MYB Promotes Desmoplasia in Pancreatic Cancer through Direct Transcriptional Up-regulation and Cooperative Action of Sonic Hedgehog and Adrenomedullin*

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Extensive desmoplasia is a prominent pathological characteristic of pancreatic cancer (PC) that not only impacts tumor development, but therapeutic outcome as well. Recently, we demonstrated a novel role of MYB, an oncogenic transcription factor, in PC growth and metastasis. Here we studied its effect on pancreatic tumor histopathology and associated molecular and biological mechanisms. Tumor-xenografts derived from orthotopic-inoculation of MYB-overexpressing PC cells exhibited far-greater desmoplasia in histological analyses compared with those derived from MYB-silenced PC cells. These findings were further confirmed by immunostaining of tumor-xenograft sections with collagen-I, fibronectin (major extracellular-matrix proteins), and α-SMA (well-characterized marker of myofibroblasts or activated pancreatic stellate cells (PSCs)). Likewise, MYB-overexpressing PC cells provided significantly greater growth benefit to PSCs in a co-culture system as compared with the MYB-silenced cells. Interrogation of deep-sequencing data from MYB-overexpressing versus -silenced PC cells identified Sonic-hedgehog (SHH) and Adrenomedullin (ADM) as two differentially-expressed genes among others, which encode for secretory ligands involved in tumor-stromal cross-talk. In-silico analyses predicted putative MYB-binding sites in SHH and ADM promoters, which was later confirmed by chromatin-immunoprecipitation. A cooperative role of SHH and ADM in growth promotion of PSCs was confirmed in co-culture by using their specific-inhibitors and exogenous recombiant-proteins. Importantly, while SHH acted exclusively in a paracrine fashion on PSCs and influenced the growth of PC cells only indirectly, ADM could directly impact the growth of both PC cells and PSCs. In summary, we identified MYB as novel regulator of pancreatic tumor desmoplasia, which is suggestive of its diverse roles in PC pathobiology.

Pancreatic cancer (PC) is one of the most lethal malignancies in the United States, with a dismal median survival rate of 2–8 months after diagnosis and a 5-year overall survival rate of ~7% (1). According to an estimate by the American Cancer Society, it is expected to overtake breast cancer to become the third leading cause of cancer-related death in the United States (2). Nearly 53,070 patients are expected to be diagnosed with PC this year and ~41,780 will succumb to it (1). This continued increase in mortality rates is alarming and necessitates that we generate a better understanding of PC biology by identifying key molecular determinants of its pathological phenotypes, so that novel and more effective mechanism-based therapeutic approaches can be developed.

Pancreatic tumors are highly desmoplastic in nature exhibiting the presence of dense, fibrous connective tissue surrounding the tumor cells (3, 4). Desmoplasia is characterized by an increase in the presence of α-smooth muscle actin (α-SMA)-positive fibroblasts (myofibroblasts or activated pancreatic stellate cells) along with extensive deposition of extracellular matrix (ECM) proteins (5). This desmoplastic reaction not only gives pancreatic tumors their characteristic histoarchitecture, but is suggested to play important roles in disease progression and chemoresistance (6). Pancreatic stellate cells (PSCs) are the major cellular component of the desmoplastic stroma that synthesizes and secretes several ECM components within tumor microenvironment (7–10). Pancreatic tumor cell-derived signaling ligands have been shown to promote proliferation and differentiation of PSCs, and targeting of these signaling nodes is suggested to improve the efficacy of chemotherapy in preclinical models (5, 6).

MYB, a cellular progenitor of ν-Myb oncogene, encodes an oncogenic transcription factor that is shown to regulate a variety of cellular function by controlling expression of a wide array of genes (11). Genetic alterations of MYB in humans were first reported in acute myelogenous leukemia (12). However, in subsequent years, several studies have demonstrated genetic alterations or deregulated MYB expression in many other human cancers as well, including pancreatic cancer (13–16). Recently, we demonstrated that MYB serves as a novel regulator of pan-

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†‡§ The abbreviations used are: PC, pancreatic cancer; SMA, smooth muscle actin; ECM, extracellular matrix; PSC, pancreatic stellate cells; SHH, Sonic-hedgehog; ADM, Adrenomedullin.
creatic tumor growth and metastasis (17). Its expression was reported in majority of PC tissues and cell lines, while it remained largely undetectable in normal pancreatic cells. We also identified several gene targets of MYB that may mediate its impact on PC pathogenesis. However, its in-depth mechanistic involvement and diverse effects on pancreatic tumor phenotypes are not yet well understood.

Here, we provide evidence supporting a role of MYB in pancreatic tumor desmoplasia. We show that the tumors derived from MYB-overexpressing PC cells exhibit greater desmoplasia, characterized by the expression of myofibroblast marker (α-SMA) and extracellular-matrix proteins (collagen I and fibronectin). We also establish SHH (Sonic hedgehog) and ADM (Adrenomedullin) as direct transcriptional targets of MYB that mediate its effects on the growth of pancreatic tumor and stellate cells via autocrine and/or paracrine signaling. Together, our studies establish MYB as a key driver of PC progression and metastasis by directly impacting the tumor cells as well as by altering the tumor microenvironment.

Results

Tumors Derived from MYB-overexpressing Pancreatic Cancer Cells Exhibit High Desmoplasia—We performed histopathological analyses of tumor xenografs derived from recently developed orthotopic mouse model of pancreatic cancer (17). Since tumors were derived from MYB-overexpressing and -silenced cells, we observed expected differences in MYB expression in tumor xenograft sections as well (data not shown). When stained with H&E, tumor sections from high MYB-expressing group exhibited dense desmoplastic regions surrounding the pancreatic tumor cells, while only minimal desmoplasia was observed in that of low MYB-expressing group (Fig. 1A). This observation was further confirmed by immunostaining the tumor sections for collagen I, fibronectin, and α-SMA, which are well-characterized markers of desmoplasia (5). A dramatic decrease in the expression of collagen I, fibronectin, and α-SMA was detected in tumors generated from MiaPaCa-shMYB cells compared with those derived from MiaPaCa-NT-Scr cells (Fig. 1, B–D). Likewise, we also observed high desmoplasia associated with elevated expression of collagen I and α-SMA in tumors derived from MYB-overexpressing BxPC3-MYB cells (data not shown). Together, these findings suggest that MYB may serve as a novel regulator of pancreatic tumor desmoplasia.

MYB Promotes Pancreatic Tumor Desmoplasia

FIGURE 1. MYB induces desmoplasia in pancreatic tumors. A, tissue sections of orthotopically developed pancreatic tumors from high (MiaPaCa-NT-Scr) and low (MiaPaCa-shMYB) MYB-expressing PC cells were deparaffinized, rehydrated, and stained with H&E to study their histopathological characteristics. B–D, deparaffinized and rehydrated tumor tissue sections were incubated overnight with (B) collagen-I, (C), fibronectin, and (D) α-SMA specific antibodies. Subsequently, sections were incubated at room temperature with respective polymer and probe, and immunoreactivity was visualized by incubation with DAB Chromagen followed by hematoxylin counterstain. Arrows indicate the desmoplastic region surrounding the tumor cells.
MYB Promotes Pancreatic Tumor Desmoplasia

ADM and SHH Are Novel Direct Transcriptional Targets of MYB in Pancreatic Cancer Cells—To understand the molecular basis of MYB-potentiated phenotypic changes, we interrogated our differential gene expression data from deep sequencing analysis of MYB-expressing and -silenced pancreatic tumor cells (GEO accession number GSE61290). Among several differentially expressed genes, we found a decreased expression of ADM (adrenomedullin) and SHH (sonic hedgehog) in MYB-silenced cells, which have previously been implicated in the development of desmoplasia in pancreatic tumors (5, 18). To ascertain the effect of MYB silencing on ADM and SHH, we analyzed their expression in MYB-expressing (MiaPaCa-NT-Scr) and -silenced (MiaPaCa-shMYB) cells as well as MYB-null BxPC3 cells and ectopically MYB-expressing BxPC3-MYB cells at both mRNA (by quantitative RT-PCR) and protein (by immunoblot) levels. We observed enhanced expression of both mRNA and protein (by immunoblot assay, β-actin was used as internal control) in cells treated with 5E1 (Fig. 5A) treatments. In parallel, the co-culture of PSCs with low MYB-expressing cells was treated with recombinant human ADM (1–52) and r-SHH (84.2%, BxPC3-MYB and MiaPaCa-shMYB) (Fig. 5A). Likewise, a greater decrease in growth induction of PSCs co-cultured with high MYB-expressing cells (BxPC3-MYB and MiaPaCa-NT-Scr) and low (BxPC3-Neo and MiaPaCa-shMYB) MYB-expressing cells was isolated and the expression of α-SMA was examined by immunoblot assay. A greater effect on the growth of PSCs was observed when the co-culture was treated simultaneously with ADM (22–52) and 5E1 treatments. In parallel, the co-culture of PSCs with low MYB-expressing cells was observed upon treatment with ADM (22–52) (~63 and ~65%, respectively) as compared with that observed upon treatment with 5E1 (~48 and ~50%, respectively) (Fig. 5B). A more potent inhibition of growth induction of both the PCCs (Fig. 5A) and PSCs (Fig. 5B) was found when the co-culture was treated simultaneously with ADM (22–52) and 5E1. Growth of BxPC3-Neo cells co-cultured with PSCs was increased by 12.3 and 15.3% upon treatment with ADM (1–52) and r-SHH, respectively, while slightly higher effect (19.8%) was observed upon their co-treatments (Fig. 5C). Likewise, the growth of PSCs co-cultured with low MYB-expressing cells also increased significantly upon treatment with ADM (1–52) (64.7%, BxPC3-Neo; 85.4%, MiaPaCa-shMYB); or r-SHH (77.3%, BxPC3-Neo; 76.5%, MiaPaCa-shMYB) (Fig. 5D). A greater effect on the growth of PSCs was observed when the co-culture was simultaneously treated with ADM (1–52) and r-SHH (84.2%, BxPC3-Neo; 90.8%, MiaPaCa-shMYB) (Fig. 5D).

To ascertain if the impact of ADM and SHH was solely paracrine or involve an autocrine loop as well, we treated high MYB-

FIGURE 2. MYB promotes growth of pancreatic stellate cells. A, pancreatic stellate cells (PSCs; $2 \times 10^5$/well) were seeded in the 6-well plates, and pancreatic cancer cells (PCCs; $1 \times 10^5$/well) were seeded into insert chamber. After 24 of culturing, insert having PCCs was placed over the well containing PSCs and allowed to grow for 144 h with medium replacement after every 48 h. Induction of growth in PSCs relative to respective monocultures (PSCs/PSCs) was examined by counting the number of viable cells. B, PCCs ($1 \times 10^5$/well) were seeded into insert chamber and growth of PCCs was examined by counting the number of viable cells after 144 h as described above. For control, both well and insert chambers were seeded with PCCs. Data presented as mean ± S.D., n = 3; * (p < 0.05). C, total protein from PSCs co-cultured with high (BxPC3-MYB and MiaPaCa-NT-Scr) and low (BxPC3-Neo and MiaPaCa-shMYB) MYB-expressing cells was isolated and the expression of α-SMA was examined by immunoblot assay. β-actin was used as internal control.

4 Transcript profiling: GEO accession number GSE61290.
expressing PC cells with ADM-(22–52) and 5E1 alone or in combination. Similarly, we also treated low MYB-expressing cells with ADM-(1–52) and/or r-SHH. We observed that the

growth of high MYB-expressing PC cells was inhibited (22.3%, BxPC3-MYB; 23.9%, MiaPaCa-NT-Scr) when treated with ADM-(22–52); however, limited effect was observed upon

FIGURE 3. MYB enhances the expression of SHH and ADM in pancreatic cancer cells. A–B, total RNA was isolated from BxPC3-Neo/BxPC3-MYB and MiaPaCa-NT-Scr/MiaPaCa-shMYB PC cells and expression of (A) ADM and (B) SHH was examined by q-RT-PCR using specific primer sets. GAPDH served as an internal control. Bars represent mean ± S.D., n = 3; *, p < 0.05. C, total protein from PC cells was isolated, resolved by SDS-PAGE and the expression of ADM and SHH was examined by immunoblot assays. β-actin was used as loading control. D–E, PC cells (1 × 10⁶/well) were grown in regular culture condition for 24 h. Subsequently, medium was replaced with 2% FBS-containing medium and allowed to grow for next 48 h. Thereafter, culture supernatants were collected and centrifuged to clear the cellular debris and levels of (D) ADM and (E) SHH were measured using respective ELISA kits. Expression levels of ADM and SHH were normalized with cell number at end point, and data shown as mean ± S.D., n = 3; *, p < 0.05. F, tissue sections of orthotopically developed tumors from MiaPaCa-NT-Scr/MiaPaCa-shMYB PC cells were incubated overnight with ADM or SHH specific antibodies at 4 °C. Thereafter, sections were incubated at room temperature with respective polymer and probe, and immunoreactivity was visualized by using DAB Chromogen followed by hematoxylin counterstain.

FIGURE 4. MYB regulates SHH and ADM via direct binding to their promoter regions. A, localization of putative MYB binding sites (thick black bars) in human ADM (hADM) and human SHH (hSHH) promoter region. Arrows indicate the complementary sites for the forward and reverse primers in the flanking region of MYB binding site(s). B–C, DNA-protein was cross-linked with formaldehyde. Cross-linked chromatin was sheared and subjected to immunoprecipitation using anti-MYB or normal rabbit IgG (as control). PCR was performed using specific primers sets flanking the MYB-binding sites within the (B) ADM and (C) SHH promoter regions. Input DNA (without immunoprecipitation) and normal IgG-precipitated DNA were used as positive and negative controls, respectively. Bars represent mean ± S.D., n = 3; *, p < 0.05.
treatment with 5E1 (Fig. 6A). Interestingly, when we treated low MYB-expressing cells with either ADM-(1–52) or r-SHH or combination, we did not observe much induction in their growth (7.9, 6.5, 10.6% in BxPC3-Neo and 8.6, 6.4, 13.1% in MiaPaCa-shMYB, respectively) (Fig. 6B). Treatment of PSCs with ADM-(1–52), r-SHH, ADM-(22–52) and 5E1 alone or in combination confirmed their growth response to both ADM and SHH (Fig. 6C). Taken together, data suggest that ADM and SHH cooperatively mediate the effect of MYB on the growth of pancreatic stellate as well as tumor cells via paracrine and/or autocrine signaling.

Discussion

Although some doubts have been raised regarding the functional significance of extensive desmoplasia in PC pathobiology, the fact remains that it is a unique feature of PC and co-evolves with the malignant disease during the course of its progression (6, 9, 10, 19, 20). Therefore, it remains of utmost importance to understand the molecular basis of its evolution as well as its biological importance in PC development. Our novel findings provide compelling data to suggest a role of MYB in pancreatic tumor desmoplasia, and thus further adding to its pathobiological significance in PC.

We recently reported a novel role of MYB in pancreatic tumor growth and metastasis (17). These effects were suggested to involve a positive impact of MYB on cell-cycle progression, apoptosis-resistance, as well as malignant features of pancreatic tumor cells via targeting of functionally relevant gene targets. The data presented herein demonstrate that MYB-overexpressing pancreatic tumors also differ in their histopathology. Tumors derived from MYB-expressing PC cells were highly fibrotic, as demonstrated by the immunostaining of ECM proteins, collagen I, and fibronectin, while only minimal reactivity was reported in tumors derived from MYB-silenced PC cells.

Both collagen I and fibronectin are not only major components of pancreatic tumor-associated stroma, but they have also been shown to promote cancer development by inducing EMT and invasiveness of pancreatic tumor cells (21–23). A major source of these ECM proteins is myofibroblasts, which originate from activated pancreatic stellate cells (PSCs) (7, 8). Accordingly, our
immunohistochemical data demonstrated high levels of smooth muscle α-actin (α-SMA), a well-established marker of myofibroblasts, within desmoplastic stroma of MYB-overexpressing pancreatic tumors. This also suggested that MYB-overexpressing PC cells interact differentially with PSCs to promote desmoplastic reaction during tumor development.

Positive interaction of MYB-overexpressing pancreatic tumor cells with PSCs was also confirmed in our in vitro co-culture studies. MYB-overexpressing PC cells not only promoted the growth of PSCs in co-culture, but also induced the expression of α-SMA suggesting their activation and differentiation into myofibroblasts. These effects were less pronounced in MYB-silenced or MYB-null cells thus supporting a role of MYB in this tumor-stromal cross-talk. However, stimuli for PSCs should come from the changes in its microenvironment (24–26), and specifically in this case from factors secreted by MYB-overexpressing cells. These factors were characterized to be SHH and ADM that exhibited greater expression in MYB-overexpressing cells at RNA, protein and secretome levels. More importantly, our data suggested mostly a paracrine action of SHH, while the other factor ADM, influenced the growth of both PSCs and PC cells and thus likely acted in both paracrine and autocrine manner. These findings were consistent with published data that has suggested mostly a paracrine signaling for pancreatic tumor cell-derived SHH (27). Inhibition of SHH was shown to decrease desmoplasia through direct activation of hedgehog signaling in PSCs (5), while it indirectly affected PC metastasis and lymphangiogenesis due to altered tumor microenvironment (28). In other reports, ADM has also been shown to be up-regulated in PC patients (29). It has been suggested that ADM acts directly on the tumor cells, but also influences tumor progression through its effects on endothelial cells and PSCs (18, 30). Moreover, despite some conflicting data suggesting a suppressive effect of PSCs on PC progression and survival (9, 10), our data indicate mutual benefit of pancreatic tumor and stellate cells cross-talk at the molecular level.

Several mechanisms have been reported for tumor-stromal interactions culminating into ECM remodeling (31, 32). In fact, not only the tumor cells, but also the hypoxic and inflammatory tumor microenvironment could also influence stromal composition of pancreatic tumors (23, 24, 26). Studies on bi-directional tumor-stromal cross-talk in PC have reported signaling pathways that could influence the production of SHH and ADM (27, 33). Moreover, hypoxic tumor microenvironment has also been shown to promote synthesis and secretion of SHH by pancreatic tumor cells to promote desmoplasia and thus engage in vicious hypoxia-desmoplasia loop (34). Similarly, ADM has also been reported to be up-regulated by hypoxia in pancreatic tumor cells (35). In this regard, our findings establishing MYB as a novel transcriptional regulator of SHH and ADM are of great significance and indicative of important roles of MYB as a mediator in active cross-talk between tumor cells and a variety of factors within the tumor microenvironment. It is likely that MYB, either mediate the tumor-tumor microenvironment signaling or act in cooperation to promote positive tumor-stromal interaction, and thus facilitate pancreatic tumor development.

In summary, we have identified important role of MYB in the growth promotion and differentiation of pancreatic stellate cells through directly regulating SHH and ADM. We have also demonstrated that ADM and SHH cooperatively mediate the effect of MYB on the growth of pancreatic tumor and stellate cells via paracrine and/or autocrine signaling. These findings establishing MYB as a novel regulator of pancreatic tumor desmoplasia are of great significance from the molecular pathogenesis standpoint and provide us novel insight into PC pathobiology.

**Experimental Procedures**

**Cell Lines and Culture Conditions**—All the pancreatic cancer cell lines used in this study were procured and maintained as previously described (17). Pancreatic stellate cells (generously gifted by Dr. P. K. Singh, Eppley Cancer Institute, Omaha, NE) were maintained in DMEM supplemented with 20% FBS and 100 μM each of penicillin and streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C. All the cells were tested and determined to be free of mycoplasma every month and prior to beginning of any functional assay.

**Reagents, Gene Constructs, and Antibodies**—The following reagents were used: Roswell Park Memorial Institute medium (RPMI 1640); Dulbecco’s Modified Eagle Medium (DMEM); fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA); penicillin and streptomycin (Invitrogen, Carlsbad, CA); Adrenomedullin (Human)-ELISA Kit (Phoenix Pharmaceuticals, Burlingame, CA); MycoSensorPCR assay kit (Stratagene, La Jolla, CA); FuGENE transfection reagent (Roche, Indianapolis, IN); a chromatin immunoprecipitation assay (ChIP) kit (Active Motif, Carlsbad, CA); Western blotting SuperSignal West Femto Maximum sensitivity substrate kit (Thermo Scientific, Logan, UT); immunohistochemical analysis reagent EZ-Dewax (Bigenex, Fremont, CA); background sniper, polymer and probe (Biocare Medical, Concord, CA). The following antibodies were used: α-SMA (1:100, rabbit polyclonal; S0010) (Epitomics, Burlingame, CA), ADM (1:1000, rabbit polyclonal; ab69117), fibroactin (ab6328) and collagen I (ab88147) (1:100, mouse monoclonal) and SHH (1:1000; rabbit monoclonal; ab53281) (Abcam, Cambridge, MA), mouse monoclonal biotinylated anti-β-actin (1:20,000; A3854; Sigma-Aldrich) and horseradish peroxidase (HRP)-labeled secondary antibodies (1:2000; Santa Cruz Biotechnology).

**Western Blotting Analysis**—Western blotting was performed using standard procedures as described earlier (36, 37). Briefly, cell lysates were resolved on 10% polyacrylamide gels and transferred to PVDF membranes. Blots were subjected to a standard immunodetection procedure using specific antibodies against various proteins and visualized using SuperSignal West Femto Maximum sensitivity substrate kit with a LAS-3000 image analyzer (Fuji Photo Film Co., Tokyo, Japan).

**RNA Isolation, cDNA Synthesis, and Quantitative Real Time-PCR (qRT-PCR)**—Total RNA was extracted using TRizol reagent. Two microgram of total RNA was used for cDNA synthesis using the High Capacity complementary DNA reverse transcription kit following manufacturer’s instructions. Subsequently, quantitative RT-PCR was performed in 96-well plates using cDNA as a template and SYBR Green Mas-
MYB Promotes Pancreatic Tumor Desmoplasia

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MYB Promotes Pancreatic Tumor Desmoplasia

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