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Original Article

Pancreatic cancer cell-derived semaphorin 3A promotes neuron recruitment to accelerate tumor growth and dissemination

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Abstract: Perineural invasion and neurogenesis are frequently observed in pancreatic ductal adenocarcinoma (PDAC), and they are associated with a poor prognosis. Axon guidance factor semaphorin 3A (SEMA3A) is upregulated in PDAC. However, it remains unclear whether cancer-derived SEMA3A influences nerve innervation and pancreatic tumorigenesis. *In silico* analyses were performed using PROGgene and NetworkAnalyst to clarify the importance of SEMA3A and its receptors, plexin A1 (PLXNA1) and neuropilin 2 (NRP2), in pancreatic cancer. *In vitro* assays, including migration, neurite outgrowth, and 3D recruitment, were performed to study the effects of SEMA3A on neuronal behaviors. Additionally, an orthotopic animal study using C57BL/6 mice was performed to validate the *in vitro* findings. Expression of SEMA3A and its receptors predicted worse prognosis for PDAC. Cancer-derived SEMA3A promoted neural migration, neurite outgrowth, and neural recruitment. Furthermore, SEMA3A-induced effects depended on PLXNA1, NRP2, and MAPK activation. Trametinib, an approved MAPK kinase (MEK) inhibitor, counteracted SEMA3A-enhanced neuronal activity *in vitro*. Inhibition of SEMA3A by shRNA in pancreatic cancer cells resulted in decreased neural recruitment, tumor growth, and dissemination *in vivo*. Our results suggested that cancer-secreted SEMA3A plays an important role in promoting neo-neurogenesis and progression of PDAC.

Keywords: Neuron, neuropilin, pancreatic ductal adenocarcinoma, plexin A1, semaphorin 3A

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a life-threatening malignancy with a five-year survival rate of 8% [1, 2]. More than 80% of the patients are diagnosed at late stages with local or distant metastases, and systemic drug treatment is the primary clinical intervention. However, direct targeting of PDAC is difficult because of its fibrotic and cold immunological characteristics. The matrix-enriched tumor microenvironment, known as the desmoplastic stroma, impedes the penetration of blood vessels and hinders the delivery of chemotherapeutic drugs to tumors. In addition, cancer-associated fibroblasts (CAFs) in the stroma produce a number of cytokines and growth factors to stimulate cancer cell proliferation and increase resistance to chemotherapeutic drugs [3, 4]. Therefore, only a small proportion of patients with PDAC respond to chemotherapy. Furthermore, the efficacy of immunotherapy has been disappointing. The unsatisfactory therapeutic effects of immunotherapy in PDAC can be attributed to the following: (1) lack of infiltration of immune cells because of intense desmoplasia and hypovascularity [3]; (2) accumulation of myeloid-derived suppressor cells (MDSCs), a heterogeneous group of immature myeloid cells that exhibit strong immune-suppressive activity in the stroma [4]; and (3) release of immune-inhibitory cytokines or che-

mokines from cancer cells and stromal cells, which leads to inactivation of immune cell function [5]. Notably, targeting the stroma could be an alternative strategy to treat PDAC. However, the results of clinical trials of stroma-targeting therapies for PDAC have not been successful, although data from preclinical studies have been encouraging [6]. The importance of neuronal cells in PDAC remains largely unexplored when compared with the well-characterized functions of CAFs and MDSCs. Whether neuronal cell signaling is a potential candidate for stroma-targeting therapy is a topic of interest.

Semaphorins (SEMAs) are a large family of signaling molecules involved in the regulation of diverse biological processes. To date, 20 vertebrate SEMA genes have been cloned. Based on protein sequences and structural similarities, they are grouped into five classes [7]. Members of class 3 SEMAs are secreted proteins, whereas members of the other classes are membrane-bound molecules. These signaling molecules bind to two types of cognate receptors: the plexin (PLXN) and neuropilin (NRP) families. Nine members of the PLXN family (PLXN A1-A4, B1-B3, C1, and D1) and two members of the NRP family (NRP1 and NRP2) have been identified in vertebrates. The interaction between SEMAs and PLXNs/NRPs creates a complex signaling network that modulates the functions and behaviors of various cell types under physiological and pathological conditions. Furthermore, SEMAs have emerged as potential clinical biomarkers and therapeutic targets in various cancers [8]. Moreover, SEMAs have been shown to be key players in controlling phenotypic alterations and functional remodeling in cancer cells.

SEMA3A was first identified as a nerve guidance factor that participates in the development and patterning of multiple organs such as the neuronal system, cardiovascular system, lung, and kidney [9-11]. Emerging evidence suggests that SEMA3A has an oncogenic activity in PDAC. Muller *et al.* demonstrated that SEMA3A upregulation was associated with poor prognosis in patients with PDAC [12]. An *in vitro* assay revealed that SEMA3A significantly enhanced the invasive ability of pancreatic cancer cells. Haider *et al.* used a 36-gene signature to predict clinical outcomes in patients with PDAC and found that higher SEMA3A expression was associated with shorter survival [13]. Genomic profiling of 142 PDAC tumors using exome sequencing revealed significant alterations in genes involved in the axon guidance pathway and an increase in SEMA3A expression [14]. In contrast, two previous studies have demonstrated that SEMA3A is a potent endogenous angiogenesis inhibitor that induces the normalization of tumor vasculature and suppresses the growth of pancreatic neuroendocrine tumors [15, 16]. Pancreatic neuroendocrine tumors are rare types of pancreatic cancer that develop from a group of specific neuroendocrine cells in the pancreas. The origin and characteristics of this tumor differ from those of PDAC. Pancreatic neuroendocrine tumors are hypervascular cancers with intense blood vessel distribution and high drug penetration efficiency. Therefore, the role of SEMA3A in PDAC remains unclear.

Nerve trunk is frequently invaded by pancreatic cancer cells, and perineural invasion is strongly correlated with disease progression [17, 18]. Neuronal cells can be recruited by pancreatic cancer cells, and they subsequently participate in tumor development [18]. Because SEMA3A is a key molecule in axon guidance, we hypothesized that SEMA3A may promote nerve innervation in PDAC tumors to accelerate tumor growth and dissemination. In this study, we analyzed the association between SEMA3A and its receptors and the outcome in patients with PDAC using a public database and validated the findings in our in-house cohort. *In vitro* and *in vivo* assays were performed to elucidate the underlying mechanism. Moreover, we identified potential drug that could inhibit SEMA3A-overexpressing PDAC. Collectively, our results elucidated a new molecular basis through which SEMA3A promotes PDAC growth, and provided a novel strategy to target PDAC with high SEMA3A expression.

Materials and methods

Bioinformatic analyses

The human secretome was analyzed using the Human Protein Atlas [19] (https://www.proteinatlas.org/) and subjected to prognosis prediction in PDAC using PROGgene [20] (http://www.progtools.net/gene/). SEMA3A-coexpressed gene signature in the PAAD dataset of The Cancer Genome Atlas (TCGA) was extracted from cBioPortal [21] (https://www.cbioportal.org/) and subjected to pathway analysis using

NetworkAnalyst [22] (https://www.networkanalyst.ca/).

RNA sequencing of PDAC specimens

RNA sequencing analysis of 105 PDAC specimens was performed and this study was approved by the Institutional Review Board of the National Cheng Kung University Hospital (B-ER-110-420). Patient anonymity was preserved. Libraries were constructed and loaded onto Illumina NovaSeq system (Illumina, San Diego, CA, USA) and sequencing was performed using a 2 × 150 paired-end configuration.

Cell lines and reagents

The human pancreatic cancer cell line, MIA PaCa-2, was obtained from ATCC and cultured in DMEM from HyClone (Logan, Utah, USA). The mouse pancreatic cancer cell line, KPC, was cultured in RPMI (HyClone). The human neuroblastoma cell line, SH-SY5Y, was kindly provided by Dr. Ju-Ming Wang (Department of Biotechnology and Bioindustry Sciences, National Cheng Kung University, Tainan, Taiwan) and was cultured in DMEM/F12 (HyClone). The mouse neuroblastoma cell line, Neuro-2a, was kindly provided by Dr. Yu-Min Kuo (Department of Cell Biology and Anatomy, National Cheng Kung University, Tainan, Taiwan) and was cultured in MEM (HyClone). All media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (both from HyClone). For antibody blockage assay, cells were treated with 2 μg/mL control rabbit IgG (cat No. 10201, LEADGENE, Tainan, Taiwan) or goat IgG (cat No. 10301, LEADGENE), SEMA3A antibody (cat No. GTX130671, GeneTex, Hsinchu, Taiwan), PLXNA1 antibody (cat No. AF4309, R&D Systems, Minneapolis, MN, USA), or NRP2 antibody (cat No. AF2215, R&D Systems) for 24 h. The SEMA3A peptide (cat No. MBS474063) was purchased from MyBioSource (San Diego, CA, USA). Retinoic acid (R2625) was purchased from Sigma/Merck (Darmstadt, Germany).

RNA interference

shRNAs against mouse SEMA3A and PLXNA1 were obtained from RNA Technology Core (Academia Sinica, Taipei, Taiwan). Subsequently, 2 μg of shRNA plasmid was transfected into indicated cells along with 3 μL of Hyfect reagent (LEADGENE). After 48 h, the transfected cells were harvested for analysis. The target sequence for shSEMA3A is CCCAGTGTTTCCTATAAATAA, for shPLXNA1-89 is CCGAGGTGAAGTACAACTATA, and for shPLXNA1-90 is CCTCTATGCTATGACGGAGAA.

RNA extraction and polymerase chain reaction

Cells were lysed in TRIzol reagent (cat No. 15596018, Thermo Fisher Scientific, Waltham, MA, USA), and total RNA was extracted according to the manufacturer's instructions. RNA concentration was quantified using NanoDrop spectrophotometer (Thermo Fisher Scientific). Subsequently, cDNA was prepared by reverse transcription of 2 μg RNA using ReverTra Ace (cat No. TY-TRT-101, PURIGO, Taipei, Taiwan), and the cDNA was amplified using 2× Taq Master Mix (cat No. P111, Vazyme, Nanjing, China) under the following conditions: 98°C, 20 s; 65°C, 1 min; 72°C, 1 min for 35 cycles. The end product was subjected to electrophoresis at 100 V for 15 min using a 1% agarose gel. The primer sequences are: m-SEMA3A-F, CAGCCATGTACAACCCAGTG; m-SEMA3A-R, ACGGTTCCAACATCTGTTCC; m-PLXNA1-F, GTGTGTGGATAGCCATCA; m-PLXNA1-R, CCAGCCTCTCGAACACT; m-GAPDH-F, AGGTCGGTGTGAACGGATTTG; and m-GAPDH-R, TGTAGACCATGTAGTTGAGGTCA.

Western blotting

Western blotting was performed as previously described [23]. The antibodies against SEMA3A (cat No. GTX130671), phospho-ERK (cat No. GTX24819), ERK (cat No. GTX134462), and GAPDH (cat No. GTX627408) were obtained from GeneTex.

Immunofluorescence and immunohistochemistry

Immunofluorescence and immunohistochemistry were performed as previously described [23].

Flow cytometry

Flow cytometry was performed as previously described [23].

MTT assay

Cells were seeded in 96-well plates and treated with the indicated medium, and cell proliferation was monitored for 3 days. After treatment, the cells were stained with 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-

zolium bromide (MTT, M2128, Sigma) for 3 h and dissolved in DMSO. The end product was measured at OD_{550} using FlexStation 3 (Molecular Probes/Thermo Fisher Scientific).

Migration assay

Migration assay was performed as previously described [24].

Neurite outgrowth assay

Neural differentiation was induced by treating SH-SY5Y and Neuro-2a with 10 μM retinoic acid for 6 days (two cycles of three-day treatment). Differentiated SH-SY5Y and differentiated Neuro-2a were then treated with the medium of the indicated conditions and subjected to neurite outgrowth assay. Neurites longer than half the cell body diameter [25, 26] were counted, and the percentage of neurite-bearing cells was analyzed.

3D recruitment assay

The 3D recruitment assay was performed as described previously [27] with minor modifications. Briefly, pancreatic cancer cells were stained with the green fluorescent dye, DiO (cat No. 60011, Biotium, Fremont, CA, USA) and neural cells were stained with the red fluorescent dye, DiI (cat No. 60010, Biotium) at a concentration of 5 μM in PBS for 20 min. Stained pancreatic cancer cells were resuspended in 20 μL Matrigel (cat No. 354234, BD, Franklin Lakes, NJ, USA) and seeded onto the center of six-well plates. This mixture was allowed to solidify for 7 min, and medium containing stained neuronal cells was added. Cell recruitment under 3D condition was observed using a fluorescence microscope after 24 h. The number of neuronal cells that migrated toward the Matrigel-embedded pancreatic cancer cells was counted.

Orthotopic mouse model

Animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of the National Health Research Institutes (approval number 109029). Six to eight-week-old C57BL/6 mice obtained from the National Laboratory Animal Center were orthotopically injected with control or shSEMA3A KPC cells. Briefly, mice were anesthetized using 2.5% isoflurane and injected intraperitoneally with ketoprofen at a concentration of 1 mg/mL. Fur was removed using an electric shaver, and the incision site was cleaned with alcohol pads and povidone-iodine. A small incision was made on the skin and peritoneum of the left lateral flank to exteriorize the pancreas. Subsequently, 200,000 KPC cells in 50 μL PBS were injected orthotopically using a 29G insulin syringe into the pancreas. The peritoneum was closed using suture with a No. 4 catgut and the skin was closed using clips. Wound healing and food and water intakes were monitored daily. After three weeks, the mice were sacrificed, and tissues were harvested for analysis. There were four and three mice in the control and shSEMA3A groups, respectively.

Statistical analysis

Statistical analysis was performed using GraphPad (San Diego, CA, USA). Statistical differences between the control and experimental groups were calculated using Student's *t*-test or Chi-square test (for RNA sequencing), and $P<0.05$ was considered statistically significant.

Results

SEMA3A and its receptors predict poor prognosis in patients with PDAC

To study the importance of neuronal factors in PDAC, we extracted the human secretome from The Human Protein Atlas [19] and applied it to prognosis prediction using PROGgene [20]. Notably, 44 factors were involved in neuronal regulation among 2,933 candidates, and only seven of them predicted poor prognosis in patients with PDAC (**Figure 1A**). These factors included ADAM metallopeptidase domain 9, cardiotrophin-like cytokine factor 1, SEMA3A, KiSS-1 metastasis suppressor, laminin subunit beta 2, matrix metallopeptidase 2, and NRP2 (**Figure 1A**). We focused on SEMA3A and its receptor, NRP2, and suggested a potential role of SEMA3A/NRP2 signaling in pancreatic tumorigenesis. The expression of SEMA3A and NRP2 significantly correlated with worsened survival in patients with PDAC (**Figure 1B**, **1C**). In addition to NRP, the PXLN family comprises another group of SEMA3A receptors. Among the PXLN family members, only PLXNA1 was associated with shorter survival in patients with PDAC (**Figure 1D**), suggesting that SEMA3A and its receptors are associated with poor prognosis in PDAC. To validate above findings

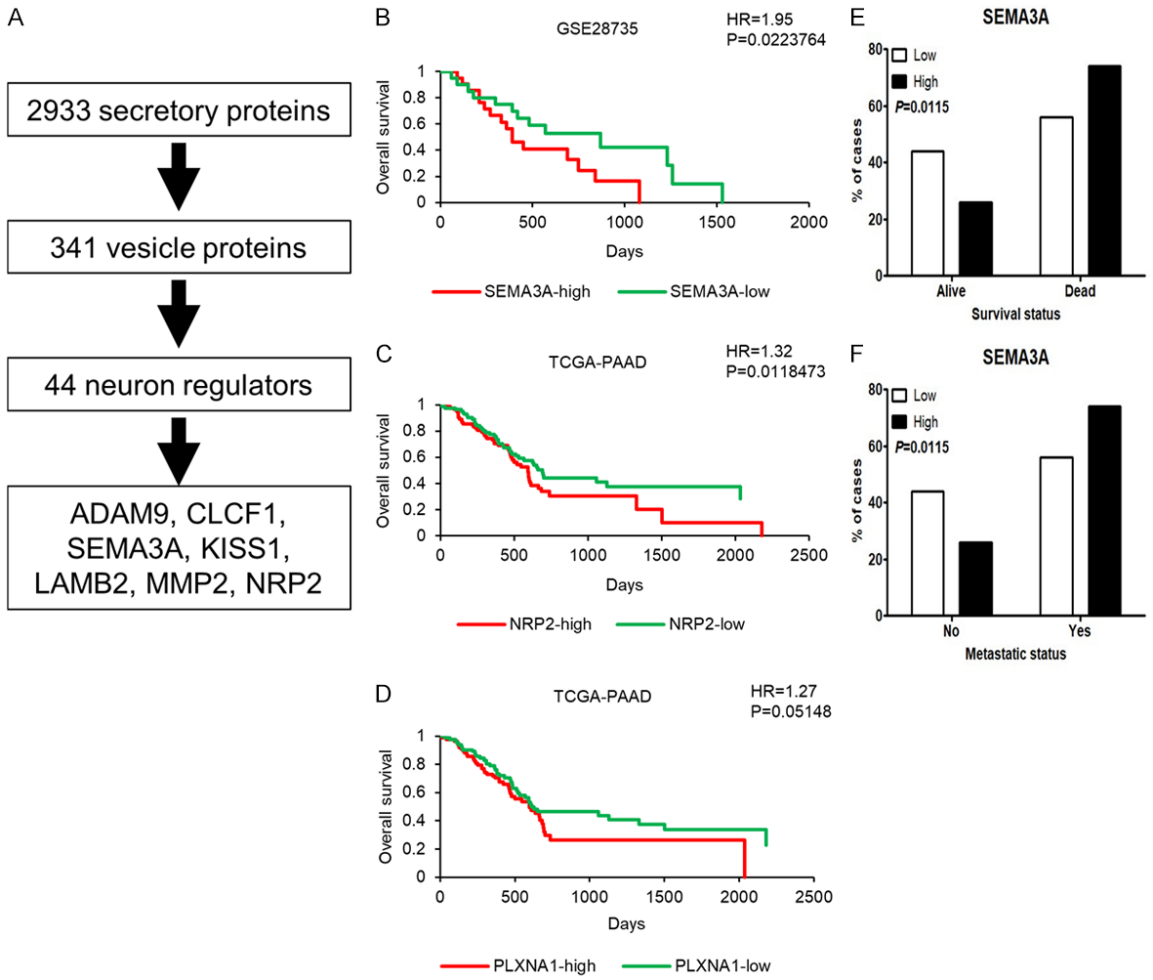

Figure 1. SEMA3A and its receptors predict poor prognosis in patients with PDAC. (A) 2933 secretory factors were filtered based on whether they are vesicle proteins and neural regulation-related proteins, and SEMA3A was selected as a candidate factor. (B) Prognostic abilities of SEMA3A and its receptors (C) NRP2 and (D) PLXNA1 were analyzed using PROGgene. (E, F) In addition, prognostic ability of SEMA3A in our own dataset was analyzed.

in the public database, RNA sequencing was performed on tumors from 105 patients with PDAC. An increased expression of SEMA3A was observed in patients with shorter survival (**Figure 1E**) and metastatic tumors (**Figure 1F**), indicating the importance of SEMA3A in PDAC.

High SEMA3A expression is associated with axon guidance and mitogen-activated protein kinase (MAPK) activation

In addition to prognostic analysis, we dissected the pathways involved in SEMA3A-high pancreatic cancer in the TCGA dataset using NetworkAnalyst [22, 23]. The results showed that SEMA3A is linked to axon guidance, based on analyses of the Kyoto Encyclopedia of Genes and Genomes (KEGG) (**Figure 2A**) and Reactome (**Figure 2B**). In addition, SEMA3A-associated MAPK activation and migration could be involved in neuronal regulation during tumorigenesis (**Figure 2C-F**). These *in silico* results were subsequently confirmed by both *in vitro* and *in vivo* assays.

Pancreatic cancer-derived SEMA3A promotes neuron migration and neurite outgrowth in a PLXNA1/NRP2-dependent manner

SEMA3A is a key regulator of neuronal regulation; therefore, we examined whether neuronal cells are targets for SEMA3A to influence PDAC progression. First, we confirmed that SEMA3A was secreted by human (MIA PaCa-2) and mouse (KPC) pancreatic cancer cell lines. Following pancreatic cancer cell culture, there

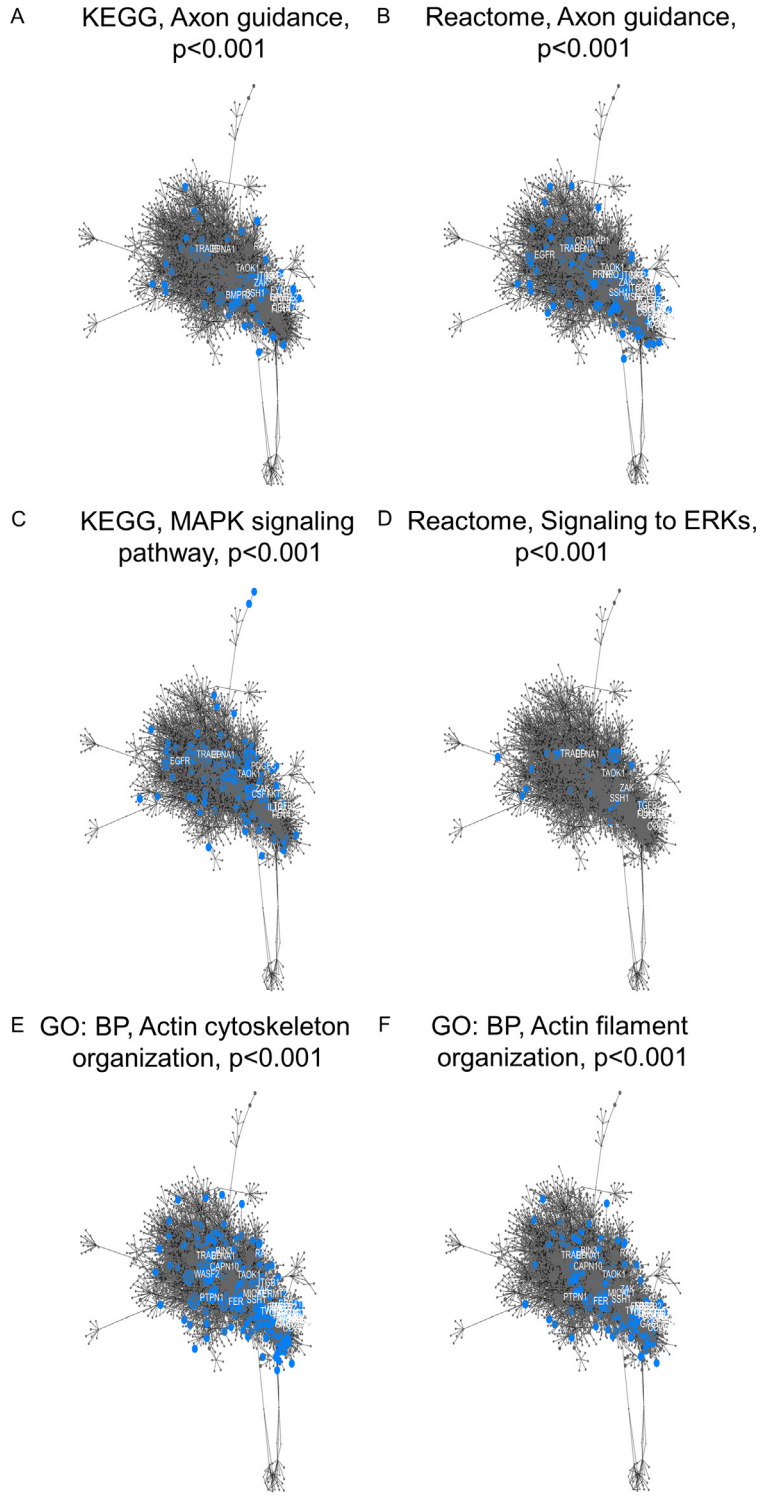

Figure 2. Gene signatures positively correlated with SEMA3A expression in PDAC. SEMA3A coexpression signature in TCGA PAAD was extracted from cBioPortal and analyzed with NetworkAnalyst in KEGG, Reactome, Gene Ontology: Biological Pathway (GO: BP) datasets for enriched pathways and statistical significances. A. Enrichment of axon guidance pathway in KEGG, P<0.001; B. Enrichment of axon guidance pathway in Reactome, P<0.001; C. Enrichment of MAPK signaling pathway in KEGG, P<0.001; D. Enrichment of signaling to ERKs in Reactome, P<0.001; E. Enrichment of actin cytoskeleton organization in GO: BP, P<0.001; F. Enrichment of actin filament organization in GO: BP, P<0.001.

was a significant increase in SEMA3A in the conditioned medium, suggesting the secretion of this protein by PDAC cells (**Figure 3A**, **3B**, Supplementary Figure 1A, 1B). Treatment of PDAC-conditioned medium onto differentiated SH-SY5Y (human) and Neuro-2a (mouse) cells did not change the proliferation of these neuronal cells (**Figure 3C**, **3D**). However, neuronal cell migration was significantly enhanced (**Figure 3E**, **3F**). In addition, neurite outgrowth, demonstrated by the extension of tubulin beta 3 class III (TUBB3)-positive neurites, increased after treatment with PDAC-conditioned medium (**Figure 3G**, **3H**). To verify whether the neuron-modulating effect was SEMA3A-dependent, we used antibody neutralization and shRNA knockdown to inhibit SEMA3A expression. Depletion of SEMA3A using neutralizing antibody significantly decreased the enhancement of migration (**Figure 4A**, Supplementary Figure 3A) and neurite outgrowth (**Figure 4B**, Supplementary Figure 3B) induced by MIA PaCa-2-conditioned medium in differentiated SH-SY5Y cells. Conversely, the addition of a SEMA3A peptide, which could block the neutralization activity of the SEMA3A antibody, completely reversed this effect (**Figure 4A**, **4B**, Supplementary Figure 3). These results confirmed the specificity of the SEMA3A antibody and the importance of PDAC-derived SEMA3A in promoting neuronal motility. Subsequently, we investigated the effect of the SEMA3A antibody on the recruitment of neuronal cells by PDAC in 3D culture. The movement of neuronal cells toward PDAC

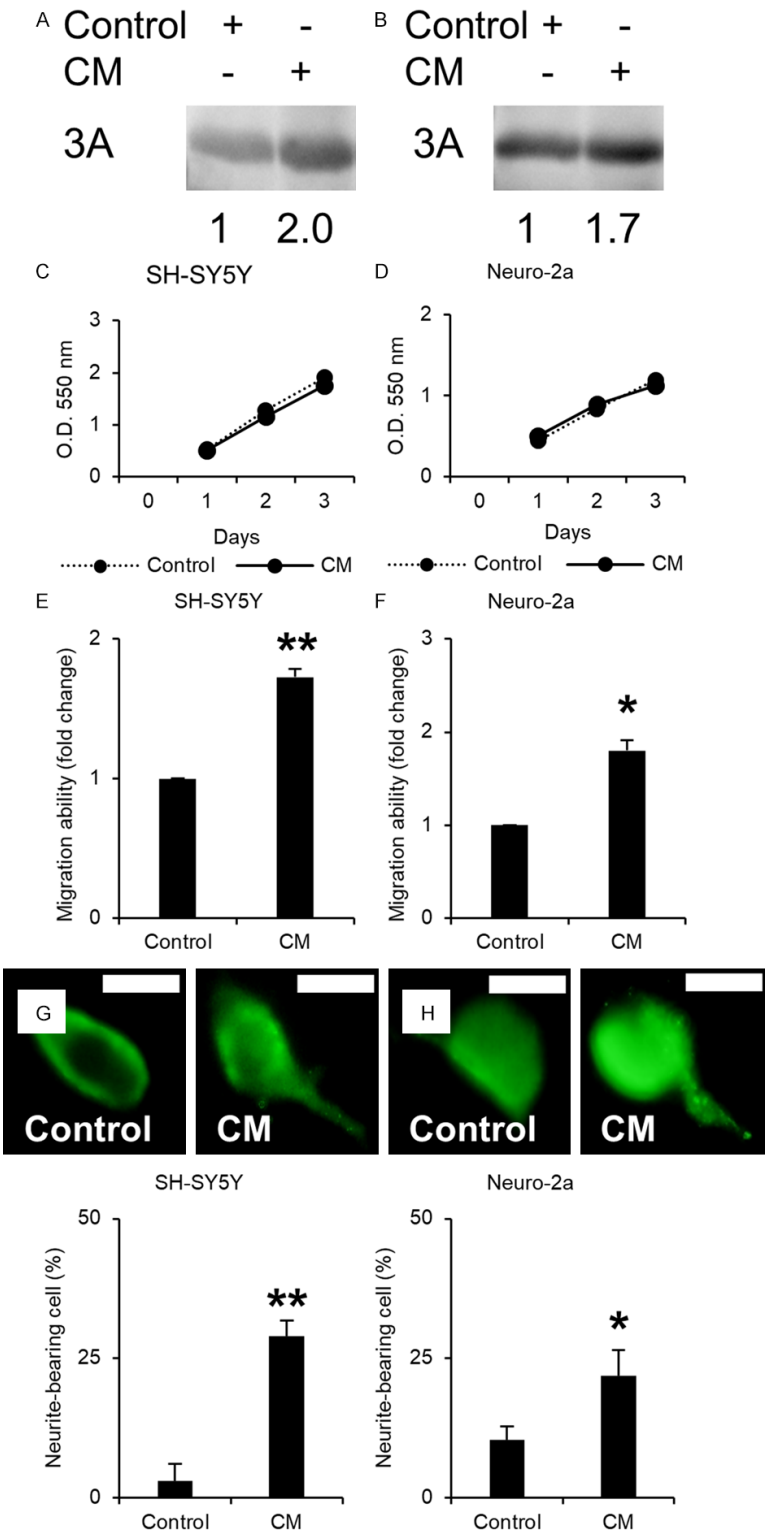

Figure 3. Pancreatic cancer cells express SEMA3A and increase neural migration and neurite outgrowth. Control medium and PDAC-conditioned medium from (A) MIA PaCa-2 or (B) KPC were subjected to Western blotting to detect SEMA3A level. The effect of PDAC-conditioned medium on (C, D) proliferation, (E, F) migration, and (G, H) neurite outgrowth in neuronal cells was analyzed. Green fluorescence in (G) and (H) indicates TUBB3 staining. For differentiated SH-SY5Y cells, control condition is 10% FBS DMEM (MIA PaCa-2 culture medium), and CM condition is MIA PaCa-2-conditioned 10% FBS DMEM. For differentiated Neuro-2a cells, control condition is 10% FBS RPMI (KPC culture medium), and CM condition is KPC-conditioned 10% FBS RPMI. *, P<0.05; **, P<0.01. Scale bar, 5 μm. Error bars represent standard error of the mean (SEM).

cells decreased after the addition of the SEMA3A antibody (**Figure 4C**). To validate the results of the antibody blockage study, we inhibited SEMA3A expression in mouse KPC cells using shRNA (**Figure 4D**, Supplementary Figure 1C) and repeated the experiments. The data showed that SEMA3A knockdown reduced KPC-induced neural migration (**Figure 4D**, Supplementary Figure 4A), neurite outgrowth (**Figure 4E**, Supplementary Figure 4B), and neural recruitment (**Figure 4F**, Supplementary Figure 4C).

Furthermore, we investigated whether the effect of SEMA3A on neural cells is mediated by its cognate receptors. Flow cytometry analysis and immunofluorescence staining confirmed the expression of PLXNA1 and NRP2 on the surface of differentiated SH-SY5Y cells (**Figure 5A**, Supplementary Figure 2). The addition of blocking antibodies against these two receptors significantly reduced the migration (**Figure 5B**, Supplementary Figure 5A, 5D) and neurite outgrowth (**Figure 5C**, Supplementary Figure 5B, 5E) of differentiated SH-SY5Y cells induced by the conditioned medium from MIA PaCa-2 cells. Additionally, recruitment of differentiated SH-SY5Y cells by MIA PaCa-2 cells in 3D culture was suppressed

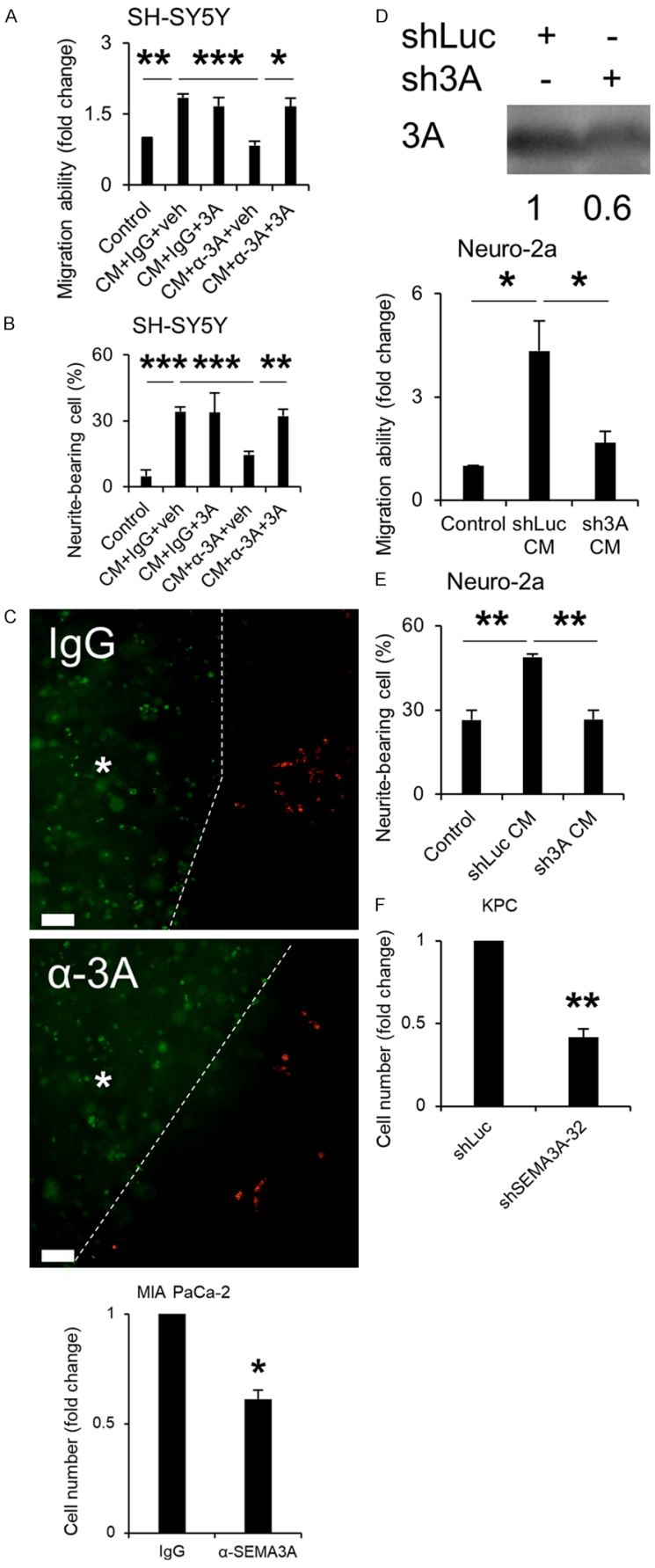

Figure 4. SEMA3A blockage decreases PDAC-induced migration and recruitment of neuronal cells. Control IgG or SEMA3A antibody was added into the conditioned medium from MIA PaCa-2, in combination with vehicle or SEMA3A peptide, and used to treat differentiated SH-SY5Y. Subsequent effects of these combinations on (A) migration, (B) neurite outgrowth, and (C) 3D recruitment were analyzed. (C) MIA PaCa-2 cells were labeled with the green fluorescence membrane dye, DiO, and differentiated SH-SY5Y cells were labeled with the red fluorescence membrane dye, DiI. (D) The expression of SEMA3A in mouse KPC was inhibited using shRNA. Conditioned media from control- or SEMA3A-depleted KPCs were used to treat differentiated Neuro-2a cells and the effects on (D) migration, (E) neurite outgrowth and (F) 3D recruitment were assayed. *, P<0.05; **, P<0.01; ***, P<0.001. Scale bar, 100 μm. Error bars represent SEM.

(**Figure 5D**, Supplementary Figure 5C, 5F) after the administration of antibodies against PLXNA1 or NRP2. In differentiated Neuro-2a cells, knockdown of PLXNA1 using two independent shRNAs effectively inhibited its expression (**Figure 5E**). PLXNA1 depletion in differentiated Neuro-2a cells markedly reduced its response to KPC-conditioned medium in terms of migration (**Figure 5F**, Supplementary Figure 5G) and neurite outgrowth (**Figure 5G**, Supplementary Figure 5H). In addition, shPLXNA1-Neuro-2a cells showed decreased recruitment by KPC in 3D culture (**Figure 5H**, Supplementary Figure 5I). NRP2 expression in differentiated Neuro-2a cells was confirmed using flow cytometry (**Figure 5I**). Inhibition of NRP2 using a blocking antibody suppressed the migration (**Figure 5J**, Supplementary Figure 5J), neurite outgrowth (**Figure 5K**,

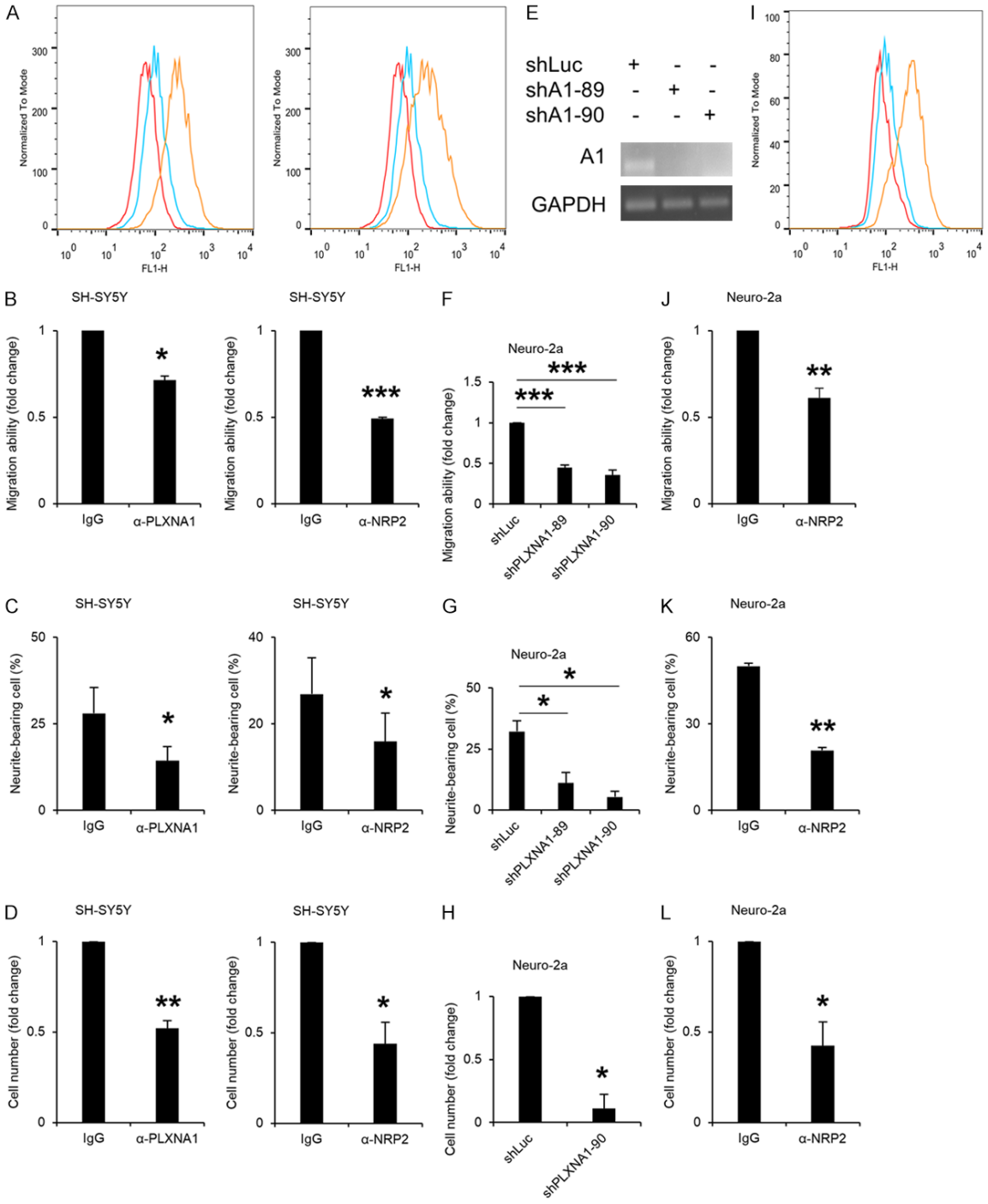

Figure 5. Inhibition of PLXNA1 and NRP2 on neuronal cells reduces SEMA3A-induced migration, neurite outgrowth, and neural recruitment. (A) Protein level of PLXNA1 and NRP2 on the surface of differentiated SH-SY5Y was determined using flow cytometry [red, unstain; blue, 2'Ab only; orange, α-PLXNA1 (left) or α-NRP2 (right)]. Differentiated SH-SY5Y was treated with control IgG, α-PLXNA1, or α-NRP2, and the effects of these treatments on (B) migration, (C) neurite outgrowth, and (D) 3D recruitment were analyzed. (E) Expression of PLXNA1 in differentiated Neuro-2a cells was inhibited using shRNA and the level of PLXNA1 was determined using RT-PCR. The effect of PLXNA1 depletion on KPC-conditioned medium-induced (F) migration, (G) neurite outgrowth, and (H) 3D recruitment of differentiated Neuro-2a cells was analyzed. (I) Expression of NRP2 on the surface of differentiated Neuro-2a cells was assayed using flow cytometry (red, unstain; blue, 2'Ab only; orange, α-NRP2). The effect of NRP2 inhibition through neutralizing antibody on KPC-conditioned medium-induced (J) migration, (K) neurite outgrowth, and (L) 3D recruitment of differentiated Neuro-2a cells was analyzed. *, P<0.05; **, P<0.01; ***, P<0.001. Error bars represent SEM.

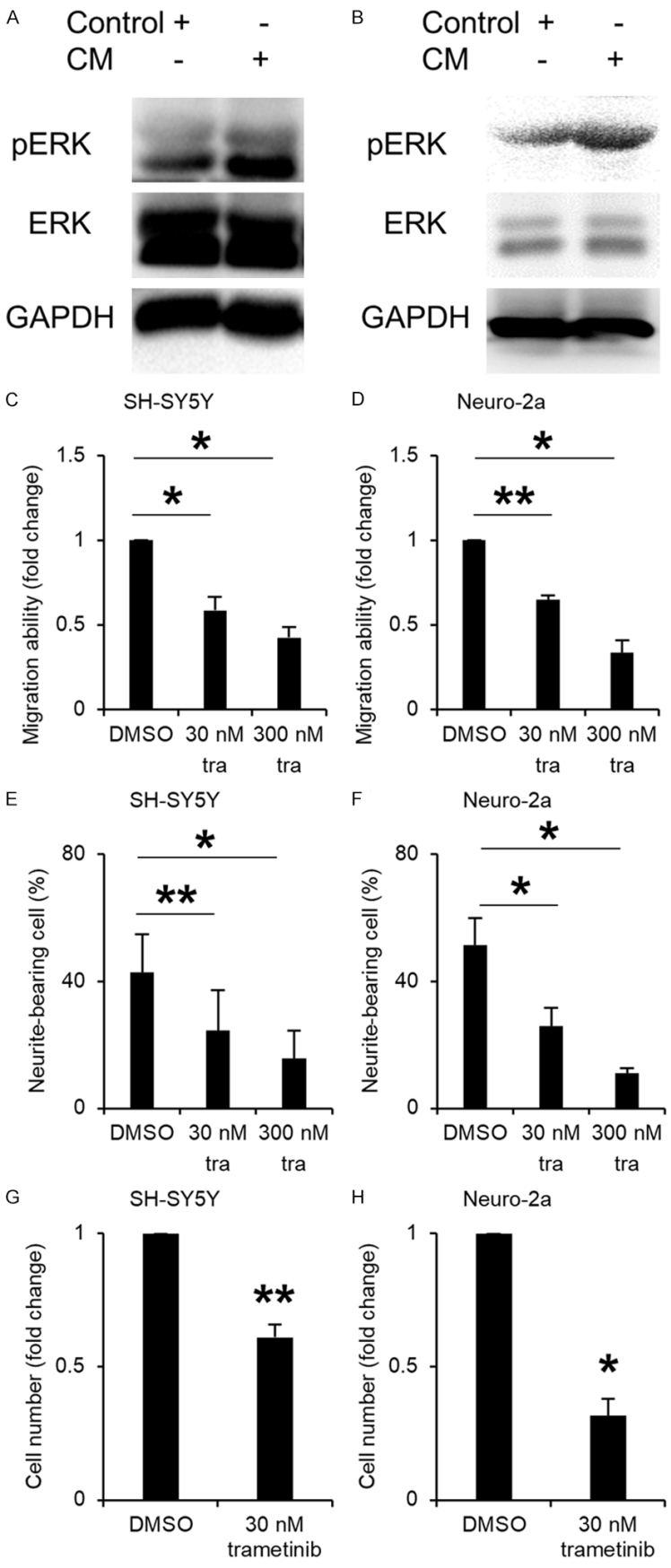

Figure 6. SEMA3A activates MEK in neuronal cells to promote their motility. Differentiated SH-SY5Y and Neuro-2a cells were treated with conditioned medium of MIA PaCa-2 or KPC in the presence or absence of the MEK inhibitor, trametinib. The effect of conditioned medium on ERK phosphorylation in (A) differentiated SH-SY5Y cells and (B) differentiated Neuro-2a cells was investigated using Western blotting. In addition, the effect of trametinib on conditioned medium-induced (C, D) migration, (E, F) neurite outgrowth, and (G, H) 3D recruitment was analyzed. *, P<0.05; **, P<0.01. Error bars represent SEM.

Supplementary Figure 5K), and 3D recruitment (**Figure 5L**, Supplementary Figure 5L) of differentiated Neuro-2a cells, even after stimulation with KPC-conditioned medium. These data confirmed the role of PDAC-derived SEMA3A in promoting neuronal motility.

MAPK signaling is a potential target in PDAC-derived SEMA3A-induced neuronal alterations

The *in silico* analysis suggested the involvement of SEMA3A-associated MAPK activation in neuronal regulation during PDAC tumorigenesis (**Figure 2C**, **2D**). In addition, the prediction of potential therapeutics by cBioPortal and L1000CDS[2] indicated that MAPK kinase (MEK) inhibitor (300 nM trametinib) may be effective. Notably, treatment with PDAC-conditioned medium stimulated the activation of extracellular signal-regulated kinases (ERK) 1 and 2, two major members of the MAPK family, in differentiated SH-SY5Y and Neuro-2a cells (**Figure 6A**, **6B**). **Figure 6B** showed the original Western blotting image at the default lowest contrast.

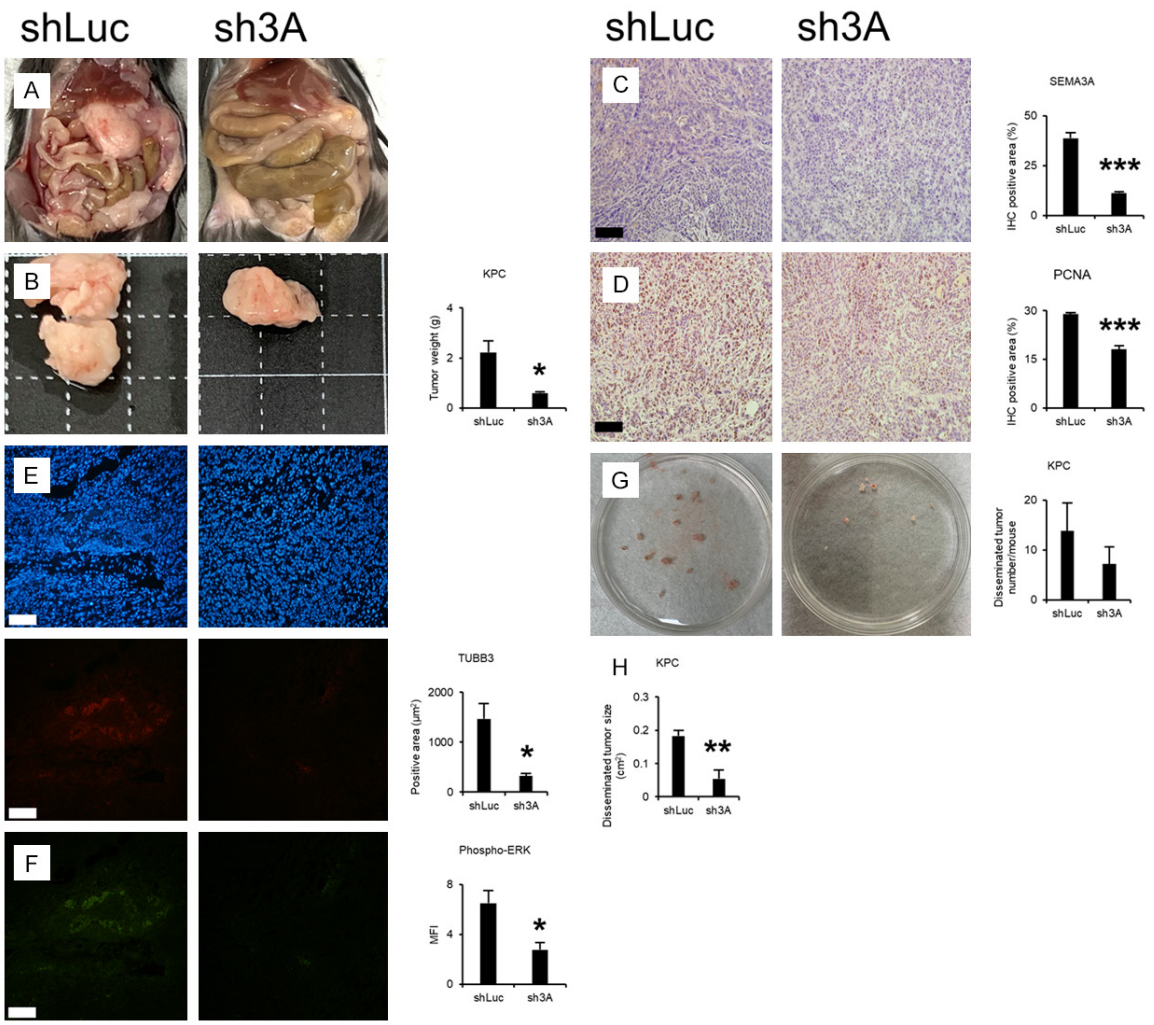

Figure 7. Inhibition of SEMA3A reduces pancreatic tumor growth, nerve innervation, and metastasis in an orthotopic mouse model. Control shLuc- or shSEMA3A-KPC cells (2×10^5) were orthotopically injected into the pancreas of C57BL/6 mice. Three weeks post-inoculation the mice were sacrificed and tissues were harvested. (A, B) Tumor size and expressions of (C) SEMA3A, (D) PCNA, (E) TUBB3, and (F) phospho-ERK were compared between control and SEMA3A-depleted groups. In addition, the (G) number and (H) size of the metastatic nodes found in the abdomen were compared. *, P<0.05; **, P<0.01; ***, P<0.001. Scale bar, 50 μm. MFI, mean fluorescence intensity. Error bars represent SEM.

Previous studies suggested that phospho-ERK-1 is less detectable in Neuro-2a cells [28-30], which is in line with our result. In addition, the FDA-approved MEK inhibitor trametinib at a clinical concentration (30 nM) significantly inhibited the PDAC-conditioned medium-enhanced migration of differentiated SH-SY5Y and Neuro-2a cells (**Figure 6C**, **6D**, Supplementary Figure 6A, 6D). Following trametinib administration, the neurite outgrowth triggered by PDAC-conditioned medium was suppressed (**Figure 6E**, **6F**, Supplementary Figure 6B, 6E), and neural recruitment in 3D culture was attenuated (**Figure 6G**, **6H**, Supplementary Figure 6C, 6F). These results demonstrated that the MEK inhibitor is a potential drug for inhibiting PDAC-induced neuronal alterations.

SEMA3A blockage displayed anti-tumor effect in orthotopic pancreatic cancer mouse model

To verify the oncogenic effect of PDAC-derived SEMA3A *in vivo*, we orthotopically injected control or SEMA3A-depleted KPC into the pancreas of C57BL/6 mice and sacrificed them three weeks post-inoculation. We observed that SEMA3A knockdown resulted in smaller tumors (**Figure 7A**, **7B**). Immunohistochemical an-

alysis confirmed a decrease in SEMA3A expression in tumors generated from SEMA3A-depleted KPC (**Figure 7C**). This was accompanied by reduced proliferation, as demonstrated by the decreased proliferating cell nuclear antigen (PCNA) staining (**Figure 7D**). Additionally, the recruitment of neuronal cells and ERK activation in neuronal cells in the SEMA3A-depleted tumors, as determined by TUBB3 and phospho-ERK levels, were decreased (**Figure 7E**, **7F**). Moreover, the number and size of disseminated tumors were reduced in the shSEMA3A group (**Figure 7G**, **7H**), suggesting that SEMA3A promoted tumor metastasis, perhaps via the enhancement of neuronal modulation.

Discussion

Under physiological conditions, SEMA3A participates in multiple biological functions in various cell types. The importance of SEMA3A in the spatial configuration of neurites and its dependence on multiple signaling pathways have been reviewed recently [31]. Daneshvar *et al.* demonstrated that SEMA3A colocalizes with S100B, an important factor produced by terminal Schwann cells, to regulate muscle regeneration after injury [32]. Under these conditions, SEMA3A may control neuronal innervation to promote functional recovery of muscles. Changes in SEMA3A and S100B levels were determined by the type and duration of injury, indicating an intimate crosstalk between these two factors *in vivo*. Chang *et al.* showed that the expression of SEMA3A was reduced in stem cells of the periodontal ligament under hypoxia. When hypoxia was inhibited by apigenin, a HIF1α inhibitor, this phenomenon was reversed [33]. In contrast, the addition of SEMA3A counteracted hypoxia-induced inhibition of periodontal ligament stem cell function. Therefore, SEMA3A may play a key role in maintaining the biological activity of these stem cells. Liu *et al.* reported that nickel stimulation induced SEMA3A expression in mouse skin cells, and silencing SEMA3A suppressed nickel-activated TNF-α release and MAPK activation [34]. In the mouse model, SEMA3A upregulation was observed at nickel-treated sites. In addition, the number of immune cells, including lymphocytes, macrophages, and dendritic cells, increased after nickel stimulation, a phenomenon not observed in SEMA3A conditional knockout mice. These results indicated the crucial role of SEMA3A in nickel-induced allergy. Therefore, SEMA3A is an important physiological signaling molecule.

Recent studies have demonstrated that SEMA3A participates in the tumorigenesis of various cancers. Lavi *et al.* found that SEMA3A expression was decreased in patients with multiple myeloma compared to that in healthy donors. In addition, modified furin-resistant SEMA3A decreased disease progression in a mouse model. Furthermore, cancer cell entry into the bone marrow was suppressed in the furin-resistant SEMA3A group, suggesting that SEMA3A may function as a tumor suppressor in multiple myeloma [35]. SEMA3A is increased in patients with glioblastoma and is associated with poor prognosis. The application of a SEMA3A therapeutic antibody suppressed tumorigenesis in a patient-derived xenograft model by reducing ERK phosphorylation [36]. In oropharyngeal carcinoma, SEMA3A was upregulated in tumor tissues and was associated with shorter survival, irrespective of the papillomavirus infection status [37]. Interestingly, SEMA3A plays a context-dependent role during tumorigenesis through its association with different germ layers [35-37], implying that SEMA3A may act as an oncogene or a tumor suppressor in human cancers in a context-dependent manner.

The pancreas is innervated by sympathetic, parasympathetic, and sensory nerves [38-40]. Sympathetic and parasympathetic nerves modulate the release of insulin and glucagon from endocrine cells, and digestive enzymes from exocrine cells, whereas sensory nerves mediate pain perception in pancreatic diseases. In human PDAC tissues, an increase in nerve innervation is generally observed, and this correlates with worse outcome [41]. However, the mechanisms by which PDAC cells promote the establishment of a nerve-rich tumor microenvironment remain to be elucidated. Several factors, including nerve growth factor (NGF), glial cell-derived neurotrophic factor (GDNF), chemokines, and cell adhesion molecules, have been implicated in this process [42]. For example, the expression of NGF and its receptors is upregulated in PDAC and associated with increased nerve innervation in tumors [43, 44]. In addition, the inhibition of NGF signaling effectively suppressed PDAC-induced neural

invasion [45]. SEMA3A is expressed in PDAC tumor tissues; however, the functional role of this signaling molecule in pancreatic tumorigenesis remains unknown. In this study, we provided *in vitro* and *in vivo* evidence that pancreatic cancer cells secrete SEMA3A to promote neuronal recruitment and enhance tumor growth and dissemination.

The possibility of using SEMA3A as a molecular target for therapy is under intensive investigation. Sang *et al.* found that SM-345431 (also known as vinaxanthone), a dual inhibitor of SEMA3A and phospholipase C, decreases the severity of renal diseases [46, 47]. In a doxorubicin-induced renal injury mouse model, SEMA3A expression was increased, and its inhibition by SM-345431 reduced podocyte apoptosis via JNK suppression. In another unilateral ureteral obstruction mouse model of renal fibrosis, SEMA3A was upregulated, and SM-345431 reduced renal fibrosis *in vivo* [47]. Yamazaki *et al.* found that SM-345431 preserved the corneal nerve density and tear secretion in a dry eye mouse model [48]. Hira *et al.* showed that SM-345431 improved the recovery of mice from stroke induced by middle cerebral artery occlusion [49]. In this study, we showed that MAPK activation was crucial for SEMA3A-induced neurite outgrowth and motility in neuronal cells and that the MEK inhibitor suppressed SEMA3A-induced neural recruitment during pancreatic tumorigenesis. In preclinical studies, MEK inhibitors reduced PDAC growth in a patient-derived xenograft model [50, 51]. However, no improvement in overall survival and progression-free survival was observed in 160 patients with PDAC treated with gemcitabine in combination with MEK inhibitors [52]. Notably, no patient selection was performed in this clinical trial. Recent studies have shown that PDAC patients harboring $KRAS^{G12C}$, $KRAS^{G12R}$, or BRAF mutations are more sensitive to MEK inhibitors [53-55]. Our results suggested another possible benefit of MEK inhibitors in suppressing the neural invasion and dissemination of SEMA3A-high PDAC.

In conclusion, we identified PDAC-derived SEMA3A as an important mediator that enhanced nerve innervation via PLXNA1, NRP2, and MAPK in neural cells in pancreatic tumors, and that the increased neuronal invasion implied accelerated PDAC metastasis.

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Disclosure of conflict of interest

None.

Abbreviations

PDAC, pancreatic ductal adenocarcinoma; SEMA3A, semaphorin 3A; PLXNA1, plexin A1; NRP2, neuropilin 2; MAPK, mitogen-activated protein kinase.

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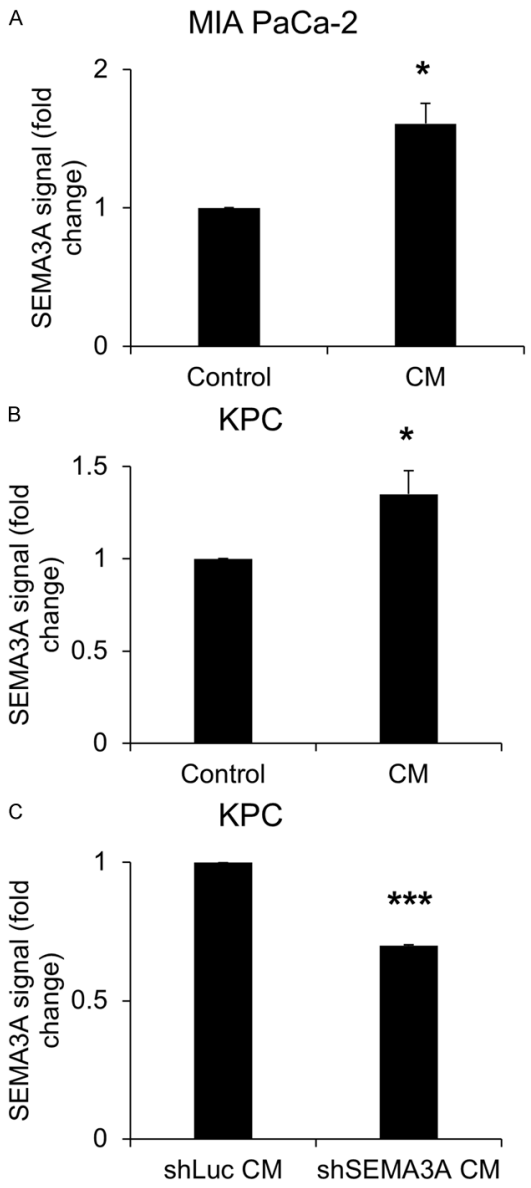
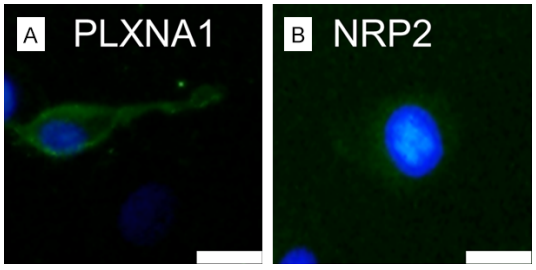
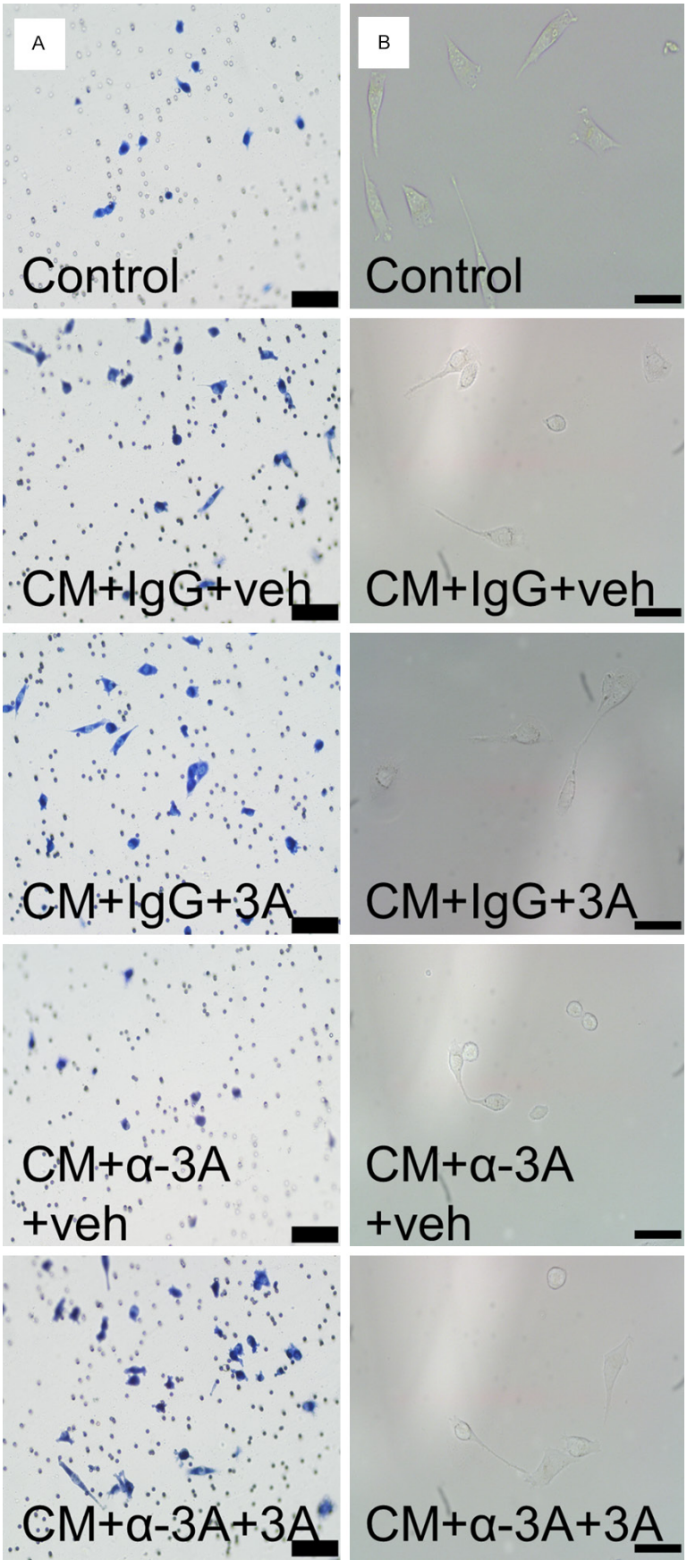


Supplementary Figure 1. SEMA3A enrichment in PDAC-conditioned medium. SEMA3A signal in Western blotting with the same amount of loading protein from each condition was compared as (A) control DMEM medium versus MIA PaCa-2-conditioned medium, (B) control RPMI medium versus KPC-conditioned medium, and (C) shLuc-KPC-conditioned medium versus shSEMA3A-KPC-conditioned medium. *, P<0.05; ***, P<0.001. Error bars represent SEM.



Supplementary Figure 2. PLXNA1 and NRP2 expressions in differentiated SH-SY5Y. In addition to flow cytometry, immunofluorescence was applied to confirm the membranous expressions of (A) PLXNA1 and (B) NRP2 in differentiated SH-SY5Y. Scale bar, 5 μm.


A
B
Control
Control
CM+IgG+veh
CM+IgG+veh
CM+IgG+3A
CM+IgG+3A
CM+α-3A
+veh
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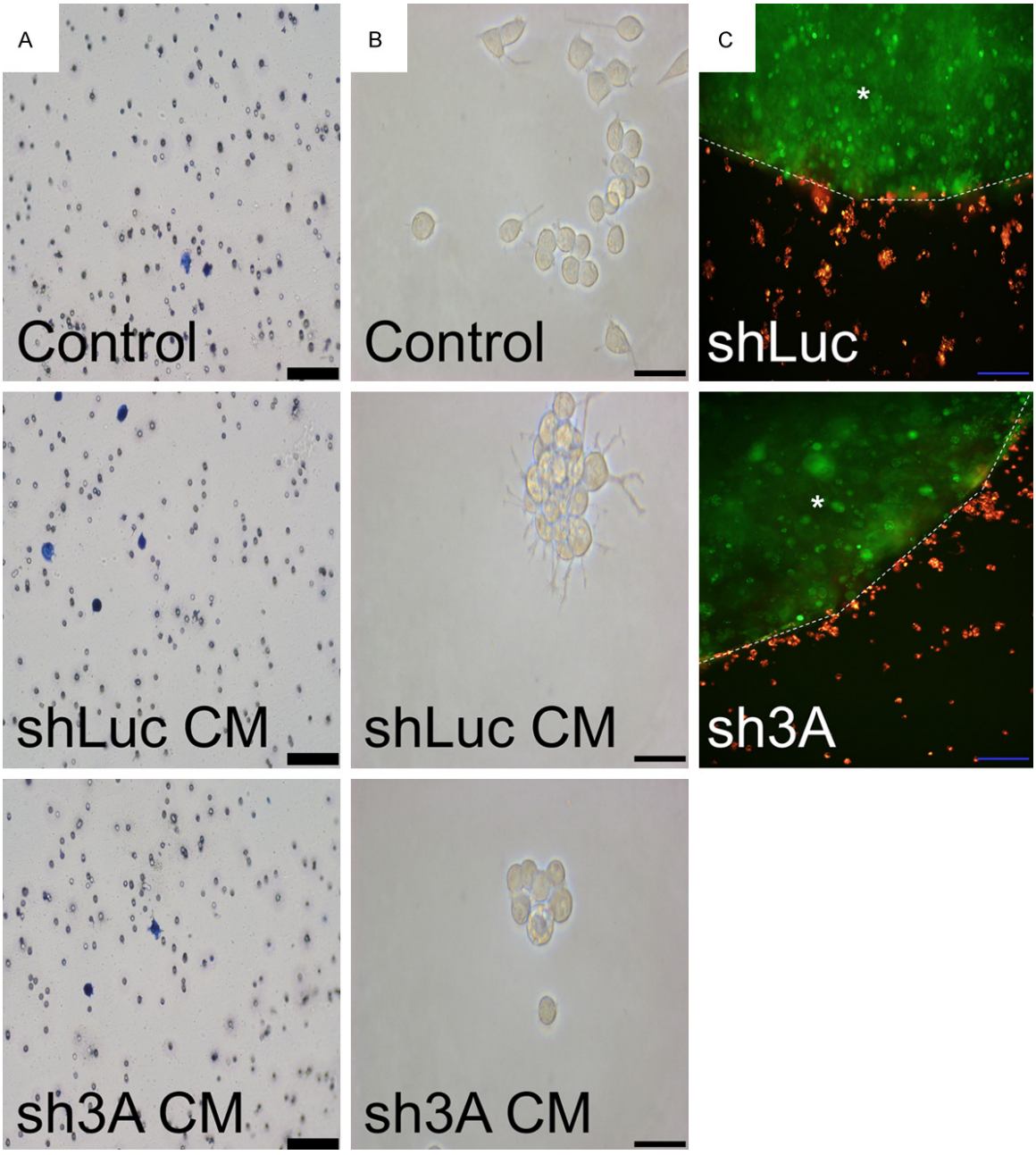
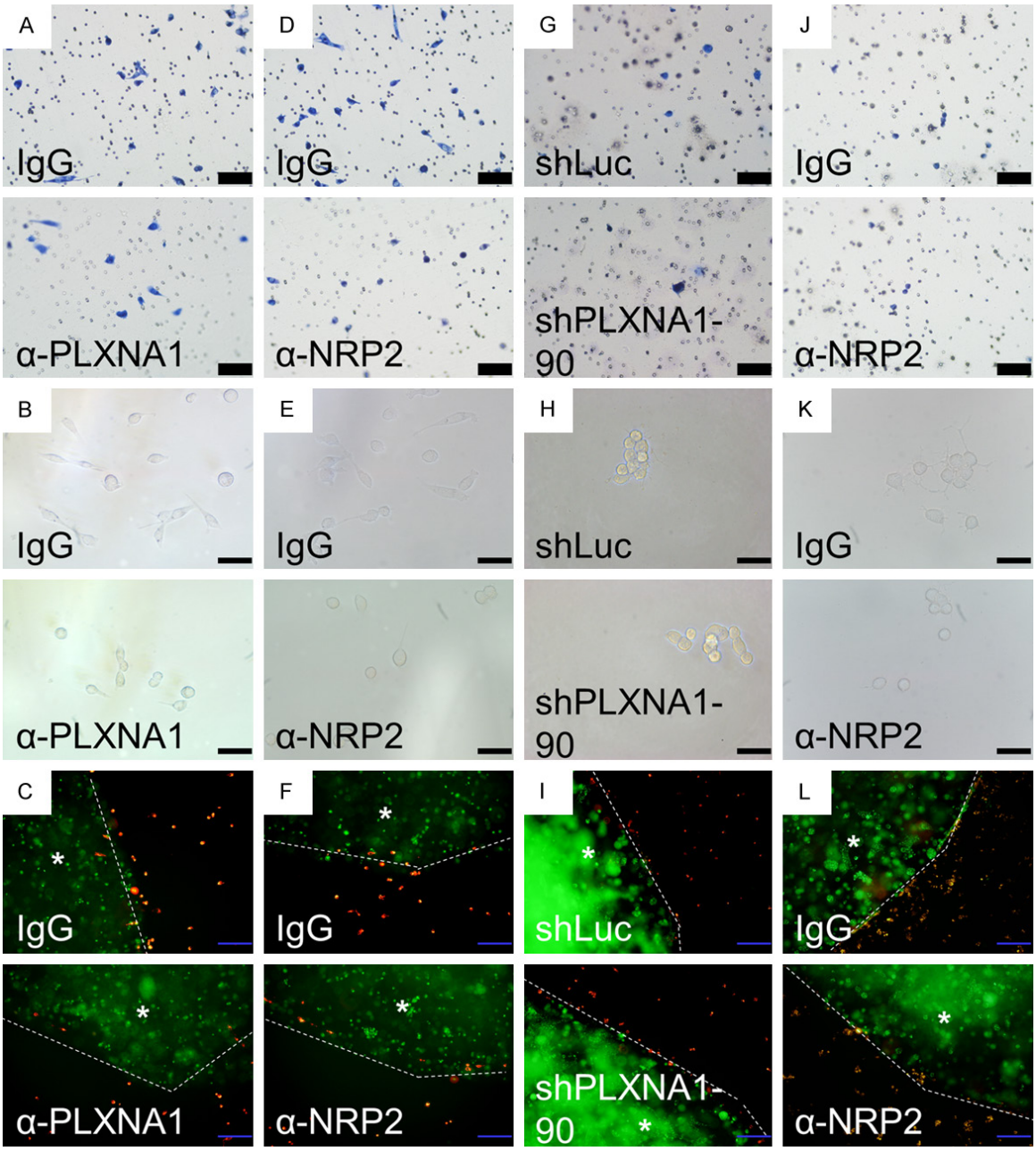
+veh
CM+α-3A+3A
CM+α-3A+3A

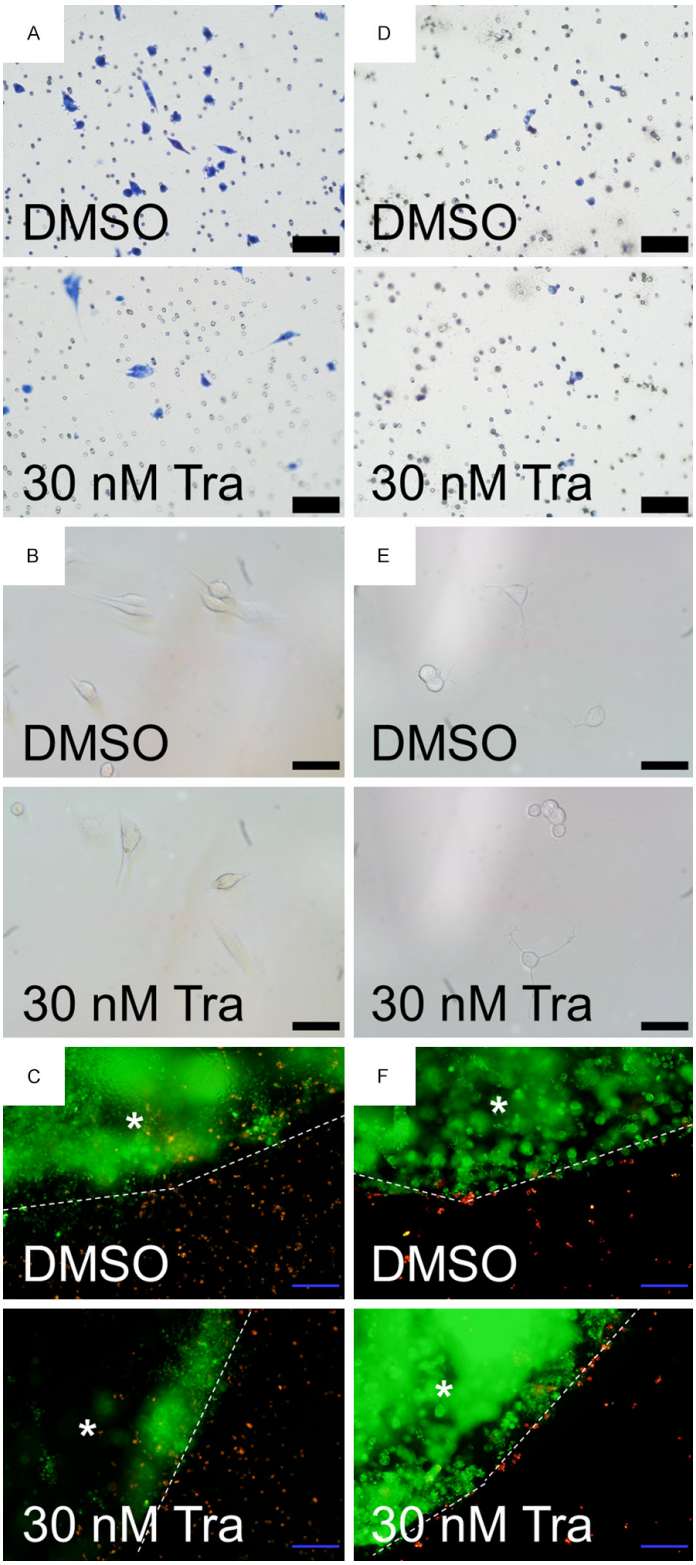
Supplementary Figure 3. Effect of MIA PaCa-2-expressed SEMA3A on migration and neurite outgrowth in differentiated SH-SY5Y. Representative images for MIA PaCa-2-expressed SEMA3A (in conditioned medium), with or without SEMA3A antibody or peptide, on (A) migration and (B) neurite outgrowth in differentiated SH-SY5Y. Scale bar for (A), 50 μm; scale bar for (B), 25 μm.



Supplementary Figure 4. Effect of KPC-expressed SEMA3A on neural modulations in differentiated Neuro-2a cells. Representative images for the effects of control medium, shLuc-KPC-conditioned medium, or shSEMA3A-KPC-conditioned medium on (A) migration, (B) neurite outgrowth and (C) 3D recruitment in differentiated Neuro-2a cells. In (C) KPC were labeled with the green fluorescence membrane dye, DiO, and differentiated Neuro-2a cells were labeled with red fluorescence membrane dye, DiI. Scale bar for (A), 50 μm; scale bar for (B), 25 μm; scale bar for (C), 100 μm.



Supplementary Figure 5. PLXNA1- and NRP2-modulated neural behaviors under SEMA3A stimulation. (A-F) Differentiated SH-SY5Y cells and (G-L) differentiated Neuro-2a cells were subjected to blockages of (A-C, G-I) PLXNA1 or (D-F, J-L) NRP2, and their effects on (A, D, G and J) migration, (B, E, H and K) neurite outgrowth, and (C, F, I and L) 3D recruitment were observed. In (C, F) MIA PaCa-2 cells were labeled with the green fluorescence membrane dye, DiO, and differentiated SH-SY5Y cells were labeled with the red fluorescence membrane dye, DiI. (I, L) KPC were labeled with the green fluorescence membrane dye, DiO, and differentiated Neuro-2a cells were labeled with the red fluorescence membrane dye, DiI. Scale bar for (A, D, G, J), 50 μm; scale bar for (B, E, H, K), 25 μm; scale bar for (C, F, I, L), 100 μm.



Supplementary Figure 6. MEKi-counteracted neural activation post SEMA3A stimulation. MEKi-counteracted neural activation post SEMA3A stimulation (with PDAC-conditioned medium) in terms of (A, D) migration, (B, E) neurite outgrowth, and (C, F) 3D recruitment in (A-C) differentiated SH-SY5Y and (D-F) differentiated Neuro-2a cells. (C) MIA PaCa-2 cells were labeled with the green fluorescence membrane dye, DiO, and differentiated SH-SY5Y cells were labeled with the red fluorescence membrane dye, DiI. (F) KPC were labeled with the green fluorescence membrane dye, DiO, and differentiated Neuro-2a cells were labeled with the red fluorescence membrane dye, DiI. Scale bar for (A, D), 50 μm; scale bar for (B, E), 25 μm; scale bar for (C, F), 100 μm.