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Chronic stress accelerates pancreatic cancer growth and invasion: A critical role for beta-adrenergic signaling in the pancreatic microenvironment

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ABSTRACT

Pancreatic cancer cells intimately interact with a complex microenvironment that influences pancreatic cancer progression. The pancreas is innervated by fibers of the sympathetic nervous system (SNS) and pancreatic cancer cells have receptors for SNS neurotransmitters which suggests that pancreatic cancer may be sensitive to neural signaling. *In vitro* and non-orthotopic *in vivo* studies showed that neural signaling modulates tumour cell behavior. However the effect of SNS signaling on tumor progression within the pancreatic microenvironment has not previously been investigated. To address this, we used *in vivo* optical imaging to non-invasively track growth and dissemination of primary pancreatic cancer using an orthotopic mouse model that replicates the complex interaction between pancreatic tumor cells and their microenvironment. Stress-induced neural activation increased primary tumor growth and tumor cell dissemination to normal adjacent pancreas. These effects were associated with increased expression of invasion genes by tumor cells and pancreatic stromal cells. Pharmacological activation of β-adrenergic signaling induced similar effects to chronic stress, and pharmacological β-blockade reversed the effects of chronic stress on pancreatic cancer progression. These findings indicate that neural β-adrenergic signaling regulates pancreatic cancer progression and suggest β-blockade as a novel strategy to complement existing therapies for pancreatic cancer.

1. Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death, with approximately 40,000 new cases in the US each year (Siegel et al., 2012). The overall survival rate is less than 5% and effective strategies to improve clinical outcomes are critically needed. Survival from pancreatic cancer depends on successful resection of the primary tumor with 5-year survival rates of 20–30% reported in specialized centers (Gaedcke, 2010). However, even when surgery results in tumor-free resection margins recurrence rates are high and most patients eventually die of local tumor recurrence and metastatic disease (Kleeff, 2007; Hishinuma, 2006; Han, 2006). Additionally, many patients are diagnosed with advanced stage pancreatic cancer, which limits surgical treatment (Kazanjian, 2008; Bilimoria, 2007). Gemcitabine is the standard-of-care chemotherapy regimen for locally advanced and metastatic pancreatic cancer, but the overall prolongation of survival is disappointingly small (5–7 months) (Storniolo, 1999; Herrmann, 2007; Rougier, 2013). Recent trials of combination chemotherapies and targeted therapeutic strategies including FOLFIRINOX, nab-Paclitaxel and the EGFR inhibitor, erlotinib, have shown additional survival benefit of only days to weeks, with increased toxicity that limits their use to otherwise fit patients (Conroy, 2011; Moore,

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2007; Von Hoff, 2013). Development of improved therapeutic strategies to treat pancreatic cancer requires a more detailed understanding of the molecular and cellular mediators that influence pancreatic cancer growth and dissemination.

The limited success of existing therapeutic strategies may be due, in part, to our poor understanding of the effect of cross-talk between pancreatic tumor cells and their surrounding stroma on cancer progression (Feig, 2012; Demir et al., 2012; Vonlaufen, 2008). Pancreatic adenocarcinomas interact with a complex stroma that includes nerve fibers, infiltrating immune cells, endothelial cells, and pancreatic stellate cells that secrete abundant extracellular matrix (Demir et al., 2012; Vonlaufen, 2008; Apte et al., 2012; Hamada et al., 2013; Richins, 1945). These stromal cells exert a critical influence on pancreatic cancer progression (Demir et al., 2012; Vonlaufen, 2008; Apte et al., 2012; Hamada et al., 2013; Farrow et al., 2008). However, many therapeutic strategies were developed in cell-based and non-orthotopic disease models that fail to reflect the complexity of *in vivo* interactions (Feig, 2012; Johnson, 2001). Identification of new targets for novel therapies to slow or prevent pancreatic cancer requires studies in disease models that better recapitulate interactions between tumor cells and the pancreatic microenvironment.

The pancreas is innervated by fibers of the sympathetic nervous system (SNS), which regulate pancreatic functions including release of insulin by islet cells and release of digestive enzymes by acinar cells (Richins, 1945; Holmgren and Olsson, 2011). SNS fibers are activated during chronic stress and release catecholaminergic neurotransmitters that act on adrenoceptors to modulate cell behavior. β-adrenoceptors are present on pancreatic tumor cells and *in vitro* studies suggest that tumor cell behavior may be sensitive to β-adrenergic signaling (Guo, 2009; Schuller and Al-Wadei, 2010; Zhang, 2010). However, the effect of stress-induced β-adrenergic signaling on cancer progression within the complex pancreatic microenvironment has not been investigated. To address this we used an orthotopic model of human pancreatic cancer to investigate the effect of chronic stress on primary tumor growth and tumor cell dissemination within the pancreatic microenvironment.

2. Methods

2.1. Orthotopic pancreatic cancer model

The human pancreatic ductal adenocarcinoma cell lines Panc-1, HPAF-II and Capan-1 were obtained from the American Type Culture Collection, and maintained at 37 °C, 5% CO_2. These cell lines were chosen because they have mutated *TP53* and *KRAS*, which are common driver mutations in pancreatic cancer and because they range from well differentiated (Capan-1) to moderately and highly undifferentiated (HPAF-II and Panc-1, respectively) (Yachida, 2012). Panc-1 cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Bovogen Biologicals) and 1% penicillin–streptomycin (Sigma–Aldrich). HPAF-II cells were cultured in RPMI (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Capan-1 cells were cultured in DMEM supplemented with 20% fetal bovine serum and 1% penicillin–streptomycin. To model human pancreatic cancer, 4×10^5 tumor cells in Matrigel (BD Bioscience) were injected into the tail of the pancreas of six week old female BALB/c-Foxn1nu nude athymic mice (The University of Adelaide, Australia) by laparotomy as previously described (Chai, 2013). Panc-1 was chosen for *in vivo* studies as tumors are poorly differentiated which is characteristic of patients who are diagnosed with advanced stage and grade (Chai, 2013; Hotz, 2003). To track tumor progression *in vivo*, cell lines were transduced with FUhLucW construct that expresses firefly luciferase under control of the ubiquitin C promoter (Morizono, 2005). Tumor progression was monitored longitudinally over 42 days by *in vivo* and *ex vivo* optical bioluminescent imaging using an IVIS Lumina ll system (Perkin Elmer) as described previously (Chai, 2013; Sloan, 2010). The presence of tumor cell dissemination beyond the tumor margins and into adjacent normal pancreas and metastasis to distant organs was measured by *ex vivo* optical imaging using long exposure times (>60 s), and confirmed by hematoxylin and eosin staining. Findings were validated in 2–4 independent experiments. All procedures were conducted in accordance with protocols approved by Institutional Animal Care and Use Committee of Monash University.

2.2. Chronic stress

Mice were randomly assigned to home cage control conditions (control) or 2 h per day restraint (stress) for 28 days commencing 7 days before tumor cell injection. Mice were restrained in a confined space that prevented them from moving freely but did not press on them (Thaker, 2006). This paradigm has been shown to induce chronic stress as shown by neuroendocrine activation (Thaker, 2006; Manni, 2008), weight loss (Smagin, 1999) (Supplementary Fig. 1), and anxiety-like behaviors (Hermann, 1994) but does not cause pain or wounding (Sheridan, 2004).

2.3. Pharmacological studies

For β-adrenergic antagonist studies 10 mg/kg/day (R/S)-propranolol (treatment) or water vehicle (placebo) was delivered to mice subcutaneously by osmotic minipump (Model 1004, Alzet). Propranolol was delivered for the duration of the experiment commencing seven days prior to tumor cell injection, with pumps replaced 24 days after implantation. Drug plasma concentration was assessed 20 days after pump implantation by UPLC-MS using a Micromass Quattro Premier coupled to an Acquity UPLC (Waters). For β-adrenergic agonist studies, 5 mg/kg/day (S/S)-isoproterenol (treatment) or 1 mM HCl vehicle (placebo) was delivered subcutaneously to mice via osmotic minipump (Model 1002, Alzet). Isoproterenol was delivered for 28 days, commencing seven days prior to tumor cell injection, with pumps replaced 14 days after implantation. Mice were maintained in their home cage for the duration of experiments that included isoproterenol treatment.

2.4. Invasion assay

2.5×10^5 pancreatic cancer cells in serum free culture medium were seeded into the top well of a transwell chamber with 8.0 μm pores (BD Falcon) that was coated with 15 mg/mL Matrigel. Cells were allowed to migrate towards medium containing 20% serum for 22 h and then stained with DAPI. Cells that had migrated to the underside of the membrane were counted.

2.5. Proliferation assay

The influence of isoproterenol on proliferation was assessed using the CellTiter 96® AQueous One Proliferation Assay (Promega). 8×10^3 cells were seeded into a 96-well plate and assayed over 120 h, according to manufacturer's instructions.

2.6. Gene expression studies

RNA was isolated from cell lines or primary pancreatic tumors using RNeasy Mini Kit (Qiagen). Transcript levels were quantified by RT-PCR using iScript One-Step RT-PCR kit (Biorad) and species-specific Taqman probes (Applied Biosystems) to identify

human (tumor cell) vs mouse (stromal cell) genes (*ADRB1* Hs02330048_s1, Mm00431701_s1; *ADRB2* Hs00240532_s1, Mm02524224_s1; *MMP2* Hs01548727_m1, Mm00439498_m1; *MMP9* Hs00234579_m1, Mm00442991_m1) with 50 amplification cycles of 15 s strand separation at 95 °C and 60 s annealing and extension at 60 °C. Samples were analyzed in triplicate and expression was normalized to *RPL30* expression (Hs00265497_m1, Mm01613252_g1) (Rubie, 2005).

2.7. cAMP assay

5×10^5 cells were seeded into a 96-well transparent plate and cultured overnight. Cells were washed with PBS and incubated in stimulation buffer (phenol-free DMEM, 0.1% BSA, 1 mM IBMX) at 37 °C for 60 min. Agonists were added for 30 min before cells were lysed with ice-cold 100% ethanol and rehydrated with lysis buffer (0.01% BSA, 5 mM HEPES, 0.3% Tween20). Cell lysates were incubated with AlphaScreen™ beads diluted in lysis buffer, followed by incubation with donor beads, and fluorescence signal was measured with a Fusion plate reader (Perkin Elmer). cAMP accumulation was expressed as a fraction of maximal stimulation induced by 10 μM forskolin.

2.8. Immunostaining

Cells were grown on slides, fixed in −20 °C acetone then incubated with antibodies against β2-adrenoceptor (H-20 rabbit polyclonal diluted 1:150, Santa Cruz Biotechnologies) for 16 h at 4 °C, followed by incubation with fluorescent Alexa-conjugated secondary antibodies (Invitrogen) and DAPI nuclear stain (Sigma). Immunostaining was imaged using an inverted microscope with fluorescence filters (Olympus). De-identified archival patient samples were obtained from Bern University Hospital in accordance with protocols approved by the Institutional Human Research Ethic Committee. Samples were dewaxed and incubated with antibodies as above and visualized by reaction with diaminobenzidine peroxidase (Vector) with hematoxylin counterstain.

2.9. Statistical analyses

Student's *t* test analyzed the effect of stress or isoproterenol on size and frequency of tumor cell dissemination and metastasis, and differences in gene expression levels. Data are presented as mean ± standard error. To determine the effect of stress on the longitudinal growth trajectory of tumors, and whether those effects were modified by pharmacological interventions that targeted β-adrenoreceptors, we examined the stress × treatment interaction term in a 2 (control vs stress) × 2 (treatment vs placebo) experimental design in the context of mixed-effects linear model analysis (Demidenko, 2004). Data were analysed according to the model: $y_{ijt} = \alpha + \nu t_{ij} + \beta_1 d_{i1} t_{ij} + \beta_2 d_{i2} t_{ij} + \beta_3 d_{i3} t_{ij} + a_i + b_i t_{ij} + \varepsilon_{ijt}$ where: y_i is the tumor-specific luciferase activity for the ith mouse on the logarithmic scale, t is the time (days of followup), α is the intercept parameter, ν is the common growth rate parameter, d_{ij} ($j = 1,\ldots,3$) are binary variables so that $d_{ij} = 1$ if the ith mouse belongs to the jth group and 0 otherwise; a_i and b_i are independent mouse-specific random effects, which we assume to be normally distributed; and ε_i is the error term. Estimated parameters are presented in Supplementary Table 1 where Model 1 (Fig. 3C) and Model 2 (Fig. 1C) have the same structure but Model 2 sets $\beta_2 = \beta_3 = 0$ for analysis of two (control and stress) groups. Parameter estimates were computed in R programming environment using the package *nlme* (Pinheiro and Bates, 2000).

3. Results

3.1. Chronic stress increases pancreatic cancer progression

To assess the effects of chronic stress on cancer progression within the pancreatic microenvironment, we used bioluminescence imaging to monitor primary tumor growth and metastatic dissemination in an orthotopic mouse model of human pancreatic cancer. To investigate the effect of chronic stress in the context of crosstalk between cancer cells and pancreatic stromal cells, luciferase-tagged Panc-1 cells were injected into the pancreas by laparotomy (Chai, 2013). Chronic stress was induced by subjecting mice to repeated daily restraint (Fig. 1A), which up regulates adrenergic stress response pathways as indicated by weight loss and increased tissue catecholamine levels (Supplementary Fig. 1) (Thaker, 2006). Longitudinal analysis found that stress increased the rate of pancreatic tumor growth by 10.92% ± 3.07 per day compared to mice maintained in their home cage ($p < .01$) (Fig. 1B). The effect of stress on primary tumor bioluminescence was apparent by day 21 after tumor cell injection and resulted in >10-fold increased tumor-specific bioluminescence at day 42 ($p < 0.001$) (Fig. 1B and C).

Pancreatic cancer morbidity and mortality is induced by primary tumor growth, tumor cell invasion of adjacent normal pancreas which may seed recurrence after resection, as well as metastatic dissemination to distant organs. To investigate the effect of stress on each of these contributors to disease progression, we evaluated primary tumor mass, tumor cell dissemination into adjacent normal pancreas and frequency of distant metastasis. Chronic stress increased primary tumor mass by 5-fold (7.5 mg ± 5 vs. 41 mg ± 13; $p = 0.03$) (Fig. 1D). To investigate the effect of stress on tumor cell dissemination beyond resection margins and into surrounding normal pancreas, the primary tumor was identified in the pancreatic tail adjacent to the spleen (Fig. 2A, left panel) and resected with 2 mm margins. R0 resection margins were confirmed by bioluminescence imaging (Fig. 2A, middle panel). The remaining pancreas was imaged *ex vivo* to identify disseminated luciferase-positive tumor cells (Fig. 2A, middle panel). Stress increased bioluminescence in adjacent normal pancreas by >10-fold ($p = 0.004$) (Fig. 2B). Local invasion of tumor cells in the pancreatic microenvironment was confirmed by histology (Fig. 2A, right panel). In addition to effects within the pancreas, stress induced metastatic dissemination of tumor cells to distant organs in 50% of mice compared to control where no distant metastasis was detected (Figs. 2C and 1B). Bioluminescence imaging and histological analyses confirmed the presence of liver metastasis (Fig. 2D), which also occurs in patients with pancreatic cancer (Michalski, 2008). These findings indicate that chronic stress promotes pancreatic cancer progression by accelerating primary tumor growth and dissemination of tumor cells within the pancreas and by inducing metastatic colonization of distant organs.

3.2. Beta-blockade slows pancreatic cancer progression

Chronic stress was found to accelerate progression of other solid tumor types and hematological malignancies through β-adrenergic signaling pathways (Sloan, 2010; Thaker, 2006; Lamkin, 2012; Ben-Eliyahu, 1999; Madden et al., 2011; Schuller, 2010). To investigate the role of β-adrenergic signaling in pancreatic tumor growth and dissemination, mice were treated with the non-selective β-blocker propranolol to block signaling through β-adrenoceptors. LC–MS analysis confirmed systemic exposure of propranolol was successfully maintained throughout the treatment period, with an average propranolol plasma concentration 41 ng/mL (range: 27–76 ng/mL). Consistent with previous findings

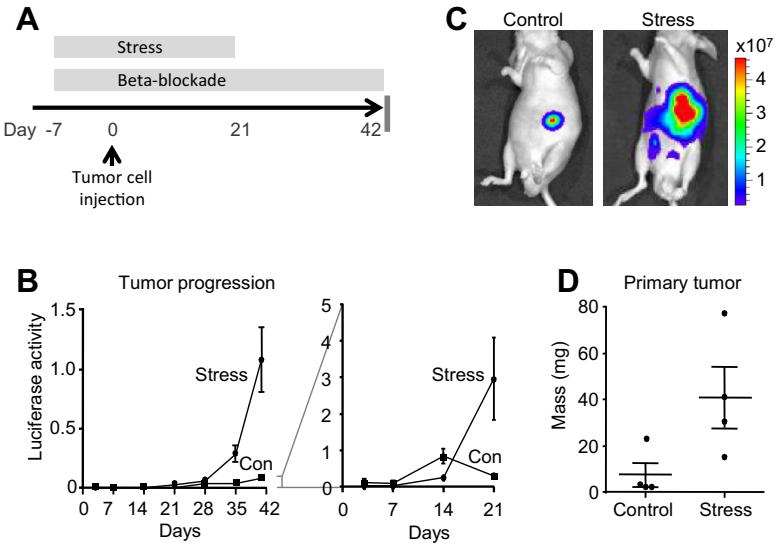

Fig. 1. Chronic stress increased pancreatic tumor progression. (A) Mice were exposed to chronic stress (daily restraint) vs home cage control conditions for 2 h per day for 28 days commencing 7 days prior to tumor cell injection. Where described, mice were treated with β-blockers for the duration of the experiment. (B) Primary tumor size was measured over time by non-invasive bioluminescence imaging. Luciferase activity: $\times 10^{10}$ p/s. Inset shows increased resolution over days 0–21, luciferase activity: $\times 10^{8}$ p/s. (C). Representative images of tumor-specific bioluminescence in mice exposed to control vs stress conditions. Luminescence scale: p/s/cm^2/sr. (D). Primary tumor mass at 42 days after tumor cell injection. Data shown are representative of three replicate experiments.

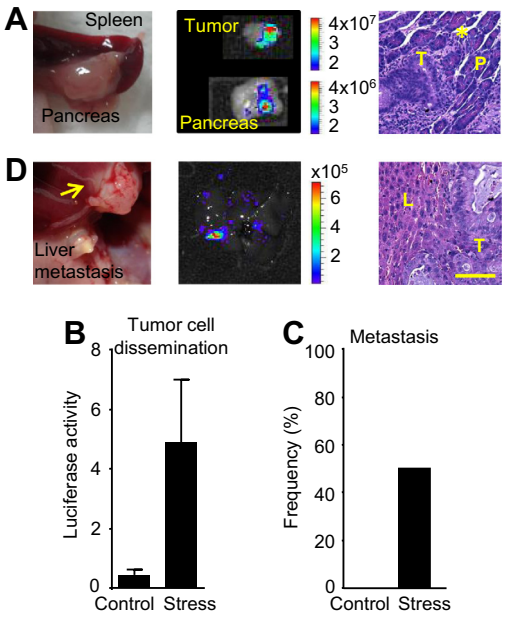

Fig. 2. Stress increased metastatic dissemination from primary pancreatic tumors. (A) Representative images of intact pancreatic tumor (left panel) and resected primary tumor and adjacent normal pancreas with bioluminescent tumor signal (middle panel, luminescence scale: p/s/cm^2/sr). Right panel: Hematoxylin and eosin staining of pancreatic tumor (T) grown orthotopically in normal pancreas (P). Asterisk shows region of local invasion. Scale bar: 150 μm. (B). Tumor cell dissemination into adjacent normal pancreas was quantified by bioluminescence imaging after resection of the primary tumor. Luciferase activity: $\times 10^{6}$ p/s. (C) Metastasis was quantified in control vs stressed mice at 42 days after tumor cell injection. (D) Pancreatic tumor metastasis in liver (left panel, arrow: metastasis, scale: 2 mm). Middle panel: Metastasis was confirmed by bioluminescence imaging (luminescence scale: $\times 10^{5}$ p/s/cm^2/sr). Right panel: Hematoxylin and eosin staining of liver metastasis (T: tumor, L: liver, scale: 100 μm).

(Fig. 1), stress increased the pancreatic tumor growth rate by 7.58% (±1.73) per day ($p < .001$). Treatment with propranolol reversed the effect of stress on pancreatic tumor growth rate by 2.83% (±1.73), resulting in a 60% decrease in tumor-specific bioluminescence on day 42 ($p = .016$)(Fig. 3A and B). These findings suggest that stress acts through β-adrenergic signaling to modulate pancreatic cancer progression. Consistent with that hypothesis, propranolol blocked the effect of stress on primary tumor growth (41 mg ± 13 vs. 21 mg ± 5) and on tumor cell dissemination through adjacent normal pancreas (4.8×10^6 p/s ± 2.1×10^6 vs $2.9 \times 10^4 \pm 0.7 \times 10^4$) ($p = 0.05$) (Fig. 3C and D). Beta-blockade did not modulate the effect of stress on metastasis in the timeframe of this investigation (Supplementary Fig. 2).

To investigate if β-adrenergic signaling is sufficient to increase pancreatic cancer progression, mice were treated with β-adrenoceptor agonist isoproterenol vs vehicle control. Isoproterenol increased the rate of primary tumor growth (Fig. 4A and B), resulting in 1.9-fold increase in primary tumor mass at 42 days after tumor cell injection ($p = 0.04$) (Fig. 4C). Isoproterenol treatment increased tumor cell dissemination into the adjacent pancreas by 3.9-fold ($p = 0.004$) (Fig. 4D), and induced metastatic dissemination to distant organs in 50% of mice (Fig. 4E). These findings indicate that pharmacological β-adrenoceptor activation is sufficient to accelerate pancreatic cancer progression and show that β-adrenergic signaling is critical for the effects of chronic stress on primary tumor growth and tumor cell dissemination to the surrounding pancreatic microenvironment.

3.3. Beta-adrenergic signaling regulates pancreatic cancer cell invasion

To begin to explore the extent to which stress acts directly on tumor cells versus on the surrounding pancreatic microenvironment, we examined the effect of β-adrenoceptor activation on tumor cell proliferation and invasion in the absence of the pancreatic microenvironment. Immunostaining showed β-adrenoceptors on pancreatic cancer cell lines and on pancreatic cancer cells in pancreatic tumor samples from patients (Fig. 5A). Quantitative RT-PCR confirmed expression of transcripts for β1- and β2-adrenoceptors in each cell line (Fig. 5B, Supplementary Fig. 3A). To confirm functional coupling of β-adrenoceptors to intracellular signaling pathways, tumor cells were treated with isoproterenol and the effect on cAMP levels was assayed. Isoproterenol induced a dose dependent accumulation of cAMP in tumor cells (Fig. 5C,

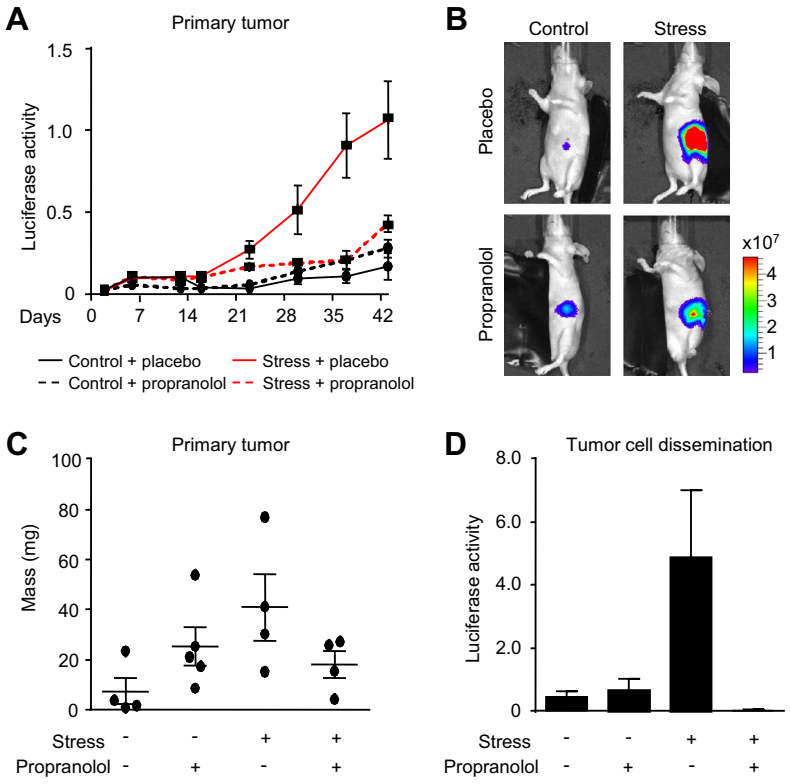

Fig. 3. Beta-blockade reversed stress-enhanced pancreatic cancer progression. (A) Tumor progression was tracked using non-invasive bioluminescence imaging in mice that were exposed to control (black) vs. stress conditions (red) and treated with propranolol (dotted line) or placebo (solid line). Luciferase activity: $\times 10^8$ p/s. (B) Representative images of mice taken on day 42 after tumor cell injection. Mice were exposed to control vs. stress conditions and treated with propranolol vs. placebo. Black tape in each panel covered auto-luminescent osmotic minipumps used for drug delivery. Luminescence scale: p/s/cm^2/sr. (C) Primary tumor mass was determined on day 42 after tumor cell injection. (D) The magnitude of tumor cell invasion into pancreas adjacent to the primary tumor was quantified by *ex vivo* bioluminescence imaging after surgical resection of the primary tumor. Luciferase activity: $\times 10^6$ p/s.

Supplementary Fig. 3B), suggesting that pancreatic cancer cells are responsive to β-adrenoceptor signaling. However, despite functional receptor coupling to downstream signaling pathways, isoproterenol treatment did not modulate proliferation of cultured tumor cells (Supplementary Fig. 4). This indicates that β-adrenergic signaling is insufficient to increase pancreatic cancer cell proliferation and suggests that the effects of stress on primary tumor growth *in vivo* (Fig. 1C) may require additional factors (e.g. β-adrenergic regulated stromal-derived growth factors) (Schuller and Al-Wadei, 2010; Zhang, 2010; Schuller, 2007; Askari et al., 2005; Chan et al., 2008).

To investigate if β-adrenoceptor signaling directly to tumor cells is sufficient to modulate tumor cell invasion, cells were treated with isoproterenol and the effects on expression of invasion related genes and on basement membrane invasion were assayed. Isoproterenol induced modest expression of genes involved in tumor cell invasion including matrix metalloprotease 2 (*MMP2*) and *MMP9* (2-fold to 4-fold increase, $p < 0.01$), and this was associated with increased invasion through Matrigel ($p < 0.01$)(Fig. 5D). Tumor cell invasion was blocked by propranolol, indicating a requirement for β-adrenoceptor signaling. However, in contrast to the modest effects observed in cultured tumor cells, chronic stress significantly up regulated expression of invasion genes in the pancreatic microenvironment. Species-specific qRT-PCR analyses found that stress preferentially up-regulated tumor cell *MMP9* expression in primary pancreatic tumors (54-fold increase vs. control, $p < .001$) and stromal cell *MMP2* expression (>100-fold increase, $p < 0.01$) in primary pancreatic tumors (Fig. 5E), consistent with patterns of *MMP* expression observed in pancreatic tumors from patients (Maatta, 2000). Collectively, these findings suggest that β-adrenoceptor signaling directly to pancreatic cancer cells may impact invasion, and emphasize the importance of the pancreatic microenvironment in regulating tumor cell proliferation and invasion.

4. Discussion

These studies found that chronic stress acts through β-adrenergic signaling pathways to increase pancreatic tumor growth and invasion. In the context of the pancreatic microenvironment, β-adrenergic signaling accelerated growth of primary pancreatic tumors and significantly enhanced tumor cell dissemination through adjacent normal pancreas and to distant organs. Even with potentially curative R0 resection, prognosis of patients with pancreatic cancer is exceptionally poor and autopsy results suggest that tumor recurrence approaches 100% (Hishinuma, 2006; Sperti, 1997; Takahashi, 1995). These findings raise the possibility that chronic stress may contribute to pancreatic tumor recurrence by facilitating dissemination of tumor cells into adjacent normal pancreas where they may seed recurrent tumor growth and metastasis even after resection of the primary tumor. Pharmacologic blockade of β-adrenergic signaling with propranolol stopped tumor cell invasion of adjacent pancreas, which suggests that β-blockers may complement existing chemotherapeutic strategies to slow or prevent pancreatic tumor growth and invasion, and improve survival of patients with pancreatic cancer.

Beta-adrenergic regulation of primary pancreatic tumor growth contrasts with neural regulation of other solid tumor types including breast cancer where β-adrenoceptor signaling did not in-

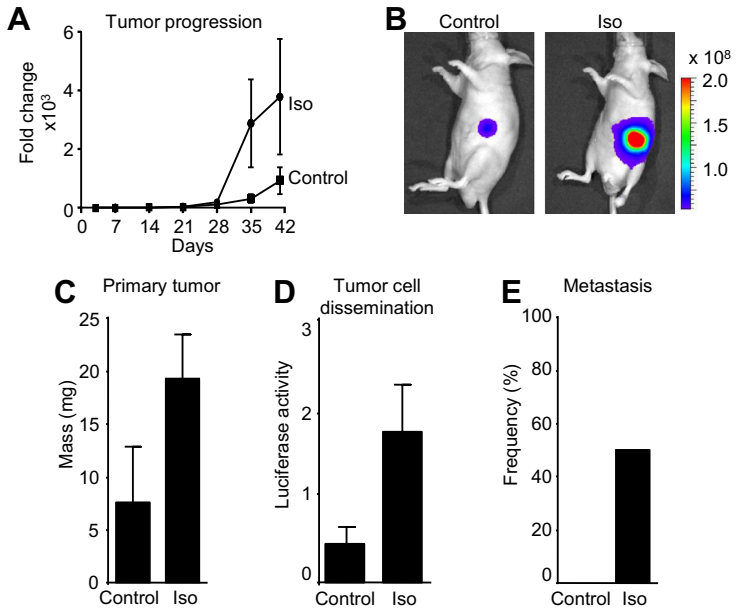

Fig. 4. Beta-adrenergic signaling is sufficient to accelerate pancreatic cancer progression. (A) Tumor progression was tracked using non-invasive bioluminescence imaging in mice treated with isoproterenol (iso) vs. placebo (control). *Y*-axis: fold change over day 0 luciferase activity. (B) Representative images of tumor-specific bioluminescence in mice treated with iso vs control. Luminescence scale: p/s/cm2/sr. (C) Primary tumor mass was determined on day 42 after tumor cell injection in mice treated with iso vs control. (D) The magnitude of tumor cell dissemination into pancreas adjacent to the primary tumor was quantified by *ex vivo* imaging after surgical resection of the primary tumor. Luciferase activity: $\times 10^6$ p/s. (E). The frequency of metastasis-bearing mice was determined at 42 days after tumor cell injection.

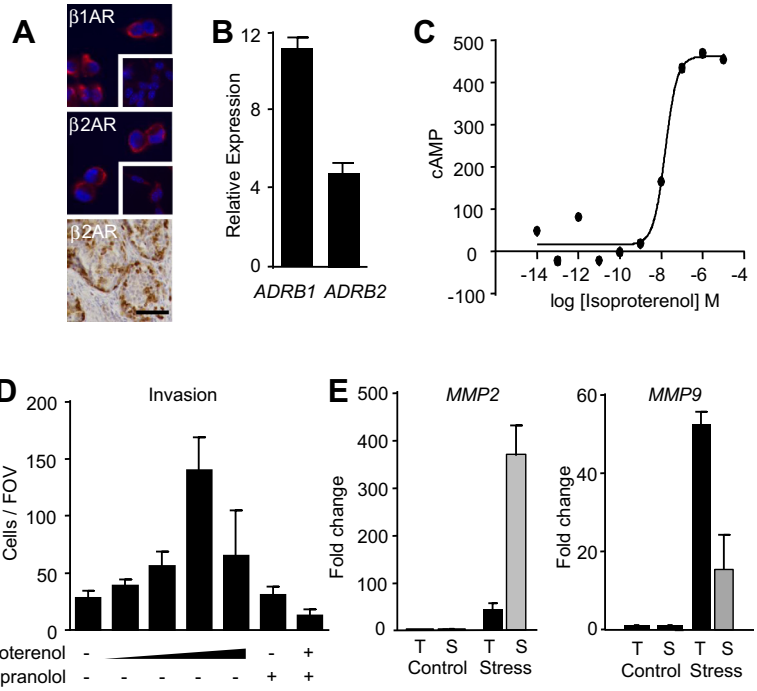

Fig. 5. Beta-adrenergic signaling induced pancreatic cancer cell invasion. (A) Immunostaining for β1-adrenoceptor (β1AR) and β2-adrenoceptor (β1AR, red) on Panc-1 pancreatic cancer cells (Upper and middle panel, blue: DAPI, inset: isotype control, scale bar: 5 μm) and archived human pancreatic cancer (Lower panel, scale bar: 100 μm). (B) Quantitative RT-PCR analyses of *ADRB1 and ADRB2* expression on Panc-1 cells, normalized to *RPL30* expression. (C) cAMP accumulation in Panc-1 cells presented as percent relative to maximal stimulation by 10 μM forskolin. (D) Matrigel invasion by Panc-1 cells treated with increasing concentration of isoproterenol (0, 0.01–10 μM), or with 10 μM propranolol ± 10 μM isoproterenol. (E) Quantitative RT-PCR analyses of tumor cell (T) or stromal cell (S) *MMP2* and *MMP9* expression in primary pancreatic tumors.

crease primary tumor size but selectively accelerated metastatic dissemination (Sloan, 2010; Madden et al., 2011; Perez Pinero, 2012). Pancreatic tumors frequently arise in the head of the pancreas where their growth may obstruct the bile duct and pancreatic duct, leading to jaundice, pruritus and liver metastasis (Bond-Smith, 2012). This suggests that β-adrenergic regulation of primary pancreatic tumor growth – in addition to its effects on tumor cell invasion and dissemination – may contribute to the morbidity and mortality associated with pancreatic cancer. The physiological mechanisms for these differential effects on primary tumor growth

are unclear but may reflect how stress signals are delivered to the tumor microenvironment and which stromal cells are responsive to those signals. In the context of breast cancer, chronic stress modulates the tumor microenvironment by recruiting M2 macrophages to primary tumors, which supports a switch to pro-metastatic gene expression (Sloan, 2010; Madden et al., 2011; Perez Pinero, 2012). Unlike breast, pancreas is densely innervated by SNS fibers, and it is possible that direct neurotransmitter activation of pancreatic stromal cell types such as pancreatic stellate cells may impact primary tumor growth. Pancreatic stellate cells are fibroblast-like cells specific to the pancreas that contribute to inflammation and tumorigenesis (Vonlaufen, 2008; Mace, 2013). Pancreatic stellate cells produce cytokines and growth factors and induce a desmoplastic reaction that has been implicated in chemoresistance (Apte, 2013). Pancreatic stellate cells are closely related to hepatic stellate cells, which express β-adrenoceptors and are sensitive to catecholaminergic neurotransmitter signaling (Sigala, 2013). The effect of β-adrenoceptor signaling on pancreatic stellate cells is yet to be investigated and may provide insight to the effects of chronic stress on primary pancreatic cancer growth.

To fully understand the impact of chronic stress on pancreatic cancer progression, it will be important to further investigate the effects of β-adrenergic signaling to tumor cells versus stromal cells in the pancreatic microenvironment. Matrix metalloproteases facilitate tumor cell invasion and contribute to pancreatic cancer progression. MMP2 and MMP9 are differentially regulated by pancreatic tumor cells and pancreatic stromal cells in patient samples (Maatta, 2000). However the physiological factors that modulate pancreatic cancer MMP expression are unknown. Our findings identify chronic stress as a novel regulator of MMP expression, which selectively up-regulated MMP9 in tumor cells and MMP2 in stromal cells in the pancreatic microenvironment. These findings suggest beta-blockade as a pharmacological intervention to limit expression of invasive genes and to prevent pancreatic cancer cell dissemination (Fig. 3D).

Use of an orthotopic model of pancreatic cancer allowed the first investigation of the effects of chronic stress on tumor development and progression in the pancreatic microenvironment. In contrast, interpretation of previous studies that investigated pancreatic tumor growth in flank was limited by the context of non-physiological intercellular interactions (Lin, 2012; Al-Wadei, 2012; Schuller, 2011; Al-Wadei et al., 2009). To better understand β-adrenergic regulation of pancreatic cancer onset it will be important to investigate the effects of chronic stress in transgenic models that spontaneously develop pancreatic cancer (Herreros-Villanueva, 2012). Modified study design will also be required to investigate β-adrenergic regulation of pancreatic cancer metastasis. Both physiologic and pharmacologic β-adrenergic activation induced metastasis (Figs. 2C and D, 4E). However, β-blockade did not modulate this effect in the short (six week) timeframe of these studies. This study design was chosen to focus on events that occur early in tumor development as studies of other tumor types suggest that this time point is sensitive to β-adrenergic signaling (Sloan, 2010; Thaker, 2006; Lamkin, 2012). However, this design limited investigation of metastatic dissemination from pancreas to distant organs as few metastases arose during the six week timeframe of tumor growth. In future studies, a modified design that includes surgical resection of the primary tumor and longitudinal imaging follow up of tumor recurrence and metastasis would better facilitate assessment of β-adrenergic regulation of tumor recurrence and metastasis (Chai, 2013).

As primary tumor growth and tumor cell invasion of surrounding pancreas impact patient survival, the findings of the current study have important implications for management of chronic stress in patients with pancreatic cancer. Diagnosis of pancreatic cancer is associated with high levels of distress (Carlson, 2004; Zabora, 2001), and beta-blocker treatment may be one strategy to reduce distress (Lindgren, 2013). Findings presented here suggest that in addition to improving quality of life, therapeutic intervention of β-adrenergic stress response pathways might also affect cancer progression. To translate these findings it will be important to prospectively investigate the effect of β-blockade on disease progression in pancreatic cancer patients and to define the patient populations that will optimally benefit from adjuvant beta-blocker therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2014.02.019.

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