Microenvironment and Immunology

Autocrine Motility Factor Modulates EGF-Mediated Invasion Signaling

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Abstract

Autocrine motility factor (AMF) enhances invasion by breast cancer cells, but how its secretion and effector signaling are controlled in the tumor microenvironment is not fully understood. In this study, we investigated these issues with a chimeric AMF that is secreted at high levels through a canonical endoplasmic reticulum (ER)/Golgi pathway. Using this tool, we found that AMF enhances tumor cell motility by activating AKT/ERK, altering actin organization, and stimulating β-catenin/TCF and activating protein 1 transcription. EGF enhanced secretion of AMF through its casein kinase II–mediated phosphorylation. RNA interference–mediated attenuation of AMF expression inhibited EGF-induced invasion by suppressing extracellular signal–regulated kinase signaling. Conversely, exogenous AMF overcame the inhibitory effect of EGF receptor inhibitor gefitinib on invasive motility by activating HER2 signaling. Taken together, our findings show how AMF modulates EGF-induced invasion while affecting acquired resistance to cytotoxic drugs in the tumor microenvironment.

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Introduction

Autocrine motility factor (AMF) was originally isolated in the conditioned medium of melanoma cancer cells and stimulates both direct and random migration (1). Before the identification of its primary sequence, it was also known by other names: neuroleukin promoting growth of embryonic spinal and sensory neurons, and maturation factor mediating differentiation of myeloid leukemia cells (2, 3). Several researchers independently identified that secreted AMF has the same sequence as phosphoglucone isomerase (PGI, GPI), which catalyzes the reversible isomerization of glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) in glycolysis and gluconeogenesis (4). AMF belongs to the orphan C-X-X-C cytokine family. Other members of the C-X-X-C family include oxidoreductases, for example, macrophage migration inhibitory factor (MIF), and thioredoxin (5, 6). These C-X-X-C proteins bind to more than one receptor or partner; for example, MIF binds to CXCR2, CXCR4, and CD74 (7, 8). Likewise, AMF binds to AMFR/gp78 as a G-protein–coupled receptor and HER2, leading to the activation of the PI3K/AKT and MAPK/ERK pathways in HER2-expressing breast cancer cells (9, 10).

Moreover, C-X-X-C members lack a secretion leader sequence governing endoplasmic reticulum (ER)/Golgi–dependent secretion and might be secreted via nonconventional secretion. Of note, clinical observations have revealed an aberrant secretion of PGI/AMF into the blood and urine of patients with cancer, as well as the circulation and synovial fluids of patients with rheumatoid arthritis (4, 11).

Functional studies of AMF-transfected breast cancer cells (MCF10A) revealed the contribution of AMF to epithelial-to-mesenchymal transition (EMT) via downstream regulation of miRNA and the switch of EMT gene markers (12). Furthermore, silencing of AMF expression inhibits anchorage-independent growth of tumor cells and tumor growth in nude mice (13). Previously, AMF studies have addressed the molecular characteristics of its cytokine properties and downstream molecular networks, but failed to resolve its linkage to other tumor-associated growth factors facilitating oncogenic signaling pathways.

Cancer invasion is a coordinated process involving dynamic regulation of cell–cell adhesion, extracellular matrix (ECM) degradation and adhesion (14, 15). Extrinsic stimulation of growth factors, including EGF and TGF-β, induces tumor cell invasion, although cancer cells have intrinsic and oncogenic mutations to drive tumor development (16). In this aspect of extrinsic modulation of cancer progression, it is meaningful to understand how endogenous AMF secretion is regulated and linked to growth factor–induced invasion in breast carcinoma cells, because therapeutically targeting a single signaling pathway is not completely effective in many cases (17, 18).

The objective of this study was to determine the secretory mechanisms of AMF upon microenvironmental stimulus. We show here that AMF is secreted from human breast cancer cells following serine phosphorylation by casein kinase II (CKII) in

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response to EGF, and suggest that it cooperates with EGF in the induction of cell invasion.

Materials and Methods

Cell culture and synchronization

T47D, MDA-MB-231, SKBR3 breast cancer, and EBNA 293 cells (American Type Culture Collection) were cultured at 37°C with 5% CO2 in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% FBS (Atlanta Biological). All experiments were performed at exponential growth and cell synchronization was achieved by serum-free medium for 16 hours.

Chemicals and antibodies

The BD Matrigel Basement Membrane Matrix, BD BioCoat BD Matrigel Invasion Chamber, and β-catenin were purchased from BD Transduction Laboratories. Monoclonal anti-PIGI (12F9A6; Pfizer) and rabbit anti-PIGI (H300; Santa Cruz Biotechnology) antibodies were used for Western blot analysis and immunoprecipitation (IP). Anti-p-AKT (Ser473), AKT, p-EGFR, EGF receptor (EGFR), and p-HER2 antibodies, Wortmannin [phosphoinositide 3-kinase (PI3K) inhibitor], and U0126 (MEK1/2 inhibitor) were from Cell Signaling Technology. Anti-vimentin, c-jun, p-ERK (E-4), ERK1/2(MK1), HER2 antibodies, TBCA [(E)-3-(2,3,4,5-tetrabromophenyl) acrylic acid], CKII inhibitor I (TBB) were purchased from Santa Cruz Biotechnology. EGFR receptor (EGFR), and p-HER2 antibodies, Wortmannin (MEK1/2 inhibitor) were from Cell Signaling Technology antibodies were used for Western blot analysis and immunoprecipitation (IP). Anti-p-AKT (Ser473), AKT, p-EGFR, EGF receptor (EGFR), and p-HER2 antibodies, Wortmannin [phosphoinositide 3-kinase (PI3K) inhibitor], and U0126 (MEK1/2 inhibitor) were from Cell Signaling Technology. Anti-vimentin, c-jun, p-ERK (E-4), ERK1/2(MK1), HER2 antibodies, TBCA [(E)-3-(2,3,4,5-tetrabromophenyl) acrylic acid], CKII inhibitor I (TBB) were purchased from Santa Cruz Biotechnology. Anti-vimentin, c-jun, p-ERK (E-4), ERK1/2(MK1), HER2 antibodies, TBCA [(E)-3-(2,3,4,5-tetrabromophenyl) acrylic acid], CKII inhibitor I (TBB) were purchased from Santa Cruz Biotechnology.

Plasmids and transfection

We performed overlapping PCR after achievement of two fragments, including signal peptide immunoglobulin K (Igk) fragment and Flag-fused human PGI/AMF product, followed by primer sets: EcoRI-signal-Igk-F, 5′-GAATTCCGGAACCACT-GGAGACAGACACACTCCTGCTATGGGTAC-3′; Igk-signal-R, 5′-GGTCACCATGGCAGAATTACCTGCTATGGGTAC-3′; Flag-signal-F, 5′-GCTCTAGATTATTGGACTCTGG-3′; Flag-signal-R, 5′-GGTTCCACTGGTGAC-3′; Flag-signal-F, 5′-GGTTCCACTGGTGAC-3′; Flag-signal-R, 5′-GGTTCCACTGGTGAC-3′; Flag-signal-F, 5′-GGTTCCACTGGTGAC-3′; Flag-signal-R, 5′-GGTTCCACTGGTGAC-3′. The PCR products of sp-flag-AMF fragment were cloned into tet-on expression vector (Clontech). T47D cells were transfected with Lipofectamine LTX Reagent (Invitrogen) and selected 3 weeks in antibiotics for mixed populations of stable clones.

Western blot analysis and IP

The cells were extracted in lysis buffer [20 mmol/L Tris–HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% NP-40, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, and proteases inhibitors (Roche)]. After bicinchoninic acid (BCA) protein assay (Pierce), 25 to 50 μg of total lysate was loaded and immuno-blotted for regular Western blot analysis. Of note, 500 μg of lysates was used for IP with appropriate antibodies for 16 hours at 4°C, washed with lysis buffer, and subjected to immunoblotting.

PGI enzymatic activity

The enzymatic activities of immunoaffinity-purified AMFs were measured as described previously (19). Briefly, we preincubated a reaction mixture consisting of 0.1 mol/L Tris (pH 8.5), 4 mmol/L F6P, 0.5 mmol/L NADPH, and 1 U of G6P dehydrogenase (G6PD) for 10 minutes, and purified AMF was added to the mixture and its enzymatic activity was immediately monitored at A340 nm using a Shimadzu spectrophotometer.

AMF secretion in conditioned media

Confluent cells were cultured for 3 days in completed medium, 10% FBS, and then were starved with serum-free medium with inhibitors or EGF. The supernatant was filtered through a 30-kDa cutoff filter (Millipore). AMF secretions were measured in Western blot analysis, after normalization and loading, dependent on cell number.

Luciferase assay

Reporter assays were performed as previously mentioned (20). Briefly, cells were plated onto 6-well plates (21). Two days following transfection, luciferase and Renilla activity of lysates was measured with Dual-Glo Luciferase Reagents (Promega). Luciferase activities were normalized against Renilla activity, and relative ratios for each transfection were calculated and represented. Experiments were performed on at least two independent occasions, and error bars indicate SE.

Transwell assay

Transwell (Corning Costar) was used for migration assay and BD BioCoat BD Matrigel Invasion Chamber was used for invasion assay. A total of 5 × 104 cells in serum-free medium were introduced into the upper compartment of Transwell chambers (8-mm pore) and were allowed to migrate to the lower chambers with 10% FBS, Matrigel, or EGF (100 ng/mL) for 24 Migrations. Migrated or invaded cells were fixed and stained with the Hema-3 Stain Kit (Fisher). Each condition was assayed in triplicate, and each experiment was repeated three times.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked in 1% bovine serum albumin/PBS, incubated with primary antibodies, and then incubated with secondary antibodies. After each antibody treatment, cells were washed three times with 0.1% bovine serum albumin/PBS each. Next, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI), washed, and mounted on a glass slide with 80% glycerol. Fluorescent images were acquired using a color Sony camera connected to a fluorescence Olympus microscope.

Statistical analysis

Data from triplicate experiments are expressed as mean ± SD or SE. Comparisons between the groups were determined
Results

Signal peptide-fused AMF enhances cell motility via activation of PI3K/AKT and MAPK/ERK

AMF plays a role in glucose metabolism and enhances cell migration. To differentiate between intracellular and extracellular AMF, we have developed a chimeric AMF construct needed for AMF over secretion and dependence on the canonical pathway by adding a secretion leader sequence (sp; Fig. 1A). In addition, an inducible promoter (tet-on system) was used to avoid a permanently adapted phenotype, which might be generated by constitutive promoter-driven AMF expression during the selection and cell cloning processes. T47D breast cancer cells were chosen because they were AKT/ERK–activated by purified AMF (10). We had T47D cells stably transfected with sp-Flag-AMF and its counterpart of Flag-AMF (Fig. 1A), and the migration was tested thereafter. We detected a significant secretion of sp-Flag-AMF (Fig. 1B) as compared with Flag-AMF transfectants, which failed to secrete AMF under the conditions tested. Cell motility was enhanced in cells transfected with chimeric AMF, indicating no difference from endogenous AMF, regardless of the route of their secretion (Fig. 1C). To examine the activation of cellular signaling pathways by chimeric AMF secretion as observed in endogenous AMF-treated cells (10), a time-course experiment was performed and showed that overexpression of chimeric AMF activates AKT and extracellular signal–regulated kinase (ERK; Fig. 1D), which was not activated with doxycycline in control cells (data not shown), indicating a similar function of chimeric AMF and endogenous AMF in migration and AKT/ERK activation.

Enzyme activity of PGI/AMF is not indispensable for AKT/ERK activation

Previously, it was suggested that AMF-induced cell motility required an intact of the sugar-binding domain of AMF for its cytokine function based on the data of migration suppression by erythrose 4-phosphate (E4P), which binds to the active site of GPI isomerase (1). However, mutant constructs of the C-X-X-C motif of AMF lose isomerase activity but maintain characteristic of enhanced motility (22). Therefore, we questioned whether canonically secreted sp-Flag-AMF retains the PGI isomerase activity. To rule out the possible background of endogenously secreted AMF, we purified sp-Flag-AMF by affinity chromatography with anti-Flag antibodies (Fig. 2A) and found a loss of isomerase activity in sp-Flag-AMF, unlike Flag-AMF (Fig. 2B), implying that the isomerase activity of chimeric AMF is not indispensable for AKT/ERK activation. We returned to the main question whether extracellular isomerase activity of endogenous AMF is not essential to AKT/ERK activation. We pretreated purified AMF with E4P to inhibit isomerase activity as it previously reported to be competitive inhibitor against G6P (Fig. 2C, left). Interestingly, E4P-pretreated AMF still stimulates cells, leading to p-AKT/p-ERK elevation, indicating no effect of E4P on AKT/ERK activation (Fig. 2C, right). To clarify the effect of E4P on cell migration, we performed migration assay in T47D/sp-Flag-AMF cells due to loss of enzymatic activity of sp-Flag-AMF. The results exhibit inhibitory effect of E4P upon noninduced condition as well as the condition expressing sp-Flag-AMF, indicating indirect effect of E4P on cell migration (Fig. 2D, left). We addressed a raising possibility whether E4P inhibits intracellular PGI/AMF activity in glycolytic metabolism instead of E4P effect on extracellular AMF. After E4P-treated cells were severally washed to avoid E4P contamination in lysate,
intracellular G6P isomerase activity was measured (Fig. 2D, right). In conclusion, the carbohydrate-binding motif of AMF is indispensable for AKT/ERK activation, and E4P at least inhibits migration via intracellular AMF binding. However, we cannot still exclude the possibility that AMF enhances migration, dependent on the carbohydrate-binding motif, because migration is a dynamic and complex process, not only dependent on kinase activation such as AKT/ERK.

Chimeric AMF activates β-catenin/TCF and AP-1 transcription, a marker of motility and invasion

Next, we questioned whether chimeric AMF affects gene expression, which is indeed associated with cell migration. As ectopic expression of AMF disrupts cell–cell adherent junctions via dissociation of E-cadherin/β-catenin complex in MCF10A cells (12), we used TOPFLASH reporter of Wnt signaling in which nuclear translocation of β-catenin initiates Wnt-responsive gene expression after associating with T-cell factor (TCF) in the nucleus (23). In addition, we investigated activating protein 1 (AP-1) transcription, which is composed of the c-jun/c-fos regulator complex, because it is mainly regulated by the mitogen—activated protein kinase (MAPK) signaling pathway and promotes the expression of matrix metalloproteinases (MMP), such as MMP1 and MMP3, required for ECM degradation (24). T47D/sp-Flag-AMF cells were cotransfected with reporters and/or dominant-negative TCF4 (DN-TCF) and DN-c-jun. The results revealed that AMF induced both TOPFLASH and AP-1 transcriptions but the transcriptions were suppressed by cotransfection of dominant-negative constructs (Fig. 3A). Next, a supporting experiment was performed for confirming elevated levels of nuclear β-catenin and c-jun (Fig. 3B). As EGF/EGFR can activate β-catenin via a receptor tyrosine kinase–PI3K/AKT pathway in invasion and metastasis of cancer cells (25) and chimeric AMF can also induce PI3K/AKT, we addressed whether EGF/EGFR and/or PI3K/AKT is
associated with the AMF-activated β-catenin pathway. The results showed that EGFR inhibitor did not affect AMF-induced translocation of β-catenin (Supplementary Fig. S1) and Wortmannin, covalent inhibitor of PI3K showed significant reduction of translocated β-catenin along with abrogation of p-AKT in chimeric AMF-expressed cells (Fig. 3C). In conclusion, sp-Flag-AMF induces cell motility through AKT/ERK signaling pathways as well as β-catenin/TCF and AP-1 transcriptional activations. Next, immunocytochemistry was executed to further analyze the process of cell motility induced by AMF (Fig. 3D). AMF-secreting cells along with doxycycline induction showed straight organization of actin filaments along with lateral stretching cell shape, compared with the round shape of control cells. However, nuclear β-catenin and loss of cell-to-cell contact were not significant, indicating that AMF alone is not sufficient for inducing EMT, showing cell-scattering effect (15). Thus, the data suggested that AMF plays a role of modifier in the EMT process and might coordinate with extrinsic growth factors relative to EMT and invasion (26).

EGF enhances endogenous AMF secretion in breast cancer cells

Although the above overexpression and chimeric studies delineate some of the biochemical functions of AMF, it remains unknown whether the mechanism underlying AMF secretion is linked to any other growth factor(s), which is implicated in EMT and cell invasion processes in the tumor microenvironment. Previously, we have reported that HER2 (ErBB2) knock-down and Herceptin (anti-HER2 antibodies) inhibit endogenous AMF secretion, and served as a clue suggesting that AMF secretion might be regulated by the HER family downstream pathways (27). Therefore, we performed pharmacologic inhibition studies of cell signal transduction to clarify the signaling cascade responsible for AMF secretion in HER2-overexpressing SKBR3 cells. As shown in Fig. 4A, AMF secretion was significantly reduced by the MAP-ERK kinase (MEK) inhibitor (U0126), lapatinib (EGFR, HER2 dual inhibitor), and gefitinib (EGFR inhibitor), indicating that the intracellular MEK pathway of HER family downstream
is responsible for AMF secretion. As these inhibitors usually affect cell growth status, we wanted to know whether AMF secretion is positively upregulated by MEK kinase relative to HER family signal transduction. We used EGF ligand and examined its effect on AMF secretion in AMF low-secreting T47D cells, resulting in enhancement of AMF secretion (Fig. 4B). Meanwhile, this inhibition and increase of AMF secretions were unlikely to be affected by cell numbers and cell growth, because of our measuring AMF secretion within a 24-hour time frame and similar cell numbers in each group.

**CKII kinase interacts with AMF under EGF stimulus**

Previously, in vitro kinase assay showed that CKII serine/threonine kinase phosphorylates AMF (28). Of note, it was reported that CKII is ubiquitously expressed and activated in response to various growth factor stimuli, including EGF (29). Therefore, we hypothesized that CKII endogenously interacts with AMF and promotes its secretion following EGF stimulus. Initially, AMF-V5 was transfected into EBNA293 cells to examine interaction of AMF-V5 and CKII. Expectedly, reciprocal IP studies showed that AMF-V5 binds to CKII (Fig. 4C). The results encouraged us to explore endogenous interaction of AMF and CKII upon EGF stimulus. Obviously, time-course IP with anti-CKII antibodies showed that CKII interacts with AMF in a time-dependent manner, resulting in serine phosphorylation of AMF (Fig. 4D). Reciprocally, it makes for convincing data that EGF induces interaction of CKII with AMF and gefitinib, EGFR inhibitor abolished interaction of CKII and AMF (Fig. 4E). Next, we wondered whether CKII is responsible for AMF secretion. We used AMF high-secreting MDA-MB 231 cells to examine inhibition effect of CKII on AMF secretion. We expected the reduction of AMF secretion under pharmacologic inhibition of CKII, resulting in inhibition of AMF secretion by CKII inhibitors. Also, reduction of AMF secretion was observed under CKII knockdown (Fig. 5A). Because CKII-mediated AMF secretion occupies a position downstream of EGF (an invasion inducer), we wanted to determine the endogenous contribution of AMF secretion to EGF-induced invasion. Interestingly, AMF knockdown suppresses EGF-induced invasion and EGF-induced AP-1 transcription, indicating AMF impact and downstream modulation of EGF/EGFR (Fig. 5B). To understand how AMF knockdown affects the downstream signaling pathways of EGF/EGFR, we investigated the change of p-AKT/p-ERK levels. In response to EGF, we did not find significant differences of p-AKT in AMF...
knockdown cells (Fig. 5C), implying the influence of PI3K constitutive mutation (H1047R) in T47D cells (30). However, we observed that p-ERK half-life is shortened upon AMF knockdown in EGF-stimulated cells. Next, we performed IP to delineate how AMF knockdown in influences on EGF-activated EGFR as well as HER2. The results exhibit that AMF knockdown reduces p-HER2 without EGF stimulus, which is not completely overridden with EGF stimulus (Fig. 5D). It indicates that EGF-induced invasion requires HER2 activation by AMF. To distinguish whether knockdown of intracellular AMF or extracellular AMF affects EGF-induced invasion, we performed add-back experiments with exogenous AMF. The results showed that exogenous AMF did not completely rescue cell motility in AMF knockdown cells (Fig. 5E), regardless of the restoration of HER2 and ERK activation (Fig. 5F). It is consistent with the observation of intracellular AMF suppression by E4P (Fig. 2E). Intracellular PGI/AMF activity or protein level might be a prerequisite for cell motility. It is unclear whether or not reduced PGI/AMF isomerase activity affects glucose metabolism and energy generation during migration. In conclusion, we suggest the EGF–AMF axis for a positive feedback loop of AKT/ERK activation, in which exogenous EGF ligands stimulate cancer cells and thereby increase AMF secretion. AMF activates more AKT/ERK pathway via HER2 in an autocrine manner during cancer invasion.

**AMF overcomes gefitinib-suppressed invasiveness in breast cancer cells**

Breast cancer cells frequently exhibit intrinsic and acquired resistance to tyrosine kinase inhibitors in the HER family, and because AMF secretion is regulated by CKII kinase, which can

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Figure 5. **AMF knockdown suppresses EGF-induced invasiveness.** A, MDA-MB 231 cells were treated with CKII inhibitor (TBB or TBCA) at indicated concentration for 16 hours. Intracellular and extracellular AMF levels were determined in immunoblot (left). The cells were treated with siRNA-CKII and then secreted AMF level was analyzed (right). Bars indicate the mean ± SE of three independent experiments (*, P < 0.05). B, after treatment with siRNA-AMF for 48 hours, T47D cells were counted and loaded into upper side of Transwell and chemoattractant of EGF (100 ng/mL) was added at bottom wells (left). --, vehicle or siRNA-control. AP-1 transcriptional activity was determined in T47D cells, which were pretreated with siRNA-AMF for 24 hours and then transfected with AP-1 reporter for 24 hours (right). Values, mean ± SE of three replicates (*, P < 0.05). C, effect of AMF knockdown on EGF-induced p-AKT/p-ERK. After T47D cells were transfected with siRNA-AMF and starved in serum-free medium for 16 hours, EGF was treated as indicated time points. D, IP with anti-EGFR or HER2 in MDA-MB-231 cells transfected with siRNA-AMF or control siRNA for 48 hours and starved, stimulated with EGF for 5 minutes. E, rescue experiment of invasion with exogenous AMF in AMF knockdown MDA-MB-231 cells. The cells were transfected with siRNA-AMF for 48 hours and subjected to Transwell assay (*, P < 0.05). F, AMF knockdown cells were stimulated with purified AMF and then IP was performed to analyze p-HER2 level.
be activated by other receptor tyrosine kinases and growth factors, while AMF can activate HER2, resulting in PI3K activation (10), it might be involved in the resistance against the tyrosine kinase inhibitor of EGFR (gefitinib). Thus, to establish whether AMF plays a role in bypassing EGFR in therapy with gefitinib, we treated T47D/sp-Flag-AMF with gefitinib. We found that gefitinib suppressed EGF-induced p-AKT/p-ERK, but chimeric AMF oversecretion overcame gefitinib-inhibited AKT/ERK activation (Fig. 6A). Consistently, chimeric AMF oversecretion recovered invasiveness reduced by gefitinib (Fig. 6B). Next, we addressed that these findings were reproducible at purified AMF treatment. These results highlight that AMFs partially restore the growth and invasion in MDA-MB-231 cells inhibited by gefitinib (Fig. 6C and D), and that HER2 activation is in part responsible for the restoration effect of AMF (Fig. 6E).

Discussion

Previously, the experimental attempts focused on the biochemical characteristics and cytokine activity of AMF, which displays enhancement of migration in several cancer cells. However, as cancer cell motility, EMT, and invasion are a coordinated process of metastasis, in which the signaling regulation is highly complex, involving activation of numerous different pathways, this process frequently involves microenvironment cross-talk among Wnt, EGF, TGF-β, and Notch, etc. (16, 31, 32). Thus, we addressed here the role of AMF and regulation of its secretion through oncogenic networks and cross-talk with their extrinsic factors in breast cancer cells. We demonstrated that AMF secretion is regulated by EGF/EGFR through CKII interaction and thereby promotes EGFR-induced invasiveness. Our studies suggest a possible mechanism underlying the positive feedback of AMF on extrinsic EGF-induced invasion to enhance PI3K/MAPK signaling pathways in an autocrine manner of AMF during tumor invasion and metastasis. Moreover, it is an attractive observation that AMF is able to overcome gefitinib-inhibited invasion, although AMF secretion is partially dependent on EGF signaling and CKII mediation. Especially, non–small-cell lung cancers (NSCLC) are frequently associated with EGFR genetic alteration (17, 33). However, PTEN mutation or c-Met amplification is proposed to provide initial or acquired resistance to gefitinib (17, 33). Given that AMF oversecreting cells show resistance to gefitinib in breast cancer cells, AMF might be involved in resistance to EGFR inhibitor in lung cancers. Accordingly, it might be important to examine the relationship of endogenous AMF secretion geared at gefitinib-resistant NSCLC. In respect to cancer targeting, CKII is a candidate to block AMF secretion. It is doubtful because CKII is a ubiquitous kinase and has been shown to regulate numerous growth-related proteins as intracellular substrates in both normal and cancer cells (29). It may be argued that gefitinib is sufficient for blocking AMF secretion. However, gefitinib treatment clinically shows application limitation and resistance (17). Indeed, because AMF secretion is partially downregulated by gefitinib, it remains elusive which microenvironment signals enhance AMF secretion. Therefore, AMF targeting can offer therapeutic benefits

**Figure 6.** AMF overexpression overcomes gefitinib-inhibited invasion. A, effect of AMF on gefitinib-pretreated T47D/sp-Flag-AMF cells. B, T47D/sp-FlagAMF cells were subjected to Transwell assay with EGF or doxycycline induction. C, the growth of MDA-MB-231 cells was measured under gefitinib and AMF treatment using MTT assay. D, MDA-MB-231 cells were subjected to Transwell assay. Each bottom side contained gefitinib or purified AMF as indicated. Photographs show settle-down cells in bottom wells after cell invasion (\(^{-}\)P < 0.05). E, MDA-MB-231 cells were pretreated with gefitinib and then stimulated with EGF or AMF for 5 minutes, respectively. Phosphorylation of EGFR and HER2 were analyzed with IP.
against the gefitinib-resistant phenomena in combination therapy.

It has been difficult to delineate AMF therapeutic potential in vivo, because AMF overexpression affects both intracellular and extracellular roles of AMF. Therefore, we have constructed a secretion leader sequence-fused to AMF cDNA to enhance secretion via the classical secretion pathway, resulting in AKT/ERK activation and augmentation of cell motility, similar to endogenous AMF function. For this reason, a classical secretion-dependent construct might be a useful tool to study its therapeutic potential in a xenograft model injected with cancer cells containing the chimeric AMF, as well as other cytokines and interleukins that are secreted via a nonclassical mode.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


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