation and migration of OSCC cells. A, C and E, OC3 cells; B, D and F, SAS cells. A and B, growth curve; C and D, migration; E and F, anchorage-independent growth. Values are expressed as means ± S.D. from three individual analysis. CTL, control; *, p < 0.05; **, p < 0.01.
Figure 3. asNHOF supernatant activated IGFR and AKT and up-regulated cyclin D1 expression of OSCC cells. A, SAS cells. A significant increase in IGFR phosphorylation and AKT phosphorylation can be noted at 15 min and 30 min, respectively, following asNHOF supernatant treatment. B, Cyclin D1 expression. Strong induction of cyclin D1 expression following asNHOF treatment for 48 h can be noted in OC3 cells. CTL, control.

Figure 4. asNHOF supernatant enhanced the anchorage-independent growth of SAS cells. Representative illustrations of colonies after various treatments are shown. CTL, control.

Figure 5. Quantitation of SAS cell colonies after various treatments. A and C, total colonies. B and D, large colonies. CTL, control; *, p < 0.05; **, p < 0.01; ***, p < 0.001; comparison to controls. #, p < 0.05; ###, p < 0.001; comparison to cells without OA-Hy or LY294002 treatment. Δ, p < 0.05; ΔΔ, p < 0.01; comparison to cells with pre-immune IgG treatment.

Figure 6. Co-injection of asNHOF enhanced the tumorigenesis of SAS cells. A, SAS. Co-injection of SAS cells with asNHOF induced larger tumors compared
to pNHOF co-injection and SAS alone injection. Injection of asNHOF alone did not induce tumors. B, OECM-1. Co-injection of OECM-1 cells with asNHOF significantly induced tumorigenesis of the OECM-1 cells, but from day 15 to day 23. However, additional injection of asNHOF to the tumors at day 32 (arrow) in two mice did not revert the recession. Injection of OECM-1 alone or OECM-1 and pNHOF co-injection did not induce tumorigenesis. SAS alone injection in this experiment was used as a control. (+), OECM-1 xenographic tumors with second asNHOF injection; (-), OECM-1 xenographic tumors without second asNHOF injection; Values are expressed as means ± S.D. from six mice in each group. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Hsuan-hsuan Lu, Fig. 1

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### C

![Graph C](image)

### D

![Graph D](image)
Hsuan-hsuan Lu, Fig. 2

A and B: Graphs showing cell number (10^3) over Days, with different conditions.

C and D: Graphs showing migration (Folds) with different conditions.

Conditions:
- Control
- MMP-2
- asNHOF
- pNHOF
- OA-Hy

Migration (Folds) and Cell number (10^3) are indicated with asterisks (*) and double asterisks (**) for statistical significance.
**Figure 3**

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<th>2 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D1</td>
<td>1.34</td>
<td>2.64</td>
</tr>
<tr>
<td>GAPDH</td>
<td>1.34</td>
<td>2.64</td>
</tr>
<tr>
<td>OC3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Hsuan-hsuan Lu**
Hsuan-hsuan Lu, Fig. 4

<table>
<thead>
<tr>
<th></th>
<th>CTL</th>
<th>MMP-2</th>
<th>asNHOF</th>
<th>pNHOF</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ DMSO</td>
<td><img src="ctl_dms.jpg" alt="Image" /></td>
<td><img src="mmp2_dms.jpg" alt="Image" /></td>
<td><img src="asn_dms.jpg" alt="Image" /></td>
<td><img src="pn_dms.jpg" alt="Image" /></td>
</tr>
<tr>
<td>+ OA-Hy</td>
<td><img src="ctl_oa_hy.jpg" alt="Image" /></td>
<td><img src="mmp2_oa_hy.jpg" alt="Image" /></td>
<td><img src="asn_oa_hy.jpg" alt="Image" /></td>
<td><img src="pn_oa_hy.jpg" alt="Image" /></td>
</tr>
<tr>
<td>+ MMP-2 Ab</td>
<td><img src="ctl_mmp2_ab.jpg" alt="Image" /></td>
<td><img src="mmp2_mmp2_ab.jpg" alt="Image" /></td>
<td><img src="asn_mmp2_ab.jpg" alt="Image" /></td>
<td><img src="pn_mmp2_ab.jpg" alt="Image" /></td>
</tr>
<tr>
<td>+ LY294002</td>
<td><img src="ctl_ly.jpg" alt="Image" /></td>
<td><img src="mmp2_ly.jpg" alt="Image" /></td>
<td><img src="asn_ly.jpg" alt="Image" /></td>
<td><img src="pn_ly.jpg" alt="Image" /></td>
</tr>
</tbody>
</table>
**Hsuan-hsuan Lu, Fig. 5**

**A**
- Colony numbers (Folds)
- MMP-2 asNHOF pNHOF OA-Hy IgG MMP-2 Ab
- Colony numbers (≥ 100 µm/5 fields)

**B**
- Colony numbers (≥ 100 µm/5 fields)
- MMP-2 asNHOF pNHOF OA-Hy IgG MMP-2 Ab

**C**
- Colony numbers (Folds)
- MMP-2 asNHOF 
- Colony numbers (≥ 100 µm/5 fields)

**D**
- Colony numbers (≥ 100 µm/5 fields)
- MMP-2 asNHOF LY294002 (µM)
- Colony numbers (≥ 100 µm/5 fields)
Hsuan-hsuan Lu, Fig. 6

A

Tumor size (10 mm³)

SAS
asNHOF
SAS+pNHOF
SAS+asNHOF

Days

B

Tumor size (mm³ x10)

SAS
OECM-1
OECM-1 + pNHOF
OECM-1 + asNHOF (+)
OECM-1 + asNHOF (-)

Days