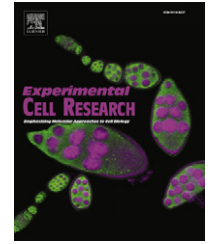


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## Research Article

# Tumor cell and carcinoma-associated fibroblast interaction regulates matrix metalloproteinases and their inhibitors in oral squamous cell carcinoma

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## ABSTRACT

Co-culture of periodontal ligament (PDL) fibroblasts and SCC-25 oral squamous carcinoma cells (OSCC), results in conversion of PDLs into carcinoma-associated fibroblasts (CAFs). Paracrine circuits between CAFs and OSCC cells were hypothesized to regulate the gene expression of matrix remodeling enzymes in their co-culture, which was performed for 7 days, followed by analysis of the mRNA/protein expression and activity of metalloproteinases (MMPs), their tissue inhibitors (TIMPs) and other relevant genes. Interleukin1- $\beta$ , transforming growth factor- $\beta$ 1, fibronectin and  $\alpha$ v $\beta$ 6 integrin have shown to be involved in the regulation of the MMP and TIMP gene expression in co-culture of CAFs and tumor cells. In addition, these cells also cooperated in activation of MMP pro-enzymes. It is particularly interesting that the fibroblast-produced inactive MMP-2 has been activated by the tumor-cell-produced membrane-type 1 matrix metalloproteinase (MT1-MMP). The crosstalk between cancer- and the surrounding fibroblast stromal-cells is essential for the fine tuning of cancer cells invasivity.

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## Introduction

One of the most predictive factors of poor clinical outcome of head and neck squamous cell carcinoma (HNSCC) is the presence of regional lymph node metastasis, and nodal status of the neck plays a decisive role in the choice of treatment [1]. Hensen et al. recently reported an independent gene expression analysis of metastasized

versus non-metastasized HNSCC. This analysis revealed differentially expressed gene sets involved in the progression of HNSCC, including extracellular matrix (ECM) remodeling- (i.e. matrix metalloproteinases, (MMPs)), hypoxia- and angiogenesis-related genes [2]. Interestingly, a similar gene profiling assay performed over 10 years ago by Villaret et al. also showed overexpressed matrix metalloproteinases in head and neck squamous cell carcinoma.

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Abbreviations: CAFs, carcinoma-associated fibroblasts; COX-2, prostaglandin-endoperoxide synthase 2; DEX, dexamethasone; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; FBS, foetal bovine serum; FN, fibronectin; HNSCC, head and neck squamous cell carcinoma; IL, interleukin; IMVD, intratumoral microvessel density; LTBP-1, latent-transforming growth factor beta-binding protein; 1MMP, matrix metalloproteinase; MT1-MMP, membrane-type 1 matrix metalloproteinase; OSCC, oral squamous cell carcinoma; PDL, periodontal ligament (PDL) fibroblasts; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; TIMP, tissue inhibitor of metalloproteinases.

ma tumor tissues [3]. By regulating matrix metalloproteinase (MMP) activity and controlling the breakdown of ECM components, also tissue inhibitors of metalloproteinases (TIMPs) play an important role in the process of tumor invasion and metastasis [4]. TIMPs not only inhibit the catalytic activity of MMPs, but also are able to act as growth factors and are involved in the activation or inactivation of MMPs [5]. The signaling pathways and circles that regulate MMPs and TIMPs are not fully understood.

Carcinoma-associated fibroblasts (CAFs) are able to promote the growth of carcinoma cells [6]. CAFs induce an epithelial-to-mesenchymal transition (EMT) in epithelial tumor cells, which is a major biological process in invasion of squamous cell carcinoma [7], progression and metastasis. During this process invasive tumor cells tend to lose their epithelial antigens [8], their epithelial cell polarity and morphology, and acquire mesenchymal and stemness-related features [9,10]. In our recent reports we have described a co-culture model of periodontal ligament (PDL) fibroblasts and SCC-25 oral squamous carcinoma cells (OSCC), which resulted in conversion of normal fibroblasts into CAFs. In the same model EMT occurred in SCC-25 cells [11]. Moreover, we have described that SCC-25 cells produce active, processed IL-1 $\beta$ , and PDL fibroblasts possess receptor for it, whose expression is increased in the presence of SCC-25 tumor cells. Upon interaction with SCC-25 cells active IL-1 $\beta$  signaling occurs in co-cultured fibroblasts leading to induction of several genes involved in tumor progression, including interleukin-6 (IL-6) and prostaglandin-endoperoxide synthase 2 (COX-2) [12]. As we reported before, dynamic interaction between CAFs and tumor cells of HNSCC dictates gene expression changes in the interacting cells, which covers major events of tumor progression. Here, we hypothesize that the paracrine interplay between SCC-25 carcinoma cells and CAFs provides a mechanistic background for the gene regulation of MMPs and TIMPs, which contributes to poor clinical outcome of HNSCC. Traces of several regulatory pathways in the paracrine interplay between SCC-25 carcinoma cells and CAFs were acknowledged to determine the gene expression and the activation of matrix remodeling enzymes and their inhibitors.

## Materials and methods

### Cell lines

PDL fibroblasts [11–13] were isolated from periodontal ligament (PDL) and received from Prof. Dr. Miosge (Department of Prosthodontics, Georg-August-University, Göttingen, Germany) [13], they were routinely cultured in DMEM-low glucose (PAA, Pasching, Austria) supplemented with 10% foetal bovine serum (FBS) (PAA), 2 mM L-glutamine, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. SCC-25 cells were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany, ref. ACC 617), and were routinely cultured in DMEM/F12 (PAA) supplemented with 10% FBS (PAA), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin [11,12].

### Co-culture

Co-culture between SCC-25 cells and PDL fibroblasts was described in details before [11,12]. PDL fibroblasts were plated into cell culture inserts and SCC-25 cells into the bottom of 6-well

plates [11,12], they were cultured in 1:1 mixed medium of DMEM-low glucose and DMEM/F12, supplemented with 10% FBS, and also the single cultured control cells were cultured in the same medium in order to have the same glucose concentration and glycemic conditions in control and co-cultured conditions. The medium in the co-cultures and in the controls was changed after 3 days. After letting them grow in co-culture for 7 days, the cells in the inserts and in the wells were used for RNA isolation [11] or for protein fractionation [12]. For gelatinase zymogram assay, the media in the inserts and in the six-well-plates were replaced with low-serum (0.3% FBS)-containing medium (a 1:1 mixed medium of DMEM-low glucose and DMEM/F12 containing 0.3% FBS) for the last 24 h. The conditioned medium was collected from the control and co-cultured cells for gelatinase zymogram assay and for combined immunoprecipitation–western blot [12], the potential disturbing effect of serum-metalloproteinases was minimized in this way. In a further run of experiments, co-cultured cells were treated with  $10^{-6}$  mol/L dexamethasone (Sigma, Vienna, Austria) as previously reported [12,14].

### Treatment of the cells with IL-1 $\beta$

$2 \times 10^5$ /ml PDLs or SCC-25 cells were plated in 10% FBS-supplemented-DMEM-low-glucose or in 10% FBS-supplemented-DMEM/F12 (PAA) in 10 cm culture plates (Unilab, Innsbruck, Austria). After 48 h, medium was replaced by 0.3% FBS-containing-medium. Cells were treated with IL-1 $\beta$  at 0.015–1.5 ng/ml concentration range for 4, 8 and 24 h as described before [12,15].

### RNA extraction, reverse transcription and real-time RT-PCR

Total RNA was isolated and reverse transcribed from control and co-cultured cells as described before, real-time RT-PCR was done as referenced [11,16]. Several human PCR primers were published previously:  $\beta$ -actin [17], MMP-1 [18], MMP-3 [19], MMP-7 [20], MMP-9 [21], MMP-13 [22], TIMP-1 [23], TIMP-2 [24], TIMP-3 [25], TGF- $\beta$ 1 [26], Integrin  $\alpha$ v [27]; fibronectin (FN) and latent-transforming growth factor beta-binding protein-1 (LTBP-1) primers were taken from the NCI, Primer Viewer Database, ref. nos. 11092, and 10108 respectively. Other primers are listed in Supplementary Document 1.  $\beta$ -Actin functioned well as a housekeeping gene [11], and did not show significant changes in co-cultured conditions compared to controls. The relative gene expression was calculated as previously reported [16].

### Protein expression and activity measurements

For the analysis of integrin  $\alpha$ v, non-nuclear protein fractions were subjected to western blot analysis [11] as described previously [12,28]. Identical equal protein containing 20  $\mu$ l samples were loaded for western blot, which was performed either with the JLA20  $\beta$ -actin antibody (in 1:100 dilution), purchased from Developmental Studies Hybridoma Bank (Iowa city, Iowa, USA) as a loading control [29], or with rabbit polyclonal integrin  $\alpha$ v antibody (#4705, Cell Signaling Technology, Danvers, MA, USA, in 1:1000 dilution).

Conditioned media were collected from both the fibroblast and the SCC-25 side of the co-culture and also from the control cells.

From the supernatant (conditioned media) of the control cells and from both sides of the co-culture 20  $\mu$ l medium was used from all samples for gelatinase zymogram, and 200  $\mu$ l samples were used for immunoprecipitation [12] with 2  $\mu$ l of rabbit poly- or monoclonal antibodies (anti-MMP-2 #4022; anti-TIMP-1 #8946; anti-TIMP-3 #5673, Cell Signaling Technology). The immunoprecipitated samples were taken in 50  $\mu$ l Laemmli-sample-buffer [30] and 20  $\mu$ l/well was loaded to 9 or 12% acrylamide gels, followed by western blot [11] with the above-mentioned antibodies. Comparable intracellular protein extracts were reacted with the JLA20  $\beta$ -actin antibody as loading controls. Cells after co-culture and controls were scraped into 500  $\mu$ l lysis buffer [31] (50 mM TRIS HCl (pH 7.5), 500 mM NaCl, 5 mM  $\text{CaCl}_2$ )/well or /insert. Protein concentration was determined in the cell lysates, and 10  $\mu$ g proteins were loaded to the gelatin-containing gels from the lysates. The gelatinase assay was performed as described previously [31]. Supernatants represented conditioned medium of the cells, which was pipetted off directly after completion of culture, lysates represented cells lysed after removal of conditioned medium, which contained molecules bound on the cell surface, membrane-, cytoplasmic- and cell nuclear-fractions inclusive. Both supernatants and lysates were subjected to polyacrylamide gel electrophoresis using gelatin-containing 10% polyacrylamide gels [31]. Gelatinase activity is recognized as white bands in colloidal Coomassie Brilliant Blue G-250 (Roth, Karlsruhe Germany)-stained gels. Density of the detected bands was measured by the Image J software [11,16].

### Immunocytochemistry and confocal microscopy

IBidi Dishes (Martinsried, Germany) with control and co-cultured PDLs were used for fibronectin (primary antibody 1:40 diluted (5  $\mu$ g/ml); Santa Cruz Biotech., Santa Cruz, CA, USA, Cat. No. sc59826, clone IST-9) immunocytochemistry after fixation with methanol for 20 min at  $-20^\circ\text{C}$ , as described previously [12]. Immunostained Ibi dishes were evaluated by confocal microscopy [12].

### ELISA

Conditioned media were collected from both the fibroblast and the SCC-25 side of the co-culture and also from the control cells. From the supernatant (conditioned media) of the control cells and from both sides of the co-culture 100  $\mu$ l medium was used from all samples for quantitative TGF- $\beta$ 1 detection using the RayBio human TGF- $\beta$ 1 ELISA Kit (RayBiotech, Norcross, GA, USA).

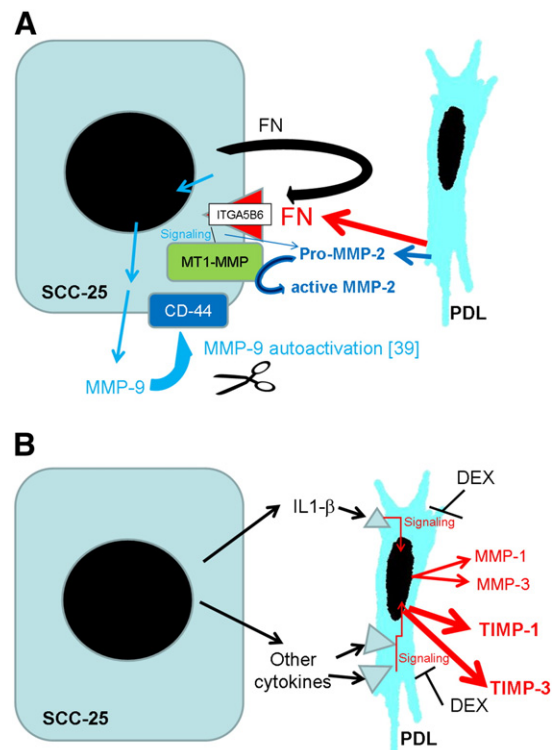
### Statistical analysis

Each experiment was performed in three independent sets containing at least three biological repeats/set. The relative gene-expression results were tested for normal distribution by D'Agostino and Pearson omnibus normality test using the Graphpad Prism 4.03 (Graphpad Software Inc). Significance of changes in co-culture vs. controls was tested by non-parametric tests (Mann-Whitney) and Students' t-tests depending on the distribution of the data. The independent experimental sets were then compared for reproducibility. Only reproducible changes with a  $p < 0.05$  level [11,12] were considered as significant.

## Results

### Model of the regulation of the paracrine interplay between SCC-25 cells and CAFs

A continuous interaction between tumor cells and fibroblasts was hypothesized in the regulation of MMP and TIMP expression and activity. The expression of MMPs and TIMPs was expected in the PDL fibroblasts, whose regulation was hypothesized to occur via inflammatory cytokines, including IL1- $\beta$  [32], produced by SCC-25



**Fig. 1 – Summary of the suggested mechanism for the regulation of MMPs and TIMPs in the paracrine interplay between SCC-25 cells and fibroblasts.** MMP-9 showed a tumor specific expression, regulated presumably by the fibronectin ITGA5B6 pathway. The ITGA5 was inducible in both SCC-25 and PDL fibroblasts in co-culture, but ITGB6 expression was tumor (SCC-25) specific. Based on a previous report [44], MMP-9 might be activated in interaction with CD-44, and according to our gelatinase assay results, it remains bound with the tumor cells (A). The results of this study suggest that MMP-2 is secreted in its pro- (inactive-) form by CAFs surrounding the tumor cells, and at a lower extent also by the tumor cells themselves. Activation of MMP-2 either requires MT1-MMP localized on the SCC-25 cancer cells [34], or integrins, where the involvement of  $\alpha$  integrins (ITGA5) is expected (A). MMPs-1, -3 and TIMPs-1, -3 are produced in the PDL fibroblasts, and their expression might be regulated by inflammatory cytokines, including IL1- $\beta$  produced by SCC-25 cells. The gene expression of MMP-1, MMP-2, TIMP-1 and TIMP-3 was reduced by dexamethasone (DEX) (B).

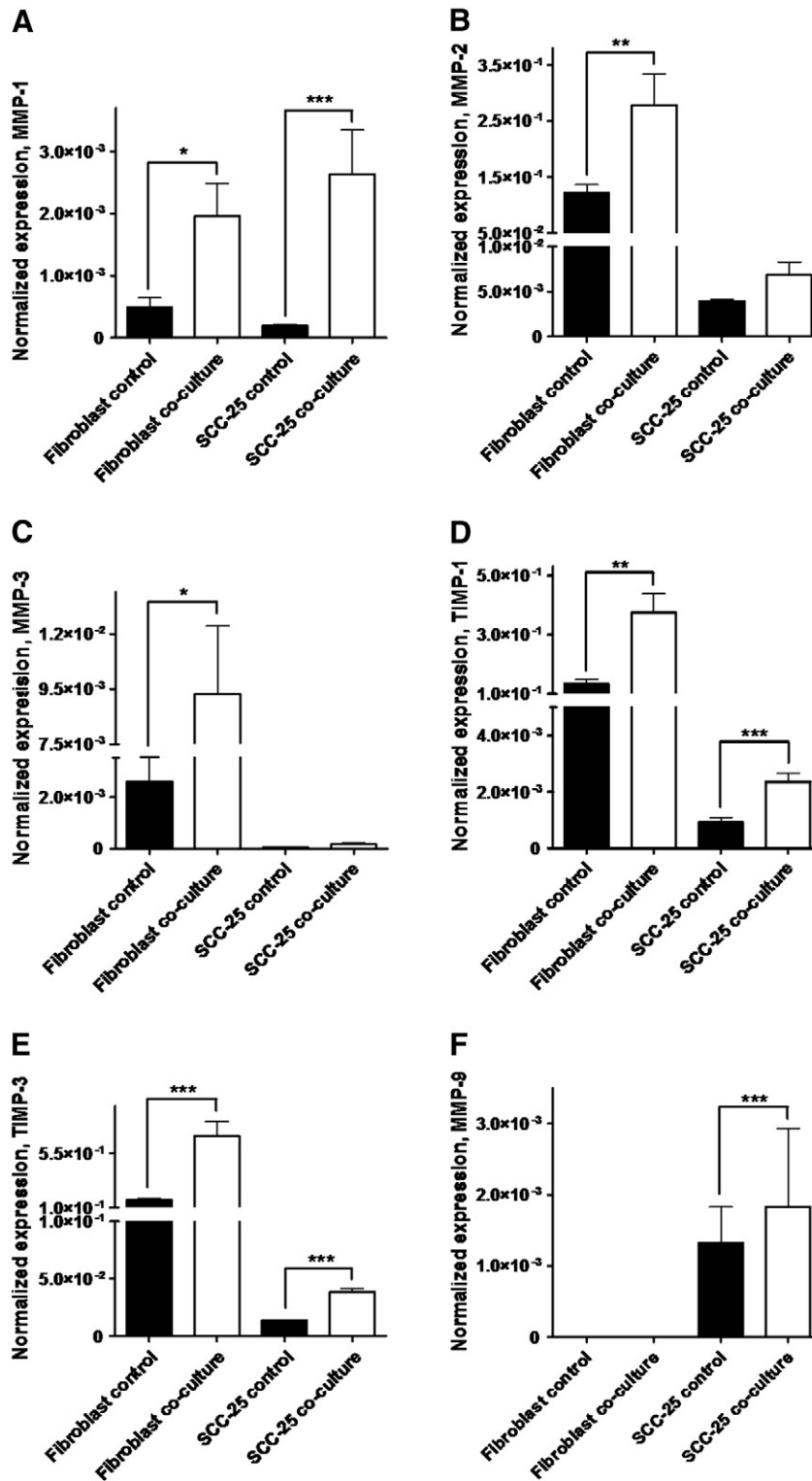


Fig. 2 – mRNA expression of MMP-1 (A), MMP-2 (B), MMP-3 (C), TIMP-1 (D), TIMP-3 (E) and MMP-9 (F) in PDL fibroblasts and SCC-25 cells in control and co-cultured conditions. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ .

cells. In the regulation of MMP-9 gene expression the fibronectin-integrin  $\alpha\beta6$  (ITGA5B6) pathway was hypothesized. Another possible pathway, especially in regulation of TIMP expression, is the TGF- $\beta$ 1-pathway [31,32]; the expression of TGF- $\beta$ 1 was expected in the fibroblasts and in the tumor cells (Fig. 1).

#### *Fibroblast contribution to the MMP and TIMP expression in co-culture of SCC-25 cells and CAFs*

Gene expression of MMPs-1, -2, -3, -7, -9 and -13 and TIMPs 1–3 was investigated in fibroblasts (PDLs) in control and co-cultured



**Table 1 – Fibroblast contribution to the MMP and TIMP expression in co-culture of SCC-25 cells and CAFs.**

Gene:	n	Change in fibroblast co-culture related to fibroblast control	Test	p
MMP-1	20	4 ± 1-times up	Mann–Whitney	p = 0.012
MMP-2	20	2.3 ± 0.4-times up	Mann–Whitney	p = 0.0047
MMP-3	20	3.15 ± 0.6-times up	Unpaired t test with Welch's correction	p = 0.02
MMP-7	20	Very low expression		
MMP-9	20	Not detected		
MT1-MMP	20	Not detected		
TIMP-1	20	3.15 ± 0.6-times up	Mann–Whitney	p = 0.014
TIMP-2	20	No change	Mann–Whitney	p = 0.4
TIMP-3	20	5 ± 0.8-times up	Unpaired t test with Welch's correction	p = 0.008

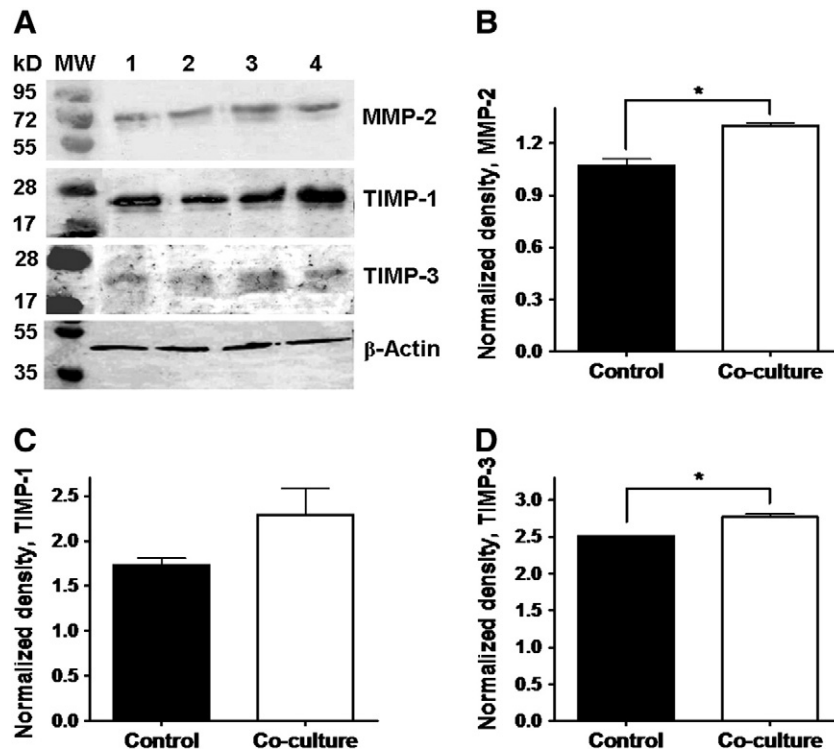
conditions. MMPs-1–3 and TIMPs-1 and -3 were constitutively expressed in PDLs, and their gene expression was significantly upregulated in co-cultured PDLs (Figs. 2A–E; Table 1). The upregulation of MMP-2, TIMP-1 and TIMP-3 gene expression in co-cultured PDLs has also been confirmed at protein level (Figs. 3A–D). MMPs-7, -9 (Table 1) and -13 were not detected in PDLs. TIMP-2 was also constitutively expressed in PDLs, and in co-culture it was not significantly regulated (Table 1).

### Tumor cell contribution to the MMP and TIMP expression in co-culture of SCC-25 cells and CAFs

MMP-1 at low levels and MMP-9 at higher levels were constitutively expressed in SSC-25 cells. In co-culture, their gene expression was significantly upregulated compared to controls (Figs. 2A, F; Table 2). Membrane-type 1 matrix metalloproteinase (MT1-MMP) was also constitutively expressed in SSC-25 cells, in co-culture, no significant regulation was observed in its gene expression (Table 2). Although the expression compared to PDLs in SCC-25 cells was low, TIMP-1 and TIMP-3 showed significant upregulation in co-cultured SCC-25 cells (Figs. 2D–E, Table 2).

### Regulation of MMPs and TIMPs expression in co-cultured fibroblasts

Based on our previous study, we investigated, if IL-1 $\beta$  [12,15] contributed to the upregulation of MMPs-1–3 in PDLs. 24 h treatment of PDLs with IL-1 $\beta$  at 1.5 ng/ml induced a significant upregulation of MMP-1 (8.8 ± 1.8-times; p = 0.003 using unpaired *t*-test with Welch's correction) and of MMP-3 (2.1 ± 0.3-times, p = 0.012 using unpaired *t*-test with Welch's correction), while MMP-2 was not upregulated. Similar to the gene expression of MMPs, that of TIMPs was also investigated in 4–24 h treatment of PDLs with IL-1 $\beta$  at 0.015–1.5 ng/ml. IL-1 $\beta$  did not change significantly the gene expression of TIMP-1 and TIMP-3 in fibroblasts (p = 0.8 and 0.7 respectively, by one way analysis of variance). In a further run of experiments, co-cultures were treated with



**Fig. 3 – Western blot analysis of MMP-2 (A, B), TIMP-1 (A, C) and TIMP-3 (A, D) related to loading control ( $\beta$ -actin) in PDL fibroblasts in control (1–2) and co-cultured (3–4) conditions. Densitometry of the detected bands normalized to the  $\beta$ -actin band densities. MMP-2 (B), TIMP-1 (C), TIMP-3 (D), \*: p < 0.05.**

$10^{-6}$  mol/L dexamethasone (DEX) as previously reported [12,14]. The gene expression of both TIMP-1 and TIMP-3 showed significant ( $p < 0.01$  using unpaired *t*-test with Welch's correction) decrease in DEX-treated co-culture compared to normal one ( $0.4 \pm 0.1$  (TIMP-1) and  $0.3 \pm 0.1$  TIMP-3 of the normal co-culture after DEX treatment).

Another possible regulatory way of TIMPs expression is the TGF- $\beta$ 1-pathway [31,32]. At mRNA level in PDLs and SCC-25 cells TGF- $\beta$ 1 was expressed at comparable levels (Fig. 4A), and it was significantly ( $p < 0.002$ , using Mann-Whitney test) upregulated in co-culture in both of them ( $2.4 \pm 0.6$ -times in fibroblasts and  $1.6 \pm 0.1$ -times in SCC-25 cells) (Fig. 4A). At protein level the TGF- $\beta$ 1 expression in PDLs and SCC-25 cells was also comparable ( $8.5$ – $37.8$  pg/ml). In co-culture, there was no significant regulation either in supernatants of fibroblasts or SCC-25 cells compared with controls ( $p > 0.05$  with non-parametric tests), and a sustained expression of TGF- $\beta$ 1 was detectable in co-culture using ELISA. The receptor TGFBR2 was constitutively expressed in PDLs, in co-culture its gene expression was significantly ( $p < 0.05$ , using Student's *t*-test),  $1.7 \pm 0.3$ -times increased in them (Fig. 4B). For the activation of TGF- $\beta$ 1: latent TGF $\beta$ -binding protein-1 (LTBP-1) is required [33], which was constitutively expressed in high levels in PDLs (Fig. 4C), in co-culture, its gene expression has not changed significantly ( $p = 0.26$ , using Mann-Whitney test) in them. Nevertheless, in SCC-25 cells there is a significant ( $p < 10^{-4}$ , using Mann-Whitney test),  $3.3 \pm 0.5$ -times increase (Fig. 4C) in co-culture. TIEG (TGF- $\beta$ 1-inducible early response gene) is used as a marker for the efficacy and activity of TGF- $\beta$ 1 [31]. Constitutive TIEG expression was detected in both PDLs and SCC-25 cells, and PDLs showed a significant ( $p < 0.05$ , using Mann-Whitney test),  $2.8 \pm 0.6$ -times upregulation in co-culture (Fig. 4D). The gene expression of TIMPs-1–3 and MMP-2 was compared with that of TGFBR2 and TIEG in co-cultured vs. control PDLs, where both TGFBR2 and TIEG showed highly significant correlation with TIMPs-1–3 and MMP-2 (correlation coefficients over 0.66,  $p < 10^{-3}$ , Supplementary Document 2).

### Regulation of MMP and TIMP expression in co-cultured tumor cells

MMP-1 and MMP-9 were constitutively expressed in SCC-25 cells, and their gene expression was upregulated in co-cultured SCC-25 cells (Figs. 2A and F). Previously [12], we have shown that the IL-1 receptor is much lower expressed in SCC-25 cells than in PDL

fibroblasts. Nevertheless, we have investigated if MMP-1 and MMP-9 are regulated by IL-1 $\beta$  in SCC-25 cells. Using the same time and concentration ranges for the IL-1 $\beta$ -treatment as for PDLs, there were no significant changes compared to control in SCC-25 cells ( $p = 0.7$  for MMP-1,  $p = 0.24$  for MMP-9 using non-parametric tests).

It was previously reported that fibronectin, via the  $\alpha$ v $\beta$ 6 integrin receptor (ITGA5B6) might upregulate MMP-9 gene expression [32]. Since IL-1 $\beta$ -treatment did not significantly contribute to MMP-9 gene expression in SCC-25 cells, it was investigated, if fibronectin, via ITGA5B6 receptor might have a contribution to MMP-9 regulation. Integrin  $\alpha$ v (ITGA5) was constitutively expressed in both PDLs and SCC-25 cells (Fig. 5A). In co-culture, it was significantly upregulated in both of them ( $2.9 \pm 1$ -times in fibroblasts and  $2 \pm 0.3$ -times in SCC-25 cells, using Mann-Whitney test) (Fig. 5A), which has been confirmed at the protein level as well (Fig. 5D). Integrin  $\beta$ 6 (ITGB6) was expressed only in SCC-25 cells, and its gene expression remained unchanged in co-culture ( $p = 0.1$ , using Mann-Whitney test, Fig. 5B). In SCC-25 cells both  $\alpha$ v and  $\beta$ 6 integrins were present, their expression levels were comparable with the one of MMP-9 (Figs. 2F, 5A–B), and the SCC-25-specific expression of ITGB6 was also comparable with the SCC-25-specific expression of MMP-9.

Fibronectin, the ligand, was constitutively highly expressed in PDLs (Figs. 5C, E), and  $29.9 \pm 0.3$ -times lower in SCC-25 cells, in co-culture its gene expression did not change significantly (Fig. 5C, using parametric and non parametric tests). At the protein level using an antibody specific for the EDA-domain of fibronectin it was detected only in the PDL fibroblasts (Fig. 5E).

### MMP-2 and -9 gelatinase activity in the co-culture of SCC-25 cells and fibroblasts

MMP-2 and -9 were functionally analysed in gelatinase zymography. Both supernatants and cell lysates of controls and co-cultures (of both the SCC-25- and PDL-side) were used in this assay. Active and inactive bands of MMP-9 and MMP-2 were recognized, and active bands of these enzymes were found at lower molecular weight. 5  $\mu$ l of fetal bovine serum was used as positive control (Figs. 6A–B, FBS lane).

In the cell lysates of PDL controls and co-culture only the inactive pro-MMP-2 was recognized, which showed comparable bands (Fig. 6A, lanes 1–2 and 5–6). In the cell lysates of SCC-25 control (Fig. 6A, lanes 3–4), three bands were recognized, the inactive pro-MMP-9, the functional active MMP-9 and the inactive,

**Table 2 – Tumor cell contribution to the MMP and TIMP expression in co-culture of SCC-25 cells and CAFs.**

Gene:	n	Change in SCC-25 co-culture vs. control	Test	p
MMP-1	20	$13.8 \pm 3.7$ -times up	Mann-Whitney	$p < 10^{-4}$
MMP-2	20	$1.73 \pm 0.36$ -times up	Unpaired <i>t</i> -test	$p = 0.0528$
MMP-3	20	Low expression		
MMP-7	20	$11.61 \pm 3.9$ -times up	Mann-Whitney	$p = 0.052$
MMP-9	20	$1.35 \pm 0.5$ -times up	Mann-Whitney	$p = 0.002$
MT1-MMP	20	No change	Mann-Whitney	$p = 0.36$
TIMP-1	20	$2.56 \pm 0.3$ -times up	Mann-Whitney	$p < 10^{-4}$
TIMP-2	20	No change	Mann-Whitney	$p = 0.13$
<b>TIMP-3</b>	20	$2.8 \pm 0.3$ -times up	Student's <i>t</i> -test	$p < 10^{-4}$

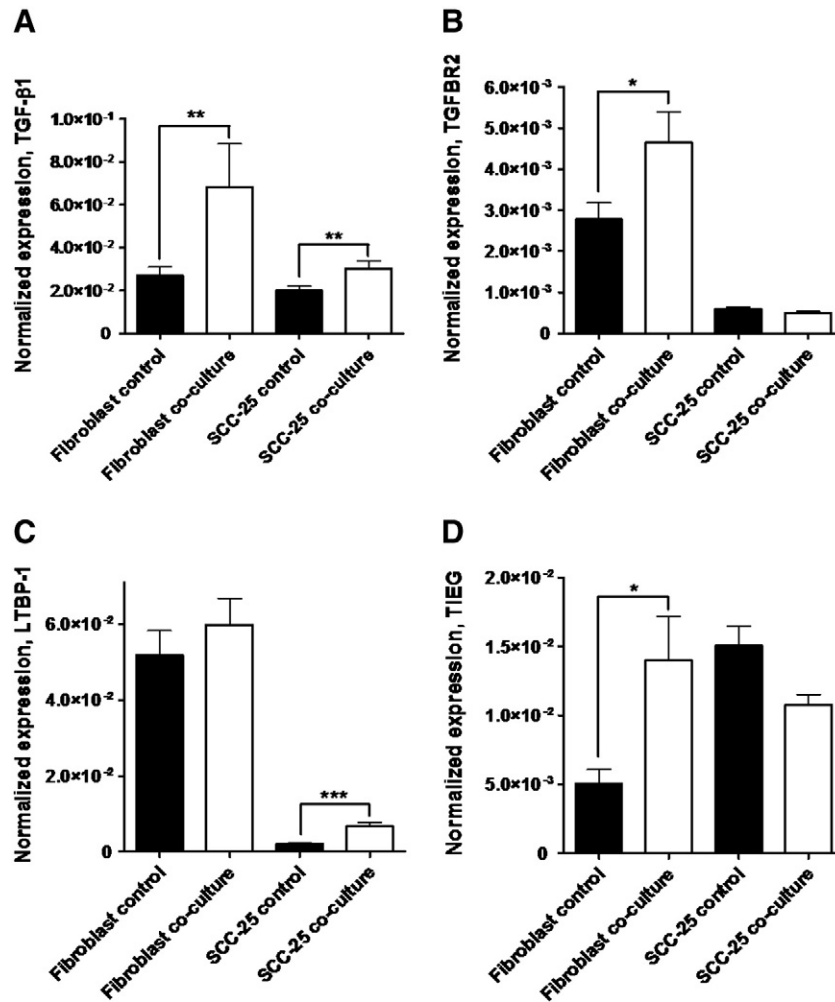


Fig. 4 – mRNA expression of TGF-β1 (A), TGFBR2 (B), LTBP-1 (C) and TIEG (D) in fibroblasts and SCC-25 cells in control and co-cultured conditions. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ .

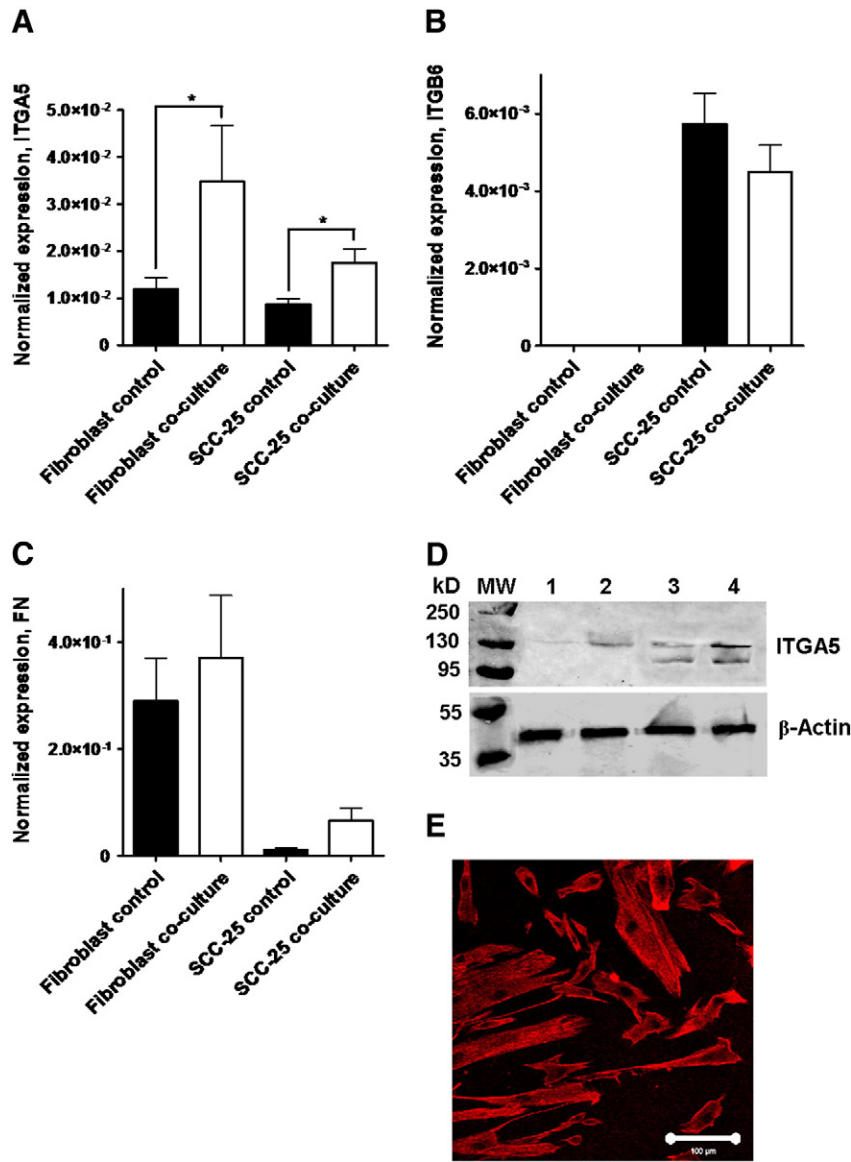
pro-MMP-2. In co-culture both the pro- and the active MMP-9 band intensity increased ( $1.57 \pm 0.04$  and  $2.72 \pm 0.3$ -times respectively; Fig. 6A, lanes 7–8, Figs. 6C, D), while pro-MMP-2-inactive showed comparable bands in the cell lysate of control and co-cultured SCC-25 (Fig. 6A lanes 3–4 and 7–8). In the supernatants of PDL controls only the inactive pro-MMP-2 was recognized (Fig. 6B, lanes 1–2). In co-cultured PDLs inactive pro-MMP-9 appeared and the inactive pro-MMP-2 bands decreased (Fig. 6B, lanes 5–6, Fig. 6E). In the supernatants of SCC-25 control three bands were recognized, pro-MMP-9 inactive, pro-MMP-2 inactive and MMP-2 active (Fig. 6B, lanes 3–4). In co-culture the inactive pro-MMP-9 band intensity increased ( $1.34 \pm 0.01$ -times; Fig. 6B, lanes 7–8), both pro-MMP-2-inactive and MMP-2 active bands intensity increased significantly ( $p < 10^{-3}$  using Mann–Whitney test) ( $1.38 \pm 0.02$ -times and  $1.27 \pm 0.02$ -times Fig. 6B, lanes 7–8, Figs. 6E, F).

Taken together, MMP-9 showed tumor cell specific function, its active form was only detected in/on the SCC-25 cells. MMP-2 showed fibroblast and tumor cell production (Fig. 6A), its active form was secreted, not found in cell lysates, and was present only at the side of tumor cells. A co-culture led to increased representation of active MMP-9 and MMP-2 either directly at the tumor cells, or in their vicinity.

## Discussion

MMPs play a complex role in tumor progression and metastasis. They facilitate invasion by degrading components of the ECM and there is also evidence that they are involved in angiogenesis. Tokumaru et al. earlier showed that MMP-2 is activated by MT1-MMP, which is localized on the cancer cells (Fig. 1A) [34]. MT1-MMP is constitutively expressed in SCC-25 cells, not regulated in co-culture with PDLs, and is not expressed in PDLs in control and co-cultured conditions. MMP-2 [35,36] and -9 are involved in the invasion process of oral cancer, and MMP-9 is related to poor prognosis in the subset of patients without neck node metastasis [37]. However, MMPs are not only produced by tumor cells but also by stromal cells and tumor infiltrating leucocytes, especially along the invasive front of the tumor [37–39]. Tumors with strong MMP-2 expression in stromal fibroblasts showed a significantly higher intratumoral microvessel density (IMVD). In addition, postoperative prognosis of strong stromal MMP-2 patients was significantly poorer than that of weak stromal MMP-2 patients [40].

The results of this study suggest that MMP-2 is secreted in its pro- (inactive-) form mainly by CAFs surrounding the tumor cells,



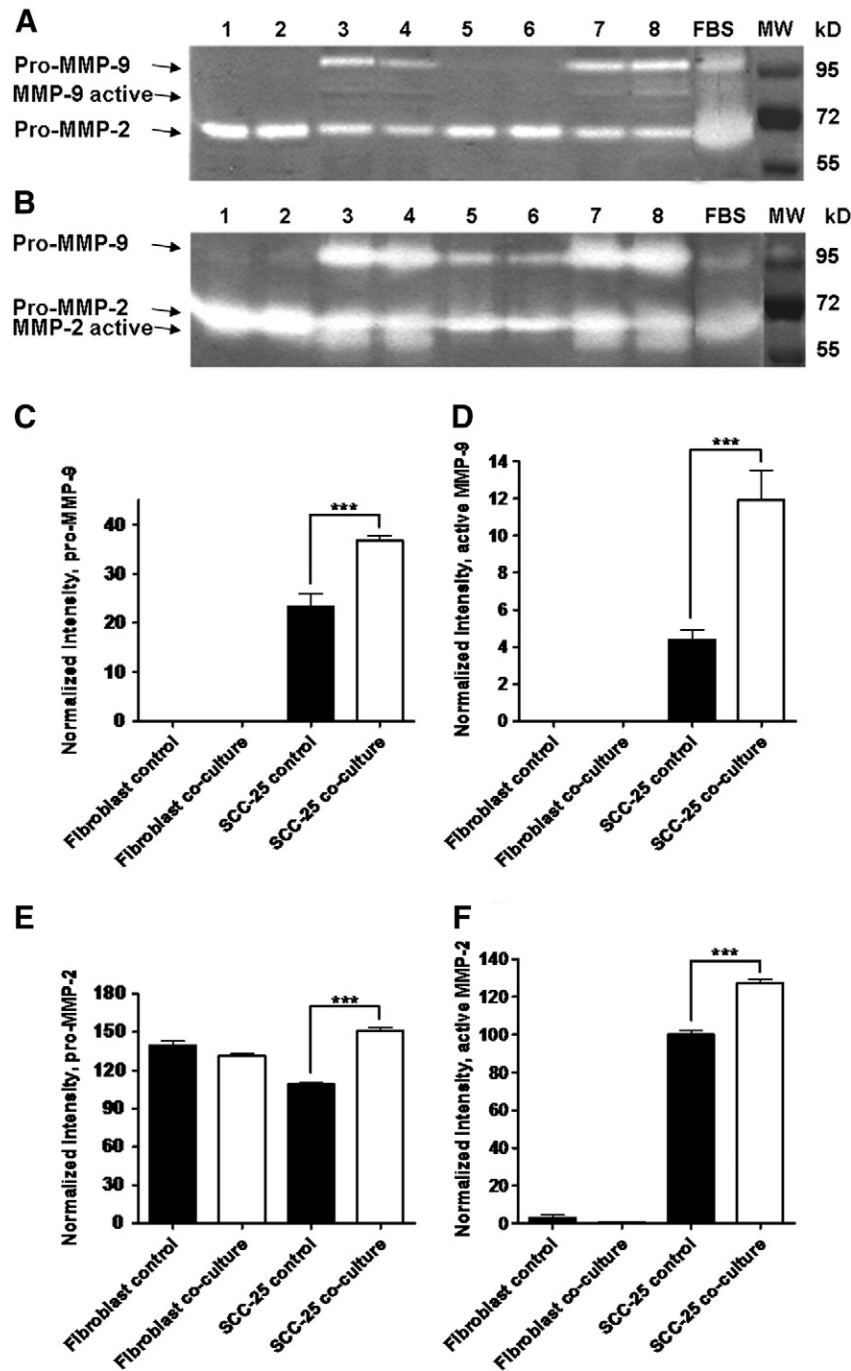
**Fig. 5** – mRNA expression of ITGA5 (A), ITGB6 (B) and fibronectin (C), in PDL fibroblasts and SCC-25 cells in control and co-cultured conditions. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ . Western blot of ITGA5 and loading control  $\beta$ -actin (D) in nuclear-free extracts of control (1) and co-cultured (2) fibroblasts, control (3) and co-cultured (4) SCC-25 cells. Confocal microscopy of EDA-fibronectin (red detected by Alexa fluor 647–conjugated anti-mouse IgG) in PDLs, bar: 100  $\mu$ m.

and at a lower extent also by the tumor cells themselves (Figs. 6A, 1A). Activation of MMP-2 either requires MT1-MMP localized on the cancer cells (Table 2) [34], or integrins, where the involvement of  $\alpha$ v integrins (ITGA5, Figs. 5A, 1A) was reported in more papers [41,42]. Our results show that the main contribution to the activation of MMP-2 resides in the tumor cells, and might be due to the constitutive expressed tumor-specific MT1-MMP. Nevertheless, CAFs play an essential role not only in the production of pro-MMP-2, but also in influencing the activation of MMP-2 by tumor cells. On the one hand ITGA5 is upregulated in CAFs, on the other hand, the interplay between CAFs and tumor cells contributes to the upregulation of ITGA5 also in the tumor cells (Fig. 5A). Interestingly, pro-MMP-2 is highly expressed in fibroblasts, but its activation only occurred in the presence of tumor cells (Figs. 6A–B). For the upregulation of MMP-2 also the interaction between

fibroblasts and carcinoma cells was required, where the involvement of the TGF- $\beta$ 1-pathway was recognized (Supplementary Document 2) [43]. The paracrine interaction between oral SCC cells and CAFs leads to the upregulation of other MMPs as of MMP-1 and MMP-3. The clear involvement of tumor-produced IL-1 $\beta$  in this process has been evidenced by the results of this work (Fig. 1B), where the induction effect was reproduced in IL-1 $\beta$ -treatments of fibroblasts. Several lines of evidence indicate that fibroblasts might facilitate the invasion of SCC cells by expressing MMPs on their own in response to tumor-cell-produced cytokines (i.e. IL-1 $\beta$ ), to TGF- $\beta$ 1 or to integrin–fibronectin interactions [41]. The tumor cells are capable of activating pro-MMPs.

In contrast to pro-MMP-2, pro-MMP-9 is produced by oral carcinoma cells, not by fibroblasts. Pro-MMP-9 is activated on the surface of tumor cells (Fig. 1A). The paracrine interaction between





**Fig. 6** – Detection of MMP-2 and MMP-9 gelatinase activity in cell lysate (A) and supernatant (B) of fibroblasts and SCC-25 cells by combined gelatinase zymography. 1–2: fibroblast control, 3–4: SCC-25 control, 5–6: fibroblast co-culture, 7–8: SCC-25 co-culture. Densitometry of MMP-2 and MMP-9 gelatinase zymography (C–F). C and D represent the densitometry of pro-MMP-9 (C) and active MMP-9 (D) bands in cell lysates. E and F show the densitometry of pro-MMP-2 (E) and active MMP-2 (F) form from supernatants.

CAFs and tumor cells also resulted in the upregulation of MMP-9 in oral carcinoma cells, where fibronectin, and its receptor,  $\alpha\text{v}\beta\text{6}$  integrin [32] as well as CD-44 [44] could have been involved (Fig. 1A).

Interestingly, besides their MMPs expression, fibroblasts produce several-fold higher amounts of inhibitors of metalloproteinases (i.e. TIMPs) whose gene expression (especially of TIMP-1 and TIMP-3) is also similarly upregulated in response to the paracrine

circuits between CAFs and oral carcinoma cells (Fig. 1B). In this regulation, the inhibitory effect of dexamethasone suggests involvement of inflammatory cytokines (Fig. 1B) (dexamethasone was also able to inhibit the upregulation of MMPs-1–2 in co-cultured fibroblasts, not shown), although, treatment of PDLs with IL-1 $\beta$  did not change significantly the gene expression of TIMP-1 and TIMP-3. In this regard, IL-1 $\beta$  might induce MMPs, but TIMPs are not upregulated at the same time, which might lead to excess of matrix

degrading enzymes over their inhibitors. In fact, all TIMPs inhibit active forms of all MMPs [45], in addition, they might contribute to activation of pro-MMPs, as TIMP-1 to the activation of MMP-9 [46] and TIMP-2 to the activation of MMP-2 [47].

Inflammatory cytokines could be, as mentioned before, selective in activation of MMPs and TIMPs, in addition, the TGF- $\beta$ 1-pathway is also involved in the regulation of TIMP expression in CAFs (Supplementary Document 2). A tissue of progressive tumor cells and CAFs is characterized by high expression of TIMPs in fibroblasts, and also by a significant TIMP expression in tumor cells. In fact, in co-cultured SCC-25 cells MMP-1, MMP-2 and MMP-9 were higher expressed than TIMP-1 or TIMP-2. These results allow the assumption that; matrix in tumor tissue is probably not digested by CAFs. In contrast, MMPs are also produced by tumor cells; moreover, pro-MMP-9 was tumor-cell-specific, not found in fibroblasts or CAFs. At the same time, tumor cells possess the tools for activation of pro-MMPs, either as MT-MMPs, or TIMPs (as TIMP-2 for MMP-2 [47]). The MMP activation might occur at the surface of tumor cells or nearby individual tumor cells. The relation of MMP/TIMP expression at this area allows activation of MMPs at the surface of tumor cells, which has been also seen experimentally using gelatinase assays, finding active MMPs at the side of tumor cells in co-culture. The tumor cell expression of TIMP-3 is relatively high, but this TIMP is sequestered bound to extracellular matrix [45], and will probably not be located close to the surface of tumor cells. In co-culture, the diffusion of released MMPs is freely allowed by the 0.4  $\mu$ m cell-culture inserts, but the tumor-cell-surface-associated activation provides a continuous excess concentration of active MMPs by the direct surface of tumor cells.

A coordination of the MMP-TIMP expression and MMP activation between progressing OSCC tumor cells and CAFs leads to local invasion potential of tumor cells, without a serious impact on whole organ tissue remodeling.

## Conclusion

Several lines of evidence indicate that fibroblasts might facilitate the invasion of OSCC cells, in part by paracrine regulatory circuits, or by increasing the proteolytic activity of latent metalloproteinases on the surfaces of OSCC cells.

Supplementary data to this article can be found online at doi:10.1016/j.yexcr.2012.03.023.

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