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KISS1 over-expression suppresses metastasis of pancreatic adenocarcinoma in a xenograft mouse model

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Abstract

Identifying molecular targets for treatment of pancreatic cancer metastasis is critical due to the high frequency of dissemination prior to diagnosis of this lethal disease. Because the KISS1 metastasis suppressor is expressed at reduced levels in advanced pancreatic cancer, we hypothesized that re-expression of KISS1 would reduce metastases. Highly metastatic S2VP10 cells expressing luciferase (S2VP10L) were transfected with a FLAG-tagged version of KISS1 (KFM), KFMΔSS (with deleted secretion signal sequence), or pcDNA3 control plasmid (CP) and expression was confirmed by RTQ-PCR. SCID mice were implanted orthotopically with S2VP10L cells or transfectants and tumor growth and metastases were monitored using bioluminescence imaging. Mice with S2VP10L-KISS1 tumors developed fewer liver (98%) and lung (99%) metastases than S2VP10L. Unexpectedly, mice with S2VP10L-KFMΔSS tumors also had reduced liver and lung metastases, but had more metastases than mice with S2VP10L-KISS. KISS1 protein was found in the cytoplasm of both KFMΔSS and KISS1-expressing orthotopic tumors by immunohistochemistry. Metastases were not found in lungs of mice with S2VP10L-KISS1 tumors; whereas, KFMΔSS lung sections had regions of concentrated KISS1 staining, suggesting that secretion of KISS1 is needed to reduce metastasis significantly.

These data suggest induction of KISS1 expression has potential as an adjuvant treatment for pancreatic cancer.

Keywords

KISS1; metastasis suppressor; pancreatic adenocarcinoma

INTRODUCTION

Human pancreatic cancer has a very poor prognosis and is currently the fourth leading cause of cancer death in the United States (1). Pancreatic adenocarcinoma patients have a median survival of <6 months and a dismal 5-year survival rate of 5% (2,3). The lethal nature of pancreatic adenocarcinoma is strongly associated with its early metastases to distant organs. The aggressive, but largely asymptomatic, nature of this disease leads to late clinical presentation which, unfortunately, remains mostly incurable (4). Conventional therapies such as chemotherapy, radiation, and surgery, are largely ineffective in treating pancreatic adenocarcinoma; therefore, novel treatment strategies are desperately needed (5). Since dissemination has usually occurred before clinical detection, preventing the proliferation of already disseminated cells (*i.e.*, colonization) offers promise for patients with pancreatic adenocarcinoma.

Metastasis suppressors represent a potential new strategy for treatment of locally advanced and metastatic pancreatic cancer. Metastasis suppressors, by definition, inhibit metastasis without blocking orthotopic tumor growth (6). Metastasis suppressors are found in every cellular compartment, including some that are secreted. Each regulates one or more steps in the metastatic cascade, including regulation of signaling pathways that inhibit proliferation at secondary sites (7-9). Many also exhibit an inverse correlation with tumor grade and the likelihood of recurrence with metastatic disease (10).

Nascent KISS1 is a secreted suppressor of metastases and is processed into a number of peptides, called kisspeptins (11). A 54-amino acid polypeptide (metastatin or kisspeptin-54 (kp-54)) is the ligand for a G-protein coupled receptor KISS1R, alternatively known as GPR54, hOT7T175 or AX0R12 (6,9,12). Re-expression of KISS1 inhibits metastasis of human melanoma, breast, and ovarian cancer in xenograft models while still allowing growth of orthotopic tumors *in vivo* (11,12). KISS1 expression has been reported to be significantly lower in human pancreatic cancer tissue than in normal pancreas and to be associated with advanced disease (13-14). Although KISS1 expression is inversely associated with disease progression by immunohistochemistry or *in situ* hybridization analyses, no statistically significant correlation has been yet found between plasma levels of secreted KISS1 (plasma metastatin-L1) and tumor grade (15).

The aim of this study was to examine whether re-expression of KISS1 in pancreatic cancer cells suppresses metastasis of the human pancreatic cancer cell line S2VP10 in an orthotopic mouse xenograft model.

MATERIALS AND METHODS

Cells and cell culture

A metastatic subclone of the SUI-2 pancreatic adenocarcinoma cell line, S2VP10, was provided by Dr. Michael A. Hollingsworth at the University of Nebraska (16-18). Cells were grown in Dulbecco's-modified Eagle's medium containing 4.5 g/L glucose (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine and were maintained

in a humidified 5% CO₂ atmosphere at 37°C. S2VP10 cells were transduced with a non-replicating retrovirus containing firefly luciferase gene (Stratagene, La Jolla, CA). A single cell clone, S2VP10L, was isolated and bioluminescence signal was confirmed using the IVIS 100 bioluminescence system (Caliper, Hopkinton, MA). The growth rates of parental S2VP10 cells and S2VP10L were identical. We have been unable to identify another pancreatic xenograft model, including L3.6pl, whose rate of metastasis is adequate for our study.

Parental cell lines were analyzed for expression of KISS1R and KISS1 by real-time RT-PCR, flow cytometry and ELISA using a monoclonal antibody recognizing KISS1 that was developed by the UAB Tissue Characterization and Immunoreagent Resource, as described below.

KISS1 transfection

Plasmids containing an internal FLAG epitope and either KISS1 (KFM) or a deleted secretion sequence (KFMASS) were previously generated using pcDNA3 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions (19). The presence of the internal FLAG epitope did not modify KP-54 function (19). Transfected cells were selected with G418 for 3 passages. S2VP10L cells transfected with control plasmid pCMV served as a negative control (S2VP10L-CP). For conceptual simplicity, we will use KISS1 (for KFM) and KFMASS, respectively.

Detection of KISS1 and KISS1R

For RTQ-PCR, mRNA was isolated from cells or tissue using Trizol (Invitrogen, Carlsbad, CA), according to manufacturer's instructions and RNA was stored at -80°C up to 6 months prior to analysis. RNA quantity was assessed with a Nanodrop (Thermo Scientific, Wilmington, DE). Primers for KISS1 (Hs00158486m1), KISS1R (Hs00261399m1), S9 (Hs00396989m1) and 18S (Hs99999901s1) were purchased from ABI, Foster City, CA. The Taqman One Step RT-PCR (ABI) was used for the reaction according to protocols. Samples were measured in an ABI7500 Real Time PCR system. Samples were measured in triplicate (20). Normalization was performed using S9 for cultured cells and 18S for tissue samples.

To examine KISS1R expression on SVP10L cells and their respective variants flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The following antibodies were used: anti-KISS1R (LifeSpan Biosciences; #LS-A1929), anti-rabbit IgG-FITC (Sigma, #F-9887) and rabbit IgG (Abcam) was used as an isotype control. Cell preparations were stained in FACS buffer (HBSS with 3% FBS). Living cells were distinguished from dead cells using propidium iodide (PI) uptake.

Human pancreatic cancer orthotopic xenograft mouse models

Female severe combined immunodeficient (SCID) mice 2 months of age (Frederick Animal Production Program, Frederick, MD) were allowed to acclimate for 4 weeks before use. Strict adherence to the UAB Institutional Animal Care and Use Committee (IACUC) approved protocol was maintained throughout the study. Orthotopic cell implantation followed the procedure in DeRosier *et al.* (21). Briefly, mice were anesthetized with *i.p.* xylazine and ketamine. A 1-cm incision was made in the left upper abdominal quadrant in a sterile hood. The spleen was located and used to indirectly position the tail of the pancreas, carefully avoiding direct pancreatic manipulation. Mice (8/group) received S2VP10L, S2VP10L-CP, S2VP10L-KFMASS1, or S2VP10L-KISS1 cells. The cellular suspension was stored on ice in a sterile tube and then drawn up using a 28-gauge needle to aliquot 1×10^5 cells/40 μ l which was injected into the tail of the pancreas. A sterile cotton tipped applicator was used to cover the injection site for 30 s to prevent peritoneal leakage. The organs were returned to the abdomen and the skin and peritoneum were closed in a single layer closure with 5-0 Prolene

sutures. Animals recovered on a warming blanket and received liquid acetaminophen for 24 h with food and water *ad libitum*.

Metastasis monitoring by bioluminescence imaging

Bioluminescence imaging was used to assess orthotopic implantation in all mice immediately following surgery with the IVIS-100 bioluminescence imaging system (Caliper). Mice with detectable leakage from the pancreas were removed from the study. Mice were injected *i.p.* with 2.5 mg luciferin 10 min prior to imaging. Each mouse was imaged weekly to monitor orthotopic tumor and metastatic growth. Region of interest (ROI) analysis was used to measure light emitted for both orthotopic and metastatic sites using the IVIS Living Image software.

Experimental metastasis

Mice were implanted orthotopically with S2VP10L, S2VP10L-CP, S2VP10L-KFMΔSS 1, or S2VP10L-KISS1 cells as indicated above, and study group was reduced to 6/group due to detectable cell leakage from the pancreas in some mice. Lung, liver, diaphragm, and pancreas were removed and examined with bioluminescence imaging 15 and 30 days post-injection (3 mice/treatment). Bioluminescent signal was quantified using the Region of Interest (ROI) method for each organ and means were compared using ANOVA test. To determine background luminescence signal, organs were removed from 3 naïve mice and imaged using the ROI method. The signal obtained in the organs of naïve mice were: lung (1.92×10^2 counts/s), liver (2.50×10^2 counts/s), and diaphragm (2.32×10^2 counts/s). Immediately following bioluminescence imaging, organs were divided into portions for histology or RTQ-PCR analyses.

For histologic analyses, tumor xenografts as well as other tissues were harvested 15 and 30 days after cell injection, formalin fixed, and processed into paraffin blocks using standard methods. Metastases were detected using a monoclonal mouse anti-KISS1 antibody (6a4.27) generated at the UAB Tissue Characterization and Immunoreagent Resource. Antibody specificity was determined by co-localization of 6a4.27 staining with anti-FLAG staining on immunoblots as well as recognition of mass spectrometry-verified kisspeptins (B.H. Beck, K.T. Nash, and D.R. Welch, unpublished observations).

Formalin-fixed, paraffin-embedded tumors were cut into 4 μ m sections and attached to slides. After deparaffinization and rehydration, antigen retrieval was preformed in a pressure cooker 121°C for 20 min in pH 8 EDTA buffer (luciferase or CD31 IHC) or pH 9 EDTA buffer (KISS1 IHC). After H₂O₂ quench and blocking with 3% goat serum, slides were incubated with the primary antibody at 1:1000 dilution (luciferase (Chemicon, Billerica, MA) or CD31 (Abcam Cambridge, MA) or 10 μ g/ml (KISS1) for 20 min followed by Signet HRP label for 20 min and ScyTeck DAB (Logan, UT) for 7 min. Slides were counterstained with hematoxylin. Sections were viewed using a Nikon microscope and photographs were acquired using a Spot Camera (Diagnostic Instruments). Luciferase and KISS1 staining were based on 3 images per group.

Statistics

Organ metastasis levels were compared among groups for each organ using ANOVA with SAS software. When the ANOVA was statistically significant ($P < 0.05$), Tukey's test was used to determine the source of the difference.

RESULTS

Confirmation of KISS1 gene transfection and KISS1R expression

KISS1 and KISS1R expression were examined in stably transfected S2VP10L cells by RTQ-PCR (KISS1 and KISS1R), ELISA (KISS1) and flow cytometry (KISS1R) (Figure 1). KISS1 and KFMΔSS mRNA levels were higher in transfected S2VP10L cells than S2VP10L-CP or non-transfected control cells. S2VP10L-KFMΔSS cells had 10-fold higher KISS1 mRNA than S2VP10L-KISS1 cells. Consistent with other cells that have been suppressed for metastasis following re-expression of KISS1, S2VP10L cells and derivative cells did not express KISS1R (Figure 1).

KISS1 expression suppresses metastatic ability *in vivo*

To test the effect of KISS1 on metastasis, an orthotopic pancreatic cancer mouse model and bioluminescence imaging were used. At day 15 post-tumor cell injection, S2VP10L and S2VP10L-CP tumor-bearing mice had similar tumor growth and metastatic efficiency (Figure 2). Mice injected with S2VP10L-KFMΔSS 1 cells developed fewer pulmonary (50% of both controls), hepatic (95% of both controls), and diaphragmatic (97% of both controls) metastasis compared to mice implanted with parental (S2VP10L) or vector-only (S2VP10L-CP) control cells ($P=0.04$, $P=0.009$, and $P=0.006$ respectively). S2VP10-KISS1 tumor bearing mice had reduced metastases in lung (90%), liver (99%), and diaphragm (97%) ($P=0.007$, $P=0.002$, and $P=0.006$ respectively). As predicted, mice implanted with S2VP10L-KISS1 cells developed fewer metastases in lung (77%) and liver (23%) compared to mice implanted with S2VP10L-KFMΔSS 1 cells ($P=0.04$ and $P=0.034$, respectively). Interestingly, mice implanted with S2VP10L-KISS1 cells did not have reduced diaphragmatic metastases compared to S2VP10L-KFMΔSS 1 cells.

At day 30 post cell implant, the effects of KISS1 were even more pronounced. KISS1-expressing S2VP10L cells had 99% fewer pulmonary ($P<0.013$) and 98% fewer hepatic ($P<0.001$) metastases than control S2VP10L implanted mice detectable by bioluminescence. At day 30, orthotopic tumor burdens were indistinguishable amongst all groups ($P=0.53$) (Figure 3). Mice with S2VP10L-KFMΔSS tumors had significantly reduced pulmonary metastasis (77%) and hepatic metastasis (81%) compared to S2VP10L-CP and S2VP10L injected mice ($P=0.04$ and $P=0.009$, respectively). Mice implanted with S2VP10L cells expressing KISS1 had significantly fewer lung and liver metastases than mice implanted with S2VP10L-KFMΔSS cells ($P=0.03$ and $P=0.04$, respectively).

Mice injected with S2VP10L, S2VP10L-CP, or S2VP10L-KFMΔSS cells developed macroscopic liver, lung, and diaphragm metastases that were detectable using bioluminescence. Metastases were confirmed by immunohistochemical staining of luciferase (Figure 4). The bioluminescence signals corresponded proportionately to metastatic burden extrapolated from organ sections (Figures 3 & 4). Strikingly, pulmonary or hepatic metastases could not be detected by immunohistochemistry in S2VP10L-KISS1 (Figure 4).

KISS1 expression confirmed by histology and RTQ-PCR

KISS1 protein was visualized by immunohistochemistry in the cytoplasm of cells in the S2VP10L-KISS1 and -KFMΔSS pancreatic tumors at days 15 (data not shown) and 30 post-tumor cell injection (Figure 5). KISS1 protein was elevated in S2VP10L-KISS1 tumor sections as compared to S2VP10L controls. KISS1 protein appeared to be more concentrated in the cytoplasm of S2VP10L-KFMΔSS tumors than tumors expressing full-length KISS1, which is consistent with loss of the secretion signal sequence. In mice implanted with S2VP10L and S2VP10L-CP cells, pulmonary metastatic lesions showed similar low cytoplasmic KISS1 protein levels as observed in the corresponding orthotopic tumors. Likewise, mice with

S2VP10L-KFMASS tumors expressed readily detectable KISS1 protein in pulmonary metastases. Mice injected with S2VP10L-KISS1 did not develop detectable pulmonary metastases (Figures 3 & 4). The antibody also recognizes murine KISS1, so it is challenging to detect tumor cells amongst the background staining in normal lung.

To assess KISS1 expression in orthotopic and ectopic cells, mRNA was isolated from intra-pancreatic and lung tumor tissues (Figure 6). S2VP10L and S2VP10L-CP pancreatic tumors had the lowest KISS1 levels; whereas KISS1 levels of S2VP10L-KFMASS tumors were 3,500X higher and S2VP10L-KISS1 was 3,000X higher than controls. KISS1 mRNA levels in the pancreatic tumors were roughly proportionate to *in vitro* levels (Figure 1). KISS1 mRNA and KISS1 protein expression (by IHC) corresponded well in pancreatic tumors (Figures 5 & 6). KISS1 mRNA levels in lung samples were similar among treatment groups (Figure 5 & 6) as well as normal mouse lung (data not shown).

Orthotopic Tumor growth assessed by bioluminescence imaging

Orthotopic tumor growth was assessed longitudinally by bioluminescence imaging. All mice had similar pancreatic luciferase signals after tumor cell injection (Figure 7). The pancreatic tumors of S2VP10L-KISS1-injected mice had significantly reduced signal compared to controls or S2VP10L-KFMASS tumors at day 7 ($P=0.04$), but, by day 14, the signals were again similar (Figure 7). Tumor burden, as indicated by the luciferase signal, was not significantly different for S2VP10L-KISS1 pancreatic tumors compared to the other tumors at either day 14 or 30 post-tumor cell injection (Figures 2, 3, & 7).

DISCUSSION

The majority of patients with pancreatic cancer already have metastases at the time of diagnosis and very few survive five years (3,23). In an initial report by Masui on 30 patients, KISS1 levels were significantly lower in pancreatic carcinomas than normal pancreatic tissue, a finding later confirmed in 53 patients (13-14). Generally, cancer patients with higher expression of KP-54 and KISS1R had longer survival (22-25). Taken together, these facts coupled with KISS1 being a secreted protein, makes KISS1 an interesting candidate for suppression of pancreatic metastasis. Our study is the first to examine the effects of over-expressing KISS1 in pancreatic cancer *in vivo*. As hypothesized, KISS1 over-expression significantly suppressed pancreatic carcinoma metastasis to lung and liver (99% and 98%, respectively (Figure 3)). These findings provide a further basis to study the actions and mechanisms of KISS1 and its potential as a new target for prevention and/or progression of metastasis.

KISS1 re-expression in ovarian and melanoma cancer cells reduced metastasis (50% ovarian and 90% melanoma) (11,19). Although secretion of KISS1 was required for metastasis suppressor activity in the melanoma model (19), KISS1 suppressed pancreatic carcinoma metastasis in our orthotopic peritoneal model. Whereas the findings in melanoma precluded an intracrine loop, the findings in pancreatic adenocarcinoma leave open this possibility, albeit all of the cells suppressed for metastasis failed to express detectable KISS1R. We acknowledge the possibility that an undetectable fraction of KISS1 might be secreted. Likewise, as we have speculated previously, other biologically active fragments of KISS1 (*i.e.*, besides KP-54) may be released into the surrounding milieu. Experiments to ascertain this possibility are underway.

Consistent with the functional definition of metastasis suppressors, KISS1 did not completely block orthotopic tumor growth. In the S2VP10L model, orthotopic tumors did develop, but not without a transient delay during the first week after injection (Figure 7). Because orthotopic pancreatic tumors were of similarly sized by day 30, any initial delay in the primary tumor growth is unlikely explain differences in metastatic outgrowth observed at the time of

experiment termination. An explanation for this initial growth delay is not readily apparent, as differences in angiogenesis (*i.e.*, blood vessel density as measured by anti-CD31 staining) were not observed (Figure 8). The effect of KISS1 on metastatic tumor outgrowth beyond 30 days was not possible because all animals had to be euthanized because of ethical considerations.

Although the lung samples of S2VP10L-KISS1 mice had a detectable, but weak, bioluminescence signal (Figure 3), metastatic lesions were not observed by IHC in step sections. These findings are consistent with prior publications in breast and melanoma models that KISS1-expressing cells are able to disseminate, but not colonize ectopic sites. This property suggests that KISS1 could be useful for controlling pancreatic tumor cells that have all-too-often previously seeded other tissues before initial diagnosis.

In summary, identification of KISS1 as a metastasis suppressor in pancreatic cancer has expanded the horizon of our understanding of the function of this gene. KISS1 and its signaling pathways may represent new targets for the treatment of metastatic pancreatic cancer. Strategies could be developed to target metastatic pancreatic tumors by the delivery of KISS1 and/or kisspeptins which may result in static disease. Re-expression of KISS1 may prove to be a valuable treatment to suppress metastasis of pancreatic cancer.

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Figure 1.

KISS1 gene transfection and KISS1R expression. (A) Cells were transfected with plasmids containing KISS1, KFM Δ SS, or control (CP). KISS1 mRNA levels were determined by RTQ-PCR. KISS1 levels relative to S2VP10L are shown. (B) SVP10L cells express very low or no KISS1R mRNA (Bar Graph showing real-time quantitative RT-PCR) or protein (Flow cytometry). T47D human breast cancer cells, which express low levels of KISS1R, are included as a positive control.

Figure 2.

At day 15 post implant, S2VP10L and S2VP10L-CP mice had similar primary and metastasis tumor levels in all organs sampled as indicated by bioluminescence imaging. S2VP10L-KFMΔSS had reduced pulmonary metastasis (50%) and hepatic metastasis (95%) as compared to controls ($P=0.04$ and $P=0.009$). S2VP10-KISS1 mice had reduced metastasis by 90% in lung and 99% in liver as compared to controls ($P=0.007$ and $P=0.002$, respectively). Group means and standard deviations are presented.

Figure 3.

Metastasis measured by bioluminescence imaging in mice and mouse organs 30 days post implant. Tissues (Lu=Lung; Li=liver, D=diaphragm; and P=pancreas) were removed 30 days post implant. S2VP10L-KISS1 had reduced lung metastasis ($P<0.013$), liver metastasis ($P<0.001$), and disease in the diaphragm ($P<0.008$) as compared to either S2VP10L-CP or S2VP10L. Pancreas levels were similar among all groups ($P=0.553$). Both S2VP10L and S2VP10L-CP had extensive metastasis in each organ as identified in whole body images of mouse and specific organs. S2VP10L-KISS1 mice had minimal metastasis in whole body of mice or in specific organs. **Left** and **Center** panels contain a **single representative mouse** and organs from this representative mouse. In contrast, group mean and standard deviation are presented in **Right** panel.

Figure 4. Immunohistochemistry confirmed luciferase positive metastasis levels in lung sections 30 days post tumor cell injection (bar=100 μ m). Representative S2VP10L (**A**) and S2VP10L-CP (**B**) lung sections had multiple metastases (indicated by arrows). S2VP10L-KFM Δ SS (**C**) had moderate lung metastases (indicated by arrows). S2VP10L-KISS1 (**D**) had no detectable metastases.

Figure 5.

Immunohistochemistry confirmed KISS1 protein levels in pancreatic tumor sections (bar=50 μ m) and lung sections (bar=100 μ m) at day 30 post tumor cell injection. **A)** Representative S2VP10L pancreatic tumor sections had low levels of KISS1 protein (indicated by brown staining). **B)** S2VP10L-KFMA Δ SS had high KISS1 levels (indicated by dark brown staining) and concentrated KISS1 levels in some cells which suggests that KISS1 is not secreted in some cells containing the KFMA Δ SS plasmid. **C)** S2VP10L-KISS1 had weak cytoplasmic staining and cell membrane staining (indicated by brown staining), but did not contain a high concentration of KISS1 as found in S2VP10L-KFMA Δ SS 1 which suggests that KISS1 is secreted. **D)** S2VP10L metastasis in the lung contained low KISS1 protein levels. **E)** S2VP10L-KFMA Δ SS 1 lung sections had concentrated KISS1 protein staining in metastatic nodules. **F)** S2VP10L-KISS1 lung section had no detectable metastasis and the lung had similar KISS1 staining to normal mouse lung.

Figure 6.

KISS1 mRNA levels were determined in tissue from mice at day 30 post tumor cell injection. RNA levels are expressed on a per cell basis. S2VP10L and S2VP10L-CP had low levels of KISS1 mRNA in pancreatic samples. S2VP10L-KFMΔSS 1 and S2VP10L-KISS1 had higher levels of KISS1 mRNA in pancreatic samples. Lung samples among the 4 groups had similar KISS1 mRNA levels ($P>0.05$). Group means and standard deviations are presented.

Figure 7.

Tumor growth monitored by bioluminescence imaging. Immediately after cell injection, tumors were measured using bioluminescence imaging. Tumor growth was monitored weekly and luciferase signal was normalized. All tumors had similar luciferase signal after implantation. **Left** panel contains a representative mouse for each group. In contrast, group mean and standard deviation are presented in **Right** panel. S2VP10L-KISS1 pancreatic tumors had decreased signal at day 7 in comparison to controls ($P=0.04$). S2VP10L-KFMΔSS pancreatic tumors grew at similar rates (day 7 and 14) as the control (S2VP10L), but by day 14 had reduced metastasis levels. At day 14, S2VP10L-KISS1 pancreatic tumors were similar to control pancreatic tumors, but had no detectable levels of metastasis.

Figure 8. Blood vessel density was examined using immunohistochemistry staining for CD 31 in tumor sections (bar=50μm). Representative S2VP10L (A) and S2VP10L-CP (B) S2VP10L-KFMASS (C) S2VP10L-KISS1 (D) had no detectable differences in blood vessel density.