Tumor cell-derived angiopoietin-like protein ANGPTL2 is a critical driver of metastasis

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Abstract

Strategies to inhibit metastasis have been mainly unsuccessful in part due to insufficient mechanistic understanding. Here we report evidence of critical role for the angiopoietin-like protein ANGPTL2 in metastatic progression. In mice, Angptl2 has been implicated in inflammatory carcinogenesis but it has not been studied in human tumors. In lung cancer patients, elevated levels of ANGPTL2 expression in tumor cells within the primary tumor were associated with a reduction in the period of disease-free survival after surgical resection. Transcription factors NFATc, ATF2 and c-Jun upregulated in aggressive tumor cells promoted increased Angptl2 expression. Most notably, tumor-cell derived ANGPTL2 increased in vitro motility and invasion in an autocrine/paracrine manner, conferring an aggressive metastatic tumor phenotype. In xenograft mouse models, tumor-cell derived ANGPTL2 accelerated metastasis and shortened survival, whereas attenuating ANGPTL2 expression in tumor cells blunted metastasis and extended survival. Overall, our findings demonstrated that tumor-cell derived ANGPTL2 drives metastasis and provided an initial proof of concept for blockade of its action as a strategy to antagonize the metastatic process.
Introduction

Cancer is a leading cause of death and accounts for 7.6 million deaths (approximately 13% of all deaths) worldwide (1). Although both diagnosis and therapeutic modalities used to treat cancer have remarkably improved, tumor metastasis still represents a major cause of cancer mortality (2, 3). Therefore, identification of mechanisms underlying metastasis is essential to understand the pathophysiology of this lethal condition and identify novel therapeutic targets.

Inflammation plays key roles at various stages of tumor development, including initiation, growth, invasion, and metastasis (4). Recently, we found that angiopoietin-like protein 2 (ANGPTL2) increases inflammatory carcinogenesis in a chemically-induced skin squamous cell carcinoma (SCC) mouse model through enhanced susceptibility to “pre-neoplastic change” and “malignant conversion” (5). In addition, we also reported that ANGPTL2 expression in tumor cells is highly correlated with the frequency of tumor cell metastasis to distant organs and lymph nodes through increased tumor angiogenesis and tumor cell epithelial-to-mesenchymal transitions (EMT) (5). However, it has been obscure whether ANGPTL2 contributes to human cancer pathogenesis.

The nuclear factor of activated T-cell (NFATc) consists of five members (NFATc1–c4 and NFAT5). Among these factors, NFATc1–c4 function in tumor cell development and metastasis (6, 7). For example, NFATc1 and NFATc3 contribute to the pathogenesis of melanoma and pancreatic cancer (8, 9), NFATc2 plays in breast cancer cell migration and invasion (10), and NFATc4 promotes breast cancer cell growth (11).
In the present study, we investigated the role of ANGPTL2 in human tumor cells, and found that lung cancer patients showing high ANGPTL2 expression in cells within the primary tumor sites showed poor prognosis in terms of disease-free survival. Furthermore, we found that Angptl2 expression in tumor cells is induced by NFATc. Tumor cell-derived ANGPTL2 enhanced tumor cell motility and invasive capacity and increased tumor angiogenesis. Tumor cell-derived ANGPTL2 also accelerated metastasis and shortened survival periods in tumor cell-implanted mouse models. By contrast, decreasing ANGPTL2 levels in tumor cells attenuated metastasis and prolonged survival periods. Collectively, our findings provide strong evidence that tumor cell-derived ANGPTL2 worsens clinical prognosis and suggest that blocking ANGPTL2 could represent a novel therapeutic strategy to inhibit tumor metastasis.
Materials and Methods

Quantitation of ANGPTL2 protein by ELISA

ANGPTL2 concentrations in tissue lysates or in culture medium from tumor cells were estimated by an ANGPTL2 Assay kit (IBL), as described (12, 13). For tissue lysates proteins were extracted from 2 mg of tumor or non-tumor tissue and dissolved in 10 ml lysis buffer (300 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, and 1mM EDTA).

Immunohistochemistry and in situ hybridization

Immunohistochemical and in situ hybridization analyses were performed as described (12, 14). Antibodies for CD44 (Abcam), paxillin (BD Bioscience) and rhodamine-phalloidin (Molecular Probes) were purchased. We also used an anti-ANGPTL2 antibody that we generated (12, 14).

Cell lines and cell culture

The human lung cancer cell lines NCI-H460 and NCI-H460-LNM35 cells, as previously described (15), were provided by Dr. Takashi Takahashi (Nagoya University, Japan). H460 and LNM35 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The human breast adenocarcinoma cell lines T47D, MDA-MB453 and MDA-MB231 were purchased from the American Type Culture Collection (ATCC) that carries out cell line characterizations and passaged in our laboratory for fewer than 6 months after receipt. Detailed information regarding establishment of stable cell lines is provided in Supplementary Methods.
Luciferase assay

H460 cells were incubated for 48 hours after co-transfection with indicated expression and reporter plasmids and phRL-TK vector (Promega). Luciferase activities were determined using a Dual-Luciferase Reporter Assay System (Promega). For some experiments, transfected cells were incubated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) or with 10 ng/ml PMA plus 1 μM ionomycin (PMA/Ion) for 24 hours. Details relevant to expression and reporter plasmids are provided in Supplementary Methods.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed as described (12). Oligonucleotide primers are listed in Supplementary Table S1.

Immunoblot analysis

Immunoblot analysis was performed as described (5). Antibodies used were against NFATc2 (BD Bioscience), ATF2 (N-96, Santa Cruz), c-Jun (D, Santa Cruz) and ANGPTL2 (13).

Cell invasion assay

Cell invasiveness was estimated using a 96-well BME Cell Invasion Assay kit (Trevigen) according to the manufacturer’s protocol. Fluorescence was measured using a Fluoroskan Ascent fluorometer (Thermo Labsystems).

Time-lapse microscopy and FRET imaging
Cell migration was monitored by time-lapse microscopy using an Olympus IX-81 inverted microscope with a 20x objective lens. Phase contrast images were collected with CoolSNAP-HQ (Roper Scientific) at 3 min intervals. A series of time-lapse images was converted to video format using MetaMorph 6.1 software. Cell motility was quantified using MetaMorph 6.1 software. FRET imaging was performed as described (13).

**Human studies**

All studies were approved by the Ethics Committee of Kumamoto University, Keio University, or the Tokyo Metropolitan Cancer and Infectious Diseases Center of Komagome Hospital. Written informed consent was obtained from each subject. Patient information is provided in Supplementary Methods.

**Animal studies**

All experiments were performed according to guidelines of Institutional Animal Committee of Kumamoto University. Details relevant to the mouse model are provided in Supplementary Methods.

**Statistics**

The Kaplan-Meier log-rank test was used to analyze survival data of mice and patients using JMP7 software (SAS Institute). Data presented as means ± standard deviation (SD) or means ± standard error of mean (SEM) were analyzed using Student’s t-test or analysis of variance (ANOVA). A $P$ value of less than 0.05 was considered significant.
Results

ANGPTL2 levels within primary tumor sites correlate with poor disease-free survival

To examine whether ANGPTL2 is expressed in human tumor tissues, we compared ANGPTL2 protein levels in lung tissues extracted from primary tumor sites in non-small-cell lung cancer (NSCLC) patients to those from peripheral non-tumor lung tissue using an enzyme-linked immunosorbent assay (ELISA). ANGPTL2 protein levels in primary tumor sites were significantly greater than those seen in non-tumor lung tissue (Fig. 1A). Given the variation in ANGPTL2 expression in tumor tissue (Fig. 1A), we examined ANGPTL2 expression in lung tumor tissues by immunohistochemistry. Accordingly, we observed wide variation in the proportion of cells within a tumor that express ANGPTL2 (Fig. 1B, Supplementary Fig. S1). In situ hybridization analysis (12) confirmed that tumor cells express Angptl2 mRNA (Fig. 1C), indicating that tumor cells are the likely source of ANGPTL2 protein. In contrast to the variation in the proportion of ANGPTL2-expressing tumor cells within the primary tumor site, we observed high and homogeneous expression of ANGPTL2 in tumor cells within metastasized tumor sites, including 3 brain tissues and 5 lymph nodes taken from 8 lung cancer patients at autopsy (Supplementary Fig. S2), suggesting that ANGPTL2-positive tumor cells exhibit high metastatic capacity. We next divided NSCLC patients into two groups based on the percentage of ANGPTL2-positive tumor cells within the primary tumor site: the high group was defined as showing greater than 20% of ANGPTL2-positive tumor cells and the low group showed fewer than 20%. Patients in the high group showed a shortened period of disease-free survival after surgery compared to the low group based on two
NFATc induces Angptl2 expression in lung cancer cells

To investigate tumor cell regulation of Angptl2 expression, we constructed luciferase reporter plasmids containing an Angptl2 regulatory region that we identified from the human NSCLC cell line NCI-H460 (H460) (Supplementary Fig. S4A). An F4 construct containing nucleotides –168 to +98 relative to the transcription start site of human Angptl2 showed high reporter activity in H460 cells, while activity of an F5 construct (containing –21 to +98) showed significantly decreased activity, indicating that F4 contains elements regulate Angptl2 expression in this context (Supplementary Fig. S4A). In that region we identified potential binding sites for ATF/CREB, NF-κB, and NFAT (top in Fig. 2A). To investigate whether these factors affect Angptl2 expression, we transfected H460 cells with the F4 construct plus expression vectors encoding CREB, ATF2, ATF3, NF-κB, or a constitutively active form of NFATc (CA-NFATc). The CA-NFATc vector increased Angptl2 reporter activity relative to a pcDNA3.1 control plasmid, whereas the other factors had no effect (Fig. 2A). Treatment of cells with a combination of PMA and ionomycin (PMA/Ion) stimulates calcium/calcineurin-dependent NFATc activation and induces its nuclear translocation (6, 16, 17). PMA/Ion treatment of H460 cells significantly increased Angptl2 mRNA expression compared with controls, while treatment with the calcineurin inhibitor cyclosporin A (CsA) significantly blocked the PMA/Ion effect (Fig. 2B). These results suggest that NFATc functions in Angptl2 expression in lung cancer cells.
ATF2 and c-Jun enhance NFATc-dependent Angptl2 expression in lung cancer cells

It has been reported that AP-1 components, c-Jun or c-Fos, form a stable heterodimer with ATF2, ATF3, or ATF4, and bind to the ATF/CREB site (18, 19). NFATc and AP-1 heterodimers form a transcriptional complex that synergistically activates target genes (20-22). We found that reporter activity of F4 constructs containing a mutant ATF/CREB site was markedly decreased relative to wildtype F4 constructs (Supplementary Fig. S4B), suggesting that ATF/CREB site is required for Angptl2 expression. We observed that CA-NFATc-dependent induction of Angptl2 reporter activity was also significantly suppressed in F4 constructs bearing a mutant compared to a wildtype ATF/CREB site (Fig. 2C). Furthermore, PMA/Ion treatment increased F4 reporter activity, an effect significantly decreased when we used F4 constructs harboring a mutant ATF/CREB site (Fig. 2D). Overall, it could be possible that NFATc forms a complex with AP-1 heterodimers that enhances NFATc-dependent induction of Angptl2 expression through the ATF/CREB site.

To investigate whether ATF family proteins may bind to ATF/CREB site on the Angptl2 promoter region, we performed an electrophoretic mobilityshift assay (EMSA) by using radiolabeled synthetic oligoDNA probe containing DNA sequences of ATF/CREB site on the Angptl2 promoter region (Supplementary Fig. S4C-E). PMA/Ion treatment induced formation of a specific DNA-protein complex (arrowhead in Supplementary Fig. S4C-E), which was inhibited in the presence of unlabeled ATF/CREB competitors (Supplementary Fig. S4C) or by preincubation with an anti-ATF2 antibody (lane 4 in Supplementary Fig. S4D and E). These findings suggest that ATF2 binds to the Angptl2 promoter.
ATF/CREB site. Preincubation with anti-c-Jun antibody reduced DNA-protein complex formation and promoted emergence of a supershifted band (lane 5, arrow in Supplementary Fig. S4E). Preincubation with an anti-NFATc antibody or addition of unlabeled competitor containing the consensus NFATc binding site partially reduced levels of the DNA-protein complex (Supplementary Fig. S4C and lane 6 in Supplementary Fig. S4E). Preincubation with both anti-c-Jun and anti-ATF2 antibodies (lane 7 in Supplementary Fig. S4E) or both anti-c-Jun and anti-NFATc antibodies (lane 8 in Supplementary Fig. S4E) markedly reduced levels of the DNA-protein complex and the supershifted band. To assay transcription, we co-transfected the F4 construct into H460 cells with expression vectors encoding CA-NFATc, ATF2, c-Jun, c-Fos, or a combination of these vectors and measured luciferase activity (Fig. 2E). Expression of the ATF2/c-Jun combination enhanced Angptl2 reporter activity relative to controls. CA-NFATc-dependent induction of reporter activity was augmented by c-Jun co-expression. Angptl2 reporter activity was also markedly increased by co-expression of CA-NFATc/ATF2/c-Jun. However, CA-NFATc/ATF2-dependent or CA-NFATc/c-Jun-dependent induction of reporter activity was not enhanced by c-Fos co-expression. Furthermore, CA-NFATc/ATF2/c-Jun-dependent reporter activity was significantly suppressed when we employed an F4 construct containing a mutant ATF/CREB site (Fig. 2F). To determine whether endogenous NFATc, ATF2 and c-Jun bind to the human Angptl2 promoter, we employed chromatin immunoprecipitation (ChIP) assays in H460 cells (Supplementary Fig. S5A). PMA/Ion treatment of cells resulted in increased NFATc and ATF2 binding to the human Angptl2 promoter region. Previous studies report that c-Jun is highly expressed and activated in
NSCLC cell lines (23, 24). c-Jun binding to the human Angptl2 promoter region was observed in PMA/Ion-untreated H460 cells, whereas that binding level was unchanged in PMA/Ion-treated cells (Supplementary Fig. S5A). Taken together, these observations demonstrate that NFATc plays an important role in Angptl2 induction and that an ATF2/c-Jun complex likely enhances NFATc-dependent Angptl2 induction through the ATF/CREB site.

**NFATc2 knockdown decreases Angptl2 expression in tumor cells**

The NCI-H460-LNM35 (LNM35) line was established by *in vivo* selection as a highly metastatic subline of the human large cell carcinoma of the lung, NCI-H460 (15). We found that that severe immuno-deficient Jak3-deficient NOD-SCID (NOJ) mice (25) subcutaneously injected with LNM35 cells showed shortened survival periods due to high frequency metastasis to lung and lymph nodes compared to mice injected with H460 cells (Supplementary Fig. S6). Interestingly, Angptl2 mRNA is more abundant in cultured LNM35 cells compared to H460 cells (Fig. 3A). We found that NFATc1, NFATc2, and NFATc3 increased Angptl2 promoter activity in the H460 cells (Supplementary Fig. S5B and C). NFATc2 expression in LNM35 cells was significantly higher than that seen in H460 cells, while expression of NFATc1, NFATc3 and NFATc4 was equivalent in both lines (Fig. 3B). NFATc2 protein levels were also markedly increased in LNM35 cells, but ATF2 and c-Jun protein levels were equivalent (Fig. 3C). To examine whether NFATc2 is required for Angptl2 expression in LNM35 cells, we established two LNM35 cell lines each stably expressing a different miRNAi expression vector designed to knockdown NFATc2 (LNM35/miNFATc2-1 and LNM35/miNFATc2-2) (Fig. 3D). Angptl2
expression levels in both lines were significantly decreased compared with control cells and comparable to those seen in H460 cells (Fig. 3E). Invasive activity of LNM35/miNFATc2 cells was decreased compared with controls (Supplementary Fig. S7). These observations suggest that NFATc2 is important for Angptl2 expression and acquisition of tumor invasivity.

**Angptl2-expressing tumor cells contribute to increased tumor metastasis**

As indicated in Fig.1 and Supplementary Fig. S2, ANGPTL2-positive tumor cells exhibit higher metastatic capacity than ANGPTL2-negative cells. We therefore generated two independent H460 lines constitutively expressing Angptl2 (H460/Angptl2-1 and -2) and an H460 line expressing a control vector (H460/Cont) (Fig. 4A). Although no differences were observed in *in vitro* growth among all three lines (Supplementary Fig. S8A), *in vitro* invasive capacity of the two H460/Angptl2 lines was significantly greater than that seen in H460/Cont (Fig. 4B). We next analyzed tumor development after subcutaneous injection of either the three lines into mice. Despite equivalence in *in vivo* tumor growth observed among the two H460/Angptl2 lines and H460/Cont (Supplementary Fig. S8B and C), lung metastasis was more severe in mice bearing H460/Angptl2-1 tumors than in H460/Cont controls (Fig. 4C and D). Tumor angiogenesis was also increased in mice bearing H460/Angptl2-1 compared to H460/Cont cells (Supplementary Fig. S8D and E), a finding consistent with our recent report (5). Finally, the survival period of mice bearing H460/Angptl2 tumors was significantly shortened compared to H460/Cont mice (Fig. 4E).

**Angptl2 increases tumor cell motility by activating Rac**
Time-lapse imaging indicated that H460/Angptl2-1 cells are significantly more motile than are H460/Cont cells (Fig. 5A and B, Supplementary Movie S1 and 2). Histological analysis indicated that H460/Angptl2-1 cells exhibit a polarized morphology associated with motility, as evidenced by formation of actin-rich lamellipodial protrusions and assembly of paxillin-marked focal complexes at the leading edge, whereas H460/Cont cells displayed non-polarized morphology with uniform distribution of F-actin and paxillin at the cell periphery (Fig. 5C and D). Since activation of Rac1, a Rho family GTPase, at a cell’s leading edge is required to form lamellipodial protrusions required for cell migration (26), we asked whether ANGPTL2 activates Rac1 using a FRET probe to visualize Rac1 activation in H460/Angptl2-1 and H460/Cont cells. Rac1 was potently activated at the leading edge of migrating H460/Angptl2-1 cells, whereas no polarized Rac1 activation was observed in H460/Cont cells (Fig. 5E, Supplementary Movie S3 and 4). Collectively, these data suggest that tumor cell-derived ANGPTL2 promotes actin reorganization and accelerates cell migration via Rac1 in an autocrine and/or paracrine manner.

**Angptl2 knockdown suppresses tumor metastasis**

Because LNM35 cells express Angptl2 abundantly and exhibit high metastatic capacity due to enhanced polarized morphology compared to H460 cells (Fig. 3, Supplementary Fig. S6 and 9), we examined the effect of Angptl2 knockdown in LNM35 cells. Of four candidate miR RNAi expression vectors (miAngptl2-a, -b, -c, and -d) recommended by the Invitrogen BLOCK-iT™ miR RNAi system, miAngptl2-b significantly decreased Angptl2 expression compared to a LNM35 line harboring LacZ knockdown (LNM35/miLacZ) used as a control
(Supplementary Fig. S10). We therefore generated two independent Angptl2 knockdown lines: LNM35/miAngptl2-b1 and -b2 (Supplementary Fig. S11A). In vitro invasive ability was significantly decreased in both lines compared to LNM35/miLacZ cells, whereas no differences in in vitro cell growth were observed among knockdown and control lines (Supplementary Fig. S11B and C). In addition, both knockdown lines displayed less-polarized morphology and more uniform distribution of F-actin and paxillin compared with LNM35/miLacZ cells (Supplementary Fig. S12). Mice bearing both Angptl2 knockdown lines showed no significant difference in tumor growth but exhibited markedly decreased lung metastases compared to mice bearing LNM35/miLacZ cells (Supplementary Fig. S11D-G). Survival times were also extended in mice injected with knockdown versus control lines (Supplementary Fig. S11H). These results suggest that tumor cell-derived ANGPTL2 increases metastasis and that decreased ANGPTL2 expression in tumor cells can attenuate that effect.

**Tumor cell-derived ANGPTL2 enhances lung metastasis in mice bearing breast cancer cells**

To investigate whether tumor cell-derived ANGPTL2 enhances metastasis in other cancer types, we examined ANGPTL2 expression and function in the human breast cancer lines T47D, MDA-MB453 and MDA-MB231. Only MDA-MB231, which shows an aggressive metastatic phenotype (27), abundantly expressed and secreted ANGPTL2 (Fig. 6A and B). MDA-MB231 cells also expressed NFATc2, ATF2 and c-Jun (Fig. 6A), while MDA-MB231 cells stably expressing NFATc2 RNAi (MB231/miNFATc2) showed significantly decreased Angptl2 mRNA and protein secretion compared to control
MB231/miLacZ cells (Fig. 6C, Supplementary Fig. S13A and B). We established two independent MDA-MB231 lines expressing miR RNAi (miAngptl2-b) to knockdown Angptl2 (MB231/miAngptl2-b1 and -2) and a control LacZ RNAi line (MB231/miLacZ). In knockdown lines cellular Angptl2 mRNA levels and levels of ANGPTL2 protein in the culture medium were significantly decreased compared to those seen in controls (Supplementary Fig. S13C). All three lines showed similar in vitro growth (Supplementary Fig. S13D), whereas in vitro invasive capacity was significantly decreased in knockdown compared to control cells (Supplementary Fig. S13E).

Next, we performed in vivo xenograft tumor experiments by implanting various lines of MDA-MB231 cells into the mouse mammary fat pad. For imaging purposes, we induced a luciferase expression vector into MB231/miAngptl2-b1 and -2 and control MB231/miLacZ cells to create MB231/miAngptl2-b1/luc, MB231/miAngptl2-b2/luc, and MB231/miLacZ/luc, respectively. No difference in in vivo tumor growth was seen among the three lines (Fig. 6D, Supplementary Fig. S13F). By contrast, lung metastasis was observed in mice bearing MB231/miLacZ/luc 5 weeks after tumor implantation, whereas fewer metastatic sites at the same time point was observed in mice bearing MB231/miAngptl2-b1/luc. To see equivalent lung metastasis after tumor implantation in mice bearing MB231/miAngptl2-b1/luc required eight weeks (Fig. 6D). Immunohistochemistry with an anti-CD44 antibody, which detects MDA-MB231 cells (27), showed statistically significant decreases in lung colonization of MB231/miAngptl2-b1 compared to MB231/miLacZ cells (Fig. 6E and F). Moreover, decreased tumor angiogenesis was observed in mice bearing MB231/miAngptl2-b1 compared to MB231/miLacZ cells (Supplementary Fig.
S14). Mice bearing the two MB231/miAngptl2 lines also showed prolonged survival periods compared with controls (Fig. 6G), suggesting that tumor cell-derived ANGPTL2 accelerates tumor metastasis in breast as well as in lung cancer.
Discussion

Here, we show that tumor cells expressing ANGPTL2 exhibit high metastatic potential through acquisition of invasive and high cell motility phenotypes in an autocrine/paracrine manner. We also demonstrated that NFATc, ATF2 and c-Jun induce Angptl2 expression, providing a mechanism for tumor cell ANGPTL2 induction. These findings are consistent with previous reports showing that activation of ATF/CREB family proteins and/or the calcineurin/NFATc pathway occurs in aggressively advanced tumors (6, 19, 28, 29).

We have reported that obese adipose tissue-related ER stress increases ANGPTL2 secretion or expression in adipocytes (13). ER stress is easily induced by stresses such as hypoxia, oxidative stress, hypoglycemia, and viral infection, all commonly observed in primary tumor microenvironment (30). We found Angptl2 mRNA levels in tumor cells were significantly increased under hypoxia and undernutrition (Supplementary Fig. S15A). In addition, increased ANGPTL2 expression was detected in tumor cells in hypoxic regions (Supplementary Fig. S15B), suggesting that tumor microenvironmental factors, such as hypoxia and/or undernutrition, induce ANGPTL2 expression in tumor cells. Cytoplasmic calcium concentrations increase due to ER stress-dependent calcium release from the ER (31) and activate the serine/threonine phosphatase calcineurin, which in turn dephosphorylates NFATc proteins and triggers their nuclear accumulation (32). NFATc function has been extensively studied in the immune system, but there is increased interest in NFATc activity in cancer (6). We speculate that tumor cell-autonomous responses to the microenvironment, such as activation of the ER stress/calcineurin/NFATc pathway and/or
ATF/CREB family proteins, induce Angptl2 expression in tumor cells, resulting in acquisition of aggressively metastatic tumor phenotypes.

Interestingly, a recent report suggests that ANGPTL2 might function in tumor refractoriness to anti-VEGF therapy (33). On the other hand, VEGF reportedly activates an inhibitor of calcineurin/NFATc signaling, namely, the Down syndrome critical region gene 1 (DSCR1) (34, 35). Taken together with these two reports, our findings suggest that in tumors refractory to anti-VEGF treatment, suppression of VEGF signaling may inactivate DSCR1 and thereby activate NFATc, resulting in increased ANGPTL2 expression and ANGPTL2-dependent tumor progression. Overall, we propose that the tumor microenvironment activates NFATc and ATF/CREB family proteins in tumor cells, resulting in ANGPTL2 induction and subsequent tumor metastasis.

Based on the results of EMSA and ChIP assays, we suggested that NFATc, ATF2, and c-Jun form a complex and bind to the Angptl2 promoter region in PMA/Iron-treated H460 cells. CA-NFATc-dependent induction of the Angptl2 reporter activity was augmented by c-Jun co-expression, and the augmentation was significantly enhanced by ATF2 co-expression. In contrast, ATF2 alone did not induce the Angptl2 reporter activity. Therefore, we consider that ATF2 would be important to form a complex with NFATc and c-Jun to induce the Angptl2 reporter activity.

We previously reported that ANGPTL2 increases angiogenesis through Rac activation in endothelial cells (13). Consistently, here we found that tumor cell-derived ANGPTL2 increases tumor angiogenesis. Interestingly, we also found that ANGPTL2 directly accelerates tumor cell motility through Rac activation in tumor cells. We observed that EMT occurs prominently in
Angptl2-expressing human lung cancer cells (Supplementary Fig. S16 and 17) as well as in a chemically-induced mouse SCC expressing Angptl2 (5). Two types of tumor cell movements have been described: a Rac-dependent mesenchymal mode and a Rho-dependent amoeboid mode (36), suggesting that ANGPTL2 induces cell motility in the mesenchymal mode. Since the EMT in tumor cells decreases intercellular adhesion, cell motility is enhanced, resulting in tumor invasion and metastasis. Thus, ANGPTL2 might be a key factor in increasing cell motility in tumor cells with mesenchymal characteristics. Overall, we demonstrate that tumor cell-derived ANGPTL2 accelerates tumor metastasis through increasing tumor cell migration in an autocrine/paracrine manner in addition to enhancing tumor angiogenesis, as described here and reported previously (5) (Supplementary Fig. S18). Based on these findings we propose that ANGPTL2 regulates tumor metastasis in human lung and breast cancer. These studies could form the basis of new therapeutic strategies to antagonize tumor metastasis.
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Figure Legends

Figure 1. ANGPTL2 expression in tumor cells correlates with poor lung cancer prognosis. A, ANGPTL2 protein levels in lung tumor tissues (n = 38) and corresponding peripheral non-tumor tissues (n = 9). B, ANGPTL2 immunostaining within the primary tumor. Groups from NSCLC patients were defined as ANGPTL2 high and low, respectively. Scale bars, 100 μm. C, Representative images of Angptl2 mRNA and protein in serial tumor tissue sections from primary NSCLC. Scale bars, 100 μm. D-E, cohort of probability of disease-free survival (D) and over-all survival (E) with ANGPTL2 high (n = 66) and low (n = 48) groups (P < 0.01 and P = 0.09 by log-rank test). *P < 0.05

Figure 2. NFATc, ATF2 and c-Jun induce Angptl2 expression. A, Top: Schematic diagram of the F4 construct and locations of putative transcription factor binding sites. The white circle, gray circles and white box indicate putative ATF/CREB site, NF-κB sites, and NFAT sites, respectively. Luc, luciferase. Bottom: Comparison of relative luciferase activity among H460 cells co-transfected with the F4 construct plus CREB, ATF2, ATF3, NF-κB (p65), or CA-NFATc expression plasmids. Reporter activity of cells co-transfected with pcDNA3.1 was set at 1. B, Relative Angptl2 mRNA levels in H460 cells treated with PMA and ionomycin (PMA/Ion) with or without cyclosporin A (CsA). Levels in untreated cells (controls) were set at 1. C, Top: Schematic diagram of wildtype and mutant F4 constructs. Bottom: Comparison of relative luciferase activity among H460 cells co-transfected with wildtype or mutant F4 constructs plus pcDNA3.1 or CA-NFATc expression plasmids. Reporter activity of cells co-transfected with wildtype F4 and pcDNA3.1 was set at 1. D, Comparison of
relative luciferase activity among H460 cells transfected with wildtype or mutant F4 constructs and treated with PMA/Ion. Reporter activity of cells transfected with the wildtype F4 construct but not treated with PMA/Ion was set at 1. E, Comparison of relative luciferase activity among H460 cells co-transfected with F4 plus ATF2, c-Jun, CA-NFAT, or c-Fos expression plasmids or with a combination thereof. Reporter activity of cells cotransfected with pcDNA3.1 was set at 1. F. Comparison of relative luciferase activity among H460 cells co-transfected with wildtype or mutant F4 constructs plus indicated expression plasmids (pcDNA3.1 or a CA-NFAT/ATF-2/c-Jun combination). Reporter activity of cells co-transfected with wildtype F4 plus pcDNA3.1 was set at 1. All experiments were performed at least three times. Error bars show SEM. *$P < 0.05$, **$P < 0.01$. n.s., no statistical difference.

Figure 3. NFATc2 knockdown decreases Angptl2 expression. A, Comparison of relative Angptl2 mRNA levels between H460 and LNM35 cells. Data from H460 was set at 1. B, Comparison of relative NFATc mRNA levels between H460 and LNM35 cells. Data from H460 was set at 1. C, Left: Representative image of immunoblotting analysis of NFATc2, ATF2, and c-Jun protein levels in H460 and LNM35 cells. Hsc70 served as control. Right: Quantitative protein levels of NFATc2, ATF2, and c-Jun relative to Hsc70. Data from H460 was set at 1, respectively. D, Representative image of immunoblotting of NFATc2 protein levels in indicated cells. E, Comparison of Angptl2 mRNA levels among indicated cells. Data from LNM35/miLacZ was set to 100%. All experiments were performed more than three times. Error bars show SEM. *$P < 0.05$, **$P < 0.01$. n.s., no statistical difference.
Figure 4. Increased Angptl2 expression in tumor cells enhances tumor metastasis. A, Relative Angptl2 mRNA levels among indicated cells. Data from H460/Cont was set at 1. B, Relative invasive capacity of indicated cells. Data from H460/Cont was set at 1. C, Representative H.E.-stained images of tumor metastasis (arrows) to lung 4 weeks after injection with H460/Cont (top) or H460/Angptl2-1 (bottom) cells. Scale bar, 200 μm. D, Quantitative analysis of (C) for severity of metastasis (n = 12). Relative number (left) and area (right) of tumor metastases in lung tissue among indicated cells. Data from H460/Cont was set at 1. E, Kaplan-Meier survival curves of mice bearing tumors derived from H460/Cont (n = 10), H460/Angptl2-1 (n = 10), and H460/Angptl2-2 (n = 7) cells. All experiments were performed more than three times. Error bars show SD. *P < 0.05, **P < 0.01.

Figure 5. Angptl2 expression positively correlates with cell motility. A, Migration tracks of 20 H460/Cont (left) and H460/Angptl2-1 (right) cells monitored for 5 hours. B, Quantitative comparison of the extent of movement of individual tumor cells. C, Representative fluorescent immunostaining images for F-actin (left) and paxillin (middle) of H460/Cont (top) and H460/Angptl2-1 (bottom) cells. Arrowheads indicate the leading edge. Scale bar, 20 μm. D, Quantitative analysis of polarized cells. E, FRET imaging of Rac activity. Arrowheads indicate site of activated Rac.

Figure 6. Angptl2 knockdown in breast cancer cells reduces metastatic capacity. A, Representative image of immunoblotting of ANGPTL2, NFATc2,
ATF2, and c-Jun protein levels in indicated cells. B, Comparison of ANGPTL2 levels in culture medium of indicated cells. n.d., not detected. C, Comparison of relative Angptl2 mRNA levels in indicated cells. Data from MB231/miLacZ was set at 1. D, Representative bioluminescence images of mice bearing MB231/miLacZ/luc or MB231/miAngptl2-b1/luc cells. Images were taken at indicated time points after xenografting (n = 12). E, Representative microscopic images of tumor cells metastasized to lung, as recognized by anti-CD44 immunostaining, 5 (left) and 8 (right) weeks after implantation with MB231/miLacZ (top) or MB231/miAngptl2-b1 (bottom) cells. Scale bar, 500 μm. F, Quantitative analysis of (E) for severity of metastasis (n = 10). Relative area of tumor metastases in lung among the three cell lines at 5 (left) and 8 (right) weeks is shown. Data from MB231/miLacZ was set at 1. G, Kaplan-Meier survival curves of mice bearing tumors derived from MB231/miLacZ (n = 12), MB231/miAngptl2-b1 (n = 10), or MB231/miAngptl2-b2 (n = 10) cells. All experiments were performed at least three times. Error bars show SD. **P < 0.01.
Figure 1 (Endo M. et al)
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Figure 3 (Endo M. et al)
Figure 4 (Endo M. et al)
Figure 5 (Endo M. et al.)
Figure 6 (Endo M. et al)
Tumor cell-derived angiopoietin-like protein ANGPTL2 is a critical driver of metastasis

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