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Neuroimmune semaphorin 4A in cancer angiogenesis and inflammation

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Abstract

Neuroimmune semaphorin 4A (Sema4A), a member of semaphorin family of transmembrane and secreted proteins, is an important regulator of neuronal and immune functions. In the nervous system, Sema4A primarily regulates the functional activity of neurons serving as an axon guidance molecule. In the immune system, Sema4A regulates immune cell activation and function granting a fine tuning of immune response. Recent studies have shown a dysregulation of Sema4A expression in several types of cancer such as hepatocellular carcinoma, colorectal and breast cancers. Cancers have been associated with abnormal angiogenesis. The function of Sema4A in angiogenesis and cancer is not defined. Recent studies have demonstrated Sema4A expression and function in endothelial cells. However, the results of these studies are controversial as they report either pro – or anti-angiogenic Sema4A effects depending on the experimental settings. In this mini-review, we discuss these findings as well as our data on Sema4A regulation of inflammation and angiogenesis, which both are important pathologic processes underlining tumorigenesis and tumor metastasis. Understanding the role of Sema4A in those processes may guide the development of improved therapeutic treatments for cancer.

Introduction

Angiogenesis is a complex physiologic process which is tightly controlled by several proteins such as VEGF, FGF, PDGF, angiopoietin-1 and -2, ephrin-B2, and others (1). Under physiological conditions the blood vessels in adults are already formed and rarely branch or sprout (1, 2). However, when a blood vessel is damaged, a complex repair process is being activated where several types of cells and signals coordinate the functions of endothelial and muscle cells involved in repair (1-3). There is a number of important steps in angiogenesis, namely: 1. protease production which includes matrix metalloproteases (MMPs), a desintegrin and metalloprotease domain (ADAMs), a desintegrin and metalloprotease domain with trombospondin motif (ADAMTs), cysteine proteases such as cathepsins, and serine proteases such as tissue plasminogen activator (tPA); 2. endothelial cell migration and proliferation; 3. vascular tube formation; 4. connections of newly formed tubes; 5. synthesis of a new basement membrane; and 6. incorporation of pericytes and smooth muscle cells (1, 3). In addition to pro-angiogenic stimuli named above, several angiogenesis inhibitors, such as angiostatin, endostatin, vasostatin, TIMP, platelet factor-4, osteopontin and others , halt angiogenesis by stopping a formation of new blood vessels or even promoting a blood vessel removal (1, 3). Those opposing stimuli tightly regulate vascular homeostasis.

Angiogenesis is also a vitally important process for tumor development and progression (3). In order to grow, tumor needs oxygen and nutrients which are being supplied by new blood vessels. These vessels can be formed by an influence of angiogenic factors made by tumor cells themselves or by other cells in tumor surrounding which are being stimulated by tumor cells to generate such factors (3). The inhibitors of angiogenesis have been long considered as clinically important cancer-fighting agents. Most FDA approved and currently used in clinical practice

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anti-cancer therapeutics with anti-angiogenic effects are based on either inhibition or blockade of VEGF and its receptors. These include Axitinib (tyrosine kinase inhibitor selective to VEGF-R1, - R2, -R3) for renal cell carcinoma (4), Bevacizumab (anti-VEGF humanized Ab) for several types of cancer, including lung, colorectal and cervical cancers (5), Sunitinib (triple-blocker, Abs to VEGF-R2, PDGF-Rb and c-kit) for gastrointestinal stromal tumor, pancreatic and renal cancers (6), and several others (1, 3). However, more recent studies have shown some alarming side-effects in patients being treated with these drugs (7-9). These undesirable consequences include an acute aortic dissection in a patient with liver tumors after a six round of sunitinib (7) or a jaw necrosis after axitinib treatment of a patient with renal cell carcinoma (9). In some cases, the use of bevacizumab in patients with prostate cancer led to a confirmed anti-tumoral activity without a concominant improvement in survival (8). Moreover, targeting just one pathway in angiogenesis, f.e. VEGF, could be insufficient to disrupt cancer angiogenesis as other VEGF-unrelated pathways would stay intact. In addition to that, VEGF itself acting in tissues induces the expression of other molecules which can express either pro- or anti-angiogenic qualities thus promoting or compensating its direct effects. As an example of the above scenario, we previously have shown that the lung tissue VEGF expression induced a local inflammatory response characterized, in part, by a formation of new blood vessels, lung resident cell activation, and their upregulated expression of several neuroimmune proteins (10, 11). Among those upregulated proteins in lung DC was a member of Class IV semaphorin subfamily Sema4A. Thus, VEGF-induced lung tissue alterations can be, at least in part, Sema4A-mediated. However, if Sema4A acts as anti- or pro-angiogenic factor, remains to be determined as currently available publications examining its function came to the opposite conclusions.

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The previously published data have shown that Sema4A is preferentially expressed on DC and B cells in the immune system (12, 13). Sema4A has seven currently known receptors, Tim-2 (T cell, Ig domain, mucin domain-2) (14, 15), NRP-1 (Neuropilin -1) (16), Plexin B1 (17), Plexin B2 (17), Plexin B3 (17), Plexin D1 (18), and most recently cloned ILT-4 (19). The initial studies have suggested that Plexin molecules are expressed on non-immune cells, whereas Tim-2 and ILT-4 expression was highly restricted to activated mouse and human CD4+ T cells, correspondingly (14, 15, 19), and NRP-1 expression was detected on mouse Treg cells (16). However, later we and others have demonstrated Plexin B1 and/or D1 expression in the immune system, particularly on DC (10, 20), T cells (21) and Treg cells (our unpublished observations). As Sema4A regulates the immune response to different antigens such as allergens (19, 22, 23), infectious agents (14, 21, 24), and tissue-derived factors in autoimmunity (15, 25-28), it is feasible to conclude that it plays a significant role in anti-tumor immunity, what have not been assessed as yet.

Sema4A-receptor pathways form a complex system of intracellular and extracellular signals which regulate different physiological and pathological tissue processes. For example, Sema4A regulates a proper retina formation (29), correct guidance of hippocampal neurons (30), angiogenesis (18, 31), and adaptive immune response (12-14, 16, 19). On the other hand, the Sema4A pathways are dysregulated in different diseases such as retinal degenerative diseases (retinitis pigmentosa type 35 and cone-rod dystrophy type 10) (29), allergy (10, 14, 19, 22, 23), infectious (14, 32) and autoimmune diseases (14, 26, 28), and certain types of cancer (16, 33). The individual impact of each Sema4A-receptor pair in disease pathogenesis and/or progression needs to be dissected separately and then a whole picture of Sema4A impact could be envisioned. This could be done, first of all, *in vitro* by applying the receptor knock-out or

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specific receptor blocking techniques in cells of interest, and *in vivo* using individual Sema4A receptor-deficient mice and their inter-crosses in the experimental models of certain diseases.

Sema4A and anti-angiogenic therapy in cancer

Tumor progression and metastasis require a growth in local tumor angiogenesis where new blood vessels are being formed in order to supply cancer cells with growth nutrients. Tumor cells themselves and tumor-associated stroma secrete angiogenesis-promoting factors such as angiopoietin-2, follistatin, G-CSF, HGF, IL-8, leptin, PDGF-BB, PECAM-1, VEGF, MMP-1, -2, -3, -7, -9, -10, -12 and -13 (34). It has been shown that VEGF mRNA expression was mainly targeted to primary colorectal tumor cells whereas angiopoietin-2 and HGF mRNA expression was targeted to tumor-adjacent stromal cells (34). Interestingly enough, recent studies have shown that many tissue-specific tumors can grow alongside the blood vessels without a formation of new ones (35) thus abating effects of anti-angiogenic therapies in such tumors. Nevertheless, several angiogenic factors such as VEGF-A, VEGF-B, angiopoietin -1, osteopontin, fibroblast growth factor, MMPs and others currently serve as targets in cancer treatment with FDA-approved inhibitors which all are being used in conjunction with chemotherapy (1, 3).

The main target for angiogenesis-based cancer therapy is VEGF (36). Currently, there are several small molecule inhibitors and monoclonal Abs targeting VEGF-A pathway with their side-effects analyzed and reported (36). Bevacizumab (Avastin, recombinant humanized monoclonal Ab to VEGF) is currently being used for treatment of metastatic colorectal cancer,

non-sqamous non-small cell lung cancer, glioblastoma, metastatic renal cell carcinoma, metastatic or recurrent cervical cancer (in combination with chemotherapy), platinum-resistant recurrent epithelial ovarian, fallopian tube, or primary peritoneal cancer in combination with chemotherapy. Cabozantinib (Cabometyx, Cometriq, a small molecule inhibitor of the tyrosine kinases c-Met and VEGFR2) and Pazopanib (Votrient, an inhibitor of three VEGF receptors) are being used in therapy of advanced renal carcinoma. This type of cancer is also being treated by Sorafenib (Nexavar, a small inhibitor of several tyrosine protein kinases, including VEGFR) which also showed therapeutic effects toward un-resectable advanced hepatocellular carcinoma and progressive differentiated radioactive iodine-resistant thyroid carcinoma. More recently developed Zif-Aflibercept (Eylea, Zaltrap, VEGF-Trap, a hybrid fusion protein of VEGFR-1 and VEGFR-2 binding domains) is being used for metastatic colorectal cancer that is resistant to an oxaliplatin-containing regimen.

The key side effect of anti-VEGF therapy includes an interference with normal angiogenesis process where the wound healing is highly disrupted being either delayed or incomplete. Indeed, when patients with metastatic colorectal carcinoma were treated with bevacizumab (Avastin, anti-VEGF-A mAb) they showed an impaired wound healing and postoperative wound complications (37). The reported side effects for Bevacizumab include sensory neuropathy, hypertension, fatigue, and neutropenia (36). Neutropenia and hypertension were also reported for Ramucirumab use in addition to an increased risk of pneumonia. Thus, hypertension is the most known side-effect of VEGF inhibition as the ability of VEGF to decrease a blood pressure is well-documented. Other reported problems include an increased risk of arterial thromboembolic events caused by a disturbed regenerative capacity of endothelial cells (36).

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As Sema4A is downstream of VEGF-induced signaling in the lung tissues (10, 11), its effects on angiogenesis and tumor progression were of our and other's significant research interest. The role of Sema4A in angiogenesis has been previously evaluated *in vitro* and *in vivo* using either Sema4A-Fc fusion protein, recombinant human Sema4A, or/and Sema4A$^{-/-}$ mice (18, 38). In developing mouse embryos, a co-expression of Sema4A and Plexin D1 in the intersomitic blood vessels was detected what suggested the potential role of this ligand-receptor pair in vascular formation (18). To evaluate such effect, the authors studied HUVEC migration in transwell chamber using VEGF alone or in combination with several semaphorins. They have found that VEGF-induced cell migration was suppressed by Sema4A-Fc. Furthermore, Sema4A-FC and Sema3E-Fc, but not Sema4D-Fc, inhibited VEGF-induced tubular structure formation by HUVEC in the *in vitro* angiogenesis assay. Interestingly enough, Sema4D showed the opposite to Sema4A effect on HUVEC although these two semaphorins share Plexin D1 receptor (39, 40). This suggests the potential competition for the receptor binding between two semaphorins. However, it has been reported previously that the binding sites on Plexin D1 are different for each individual semaphorin ligand (40). Nevertheless, there is a possibility that the binding of one Sema4 molecule can induce Plexin D1 modification leading to another Sema4 molecule binding site to be hidden or inaccessible. Another study, however, has shown that a pro-angiogenic effect of Sema4D on endothelial cells is mediated by a different plexin family member, namely Plexin B1 (41), which is also a binding partner for Sema4A (38, 39). The signaling events occurring in endothelial cells under Sema4D exposure were dependent on a COOH-terminal PDZ-binding motif of Plexin B1, which binds two guanine nucleotide exchange factors for the small GTPase Rho, PDZ-RhoGEF and LARG, and were mediated by activation of

Rho-initiated pathways. The signaling events under Sema4A exposure have never been examined in details.

The *in vivo* effect of rSema4A on vascularization in chick embryos has proven its indispensable role in blood vessel formation (18). Chorioallantoic membrane (CAM) assays were used to evaluate such effect where gelatin sponges were inserted into chick embryos for three days. When examined thereafter, pre-treated with rSema4A sponges contained lower numbers of preformed blood vessels as compared to isotype control-pretreated sponges thus again proving the inhibitory role of Sema4A in angiogenesis. Pre-treatment of HUVEC with siRNA specific for individual Plexin family members, such as Plexin B1, D1, and A1, before rSema4A exposure determined Plexin D1 as its functional receptor on endothelial cells which mediates its anti-angiogenic activity (18). All of the discussed above results define Sema4A as a potent anti-angiogenic molecule and pave the way to its evaluation in cancer immunotherapy. However, a recent research by Meda and associates (31) has shown a pro-angiogenic role of Sema4A ligating Plexin D1 on macrophages and stimulating their migration, VEGF-A production and VEGF-R1 expression. Moreover, this Sema4A-VEGF-A pathway has been shown to be involved in macrophage activation and recruitment during inflammatory processes such as the experimental models of peritonitis and cardiac inflammation. Thus, considering the discussed here opposite effects of Sema4A on endothelial cells and macrophages, the identification of additional mechanisms of its action should be an important focus of future research aimed to develop of Sema4A-based therapeutic strategies to target cancer angiogenesis.

We previously reported that VEGF expression in lungs induces potent angiogenesis and edema formation (11). Staining of mouse lung tissues with Lycopersicon esculentum lectin has demonstrated a normal arrangement of blood vessels in the tracheas and intrapulmonary bronchi

of wild-type mice. These blood vessels formed cascades with capillaries crossing between arterioles and venules. In contrast, we observed multiple endothelial sprouts, mostly arising from the venules, in VEGF transgenic mice as early as on day 3 of transgene expression induction. The vascular density (the percent of the airway covered with vessels) reached its maximum on day 7 and remained elevated for at least a month thereafter. The newly formed blood vessels were larger than the capillaries of the VEGF-unaffected control airways. The endothelial cells of these vessels were thin, had occasional fenestrations, and were enveloped by pericyte processes and basement membranes. Besides angiogenesis, we studied the effect of lung VEGF expression on local immune cells. We have shown that lung DC were activated by VEGF-A and upregulated of Sema4A and Plexin D1 expression (10). Thus, for DC and macrophages, there is a positive feedback loop between VEGF-A and Sema4A which bind the corresponding receptors, Plexin D1 and VEGF-R1, and mediate this loop's signaling pathways. However, as it has been shown previously and stated in the Introduction section here, Sema4A uses different receptors on different cell types to regulate their activation and function. For example, it uses Neuropilin-1 to mediate mouse Treg cell's phenotype stabilization and function (16), Plexin B1 to induce such effect in human Treg cells (our unpublished observations), Tim-2 to co-stimulate mouse CD4+ T cells into Th1 phenotype (14, 15), Plexin B2 for an optimal differentiation of CD8+ T cells (21), and ILT-4 to co-stimulate human CD4+ T cells into Th2 phenotype in vitro (19). We did not detect Plexin B1 or Tim-2 expression on lung endothelial cells in mouse tissues either in steady-state or inflammatory conditions (10). However, no such study was performed for human lung tissues.

We analyzed the expression of Sema4A and Plexin D1 on human lung cancer tissue arrays using immunohistochemistry with corresponding Abs (Fig. 1). We have found that blood

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vessels in cancer-associated inflammatory sights expressed both molecules (marked with red arrows on Fig. 1). Thus, it is quite possible that Sema4A exerts its pro- or anti-angiogenic activity on pulmonary endothelial cells through Plexin D1 receptor. This statement, however, requires an extended focused testing.

We were interested to define Sema4A effect on VEGF-induced lung vascularization and inflammation. The main question was if Sema4A further deepens VEGF-induced lung pathologies acting as a pro-angiogenic and pro-inflammatory factor similarly to described earlier effects of fatty acid binding protein 4 (FABP4, adipocyte-FABP, aP2) (42), or it is being produced as a compensatory protective molecule aimed to diminish or dampen VEGF-mediated tissue damages.

Sema4A and anti-inflammatory therapy in cancer

We previously reported that lung VEGF-A expression induced local conventional DC (cDC) maturation and direction toward DC2 phenotype (11). These VEGF-stimulated cDC upregulated Sema4A expression (10). To assess the role of Sema4A in allergen-induced lung inflammation, we used OVA model of asthma in Sema4A$^{-/-}$ mice where we found an exaggerated lung allergic response as compared to WT mice (22). This suggests that Sema4A is a suppressive molecule for the *in vivo* Th2 response. We next crossed VEGF tg mice (11) with Sema4A$^{-/-}$ mice (14) and have found that this semaphorin deficiency led to an increased inflammatory cell infiltration in the lungs of VEGF tg mice when transgene expression is turned on by doxycycline-containing water (Fig. 2). As we have shown previously, lung bronchial epithelial expression of VEGF transgene lead to an asthma-like phenotype with inflammation,

parenchymal remodeling, increased vascularization, edema formation, mucous cell and myocyte hyperplasia and airway hyperreactivity (11). The observed lung tissue inflammatory response and vascularization in our VEGF tg/Sema4A$^{-/-}$ mice were more pronounced than those found in transgenic mice alone (Fig. 2). In addition, we observed higher local levels of Th2 cytokine IL-13 in VEGF tg mice with Sema4A deficiency (Fig. 2 B). In fact, IL-13 was a signature Th2 cytokine, in contrast to unchanged levels of IL-4 and IL-5, upregulated in the Sema4A$^{-/-}$ lungs and spleens after allergen exposure (22). Based on the well-established role of VEGF in angiogenesis and tumor pathogenesis, our preliminary data for the mouse models of experimental asthma in Sema4A$^{-/-}$ and VEGF tg/Sema4A$^{-/-}$ mice, and the discussed above publications on the Sema4A inhibitory role in VEGF-induced angiogenesis, we suggest that Sema4A may act as a tumor suppressor interfering at least with three critical pathways in tumor development, progression, and metastasis: 1. immune cell activation and function; 2, inflammation, and 3. angiogenesis.

Based on all of the above, Sema4A is a suppressive molecule for both, allergen-induced and VEGF-mediated lung tissue responses what makes it an attractive target for allergic disease immunotherapy. Indeed, when recombinant Sema4A protein was introduced into the allergic murine lungs, it significantly suppressed all features of an inflammatory Th2 response such as lung eosinophilia, mucus hypersecretion, proinflammatory and Th2 cytokine production (22). We and others have shown that Sema4A affects Treg cells *in vitro* and *in vivo* (16, 22) as Treg cell local lung number decreases under inflammation by Sema4A deficiency (22). Moreover, Sema4A acting through NRP-1 in mice (16) and Plexin B1 in humans (our unpublished observation) stabilizes Treg cell number and function. Therefore, Sema4A serves as a downregulatory molecule for allergic diseases suppressing allergen-dependent and -independent

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responses, in part, by upregulating Treg cell response. However, a recent article by Lu and colleagues (19) has demonstrated a costimulatory effect of Sema4A for T cell, especially Th2 cell, activation and function. Further studies are warranted to elucidate Sema4A-ILT-4 roles in different diseases including cancer.

As a translational part of our research, we obtained human lung cancer tissue arrays (Z7020065, BioChain) and assessed them for Sema4A and corresponding receptor expression using commercially available Abs. These tissue arrays constituted of: 1. adenocarcinoma, stages I to III, 2. bronchioalveolar carcinoma, 3. papillary carcinoma, 4.squamous cell carcinoma, and 4. small cell lung cancer. We have found a low to absent Sema4A expression in bronchioalveolar, papillary, and small cell lung cancer but the cancer stage-dependent increased levels of Sema4A in adenocarcinoma and squamous cell carcinoma (Fig. 3). This observation supports a previous notion that the effects of different semaphorins on cancer progression are broad and context-dependent (46). For example, Sema3A has been shown to display either pro- or anti-malignant role in different types of cancer (46). Given the many potential impacts of Sema4A on tumors, its detailed investigation will be beneficial for basic and clinical cancer research.

Conclusion

The presented and discussed here data show that Sema4A function in tumorigenesis and metastasis most probably is a protective and VEGF-opposing one. Previously published data suggested that semaphorin molecules, specifically Sema3B and Sema3F, could act as tumor suppressors as they bind antagonistically to NRP-1 and NRP-2, which are also co-receptors for

VEGF, and thus inhibit angiogenesis (43). More detailed examination of these semaphorins actions had supported a suppressive role for Sema3B in lung and renal cancers (44) and for Sema3F in oral squamous cell carcinoma (45). Nevertheless, the mechanisms of Sema4A action in cancer need to be carefully dissected in details. The only currently proven association of Sema4A mutation with Familial colorectal cancer type X (FCCTX) was reported in 2014 (33, 34). Surprisingly, no new data for other types of cancer has been shown since then. Considering multiple receptors translating Sema4A effects into different cells, the individual and/or dominating receptor function needs to be detected and analyzed first. Therefore, additional functions of Sema4A are likely to emerge in the near future.

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Figure 1

Sema4A and Plexin D1 are expressed on endothelial cells in cancer-related lung inflammation

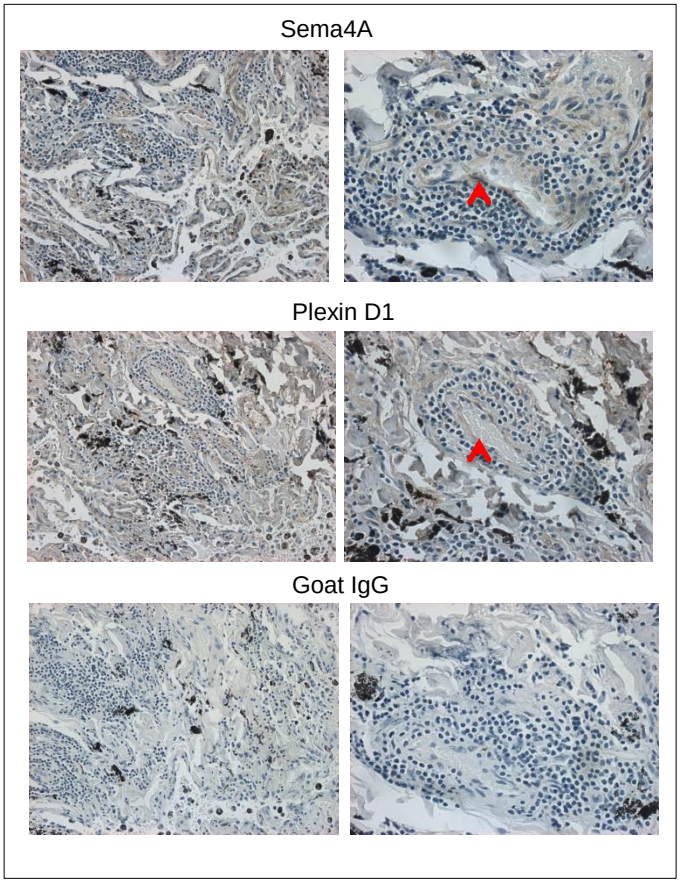

Figure 2. Immunohistochemistry of human lung cancer-adjoined tissue on BioChain arrays was performed as a four-steps assay . Primary Ab for Sema4A (sc-46258) and Plexin D1 (E-13) were obtained from Santa Cruz Biotech. Biotynilated rabbit anti-goat IgG was used as a secondary Ab. Panels on the left represent x20 magnification, panels on the right show a magnification of x40.

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Figure 2

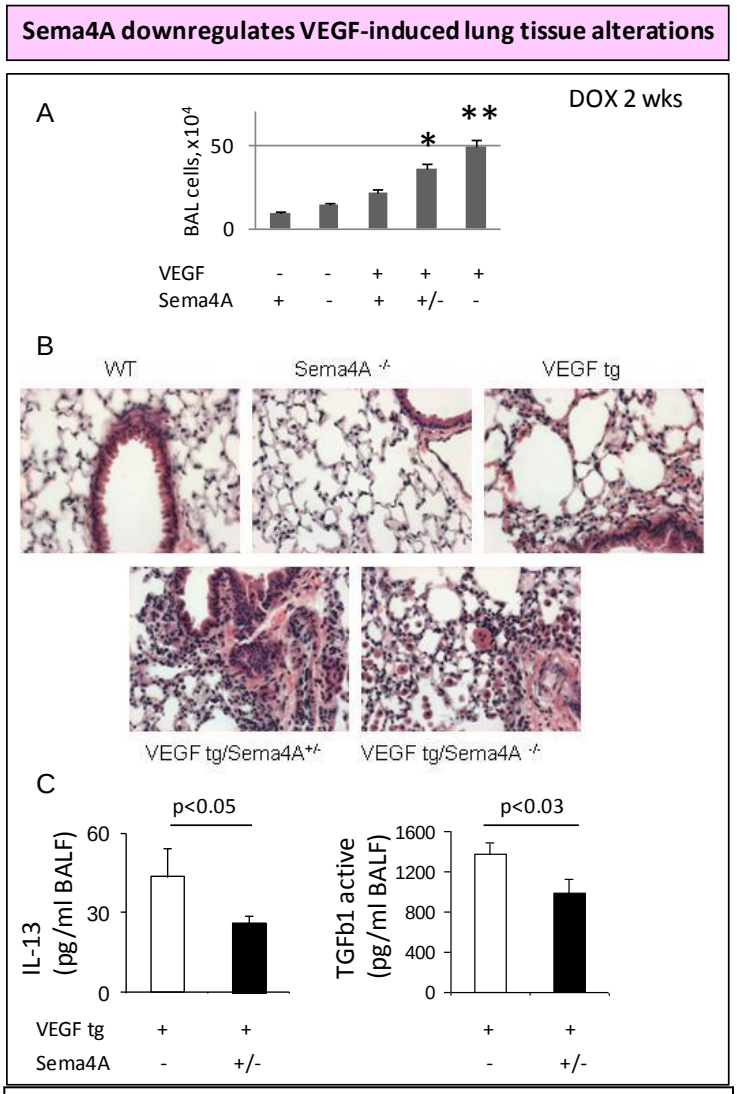

Figure 2. Increase in lung pathology in VEGF tg mice with Sema4A deficiency. (A) Broncho-alveolar lavage (BAL) fluids were harvested and the total cell counts were performed with hemacytometer (33). *p<0.03, VEGF tg/Sema4A$^{+/-}$ mice vs VEGF tg mice; **p<0.05, VEGF tg/Sema4A$^{-/-}$ mice vs VEGF tg mice. (B) Lung sections were stained with H&E (magnification 40x). (C) IL-13 and TGFβ1 levels in BAL fluids were measured by ELISA (Quantikine).

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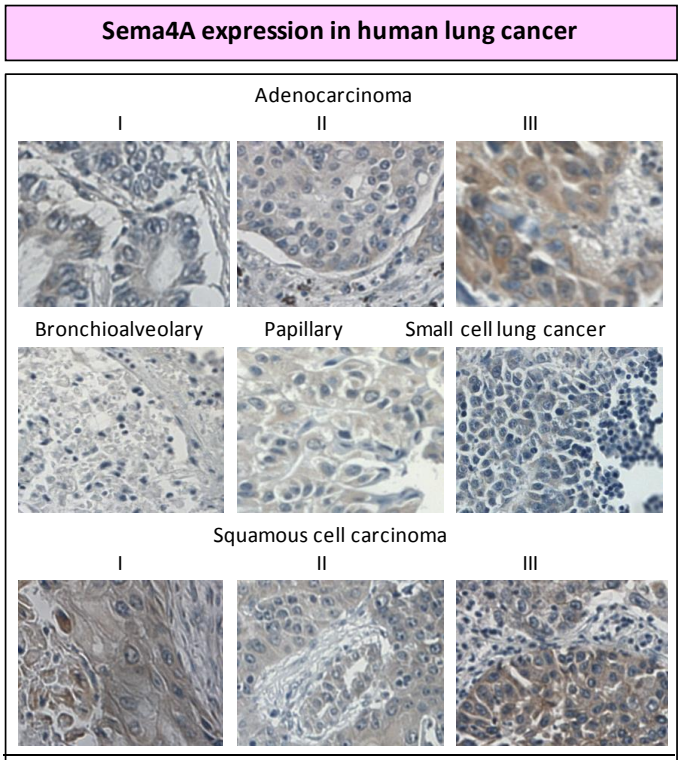

Figure 2. Immunohistochemistry of human lung cancerous tissue arrays (BioChain) was visualized via a four-step staining procedure using anti-Sema4A (sc-46258, Santa Cruz) as primary Ab, biotinylated rabbit anti-goat IgG (sc-2774) as secondary Ab, streptavidin-HRP (Abcam) as detection enzyme, and DAB peroxidase substrate kit (Vector) for visualization (100x). Staining specificity was compared with goat IgG control Ab stain (not shown).