Tumor-associated macrophages promote malignant progression of breast phyllodes tumors by inducing myofibroblast differentiation

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Abstract
Myofibroblast differentiation plays an important role in the malignant progression of phyllodes tumor (PT), a fast-growing neoplasm derived from periductal stromal cells of the breast. Macrophages are frequently found in close proximity with myofibroblasts, but it is uncertain whether they are involved in the myofibroblast differentiation during PT progression. Here we show that increased density of tumor associated macrophage (TAM) correlates with malignant progression of PT. We found that TAM stimulated myofibroblast differentiation and promoted the proliferation and invasion of PT cells. Furthermore, we found that levels of the chemokine CCL18 in TAM was an independent prognostic factor of PT. Mechanistic investigations showed that CCL18 promoted expression of α-smooth muscle actin, a hallmark of myofibroblast, along with the proliferation and invasion of PT cells, and that CCL18-driven myofibroblast differentiation was mediated by an NF-κB/miR-21/PTEN/AKT signaling axis. In murine xenograft models of human PT, CCL18 accelerated tumor growth, induced myofibroblast differentiation and promoted metastasis. Taken together, our findings indicated that TAM drives myofibroblast differentiation and malignant progression of PT through a CCL18-driven signaling cascade amenable to antibody disruption.
Introduction

Breast phyllodes tumor (PT) is a biphasic breast tumor composed of cellular spindle stroma with epithelial elements. It constitutes 0.3-1% of all breast tumors and 2.5% of fibroepithelial lesions of the breast (1). Currently, PTs are histologically classified as benign, borderline, or malignant on the basis of stromal cellularity, mitotic activity of stromal cells, stromal nuclear atypia, stromal overgrowth, and types of border (infiltrating or pushing). The clinical outcome of phyllodes tumors is hard to predict, with frequent local relapse and sometimes distant metastasis. Adjuvant chemotherapy or radiotherapy is not effective against phyllodes tumors (2). The potentially recurring and metastatic behavior of PTs is attributed to the characteristics of stromal cells, mainly fibroblasts (3). The normal fibroblast can acquire an "activated" phenotype, which expresses the α-smooth muscle actin (α-SMA) as a hallmark and is so called as myofibroblasts. Our previous studies have indicated myofibroblasts were the major malignant component of phyllodes tumors. The increased myofibroblast population drives the tumorigenicity of PTs. In addition, α-SMA can serve as an independent prognostic factor for phyllodes tumors with better predictive values than histological classification (4). We further demonstrated that the fibroblasts–myofibroblasts transition in PTs is driven by the elevated miR-21 (4), whereas the mechanism of miR-21 upregulation and how it drives tumorigenicity of PTs remain unknown.

It is well established that tumor associated macrophages (TAMs) are one of the most abundant cell type in tumor microenvironment (5), which are involved in tumor...
metastasis and progression (6). Clinical and epidemiological studies have shown a strong correlation between the increased TAMs density and poor prognosis in several types of cancer (6, 7), including breast cancer (8, 9). However, how TAMs impact on the malignant progression of phyllodes tumor and whether TAMs density can be a prognostic factor for phyllodes tumors are still unknown.

Macrophages are usually found in close proximity with collagen-producing myofibroblasts (9). Macrophages produce profibrotic mediators that directly activate fibroblasts, including transforming growth factor (TGF)-β1 and platelet-derived growth factor (PDGF) (10). Macrophages also provide insulin-like growth factor-1, which stimulates the proliferation and survival of myofibroblasts and promotes collagen synthesis by these cells (11). In this study, we found that TAMs are essential for driving myofibroblast differentiation (fibroblasts–myofibroblasts transition) in the malignant progression of PTs via the CCL18/NF-κB/miR-21/PTEN/AKT axis and targeting CCL18 is a promising strategy for treating phyllodes tumors.

Materials and Methods

Patients and Tissue Samples

Breast phyllodes tumor samples were obtained from 268 female patients with 167 benign, 36 borderline and 65 malignant PTs in the Breast Tumor Center, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, from January 2000 to June 2011. The patients were followed up for 8-148 months (median follow-up is 112 months).

Pathological diagnosis, as well as mitoses and stromal overgrowth status,
confirmed by two pathologists independently. All human samples were collected with
informed consent from the donors according to the International Ethical Guidelines
for Biomedical Research Involving Human Subjects (CIOMS). The study was
performed after approval by the institutional review board (IRB) of SunYat-Sen
Memorial Hospital.

**Separation and Culture of Primary Cells from Breast PTs**

PTs cells were isolated from benign and malignant PTs as previously described (4).
TAMs were isolated from eight fresh breast malignant PTs samples as previously
described (12, 13), with slight modifications. Briefly, the tissues were minced into
small (1 to 2 mm) pieces and digested with 5% fetal bovine serum Dulbecco’s
modified Eagle’s medium containing 2 mg/ml collagenase I and 2 mg/ml
hyaluronidase (Sigma) at 37°C for 2 hr. The cells were sequentially filtered through
500 μm mesh, 100 μm, and 70 μm cell strainer. The cells were then centrifuged in a
Beckman Allegra X-15R centrifuge at 2,500 rpm for 20 min with 1 ml cell suspension
above 5ml 45% Percoll (GE Healthcare) in the middle and 5ml 60% Percoll at the
bottom in a 15 ml tube. Mononuclear cells were collected from the cell layer in the
interphase between 45% and 60% Percoll. CD14+ monocytes and macrophages were
isolated by a magnetic-activated cell sorting using direct CD14 Isolation Kit (Miltenyi
Biotec) according to the manufacturer’s instructions.

**ChIP assay**

ChIP assay was performed using Pierce Agarose ChIP Kit (26156, Thermo)
according to manufacturer’s instructions. The specific sequences from
immunoprecipitated and input DNA were determined by PCR primers for miR-21
promoter upstream regions:
miR-21 promoter forward, 5'-TCCCCTCCTGGGAAGTTTC-3',
reverse, 5'-TTGGCTCTACCCCTTGT-3'.
The negative control primer:
miR21-NC-1: forward, 5'-TTCCTCATTTCCCTAAACAACAA-3'
reverse, 5'-AATCTACCAGGGATAGCCATAGTC-3'.
miR21-NC-2: forward, 5'-GAGGACTTCCCCAACTTAACTATG-3'
reverse, 5'-TTATTCTCAAGCAGCAGACCAG-3'.

Animal Experiments
All animal work was conducted in accordance with a protocol approved by the
Institutional Animal Care and Use Committee at the Medical School of Sun Yat-Sen
University. Breast PTs cells (1×10^7) mixed with MDMs (2×10^6) were inoculated into
the mammary fat pads of six-week old female nude mice. Mice are from Beijing Vital
River Laboratory Animal Technology Co., Ltd. For antibody treatment, mice were
injected with CCL18-specific neutralizing antibody (Cat.No.ab9849, Abcam,
Cambridge, MA) or Isotype-matched IgG (Abcam) via the tail vein at 1mg/kg twice
weekly after the xenografts became palpable (around 0.5cm in diameter). In some
groups, intratumor injection of M-CSF at 0.2μg/kg was performed twice weekly after
inoculating and 48 hours prior to antibody injection. Tumor growth was evaluated by
monitoring tumor volume (TV=length×width^2×0.5) every three days for eight weeks.
The animals were sacrificed when the xenografts reached 1.5 cm in diameter. Tumor
xenografts as well as the livers and lungs of mice were harvested for further evaluation. Cryosections (4μm) of the harvested organs were H&E for histological assessment, and total RNA was extracted for qRT-PCR analysis of human hypoxanthine phosphoribosyltransferase (HPRT) mRNA expression.

Statistics

The in vitro data were depicted as mean ± S.D. of three independent experiments performed in triplicate. All statistical analyses were performed using SPSS 16.0 statistical software package (SPSS, Chicago, IL, USA). Student’s t test and one-way ANOVA was used to compare CCL18 expression levels between the PTs with different tumor grades, whereas chi-square test was used to analyze the relationship between CCL18 expression and clinicopathological status. Kaplan-Meier curves and log-rank test were used to compare overall survival (OS) and disease-free survival (DFS) in different patient groups. In all cases, P<0.05 was considered statistically significant.

Results

TAMs density is associated with malignant progression of PT and CCL18 is an independent prognostic marker for PT patients

Evidence from clinical and epidemiological studies has shown a strong correlation between TAMs density and poor prognosis in several types of cancer (6, 7). To investigate whether malignant progression of PT is correlated with TAMs density, we examined the presence of TAMs in 268 PT samples, including 167 benign, 36
borderline and 65 malignant PTs. Normal breast tissues were used as control. We tested the expression of CCL18, the hallmark of TAM in paraffin-embedded PT samples by immunohistochemistry (IHC). The expression of CCL18 level in malignant PTs is significantly higher than that in benign and borderline PTs (Fig. 1A).

Previously, we have found PITPNM3 was the receptor of CCL18 on the surface of breast tumor cells (12). CCR8 was also reported as the CCL18 receptor (14). We then examined the level of PITPNM3 or CCR8 in PTs and found that PIPTNM3 was increased in malignant PTs (Fig. 1A), whereas CCR8 was not (Supplementary Fig. S1A). CCL18 is expressed in both TAMs and DCs (14). To confirm whether CCL18 positive cells were macrophages, two macrophages markers CD68 and CD163 were used. CD68 is specially expressed by macrophages as well as in tolerogenic DCs (15). CD163 is a marker of M2 macrophages. Microscopy revealed that both CD68 (Supplementary Fig. S1B) and CD163 (Fig. 1B) immunostaining were co-localized with CCL18 positive cells in breast PT tissues, showing the CCL18 positive cells were M2 like macrophages, which in fact were TAMs in the PTs.

Furthermore, we measured the CCL18 mRNA levels in fresh frozen PT tissues and primary PT cells isolated from benign, borderline or malignant PTs, using normal breast tissues as a control. Using qRT-PCR, we found that the mRNA levels of CCL18 from fresh frozen tissues were progressively increased from normal breast tissue to benign, borderline and malignant PTs (Fig. 1C). The mRNA levels of CCL18 from primary PT cells did not show an increasing tendency from benign, borderline to malignant PT cells. While TAMs isolated from the malignant PTs expressed
extremely high levels of CCL18 mRNA (Supplementary Fig. S1C). ELISA assay was used to measure the serum CCL18 level of PT patients or the secreted CCL18 level in the culture suspension of primary cells isolated from PT tumors. We found that the CCL18 protein levels in the serum of patients with malignant PTs were significantly higher than in those with benign and borderline PTs (Fig. 1D). However, primary PT cells secreted few CCL18 into the culture medium. Similar to the mRNA level, the secreted CCL18 level in the culture supernatant of the primary TAMs isolated from malignant PT tumors was much higher than in those from PTs cells (Supplementary Fig. S1D). Together, these results indicate that TAMs density is associated with the malignant progression of PTs and CCL18 is mostly produced by TAMs.

Next, we tested whether CCL18 had clinical prognostic value for PT patients. The 268 PT patients were followed up for 8-148 months (median follow-up time is 112 months). During the follow-up, 49 cases were diagnosed with recurrence, including 18 in the benign group, 9 in the borderline group and 22 in the malignant group. Additionally, 31 cases were diagnosed with metastasis, with 3 in the borderline group and 28 in the malignant group.

We then analyzed the association of CCL18 expression with the clinicopathological status of PTs (Supplementary Table S1). The expression of CCL18 increased with higher tumor grade, mitotic activity and stromal overgrowth \( p<0.001 \), but was not associated with age and the size of tumor (Supplementary Table S1). The expression of CCL18 was also more abundant in the PTs with local recurrence and distal metastasis \( p<0.001 \), Supplementary Table S1). Furthermore, Kaplan-Meier survival
curve demonstrated that patients with low CCL18 expression (Staining index, SI\leq 4, 
refers to the supplementary methods and ref(4)) have a longer overall survival (OS) 
and disease-free survival (DFS) than those with high CCL18 expression (p<0.001, Fig. 
1E and F). We also used ROC curve to evaluate the efficacy of serum CCL18 in PT 
patients as a diagnostic marker. It showed that CCL18 serum level could distinguish 
aggressive cases from benign PTs (Supplementary Fig. S1E and F). In addition, 
multivariate Cox regression analyses demonstrated that CCL18 (p<0.001), stromal 
overgrowth (p<0.001) and tumor grading (p=0.006) were independent prognostic 
predictors for local recurrence-free survival (LRFS) (Supplementary Table S2). These 
results suggest that the TAMs density is associated with malignant progression of PT, 
and the levels of CCL18 can be used to predict the outcome of PT patients.

TAMs induce myofibroblast differentiation and promote the proliferation and 
invasion of the PT cells

Macrophages are frequently found in close proximity with collagen-producing 
myofibroblasts (9), and there is strong evidence that this interaction is reciprocal (16). 
Macrophages produce profibrotic mediators that directly activate fibroblasts, 
including transforming growth factor (TGF)-β1, platelet-derived growth factor (PDGF) 
(10). Macrophages also produce insulin-like growth factor-I, which stimulates the 
proliferation and survival of myofibroblasts and promotes collagen synthesis by these 
cells (11). As we have found TAMs density is correlated with malignant progression 
of PTs and our previous studies have reported that myofibroblast differentiation is
associated with the malignant progression of PTs (4), we speculated that TAMs may play an important role in the myofibroblast differentiation of breast PTs. To demonstrate this hypothesis, we cultured primary benign PT cells with primary TAMs isolated from malignant PT patients. TAMs in breast cancers are primarily M2 cells (17). Because M2 macrophages, which can be induced from monocyte-derived macrophages (MDMs) by M-CSF (18), have been shown to induce myofibroblast transition (9), we also cultured benign PT cells with M-CSF pretreated MDMs by transwell assay. Benign PT cells were seeded into the lower chamber. MDMs or TAMs were seeded into the upper chamber of a 6-well transwell apparatus. The mRNA and protein level of α-SMA, the hallmark of myofibroblast, was detected to evaluate the phenotype change of PT cells. Compared with untreated PT cells, cells cocultured with primary TAMs or M-CSF activated MDMs had a significant higher level of α-SMA (Fig. 2A and B). The expression level of α-SMA in PT cells treated with M-CSF alone or cocultured with untreated MDMs had no obviously changes (Fig. 2A and B). Immunofluorescent (IF) staining (Fig. 2C) also showed the increased levels of α-SMA and fibroblast activation protein (FAP), which is also a marker of myofibroblast.

Since myofibroblasts are known to have an increased ability to induce collagen gel contraction (19), collagen contraction assay was used to test whether TAMs-treated PT cells function as myofibroblast. We observed that benign PT cells cocultured with TAMs or M-CSF pretreated MDMs contracted collagen gels to a much greater extent than PT cells treated with or without M-CSF or PT cells cocultured with untreated
MDMs (Fig. 2D). Together, these findings suggest that TAMs induce myofibroblast function in the PT cells.

Previous studies have reported that myofibroblasts in epithelial tumors have an increased proliferative activity and can promote cancer invasion and metastasis (20, 21). We then examined the effects of TAMs on the proliferation, migration and invasion of primary PT cells. Cell viability and clonogenic assays showed that TAMs or M-CSF pretreated MDMs increased the foci formation of PT cells (Fig. 2E), indicating that TAMs promoted the growth of PT cells. Boyden chamber assays also showed that TAMs or M-CSF pretreated MDMs significantly increased the number of migrated and invaded PT cells (Fig. 2F and G) \( (p<0.01) \). These data suggest that TAMs not only induces the myofibroblast differentiation, but also promotes their malignancy.

**CCL18 is responsible for TAM-induced myo-fibroblasts differentiation, proliferation and invasion**

Previously, we have reported that CCL18 released by TAMs promotes breast cancer metastasis, causing poor survival of breast cancer patients (22). It has been shown above that elevated CCL18 expression is also associated with poor outcome of breast PT patients, and that myofibroblast differentiation is progressively increased during the malignant progression of PT in breast. Therefore, we examined whether CCL18 induced the myofibroblast differentiation. We found that breast PT cells treated with 20ng/ml CCL18 had a significant increase in the mRNA and the protein levels of
α-SMA (Fig. 3A-C) and enhanced activity of collagen gel contraction, proliferation (Fig. 3D and E), migration and invasiveness (Fig. 3F and Supplementary Fig. S2A and B), suggesting that CCL18 induced myofibroblast differentiation and promoted the proliferation and invasion of the PT cells. Consistent with the results described above, when cocultured with primary TAMs and control IgG, the phenotype and function changes of PT cells were similar to CCL18 treated alone. On the other hand, anti-CCL18 neutralizing antibody inhibited myofibroblast differentiation, proliferation and invasion induced by TAMs (Figures 3A-F), indicating that the TAM released CCL18 is responsible for the myofibroblast differentiation of PT cells.

**CCL18 upregulates miR-21 expression by activating NF-κB**

Our previous study has shown that miR-21 expression levels are up-regulated in the malignant PTs. miR-21 not only induces the myofibroblast differentiation of PT cells, but also promotes their malignant properties, including proliferation and invasion (4). Thus we speculate that CCL18 may regulate the myofibroblast differentiation through upregulating miR-21. To further demonstrate this hypothesis, we first analyzed the expression correlation between miR-21(detected by ISH as in ref(4)) and CCL18, and revealed that the percentage of miR-21 positive cells was positively correlated with that of CCL18 producing cells in the 268 PT samples (Fig. 4A, r=0.752, p<0.001). Then we employed qRT-PCR to test the miR-21 level of PT cells in response to CCL18 treatment. Compared with the untreated group, the RNA level of miR-21 of CCL18-treated PT cells increased by 3.9-fold (Fig 4B).
A recent study showed NF-κB could bind to the promoter of miR-21 and regulate pancreatic β cells death through miR-21 (23, 24). As we have shown that CCL18 activated NF-κB pathway to induce EMT in breast cancer (25), we proposed that CCL18 might upregulate miR-21 expression via activating NF-κB. To clarify the roles of NF-κB in CCL18-induced miR-21 expression, the luciferase reporter assay was applied in PT cells. We found that NF-κB activity was escalated to a 6.2-fold increase in CCL18 treated cells. The TNF-α-treated PT cells were used as positive control (Fig 4C). Constantly, we revealed that CCL18-induced p65 nuclear translocation could be reversed by both NF-κB pathway inhibitors and siPITPNM3, suggesting CCL18 induced activation of NF-κB pathway through membrane-associated phosphatidylinositol transfer protein 3 (PITPNM3) (Supplementary Fig. S3A and B). The PITPNM3 RNAi efficiency was detected by western blot (Supplementary Fig. 3C). Again, we confirmed that CCL18 promoted NF-κB pathway via PITPNM3, as siPITPNM3 eliminated the phosphorylation of IKK and IKB in the presence of CCL18 (Supplementary Fig. S3D). Additionally, CCL18 upregulated the mRNA levels of several NF-κB target genes, including IL8, IL4, Twist, etc (Fig 4D). Collectively, these data suggest that CCL18 can enhance NF-κB transcriptional activity in PT cells.

To investigate whether NF-κB can directly regulate miR-21 expression, inhibitors of NF-κB pathway (BAY-117082, an IKK inhibitor which also interferes the ubiquitin conjugating enzymes (26), and JSH-23, an inhibitor of NF-κB nuclear translocation) were used. Both BAY-117082 and JSH-23 reversed CCL18-induced miR-21 upregulation in PT cells (Figure 4E), indicating that NF-κB activation is involved in
CCL18-mediated upregulation of miR-21. A ChIP-qPCR assay with antibodies against NF-κB p65 was used to determine if miR-21 is the direct target of NF-κB p65. CCL18 increased the binding of NF-κB p65 to the promoters of miR-21 by 12.7-fold (Fig. 4F). We next examined the functional relationships among CCL18, NF-κB and miR-21 in breast PT cells. When PT cells were treated by CCL18 for 24 hours, miR-21 promoter activity was increased by 8.6-fold (Fig. 4G). Taken together, our results indicate that CCL18 upregulates miR-21 expression via activating NF-κB.

CCL18 upregulates miR-21 expression and thus inducing myofibroblast differentiation via activating NF-κB

To investigate whether CCL18 upregulates miR-21 expression and thus induces myofibroblasts differentiation via activating NF-κB, PT cells were pretreated with BAY-117082 or JSH-23 for 1 hour or transfected with miR-21 antisense oligos (ASO) for 48 hours and then exposed to 20ng/ml CCL18 or co-cultured with TAMs for 48 hours. We found that CCL18 treatment increased the mRNA (Fig. 5A and C) and protein level of α-SMA (Fig. 5 B and D). However, the NF-κB inhibitor BAY-117082 or JSH-23 or the miR-21 inhibitor blocked the CCL18 or TAM-induced increase of α-SMA in the PT cells, indicating that myofibroblast differentiation was prevented under these conditions. Similarly, collagen contraction assay showed that CCL18 contracted collagen gels to a much greater extent than the untreated cells (P<0.01), whereas BAY, JSH or miR-21 ASO suppressed the contractile ability (P<0.01) (Fig. 5E and Supplementary Fig. S4A). We then evaluated the effect of NF-κB and miR-21
on the invasive ability of PT cells treated with CCL18 or cocultured with TAMs. Consistent with previous results, BAY, JSH and miR-21 ASO also abrogated the CCL18 or TAMs-promoted migration, invasion (Fig. 5E and Supplementary Fig. S4B) and proliferation (Fig. 5F and Supplementary Fig. S4C) of PT cells. These data suggest that CCL18 upregulates miR-21 expression and thus induces myofibroblast differentiation and promotes their tumorigenicity via activating NF-κB.

**CCL18 promotes AKT activation in myofibroblasts through NF-κB/miR21/PTEN axis**

Our previous work has revealed that mRNA of PTEN can be targeted by miR-21 in PT cells (4). As PTEN is the major negative regulator of PI3K-AKT pathway, which is most frequently mutated and inactivated in malignant tumors, we hypothesize CCL18 drives tumorigenicity of PT through activating PI3K-AKT pathway. Therefore, the phosphorylation of AKT was examined to explore whether this pathway is activated. We found that CCL18 treatment or TAM coculture decreased PTEN level and increased AKT phosphorylation followed by the elevated phosphorylated p65 (Fig.6A), suggesting the activation of PI3K-AKT pathway. When CCL18 antibody was added, the PI3K-AKT pathway kept inactive. Consistently, the NF-κB inhibitor (BAY-117082 or JSH-23) or the miR21 ASO could completely reverse the CCL18-enhanced AKT phosphorylation and increase PTEN level in PT cells (Fig.6B). These results suggested that the CCL18/NF-κB/miR-21 axis was necessary and sufficient for TAMs to activate PI3K-AKT pathway in PT cells. Then, we detected PTEN and p-AKT in human PT tumors by immunohistonchemistry and confirmed
that PTEN level decreased and p-AKT increased from benign to malignant PTs (Fig. 6C). Together, these data suggested that TAMs induced myofibroblast differentiation and promoted PT tumorigenecity through secreting CCL18 to activate PI3K-AKT pathway via NF-κB/miR-21/PTEN axis (Fig. 6D). We also explored which factor in the microenvironment promoted macrophages producing CCL18. We compared the cytokine expressed by the PT cells and found that hepatocyte growth factor (HGF) was highly secreted by malignant PT cells comparing with benign PT cells (Supplementary Fig.S5 A and B). Then a HGF-neutralized antibody was used to block the PT cell-secreted HGF in a transwell culture system with malignant PT cells and corresponding TAMs. We found that TAMs produced less CCL18 upon HGF-neutralized antibody treatment (Supplementary Fig.S5C).

M2 macrophages-secreted CCL18 accelerates tumor growth, induces myofibroblast differentiation and promotes metastasis of breast PT xenografts.

To investigate the role of CCL18 on tumor formation and progression in vivo, we inoculated benign PT cells and MDMs into the mammary fat pads of athymic nude mice (n = 10 per group), and evaluated tumor metastasis to the lungs and livers. When the xenografts became palpable, CCL18-specific neutralizing antibody (CCL18-Ab, 1mg/kg twice weekly) or isotype IgG (1mg/kg twice weekly) were injected via the tail vein twice weekly. Intratumor injection of M-CSF (0.2μg /kg twice weekly) was performed twice weekly after inoculating and 48 hr prior to antibody injection. We found that compared with MDMs inoculated alone, additional injection of M-CSF,
significantly increased the tumor formation efficiency ($p<0.01$, Fig. 7A) and accelerated tumor growth of xenografts ($p<0.01$, Fig. 7B). CCL18-specific neutralizing antibody markedly inhibited the tumor formation and lowered tumor growth of xenografts ($p<0.01$, Fig. 7A and B). These data suggest that CCL18 play an important role in the malignant transformation of breast PTs.

To further evaluate whether CCL18 regulates myofibroblast differentiation in vivo, we examined the protein levels of α-SMA, PTEN, p-Akt and Ki67 in the xenografts using immunohistochemistry (Fig. 7C). Similar to the results obtained in vitro, injection of M-CSF enhanced the levels of α-SMA p-Akt and Ki67 in the xenografts, but decreased the expression of PTEN, whereas CCL18-specific neutralizing antibody attenuated the upregulation of α-SMA, p-Akt or Ki67 in the xenografts, but increased the expression of PTEN (Fig. 7C). CD163 positive cells were significantly increased in M-CSF-treated MDMs group, indicating that M-CSF induced MDMs to M2 macrophages in the xenografts. As increased migration and invasion of tumor cells are linked with metastasis, we evaluated whether CCL18 promoted the metastasis of breast PT xenografts. Consistent with the findings in vitro, injection of M-CSF into the fat pads of the athymic nude mice activated MDMs to M2 macrophages, induced myofibroblast differentiation, promoted migration and invasion of breast PT, and significantly increased the number of metastatic nodules and human hypoxanthine-guanine-phosphoribosyltransferase (HPRT) mRNA in the livers (Fig. 7D, E and G). However, there was no significant lung metastasis observed in the mice according to histological examination (data not shown), wet weight of lung (Fig. 7F)
and human HPRT mRNA level (Fig. 7G). In addition to the M2 polarization of macrophages in the tumors, we also found that the peritoneal macrophage of the M-CSF-treated mice, especially of those from the group with the fastest tumor growth, showed M2 polarization. (Supplementary Fig.S6)

Discussion

Myofibroblasts are activated mesenchymal stromal cells that are differentiated from fibroblasts upon tissue injury. They contribute to tissue repair during wound healing by their strong contractility and extracellular matrix (ECM) secretory function. At the end stage of tissue repairing, myofibroblasts disappear by massive apoptosis (27). However, the excessive contraction and ECM protein secretion by myofibroblasts severely impair tissue repairing, such as in hypertrophic scars, scleroderma, and Dupuytren’s disease (28). In addition, continuous stimulation by toxic, infectious and metabolic agents or chronic inflammation leads to continuous myofibroblast differentiation and induces fibrosis in liver, kidney and heart (29, 30). Myofibroblasts were also found to promote invasion and metastasis of breast, pancreatic and colorectal cancer (31). In PTs, myofibroblasts derive from stromal fibroblasts and constitute the major malignant component of tumor mass. Transition of the mesenchymal fibroblasts to myofibroblasts (FMT) has been suggested as a key process in the tumorigenesis of PTs (4). TGF-β1 was shown to be able to induce FMT in fibrosis and thus played an important role in cancer progression (32, 33). In this study, we found that the infiltration of TAMs increased dramatically along with the transformation from benign to malignant PTs. TAMs in breast cancer are skewed to
M2 phenotype by secreting high levels of IL-10, CCL18 and CCL22 (25), and our previous studies have shown that the chemokine CCL18 released by TAMs induces epithelial-mesenchymal transition (EMT) and promotes metastasis of breast cancer (12). Here we went one step forward by showing that CCL18 from the TAMs of PTs is also a key regulator of FMT in myofibroblast differentiation. Blocking CCL18 by anti-CCL18 neutralizing antibody dramatically reverses the myofibroblast differentiation and thus inhibits PT growth in vivo. According to our results, the FMT in PTs is driven by CCL18 from TAMs, suggesting an essential role of TAMs in the process of FMT of PT malignant development.

It has been well documented that TAMs secrete CCL18 to promote cancer progression and metastasis, such as breast cancer (34), pancreatic cancer (35), and lung cancer (36), by activating PI3K/AKT (37), Lin 28 (38), and Nir1-ELMO1/DOC180 signaling pathways (39, 40). Our previous work revealed that CCL18 could serve as an NF-κB activator to promote breast cancer progression (25). In this study, we showed that TAMs induced myofibroblasts differentiation and promoted the malignant progression of breast phyllodes tumors via secreting CCL18. Furthermore, CCL18 level can serve as an independent prognostic factor of phyllodes tumors. The NF-κB pathway has been identified as a key regulator of fibroblast function and matrix remodeling (41). Activated NF-κB pathway is involved in maintaining myofibroblast phenotype (42, 43). However, whether NF-κB pathway is involved in the FMT process and promotes tumor progression, especially in PTs, remains obscure. In the present study, we have revealed that the NF-κB pathway was
activated by CCL18 in myofibroblast. Subsequently, the nuclear translocated p65
binds to the promoter of miR-21 gene and increases its transcription in the
myofibroblast. We have previously illustrated that miR-21 induces myofibroblast
differentiation and promotes the malignant progression of PTs (4). Thus, miR-21 is a
key downstream mediator of p65 in TAM-induced FMT of myofibroblast
differentiation.

It is well established that miR-21 is involved in cellular survival, invasiveness and
apoptosis by suppressing specific target genes as PTEN, PDCD4, RECK, TIMP3,
Smad3 et al (44-46). Our previous data suggested that Smad 7 and PTEN are targets
of miR-21 in phyllodes tumors (4). PI3K/AKT pathway is most frequently activated
in breast cancer. A case report based on whole genome sequencing revealed N-RAS
mutation with concomitant activation of PI3K/Akt/mTOR in phyllodes tumor (47),
suggesting the PI3K/AKT pathway may contribute to myofibroblast transition in PTs.
Indeed, CCL18/NF-κB/miR-21 axis between TAMs and PT cells decreases PTEN
level and further activates AKT. Therefore, an intercellular communication, where
TAMs secreted CCL18 to activate the NF-κB/miR-21/PTEN/AKT pathway in
myofibroblasts, is established (Fig 6D). Our previous data and the present study have
demonstrated that CCL18 and miR21 play major roles in myofibroblast transition of
PTs, suggesting that the above intercellular communication pathway from TAM to
myofibroblast may serve as a major driver of tumorigenicity in phyllodes tumors.

Even with surgical resection, local recurrence rate of PTs is still as high as 8% to 36%
(48). Moreover, recurrent phyllodes tumors are frequently more aggressive in
Our findings demonstrate that CCL18 drives PT progression by inducing FMT of the stromal cells. Higher CCL18 level dramatically correlates with local or distal recurrence of the malignancy and is a better prediction factor for recurrence compared with other clinical and pathological markers of PTs (Supplementary Table S1 and S2). Given that chemotherapy or radiotherapy is not effective against phyllodes tumors, there is a pressing requirement to develop new therapeutic strategy. In the present study, our in-vivo findings show that blocking CCL18 with neutralizing antibody effectively shrinks PTs in mouse xenografting models, which suggests that antagonizing the CCL18 signaling may emerge as a promising strategy to treat PTs. Together, our data suggest that the intercellular communication between TAMs and myofibroblasts via CCL18/NF-κB/miR-21/PTEN/AKT axis plays a central role in the tumorigenesis of PTs. Monitoring CCL18 level and targeting this pathway raise the possibility of precision diagnosis and treatment for breast phyllodes tumors.

References


Figure Legends

Fig. 1. TAMs density is associated with malignant progression of PTs.

(A) Immunohistochemical (IHC) staining shows both CCL18 and its corresponding receptor PITPMN3 are elevated in the malignant breast PTs. Scale bar, 20μm.

(B) Microscopy for immunostaining of normal breast tissue and breast PT tissues shows CCL18 is co-localized with CD163, the marker of M2 macrophages. Anti-CD163 antibody is labeled as red, Anti-CCL18 antibody is labeled as green. Cell nuclei are counterstained with DAPI (blue). Scale bar, 20μm.

(C) Real-time qRT-PCR demonstrates that the expression of CCL18 is increased in PT tissues. RNA were extracted from normal breast tissues or breast PTs. Bars indicate RNA expression normalized to GAPDH ± SD of 8 samples in each group. ***. p<0.001, **, p<0.01 and *, p<0.05 as compared with normal breast tissue.

(D) Serum CCL18 level is increased in the patients of breast PT, as determined by ELISA. Bars correspond to mean ± SD. ***, p<0.001, **, p<0.01 and *, p<0.05.

(E) Kaplan-Meier overall survival (OS) curve of patients with low and high CCL18 with a median follow-up period of 112 months.

(F) Kaplan-Meier disease-free survival (DFS) curve of patients with low and high CCL18 with a median follow-up period of 112 months.

Fig. 2. TAMs induce the myofibroblast differentiation and promote the growth and invasion of PT cells.

(A) The RNA level of α-SMA in benign PT cells (5x10^5/well) increases after the cells were cocultured with M-CSF (50ng/ml) pretreated MDMs (5x10^5/well) or primary
TAMs (5x10^5/well) for 3 days. Peripheral blood monocytes (PBM)s from healthy
donors were isolated and then induced to M2 phenotype by M-CSF for 6 days. 8 cases
of benign PT cells were isolated from 8 benign PT tissues. TAMs were isolated from
human malignant PT tissue. Bars correspond to means ± SD of 8 samples with
experimental triplicates for each sample.

(B) Western blot shows the protein level of α-SMA in benign PT cells isolated and
treated as in A.

(C) Immunofluorescent staining of α-SMA and FAP in benign PT cells isolated and
treated as in A. Scale bar, 20um.

(D) Collagen gel contraction is measured in PT cells isolated and treated as in A. Bars
correspond to means ± SD of 8 samples with experimental triplicates for each sample.

(E) Colony formation assays in PT cells isolated and treated as in A. Bars correspond
to means ± SD of 8 samples with experimental triplicates for each sample.

(F-G) Migration and invasion assays of PT cells isolated and treated as in (A). Scale
bar, 50um. Bars correspond to means ± SD of experimental triplicates from one case
of primary cells.

** p < 0.01 as compared with the untreated benign PT cells.

Fig. 3. TAMs induce myofibroblast differentiation and promote the growth and
invasion of PT cells via CCL18.

(A) The RNA level of α-SMA in PT cells. The primary benign PT cells were isolated
from benign PT (5x10^5/well) and were cultured alone, or treated with 20ng/ml CCL18
for 24 hours, or cocultured with primary TAMs (5x10^5/well) for 3 days. Primary
TAMs were isolated from malignant PT. Indicated control IgG (10μg/ml) or anti-CCL18 neutralizing antibody (10μg/ml) was added into the experiment simultaneously as the cells were cocultured with primary TAMs. The primary malignant PT cells (5x10⁵/well) were used as the positive control.

(B) The protein level of α-SMA in malignant and benign PT cells isolated and treated as in A.

(C) Immunofluorescent staining of α-SMA and FAP in malignant and benign PT cells isolated and treated as in A. Scale bar, 20μm.

(D) Representative images of collagen gel contraction and colony formation assay for benign and malignant PT cells isolated and treated as in A.

(E) Collagen gel contraction and colony formation was measured in PT cells isolated and treated as in A.

(F) Representative images of Boyden chamber assay for migrated and invaded PT cells isolated and treated as in A. Scale bar, 50μm.

Bars correspond to means ± SD of three samples with experimental triplicates.

Fig. 4. CCL18 upregulates miR-21 expression via activating NF-κB.

(A) Percentage of miR-21+ cells is correlated with the percentage of CCL18+ cells in breast PT samples. 268 PT samples, including 167 benign, 36 borderline and 65 malignant PT are used. Percentage of miR-21+ cells in each sample is detected by in situ hybridization. Percentage of CCL18+ cells in each sample is determined by IHC.

(B) Real-time qRT-PCR revealed that the expression of miR-21 is upregulated by
CCL18 treatment in PT cells. The primary PT cells were isolated from benign PT and then treated with CCL18 for 24 hours. The miR-21 levels were indicated as RNA expression normalized to U6. Bars correspond to mean ± SD of 8 samples with three experimental triplicates in each group, **, p<0.01 as compared with PBS group.

(C) NF-κB activity increases upon the treatment of CCL18 and TNF-α. NF-κB activity is exhibited by luciferase reporter assay. (mean ± SD, n = 8, ***, p < 0.001 and **, p < 0.01 versus PBS).

(D) Real-time qRT-PCR shows the expression of NF-κB target genes are upregulated by CCL18. Cell were isolated and treated as described in B. Bars correspond to mean ± SD of 3 samples with three experimental triplicates in each group. **: p<0.01, ***: p<0.001, versus PBS).

(E) Real-time qRT-PCR reveals that CCL18-induced miR-21 expression can be reversed by NF-κB inhibitor. Primary cells isolated from benign PT were pretreated with DMSO, BAY-117082, or JSH-23 for 1 hour and then CCL18 for 24 hours before harvest. Bars correspond to means ± SD of 3 samples with three experimental triplicates in each group. ***, p < 0.001 compared with cells treated with PBS; ##, p < 0.01 compared with cells treated with CCL18.

(F) CCL18 induces p65 to bind to miR-21 promoter. Primary benign PT cells were treated with PBS or CCL18 for 30 min. The ChIP assay was performed by using anti-p65 antibody to pulldown p65 bond DNA fragments. RT-PCR was used to determine amount of the precipitated DNA. Bar represents mean ± SD of experimental triplicates, ***, p < 0.001 versus PBS).
(G) CCL18 promotes miR21 transcription activity. Luciferase reporter assays were performed in benign PT cells transfected with reporter plasmids containing miR-21 promoter and treated with PBS or CCL18 for 24 hr. (Bar represents mean ± SD of experimental triplicates. p < 0.001 versus PBS).

**Fig.5. CCL18 upregulates miR-21 expression and thus induces myofibroblast differentiation via activating NF-κB.**

(A-B) α-SMA in benign PT cells can be induced by CCL18 and reversed by NF-κB inhibitor or miR21 ASO. Benign PTs cells were isolated as in Fig 3A and then pretreated with DMSO, BAY-117082, or JSH-23 for 1 hour or transfected with lin4 ASO (NC), lipofectamine alone (mock) or miR-21 ASO for 24 hours before treated by 20ng/ml CCL18 for 24 hours. The RNA and protein level of α-SMA in benign PT cells were determined by RT-PCR (A) or western blot (B). Bars correspond to means ± SD of experimental triplicates.***, P<0.001 versus untreated PT cells, ###, P<0.001 versus without pretreated PT cells.

(C-D) α-SMA in benign PT cells can be induced by coculture with TAMs and reversed by NF-κB inhibitor or miR-21 ASO. Benign PTs cells were isolated as in Fig 3A and then pretreated with DMSO, BAY-117082, or JSH-23 for 1 hour or transfected with lin4 ASO (NC), lipofectamine alone (mock) or miR-21 ASO for 24 hours before coculture with TAMs (5x10^5/well) for 3 days. The RNA and protein level of α-SMA in benign PT cells were determined by RT-PCR (C) or western blot (D). Bars correspond to means ± SD of experimental triplicates. ###, P<0.001 as compared with untreated PT cells;
(E) Representative images of collagen gel contraction and Boyden chamber assay for benign PT cells treated as in A and C. CCL18 or coculture with TAMs promotes the indicated ability of PT cells, while NF-κB inhibitor or miR21 ASO can reverse the enhanced malignant behaviors. Scale bar, 50μm.

(F) Colony numbers of benign PT cells treated as in A (left) and C (right).

Fig 6. CCL18 promotes AKT activation through NF-κB/miR-21/PTEN axis

(A) PTEN is decreased and p-AKT is elevated at protein level upon CCL18 treatment. Benign PT cells are cultured alone, or treated with 20ng/ml CCL18 for 24 hours, while control IgG (10μg/ml) or anti-CCL18 neutralizing antibody (10μg/ml) is introduced into the experiments simultaneously as indicated. Isolated primary malignant PT cells are used as positive control.

(B) Western blotting reveals that CCL18 induced changes of PTEN and p-AKT levels can be reversed by NF-κB inhibitor or miR-21 ASO. Benign PT cells were pretreated BAY-117082, or JSH-23 for 1 hour or transfected with lin4 ASO (NC), lipofectamine alone (mock) or miR-21 ASO for 24 hours and then treated with 20ng/ml CCL18 for 24 hours.

(C) Immunohistochemical (IHC) staining in paraffin-embedded normal breast tissue and breast PT reveals that PTEN is down-regulated and p-AKT is unregulated along with increased malignancy in PT. Scale bar, 20μm.

(D) The working model of how macrophages release CCL18 to promote PT tumorigenesis via NF-κB/miR-21/PTEN/AKT axis.

Fig 7. CCL18 accelerate tumor growth, induce myofibroblast differentiation and...
promote metastasis of breast PT xenografts.

(A) Xenografts of indicated groups in nude mice, harvested 60 days post injection. Benign PT cells (indicated as “B”) and MDMs were injected into the mammary fat pads of athymic nude mice. Mice were injected with control IgG (IgG) (1mg/kg twice weekly), or anti-CCL18 neutralizing antibody (CCL18-Ab, 1mg/kg twice weekly), via the tail vein as indicated after the xenografts became palpable. Intratumor injection of M-CSF (0.2μg /kg twice weekly) was performed twice weekly after inoculating and 48 hr prior to antibody injection. (n=10 per group). When cocul trued with M-CSF activated MDMs, the tumors from benign PT grow much faster and show a more aggressive phenotype than the other group. The CCL18 neutralizing antibody can prevent the activated MDMs enhanced aggressive manner of benign PT.

(B) Tumor size during the course of each indicated treatment. Error bars show ±SEM. (n=10 per group), **, P<0.01 versus benign PT cells inoculated alone, ##, P<0.01 versus control IgG group, with M-CSF treated.

(C) Representative images of H&E staining for xenografts and IHC staining for α-SMA, PTEN, p-Akt, ki67 and CD163 as described above. Scale bar, 50μm.

(D) Representative images of H&E staining for xenografts as described above, showing the presence or absence of liver metastasis. Scale bar, 50μm.

(E) The number of metastatic liver nodules in mice xenografted as indicated. The bars correspond to the mean ± SD. ***, P<0.001 versus benign PT cells inoculated alone. ##, P<0.01 versus control IgG group, with M-CSF treated.

(F) Wet lung weights in tumor-bearing mice. Bars correspond to mean ± SD.
(G) Expression of human HPRT mRNA relative to mouse 18S rRNA in the lungs and livers of mouse xenografts as described above, **, P<0.01 versus benign PT cells inoculated alone, ##, P<0.01 versus control IgG group, with M-CSF treated.
Figure 3

A

$\alpha$-SMA mRNA level relative to control

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B

$\alpha$-SMA

GAPDH

C

benign

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Contraction (mm)

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Tumor-associated macrophages promote malignant progression of breast phyllodes tumors by inducing myofibroblast differentiation

Yan Nie, Jia-Ning Chen, Di Huang, et al.

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