

Mesenchymal Stem Cells Promote Pancreatic Tumor Growth by Inducing Alternative Polarization of Macrophages^{1,2}

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Abstract

Pancreatic cancer is characterized by an extensive desmoplastic stroma, the functional relevance of which is poorly understood. Activated fibroblasts are a prevalent component of the stroma, and traditionally, these cells have been considered as a homogenous population derived from pancreatic stellate cells. In this study, we highlight a previously unappreciated heterogeneity of the fibroblast population within the stroma. In particular, a subset of stromal fibroblasts has characteristics of mesenchymal stem cells (MSCs). MSCs are present in the normal pancreas as well as in the carcinomatous pancreas (CA-MSCs). Here, we determine that CA-MSCs have increased tumor-promoting function compared with MSCs in normal pancreas. This ability to promote tumor growth is associated with CA-MSCs' unique ability to promote alternative macrophage polarization. Thus, our study identifies a previously uncharacterized cell population within the stroma and sheds light on tumor-promoting interactions between different components of the stroma.

Significance

Targeting the stroma is emerging as a new paradigm in pancreatic cancer; however, efforts to that effect are hampered by our limited understanding of the nature and function of stromal components. Here, we uncover previously unappreciated heterogeneity within the stroma and identify interactions among stromal components that promote tumor growth and could be targeted therapeutically.

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Introduction

Pancreatic cancer is among the deadliest of human malignancies. A prominent feature of pancreatic cancer is an extensive reactive stroma, which can comprise up to 90% of the overall tumor volume, the highest fraction of all solid, epithelial tumors (for review, see [1]). The accumulation of a desmoplastic stroma occurs from the onset of pancreatic carcinogenesis and is evident in the precursor lesions of pancreatic cancer known as *pancreatic intraepithelial neoplasia* or *PanINs* [2]. The cellular components of the stroma include fibroblasts, myofibroblasts, endothelial cells, and infiltrating immune cells [3,4]. Fibroblasts are an abundant and poorly characterized component of the stroma, thought to derive from pancreatic stellate cells. Fibroblasts within the stroma have been thought of as protumor

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agents [5], but recent studies have challenged this concept, indicating that stromal fibroblasts might act to restrain tumor growth [6,7]. This controversy might in part reflect our limited understanding of cellular components of the stroma and their individual contribution to tumorigenesis.

The healthy pancreas includes different fibroblast populations. A population of mesenchymal stem cells (P-MSCs) was identified in the normal human and mouse pancreas [8,9]; however, whether MSCs are present in pancreatic carcinoma and what their function might be during carcinogenesis remained unclear. MSCs were identified as a tumor-promoting stromal component in several epithelial cancers [10–12]. Interestingly, the manner in which MSCs promote tumorigenesis is distinct in each tumor context. In breast cancer, bone marrow–derived MSCs promote the metastasis of tumor cells through a CCL5-mediated effect [11]. In ovarian cancer, MSCs isolated from the tumor stroma secrete bone morphogenic proteins (BMPs) that increase the cancer stem cell population [12]. More recently, stroma-derived MSCs from lymphomas have been shown to secrete monocyte/macrophage chemoattractants, which in turn promote tumor growth [13].

The identification and characterization of MSCs in pancreatic tumor growth are the focus of the current study. We use a genetically engineered mouse model of pancreatic cancer, the KC mouse (Ptf1a-Cre;LSL-Kras^{G12D}) [14], that expresses an oncogenic form of Kras, thus recapitulating the most common genetic alteration in human PanINs and pancreatic cancer [15,16]. KC mice develop PanINs in a stepwise manner that recapitulates human carcinogenesis [14]. Our results show that MSC populations are present in both the normal murine pancreas (P-MSCs) and neoplastic mouse pancreas (carcinogenesis-associated MSCs, CA-MSCs). By performing functional comparisons of these two populations, we determined that CA-MSCs have an increased tumor-promoting potential, which is mediated, at least in part, by their unique ability to induce macrophage polarization to a protumor, alternatively activated status.

Results

MSCs in the Normal and in the Neoplastic Pancreas

MSCs are defined by their ability to differentiate into osteoblasts, chondrocytes, and adipocytes when exposed to appropriate differentiation media *in vitro*. To test whether we could identify an MSC population in the normal and neoplastic murine pancreas, we isolated pancreata from wild-type and littermate KC mice 3 weeks following caerulein-induced acute pancreatitis. Wild-type pancreata have completed the tissue repair process by this time, whereas in KC pancreata, extensive PanINs surrounded by desmoplastic stroma are evident (Figure 1A). Isolated bulk fibroblast populations from the pancreata were exposed to osteoblast, adipocyte, and chondrocyte differentiation media. Trilineage differentiation was observed both in the wild-type and in the KC-derived pancreata, indicating that an MSC population might exist in both settings (Figure 1B). Thus, our data confirmed the presence of an MSC population in the normal pancreas, as previously described [8,9,17], and identified a similar population in the neoplastic pancreas. MSCs have been isolated based on the expression of a panel of surface markers: CD45⁻;CD44⁺;CD49a⁺;CD73⁺;CD90⁺ [18]. To determine whether these markers were sufficient to isolate the MSC population in the pancreas, we isolated single-cell suspensions from wild-type and KC pancreata 3

weeks after the induction of pancreatitis ($n = 3$ mice/genotype) and used fluorescent-activated cell sorting to isolate and quantify cells expressing MSC markers. Although CD45⁻;CD44⁺;CD49a⁺;CD73⁺;CD90⁺ cells were present in both sample sets, their number was significantly higher in KC pancreata compared with the normal mouse pancreas (Figure 1A). To determine whether the surface markers did indeed identify the MSC population, we cultured CD44⁺;CD49a⁺;CD73⁺;CD90⁺ cells (putative MSCs, 4⁺) as well as cells negative for all markers (4^{neg}) and performed *in vitro* differentiation assays with protocols promoting osteoblast and adipocyte lineages. In differentiation media, CD45⁻;CD44⁺;CD49a⁺;CD73⁺;CD90⁺ cells from both the normal and neoplastic pancreas could differentiate into osteoblasts as determined by Alizarin Red staining of calcium deposits, and expression of the osteoblast marker alkaline phosphatase by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis (Figure 1, C and D, top row). Likewise, CD45⁻;CD44⁺;CD49a⁺;CD73⁺;CD90⁺ cells could differentiate into adipocytes as determined by Oil Red O staining of lipid droplets and expression of adipocyte marker fatty acid binding protein 4 (Fabp4) (Figure 1, C and D, bottom row). In contrast, 4^{neg} cells did not differentiate into any of the lineages (Figure 1, C and D), indicating that the combination of CD44⁺, CD49a⁺, CD73⁺ and CD90⁺ surface markers identifies a subset of multipotent cells.

Distinct Cytokine Expression Profile in CA-MSCs

Because MSCs were present both in the normal and in the neoplastic pancreas, we set out to compare their functional characteristics. Hereby, normal pancreas–derived MSCs are referred to as P-MSCs, whereas MSCs derived from the neoplastic pancreas are referred to as CA-MSCs (carcinoma-associated MSCs). In ovarian cancer, CA-MSCs are distinct from bone marrow and adipose-derived MSCs by the expression of BMP2 and BMP4 [12], and those factors confer a higher tumor-promoting ability to CA-MSCs. Thus, we measured the relative expression of BMP2 and BMP4 by qRT-PCR in isolated P-MSCs and CA-MSCs. We detected no difference in BMP2 expression, whereas BMP4 expression was decreased in CA-MSCs compared with P-MSCs (Figure S1A).

We have previously shown that pancreatic fibroblasts secrete a number of cytokines that regulate the infiltration of immune cells during pancreatic damage and repair and during carcinogenesis [19]. Thus, we measured expression of those cytokines by qRT-PCR in freshly sorted P-MSCs and CA-MSCs. Interestingly, we observed a significant increase in several cytokines known to promote tumorigenesis, including IL-6, IL-10, and TGFβ [20–24] (Figure 1E), in CA-MSCs compared with P-MSCs. Then, we measured the expression of the same subset of cytokines in cultured CA-MSCs and P-MSCs, respectively, and found a similar profile, with significantly elevated cytokines in CA-MSCs (Figure S1B). Similarly M-CSF and GM-CSF, cytokines known to regulate immune cell recruitment and function, were significantly elevated in CA-MSCs compared with P-MSCs. Finally, we compared paired bone marrow and pancreatic MSCs extracted from a control mouse (P-BM MSCs and P-MSCs) or a PanIN-bearing KC mouse (CA-BM MSCs and CA-MSCs). P-MSCs expressed higher levels of IL6, Cox-2, and IL10 than their bone marrow counterparts. CA-MSCs expressed higher levels of IL6, Cox-2, TGFβ, and IL-10 than both P-MSCs and CA-BM MSCs (Figure S1C). These data indicate that MSCs extracted from the neoplastic pancreas have unique

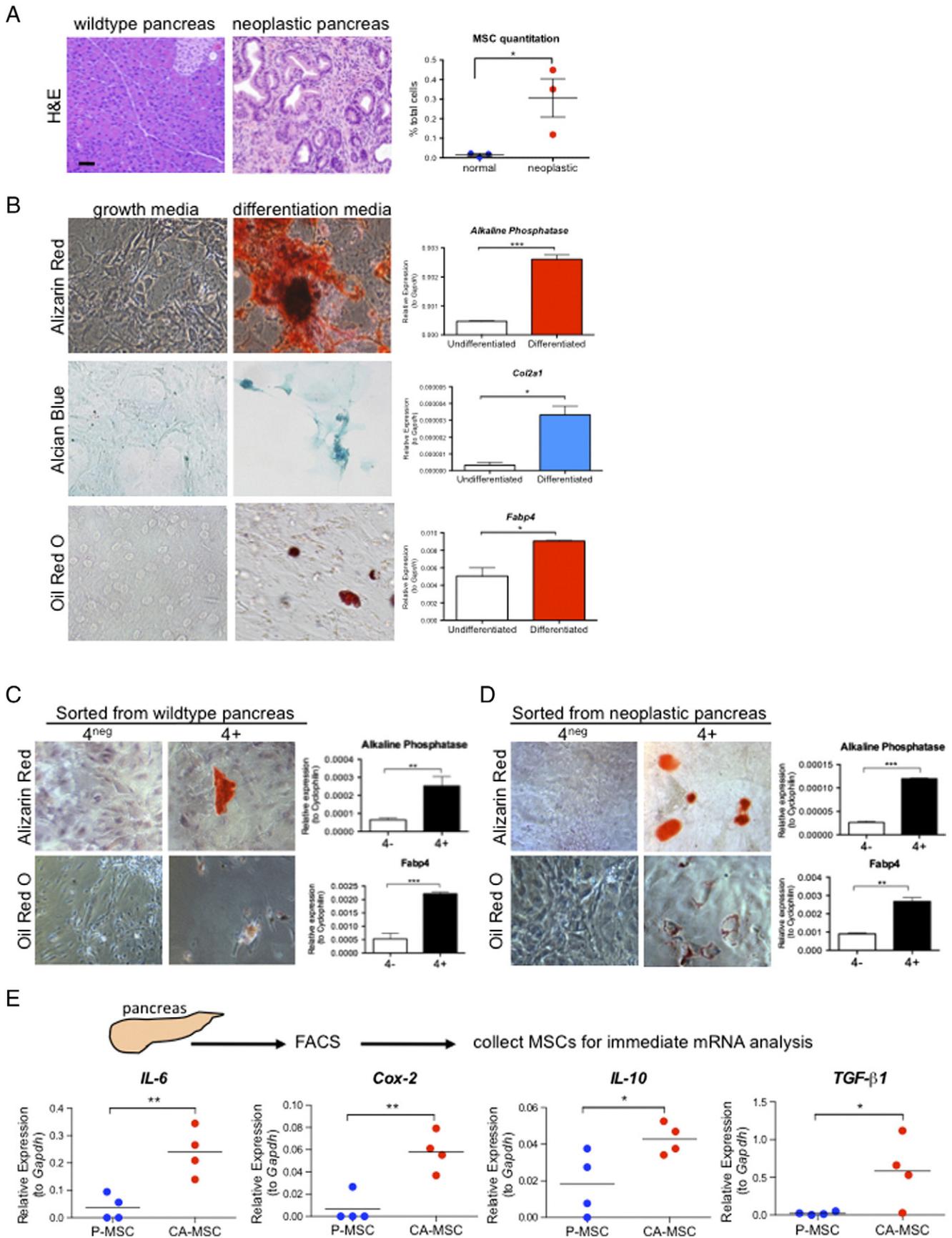


Figure 1. Multipotent stromal cells (MSCs) are present in the pancreas. (A) MSCs are increased in the neoplastic pancreas compared with the normal pancreas as quantified by flow cytometry. Scale bar for representative hematoxylin and eosin (H&E) images, 50 μ m. (B) Stromal cells isolated from the neoplastic pancreas can differentiate into osteoblasts (top row), chondrocytes (middle row), and adipocytes (bottom row). (C) P-MSCs can differentiate into bone (top row) and fat (bottom row). (D) CA-MSCs can differentiate into bone (top row) and fat (bottom row). (E) qRT-PCR on MSCs freshly sorted from the pancreas.

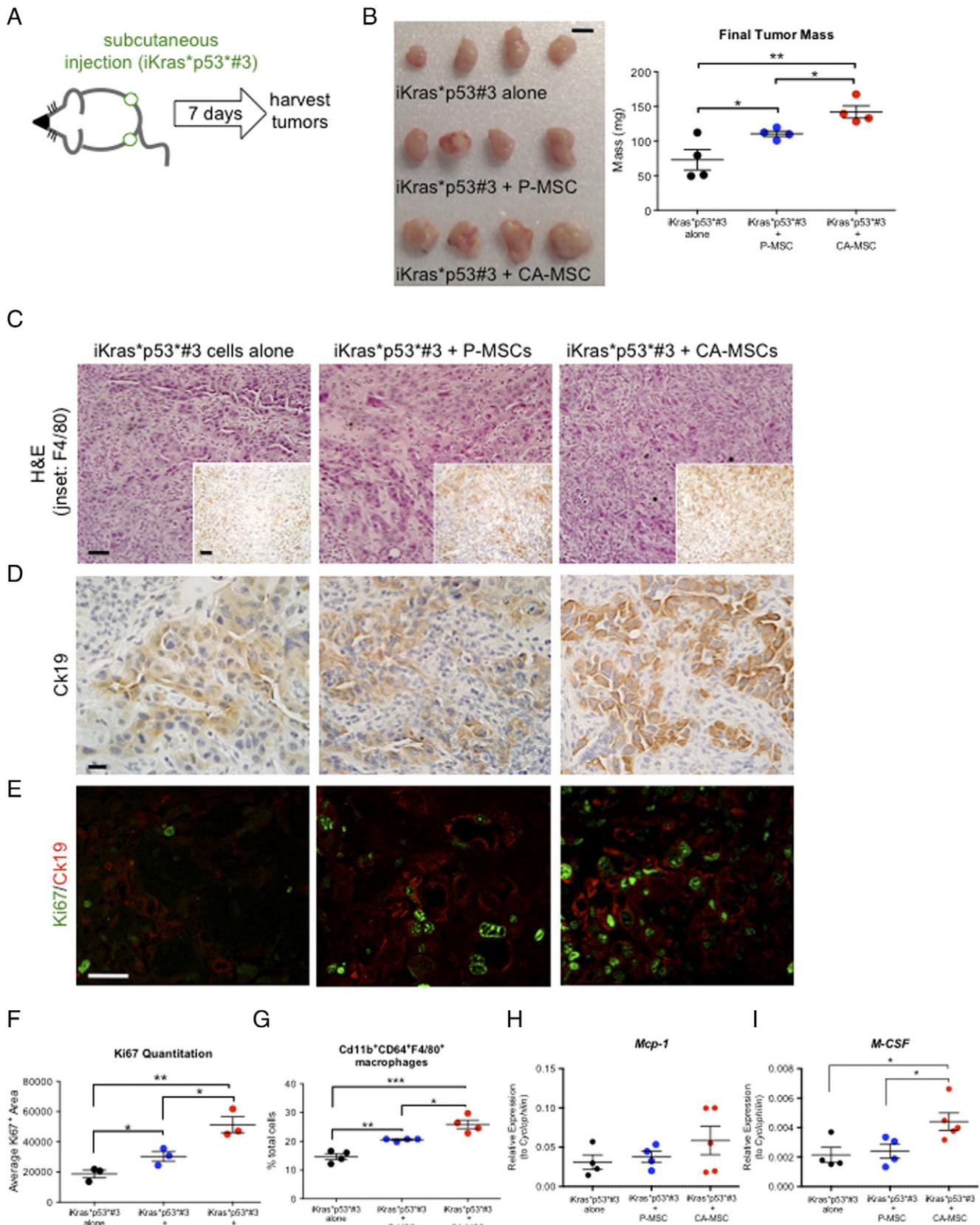


Figure 2. CA-MSCs promote tumor growth. (A) Experimental design. (B) Gross tumor morphology and final tumor weights. Scale bar represents 0.5 cm. (C) Histopathological analysis of tumors following coinjection. H&E staining; scale bar represents 50 μ m. (Inset) F4/80 staining; scale bar represents 50 μ m. (D) Immunohistochemistry for Ck19; scale bar represents 20 μ m. (E) Immunohistochemistry for Ki67 (green) and Ck19 (red); scale bar represents 20 μ m. (F) Quantitation of Ki67 staining. (G) Flow cytometry analysis of macrophages. (H) qRT-PCR analysis for *Mcp-1* and (I) *M-csf*.

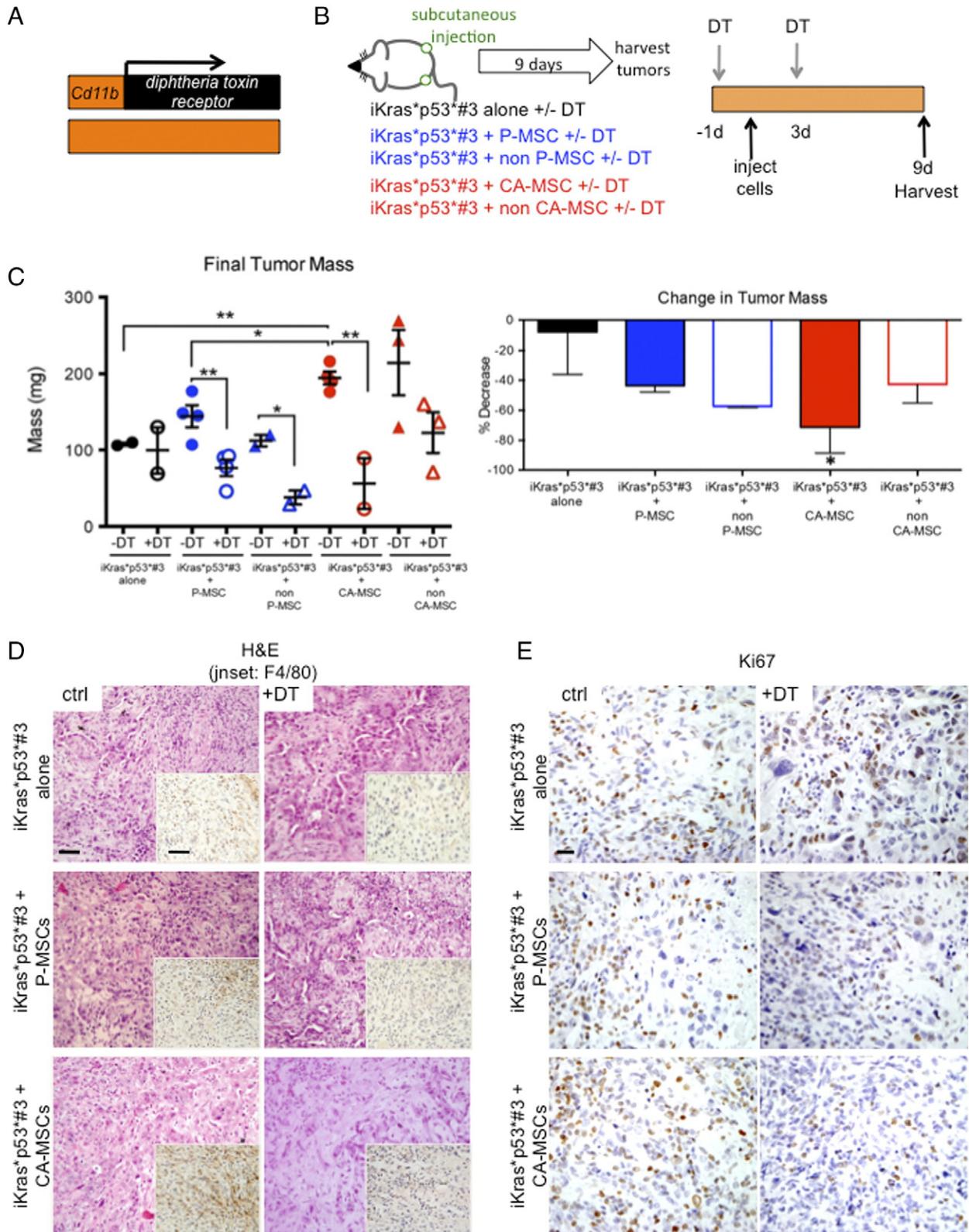


Figure 3. Myeloid cells are vital for CA-MSC–mediated tumor growth. (A) Schematic for Cd11b-DTR mouse. (B) Experimental design. (C, Left) Final tumor weights. Data are presented as mean \pm SEM. (Right) Percentage decrease in tumor weights. (D) Histopathological analysis of tumors following coinjection. H&E staining; scale bar represents 50 μ m. (Inset) F4/80 staining; scale bar represents 50 μ m. (E) Immunohistochemistry for Ki67; scale bar represents 20 μ m.

characteristics that are not shared by their counterpart extracted from the normal organ nor by MSCs extracted by different organs (in this case, the bone marrow) of a mouse bearing neoplastic changes in the pancreas.

MSCs Pancreatic Tumor Growth

To determine the functional effect of MSCs on tumor growth, we performed subcutaneous coinjections of tumor cells and MSCs into immunocompetent mice using a littermate syngeneic approach. We used tumor cells isolated from the *iKras**p53* model [25] of pancreatic cancer (*iKras**p53*#3 cells [26]) or tumor cells derived from the KPC mouse model [27] of pancreatic cancer (13442 cells). Tumor cells were injected at a 1:1 ratio with either P-MSCs or CA-MSCs (see schematic in Figures 2A and S2A). Coinjection of P-MSCs with *iKras**p53*#3 or 13442 tumor cells promoted tumor growth, but coinjection of CA-MSCs promoted even larger tumor growth (Figures 2B and S2B). The histology of all three cohorts was similar, with epithelial structures surrounded by abundant stroma (Figures 2C and S2C). Staining for Ck19 to mark tumor cells revealed increased number of tumor cells in coinjections with CA-MSCs (Figures 2D and S2D). Consistently, we detected increased intratumor proliferation—as indicated by Ki67 staining—in coinjections with CA-MSCs. Immunofluorescence analysis revealed a notable increase in proliferating tumor Ck19+ cells, indicating an increase in proliferating tumor cells (Figures 2, E and F, and S2, D and E). To determine whether MSCs were still able to promote tumor growth when injected at a lower ratio, we performed a parallel set of experiments by injecting tumor cells (13442) and MSCs at a 2:1 ratio. We found that, at this lower ratio, P-MSCs were unable to promote tumor growth, whereas in contrast, CA-MSCs still promoted tumor growth, further validating the concept that CA-MSCs have increased tumor-promoting ability (Figure S2K).

Because our data indicated that CA-MSCs have different functional characteristics than P-MSC, we expanded our characterization to include bone marrow–derived MSCs (BM-MSCs). In the absence of rigorous lineage tracing studies, it is not known whether CA-MSCs derived from P-MSCs or, at least in part, from infiltrating BM-MSCs, as observed in other disease contexts [28,29]. This is a question that warrants further studies that are beyond the scope of the current manuscript. BM-MSCs from a control mouse promoted tumor growth similarly to P-MSCs. However, CA-MSCs were able to promote significantly more tumor growth than their bone marrow–derived counterparts (Figure S2, I and J). Thus, CA-MSCs have a unique tumor promoting ability; we then set out to understand the mechanistic basis for this finding.

Given our previous observation that CA-MSCs secreted a number of cytokines that are known to regulate macrophages, we sought to determine the effect of MSC coinjection on macrophage infiltration within the tumor. Thus, we stained tissues for F4/80, a mature macrophage marker. We detected a significant increase in macrophages in the tumors derived from coinjection of tumor cells and CA-MSCs (Figures 2C and S2C, insets) compared with tumor cells alone or coinjected with P-MSCs. To quantify macrophage numbers in the subcutaneous tumors, we performed flow cytometry for macrophages defined as CD11b⁺;CD64⁺;F4/80⁺ cells. We found that tumors derived from the CA-MSC coinjection consistently had the highest population of macrophages compared with the other experimental cohorts, thus corroborating the histology (Figures 2G and S2F).

Monocyte migration to the tumor stroma and subsequent differentiation into macrophages are processes orchestrated by an

array of signaling molecules. To assess potential differences in these cytokine levels between tumor cohorts, we collected RNA from subcutaneous tumor tissue for qRT-PCR analysis. We found that expression of *Mcp-1*, a potent monocyte chemoattractant, did not differ between tumors coinjected with P-MSCs or with CA-MSCs but, at least for tumors derived from the 13442 line, was higher in the MSC-coinjected tumors than in the control group (Figures 2H and S2G). Interestingly, we detected an increase in M-CSF in coinjections with CA-MSCs (Figures 2I and S2H), a cytokine that supports the differentiation of monocytes into macrophages.

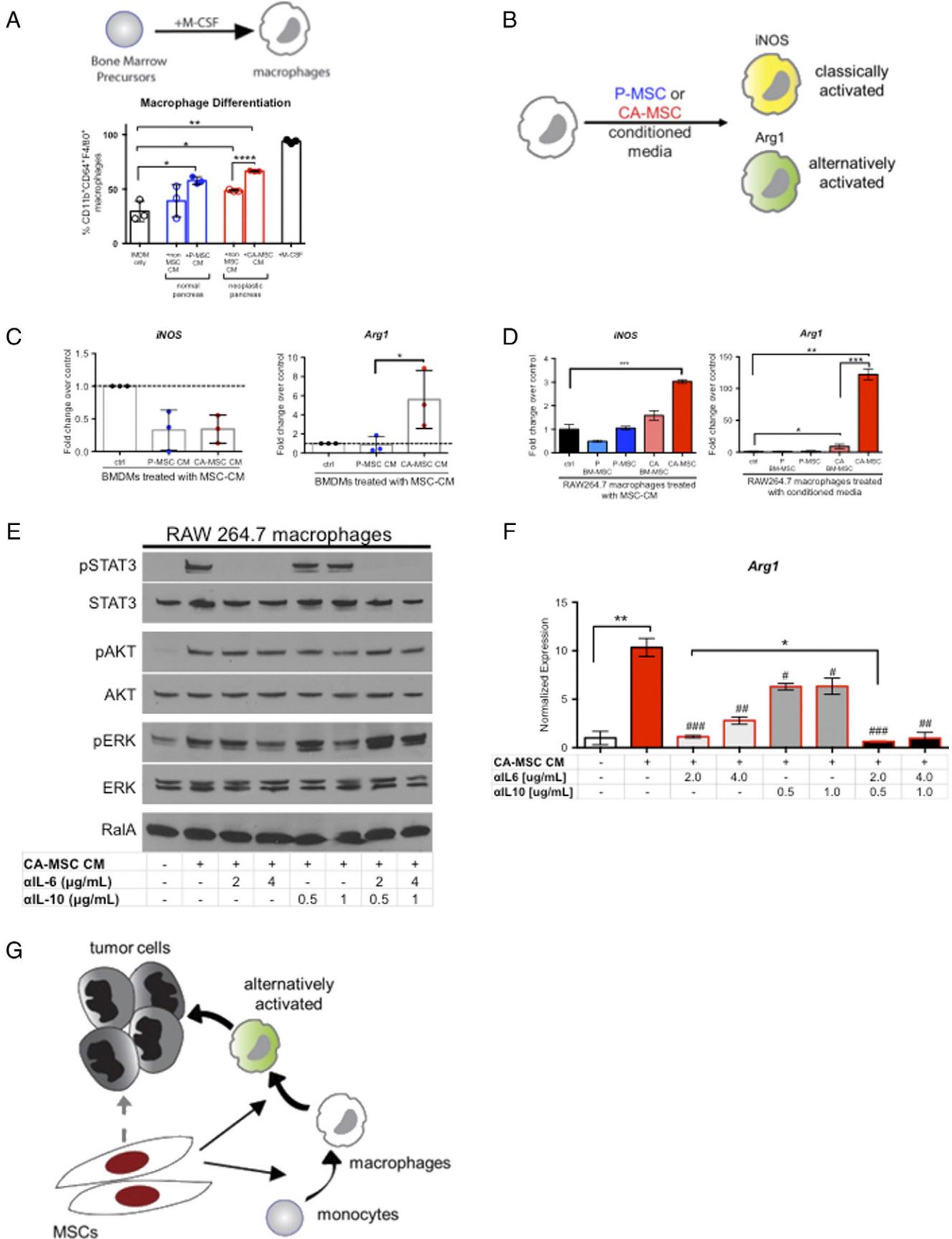
Requirement for Monocytes/Macrophages in MSC-driven tumor growth

Macrophages have been shown to promote pancreatic growth [30]. Thus, based on the observation that CA-MSCs promoted the highest infiltration of macrophages, we next tested whether this population's elevated tumor-promoting potential required macrophages. To achieve this, we coinjected tumor cells and MSCs into immunocompetent mice with concomitant depletion of monocytes and macrophages. Cd11b-DTR mice express the human diphtheria toxin receptor under the Cd11b promoter (Figure 3A). Administration of diphtheria toxin (DT) to Cd11b-DTR mice depletes all CD11b+ cells, including monocytes and macrophages [31]. We performed a series of coinjection experiments with *iKras**p53*#3 cells alone or in combination with P-MSCs or CA-MSCs. In a subset of each cohort, mice were injected with DT (Figure 3B). We confirmed DT-mediated depletion by staining tumor tissues for F4/80; in all cohorts, DT administration significantly attenuated macrophage infiltration (Figure 3D, inset). We found that tumor size was reduced by depleting CD11b+ cells in all experimental cohorts, underscoring the importance of myeloid cells in tumor growth. However, coinjections of *iKras**p53*#3 cells with CA-MSCs were significantly more susceptible to myeloid cell ablation than any other experimental cohort, both in terms of tumor mass changes (Figure 3C) and when considering the ratio of epithelial cells within the tumor (Figure 3D). The susceptibility of CA-MSCs to myeloid cell depletion was consistently observed using distinct MSC lines (Figure S3D). Whereas the tumor histology remained similar (Figure 3D), immunostaining for Ck19 revealed a reduction in the ratio of epithelial cells within the tumors (Figure S3A). Moreover, although tumors derived from coinjections with CA-MSCs are more proliferative than the other cohorts, this increase in proliferation was abrogated upon myeloid cell depletion (Figure 3E).

To determine whether the dependence on myeloid cells for tumor promotion was a unique property of CA-MSCs or rather a common feature of fibroblasts derived from the neoplastic pancreas, we performed parallel coinjection experiments with non-MSCs (CD45⁻; CD44⁻;CD49a⁻;CD73⁻;CD90⁻) sorted from the neoplastic stroma (CA-non-MSCs) (Figure S3B). Although these cells also promoted tumor growth, the extent of tumor mass reduction upon CD11b+ cell depletion was less than in coinjections with MSCs (Figure S3C). Thus, CA-MSCs have a unique dependence on myeloid cells, which led us to investigate the interaction between these cell types.

MSCs Promote Macrophage Differentiation and Polarization to an Alternatively Activated Subtype

Pancreatic cancer fibroblasts, including the FAP+ subset, are known to modulate the immune response, thus affecting tumor growth [32,33]. To directly interrogate whether MSCs directly regulate macrophage polarization, we investigated their interactions



in vitro. First, we sought to determine whether CA-MSCs promote the differentiation of monocytes into macrophages. For this purpose, we isolated and cultured mouse bone marrow precursors. After 1 week of culture in regular medium, about 25% of the cell population differentiates to macrophages, defined as CD11b⁺;CD64⁺;F4/80⁺ cells by flow cytometry. As expected based on previous studies [34], exposure to M-CSF-supplemented media led to differentiation of a uniform population of CD11b⁺;CD64⁺;F4/80⁺ macrophages (Figure 4A). We tested the effect of supplementing the culture medium with conditioned media from either P-MSCs or CA-MSCs, as well as P-non-MSCs and CA-non-MSCs (pancreatic fibroblasts or neoplastic fibroblasts). Both P-MSCs and CA-MSCs promoted macrophage differentiation in more than 50% of the bone marrow precursors, with no significant difference between the two populations. In contrast, only non-MSC fibroblasts from the neoplastic pancreas could also promote macrophage differentiation, although to a lesser extent than their MSC counterparts (Figure 4A).

We next assessed whether P-MSCs or CA-MSCs regulated macrophage polarization. First, we performed qRT-PCR to evaluate gene expression indicating either classically or alternatively activated macrophage polarization RNA collected from our subcutaneous coinjection experiments (Figure S4, A and B). We found that tumors derived from coinjection with both P-MSCs and CA-MSCs had decreased expression of *iNos*, a classically activated marker, compared with tumor cells injected alone. However, only coinjections with CA-MSCs tumor cells showed increased expression of *Arg1* and *CD206*, both markers of alternatively activated macrophages. Likewise, the expression of IL10, a cytokine known to induce alternative macrophage differentiation, was significantly increased in coinjections with CA-MSCs (Figure S4, A and B). Importantly, these changes were observed with both iKras^{*}p53^{*} and KPC tumor cells.

To test whether MSCs directly regulated macrophage polarization to an alternatively activated phenotype, we treated three independently derived cultures of bone marrow-derived macrophages (BMDMs) with conditioned media from either P-MSCs or CA-MSCs for 12 hours (Figure 4B). We found that both P-MSC- and CA-MSC-conditioned media decreased *iNOS* expression in BMDMs compared with control medium, indicating suppression of the classically activated subtype. However, only CA-MSC-conditioned medium promoted alternatively activated macrophage polarization, as determined by *Arg1* expression (Figure 4C). We then repeated this set of experiments using the mouse macrophage cell line RAW264.7 that can be polarized in culture. Conditioned medium from P-MSCs had no effect on the expression of *iNos* or *Arg1* in macrophages. In contrast, whereas conditioned medium from CA-MSCs stimulated a four-fold increase in *iNos* expression, *Arg1* expression was increased more than one-hundred-fold. We also tested the effect of conditioned media from bone marrow MSCs, which have been reported to induce *Arg1* expression in macrophages, leading to an alternatively activated phenotype [35,36]. We found that whereas CA BM-MSCs could

promote *Arg1* expression, this effect was significantly less than CA-MSCs (Figure 4D). Likewise, CA-MSCs could stimulate significantly greater *Arg1* expression than CAFs (non-CA-MSCs) (Figure S4C). Taken together, our data indicate that the ability to promote alternatively activated macrophage polarization is restricted to CA-MSCs.

As CA-MSCs expressed significantly higher *IL-6* and *IL-10* compared with P-MSCs and these cytokines are known to regulate macrophage polarization, we tested whether production of these cytokines explained the unique ability of CA-MSCs to promote macrophage alternative activation. Thus, we treated RAW264.7 macrophages with CA-MSC conditioned media with and without an IL-6-neutralizing antibody or an IL-10-neutralizing antibody and assessed *Arg1* expression. Consistent with IL-6 stimulation, CA-MSC conditioned medium increased pSTAT3(Y705) levels in RAW264.7 macrophages compared with control medium, and this increase was abrogated by the anti-IL6 antibody but not by the anti-IL10 antibody (Figure 4E). Treatment with CA-MSC conditioned medium induced *Arg1* expression in RAW264.7 macrophages; this induction was partially abrogated by anti-IL6 and, to a lesser extent, by anti-IL10 treatment (Figure 4F). Concomitant treatment with anti-IL6 and anti-IL10 antibodies completely blocked the induction of *Arg1* expression, indicating that these two cytokines might act along parallel, nonoverlapping paths. We then investigated the effect of CA-MSC conditioned medium on the expression of classically activated differentiation markers. We observed no change in *iNOS* expression but a reduction of *IL12p35* upon treatment with conditioned medium (Figure S4, D and E). This decrease was reversed with higher concentrations of either IL6 or IL10 inhibition. Taken together, our results show that both P-MSCs and CA-MSCs can induce macrophage differentiation for a precursor population, but only CA-MSCs specifically direct polarization to a tumor-promoting, alternatively activated subtype.

Discussion

Fibroblasts exist in every tissue and organ in the body and are a prevalent population within the pancreatic cancer stroma. Our understanding of their functional specificity, however, remains limited. Here, we have identified a subpopulation of fibroblasts present both within the normal pancreas and in the neoplastic pancreas, with MSC characteristics, namely, the ability to differentiate into chondrocytes, adipocytes, and osteoblasts. We determined that MSCs from the neoplastic pancreas (CA-MSCs) have a unique ability to promote tumor growth that depends on their ability to promote infiltration of monocytes/macrophages and their differentiation to an alternatively activated, tumor-promoting, phenotype (Figure 4G). Our study highlights the functional heterogeneity of stromal fibroblast populations and the different functional properties of fibroblasts derived from the normal or neoplastic organ. Furthermore, our study identifies novel interactions between the fibroblast population and the immune component of pancreatic cancer.

Figure 4. CA-MSC-derived IL6 and IL10 promote alternatively activated macrophage polarization. (A) Flow cytometry analysis of *in vitro* differentiated macrophages. Each data point represents bone marrow precursors from one mouse. (B) Experimental design. (C) qRT-PCR on BMDMs for *iNOS* (left) and *Arg1* (right). Each data point represents BMDMs from one mouse. (D) qRT-PCR on Raw264.7 macrophages for *iNOS* (left) and *Arg1* (right). (E) Western blot analysis of RAW264.7 macrophages treated with conditioned media and inhibitors. (F) qRT-PCR on RAW264.7 macrophages treated with conditioned media and inhibitors for *Arg1*. # indicates significant differences from CA-MSC-stimulated cells. (G) Working model of CA-MSC-mediated promotion of tumor growth.

Experimental Procedures

Detailed reagent information and experimental procedures are provided in Supplemental Experimental Procedures.

Statistical Analysis

Student's *t* tests were used to compare experimental cohorts, and significance was established for *P* values < .05. Significance values indicated by asterisks or pound signs are as follows: **P* < .05, ***P* < .01, ****P* < .0005, and *****P* < .0001.

Author Contributions

E. M. and M. P. d. M. conceived and designed the experiments. E. M. executed the experiments and collected the data. A. L. B., A. d. V., M. K. P., K. T. K., P. E. G., and A. d. V. assisted with data collection and analysis. R. J. B. provided expertise on the identification and characterization of MSC in the cancer stroma and shared protocols and reagents developed in his laboratory. E. M., A. L. B., P. E. G., and M. P. d. M. wrote and edited the manuscript.

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