FGF receptor-4 (FGFR4) polymorphism acts as an activity switch of a membrane type 1 matrix metalloproteinase–FGFR4 complex

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Tumor cells use membrane type 1 matrix metalloproteinase (MT1-MMP) for invasion and metastasis. However, the signaling mechanisms that underlie MT1-MMP regulation in cancer have remained unclear. Using a systematic gain-of-function kinome screen for MT1-MMP activity, we have here identified kinases that significantly enhance MT1-MMP activity in tumor cells. In particular, we discovered an MT1-MMP/FGF receptor-4 (FGFR4) membrane complex that either stimulates or suppresses MT1-MMP and FGFR4 activities, depending on a tumor progression-associated polymorphism in FGFR4. The FGFR4-R388 allele, linked to poor cancer prognosis, increased collagen invasion by decreasing lysosomal MT1-MMP degradation. FGFR4-R388 induced MT1-MMP phosphorylation and endosomal stabilization, and surprisingly, the increased MT1-MMP in return enhanced FGFR4-R388 autophosphorylation. A phosphorylation-defective MT1-MMP was stabilized on the cell surface, where it induced simultaneous FGFR4-R388 internalization and dissociation of cell–cell junctions. In contrast, the alternative FGFR4-G388 variant down-regulated MT1-MMP, and the overexpression of MT1-MMP and particularly its phosphorylation-defective mutant vice versa induced FGFR4-G388 degradation. These results provide a mechanistic basis for FGFR4-R388 function in cancer invasion.

proteolysis | signaling | MMP14 | ECM | invasion

The mechanisms of tumor cell proliferation, survival, and spread depend on the growth factor stimuli and tissue environment (1–4). Tumor cells can invade poorly cross-linked ECMs independently of proteolytic activity (4, 5). However, during invasion, growth, and metastasis, most cells of solid human tumors seem to use membrane type 1 matrix metalloproteinase (MT1-MMP, MMP14) activity on the surface of tumor cells or stromal cells to degrade cross-linked interstitial matrices or basement membranes (3, 6, 7). MT1-MMP can also regulate invasive cell functions and tissue remodeling by cleaving pericellular proteins and cell-surface receptors, as well as by serving as an activator for secreted MMPs, such as MMP-2 and MMP-13 (3, 8).

Cytokines and growth factors such as TNF-α, IL-1β, and TGF-β regulate *MT1-MMP* expression that is commonly detected in cells of mesenchymal origin during tissue remodeling (9–12). In addition, branching epithelial cells show a timely and spatially controlled MT1-MMP expression (13, 14). In various forms of human cancer, *MT1-MMP* is overexpressed in tumor cells or stromal cells, being frequently detected in the collectively invading carcinoma fronts. However, the strongest *MT1-MMP* induction in carcinoma cells often correlates with the transition of neoplastic epithelium to an aggressively invasive mesenchymal morphology (3, 4). After transcription, the proinvasive MT1-MMP activity is posttranscriptionally regulated through its cytoplasmic tail by, for example, cell surface clustering, endocytosis, and recycling coupled with the lysosomal degradation of bound inhibitors (15–19). In this way MT1-MMP can function efficiently in a sequestered pericellular tumor microenvironment, allowing it to escape inactivation by the concentrations of inhibitors that are effective against soluble MMPs but unsuccessful in clinical trials using MMP inhibitors (3, 20). Because low physiological MT1-MMP activity is essential for connective tissue homeostasis and likely more sensitive to MMP inhibition, systemic MT1-MMP inhibition may have also contributed to the musculoskeletal adverse effects observed in the trials (20–22).

Understanding upstream and MT1-MMP cooperating signaling mechanisms could help to more efficiently block tumor progression. We used a systematic kinome screen to identify the key molecules and mechanisms that control the cancer-specific MT1-MMP activity. Our study identified unique FGF receptor 4 (FGFR4)/MT1-MMP membrane complexes, in which MT1-MMP and FGFR4 are regulated in an opposite manner depending on the tumor progression–associated FGFR4 SNP (23–27). This SNP changes Gly388 to arginine in the predicted FGFR4 transmembrane domain, resulting in enhanced stability of the activated receptor (28).

Results

Identification of FGFR4 as a Unique MT1-MMP Regulator. To identify the protein kinases that regulate MT1-MMP, 564 cDNAs constituting ≈93% of all human protein kinases (29) were expressed in human HT-1080 fibrosarcoma cells. Because MT1-MMP is the main activator of secreted MMP-2 in these cells (30), proMMP-2 activation was quantified by gelatin zymography as a measure of MT1-MMP activity (Fig. 1*A*). MMP-9, the other gelatinolytic MMP in HT-1080 cell conditioned medium that is also implicated in cell invasion (8), was also quantified (Fig. 1*B*). The kinases that enhanced MMP-2 activation and proMMP-9 were mostly distinct, and none of the kinases induced MMP-9 activation (Fig. 1*A*–*C*). The 32 top kinases scored by the ratio between the activated and proenzyme forms of MMP-2 were selected for a secondary screen (Fig. 1*A*, red bars), in which 21 kinases resulted in significant, >2-fold increased proMMP-2 activation relative to the mock-transfected control (Fig. 1*D*). These kinases included both unique MT1-MMP/MMP-2 regulators and kinases acting on pathways activated by MT1-MMP–inducing stimuli (10, 12, 31). The latter group included IL-1 receptor–associated kinase (IRAK1), JNK, and p38 pathway kinases involved in IL1 and TNF-α signaling, and receptors of TGF-β family members (Fig. 1*D*). Unexpectedly, the FGFR4-R388

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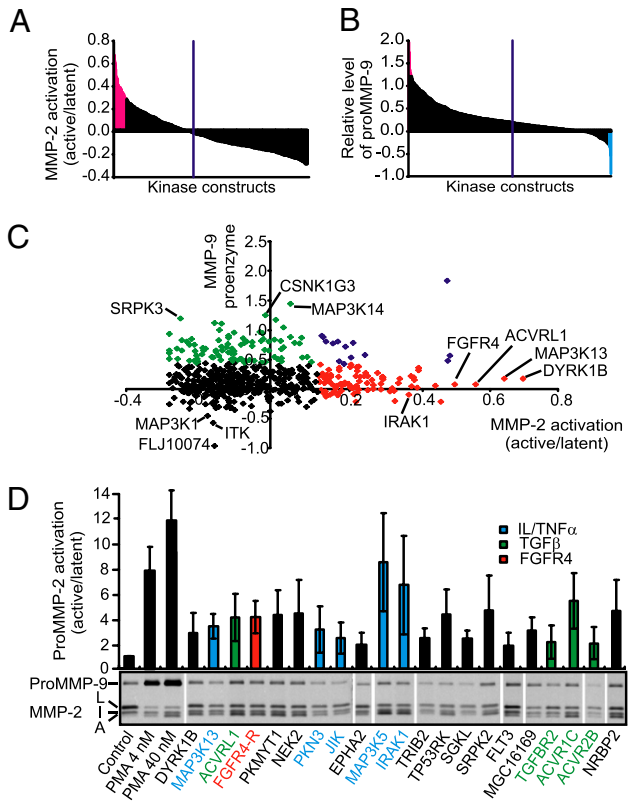

Fig. 1. Gain-of-function kinome screen for MT1-MMP regulation. (*A*) 564 cDNAs representing 480 different protein kinases were expressed in HT-1080 cells. MT1-MMP–mediated proMMP-2 activation is expressed as the levels of activated MMP-2 relative to the proenzyme in gelatin zymography, as sorted by activation score. Equal levels have been set to zero. Blue vertical bars indicate mean values. The kinases for secondary screen are indicated in red. (*B*) Quantification of MMP-9, the other gelatinolytic MMP in HT-1080 cell conditioned medium. Top proMMP-9 inducers and suppressors are indicated in red and blue, respectively. None of the kinases induced detectable MMP-9 activation. (*C*) An *x-y* plot of MMP-2 and -9 results, which indicates that the regulators of MMP-2 activation (red) and MMP-9 (green) are mostly distinct. Blue indicates the kinases that enhance MMP-2 activation and proMMP-9. The top MMP-2 and -9 regulators have been named. (*D*) In the secondary screen, FGFR4 as well as TGF-β and IL1/TNF-α pathway kinases (marked with the indicated colors) promote MT1-MMP activity. Quantification of proMMP-2 activation for the kinases increasing the activation >2-fold over control (mean ± SD, $n = 3$, $P < 0.05$) and negative images of representative zymograms (*Lower*) are shown. MMP-2 L, latent proenzyme; I, intermediate; A, active. Phorbol 12-myristate 13-acetate treatment served as a positive control.

variant linked to poor cancer prognosis also increased MMP-2 activation significantly (Fig. 1*D*), unlike the alternative FGFR4-G388 allele or the other FGFRs (Fig. S1 *A–D*).

FGFR4-R388 Risk Variant Reduces Lysosomal MT1-MMP Degradation, Whereas the Alternative FGFR4-G388 and MT1-MMP Suppress Each Other. Because *MT1-MMP* gene expression is frequently up-regulated in malignant vs. normal tissues, the effect of FGFR4-R388 on MT1-MMP transcript was quantified by quantitative PCR (qPCR) in HT-1080 cells and MDA-MB-231 human breast carcinoma cells. FGFR4-R388 had negligible effects on MT1-MMP mRNA, whereas IRAK1, the most potent hit kinase on the known MT1-MMP regulatory interleukin pathway, moderately but significantly increased MT1-MMP mRNA (Fig. S2*A*). These results suggest that FGFR4-R388 regulates MT1-MMP posttranscriptionally. Considering the reported constitutive lysosomal MT1-MMP degradation (19), we next analyzed whether FGFR4-R388 inhibits MT1-MMP degradation. As expected, the lysosomal inhibitor bafilomycin A markedly increased endogenous MT1-MMP in mock-transfected MDA-MB-231 cells that normally express undetectable levels of FGFR4-G388 (Fig. 2 *A* and *B*). In contrast, the effect of the proteasome inhibitor MG132 on MT1-MMP was minor (Fig. 2*A*). Importantly, the FGFR4-R388 risk variant increased endogenous MT1-MMP in untreated but not in bafilomycin A–treated cells (Fig. 2 *A–C*). MT1-MMP colocalization with lysosome-associated membrane protein-1 was also decreased by FGFR4-R388 (Fig. S2 *B* and *C*). In contrast, the FGFR4-G388 allele suppressed the coexpressed MT1-MMP (Fig. 2*D*). In vivo, MT1-MMP protein to mRNA ratio also showed an increasing trend in human skin biopsies from individuals carrying heterozygous and homozygous FGFR4-R388 variants relative to those having FGFR4-G388 (Fig. S2*D*).

Furthermore, MT1-MMP accumulation after bafilomycin A treatment in MDA-MB-231 cells correlated inversely with FGFR4-G388 down-regulation, which was not seen in cells expressing the FGFR4-R388 risk variant or the corresponding kinase activity-deficient (KD) proteins with an inactivating point mutation in the active site (Fig. 2*C*) (29). The normally undetectable endogenous FGFR4-G388 was also observed in the mock cells after the inhibition of endogenous MMP activity (Fig. 2*C*), suggesting that FGFR4-G388 and MT1-MMP down-regulate each other.

FGFR4 Variants Physically Interact with MT1-MMP. To test whether the opposite effects of the FGFR4 variants on MT1-MMP stability were mediated through a physical interaction, FGFR4 and MT1-MMP coimmunoprecipitation was assessed in MDA-MB-231 and COS-1 cells. Interestingly, the FGFR4-R388/MT1-MMP complexes were most abundant, but FGFR4-G388 and the respective KD proteins also coprecipitated with MT1-MMP (Fig. 2*D* and Fig. S3 *A* and *B*). Furthermore, MT1-MMP coprecipitation was detected with FGFR4 mutant proteins with deletions of the kinase domain or the C-terminal tail, as well as with FGFR2, but not with IRAK1 (Fig. S3 *C–G*). These results suggest that the interaction as such is not sufficient for MT1-MMP stabilization.

FGFR4-R388 Induced MT1-MMP Phosphorylation Is Coupled with Endosomal MT1-MMP Stabilization. The MT1-MMP cytoplasmic tail contains a single tyrosine residue that can be phosphorylated by Src (32). Because this phosphorylation has been associated

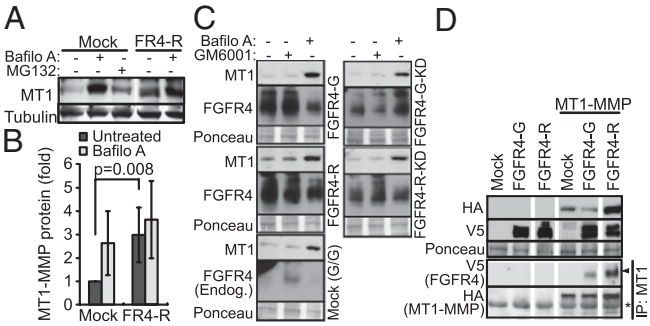

Fig. 2. The FGFR4-R388 risk variant inhibits lysosomal MT1-MMP degradation, whereas FGFR4-G388 and MT1-MMP suppress each other. (*A*) Transiently transfected FGFR4-R–expressing MDA-MB-231 cells were incubated with lysosomal inhibitor bafilomycin A (100 nM) for 16 h or with proteasome inhibitor MG132 (5 μM) for 6 h. MT1-MMP was assessed by immunoblotting. Tubulin served as a loading control. (*B*) Chart illustrates relative MT1-MMP levels in cell extracts (mean ± SD, $n = 3$). (*C*) Stable MDA-MB-231 cells expressing FGFR4-G, FGFR4-R, or the respective KD proteins were incubated with bafilomycin A or MMP inhibitor GM6001 (10 μM) for 16 h, followed by immunoblotting ($n = 3$). (*D*) FGFR4 interacts with MT1-MMP. MDA-MB-231 cells transiently transfected to express HA-tagged MT1-MMP alone or with V5-tagged FGFR4 variants were subjected to immunoblotting and immunoprecipitation as indicated ($n = 3$). Arrowhead indicates coprecipitated FGFR4 in the MT1-MMP immunocomplexes, and asterisk indicates IgG. Ponceau Red staining served as a loading control.

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with tumor cell growth and invasion (32–34), we first assessed the effects of FGFR4 on MT1-MMP phosphorylation using COS-1 cells that lack endogenous expression of these proteins. Coexpression of MT1-MMP with either allele of FGFR4 resulted in MT1-MMP tyrosyl phosphorylation coincidentally with FGFR4 autophosphorylation (Fig. S4 *A* and *B*). In contrast, MT1-MMP was not phosphorylated in cells overexpressing MT1-MMP only, FGFR4-KD, or MT1-Y/F protein in which the tyrosine residue was changed to phenylalanine (Fig. S4*C*). Furthermore, the FGFR4-R388–dependent MT1-MMP phosphorylation was inhibited by Src inhibitor PP2 (Fig. S4*D*).

In stable FGFR4-R388–expressing MDA-MB-231 cells, FGF2 treatment increased the phosphorylation of both FGFR4 and MT1-MMP (Fig. 3 *A*–*C*). Upon FGFR4-R388 activation, FGF2 also enhanced FGFR4/MT1-MMP interaction and the endosomal accumulation of MT1-MMP and FGFR4-R388 (Fig. 3 *A* and *D*). Enhanced MT1-MMP colocalized with clathrin and early endosomal antigen-1 (Fig. S5 *A* and *B*) in the FGFR4-R388–expressing cells, which is consistent with the increased stability of endocytosed MT1-MMP even in normal culture conditions. In contrast, very little colocalization of endogenous MT1-MMP and FGFR4-G388 or the kinase activity-deficient KD proteins was detected in the intracellular vesicles (Fig. S6 *A* and *B*). The FGF2 treatment increased MT1-MMP in FGFR4-R388–expressing cells with and without MMP inhibition (Fig. 3*E*), indicating that MT1-MMP proteolytic activity was not required for its stabilization by FGFR4-R388. The endosomal MT1-MMP accumulation and the levels of MT1-MMP in the FGFR4 complexes thus reflected the differential stabilities of the activated FGFR4 variants. In contrast, the FGF2-induced FGFR4-G388 suppression was inhibited by GM6001 (Fig. 3*E*), indicating that it involved proteolysis.

Unphosphorylated MT1-MMP Increases Cell–Cell Junctional Disassembly and FGFR4 Internalization. The importance of MT1-MMP phosphorylation for the function of the FGFR4/MT1-MMP complexes

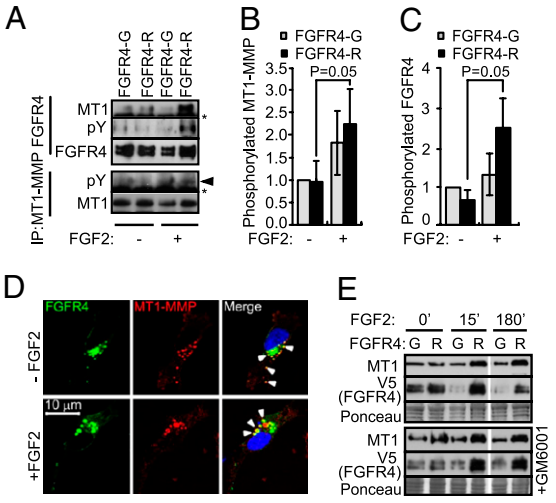

Fig. 3. Active FGFR4-R388 induces MT1-MMP phosphorylation and endosomal stabilization. (*A*) Stable MDA-MB-231 cells expressing the FGFR4 variants were incubated with FGF2 (10 ng/mL) for 15 min, followed by immunoprecipitation and immunoblotting as indicated. Arrowhead indicates phosphorylated MT1-MMP, and asterisks indicate IgG. (*B* and *C*) Chart illustrates the relative phosphorylation levels of MT1-MMP (*B*; mean ± SD, $n = 5$) and FGFR4 (*C*; mean ± SD, $n = 3$). (*D*) Confocal laser scanning micrographs of MT1-MMP (red) and FGFR4 (green) in stable FGFR4-R–expressing MDA-MB-231 cells after treatment with FGF2 as indicated. Arrowheads and yellow indicate colocalization. (*E*) Stable MDA-MB-231 cells were incubated with GM6001 (10 μM) for 16 h, followed by FGF2 treatment as indicated. Total MT1-MMP and FGFR4 levels were assessed by immunoblotting as indicated. Ponceau Red staining served as a loading control.

was assessed using MT1-Y/F (Fig. S7*A*). In contrast to the cytoplasmic domain deletion, which inhibits FGFR4-independent endocytosis of MT1-MMP (17, 18), the Y573F mutation did not alter MT1-MMP–mediated MMP-2 activation in HT-1080 cells that do not express endogenous FGFR4 (Fig. S7*B*). However, the colocalization of endogenous FGFR4-R388 with MT1-MMP in cell–cell contacts and intracellular vesicles of MDA-MB-453 breast carcinoma cells was lost by the mutation (Fig. 4*A*). The expression of the mutant protein also led to its accumulation at the cell surface, dissociation of cell–cell junctions, elongated cell morphology, and FGFR4-R388 translocation into intracellular vesicles (Fig. 4*A* and Fig. S7*C*).

The alternative FGFR4-G388 variant and MT1-MMP were detected in separate subcellular compartments of individual FGFR4-G388–overexpressing MDA-MB-231 cells (Fig. 4*B* and Fig. S7*D*). Furthermore, in MT1-Y/F and FGFR4-G388 cotransfected cells, predominantly MT1-Y/F or FGFR4-G388 was detected by immunofluorescence (Fig. 4*B* and Fig. S7*D*). GM6001 increased the colocalization of MT1-MMP and FGFR4-G388 in intracellular vesicles. Likewise, the coexpression of MT1-Y/F and FGFR4-G388 within the same cells was increased by the MMP inhibitor, indicating that MT1-Y/F activity induced FGFR4-G388 degradation.

MT1-MMP and FGFR4-R388 Activate and MT1-MMP and FGFR4-G388 Suppress Each Other. To study the mechanism of MT1-MMP and FGFR4 regulation in the complexes, MT1-E/A protein with an inactivating mutation of the active site and MT1-Y/F were coexpressed with the FGFR4 variants in MDA-MB-231 cells. Consistently with the loss of their colocalization in MDA-MB-453 cells, fewer FGFR4-R388/MT1-Y/F complexes than FGFR4-R388/MT1-MMP complexes were detected by coprecipitation, although the total MT1-Y/F protein content remained high (Fig. 4*C*). Although the total FGFR4-R388 risk variant was slightly decreased by MT1-Y/F, total and cell-surface FGFR4-G388 was notably suppressed in cells overexpressing MT1-Y/F prominently on the cell surface (Fig. 4*C* and Fig. S7*E*). At the same time, the FGFR4-G388/MT1-Y/F complexes were barely detectable (Fig. 4*C*). FGFR4-G388 was also suppressed by wild-type MT1-MMP but not by MT1-E/A (Fig. 4*C*). The MMP activity-dependent down-regulation did not, however, correlate with the appearance of proteolytic FGFR4-G388 fragments (Fig. S8 *A* and *B*). In COS-1 cells, strong MT1-MMP overexpression dramatically suppressed FGFR4-G388, but additionally the FGFR4-R388 variant was slightly decreased by the MT1-MMP coexpression (Fig. S8*B*). High concentrations of either FGFR4 variant also led to slightly decreased MT1-MMP levels (Fig. S8*C*). However, MT1-MMP did not suppress FGFR4-KD proteins (Fig. S8*B*), indicating that the FGFR4 degradation was dependent on both MT1-MMP and FGFR4 activities (Fig. 4*D*).

Importantly, the phosphorylation of the FGFR4-R388 risk allele was enhanced by MT1-MMP but not by MT1-E/A, whereas changes in FGFR4-G388 phosphorylation were less clear owing to the receptor down-regulation by MT1-MMP in MDA-MB-231 cells (Fig. 4*C*). Thus, in contrast to the reciprocally suppressive MT1-MMP/FGFR4-G388 complex, the MT1-MMP/FGFR4-R388 interaction sustains or enhances both the proteolytic and signaling activities of the complex (Fig. 4*D*).

FGFR4-R388 Risk Variant Induces Rapid MT1-MMP–Mediated Collagen Invasion. The significance of the FGFR4-R388–mediated MT1-MMP regulation in tumor cell invasion was tested in 3D collagen invasion assay. Importantly, the FGFR4-R388 risk variant increased the number of MDA-MB-231 cells that invaded >100 μm by ≈20-fold and total invasion by >4-fold (>30 μm), whereas FGFR4-R388-KD did not alter invasion (Fig. 5 *A* and *B*). The invasion was abolished by MT1-MMP knockdown (Fig. S9 *A* and *B*; ≈85% reduction of MT1-MMP mRNA by qPCR), indicating a functional link between FGFR4-R388 and MT1-MMP. FGFR4-G388 overexpression resulted in equal or even slower invasion relative to the

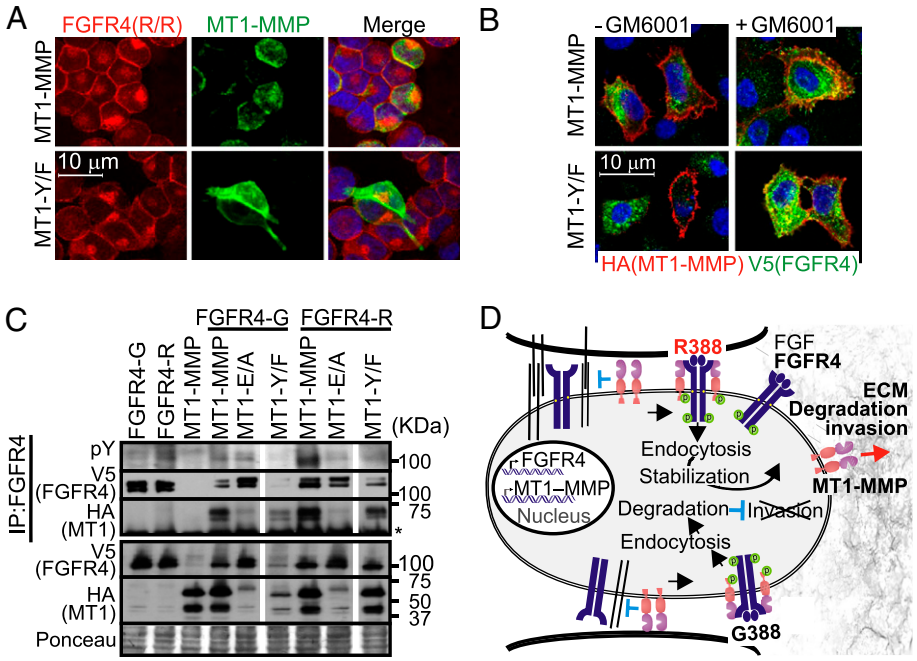

Fig. 4. Unphosphorylated cell-surface MT1-MMP induces FGFR4 internalization followed by G388R polymorphism–dependent FGFR4 regulation. (*A*) MDA-MB-453 cells (R/R; endogenous homozygous FGFR4-R388) were transfected to express wild-type MT1-MMP or the mutant MT1-Y/F protein (Fig. S7*A*), followed by immunofluorescence for FGFR4 (red) and MT1-MMP (green). (*B*) Confocal laser scanning micrographs of MT1-MMP (red) and FGFR4 (green) after MT1-MMP and MT1-Y/F overexpression in stable FGFR4-G388–expressing MDA-MB-231 cells (G/G) on collagen. The cells were treated with MMP inhibitor GM6001 (10 μM) for 16 h. Individual images of separate channels are shown in Fig. S7*D*. (*C*) HA-tagged MT1-MMP (MT1-MMP), enzymatically inactive mutant (MT1-EA), or unphosphorylated mutant (MT1-Y/F) were expressed alone or with V5-tagged FGFR4-G FGFR4-R in MDA-MB-231 cells, followed by immunoprecipitation and immunoblotting as indicated (n = 3). (*D*) Model for the function of FGFR4/MT1-MMP complexes. FGFR4 overexpression induces partially Src-dependent MT1-MMP phosphorylation and endocytosis in the membrane complexes. Differential stabilities of the activated FGFR4 variants determine whether these events result in synergistic FGFR4-R388 signaling and MT1-MMP activities promoting tumor cell invasion, or MT1-MMP down-regulation by FGFR4-G388. Upon overexpression at the cell surface, MT1-MMP promotes dissociation of cell–cell junctions and FGFR4 internalization. MT1-MMP thus induces the down-regulation of the unstable MT1-MMP–suppressive FGFR4-G388, allowing the proinvasive MT1-MMP function, whereas the activation of more stable FGFR4-R388 is promoted simultaneously with enhanced MT1-MMP activity.

mock-transfected cells (Fig. S9 *A* and *B*). Unlike the rapid invasion induced by FGFR4-R388, IRAK1 or MT1-MMP overexpression enhanced invasion mainly to the superficial layers of collagen gel (Fig. 5 *A* and *B*).

In the cells on collagen, the FGFR4-R388–induced invasion correlated with increased total and cell-surface levels of endogenous MT1-MMP (Fig. 5*C*). Both the activated 60-kDa MT1-MMP and its autocatalytically processed 43-kDa fragment that correlates with high MT1-MMP activity (15) were increased, whereas FGFR4-R388-KD protein did not markedly affect MT1-MMP (Fig. 5*C*). Notably, the increased MT1-MMP colocalized with the foci of increased gelatin proteolysis at the leading edges of the stable FGFR4-R388–expressing cells (Fig. 5*D* and Fig. S9*C*). Likewise, both MT1-MMP and FGFR4-R388 were clustered at the leading cell edges inside 3D collagen (Fig. 5*D*). FGFR4-R388- but not FGFR4-R388-KD–expressing cells also degraded and traversed a thin layer of cross-linked collagen within 3 h (Fig. 5*D* and Fig. S9*C*). The FGFR4-R388 risk variant thus enhanced pericellular ECM degradation by MT1-MMP in a polarized manner, which resulted in rapid tumor cell invasion in collagen (Fig. 4*D*).

Discussion

The ability of neoplastic cells to engage in tissue-invasive programs is critical for cancer progression (1). As one such program, many types of tumor cells up-regulate MT1-MMP that degrades covalently cross-linked networks of type IV collagen in basement membranes or fibrillar collagen in interstitial matrices (3, 35–37). Using a systematic screen for kinases that regulate MT1-MMP activity, we identified the FGFR4-R388 risk variant as a unique inducer of MT1-MMP and collagen invasion. The identification of IL and TNF pathway kinases and TGF-β receptors in the screen is consistent with the reported roles of these inflammatory mediators in MT1-MMP–mediated tissue remodeling (10, 12, 31) and also validates our screen.

Approximately half of humans carry homozygous or heterozygous FGFR4-G388R SNP variant, which has been linked to poor prognosis of patients with several types of tumors, such as adenocarcinomas of the breast, prostate, and colon, as well as head-and-neck squamous cell carcinomas and melanomas (23, 24, 38, 39). Although the corresponding mutation was found recently to increase invasion in a mouse knockin model (27), the underlying mechanisms have remained unclear (23, 25, 28, 40). We found that both FGFR4-R388 and FGFR4-G388 formed a complex with MT1-MMP and induced MT1-MMP tyrosyl phosphorylation, but they had opposite effects on MT1-MMP levels. FGFR4-R388 stabilized MT1-MMP, whereas the corresponding FGFR4-G388 down-regulated MT1-MMP. The Y573F point mutation that blocks MT1-MMP tyrosyl phosphorylation increased cell-surface MT1-MMP. However, the phosphorylation as such did not mediate MT1-MMP down-regulation by FGFR4-G388, because MT1-MMP phosphorylation was strongest during FGF2 or overexpression induced activation of the FGFR4-R388 risk allele simultaneously with the endosomal MT1-MMP stabilization.

In human dwarfism, the substitution of a hydrophobic G380 residue with a positively charged arginine in FGFR3 transmembrane region increases the kinase activity, stability, and recycling of this receptor (41, 42). Likewise, the increased stability of activated FGFR4-R388 results in its sustained autophosphorylation (28). In contrast to the MT1-MMP interaction that occurred not only with active and KD FGFR4 variants but also with FGFR2, the

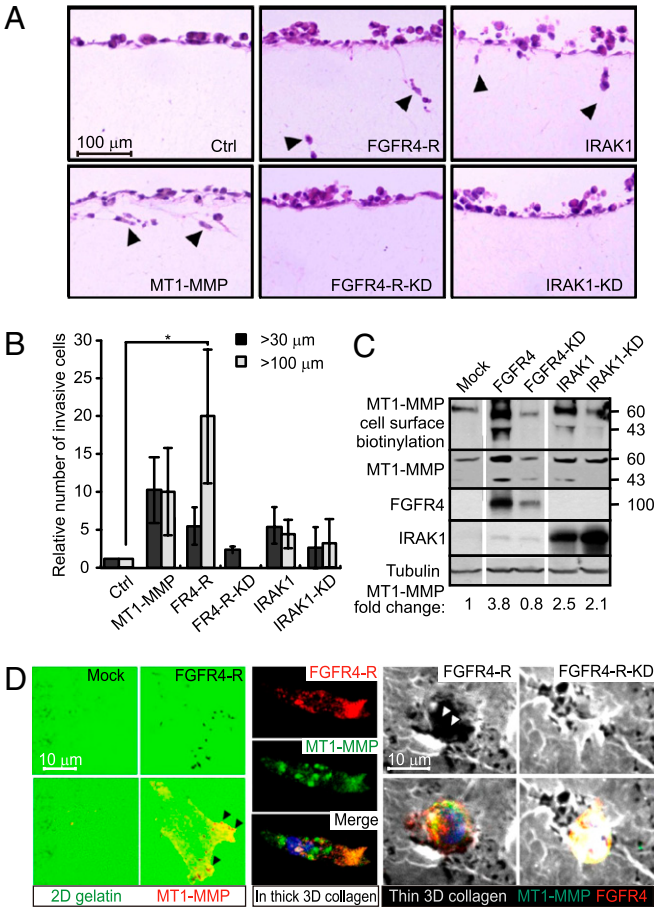

Fig. 5. FGFR4-R388 risk variant enhances collagen degradation and invasion. (*A*) MDA-MB-231 cells transiently overexpressing MT1-MMP, FGFR4-R, IRAK1, or the respective KD proteins were allowed to invade 3D type I collagen for 5 d. FGF-2 (25 ng/mL) was used as chemoattractant. Arrowheads indicate invading cells in H&E-stained cross-sections. (*B*) Quantitative results are expressed as the number of invasive cells per microscopic field that invaded >30 μm and >100 μm (mean ± SD, $n = 3$). (*C*) Cell-surface and total MT1-MMP content as well as FGFR4 and IRAK1 expression were detected in the cells on collagen. Relative levels of total MT1-MMP normalized with tubulin are indicated below each lane ($n = 3$). (*D*) Stable MDA-MB-231 cells expressing FGFR4-R and the respective KD protein were allowed to degrade Alexa-488–conjugated gelatin for 20 min (*Left*). Dark regions on bright fluorescent gelatin colocalize with MT1-MMP (red) and represent the foci of pericellular gelatin proteolysis (black arrowheads). MT1-MMP (green) and FGFR4 (red) were immunostained in cells invading in 3D collagen (*Center*). Arrowheads and yellow indicate colocalization. The cells were plated on a thin layer of 3D collagen for 3 h, followed by confocal reflection microscopy (*Right*). White arrowheads indicate degraded areas of collagen.

MT1-MMP stabilization was specific for the FGFR4-R388 risk variant. Increased FGFR4-R388 stability rather than the interaction thus correlated with the stabilization of the phosphorylated and endocytosed MT1-MMP. Therefore, the FGFR4-G388R SNP most likely alters the interactions of the activated receptor with vesicular sorting proteins similarly to FGFR3 (41) and enhances the trafficking of FGFR4/MT1-MMP complex to recycling instead of lysosomal degradation. Considering the related mutations also found in the transmembrane regions of FGFR1 and FGFR2 in bone disorders, as well as the functions of MT1-MMP and FGFRs in bone development, the potential significance of other FGFR–MT1-MMP interactions will be of interest under both physiological and pathological conditions (21, 22, 43, 44). Besides altered vesicular sorting and trafficking, MT1-MMP distribution is controlled by extracellular interactions with, for example, the ECM substrates that can stabilize MT1-MMP at the cell surface (45). Indeed, enhanced MT1-MMP was mainly localized intracellularly in the FGFR-R388–expressing MDA-MB-231 cells on plastic, whereas cell surface MT1-MMP was notably increased during rapid matrix degradation and cell invasion on collagen.

FGFR4 interacts with cell-adhesion receptors such as N-cadherin (46). Accordingly, the endogenous FGFR4-R388 risk allele was localized to the cell–cell junctions in MDA-MB-453 cells. In the reciprocal MT1-MMP/FGFR4 interaction, the cell-surface accumulated MT1-MMP-Y573F mutant down-regulated FGFR4 protein levels. This was seen as a dramatic down-regulation of FGFR4-G388 or simultaneous loss of lateral cell–cell junctions and the cell surface FGFR4-R388. Whereas MT1-MMP-Y573F stimulated FGFR4-R388 translocation into intracellular vesicles, enzymatically active wild-type MT1-MMP that colocalized with FGFR4-R388 in both cell–cell contacts and in the endosomes further promoted FGFR4-R388 activation. These results are consistent with a model whereby MT1-MMP phosphorylation and endocytosis are induced via the MT1-MMP/FGFR4 complex, whereas the cell-surface MT1-MMP promotes dissociation of cell–cell junctions in conjunction with FGFR4 phosphorylation and internalization (Fig. 4*D*). Differential stabilities of the activated FGFR4 SNP variants then determine whether these events result in synergistic ECM degradation by MT1-MMP and FGFR4-R388 signaling, or reciprocal FGFR4-G388 and MT1-MMP down-regulation.

FGFR4 is widely overexpressed in human epithelial carcinomas (26, 40, 47), where it can contribute to tumor progression by multiple mechanisms (23, 25, 27, 28, 40). Current results suggest that, depending on the level of MT1-MMP induction at the invasive tumor edges, the FGFR4-R388 risk variant–expressing cells would be expected to either sustain cell–cell adhesion and promote MT1-MMP–dependent tumor expansion and collective invasion into stroma or loose cell–cell adhesion and invade as single cells (3). The mutual suppression of FGFR4-G388 and MT1-MMP was likewise dependent on their relative levels and activities. Indeed, even the normally undetectable endogenous FGFR4-G388 in MDA-MB-231 cells became detectable after MMP inhibition. Considering the proliferative and antiapoptotic functions reported for both FGFR4 variants (26, 40), these results reveal a unique feedback mechanism for transient transition between proliferative and invasive cell phenotypes depending on local induction or cell-surface stabilization of MT1-MMP in the FGFR4-G388–expressing tumors (Fig. 4*D*). Our present results could thus help to understand mechanisms of cancer progression in individuals with either FGFR4 alleles.

Methods

Cell lines, cDNAs, antibodies, and other reagents are described in *SI Methods*.

MMP Screen. A total of 564 cDNAs of human kinases (29) were transfected to HT-1080 cells using FuGENE6 (Roche) in 96-well plates. The cells were incubated in complete medium for 24 h and in serum-free medium for 20 h. Aliquots of the conditioned medium were subjected to gelatin zymography (10). The secondary screen was performed in triplicate.

Matrix Degradation Invasion Assay. Cells on 488-Oregon-Green gelatin (2×10^4 cells/cm^2; Molecular Probes) were allowed to spread in complete medium in the presence of GM6001 (10 μM; Calbiochem) for 3 h at 37 °C. After removing GM6001, subjacent gelatin degradation was continued for 20 min. For collagen degradation, cells on thin layers of 3D collagen were incubated for 3 h at 37 °C. The fluorescence and collagen reflection confocal images were obtained by Zeiss510-DUO (Carl Zeiss). Collagen invasion was assessed essentially as previously described (35), with modifications described in *SI Methods*.

Immunoblotting, Immunoprecipitation, and Immunofluorescence. Immunofluorescence staining and cell-surface biotinylation were carried out as previously described (10, 15). Biotinylation was used to obtain results of relative MT1-MMP, MT1-Y/F, and FGFR4 cell-surface levels in Fig. 5*D* and Fig. S7. Fluorescence

images were obtained using an LSM 5 DUO confocal microscope (Carl Zeiss). Cell lysates were subjected to immunoprecipitation, SDS/PAGE, and immunoblotting (10, 15) or using anti-FGFR4 antibody–conjugated agarose (Santa Cruz Biotechnology) and anti-HA agarose affinity gels (Sigma).

Statistical Analysis. All numerical values represent mean ± SD. Statistical significance was determined using the Mann-Whitney test.

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