The pancreatic cancer secreted REG4 promotes macrophage polarization to M2 through EGFR/AKT/CREB pathway

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Abstract. In the periphery of pancreatic ductal adenocarcinoma (PDAC), high accumulation of tumor-associated macrophages (TAMs), which exhibit M2 phenotype, has been shown to be correlated with extra-pancreatic invasion, lymph vessel invasion, lymph node involvement and shortened survival time. However, mechanisms by which tumor cells educate and reprogram TAMs remain largely unclear. The phenotype of TAMs in PDAC tissues was confirmed by immunofluoresence and confocal microscopy. Human CD14+ monocytes were incubated with recombinant human REG4 (rREG4) before being stimulated with LPS and IL-10 and IL-6 were measured with ELISA. A panel of M1 and M2 genes were measured by quantitative real-time PCR. Panc1, AsPC1 and BxPC3 cells were cultured in the conditioned medium (CM) and treated with REG4. The macrophages were infected with CREB shRNA or cultured by the CM of Panc1 cells infected with REG4 shRNA. The expression of CD163, CD206 and REG4 and the phosphorylation levels of epidermal growth factor receptor (EGFR), AKT and cAMP response element-binding protein (CREB) in cells were assessed with western blotting. Cell proliferation and invasiveness were also assessed. The rREG4 or the conditioned medium of Panc1 cells which secreted REG4 induced the polarization macrophages to M2 phenotype. Treatment of human macrophages with REG4 resulted in phosphorylation of EGFR, AKT and CREB. The latter was responsible for REG4-mediated macrophage polarization to M2. The conditioned medium of macrophages treated with rREG4 promoted the proliferation and invasion of pancreatic cancer cell lines. REG4, overexpressed in PDAC and secreted by cancer cells, promoted macrophage polarization to M2, through at least in part, activation of ERK1/2 and CREB and changed the microenvironment to facilitate cancer growth and metastasis.

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Abbreviations: PDAC, pancreatic ductal adenocarcinoma; TAMs, tumor-associated macrophages; CM, conditioned medium; EGFR, epidermal growth factor receptor; CREB, cAMP response element-binding protein; REG, regenerating islet-derived; PBMC, peripheral blood mononuclear cells; FBS, fetal bovine serum; HRP, horseradish peroxidase

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease. Its aggressiveness contradicts the finding that majority of the tumor volume is not composed of neoplastic cells, but mediates the stroma/desmoplastic reaction to the cancer (1,2). In recent years, it has been well established that the desmoplastic reaction present in PDAC is not just a bystander, but also it is a source of different cellular and acellular factors that promote tumor progression, immunosuppression and metastasis (3,4). As a major immune cell type enriched in the stroma, tumor-associated macrophages (TAMs) play a key role in cancer-related inflammation, immune escape, matrix remodeling and metastasis (5-7). Increased numbers of TAMs in many cancers are linked to reduced patient survival. In PDAC, high accumulation of TAMs in the periphery of the tumor is correlated with extra-pancreatic invasion, lymph vessel invasion, lymph node involvement and shortened survival time (8). Furthermore, the phenotype of TAMs is likely to change with the stage of tumor progression. M1 macrophages are mainly abundant in chronic inflammatory sites, where tumors are initiated and start to develop (9,10). However, during cancer progression, macrophages switch to an M2-like phenotype as the tumor begins to invade, vascularize and develop (11-13). In agreement with these findings, more M2 converted macrophages are found in patients with pancreatic cancer compared to patients with chronic pancreatitis. High numbers of M2 macrophages are also associated with larger tumor size, early recurrence in the liver, local recurrence and shortened survival in patients with pancreatic cancer (8). These findings underline

the plasticity of macrophages within the pancreas depending on their activation. However, mechanisms by which tumor cells modulate the phenotype of TAMs remains largely unclear.

The regenerating islet-derived (REG) family comprises secreted proteins that play a role in tissue regeneration and inflammation in digestive organs (14-16). Several studies have shown an upregulation of REG4 in pancreatic, colorectal, gastric, gall bladder and prostate cancer tissues compared to paired normal mucosa (17-22). REG4 is highly expressed in poorly differentiated pancreatic cancer cells, but is modestly expressed in moderately and well differentiated pancreatic cancer cells (23). As a secreted protein, REG4 can be detected in the serum; however, compared with healthy controls, serum levels of REG are higher in pancreatic cancer patients (24). The secreted REG4 appears to play a role in the invasiveness of PDAC, as recombinant REG4 significantly promotes the proliferation and invasiveness of pancreatic cancer cells (23). Studies in other cancer types, such as colorectal (25-27) and gastric cancer (28-30), also indicate an important role of REG4 in cancer proliferation and metastasis. However, as a protein secreted by cancer cells, whether REG4 plays a role in the differentiation or polarization of TAMs remains largely unclear.

In the present study, we demonstrated that recombinant REG4 or the conditioned medium of Panc1 cells, which secreted relative high level of REG4, induced the polarization of macrophages to M2 phenotype. We further showed that REG4 activated the epithelial growth factor receptor (EGFR)/AKT/cAMP response element binding protein (CREB) signaling cascade, which was required for REG4-mediated M2 polarization. Finally, we showed that the conditioned medium of macrophages treated with recombinant REG4 promoted the proliferation and invasion of pancreatic cancer cell lines. These data provided the first evidence for the involvement of REG4 in the polarization of TAMs.

Materials and methods

Pancreatic cancer cell culture and conditioned medium (CM). Three pancreatic cancer cell lines, including Panc1, AsPC1 and BxPC3, were purchased from the Chinese Academy of Sciences Committee, Type Culture Collection Cell Bank. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) in 5% CO_2 atmosphere at 37˚C. All experiments were performed in glucose-free DMEM supplemented with 5 mM D-glucose, 2 mM L-glutamine and antibiotics as above (serum-free media, SFM). CM was obtained by culturing Panc1 cells to 70-80% confluence, washing twice and changing to SFM. CM was collected after 48-h incubation, residual cells removed by centrifugation and aliquots stored at -80˚C until further use.

Monocyte purification, differentiation and CM preparation. Human monocytes were isolated and purified from peripheral blood mononuclear cells (PBMC) of buffy coats obtained from healthy donors under an IRB-approved protocol according to the manufacturer's instructions (GE Healthcare). After isolation from the PBMC using a CD14⁺

selection kit (Miltenyi Biotech, GmBH, Bergisch Gladbach, Germany), monocytes were cultured at a concentration of 2x10⁵/ml in RPMI-1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 20 μM beta-mercaptoethanol (all reagents were from Life Technologies, Shanghai, China). CD14⁺ cells (>95% monocytes) were cultured for 6 days in RPMI-1640 with 40% human plasma with GM-CSF (0.5 ng/ml) under 5-10% O_2 and 5% CO_2 at 37˚C in a humidified atmosphere in a chamber flushed with N_2 under the control of a ProOX sensor and ProCO₂ regulator (BioSpherix, Ltd., Lacona, NY, USA). Cells were then activated for 24 h with REG4 (10 nM, unless otherwise indicated). On day 2, the CM was collected, centrifuged, filtered and stored at 20˚C in aliquots. These were added to pancreatic cancer cells in the dose of 20%. The study was approved by the Ethics Committee of Shanghai Tenth People's Hospital fully complied with the Treaty of Helsinki. The participants in the present study provided informed consent with the 'Ethics, consent and permissions' heading.

Lentiviral particles and cell infection. The lentiviral REG4 shRNA, targeting human REG4 (NM_001159352), was purchased from Applied Biological Materials (Richmond, BC, USA). The lentiviral CREB shRNA, targeting human CREB (NM_004379) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For lentiviral infection, cells (1x10⁵) in a 6-well plate were transfected with the lentiviral vector in the presence of 8 mg/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA). Forty-eight hours after infection, the medium was replaced with 2 ml of complete culture medium. The expression of REG4 or CREB was examined with western blot analysis.

ELISA cytokine analysis. ELISA kits for detection of secreted IL-6 and IL-10 in cell culture supernatants were purchased from R&D Systems and used according to the manufacturer's instructions.

RNA isolation, cDNA synthesis and RT-PCR. Total RNA was extracted from cells with an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions and subsequently transcribed into cDNA using PrimeScript® RT reagent kit (Bio-Rad Laboratories). Real-time PCR reactions were performed using an ABI 7900HT (Applied Biosystems) with customized primer sets for human M1 and M2 macrophage marker genes (*p40, CXCL19, IL2RA, CXCL10, p35, PLA1A, IDO, p19, CXCL11, IL6, CD1A, CXCL8, FAS, CCL5, TNFα, PDGF, CCR7, IGFBP4, IL15, AK3, TRAIL, IL18, BCL2A1, CD80, CD83, CD54, CD40, CD206, CERK, FCGR3A, CCL23, FN1, CD209, SLC11A9, LTA4H, CHN2, MS4A6A, DCL1, MSR1, FCGR1A, HRH1, CD36, CD163, EGR2, IL10, TGFβ1, IGF1, ALOX5* and *SLC4A7*). Data were analyzed using Bio-Rad's CFX Manager 2.0, and several housekeeping genes (GAPDH, HPRT, β-actin and 18S) were used for normalization. All experiments were related to untreated control. Heatmaps were generated in R (www.r-project.org/) using donor median values. Rows and columns were subjected to unsupervised clustering using the distance

function: 1-cor(t(x)) applying Spearman's correlation. The color gradients are scaled for each gene.

In vitro invasion assay. Appropriate Matrigel (Becton-Dickinson, Franklin Lakes, NJ, USA) was added into the upper chamber of the Transwell apparatus with 8-μm pore size membrane (Costar, Cambridge, MA, USA). After the Matrigel solidified at 37˚C, 1x10^5 cancer cells were seeded onto the Matrigel and incubated at 37˚C overnight. Membranes coated with Matrigel were removed with a cotton swab and fixed with 100% methanol for 10 min. Giemsa stain (Sigma Chemicals) was added to the membrane with cells for 3 h and then washed with distilled water. The number of cells attached to the lower surface of the polycarbonate filter was counted at x400 magnification under a light microscope. Each type of cell was assayed in triplicate.

Cell proliferation assay. Cells plated in a 96-well plate (2,000 cells/well) were incubated for 1-7 days at 37˚C in an atmosphere of 5% CO_2 in air. Thereafter, 20 μl of CCK-8 solution was added to each well and incubated for an additional 4 h in the same condition. Then, absorbance at 450 nm was measured using a microplate reader, wherein the absorbance value indicated the proliferative capacity.

Western blotting. Human M1 or M2 cells, growing in 6-well plates, were treated with 10 nM CCL19 or CCL21 for different time intervals. Cells were lysed in ice-cold lysis buffer containing a protease inhibitor cocktail (Roche, Basel, Switzerland). The protein extracts were denatured in a boiling water bath for 5 min and then resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes which were probed with primary antibodies overnight at 4˚C, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 60 min. Target proteins were detected with a chemiluminescence kit. Primary antibodies used include those against AKT (BD Biosciences, Franklin Lakes, NJ, USA), phospho-AKT, EGFR, phospho-EGFR, CREB (Cell Signaling Technology, Danvers, MA, USA), CD163, CD206, REG4 and β-actin (Sigma).

Immunofluoresence and confocal microscopy. All primary pancreatic cancer tissues and adjacent non-cancerous tissues were obtained from surgery with informed consent from the patients. Tissues were frozen in liquid nitrogen immediately after surgical removal. Human tumor sections (5 μm) were deparaffinized, rehydrated, and treated with 10 mM citrate buffer (pH 6.0) at 95˚C to retrieve antigens. After quenching endogenous peroxidase activity with H_2O_2 and blocking with 10% normal horse serum, the sections were incubated with an antibody mixture containing a mouse monoclonal anti-CD163 (Abcam) and a rabbit polyclonal anti-Iba1 (Abcam), followed by incubation with a mixture of Alexa Fluor® 488 goat anti-mouse IgG and Cy3-conjugated goat anti-rabbit IgG. The slides were cover slipped using a mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) to identify the nuclei. Images were observed and captured using an LSM 710 laser scanning microscope (Carl Zeiss, Oberkochen, Germany)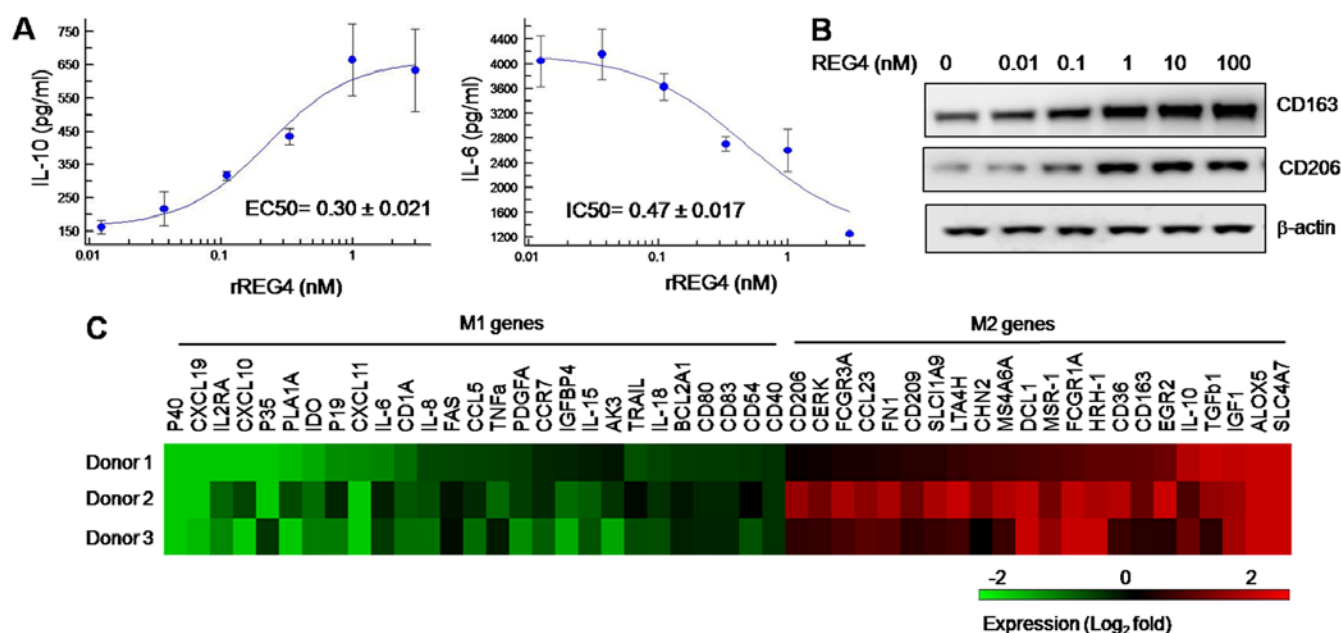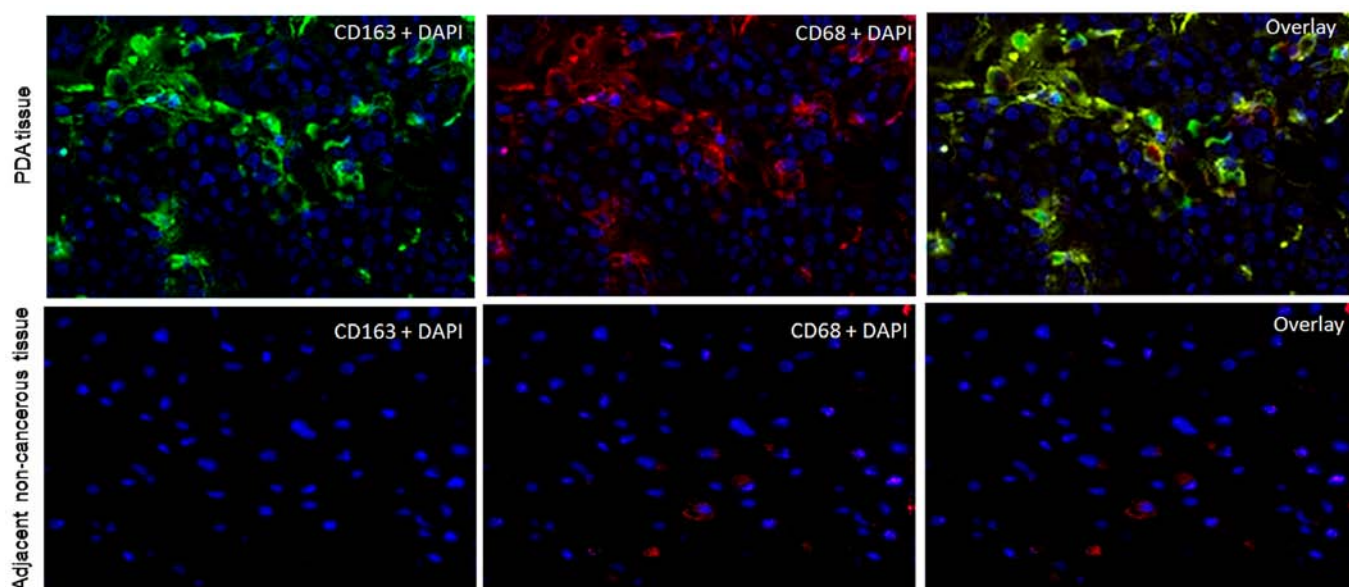 with a 40x1.3 numerical aperture oil immersion lens. All digital images were captured at the same settings. Final images were processed using Adobe PhotoShop software.

Statistical analysis. The results were analyzed using the GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA) and were presented in the form of mean ± SEM. Unpaired Student's t-test and paired Student's t-test and one-way ANOVA with Tukey's post-hoc tests were used to determine statistic significance. P-value <0.05 was considered statistically significant.

Results

TAMs are M2 type in PDAC tissue. Previous studies have shown that TAMs in PDAC tissues are predominantly M2 type, and high density of M2 type TAMs is associated with large tumor size, early recurrence in the liver, local recurrence and shortened survival time (8). To confirm the phenotype of TAMs in PDAC tissues, paraffin sections (5 μm) of PDAC tissues and adjacent non-cancerous tissues were immunostained with a mixture of anti-CD68 (pan macrophage marker) and anti-CD163 (M2 macrophage marker), and the distribution of macrophages was observed with confocal microscopy. As shown in Fig. 1, a large number of CD68 positive macrophages were observed in the PDAC tissues, and the majority of these cells expressed CD163, suggesting that these TAMs are M2 type. In contrast, though a few CD68 positive macrophages were detected in the adjacent non-cancerous tissue, these cells were negative for CD163. Thus, our data support the previous findings regarding the M2 phenotype of TAMs in PDAC tissue.

Recombinant REG4 induces macrophage polarization to M2. To examine the effect of REG4 on macrophage polarization, human CD14$^+$ monocytes from 3 different independent donors were incubated with GM-CSF for 6 days followed by incubation with various concentrations of recombinant REG4 (rREG4) for 1 day, before being stimulated with LPS (10 ng/ml) for 6 h to boost the production of cytokines. IL-10 (M2 cytokine) and IL-6 (M1 cytokine) were measured with ELISA. As shown in Fig. 2A, rREG4 dose-dependently promoted the production of IL-10 (EC_{50}=0.30±0.021 nM) and suppressed the production of IL-6 (IC_{50}=0.47±0.017) in human macrophages. To confirm that the rREG4-induced cytokine change reflects the polarization of macrophages to M2 phenotype, the GM-CSF-differentiated macrophages were incubated with or without rEG4 (1 nM) for 24 h, and the expression of CD163 and CD206, two specific M2 macrophage markers, was assessed with western blot analysis. As shown in Fig. 2B, rREG4 treatment resulted in a significant increase in the cell surface expression of CD163 and CD206. The effect of rREG4 on macrophage polarization was further confirmed with the measurement of a panel of M1 and M2 genes using quantitative real-time PCR. As shown in Fig. 2C, treatment of the GM-CSF-differentiated macrophages with rREG4 (1 nM) resulted in enhanced expression of a number of M2 genes, including CD206, CD209, CD163, IL-10 and IGF-1. Together, these data suggest that REG4 promotes macrophage polarization to M2.

Figure 1. The phenotype of TAMs in PDAC. PDAC cancer tissues (5 μm) or adjacent non-cancerous tissues were co-immunostained with an anti-CD163 (green) and an anti-CD68 (red), and counterstained with DAPI (blue). Shown are representative confocal images of 5 different PDAC tissues and corresponding adjacent non-cancerous tissues with similar results.

Figure 2. REG4 induces macrophage polarization to M2. (A) Cultured macrophages were treated with different concentrations of REG4 for 24 h before being stimulated with LPS (10 ng/ml) for 6 h. IL-10 and IL-6 in the culture medium were measured with ELISA. Data are mean ± SEM from three independent experiments. (B) Cultured macrophages were treated with different concentrations of REG4 for 24 h and the expression levels of CD163 and CD206 were measured with western blot analysis. Shown are representative images of three independent experiments with similar results. (C) Gene expression analysis of selected M1- and M2-associated transcripts in macrophages after being treated with REG4 for 24 h. Heatmaps depict relative expression levels.

REG4-induced EGFR/AKT/CREB signaling pathway is involved in macrophage polarization to M2. To examine whether an intracellular signal triggered by REG4 is involved in the effect of REG4 on M2 macrophage polarization, we measured the phosphorylation levels of EGFR, AKT and CREB. REG4 (10 nM) treatment time-dependently induced the phophorylation and activation of EGFR and AKT (Fig. 3A). Moreover, REG4 treatment induced phosphorylation of CREB (at Ser133) in a time-dependent manner, which peaked 30 min

after REG4 stimulation (Fig. 3B). Notably, the REG4-mediated phosphorylation of CREB was blocked by 10 μM of the AKT inhibitor LY294002 (Fig. 3C and D). To determine whether CREB is involved in the M2 promoting effect of REG4, the GM-CSF-differentiated macrophages were infected with lentiviral particles containing a scramble shRNA or CREB specific shRNA for 48 h before being incubated with REG4 for 24 h. Intriguingly, the lentiviral shRNA-mediated knockdown of CREB blocked rREG4-induced expression of CD163 and
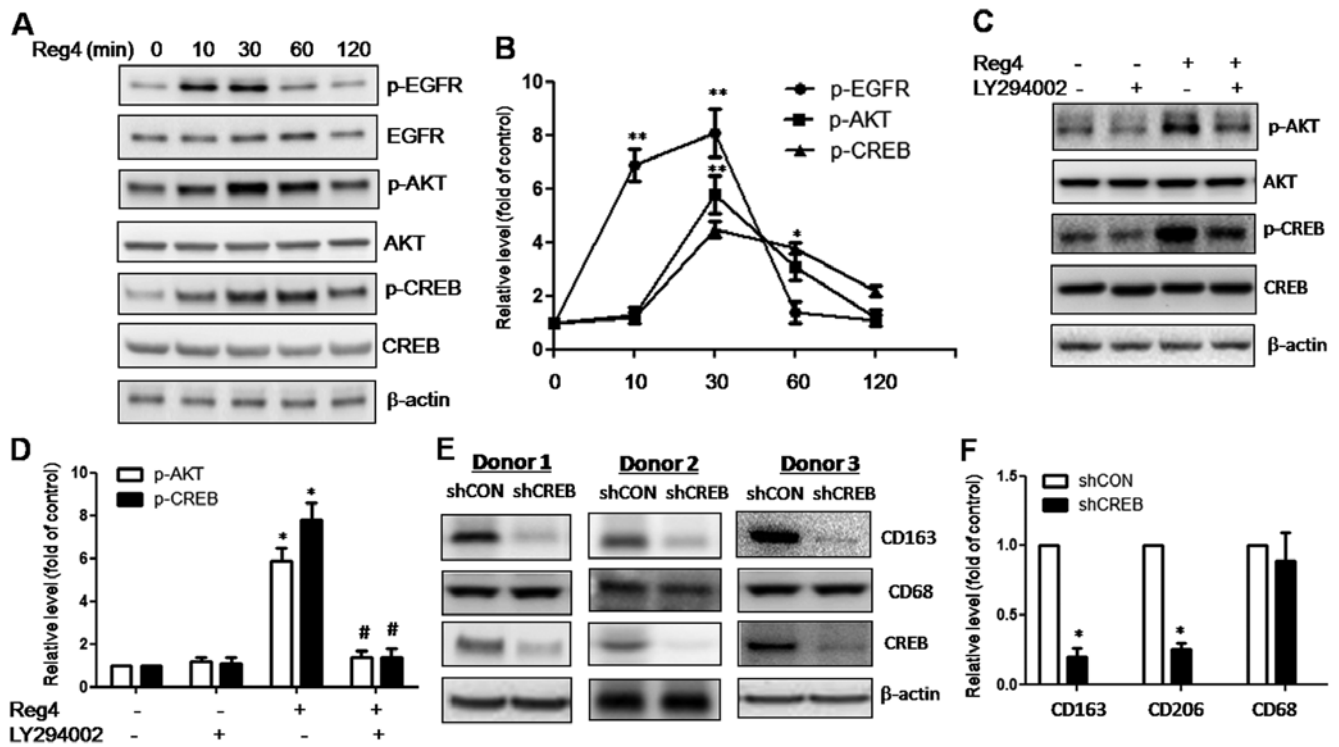
Figure 3. Involvement of EGFR/AKT/CREB pathway in REG4-mediated macrophage polarization to M2. (A) Representative western blots of phosphorylated EGFR, AKT and CREB in macrophage cultures treated with REG4 (10 nM) for different time intervals indicated. (B) Quantitative analysis of the immunoblots of p-EGFR, p-AKT and p-CREB normalized with EGFR, AKT and CREB, respectively. Data are mean ± SEM from four independent experiments. *P<0.05, **P<0.01 compared to untreated cells. (C) Representative western blotting images showing the phosphorylation of EGFR, AKT and CREB in macrophages cultures treated with or without REG4 (10 nM) for 30 min in the absence or presence of LY294002. (D) Quantitative analysis of the immunoblots of p-EGFR, p-AKT and p-CREB normalized with EGFR, AKT and CREB, respectively, in macrophages cultures treated with or without REG4 (10 nM) for 30 min in the absence or presence of LY294002. Data are mean ± SEM from four independent experiments. *P<0.05 compared to untreated cells; $^{#}$P<0.05 compared with REG4 treatment alone. (E) Representative western blots of CD163, CD68, and CREB in REG4-treated macrophages which were infected with control shRNA (shCON) or shCREB lentiviral particles. (F) Quantitative analysis of the immunoblots of CD163, CD68 and CREB in REG4-treated macrophages which were infected with control shRNA (shCON) or shCREB lentiviral particles. Data are mean ± SEM from three independent donors. *P<0.05 compared with control.

induced the expression of CD68, a specific M1 macrophage marker (Fig. 3E and F). These data suggest that activation of EGFR/AKT/CREB pathway plays an important role in REG4-mediated polarization of macrophages to M2 phenotype.

REG4 secreted from Panc1 pancreatic cancer cell line induces macrophage polarization to M2. To confirm that REG4 secreted from pancreatic cancer cells also induce macrophage polarization to M2 phenotype, three pancreatic cancer cell lines which produce different levels of REG4 were used. A previous study showed that Panc1 cells can produce high level of REG4 whereas AsPC1 cells and BxPC3 cells produce low level of REG4 (23). Using western blot analysis, we observed that Panc1 cells produced high level of REG4 in both the cells and culture medium. Compared to Panc1 cells, AsPC1 cells expressed a markedly lower level of REG4, and BxPC3 cells expressed the lowest level of REG4. Whereas, a very low level of REG4 was detected in the culture medium of AsPC1 cells, no REG4 was detected in the culture medium of BxPC3 cells (Fig. 4A). After incubation of macrophages with the CM of the three pancreatic cancer cell lines, we observed that macrophages incubated with the CM of Panc1 cells (CM$_{Panc1}$) expressed high level of CD163 and CD206 (Fig. 4B and C). By contrast, macrophages incubated with the CM of AsPC1 cells (CM$_{AsPC1}$) expressed markedly lower

level of CD163 and CD206, whereas macrophages incubated with the CM of BxPC3 cells (CM$_{BxPC3}$) had modest C163 and CD206 expression (Fig. 4C). To further prove that it is the REG4 in the CM of Panc1 cells that results in macrophage polarization to M2, REG4 in Panc1 cells was knocked down with a specific lentiviral shRNA (Fig. 4D), and the CM of Panc1 cells infected with lentiviral scramble shRNA (shCon) or REG4 shRNA (shREG4) was added to macrophage culture. As shown in Fig. 4E and F, the CM of Panc1 cells infected with lentiviral shCon induced the expression of CD163, whereas the CM of Panc1 cells infected with lentiviral shREG4 did not. These data suggest that REG4 produced by pancreatic cancer cells promoted the polarization of TAMs to M2 phenotype.

REG4-treated macrophages promote pancreatic cancer cell proliferation and invasion. To test the hypothesis that REG4-induced M2 macrophages promote pancreatic cancer cell proliferation and invasion, macrophage cultures treated with or without REG4 (1 nM) for 24 h were washed to remove REG4, followed by incubation in normal medium for 24 h. The CM of macrophages treated with vehicle (CM$_{MΦ-Veh}$) or REG4 (CM$_{MΦ-REG4}$) was added to the cultures of pancreatic cancer cell lines, Panc1, AsPC1 and BxPC3, respectively, and cell proliferation and invasiveness were assessed. The three cell
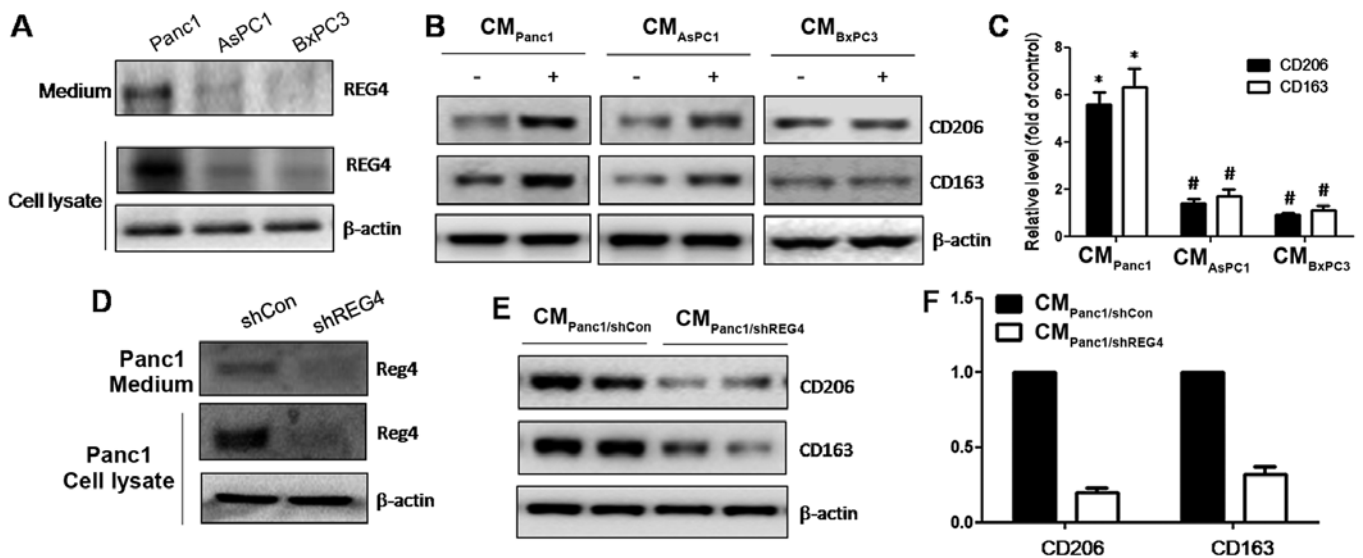
Figure 4. The conditioned medium of Panc1 cells induces macrophage polarization to M2. (A) Western blots of REG4 in the culture medium or cell lysate of Panc1, AsPC1 and BxPC3 cells. Shown are representatives of three independent experiments with similar results. (B) Representative western blots of CD206 and CD163 in macrophage cultures treated with or without the conditioned medium of Panc1, AsPC1 and BxPC3 cell cultures, respectively. (C) Quantitative analysis of the immunoblots of CD163 and CD206 in macrophage cultures treated with or without the conditioned medium of Panc1 (CM_{Panc1}), AsPC1 (CM_{AsPC1}) and BxPC3 (CM_{BxPC3}) cell cultures, respectively. Data are shown by mean ± SEM from four independent experiments. *P<0.05 compared to untreated cells; #P<0.05 compared with CM_{Panc1} treatment. (D) Western blots of REG4 in the culture medium and cell lysate of Panc1 cells infected with or without lentiviral REG4 shRNA. Shown are representatives of three independent experiments with similar results. (E) Representative western blots of CD206 and CD163 in macrophage cultures treated with the conditioned medium Panc1 cells infected with lentiviral control shRNA ($CM_{Panc1/shCon}$) or REG4 shRNA ($CM_{Panc1/shREG4}$). (F) Quantitative analysis of the immunoblots of CD163 and CD206 in macrophage cultures treated with $CM_{Panc1/shCon}$ or $CM_{Panc1/shREG4}$. *P<0.05 compared with control.
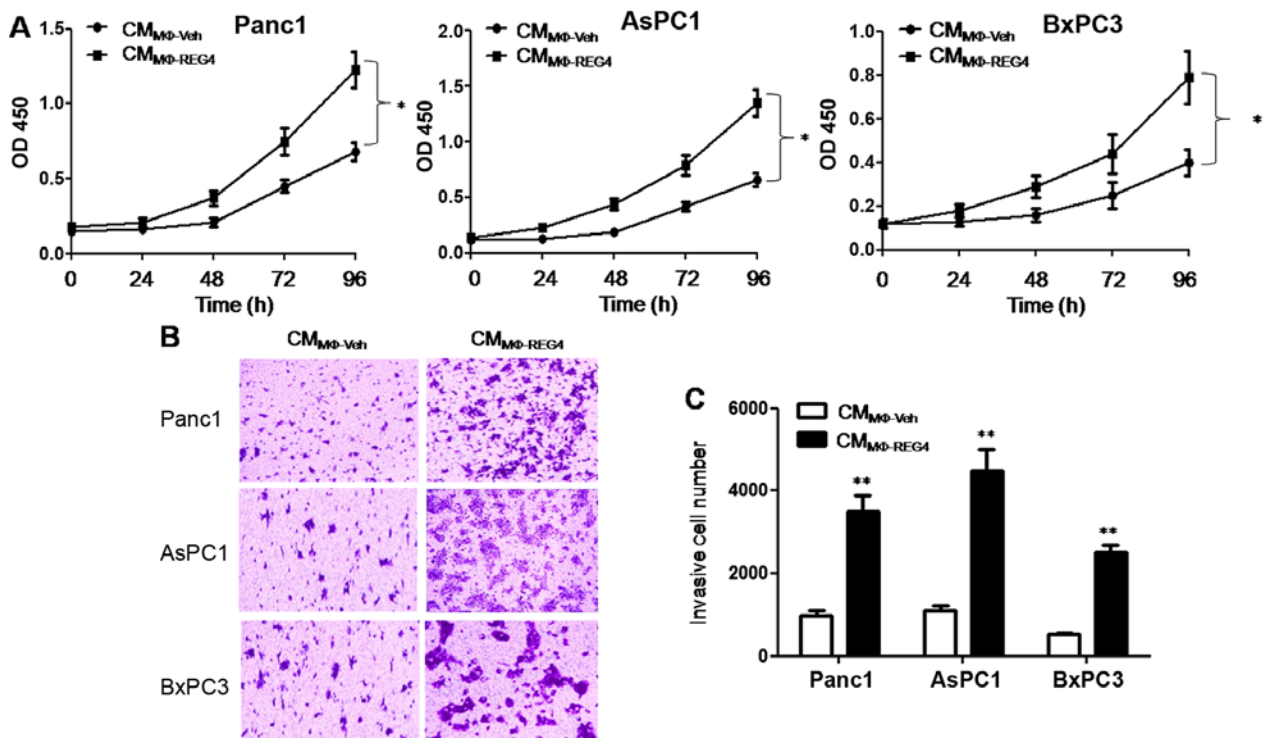

Figure 5. The conditioned medium of REG4-treated macrophages promotes pancreatic cancer cell proliferation and invasion. (A) Time-dependent proliferation of Panc1, AsPC1 and BxPC3 cells in the presence of the conditioned medium of macrophages treated with vehicle ($CM_{M\phi-Veh}$) or REG4 ($CM_{M\phi-REG4}$). (B) Representative images showing the invasiveness of Panc1, AsPC1 and BxPC3 cells treated with $CM_{M\phi-Veh}$ or $CM_{M\phi-REG4}$. (C) Quantitative analysis of the invasiveness of Panc1, AsPC1 and BxPC3 cells treated with $CM_{M\phi-Veh}$ (control) or $CM_{M\phi-REG4}$. **P<0.01 compared with control.

lines exhibited significantly increased proliferation (Fig. 5A) and invasiveness (Fig. 5B and C) after incubation with the $CM_{M\phi-REG4}$ compared to the cells incubated with the $CM_{M\phi-Veh}$ (P<0.05).

Discussion

In the present study, we demonstrated that REG4, which is overexpressed in PDAC and secreted by cancer cells, promoted macrophage polarization to M2, through at least in part, activation of ERK1/2 and CREB. Macrophages treated with REG4 significantly promoted the proliferation and invasion of pancreatic cancer cells. These data, together with the previous finding for its direct tumor-promoting role, suggest that the cancer cell-produced REG4 not only directly enhances cancer progression but also changes the microenvironment to facilitate cancer growth and metastasis.

During tumor progression, circulating monocytes and macrophages are actively recruited into tumors where they alter the tumor microenvironment to accelerate tumor progression. Macrophages shift their functional phenotypes in response to various micro-environmental signals generated from tumor and stromal cells. TAMs closely resemble the M2-polarized macrophages and are critical modulators of the tumor microenvironment (31). In support of this notion, experimental studies have shown that TAMs can provide a favorable microenvironment to promote tumor development and progression (31). Similar to that observed in other tumors, TAMs in PDAC also exhibit M2 phenotype, as observed in the present study, and previously reported (8). While mounting evidence indicates that TAMs are recruited to tumors by multiple growth factors and chemokines that are often produced by tumor cells themselves (32-37), little is known about how the tumor cells educate and reprogram the TAMs towards M2. Recent experimental results suggest that in different tumors, different factors are involved in the polarization of M2-like TAMs. For instance, in a model of human papilloma virus-driven squamous epithelium carcinogenesis, a remote control pathway involving CD4$^+$ T cells, B cells, antibodies and Fcγ receptors are responsible for the M2-like phenotype of tumor-promoting TAMs (38). In contrast, in a mammary carcinoma, Th2-derived IL-4 promotes M2 polarization and metastasis (39). In PDAC, previously the factors that promote TAMs towards M2 polarization were poorly defined. In the present study, we demonstrated for the first time that recombinant REG4 induced the expression of IL-10, CD163 and many other M2 genes in macrophages. Moreover, incubation of macrophages with the CM of Panc1 cells, which express and secret high level of REG4, resulted in elevated expression of CD163, whereas the CM of Panc1 cells infected with lentiviral REG4 shRNA did not. Thus, we conclude that REG4 plays a role in macrophage polarization to M2.

With regard to the underlying mechanism, we demonstrated that REG4-mediated M2 macrophage polarization was blocked by knockdown of CREB, which is known to be required in infiltrating macrophages for upregulation of M2-specific genes (40). We further showed that treatment of macrophages with REG4 resulted in phosphorylation of EGFR, which was recently reported to be expressed in macrophages and play a tumor-promoting role (41), and phosphorylation of AKT, and the latter was required for REG4-mediated CREB activation. Thus, we propose that REG4 induces the polarization of macrophages to M2 phenotype through, at least in part, the EGFR/AKT/CREB signaling pathway. Interestingly, increase of expression and secretion of REG4 are associated with increased levels of phosphorylated AKT and phosphorylated EGFR in gastric cancer (29), and inhibition of REG4/EGFR/Akt signaling pathway attenuates tumor growth in colorectal carcinoma (42). The tumor-secreted REG4 has been shown to promote pancreatic cancer cell proliferation and invasion (23). Given the factor that EGFR overexpression is detected in up to 90% of pancreatic tumors findings in other cancers, it is worthwhile to investigate whether REG4 directly promotes pancreatic cancer proliferation and/or metastasis through EGFR/AKT pathway. If this is the case, we would reasonably propose that REG4 plays a role in pancreatic cancer progression through both paracrine and autocrine signaling pathways.

In conclusion, we demonstrated for the first time that the tumor-secreted REG4 induced macrophage polarization to M2, through at least in part, activation of EGFR/AKT/CREB signaling pathway. The REG4-polarized macrophages promoted pancreatic cancer cell proliferation and invasion. This study shed new light on the tumor-promoting role of REG4, and suggests that targeting REG4 may offer therapeutic benefits to PDAC patients.

References

1. Farrow B, Albo D and Berger DH: The role of the tumor micro-environment in the progression of pancreatic cancer. J Surg Res 149: 319-328, 2008.
2. Lewin CS, Allen RA and Amyes SG: Mechanisms of zidovudine resistance in bacteria. J Med Microbiol 33: 235-238, 1990.
3. Coussens LM and Pollard JW: Leukocytes in mammary development and cancer. Cold Spring Harb Perspect Biol 3: 3, 2011.
4. Le DT, Lutz E, Uram JN, Sugar EA, Onners B, Solt S, Zheng L, Diaz LA Jr, Donehower RC, Jaffee EM, et al: Evaluation of ipilimumab in combination with allogeneic pancreatic tumor cells transfected with a GM-CSF gene in previously treated pancreatic cancer. J Immunother 36: 382-389, 2013.
5. Allavena P, Sica A, Solinas G, Porta C and Mantovani A: The inflammatory micro-environment in tumor progression: The role of tumor-associated macrophages. Crit Rev Oncol Hematol 66: 1-9, 2008.
6. Mantovani A, Sozzani S, Locati M, Allavena P and Sica A: Macrophage polarization: Tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 23: 549-555, 2002.
7. Sica A, Schioppa T, Mantovani A and Allavena P: Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: Potential targets of anti-cancer therapy. Eur J Cancer 42: 717-727, 2006.
8. Yoshikawa K, Mitsunaga S, Kinoshita T, Konishi M, Takahashi S, Gotohda N, Kato Y, Aizawa M and Ochiai A: Impact of tumor-associated macrophages on invasive ductal carcinoma of the pancreas head. Cancer Sci 103: 2012-2020, 2012.
9. Greten FR, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, Kagnoff MF and Karin M: IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell 118: 285-296, 2004.
10. Karin M and Greten FR: NF-kappaB: Linking inflammation and immunity to cancer development and progression. Nat Rev Immunol 5: 749-759, 2005.

11. Lin EY, Li JF, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, Qian H, Xue XN and Pollard JW: Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer Res 66: 11238-11246, 2006.

12. Qian B, Deng Y, Im JH, Muschel RJ, Zou Y, Li J, Lang RA and Pollard JW: A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. PLoS One 4: e6562, 2009.

13. Ruffell B, Affara NI and Coussens LM: Differential macrophage programming in the tumor microenvironment. Trends Immunol 33: 119-126, 2012.

14. Hartupee JC, Zhang H, Bonaldo MF, Soares MB and Dieckgraefe BK: Isolation and characterization of a cDNA encoding a novel member of the human regenerating protein family: Reg IV. Biochim Biophys Acta 1518: 287-293, 2001.

15. Watanabe T, Yonekura H, Terazono K, Yamamoto H and Okamoto H: Complete nucleotide sequence of human reg gene and its expression in normal and tumoral tissues. The reg protein, pancreatic stone protein, and pancreatic thread protein are one and the same product of the gene. J Biol Chem 265: 7432-7439, 1990.

16. Unno M, Itoh T, Watanabe T, Miyashita H, Moriizumi S, Teraoka H, Yonekura H and Okamoto H: Islet beta-cell regeneration and reg genes. Adv Exp Med Biol 321: 61-66, discussion 67-69, 1992.

17. Hayashi T, Matsubara A, Ohara S, Mita K, Hasegawa Y, Usui T, Arihiro K, Norimura S, Sentani K, Oue N, *et al*: Immunohistochemical analysis of Reg IV in urogenital organs: Frequent expression of Reg IV in prostate cancer and potential utility as serum tumor marker. Oncol Rep 21: 95-100, 2009.

18. Oue N, Hamai Y, Mitani Y, Matsumura S, Oshimo Y, Aung PP, Kuraoka K, Nakayama H and Yasui W: Gene expression profile of gastric carcinoma: Identification of genes and tags potentially involved in invasion, metastasis, and carcinogenesis by serial analysis of gene expression. Cancer Res 64: 2397-2405, 2004.

19. Oue N, Mitani Y, Aung PP, Sakakura C, Takeshima Y, Kaneko M, Noguchi T, Nakayama H and Yasui W: Expression and localization of Reg IV in human neoplastic and non-neoplastic tissues: Reg IV expression is associated with intestinal and neuroendocrine differentiation in gastric adenocarcinoma. J Pathol 207: 185-198, 2005.

20. Tamura H, Ohtsuka M, Washiro M, Kimura F, Shimizu H, Yoshidome H, Kato A, Seki N and Miyazaki M: Reg IV expression and clinicopathologic features of gallbladder carcinoma. Hum Pathol 40: 1686-1692, 2009.

21. Violette S1, Festor E, Pandrea-Vasile I, Mitchell V, Adida C, Dussaulx E, *et al*: Reg IV, a new member of the regenerating gene family, is overexpressed in colorectal carcinomas. Int J Cancer 103: 185-193, 2003.

22. Legoffic A, Calvo E, Cano C, Folch-Puy E, Barthet M, Delpero JR, Ferrés-Masó M, Dagorn JC, Closa D and Iovanna J: The *reg4* gene, amplified in the early stages of pancreatic cancer development, is a promising therapeutic target. PLoS One 4: e7495, 2009.

23. He XJ, Jiang XT, Ma YY, Xia YJ, Wang HJ, Guan TP, Shao QS and Tao HQ: REG4 contributes to the invasiveness of pancreatic cancer by upregulating MMP-7 and MMP-9. Cancer Sci 103: 2082-2091, 2012.

24. Takayama R, Nakagawa H, Sawaki A, Mizuno N, Kawai H, Tajika M, Yatabe Y, Matsuo K, Uehara R, Ono K, *et al*: Serum tumor antigen REG4 as a diagnostic biomarker in pancreatic ductal adenocarcinoma. J Gastroenterol 45: 52-59, 2010.

25. Rafa L, Dessein AF, Devisme L, Buob D, Truant S, Porchet N, Huet G, Buisine MP and Lesuffleur T: REG4 acts as a mitogenic, motility and pro-invasive factor for colon cancer cells. Int J Oncol 36: 689-698, 2010.

26. Zhang Y, Lai M, Lv B, Gu X, Wang H, Zhu Y, Zhu Y, Shao L and Wang G: Overexpression of Reg IV in colorectal adenoma. Cancer Lett 200: 69-76, 2003.

27. Bishnupuri KS, Luo Q, Sainathan SK, Kikuchi K, Sureban SM, Sabarinathan M, *et al*: Reg IV regulates normal intestinal and colorectal cancer cell susceptibility to radiation-induced apoptosis. Gastroenterology 138: 616-626.e2, 2010.

28. Tao HQ, He XJ, Ma YY, Wang HJ, Xia YJ, Ye ZY and Zhao ZS: Evaluation of REG4 for early diagnosis and prognosis of gastric cancer. Hum Pathol 42: 1401-1409, 2011.

29. Kuniyasu H, Oue N, Sasahira T, Yi L, Moriwaka Y, Shimomoto T, Fujii K, Ohmori H and Yasui W: Reg IV enhances peritoneal metastasis in gastric carcinomas. Cell Prolif 42: 110-121, 2009.

30. Miyagawa K, Sakakura C, Nakashima S, Yoshikawa T, Fukuda K, Kin S, Nakase Y, Shimomura K, Oue N, Yasui W, *et al*: Overexpression of RegIV in peritoneal dissemination of gastric cancer and its potential as A novel marker for the detection of peritoneal micrometastasis. Anticancer Res 28: 1169-1179, 2008.

31. Chanmee T, Ontong P, Konno K and Itano N: Tumor-associated macrophages as major players in the tumor microenvironment. Cancers (Basel) 6: 1670-1690, 2014.

32. Pollard JW: Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 4: 71-78, 2004.

33. Sica A and Bronte V: Altered macrophage differentiation and immune dysfunction in tumor development. J Clin Invest 117: 1155-1166, 2007.

34. Mantovani A, Bottazzi B, Colotta F, Sozzani S and Ruco L: The origin and function of tumor-associated macrophages. Immunol Today 13: 265-270, 1992.

35. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, Kaiser EA, Snyder LA and Pollard JW: CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature 475: 222-225, 2011.

36. Mantovani A, Savino B, Locati M, Zammataro L, Allavena P and Bonecchi R: The chemokine system in cancer biology and therapy. Cytokine Growth Factor Rev 21: 27-39, 2010.

37. Zhang J, Patel L and Pienta KJ: CC chemokine ligand 2 (CCL2) promotes prostate cancer tumorigenesis and metastasis. Cytokine Growth Factor Rev 21: 41-48, 2010.

38. Andreu P, Johansson M, Affara NI, Pucci F, Tan T, Junankar S, Korets L, Lam J, Tawfik D, DeNardo DG, *et al*: FcRgamma activation regulates inflammation-associated squamous carcinogenesis. Cancer Cell 17: 121-134, 2010.

39. DeNardo DG, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N and Coussens LM: CD4$^+$ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. Cancer Cell 16: 91-102, 2009.

40. Ruffell D, Mourkioti F, Gambardella A, Kirstetter P, Lopez RG, Rosenthal N and Nerlov C: A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. Proc Natl Acad Sci USA 106: 17475-17480, 2009.

41. Lanaya H, Natarajan A, Komposch K, Li L, Amberg N, Chen L, Wculek SK, Hammer M, Zenz R, Peck-Radosavljevic M, *et al*: EGFR has a tumour-promoting role in liver macrophages during hepatocellular carcinoma formation. Nat Cell Biol 16: 972-981, 2014.

42. Li YG, Ji DF, Zhong S, Zhu JX, Chen S and Hu GY: Anti-tumor effects of proteoglycan from *Phellinus linteus* by immunomodulating and inhibiting Reg IV/EGFR/Akt signaling pathway in colorectal carcinoma. Int J Biol Macromol 48: 511-517, 2011.