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p21/waf1 and smooth-muscle actin α expression in stromal fibroblasts of oral cancers

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Abstract. *Background*: Concerted alterations between stromal fibroblasts and neoplastic cells underline the carcinogenic process. Activation of alpha-smooth muscle actin (SMA) expression, a cytoskeleton protein normally expressed only in myoepithelial cells, is considered a landmark for the activation of stromal fibroblasts with little however being known regarding the mechanism governing the expression of SMA in the stroma.

Methods: We have evaluated by immunohistochemistry the expression of SMA in the stroma of oral malignant and pre-malignant lesions, in association with the expression of p53 and p21 tumor suppressors that were shown previously to be deregulated and/or mutated in stromal fibroblasts of various cancers. The effects of p21 knockdown in SMA expression and cell migration and the mRNA levels of endogenous p21 in fibroblasts co-cultured with cancer cells were also assessed.

Results: We found that both p21 and SMA expression was elevated in the stroma, but not the epithelium, of malignant as compared to pre-malignant lesions. We also noted that the expression of both was positively correlated, implying that SMA expression may be regulated by p21. Consistently with this notion we found that siRNA-mediated p21 suppression resulted in the reduction of SMA levels and also inhibited cell migration.

Conclusion: Our results show that p21 deregulation is associated with the activation of stromal fibroblasts of oral cancers by a mechanism that involves the stimulation of SMA expression.

Keywords: Stroma, p21, SMA, fibroblasts, desmoplastic reaction

1. Introduction

It is well established that during carcinogenesis stromal fibroblasts undergo certain changes, and eventually promote tumor growth [7,9]. In their transition from the normal into the cancer-associated state, at which they are defined as cancer-associated fibroblasts (CAFs), they are subjected to a myofibroblast differentiation program that is exemplified by the expression of α-smooth muscle actin (SMA) [3,5,6,16,18]. SMA positivity is considered diagnostic for fibroblast activation during tumorigenesis [7].

Despite the satisfactory characterization of CAFs, the regulation of their transition into the cancer-associated state remains poorly understood. p53 and its downstream target p21/waf1 emerge as potential regulators of fibroblasts' activation [1,20]. Cancer cells suppress fibroblasts' p53 by paracrine mechanisms while genetic ablation of p53 stimulated cancer growth [1,8,11]. Furthermore, mutations in p53 have been detected in the stroma fibroblasts of primary breast cancers, consistently with the notion that p53 mutations offer a proliferative advantage to the stromal fibroblasts [10,13,15]. Recently we showed that p21/waf1 in fibroblasts modulates the profile of tumorigenesis by non-autonomous mechanism(s) while in primary breast cancers and benign lesions deregulated p21 expression was detected [20].

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In the present study we investigated the expression of p53 and p21 in stromal fibroblasts of oral cancers and dysplastic lesions. Considering the pivotal role of SMA in fibroblast activation, we also evaluated whether p21 and/or p53 positivity is associated with SMA expression is stromal fibroblasts. Finally, we explored the consequences of modulated p21 expression in SMA levels and cell migration in primary stromal fibroblasts from oral carcinomas.

2. Materials and methods

2.1. Specimens and immunohistochemistry

A bank of 33 oral specimens consisting of 13 squamous cell carcinomas and 20 premalignant lesions (one hyperplasia and 19 dysplasias) were collected from the archives of the laboratory of Histology and Embryology, University of Athens Medical School and analyzed by immunohistochemistry for p53, p21 and SMA expression in the stroma and the epithelium. Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and immunostaining was performed by using the Kwik-DAB kit (ThermoShandon, Pittsburgh, PA, USA), following the manufacturer's instructions. Before observation, a weak counter stain with hematoxylin was performed. As positive, specimens arbitrarily bearing at least 10 positive cells per high power optic field (HPF) were classified in at least 5 fields that have been evaluated per specimen. Positivity in the pericytes has been excluded by the analysis and only positivity in the fibroblasts was taken into consideration.

2.2. Cell culture, siRNA and RT-PCR experiments

Fibroblasts were isolated using standard methods and maintained in DMEM containing 10% FBS and antibiotics/antimycotics. Suppression of p21 in the fibroblasts was achieved by transfecting wild-type mouse embryonic fibroblasts (MEFs) and human oral fibroblasts with siRNA specific for mouse (clone ID 160142, Ambion) or human (clone ID 1621, Ambion) according to the manufacturer's instruction. Cells were lysed 48 h after transfection by using RIPA reagent and total protein was subjected to western blot analysis. For western blot analysis antibodies for p21, SMA and actin were obtained from Sigma. Cell migration assay was performed by the "scratch" assay as described [12]. For the co-culture assay, 0.4 μ pore size transwells (Corning) were used at which MEFs were seeded on the bottom compartment while MCF7 cells at the top compartment. Prior to RNA extraction cells were co-cultured for the time periods indicated. For RT-PCR analysis RNA was isolated with Trizol reagent according to manufacturer's instructions, reverse transcribed into cDNA and subjected to semiquantitative PCR analysis. Primers used and cycling conditions were as follows: GAPDH (223 bp): 5′AACTTTGGCATTGTGGAAG G3′(left) and 5′AC ACATTGGGGGTAGGA ACA3′ (right), at 95°C for 30 s, 55°C at 30 s and 72°C at 30 s for 30 cycles. p21 (359 bp): 5′GTCCAATCCTGGTGATGTCC3′ (left) and 5′GCTCAGACACCAGAGTGC AA3′ (right), at 95°C for 30 s, 60°C at 30 s and 72°C for 70 s for 30 cycles. PCR products were electrophoresed into 2% agarose gel and visualized by ethidium bromide. All experiments were performed at least three times and similar results were obtained.

2.3. Statistical analysis

Immunohistochemical data were analyzed by the chi-squared test while the statistical analysis of cell migration by the Student's t-test (2-tailed, 2 parameter).

3. Results

3.1. Expression of p53, p21 and SMA in the stroma and the epithelium of primary oral cancers

Thirty-three (33) oral specimens including 13 squamous cell carcinomas and 20 premalignant lesions (one hyperplasia and 19 dysplasias) were analyzed by immunohistochemistry for p53, p21 and SMA expression in the stroma and the epithelium (Fig. 1). All antigens tested exhibited a mosaic pattern of staining in both the stroma and the epithelium, with the exception of SMA that as expected, did not stain epithelial cells but was detected in the pericytes besides the activated fibroblasts (Fig. 1). Positivity for all specimens and antigens tested ranged between 10–70 positive cells per HPF with the exception of two SCC that showed massive positivity for stromal SMA and epithelial p21, respectively.

A summary of the results is presented in Table 1. In the epithelium, consistently with previous findings a slight but insignificant increase in the number of positive specimens was detected for both p21 and p53 [14,19]. In the stroma, while p53 positivity remained

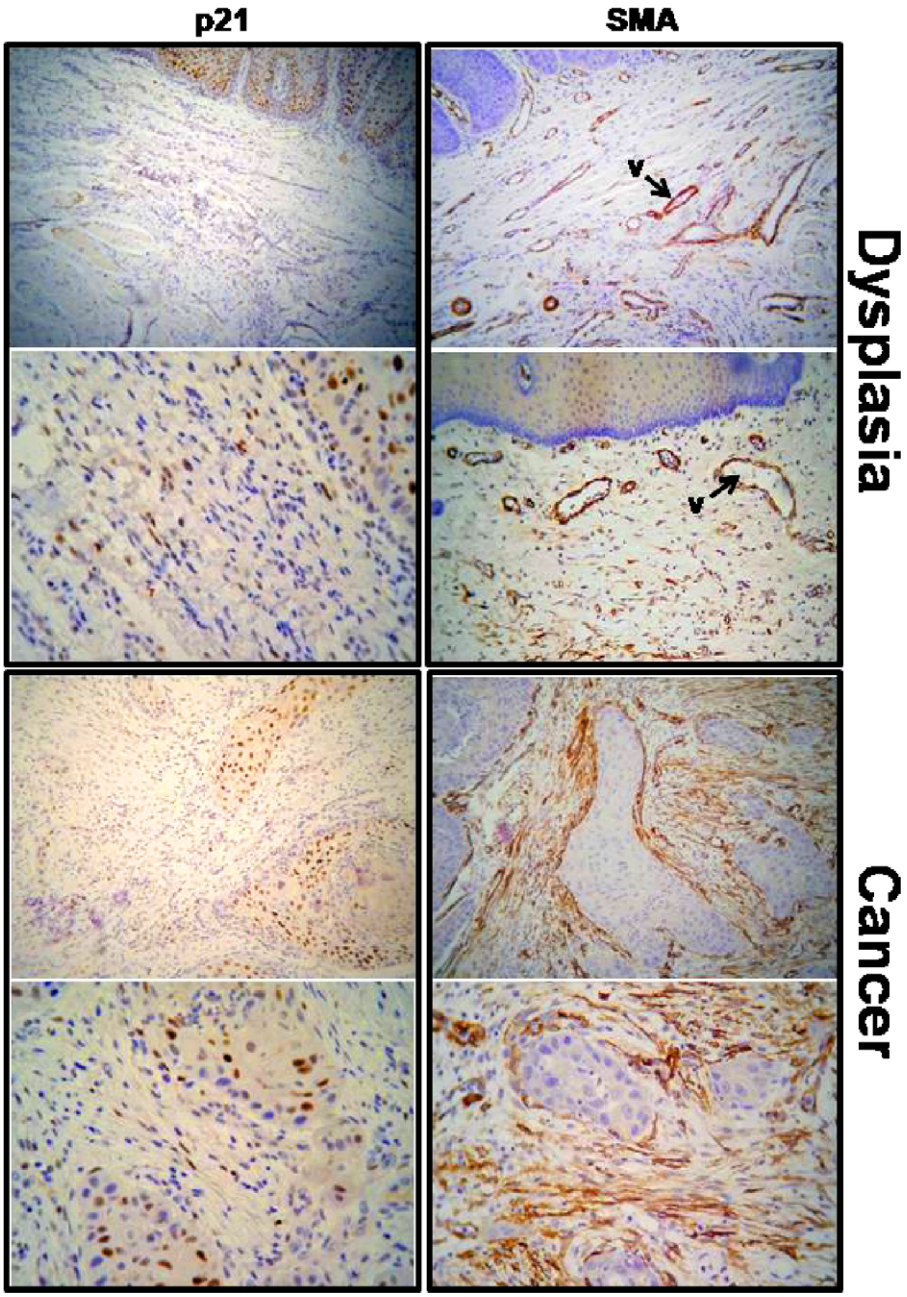

Fig. 1. Representative microphotographs of p21 and SMA immunopositivity (brown staining) in dysplastic and malignant lesions. Upper and lower panel in each set of pictures corresponds to different magnifications (objectives used 10× and 40×, respectively). Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and immunostaining was performed by using the Kwik-DAB kit (ThermoShandon, Pittsburgh, PA, USA), following the manufacturer's instructions. Before observation, a weak counter stain with hematoxylin was performed. Arrows indicate blood vessels that are positive for SMA.

essentially unaffected between the cancerous and the pre-malignant stroma, both p21 and – consistently with previous findings – SMA positivity significantly increased in the malignant as compared to that of the premalignant lesions (chi-squared test, $p = 0.005$ and $p = 0.045$, respectively).

Pair-wise comparisons between specimens co-expressing either of p53, p21 and SMA in the stroma revealed a statistically significant correlation between p21 and SMA expression (chi-squared test, $p =$

Table 1
Immunohistochemical detection of p53, p21 and SMA in the stroma and the epithelium of oral cancers and pre-malignant lesions

	Negative	Positive	p
p53 (epithelium)			
Pre-malignant	13 (65%)	7 (35%)	0.279
Malignant	6 (46%)	7 (54%)	
p21 (epithelium)			
Pre-malignant	5 (25%)	15 (75%)	0.486
Malignant	2 (15.4%)	11 (84.6%)	
p53 (stroma)			
Pre-malignant	16 (80%)	4 (20%)	0.861
Malignant	10 (76.9%)	3 (23.1%)	
p21 (stroma)			
Pre-malignant	10 (50%)	10 (50%)	0.045
Malignant	2 (15.4%)	11 (86.4%)	
SMA (stroma)			
Pre-malignant	13 (65%)	7 (35%)	0.005
Malignant	2 (15.4%)	11 (84.6%)	
	p53 negative	p53 positive	p
p53 vs. SMA (stroma)			
SMA negative	12 (36%)	3 (9%)	0.864
SMA positive	14 (43%)	4 (12%)	
p21 vs. SMA (stroma)			
SMA negative	11 (33%)	4 (12%)	0.00006
SMA positive	1 (3%)	17 (52%)	
p53 vs. p21 (stroma)			
p21 negative	9 (27%)	3 (9%)	0.657
p21 positive	17 (52%)	4 (12%)	

Notes: The percentage is shown in brackets. p-value is also indicated.

0.00006). These antigens tended to be co-expressed in the stromal fibroblasts of the specimens analyzed with 85% being present or absent *vs.* 15% expressing either protein. Despite the fact that p21 is considered a major p53 transcriptional target [4], no association was detected in the expression of these two genes, extending our previous findings on breast cancers [20] and implying the p53-independent activation of p21 in the stromal fibroblasts. Furthermore, no association between p53 and SMA expression was detected in stromal fibroblasts, despite the previous identification of SMA gene as a direct p53 transcriptional target (Table 1) [2].

3.2. *Effect of p21 knockdown in SMA expression and cell migration*

In order to test if p21 regulates SMA we have evaluated the effect of p21-knockdown by siRNA on SMA levels, in primary cultures of human fibroblasts isolated from oral SCC. Primary cultures of stromal fibroblasts were isolated by standard methods and were verified as such by the positive expression of vimentin and the negative expression of cytokeratin 18. All experiments were performed before cells reached passage 10. p21-knockdown, while not affected cell proliferation (data not shown), resulted in the suppression of SMA in the primary fibroblasts (Fig. 2). Similar effects were also found when primary MEFs were used, despite the fact that the latter appeared less sensitive than the former: while in the oral cancer fibroblasts adequate SMA suppression was obtained at 5 nM siRNA, in MEFs comparable effects were obtained at 30 nM (Fig. 2). It is noted, however, that given the different species origin and therefore siRNA used, a formal comparison between these findings cannot be performed. Notwithstanding these limitations it appears that SMA expression frequently – albeit not constantly – depends on p21 levels. Cell migration was assessed by a standard "scratch" assay [12]. As shown

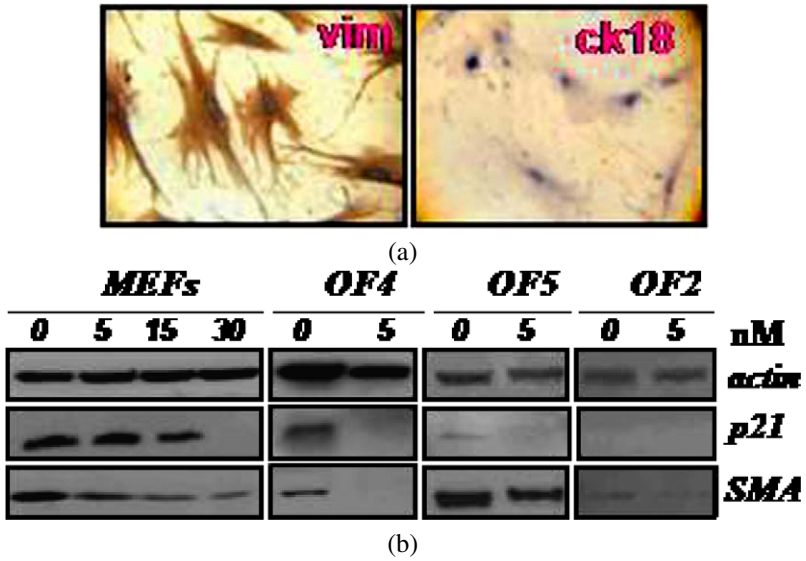

Fig. 2. Characterization and modulation of p21 expression in primary fibroblasts from oral cancers. (a) Immunostaining for vimentin (positive) and cytokeratin 18 (negative) was performed to confirm the fibroblastic identity of the cells. The cell fraction positive for vimentin exceeded 95% and was less than 5% for cytokeratin. All experiments were performed before cells reach passage number 10. (b) siRNA-mediated suppression of p21 results in the reduction of SMA protein levels in MEFs and primary fibroblasts from oral cancer (OF). Note that in OF2, baseline expression of p21 was minimal however a reduction in SMA was apparent following siRNA transfection for p21. No effect was found when cells were transfected with scrambled RNA (data not shown).

in Fig. 3, p21 suppression caused reduction in the migration ability of the cells by about 25% ($p < 0.05$), while equimolar amount of scrambled RNA had no effect in cell migration.

3.3. *P21 expression in fibroblasts co-cultured with MCF7 breast cancer cells*

We explored the expression of p21 in fibroblasts (MEFs) co-cultured with human MCF7 breast cancer cells in a transwell system (0.4 μ pore size) permitting the exchange of soluble factors, however inhibiting cell migration from one cellular compartment to the other. Therefore, MEFs were co-cultured with MCF7 breast cancer cells and the levels of p21 transcripts were monitored by RT-PCR. As shown in Fig. 3c, following the co-culture of these cells p21 expression transiently increased, picking at about 6 h and subsequently decreased below the detection limits of our assay at about 48 h.

4. Discussion

Stroma fibroblasts play an increasingly appreciated role in carcinogenesis. Given the pivotal role of SMA neo-expression in cancer-associated fibroblasts and the recently suggested role of p21/p53 in fibroblast activation, we have analyzed by immunohistochemistry the role of p21, p53 and SMA in primary oral cancers and pre-malignant lesions. We have chosen to evaluate oral lesions because occasionally they are rich in fibroblastic stroma, a feature that will facilitate our analysis. Furthermore, in the oral epithelium the predominant SMA-positive cells are the pericytes and thus the activated SMA-positive myofibroblasts will easily be identifiable.

Our results showed that both SMA and p21 positivity significantly increased in the malignant as compared to that of the premalignant lesions, implying that fibroblast activation and subsequently transition into the CAF-state is related to the concerted overexpression of SMA and p21. Consistent with this notion was the observation that positive cells were not randomly distributed in the stroma but frequently were localized adjacent to the cancerous epithelium and particularly surrounding the neoplastic epithelium, especially as regards SMA immunopositivity (Fig. 1). It is noted that in our previous study on breast cancers, an analogous pattern of p21 expression was detected at which, despite the reduction in expression at the invasive adenocarcinomas, during the earlier stages of the disease, p21 exhibited a periductal pattern of staining. Furthermore, a statistical correlation was found

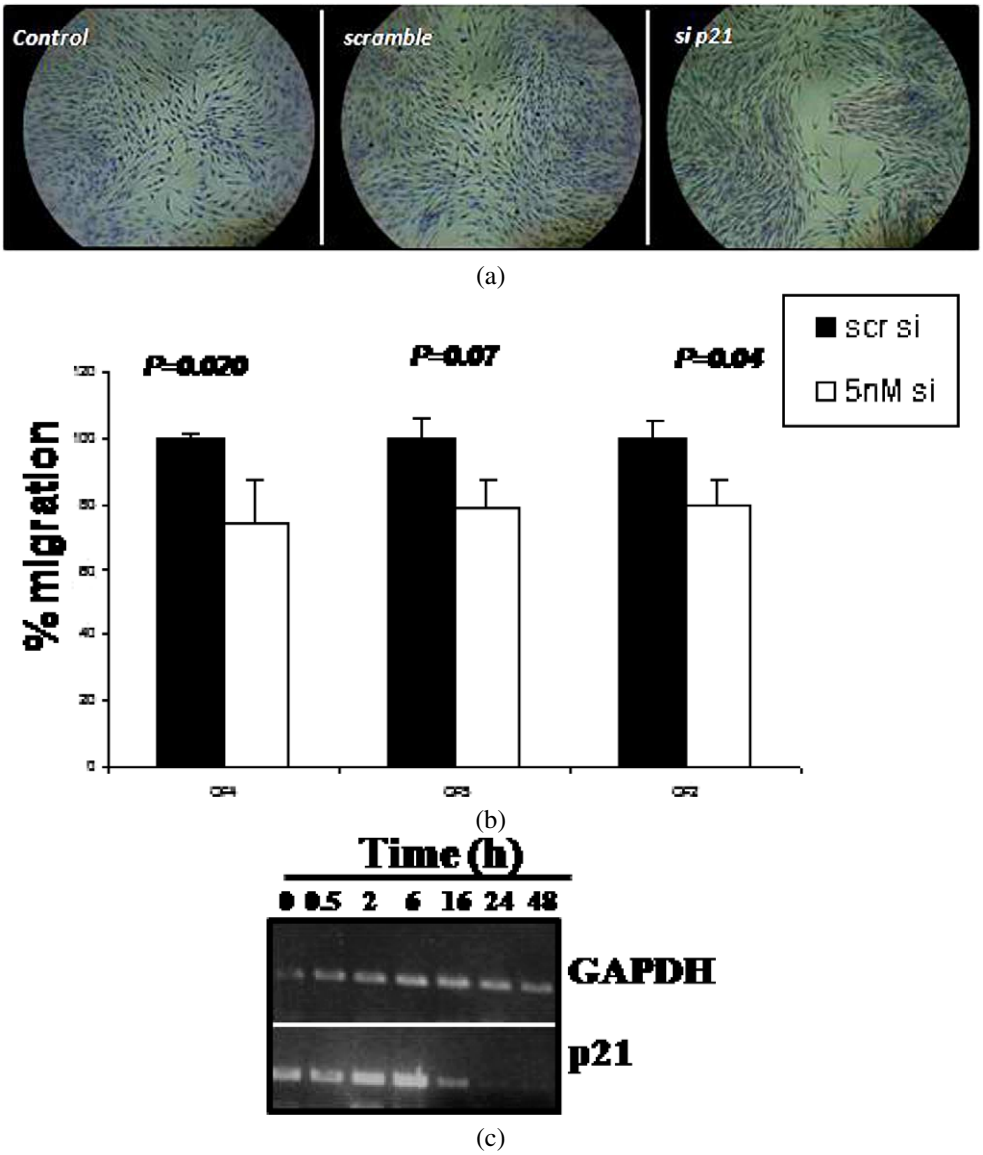

Fig. 3. (a) Representative microphotographs of cell migration during p21-knockdown by siRNA. Effect of scrambled RNA at 5 nM is also shown. (b) Graphical presentation of cell migration during p21-knockdown. p21-knockdown resulted in the decrease of cell migration. (c) RT-PCR analysis of p21 expression in fibroblasts co-cultured with MCF7 breast cancer cells in 0.4 μ pore size transwells (Corning).

between p21 and SMA expression which points to a potential regulatory role of p21 to SMA expression. Indeed, evidence for a role of p21 in SMA regulation was provided in experiments involving cardiac normal fibroblasts. In this study the p21-dependent activation of SMA gene transcription was shown to be critical for the differentiation of cardiac fibroblasts into myofibroblasts [17]. This finding, in conjunction with the correlated expression of SMA and p21 in the stroma of oral lesions, raises the possibility for the potential regulation of SMA expression by p21 in the stromal fibroblasts during carcinogenesis. In accordance with these findings we found that siRNA-mediated inhibition of p21 expression suppresses SMA levels.

While misexpression of SMA in stromal fibroblasts has been documented extensively, its precise role in stroma activation is poorly understood. Given the preferential localization of p21 and SMA-positive fibroblasts in the vicinity of the cancerous blasts of the malignant oral epithelium, we asked whether p21 affects the migration ability of the fibroblasts. Thus, primary fibroblasts from oral SCC were subjected to a migra-

tion "scratch" assay following p21-knockdown. Our results showed that p21-knockdown inhibits cell migration by about 25%. Thus, it is likely that the induction of p21 in the stromal fibroblasts is associated with the up-regulation of SMA and eventually with the stimulation of stromal fibroblasts' migration, a feature that is intrinsically related to tissue remodeling. It is noted however, that while our results are consistent with the direct regulation of SMA by p21, a wider role of p21 in the differentiation of fibroblasts during the desmoplastic reaction should not be excluded. This is supported by the fact that no effect in cell proliferation was documented, indicating that the consequences of modulated p21 expression are not directly associated with the quantity of the stromal fibroblasts but rather with their ability to migrate and affect stroma reorganization.

The aforementioned findings are consistent with an important role for p21 in stromal fibroblast activation however its biological relevance highly depends on the differential regulation of p21 expression in the stromal fibroblasts during malignant progression. Indeed, recent evidence provided by Bar et al. [1] showed that p53, which is a major p21 transcriptional regulator, is suppressed in fibroblasts by adjacent cancer cells. Consistent with these findings we found that p21 is also subjected to differential regulation in fibroblasts cocultured with cancer cells. Thus, it is likely that fibroblasts respond to the presence of cancer cells in their vicinity by modulating the transcription of p21 gene that eventually affects their migration ability. The latter is most likely associated with the mobilization of the fibroblasts close to the cancer cells and the initiation with the desmoplastic reaction. At subsequent stages of disease progression, however, it is possible that expression of p21 and SMA – and thus fibroblast mobility – are suppressed. While the latter was apparent in invasive breast adenocarcinomas (8), it was not evident in the oral lesions utilized here, probably because the latter have not reached the degree of stroma activation of the former.

Taken together our results support the notion that differential expression of p21 is important for the activation of stromal fibroblasts associating it with SMA regulation and cell migration.

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