Embelin suppresses pancreatic cancer growth by modulating tumor immune microenvironment

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1. ABSTRACT

Since pancreatic carcinoma is largely refractory to conventional therapies, development of novel agents is required for the effective treatment of pancreatic cancer. The objective of this paper was to examine the molecular mechanisms by which embelin inhibited human pancreatic cancer growth in mice by modulating tumor immune microenvironment. Embelin inhibited PANC-1 tumor growth, angiogenesis, and metastasis which were associated with suppression of Akt and Sonic Hedgehog (Shh) pathways. Embelin inhibited the expression of Bcl-2, cyclin D1, CDK2 and CDK6, IL-6 and IL-8, and induced the expression of Bax in tumor tissues. Embelin also reversed epithelial-mesenchymal transition by up-regulating E-cadherin and inhibiting the expression of Snail, Slug and Zeb1. Embelin inhibited pancreatic cancer growth in $Kras^{G12D}$ mice by modulating tumor immune microenvironment where CTL, NKT, γδT, NK, and IFNγ (Th1 type) cells were up-regulated, and Th17, PMN-MDSC, IL-6 and IL-8 (Th2 type) immune cells were inhibited. These data suggest that embelin can inhibit pancreatic cancer growth by modulating tumor immune microenvironment and Akt and Shh pathways, and inhibiting inflammation. Embelin may offer therapeutic benefits for the treatment and/or prevention of pancreatic cancer.

2. INTRODUCTION

Embelin is a polyphenolic compound derived from the fruit of *Embelia ribes* Burm plant (Myrsinaceae). It was originally discovered as an XIAP inhibitor (1), but other mode of actions have been proposed. Embelin plays a crucial role in apoptosis, cell migration and cytoskeleton remodeling (2). Recently, embelin has been shown to induce apoptosis in various cancer cells, including prostate, colon, pancreatic and lung cancer cells, chronic leukemia, and multiple myeloma cells (3-5). Furthermore, the anti-inflammatory and anti-cancer activities embelin may be mediated in part through the suppression of the STAT3 pathway (6). Embelin has also been shown to enhance the

proapoptotic effects of TRAIL (7). In spite of these findings, the molecular mechanisms by which embelin inhibits tumor microenvironment has not been examined.

Pancreatic cancer is the fourth leading cause of cancer-related mortality in the United States with an overall 5-year survival rate of <5% (8). The poor prognosis of pancreatic cancer is related with late presentation, aggressive local invasion, early metastasis, and poor response to conventional chemotherapy and radiotherapy (8, 9). Several factors are associated with increased risk for pancreatic cancer including diabetes, chronic pancreatitis, prior gastric surgery, exposure to organic solvents, radiation, smoking, and gene polymorphisms (10, 11). Heritable and acquired gene mutations have been identified in pancreatic tumors (12). The K-*Ras* oncogene is primarily mutated in codon 12 in > 90% of pancreatic tumors and the mutation results in a constitutively active form of Ras that can lead to enhanced cell proliferation (12). Mutations in the *p53,* p16, and SMAD4 have also been demonstrated in high frequencies in pancreatic cancer (13, 14). Therefore, understanding the mechanisms of pancreatic carcinogenesis, and developing effective strategies to prevent pancreatic cancer are urgently needed.

Numerous signaling pathways are misregulated in pancreatic ductal adenocarcinoma (PDAC). The PI3K/Akt signaling pathway is important in cell proliferation and survival, and it is frequently and aberrantly activated in later stages of pancreatic ductal adenocarcinoma (PDAC) (15, 16). A recent study used compounded mice where Pten conditional knockout mice [Pten(lox/lox)] were crossed with conditionally activated Kras[G12D] mice (17). These compound heterozygous mutant mice showed significantly accelerated development of acinar-to-ductal metaplasia (ADM), malignant pancreatic intraepithelial neoplasia (mPanIN), and PDAC (17). Most importantly, all mice with Kras[G12D] activation and Pten homozygous deletion got cancer. This study confirm the role for PTEN, and the resulting dysregulation of the PI3K/AKT signaling axis, in both PDAC initiation and progression, and shed additional light on the signaling mechanisms that lead to the development of ADM and subsequent mPanIN and pancreatic cancer. Similarly, we have recently demonstrated that resveratrol can inhibit pancreatic carcinogenesis in Kras[G12D] mice (18).

The Hedgehog (HH) pathway is normally involved in patterning processes in the developing embryo. Expression of the ligand Sonic Hedgehog is an early event in pancreatic carcinogenesis and correlates with the mutation of the Kras oncogene. Recent data establish a functional role for HH signaling primarily in the tumor microenvironment, where it is involved in myofibroblast differentiation and the induction of stroma-derived growth promoting molecules. Given the pro-tumorigenic functions of the abundant stromal desmoplasia typically associated with pancreatic cancer, targeting the HH pathway may be beneficial in the treatment of this deadly disease. Shh signaling begins with the binding of Shh to Patched (Ptch) receptors, resulting in loss of Ptch activity and consequent phosphorylation and posttranscriptional stabilization of

Smoothened (Smo) (19). As a result, expression of HH target genes is regulated through posttranslational activation of the Gli family of transcription factors (20). The activation of Shh via Smo can occur either by HH protein stimulation or through loss of Ptch activity. Therapeutics that target Smo and Gli can abrogate HH signaling and thus can be used for the treatment of pancreatic cancer patients.

The tumor microenvironment is the cellular environment in which the tumor cells reside with immune cells, fibroblasts, other cells, blood vessels, signaling molecules, and the extracellular matrix (ECM). The interaction between tumor and its microenvironment determines the fate of tumor progression. Immune cells that infiltrate the tumor bed are transported there by blood circulation and exert a variety of effects, either counteracting or favoring tumor outgrowth. A marked infiltration of macrophages into the stromal compartment and the generation of a desmoplastic stromal reaction is a particular characteristic of PDAC and play a key role in disease progression and its response to therapy. Stromal cells within the pancreatic cancer microenvironment produce numerous factors that support the growth and survival of malignant cells. However, how soluble factors from the stroma alter immune cell phenotype and function in the tumor microenvironment is not well understood.

Tumor-infiltrating lymphocytes (TILs) are considered as positive or negative regulator of antitumor immunity. In the present study we have examined how embelin could alter the compositions of CD4^{+} and CD8^{+} TILs. Naive CD4^{+} T cells differentiate into mature T helper 1 (Th1), Th2, Th17 or T regulatory cells (Tregs), whereas CD8^{+} cells can differentiate into CTL, NK, NKT and γδT. During drug resistance, increase infiltrations of these cells have been demonstrated. NK, NKT and γδT can exert antitumor immune surveillance. Various studies have shown that Tregs and myeloid-derived suppressor cells (MDSC) promote tumor progression by suppressing immune response and inducing tolerance at location of tumor. Suppression of IL-17A at tumor sites can promote a Th1-dominant environment, and eliminate MDSC and regulatory T cells at tumor sites. Blockade of IL-17A at tumor sites can suppress tumor growth by inhibiting angiogenesis, and CTL activation at tumor sites, suggesting a critical role of IL-17A in angiogenesis and tumor immunity.

The purpose of this study was to examine the molecular mechanisms by which embelin inhibits tumor growth, angiogenesis, and metastasis of pancreatic cancer, and alter tumor immune micro-environment. Our data showed that embelin inhibited tumor growth, angiogenesis and metastasis of PANC-1 tumors in nude mice through inhibition of Akt and Shh signaling pathways. Embelin also inhibited pancreatic cancer growth in Kras[G12D] mice by modulating tumor immune microenvironment i.e. by increasing the infiltration of CTL, NK, NKT, and γδT cells, and IFNγ (Th1 type) producing cells, and decreasing the infiltration of PMN-MDSC and Th-17 cells, and IL-6 and IL-8 (Th2 type) producing cells. Overall, our results

suggest that embelin can be used for the treatment and/or prevention of pancreatic cancer.

3. MATERIALS AND METHODS

3.1. Reagents

Antibodies against phospho-Akt, Akt, Gli1, Gli2, cyclin D1, CDK-2, CDK-6, PCNA, IL-6, IL-8, Ki67, caspase-3, PARP, Bcl-2, Bax,Cox-2, VEGF, VEGFR, MMP-2, MMP-9, TRAIL-R1/DR4, TRAIL-R2/DR5, E-Cadherin, Snail, Slug and Zeb1 and β-actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Unlabeled or fluorochrome-conjugated anti-CD3, CD4, CD8, CD25, γδT, NK1.1, CD11b, Ly6C, IFN-γ, IL-17A, IL-6, IL-8, Ly6G and isotype-matched control mAbs were obtained from eBioscience (San Diego, CA). Embelin was purchased from LKT Laboratories, Inc. (St. Paul, MN).

3.2. Antitumor activity of embelin

The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Health Science Center at Tyler. PANC-1 cells (2 X 10^6 cells mixed with Matrigel, Becton Dickinson, Bedford, MA, 50:50 ratio, in a final volume of 100 μl) were injected subcutaneously into the flanks of Balb/c *nu/nu* mice (4-6 weeks old). Balb C Nude mice were purchased from the National Cancer Institute, Frederick, MD. After tumor formation, mice were orally gavaged with embelin (0 or 40 mg/kg body weight), 5 days a week for 6 weeks, once daily. Tumor volume and body weight were recorded weekly. After completion of the experiment, tumor bearing mice were euthanized for molecular analysis of tumor.

For tumor immune microenvironment analysis, Kras[G12D] mouse (3-4 weeks old) were orally gavaged with embelin (0 or 40 mg / kg body weight) 5 days per weeks (Monday through Friday, Once daily) for 10-months. Kras[G12D] mice were generated as we described earlier (18). At the end of experiment, mice were euthanized, and pancreas and spleens were harvested.

3.3. Isolation of splenocytes and pancreatic cancer cells

Pancreas and spleens were collected from Kras[G12D] mice. Single cell suspensions from spleens were prepared by mechanical disruption. Cells were filtered through a 40-μl cell strainer, and cells were incubated in RBC lysis buffer. Remaining cells were washed three times with ice-cold PBS.

The tumor tissues were minced into small pieces and were digested with 0.05 mg/ml collagenase, 0.05 mg/ml hyaluronidase and 0.05 mg/ml DNase I for 30 min at 37 ^{0}C. Single cell suspensions were obtained by grinding the digested tissues and passing them through a 40-μl cell strainer. Tumor infiltrating lymphocytes (TILs) were isolated using Ficoll density gradient centrifugation.

3.4. Flow cytometry

For the analysis of immune cells, the cell suspension was stimulated with 20 ng/ml phorbol 12-myristate-13-acetate and 1 μg/ml ionomycin in the presence of 2 mM monensin (Sigma-Aldrich, Saint Louis). After 4 hours of culture (37°C; 5% CO_2), the cells were transferred to tubes and washed once in phosphate buffered saline (PBS). After staining, cells were fixed and permeabilized according to the manufacturer's instructions and stained with appropriate fluorescence antibody.

3.5. Western blot analysis

Western blots were performed as we described earlier (21).

3.6. Immunohistochemistry and TUNEL assay

Imunohistochemistry of tumor tissues collected was performed as we described elsewhere (22). TUNEL assays were performed as per manufacturer's instructions (Roche Applied Sciences).

3.7. Statistical analysis

Statistical analyses were performed using PRISM statistical analysis software (GrafPad Software, Inc., San Diego, CA). Group data are expressed as the mean ± standard deviation or standard error of mean. A paired samples *t*-test was used to evaluate differences in blood sample parameters on different days. Correlation coefficients were calculated using Spearman's rank method. P values <0.05 were considered to be statistically significant.

4. RESULTS

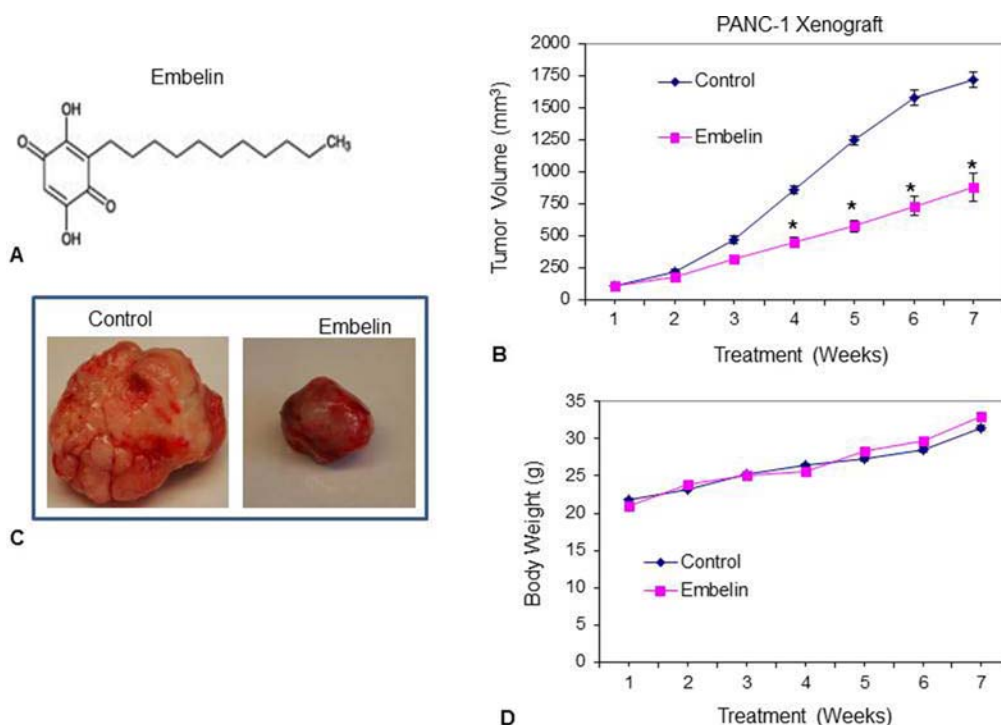### 4.1. Embelin inhibits the growth of PANC-1 xenografts in Balb C Nude mice

In order to examine the tumorigenic potential of embelin, we first examined the effects of embelin on growth of PANC-1 xenografted tumors in Balb C nude mice. As shown in Figure 1A, embelin inhibited PANC-1 pancreatic tumor growth in mice compared to that of control group. Furthermore, embelin had no effect on the body weight of PANC-1 tumor bearing mice, although mice gained weight during the treatment (Figure 1B). It is important to note that we did not observe any toxicity in the liver, spleen and intestine of mice treated with embelin. These data suggest that embelin can inhibit pancreatic xenograft growth without any toxicity to normal organ.

4.2. Embelin inhibits tumor cell proliferation, and induces apoptosis through activation of caspase-3 and cleavage of Poly (ADP-ribose) polymerase (PARP)

We next examined the effects of embelin on cell proliferation in tumor tissues derived from control and embelin treated mice using anti-PCNA and anti-Ki67 antibody (Figure 2). PCNA and Ki67 are the markers of cell proliferation. Embelin inhibited markers of cell proliferation in tumor tissues obtained from PANC-1 xenografts compared to control mice, as measured by immunohistochemistry (IHC) and the Western blot (WB) analysis (Figure 2A and B).

Caspase activation and cleavage of its substrate PARP are the hall marks of apoptosis (23). We next examined whether embelin induced tumor cell apoptosis through activation of caspase-3 and cleavage of PARP.

Figure 1. Embelin inhibits the growth of PANC-1 tumors xenografted in Balb C Nude mice. (A), Structure of embelin. (B), Tumor volume. PANC-1 cells (2×10^6 cells mixed with Matrigel, 50:50 ratio) were subcutaneously implanted into the flanks of Balb C nude mice. Tumor-bearing mice were treated with embelin (0 or 40 mg/kg body weight) through gavage (5 days per week, once daily) for 6 weeks. Tumor volume of mice was recorded weekly. Data represent the mean ± S.D. * = significantly different from control, $P < 0.05$. (C), Representative photograph of tumors obtained from vehicle and embelin treated mice. (D), Body weight of mice. Whole body weight of mice from vehicle and embelin-treated group was recorded weekly. Data represent the mean ± S.D.

Caspase-3 activation was measured by IHC and Western blot analysis using active anti-caspase-3 antibody. Embelin induced caspase-3 activation (Figure 2A and B), and apoptosis (Figure 2C). PARP is a substrate of caspase-3 (23). Embelin treatment resulted in cleavage of PARP. Activation of caspase-3 by embelin correlated with cleavage of PARP in tumor tissues. Overall, these data suggest that embelin inhibited cell proliferation and induced apoptosis in pancreatic tumor tissues through inhibition of PCNA, Ki67, and activation of caspae-3 and cleavage of PARP.

**4.3. Embelin regulates Bcl-2 family members and cell cycle proteins in tumor tissues**

The Bcl-2 family members consist of anti- and pro-apoptotic proteins (24, 25). In addition to Bcl-2 family members, cell cycle proteins also regulate cell proliferation and apoptosis (26). We therefore measured the effects of embelin on the expression of Bcl-2, Bax, Cyclin D1, CDK-2, and CDK-6 in tumor tissues (Figure 2D). Embelin inhibited the expression of anti-apoptotic protein Bcl-2 and induced the expression of pro-apoptotic protein Bax in tumor tissues. Furthermore, embelin inhibited the expression of cell cycle related proteins Cyclin-D1, CDK-2 and CDK-6 in tumor tissues obtained from PANC-1 xenografts. These data suggest that embelin can regulate cell cycle and apoptosis in tumor tissues.

4.4. Embelin inhibits angiogenesis and COX-2 expression in xenografted mice

Since angiogenesis plays a major role in tumor growth (27), we sought to measure the effects of embelin on angiogenesis by measuring the expression of VEGF and VEGFR in tumor tissues. Treatment of mice with embelin resulted in significant inhibition in VEGF and VEGFR in tumor cells obtained from PANC-1 xenografts (Figure 3).

Cyclooxygenase-2 (COX-2) has been implicated in tumorigenesis and metastasis, and promotes vascular permeability and mediates the proliferation of endothelial cells (28). We therefore examined whether embelin inhibits the expression of COX-2 in pancreatic cancer xenografts. As shown in Figure 3A and B, embelin inhibited the expression of COX-2 in pancreatic tumor tissues obtained from PANC-1 xenografts. These data suggest that embelin can suppress inflammation in tumor tissues.

Proliferation of cancer cells is under the control of a complex network of cytokines (29). Proinflammatory cytokines have been shown to enhance cancer invasion and angiogenesis (29, 30). Recent studies have demonstrated that the IL-6 and IL-8 signaling pathways play important roles in early metastasis and tumor microenvironment (29). We therefore measured the expression of Il-6 and IL-8 in
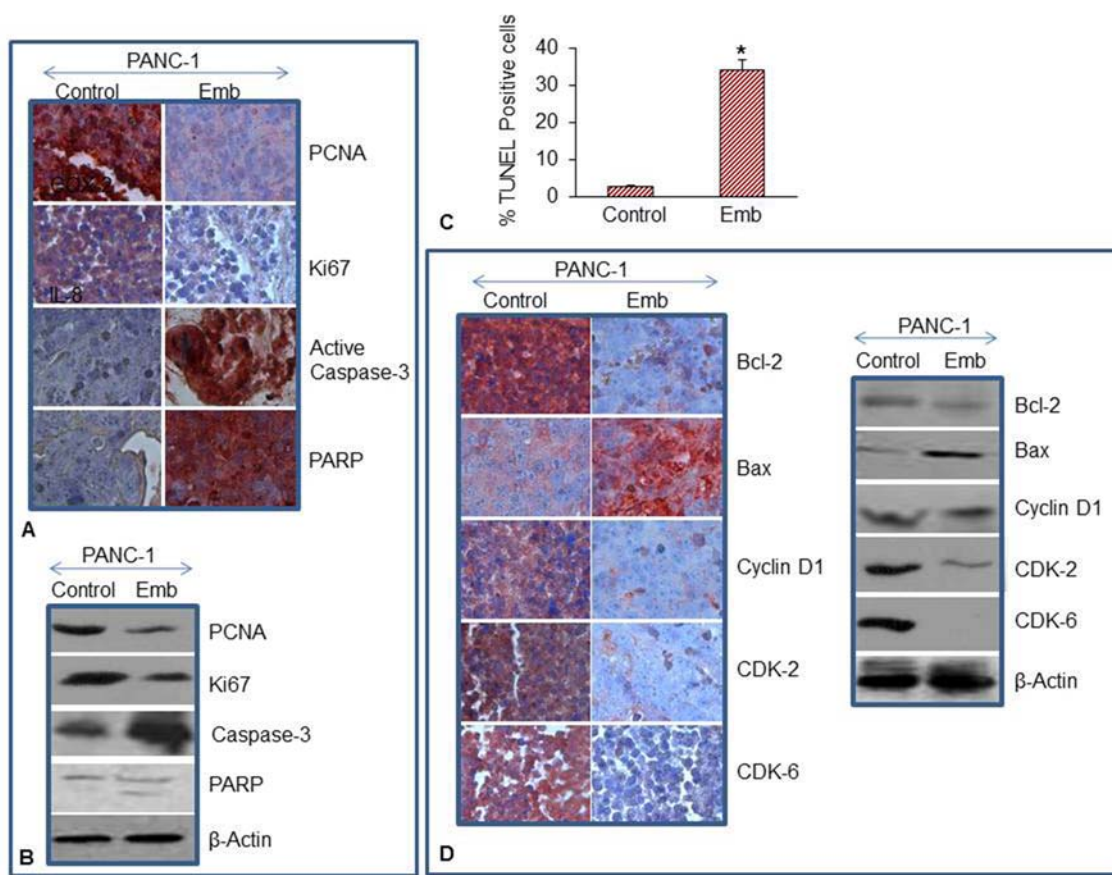
Figure 2. Effects of embelin on markers of cell proliferation, apoptosis and cell cycle. (A), Expression of PCNA, Ki67, caspase-3, and PARP in tumor tissues. Immunohistochemistry was performed to measure the expression of PCNA, Ki67, active caspase-3 and PARP in tumor tissues isolated from vehicle and embelin-treated mice. (B), Effects of embelin on markers of cell proliferation and apoptosis. Western blot analysis was performed to measure the expression of PCNA, Ki67, cleaved caspase-3 and PARP in tumor tissues. The β-actin was used as a loading control. (C), Quantification of TUNEL positive cells. Apoptosis was measured by TUNEL assay. Data represent the mean ± S.D. * = significantly different from control, P < 0.05. (D), Effects of embelin on the markers of apoptosis and cell cycle. IHC and Western blot analyses were performed to measure the expression of Bcl-2, Bax, Cyclin D1, CDK2 and CDK6 in tumor tissues obtained from vehicle and embelin-treated mice. The β-actin was used as a loading control.

tumor tissues obtained from xenografted mice. Treatment of tumor-bearing mice with embelin resulted in suppression of IL-6 and IL-8 (Figure 3A and B). These data suggest that IL-6 and IL-8 may mediate antitumor activity of embelin.

**4.5. Embelin inhibits epithelial to mesenchymal transition in tumor tissues**
The switch of tumor cells from an epithelial to a mesenchymal-like phenotype [EMT] is known to induce tumor cell motility and invasiveness, therefore promote metastasis of epithelial tumors (31). During EMT, expression of EMT transcription factors are induced, as a result the expression of E-cadherin is inhibited and the expression of N-cadherin is up-regulated. The expression of MMPs, which digest the different components of the extracellular matrix (ECM) and basement membrane, also enhanced during EMT (32). We therefore measured the effects of embelin on the expression of E-cadherin, Snail, Slug, Zeb1, MMP-2 and MMP-9 by IHC and

Western blot analysis. Treatment of mice with embelin induced the expression of E-cadherin and inhibited the expression of MMP-2, MMP-9, Snail, Slug, and Zeb-1 in tumor tissues (Figure 3C and D). Our data demonstrate that embelin can inhibit / reverse EMT by inducing the expression of E-cadherin and inhibiting its associated transcription factors and MMPs. Overall, our data demonstrate that embelin can inhibit / reverse early metastasis in pancreatic cancer.

4.6. Embelin inhibits Sonic Hedgehog and Akt pathways
Shh pathway promotes EMT by mediating a complex signaling network, and plays an important role in pancreatic cancer development and progression. We therefore sought to measure the expression of Gli1 and Gli2 transcription factors. Gli1 regulates its own expression. Embelin inhibited the expression of Gli1 and Gli2 as demonstrated by IHC and Western blot analysis (Figure 4A and B). These data suggest that embelin can inhibit tumor growth by suppressing Shh pathway.
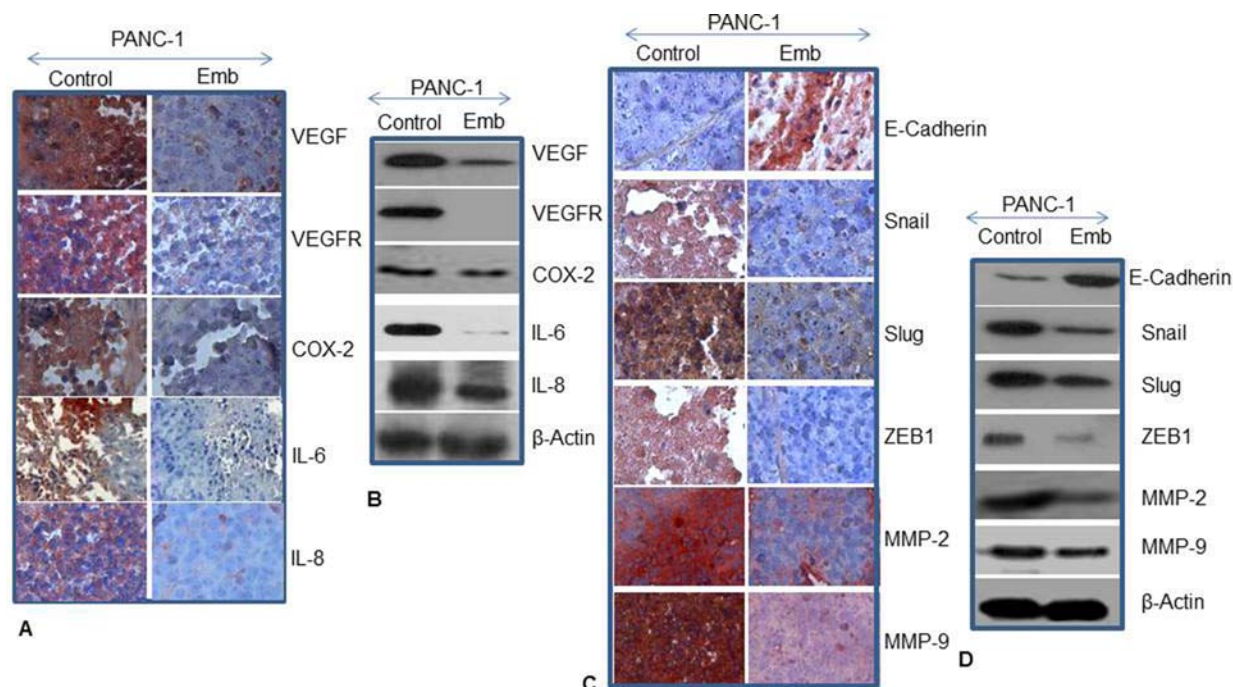
Figure 3. Effects of embelin on markers of angiogenesis and epithelial-mesenchymal transition in pancreatic tumor tissues. (A), Immunohistochemistry was performed to examine the expression of VEGF, VEGFR, COX-2, IL-6 and IL-8 in tumor tissues isolated from vehicle and embelin-treated mice. (B), Western blot analysis was performed to measure the expression of VEGF, VEGFR, COX-2, IL-6 and IL-8. The β-actin was used as a loading control. (C), Immunohistochemistry was performed to examine the expression of E-cadherin, Snail, Slug, Zeb1, MMP-2 and MMP-9 in tumor tissues isolated from vehicle and embelin-treated mice. (D), Western blot analysis was performed to measure the expression of E-cadherin, Snail, Slug, Zeb1, MMP-2 and MMP-9. The β-actin was used as a loading control.

We have previously demonstrated that TRAIL-R1/DR4 and TRAIL-R2/DR5 are the transcriptional targets of Gli (33). Up-regulation of DRs in tumor tissues may be beneficial for ligand TRAIL to induce apoptosis (33). We therefore examined the effects of embelin on the expression of TRAIL-R1/DR4 and TRAIL-R2/DR5 in tumor tissues (Figure 4A and B). Embelin up-regulated the expression of TRAIL-R1/DR4 and TRAIL-R2/DR5 in PANC-1 tumor tissues compared to untreated control. These data suggest that embelin can be combined with TRAIL to get maximum therapeutic benefits.

The PI3K/Akt signaling pathway promotes cell proliferation, survival, cell cycle progression and tumorigenesis, and it is frequently and aberrantly activated in later stages of pancreatic ductal adenocarcinoma (PDAC) (34, 35). Since PI3K/Akt pathway has been shown to regulate pancreatic carcinogenesis (34, 35), we measured the expression of phospho-Akt in tumor tissues (Figure 4A and B). Embelin inhibited the expression of pAKT in tumor tissues obtained from xenografted mice. Overall, these data suggest that embelin inhibits PI3K/Akt pathway which regulates tumor cell proliferation and carcinogenesis in pancreatic cancer.

4.7. Embelin inhibits pancreatic tumor growth and induces apoptosis in Kras^{G12D} mouse model of pancreatic cancer

We next examined the ability of embelin to inhibit tumor growth in Kras^{G12D} mouse model of pancreatic cancer. The Kras^{G12D} mouse model recapitulates pancreatic cancer development in humans, and provides a suitable microenvironment to study the effects of anticancer drugs on tumor growth while considering tumor-stroma interactions. As shown in Figure 5A, embelin inhibited pancreatic tumor growth in Kras^{G12D} mouse model of pancreatic cancer compared to that of vehicle treated mice. Embelin induced apoptosis in pancreatic tumor tissues as evidenced by increased percentage of TUNEL-positive cells as compared to tumors derived from vehicle treated mice (Figure 5B). These data suggest that embelin can inhibit pancreatic tumor growth by inducing apoptosis.

4.8. Embelin increases the infiltration of CTL, NK, NKT and γδT cells, and decreases the infiltration of PMN-MDSCs in Kras^{G12D} mouse model of pancreatic cancer

The migration of T and NK cells and access to tumor antigens is crucial for the induction of protective anti-tumor immunity. Once having entered a tumor site, T cells encounter a complex environment composed of non-
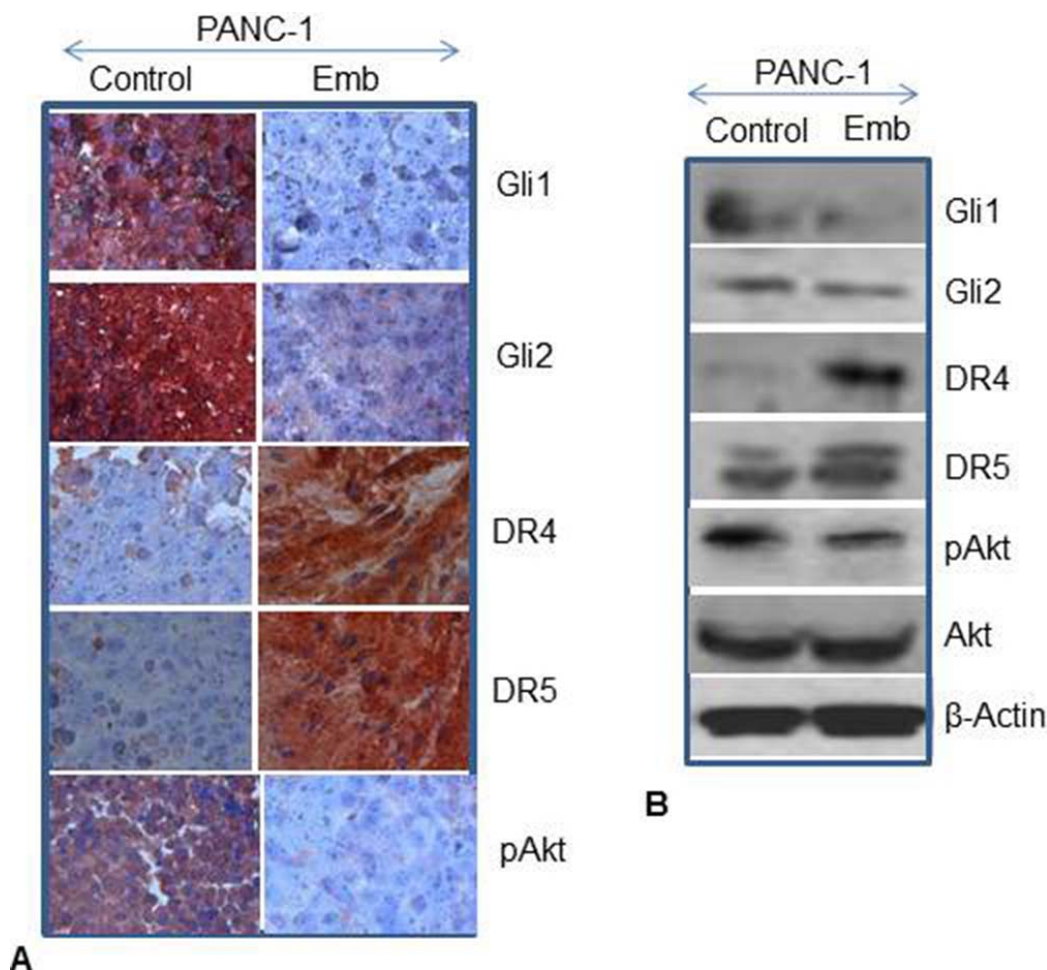
Figure 4. Effects of embelin on Sonic hedgehog and Akt pathways in pancreatic tumor tissues. (A), Immunohistochemistry was performed to measure the expression of Gli1, Gli2, TRAIL-R1/DR4, TRAIL-R2/DR5, and pAkt in tumor tissues isolated from vehicle and embelin-treated mice. (B), Western blot analysis was performed to measure the expression of Gli1, Gli2, TRAIL-R1/DR4, TRAIL-R2/DR5, pAkt, and Akt. The β-actin was used as a loading control.

tumor cells along with the extracellular matrix (ECM). Numerous studies demonstrate that a deregulated ECM favors tumor progression and metastasis. We therefore examined the ability of embelin to modulate the immune system in Kras[G12D] mouse model of pancreatic cancer by measuring the percentage of splenocytes and tumor infiltrating lymphocytes (TILs) – CTL, NK, NKT, γδT, and myeloid-derived suppressor cells (MDSCs) cells. Treatment of mice with embelin resulted in an increase in tumor infiltrating CTLs, NKs, NKTs and γδTs, and a decrease in the percentage of PMN-MDSCs compared to vehicle treated mice (Figure 6 A, B, C, D, and F). As shown in Figure 6, embelin upregulated the percentage of splenic CTLs, NKT, γδTs, NK, and polymorphonuclear granulocytic myeloid-derived suppressor cells (PMN-MDSC) compared to that of control mice. Furthermore, we have not noticed any difference in MO-MDSCs in pancreatic tumors and splenocytes. These data suggest that embelin can significantly improve the tumor immune micro-environment by increasing the infiltration of the anti-

tumor CTL, NK, NKT and γδT cells, and decreasing the population of PMN-MDSC cells in Kras[G12D] mouse model.

4.9. Embelin decreases the population of pro-inflammatory cytokine-producing cells in Kras[G12D] mice

The processes of initiation and progression of cancer may be mediated by proinflammatory cytokines. Since inflammatory cytokines play an important role in the immune-microenvironment, we next examined the effects of embelin on IL-17A^{+}, IL-6^{+}, IL-8^{+} and IFNγ^{+} cells in the splenocytes and tumor infiltrating populations in tumor-bearing mice (Figure 7). In the spleens, embelin inhibited the percentage of IL-17A^{+}, IL-6^{+} and IL-8^{+} cells, whereas the percentage of IFNγ^{+} cells were significantly increased. In the pancreas, embelin treatment of mice resulted in a decrease in tumor infiltrating IL-17A^{+}, IL-6^{+} and IL-8^{+} cells, whereas the percentage of IFNγ^{+} cells were increased. These data suggest that embelin can inhibit the pro-inflammatory cytokine-producing IL-17A^{+}, IL-6^{+}, and IL-8^{+} cells, and can increase the antitumor cytokine-producing
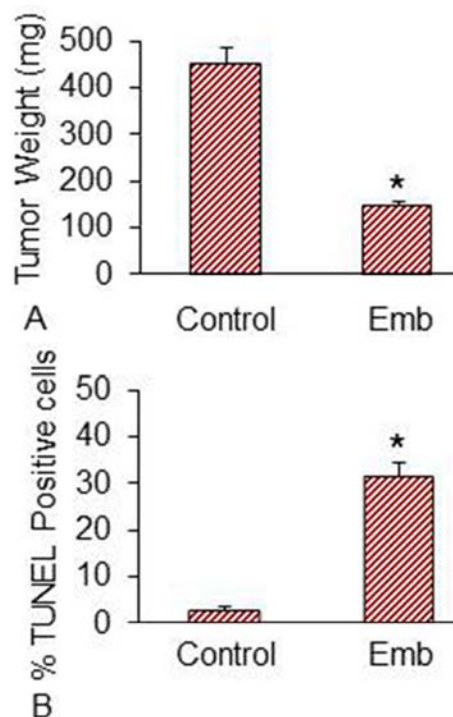
Figure 5. Embelin inhibits pancreatic cancer growth in Kras[G12D] mouse model of pancreatic cancer. (A), Pancreatic tumor weight. Kras[G12D] mice (3-4 weeks old) were gavaged with vehicle or embelin (40 mg / kg) for 10 months. At the end of experiment, mice were euthanized and pancreas weights were recorded. (B), Quantification of TUNEL positive cells. Pancreatic tissues from vehicle and embelin-treated Kras[G12D] mice were processed for TUNEL assay. Apoptosis was measured as % TUNEL positive cells. Data represent the mean ± S.D. * = significantly different from control, P < 0.05.

IFNγ^+ cells in the splenocyte and TIL populations in Kras[G12D] mouse model of pancreatic cancer.

4.10. Embelin decreases IL-17$^+$, IL-6$^+$, and IL-8$^+$ (Th2 type) cells, and increases IFNγ^+ (Th1 type) cells in Kras[G12D] mouse model of pancreatic cancer

Determination of the Th1/Th2 profile may be helpful for predicting the disease prognosis and targeting treatment strategies in pancreatic cancer patients. Since embelin modulated the tumor-immune micro-environment, we next sought to analyze the percentage of IL-17A$^+$, IL-6$^+$, IL-8$^+$ and IFN-γ^+ splenocytes and TILs that were also positive for CD3 and CD4. In the pancreas, the percentage of IL-17A$^+$, IL-6$^+$, and IL-8$^+$ TILs that were also positive for CD3 and CD4 were decreased, whereas the percentage of IFN-γ^+ cells that were CD3 and CD4 positive were significantly increased (Figure 8). Similarly in the spleen of Kras[G12D] mouse model, embelin decreased the percentage of IL-17A$^+$, IL-6$^+$, and IL-8$^+$ cells that were also positive for CD3 and CD4, whereas the percentage of IFNγ^+ cells that were CD3 and CD4 positive were significantly increased. These results suggest that embelin can inhibit inflammation by decreasing the populations of

pro-inflammatory Th17, IL-6 and IL-8 Th2 cells, and by increasing the population of antitumor immune Th1 cells, both in the TIL and splenocyte populations in Kras[G12D] mouse model of pancreatic cancer.

5. DISCUSSION

We have demonstrated for the first time that embelin can inhibit pancreatic cancer growth by modulating tumor immune microenvironment in Kras[G12D] mice. Specifically, (i) embelin inhibited PANC-1 tumor growth which was associated with suppression of Akt and Shh pathways; (ii) treatment of mice with embelin inhibited markers of angiogenesis and metastasis, (iii) embelin can significantly improve the tumor immune microenvironment by increasing the infiltration of the anti-tumor CTL, NK, NKT and $\gamma\delta$T cells, and decreasing the population of PMN-MDSC cells in Kras[G12D] mouse model, and (iv) embelin can inhibit inflammation by decreasing the proinflammatory IL-17, IL-6 and the IL-8 producing Th2 cells, and by increasing the antitumor immune IFNγ producing Th1 cells, both in the TIL and splenocyte populations of Kras[G12D] mice. Since embelin is a nontoxic compound, it can be safely used for the treatment and/or prevention of pancreatic cancer.

The PI3K/Akt and Shh signaling pathways regulate cell proliferation and survival, and are frequently and aberrantly activated in pancreatic cancer. In our study, embelin inhibited the activation of these pathways, suggesting their roles in mediating anticancer activities of embelin. Akt and Shh pathways have been demonstrated to interact together where Akt regulated Gli phosphorylation. Shh is abnormally expressed in pancreatic adenocarcinoma and its precursor lesions (PanIN). Overexpression of Shh in the pancreas of mice results in the development of abnormal tubular structures, PanIN-1 and -2 (36). The data from our laboratory demonstrated that the components of Shh pathway are highly expressed in human pancreatic cancer stem cells and pancreatic cancer cell lines, and several chemopreventive agents inhibited pancreatic cancer growth (18, 37-39). In another study, it was demonstrated that inhibition of the HH pathway decreased cell proliferation and induced apoptosis through inhibition of the PI3K/Akt pathway and cancer stem cells (40). Overall, these data suggest that inhibition of the Shh pathway may be a potential molecular target of new therapeutic strategies for human pancreatic cancer.

COX-2, an inducible isoform of COX, converts arachidonic acid to PGs which function as more than acute inflammation mediators, and contribute to chronic inflammation by inducing chemokines and resultant infiltration of inflammatory cells at affected sites. COX-2 is induced by a variety of proinflammatory cytokines, lipopolysaccharide and growth factors. Several studies have reported that COX-2 overexpression is associated with the process of carcinogenesis through increased proliferation, reduced apoptosis, and stimulation of metastases and angiogenesis in a variety of tumor types. Accumulating evidence suggests the up-regulation of COX-2 in the pancreatic cancer development (28). Thus COX-2
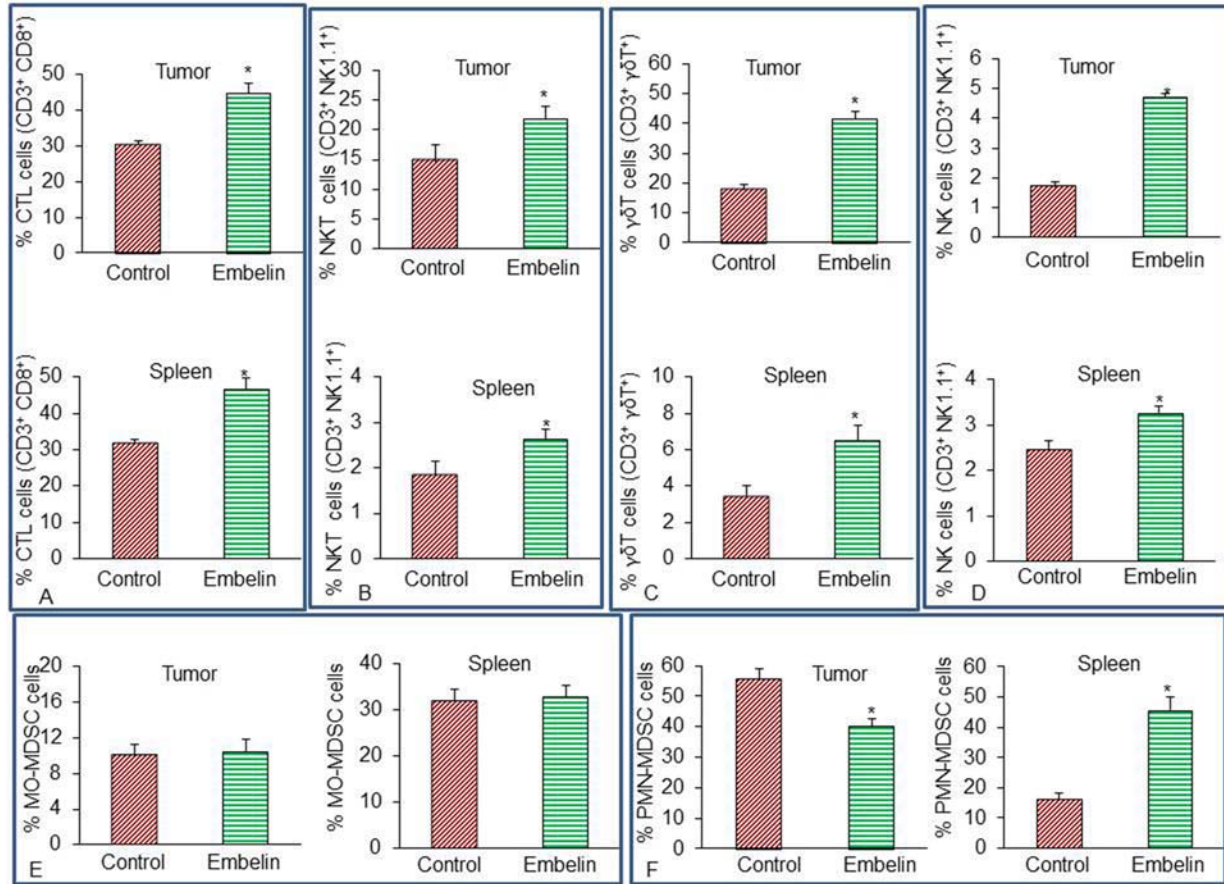
Figure 6. Effects of embelin on infiltrating CTL, NK, NKT, γδT and PMN-MDSC in Kras[G12D] mouse model of pancreatic cancer. Splenic and pancreatic tumor cell populations were isolated from Kras[G12D] mice, and immune cells were characterized as described in Materials and Methods. The data represent the percentage of target cell subset within a particular immune cell population. (A, B and C), %CTL ($CD3^+$ $CD8^+$), %NK ($CD3^+$ $NK1.1^+$) and %γδT ($CD3^+$ $γδT^+$) cells in pancreatic tumor and splenic populations of embelin- and vehicle-treated mice. (D), Tumor and splenic NK cells. The percentages of $CD3^-NK1.1^+$ cells (NK) were quantified in pancreas and spleen isolated from vehicle- and embelin-treated mice. Data represent the mean ± SD. * significantly different from respective control ($P < 0.05$). (E and F), Tumor infiltrating and splenic populations of MO-MDSC ($CD11b^+$ $LY6G^{high}$ $LY6C^-$) and PMN-MDSC ($CD11b^+$ $LY6G^{low}$ $LY6C^+$). The percentages of $LY6G^{high}$ $LY6C^-$ and $LY6G^{low}$ $LY6C^+$ cells in $CD11b^+$ populations isolated from spleen and pancreas of mice treated with vehicle and embelin were quantified. Data represent the mean ± SD. * significantly different from respective control ($P < 0.05$).

may play a role in in tumor initiation and progression through activation of the PI3K/Akt pathway. In our study, embelin inhibited the expression COX-2, in addition to suppression of Akt and Shh pathways. Therefore, simultaneous inhibition of COX-2 with other survival pathway(s) could be beneficial for the treatment and/or prevention of the pancreatic cancer.

Tumor cells undergoing EMT are also known to increase the secretion of specific factors, including cytokines, chemokines and growth factors, which could play an important role in tumor progression. During metastasis, ECM and basement membrane are degraded through the action of proteases (MMPs) (31). The increased activities of MMPs have been associated with increasing tumor metastases in pancreatic cancer. Furthermore, we have demonstrated that inhibition of the

Shh signaling pathway significantly inhibited EMT by suppressing the activation of transcription factors Snail and Slug, which were correlated with significantly reduced pancreatic cancer stem cell invasion (37-39, 41, 42), suggesting that the Shh signaling pathway is involved in early metastasis. In the present study, embelin inhibited the expression of IL-6 and IL-8 in tumor tissues. Our results also indicate the essential role of IL-6 and IL-8 signaling for the maintenance of the invasive features of tumor cells and suggest that IL-6 and IL-8 secreted by tumor cells undergoing EMT could potentiate tumor progression by inducing adjacent epithelial tumor cells into EMT. Thus, IL-6 and IL-8 signaling blockade by embelin may provide a means of inhibiting or reversing EMT. In addition to inhibiting EMT / metastasis, our data suggest that embelin can also inhibit angiogenesis by regulating VEGF/VEGFR axis. Several angiogenesis inhibiting drugs are in clinical
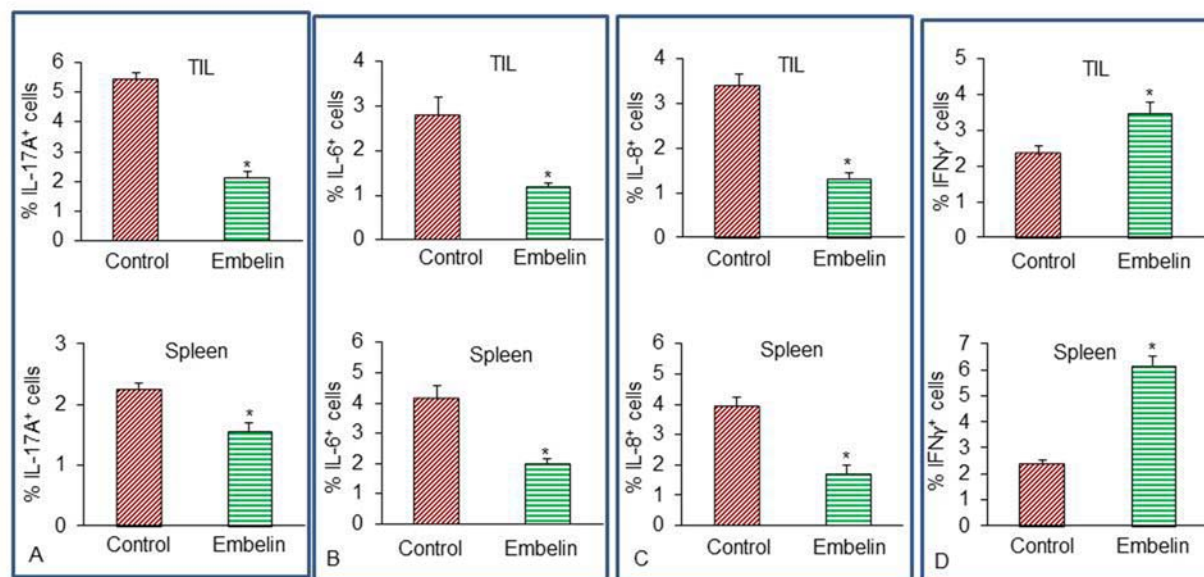
Figure 7. Effects of embelin on cytokine producing cells in Kras^{G12D} mouse model. Pancreatic and splenic cell populations were isolated as described in Materials and Methods. Cells were stimulated with ionomycin and PMA in the presence of monensin for 5 h and then stained with anti-IL-17A, anti-IL-6, anti-IL-8 and anti-IFNγ antibody. (A-D, Upper panel), The percentages of pancreatic tumor infiltrating (TILs) IL-17A$^+$, IL-6$^+$, IL-8$^+$ and IFNγ$^+$ cells were quantified. Data represent the mean ± SD. * significantly different from respective control (P < 0.05). (A-D, Lower panel), The percentages of splenic IL-17A$^+$, IL-6$^+$, IL-8$^+$ and IFNγ$^+$ cells were quantified. Data represent the mean ± SD. * significantly different from respective control (P < 0.05).

trials for the treatment of solid tumors.

The tumor microenvironment has been largely studied as a dynamic system orchestrated by inflammatory cells, including cancer cells, stroma as well as the extracellular matrix. Tumor immune microenvironment appears to play a major role in tumor growth and progression. In the present study, we have examined for the first time that embelin can alter the compositions of CD4+ (e.g. Th1, Th2, and Th17) and CD8+ CTL, NK, NKT and γδT cells, and thus modulate tumor microenvironment in Kras^{G12D} mice. Embelin significantly increased the percentage of tumor infiltrating CTL, NK, NKT and γδT cells, whereas the percentage of PMN-MDSCs was significantly decreased in Kras^{G12D} mice. Our data also demonstrated that the modulation of immune microenvironment was much higher in tumors compared to that of spleens in Kras^{G12D} mice.

It is well established that an intimate connection exists between inflammation and neoplasia. Accumulated data from animal models and human cancer patients strongly support the concept that the immune system can identify and control tumor cells through immunosurveillance. The immune system can also promote tumor progression through chronic inflammation, immunoselection of poorly immunogenic variants, and suppressing antitumor immunity. In order to examine the anti-inflammatory effects of embelin, we measured the percentage of splenocytes and TILs that were comprised of IL-17A-, IL-6-, IL-8-, and IFNγ-producing cells in Kras^{G12D} mice. Embelin significantly

inhibited the population of IL-17A$^+$, IL-6$^+$, and IL-8$^+$ cells, and increased the percentage of IFNγ$^+$ cells in both the spleen and pancreas of Kras^{G12D} mice. These data suggest that embelin can regulate inflammation by decreasing the production of pro-inflammatory cytokines (IL-17A, IL-6 and IL-8) and increasing the production of anti-tumor cytokines (IFNγ) in our Kras^{G12D} mouse model. Furthermore, in CD3$^+$CD4$^+$ splenic and tumor infiltrating cell populations, the percentage of IL-17A$^+$, IL-6$^+$ and IL-8$^+$ cells were significantly reduced, whereas the percentage of IFNγ$^+$ (Th1 type) cells were significantly increased after embelin treatment. These data confirm our findings that embelin can inhibit inflammation by decreasing the infiltration of pro-inflammatory IL-17A$^+$, IL-6$^+$ and IL-8$^+$ Th cells, and increase the population of splenic and tumor infiltrating IFNγ Th1 cells in Kras^{G12D} mice. Our study suggests that Th1/Th2 cytokine profile may be helpful for predicting the disease prognosis and targeting treatment strategies in pancreatic cancer patients.

6. CONCLUSIONS

Our study provides the first evidence that embelin can inhibit pancreatic cancer growth in mice models by modulating tumor microenvironment. Specifically, we have demonstrated that embelin inhibits PANC-1 xenografted tumor growth by suppressing Akt and Shh pathways. Embelin inhibits the production of pro-angiogenic VEGF/VEGFR, IL-6 and IL-8, and metastasis-promoting MMP-2 and MMP-9 thus blocking production of tumorigenic mediators in the microenvironment of the
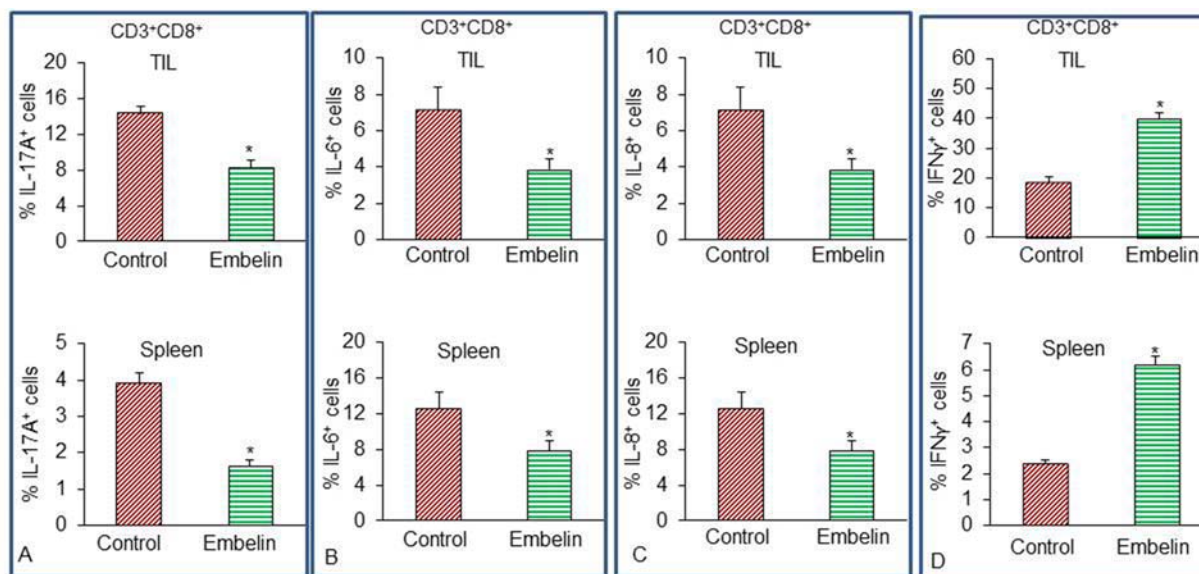
Figure 8. Effects of embelin on IL17-A$^+$, IL-6$^+$, IL-8$^+$ and IFNγ$^+$ cells in Kras^{G12D} mouse model. Splenic and pancreatic tumor cell (TIL) populations were isolated from Kras^{G12D} mouse as described in Materials and Methods. Cells were stimulated with ionomycin and PMA in the presence of monensin for 4 h and then stained with anti-CD3, anti-CD4, anti-IL-17A, anti-IL-6, anti-IL-8 and anti-IFNγ antibody. (A-D, upper panel), the percentage of CD3$^+$CD4$^+$ tumor infiltrating IL-17A$^+$, IL-6$^+$, IL-8$^+$, and IFNγ$^+$ cell populations. Data represent the mean ± SD. * significantly different from respective control (P < 0.05). (A-D, lower panel), the percentage of CD3$^+$CD4$^+$ splenic IL-17A$^+$, IL-6$^+$, IL-8$^+$ and IFNγ$^+$ cell populations. Data represent the mean ± SD. * significantly different from respective control (P < 0.05).

tumor. Embelin also inhibits EMT which plays a major role in metastasis. Furthermore, embelin inhibits pancreatic cancer growth in Kras^{G12D} mouse model of pancreatic cancer by modulating tumor immune microenvironment i.e. by increasing the infiltration of CTL, NK, NKT, γδT, and Th1 cells, and decreasing the infiltration of PMN-MDSC, IL-17A, IL-6, and IL-8 positive cells. Although we have not been able to provide precise mechanisms by which embelin regulates tumor immune microenvironment, it is clear that embelin upregulates Th1 type cells and inhibits Th2 type immune cells in Kras^{G12D} mice. Overall, our results suggest that embelin can inhibit pancreatic tumor growth and progression by modulating tumor immune microenvironment and Shh pathway, and thus can be used for the treatment and/or prevention of pancreatic cancer.

7. ACKNOWLEDGEMENTS

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