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Myofibroblasts in the stroma of oral cancer promote tumorigenesis via secretion of activin A

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SUMMARY

Myofibroblasts are essential during wound healing and are often found in the stroma of oral squamous cell carcinomas (OSCC). Although the molecular mechanisms by which myofibroblasts influence OSCC remain largely unknown, previous studies demonstrated that presence of myofibroblast in OSCC stroma is an important risk factor of patient's shortened survival. Here we showed that some growth factors are produced in higher levels by tumor-associated myofibroblasts compared to tumor-associated fibroblasts, including activin A. Myofibroblast-conditioned media containing activin A significantly increased OSCC cell proliferation and tumor volume, whereas down-regulation of activin A in the conditioned media decreased proliferation. In addition, myofibroblasts induced in vitro invasion of OSCC cells, which was accompanied by an increased production of matrix metalloproteinases (MMP). In vivo, a significant correlation between presence of myofibroblasts and activities of MMP-2 and MMP-9 was observed in OSCC samples. However, blockage of activin A synthesis by myofibroblasts did not affect invasion and MMP production by OSCC cells. Together, our data demonstrate that activin A is required for the proliferative effects of myofibroblasts on OSCC cells. We conclude that myofibroblasts in the stroma of OSCC may influence proliferation and invasion, resulting in more aggressive tumor.

Introduction

Myofibroblasts secrete extracellular matrix molecules and degrading enzymes, angiogenic and pro- and anti-inflammatory factors, and stimulate epithelial cell proliferation and invasion.[1–3] Myofibroblasts are classically involved in wound healing, but they are also found in the reactive tumor stroma.[4,5] Although the knowledge of myofibroblast participation in tumors is evolving, there has been little work investigating the role of myofibroblasts in oral squamous cell carcinomas (OSCC).

The first evidence in favor of a role for myofibroblasts in OSCC was obtained by Barth et al. in 2004,[6] and later of this year Lewis et al.[7] demonstrated that myofibroblasts induced by TGF-β1 secrete hepatocyte growth factor, which promotes OSCC invasion in vitro. Clinicopathologic studies demonstrated that myofibroblasts in the stroma of OSCC significantly correlate with lymph node metastasis, vascular, lymphatic and perineural invasion of the tumor cells, and patient's shorter survival, suggesting that myofibroblasts may assist tumor invasion and metastasis.[8–11] Moreover, it was demonstrated that mutual interactions between OSCC cells and myofibroblasts may exist, and that conditioned media from TGF-β1-induced myofibroblasts enhances cell growth of OSCC cells.[12] In this study we examine the role of myofibroblasts in tumor proliferation and invasion and identify the molecular mechanism by which myofibroblasts influence cellular proliferation. By secretion of activin A, myofibroblasts set in motion a pathway for proliferation of OSCC cells. We further show that myofibroblast-released factors, others than activin A, induce invasion and secretion of matrix metalloproteinases (MMP) by OSCC cells, and that presence of myofibroblasts in OSCCs correlates with increased tumor production of MMP-2 and MMP-9.

Material and methods

Cell cultures

Tumor-associated fibroblast and myofibroblast cell lines were established from fragments of tongue SCCs using tissue explants as described previously.[13] Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10% donor calf serum (DCS) and antibiotics at 37 °C in a 5% CO_2 air atmosphere. Individual clones were selected by cloning ring strategy and screened for α-SMA expression by western blot and flow cytometric analysis,[14] for differentiation markers

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by immunocytochemistry,[15] and for type I collagen production by ELISA.[16] All experiments were performed using cells between the 8th and 15th passages.

SCC9 and SCC25 oral cancer cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as recommended.

Preparation of the media conditioned by fibroblast and myofibroblast clones

Subconfluent monolayers (2500 cells/cm^2) of three fibroblast clones and two myofibroblast clones were washed twice with PBS and once with serum-free DMEM, and subsequently cultured in serum-free DMEM for 24 h. The conditioned media was clarified by centrifugation, aliquoted and stored at −80 °C until used to determine the effect on tumor cell proliferation or MMP production.

Proliferation assay

The effect of fibroblast and myofibroblast clones on OSCC cell proliferation was determined by bromodeoxyuridine (BrdU) incorporation index,[12] 24 h after release from a serum-starved synchrony. BrdU-labeling index, expressed as the percentage of cells labeled with BrdU, was determined by counting 500 cells in two independent reactions using the Kontron 400 image analysis system (Zeiss).

Invasion assay

The in vitro cell invasion assay was performed by using a modified Boyden chamber insert with polycarbonate filter membrane containing 8-μm pores in 24-well plates (Corning Inc., Corning, NY, USA). Matrigel (BD Biosciences, San Jose, CA, USA) was diluted to 1:1 with serum-free media and used to coat the filter membranes. In the lower chamber, monolayers of fibroblast and myofibroblast clones were plated in triplicate and cultured in serum-free DMEM. Tumor cells (1×10^5) suspended in 200 μl of serum-free DMEM/F12 were seeded onto the upper compartment of the transwell chamber. After incubation for 72 h, the media in the upper chamber was removed and the filters were fixed with 10% formalin for 15 min. The cells remaining on the upper surface of the filter membrane were then completely removed by wiping with a cotton swab, and the cells on the opposite surface of the filter membrane were stained with 1% toluidine blue in 1% borax for 5 min and then lysed with 1% SDS. Absorbance was read at 650 nm to determine relative cell number.

Elisa

For MMP quantification, OSCC cells were cultured with fibroblast or myofibroblast-conditioned media for 24 h. The media were collected, whereas the cells were fixed and used for cell count by toluidine blue stain method. In essence, microtite plate wells were coated with 100 μl of the culture medias for 2 h at room temperature. The wells were then washed and non-specific binding sites were blocked with 3% BSA in PBS for 2 h. After washing, anti-MMP-1 (diluted 1:10,000), anti-MMP-2 (diluted 1:3500), anti-MMP-9 (diluted 1:2000), and anti-MMP-13 (1:2000) antibodies in PBS were added to the wells and incubated for 2 h. All antibodies were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). After another washing step, goat anti-mouse peroxidase-conjugated IgG (Vector Labs, Burlingame, CA, USA) diluted 1:1000 in PBS was added and maintained for 1 h. The reaction was developed with 0.5 mg/ml of *o*-phenylenediamine (Sigma) in 0.5 M citric buffer pH 5.5 containing 0.01% H_2O_2 for 20 min. After terminating the reaction with 50 μl of 2 N H_2SO_4, absorbance was read at 450 nm. The values were expressed as MMPs/cell.

Real-time PCR array and quantitative real-time PCR

RNA isolation and cDNA synthesis were performed as previously described.[16] cDNAs were used to quantify human growth factor expression in a 96-well plate array (RT^2 Profiler™ PCR Array System, SABiosciences, Qiagen, Frederick, MD, USA), according to the manufacturer's instructions. Up- and down-regulated genes were defined as those whose expression level in myofibroblast clones was greater or lower than 2-fold that observed in fibroblast clones, in an average of three independent experiments with two individual clones for each group.

Quantitative reverse transcription-PCR (qRT-PCR) was performed with the StepOne Plus instrument (Applied Biosystems), and amplicons were detected by using SYBR green fluorescence method. Target genes were analyzed by using standard curves to determine relative levels of gene expression, and individual RNA samples were normalized according to the levels of GAPDH. All primers used in this study are described in Supplementary Table 1.

Small interference RNA (siRNA)

siRNA molecules to target INHBA mRNA and nontargeting negative control were purchased from Invitrogen and transfected as previously described.[17] After transfection, the efficacy of the INHBA knock down was determined by qRT-PCR and ELISA.

Tumorigenicity assay

To assess the growth of xenographic tumors in nude mice, eight 12 week-old nude mice per cell line were injected s.c. in the flank with SCC9 cells mixture with either fibroblast clones or myofibroblast clones. Tumor size was measured weekly with a calipers, and volumes are reported as mm^3, calculated by using the formula volume = 0.5 × length × width^2. At 12 week postinjection, all mice were euthanized, and tumors and multiple organs were examined grossly, collected, fixed in 10% formalin and then embedded in paraffin. Five-micrometer sections were stained with H&E for histopathologic analysis.

Immunohistochemistry and zymography

Thirty-three consecutive OSCC samples removed during tumor resection were divided into two parts: one was fixed in formalin and embedded in paraffin for H&E staining or immunohistochemistry against α-SMA,[14] while the other was used as tissue culture to zymographic analysis.[18]

Statistical analysis

Assays were performed at least three times. The Kruskal–Wallis multiple comparison test was used to test group effects at 5% significance ($p < 0.05$).

Results

Characterization of the cancer-associated myofibroblast clones

From three tongue SCCs we established several spindle-shaped cells with indented nuclei that were plated in low density, isolated with aid of cloning rings and characterized as myofibroblasts by the expression of α-SMA and elevated type I collagen synthesis. Western blot analysis revealed an excessive production of α-SMA

by myofibroblast clones, whereas fibroblasts lacked its production (Fig. 1A). Flow cytometric analysis showed that more than 90% of the cells express α-SMA in the myofibroblast clones (Fig. 1B). As a marker of myofibroblast activity, type I collagen production was evaluated. Myofibroblast clones produced significantly higher amounts of type I collagen than fibroblasts (Fig. 1C). Both fibroblast and myofibroblast clones were positive for vimentin and negative for pan-cytokeratin and CD34 (Supplementary Fig. 1). In long time culture, western blot, flow cytometric analysis and ELISA revealed constant α-SMA expression and elevated production of type I collagen. Thus, those clones were used to further characterization of myofibroblast-effects on tumor proliferation and invasion.

Myofibroblasts promote proliferation and enhance tumor volume in nude mice

The comparative effect of myofibroblast-conditioned media and fibroblast-conditioned media on OSCC proliferation was assessed by measuring BrdU incorporation into DNA. Fig. 2A shows that myofibroblast-conditioned media significantly stimulated BrdU-labeling index of both SCC9 and SCC25 oral cancer cell lines compared to fibroblast-conditioned media ($p < 0.001$ for SCC9 and $p < 0.05$ for SCC25). Interestingly, BrdU-labeling index of both oral cancer cell lines induced by myofibroblast-conditioned media was similar to those of the positive control (fresh media containing 10% of DCS). When myofibroblast cell lines were injected with SCC9 cells into nude mice, tumors formed were significantly larger than those formed by the fibroblast clones and SCC9 (Fig. 2B). Tumors formed in the presence of the myofibroblast clone 1 were significantly larger than controls from week 2 to 12 ($p < 0.001$), whereas differences for myofibroblast clone 2 was observed later, from week 6 to 12 ($p < 0.01$). No metastases were observed. As an unexpected result, SCC25 cells were not capable to induce tumors either when injected alone or when injected in association with fibroblasts or myofibroblasts.

Myofibroblasts induce invasion and synthesis of MMPs, and MMP activities are correlated with myofibroblasts in vivo

We compared the effect of factors released from fibroblasts and myofibroblasts on invasion of SCC9 and SCC25 cells. We found that myofibroblasts significantly promoted invasion of SCC9 cells compared with fibroblasts ($p < 0.0001$, Fig. 3A). Since myofibroblast clones stimulated invasion of SCC9 cells, we further investigated whether this event is accompanied by increased MMP production. Myofibroblast clones significantly induced production of MMP-1, MMP-2, MMP-9, and MMP-13 by SCC9 cells compared with fibroblast clones (Fig. 3B). Although myofibroblast clones induced MMP production by SCC25 cells, those cells were not capable to invade towards any of the stimuli (data not shown).

To investigate whether myofibroblasts are associated with highly invasive oral cancers, we used 33 samples of OSCC to examine myofibroblast presence concurrent with MMP activities. As previously reported,[8] myofibroblasts was detected by immunohistochemistry and scored as undetectable (0), scanty (1), or abundant (2), whereas MMP activities were characterized by gelatin zymography. Four major gelatinolytic bands were produced by the tumor samples, a doublet with approximately 66 and 72 kDa, which correspond to the active and latent forms of MMP-2, and two more diffuse bands with approximately 85 and 92 kDa corresponding to active and latent forms of MMP-9, respectively. These activities were attributed to MMPs since they were completely

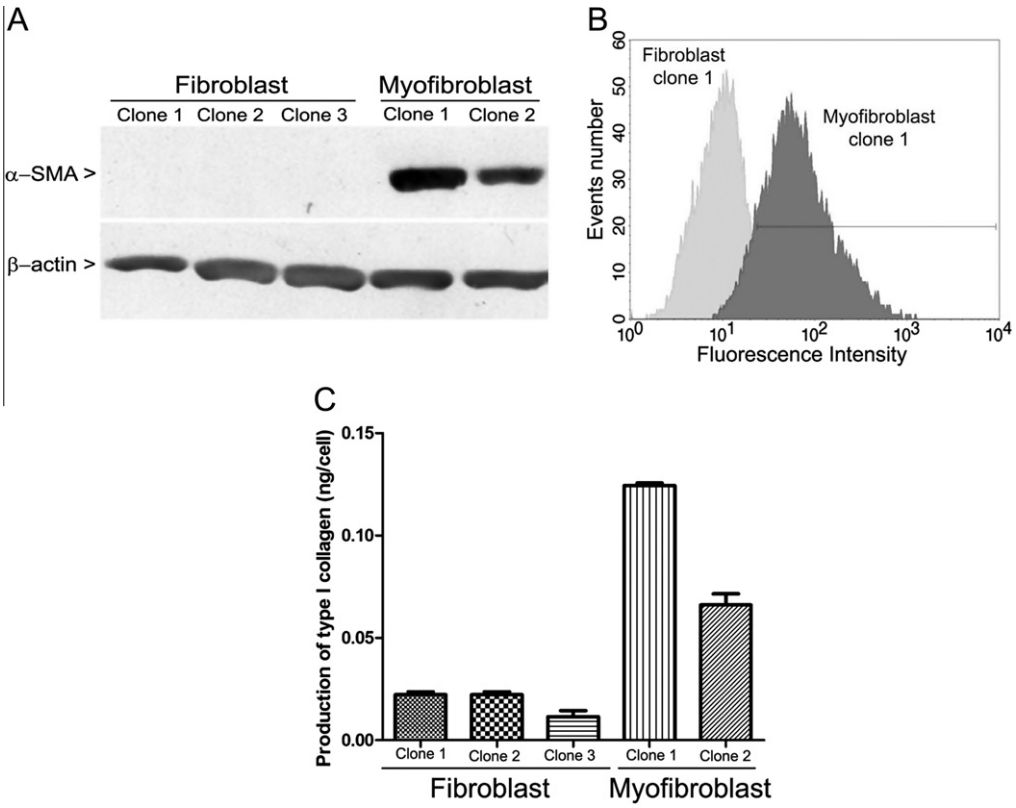

Figure 1 Characterization of the myofibroblast clones. Individual clones of OSCC-associated mesenchymal fusiform cells were cloned, screened for α-SMA and type I collagen production, and characterized as fibroblast or myofibroblast cell lines. (A) Western blot analysis showed a lack of α-SMA expression in the fibroblast cell lines, and a high expression in the myofibroblasts. (B) Representative flow cytometric analysis showed that more than 90% of the cells express α-SMA in the myofibroblast clones. (C) ELISA for type I collagen showed that myofibroblast clones produced significantly higher amounts of type I collagen than fibroblasts ($p < 0.01$).

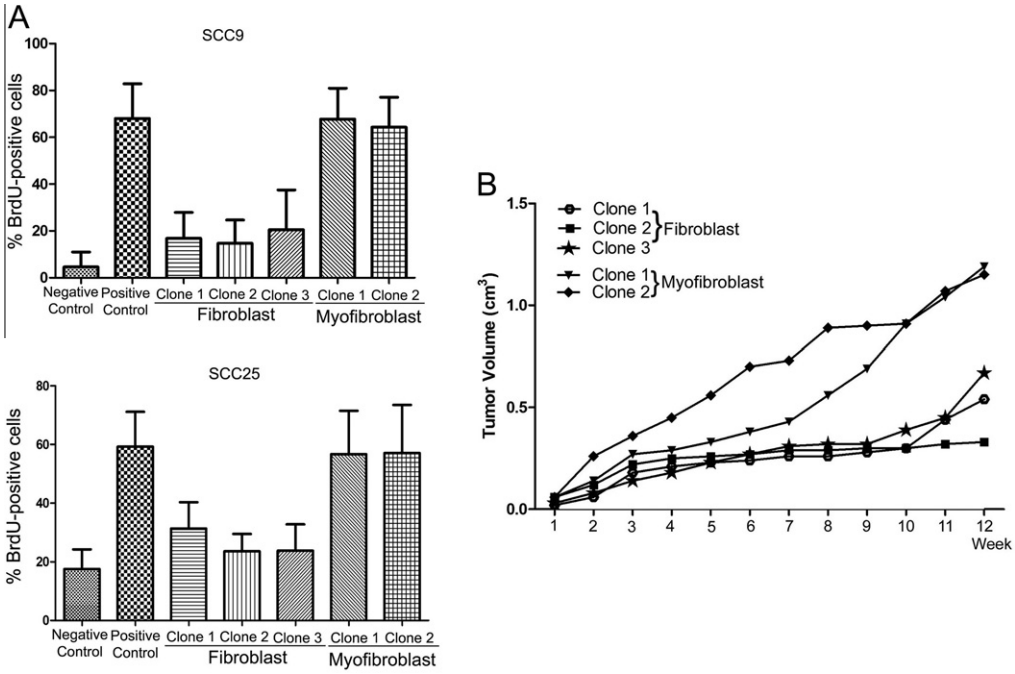

Figure 2 Myofibroblast stimulates OSCC proliferation. (A) Myofibroblast-conditioned media significantly stimulated BrdU-labeling index of both SCC9 and SCC25 oral cancer cell lines compared to fibroblast-conditioned media ($p < 0.001$ for SCC9 and $p < 0.05$ for SCC25). Negative control represented cells cultivate without FBS, whereas cells cultured in the presence of 10% FBS were used as positive control. (B) Myofibroblasts significantly increases tumor burden in nude mice. SCC9 cells with either fibroblast or myofibroblast clones were injected into the flank of 12-week-old nude mice, and tumor size was measured over a 12-week time period. Data are shown as mean ± SD. In the presence of the myofibroblast clone 1, tumors were significantly larger than controls from week 2 to 12 ($p < 0.001$), whereas differences for myofibroblast clone 2 was observed later, from week 6 to 12 ($p < 0.01$).

inhibited by 1,10-phenanthroline (Fig. 3C). We found that tumors with abundant presence of myofibroblasts showed significantly higher activities of MMP-2 and MMP-9 compared with tumors classified as negative or scanty for myofibroblasts (Fig. 3D). These data are consistent with the notion that abundant presence of myofibroblasts results in highly invasive oral tumors.

Myofibroblasts are dependent on activin A secretion to stimulate proliferation but not invasion of OSCC cells

To gain insight into the molecular mechanism by which myofibroblasts affect proliferation and invasion, we examined the expression profile of growth factors by using a quantitative real-time PCR array. When the criteria outlined in materials and methods were used, five significantly up-regulated and four down-regulated genes were identified (Table 1). All genes were reproducibly and significantly regulated in myofibroblasts as revealed by qRT-PCR. In three independent experiments we found up-regulation of *INHBA* in myofibroblast clones compared to fibroblast clones, whereas the levels of *INHBB* and *INHA* expression were quite similar. As the product of *INHBA* (activin A) has important role in both cell cycle and invasion control,[19] we focused our attention on activin A. The amount of activin A was significantly higher in the supernatants of myofibroblast clones compared with fibroblast clones (Supplementary Fig. 2).

To determine whether myofibroblast-released activin A promotes proliferation and invasion, activin A was knocked down by using siRNA. When activin A-specific oligonucleotides were used, a rapid and significant downregulation of activin A mRNA was observed and maintained for 3 days (Fig. 4A). The decrease in activin A levels was concomitant with a significant decrease in the proliferation of both SCC9 and SCC25 cells cultured with myofibroblast-conditioned media, demonstrating that activin A released by myofibroblasts regulates OSCC cell proliferation (Fig. 4B). On the other hand, downregulation of activin A on myofibroblast conditioned media did not alter SCC9 invasion (Fig. 4C). Since SCC9 invasion induced by myofibroblast clones was associated with increased MMP production, we performed ELISA for all MMPs in the conditioned media. MMP levels remained constant even in activin A downregulated cells (Fig. 4D). Together, these data suggest that myofibroblast-induced OSCC invasion and MMP synthesis are independent of activin A.

Discussion

Emergence of myofibroblasts is commonly observed in human cancers. Although through secretion of an extensive repertoire of molecules these cells are known to affect numerous processes important in both would healing and fibrosis,[20] a causal role for myofibroblasts in tumor progression remains controversial. The evidence presented in this article that myofibroblasts secrete several growth factors which induce proliferation, invasion and production of MMPs in OSCC cells in culture, and that their presence correlates with indices of MMP-2 and MMP-9 activity in OSCCs, strongly support a role for these cells on tumor progression and invasion. Furthermore, our current data, coupled with previously published work,[12] show that myofibroblasts promote proliferative activity of OSCC by upregulating activin A, a member of the transforming growth factor-β superfamily of proteins.[21] Activins are homo or heterodimeric proteins consisting of two β subunits (βA and βB), and INHBA is one of the β subunits (βA) that comprise activin A (βAβA), activin AB (βAβB) and inhibin A (αβA).[22] Activin A regulates normal embryogenesis,[21] and its dysregulation is observed in endometrial carcinoma,[23] testicular cancer,[24] esophageal squamous cell carcinoma,[25] and lung adenocarcinoma.[26] In the later, activin A overexpression promotes tumor proliferation and is associated with worse survival. Thus, elevated expression of *INHBA*, β subunits of activin A, by stromal myofibroblasts induces

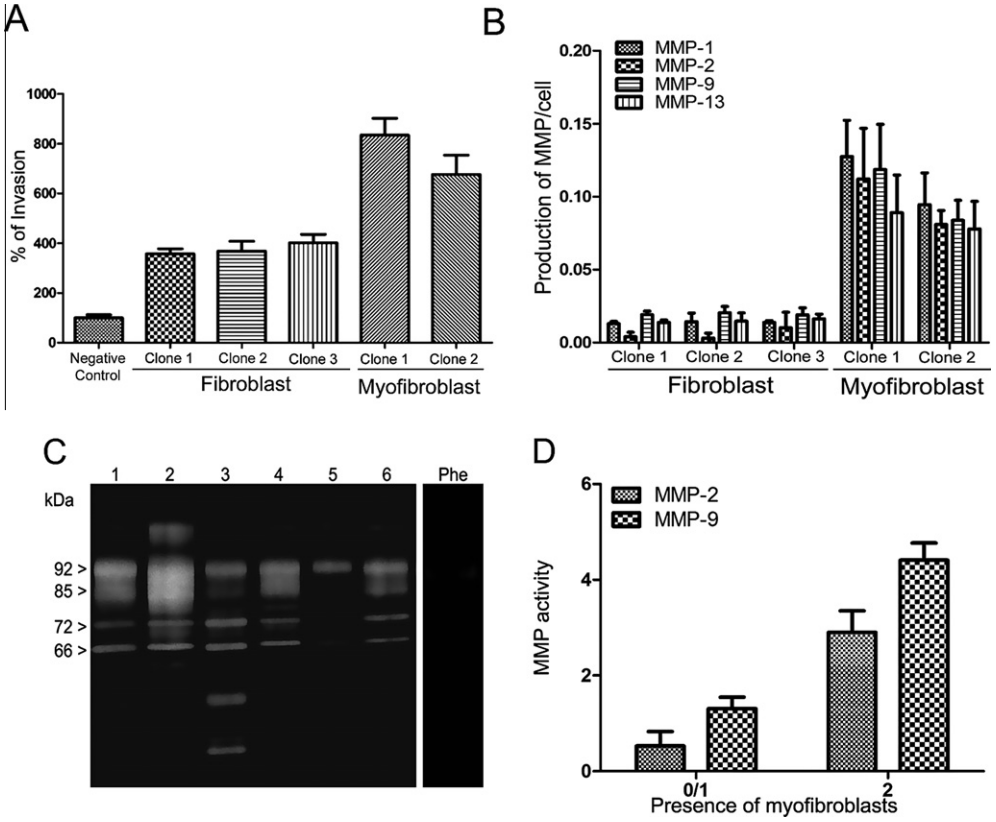

Figure 3 Myofibroblast promotes invasion and MMP synthesis of OSCC cells. (A) SCC9 cells were subjected to transwell chamber invasion assay with factors released by fibroblast or myofibroblast clones as chemotactics. Myofibroblasts significantly promoted invasion of SCC9 cells compared with fibroblasts ($p < 0.0001$). Cells cultured in the absence of chemotactic factors in the lower chamber were used as negative control. (B) Myofibroblast-induced SCC9 invasion was associated with increased production of MMP-1, MMP-2, MMP-9 and MMP-13, as revealed by ELISA ($p < 0.001$). (C) Zymographic analysis of MMPs secreted by six representative OSCC samples. Gelatinolytic activities were detected at ~66–72 kDa and ~85–92 kDa, consistent with MMP-2 and MMP-9, respectively. Lanes 1, 2, and 6 depict conditioned cell culture media from OSCCs of the tongue, lane 3 of the retromolar region, and lanes 4 and 5 of the floor of mouth. Last lane represents sample 6 incubated with 1 mM 1,10-phenanthroline, a specific MMP inhibitor. (D) Presence of myofibroblasts [from undetectable (0), scanty (1), and abundant (2)] was significantly associated with an increasing in the activity of the MMP-2 and MMP-9 of the OSCCs ($p < 0.05$ for MMP-2 and $p < 0.008$ for MMP-9).

Table 1
Up- and down-regulated genes in myofibroblasts. Values represent the fold of expression in myofibroblast clones in relation to fibroblast clones. After selection using the method outlined in materials and methods, genes were validated by qRT-PCR in three independent experiments.

Genes	Real-time PCR array	qRT-PCR	*p* Value
Up-regulated			
BMP4	7.63	4.45	0.01
FIGF	6.24	3.54	0.013
INHBA	2.09	3.06	0.003
MDK	3.44	1.80	0.007
NTF3	16.53	2.75	0.02
Down-regulated			
BMP6	−10.70	−3.95	0.001
FGF13	−7.88	−6.45	0.002
GDF10	−3.38	−9.50	0.03
IL1B	−3.65	−5.85	0.0003

tumor cell growth. Previous studies suggested that activin A have a prognostic value and may represent an important target for cancer therapy.[26–30] Blockage of activin A expression or its biological activity with specific agents, such as follistatin, monoclonal antibodies or small interference RNA molecules, may have a therapeutic potential, however, since activin A is expressed in a wide variety of human tissues, side effects are expected. To date is proposed that circulating levels of activin A may distinguish patients with breast or prostate tumors showing bone metastases than patients without metastatic diseases.[27] Moreover, inhibition of activin A activity with soluble activin receptor type IIA fusion protein suppressed osteolytic bone disease and tumor burden and increased survival in in vivo models of multiple myeloma and breast cancer-induced bone disease.[29] Thus, the present data support further investigations of the role of activin A in OSCC as well as its clinical and therapeutic implications.

Myofibroblasts are known to induce migration and invasion in a number of contexts both in normal development[31] and tumorigenesis.[3] Such a role for myofibroblasts in tumor progression is supported by observations that they are critical for invasion and metastasis of colon and prostate cancer and basal cell carcinoma,[32–34] and are associated with increased lymph node metastasis as well as poor survival in OSCC,[8,9] supporting a role for these cells in invasion and metastasis. Indeed, our in vitro system demonstrated that myofibroblasts induce secretion of MMPs with subsequent increment in OSCC cell invasion. Although we did not observe organ metastases in the xenographic model, the study was not designed to examine metastasis, and thus, it remains a possibility that myofibroblasts not only promote proliferation of cancer cells but facility tumor invasion and metastasis. This is in line with previous reports that demonstrated myofibroblasts as the main source of MMPs in some types of cancers.[11,35–37] In ameloblastomas, presence of myofibroblasts as a releasing font of MMP-2 was associated with rupture of the osseous cortical, which has been considered an important prognostic marker of ameloblastoma aggressiveness.[38] Thus, myofibroblasts not only promotes

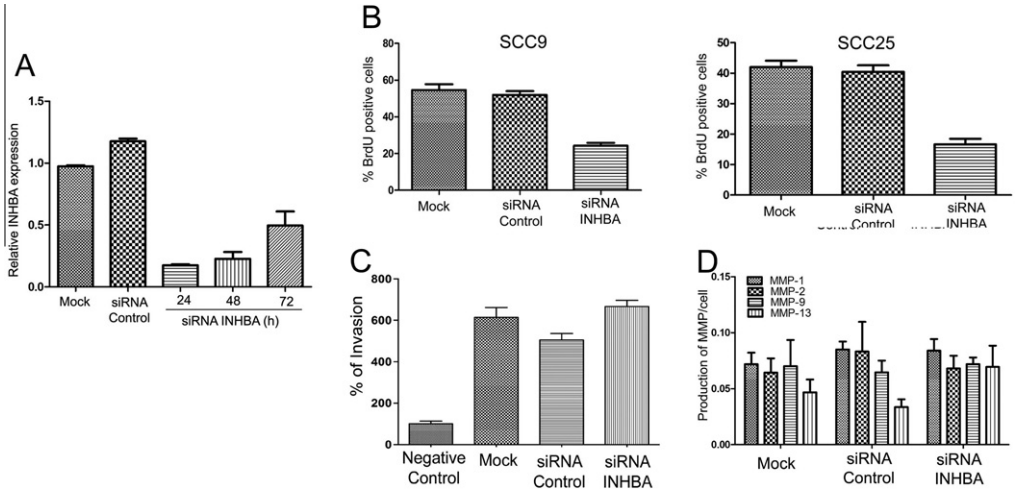

Figure 4 Inhibition of activin A production by myofibroblasts decreases effect on OSCC cell proliferation. (A) Effect of siRNA against *INHBA* in myofibroblast clone 2 over a 3-day time course. Cells were exposed to transfectant agent only (mock), siRNA control or siRNA INHBA. qRT-PCR analysis demonstrate that *INHBA* levels are significantly decreased after introduction of the specific siRNA oligonucleotides. (B) BrdU incorporation assay demonstrated a statistically significant decrease in both SCC9 and SCC25 proliferation when *INHBA* was downregulated by siRNA in myofibroblast clone 2 ($p < 0.0001$ for SCC9 and $p < 0.0001$ for SCC25). Downregulation of activin A on myofibroblast clone 2 conditioned media did not alter invasion (C) and MMP production (D) of SCC9 cells.

OSCC cell proliferation, but also is likely to contribute to subsequent stages of tumor progression.

In summary, myofibroblasts are powerful regulators affecting many cellular processes involved in tumorigenesis including proliferation, migration, invasion, and neovascularization.[39] Here, we present strong evidences that myofibroblasts in OSCC induce proliferation via secretion of activin A, and promote invasion throughout secretion of MMPs. Together with recent clinicopathological studies,[8–12] our data suggest that myofibroblast promotes tumorigenesis in OSCC, supporting its verification and monitoring as a marker of OSCC behavior.

Conflict of interest statement

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.oraloncology.2011.06.011.

References

1. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res* 1999;**59**(19):5002–11.
2. Zhang C, Fu L, Fu J, Hu L, Yang H, Rong TH, et al. Fibroblast growth factor receptor 2-positive fibroblasts provide a suitable microenvironment for tumor development and progression in esophageal carcinoma. *Clin Cancer Res* 2009;**15**(12):4017–27.
3. Franco OE, Shaw AK, Strand DW, Hayward SW. Cancer associated fibroblasts in cancer pathogenesis. *Semin Cell Dev Biol* 2010;**21**(1):33–9.
4. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 2002;349–63. 2002/05/04 ed.
5. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer* 2006;**6**(5):392–401.
6. Barth PJ, Schenck zu Schweinsberg T, Ramaswamy A, Moll R. CD34+ fibrocytes, alpha-smooth muscle antigen-positive myofibroblasts, and CD117 expression in the stroma of invasive squamous cell carcinomas of the oral cavity, pharynx, and larynx. *Virchows Arch* 2004;**444**(3):231–4.
7. Lewis MP, Lygoe KA, Nystrom ML, Anderson WP, Speight PM, Marshall JF, et al. Tumour-derived TGF-beta1 modulates myofibroblast differentiation and promotes HGF/SF-dependent invasion of squamous carcinoma cells. *Br J Cancer* 2004;**90**(4):822–32.
8. Kellermann MG, Sobral LM, da Silva SD, Zecchin KG, Graner E, Lopes MA, et al. Myofibroblasts in the stroma of oral squamous cell carcinoma are associated with poor prognosis. *Histopathology* 2007;**51**(6):849–53.
9. Kawashiri S, Tanaka A, Noguchi N, Hase T, Nakaya H, Ohara T, et al. Significance of stromal desmoplasia and myofibroblast appearance at the invasive front in squamous cell carcinoma of the oral cavity. *Head Neck* 2009;**31**(10):1346–53.
10. Marsh D, Suchak K, Moutasim KA, Vallath S, Hopper C, Jerjes W, et al. Stromal features are predictive of disease mortality in oral cancer patients. *J Pathol* 2011;**223**(4):470–81.
11. Bello IO, Vered M, Dayan D, Dobriyan A, Yahalom R, Alanen K, et al. Cancer-associated fibroblasts, a parameter of the tumor microenvironment, overcomes carcinoma-associated parameters in the prognosis of patients with mobile tongue cancer. *Oral Oncol* 2011;**47**(1):33–8.
12. Kellermann MG, Sobral LM, da Silva SD, Zecchin KG, Graner E, Lopes MA, et al. Mutual paracrine effects of oral squamous cell carcinoma cells and normal oral fibroblasts: induction of fibroblast to myofibroblast transdifferentiation and modulation of tumor cell proliferation. *Oral Oncol* 2008;**44**(5):509–17.
13. Coletta RD, Almeida OP, Graner E, Page RC, Bozzo L. Differential proliferation of fibroblasts cultured from hereditary gingival fibromatosis and normal gingiva. *J Periodontal Res* 1998;**33**(8):469–75.
14. Bitu CC, Sobral LM, Kellermann MG, Martelli-Junior H, Zecchin KG, Graner E, et al. Heterogeneous presence of myofibroblasts in hereditary gingival fibromatosis. *J Clin Periodontol* 2006;**33**(6):393–400.
15. Sobral LM, Zecchin KG, Nascimento de Aquino S, Lopes MA, Graner E, Coletta RD. Isolation and characterization of myofibroblast cell lines from oral squamous cell carcinoma. *Oncol Rep* 2011;**25**(4):1013–20.
16. Sobral LM, Montan PF, Martelli-Junior H, Graner E, Coletta RD. Opposite effects of TGF-beta1 and IFN-gamma on transdifferentiation of myofibroblast in human gingival cell cultures. *J Clin Periodontol* 2007;**34**(5):397–406.
17. De Souza Setubal Destro MF, Bitu CC, Zecchin KG, Graner E, Lopes MA, Kowalski LP, et al. Overexpression of HOXB7 homeobox gene in oral cancer induces cellular proliferation and is associated with poor prognosis. *Int J Oncol* 2010;**36**(1):141–9.
18. Coletta RD, Almeida OP, Reynolds MA, Sauk JJ. Alteration in expression of MMP-1 and MMP-2 but not TIMP-1 and TIMP-2 in hereditary gingival fibromatosis is mediated by TGF-beta 1 autocrine stimulation. *J Periodontal Res* 1999;**34**(8):457–63.
19. Chen YG, Wang Q, Lin SL, Chang CD, Chuang J, Ying SY. Activin signaling and its role in regulation of cell proliferation, apoptosis, and carcinogenesis. *Exp Biol Med (Maywood)* 2006;**231**(5):534–44.
20. Desmouliere A, Chaponnier C, Gabbiani G. Tissue repair, contraction, and the myofibroblast. *Wound Repair Regen* 2005;**13**(1):7–12.
21. Welt C, Sidis Y, Keutmann H, Schneyer A. Activins, inhibins, and follistatins: from endocrinology to signaling. A paradigm for the new millennium. *Exp Biol Med (Maywood)* 2002;**227**(9):724–52.

22. Vale W, Wiater E, Gray P, Harrison C, Bilezikjian L, Choe S. Activins and inhibins and their signaling. *Ann NY Acad Sci* 2004;**1038**:142–7.
23. Tanaka T, Toujima S, Umesaki N. Activin A inhibits growth-inhibitory signals by TGF-beta1 in differentiated human endometrial adenocarcinoma cells. *Oncol Rep* 2004;**11**(4):875–9.
24. Devouassoux-Shisheboran M, Mauduit C, Tabone E, Droz JP, Benahmed M. Growth regulatory factors and signalling proteins in testicular germ cell tumours. *APMIS* 2003;**111**(1):212–24. discussion 24.
25. Yoshinaga K, Yamashita K, Mimori K, Tanaka F, Inoue H, Mori M. Activin a causes cancer cell aggressiveness in esophageal squamous cell carcinoma cells. *Ann Surg Oncol* 2008;**15**(1):96–103.
26. Seder CW, Hartojo W, Lin L, Silvers AL, Wang Z, Thomas DG, et al. Upregulated INHBA expression may promote cell proliferation and is associated with poor survival in lung adenocarcinoma. *Neoplasia* 2009;**11**(4):388–96.
27. Leto G, Incorvaia L, Badalamenti G, Tumminello FM, Gebbia N, Flandina C, et al. Activin A circulating levels in patients with bone metastasis from breast or prostate cancer. *Clin Exp Metastasis* 2006;**23**(2):117–22.
28. Incorvaia L, Badalamenti G, Rini G, Arcara C, Fricano S, Sferrazza C, et al. MMP-2, MMP-9 and activin A blood levels in patients with breast cancer or prostate cancer metastatic to the bone. *Anticancer Res* 2007;**27**(3B):1519–25.
29. Chantry AD, Heath D, Mulivor AW, Pearsall S, Baud'huin M, Coulton L, et al. Inhibiting activin-A signaling stimulates bone formation and prevents cancer-induced bone destruction in vivo. *J Bone Miner Res* 2010;**25**(12):2633–46.
30. Chang KP, Kao HK, Liang Y, Cheng MH, Chang YL, Liu SC, et al. Overexpression of activin A in oral squamous cell carcinoma: association with poor prognosis and tumor progression. *Ann Surg Oncol* 2010;**17**(7):1945–56.
31. Serini G, Gabbiani G. Mechanisms of myofibroblast activity and phenotypic modulation. *Exp Cell Res* 1999;**250**(2):273–83.
32. De Wever O, Demetter P, Mareel M, Bracke M. Stromal myofibroblasts are drivers of invasive cancer growth. *Int J Cancer* 2008;**123**(10):2229–38.
33. Hill R, Song Y, Cardiff RD, Van Dyke T. Selective evolution of stromal mesenchyme with p53 loss in response to epithelial tumorigenesis. *Cell* 2005;**123**(6):1001–11.
34. Marsh D, Dickinson S, Neill GW, Marshall JF, Hart IR, Thomas GJ. Alpha vbeta 6 Integrin promotes the invasion of morphoeic basal cell carcinoma through stromal modulation. *Cancer Res* 2008;**68**(9):3295–303.
35. Okada A, Bellocq JP, Rouyer N, Chenard MP, Rio MC, Chambon P, et al. Membrane-type matrix metalloproteinase (MT-MMP) gene is expressed in stromal cells of human colon, breast, and head and neck carcinomas. *Proc Natl Acad Sci USA* 1995;**92**(7):2730–4.
36. Seomun Y, Kim J, Lee EH, Joo CK. Overexpression of matrix metalloproteinase-2 mediates phenotypic transformation of lens epithelial cells. *Biochem J* 2001;**358**(Pt. 1):41–8.
37. Bisson C, Blacher S, Polette M, Blanc JF, Kebers F, Desreux J, et al. Restricted expression of membrane type 1-matrix metalloproteinase by myofibroblasts adjacent to human breast cancer cells. *Int J Cancer* 2003;**105**(1):7–13.
38. Fregnani ER, Sobral LM, Alves FA, Soares FA, Kowalski LP, Coletta RD. Presence of myofibroblasts and expression of matrix metalloproteinase-2 (MMP-2) in ameloblastomas correlate with rupture of the osseous cortical. *Pathol Oncol Res* 2009;**15**(2):231–40.
39. Kunz-Schughart LA, Knuechel R. Tumor-associated fibroblasts (part II): functional impact on tumor tissue. *Histol Histopathol* 2002;**17**(2):623–37.