Tumor-Antagonizing Fibroblasts Secrete Prolargin as Tumor Suppressor in Hepatocellular Carcinoma

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Abbreviations used in this paper: HCC, hepatocellular carcinoma; CAF, cancer-associated fibroblasts; EMT, epithelial-mesenchymal transition; PRELP, prolargin, FFPE, formalin-fixed paraffin-embedded; NLF, normal liver fibroblast; CM, conditioned medium; TFA, trifluoroacetic acid; SLRP, small leucine-rich proteoglycan; MMP1, matrix metalloproteinase-1; MMP3, matrix metalloproteinase-3; PDAC pancreatic ductal adenocarcinoma; Alex, Alexander hepatoma cell line.

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Abstract

Hepatocellular carcinoma (HCC) is a difficult to cure primary liver cancer. Recent breakthroughs in cancer treatment highlight the importance of the tumor microenvironment (TME) and its ability to promote or suppress tumor development. While understanding of this dual behaviour is expanding for immune cells, little is known for other stromal cells, and notably cancer-associated fibroblasts (CAF). Here, we unveil a novel CAF tumor-antagonizing protein, prolargin, and unravel its regulation and mechanism of action in human HCC. Employing proteomics and single cell RNA sequencing data analysis we investigated prolargin expression and the cell of origin in human HCC. The significance for clinical outcome was examined in a cohort of HCC patients (N=188), while prolargin function was studied *in vivo* using orthotopic models. Biochemical studies along with structural modelling/docking were employed to elucidate the mechanism of action. Our findings show that the expression of prolargin is confined to portal fibroblast-derived CAF. Prolargin is secreted in the TME where its quantity positively correlated with patient outcome (HR=0.37; p=0.01). Aggressive HCC cells reduce prolargin levels mainly through matrix metalloprotease 3 (MMP3) activity. *In vivo*, tumors with lower prolargin expression displayed faster progression (5-fold; p=0.01) and stronger angiogenesis. Models of prolargin structure revealed a solenoid (horseshoe) folding, favouring its binding and inhibition of several growth factors, except vascular endothelial growth factor (VEGF). MMP-inhibition (batimastat) combined with VEGFR targeting (sorafenib) *in vivo* demonstrated superior tumor control compared to sorafenib treatment alone. Prolargin-expressing fibroblasts have tumor-antagonizing properties and stabilizing prolargin expression should be considered as therapeutic strategy for HCC.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of mortality from cancer worldwide.[1,2] By 2035, HCC is expected to become the second cause of cancer-related deaths (1.3 million people per year; GLOBOCAN, WHO). Despite the rising number of patients, HCC treatment remains limited, with patients facing poor survival outcomes (5-year, 18%).[3] Surgery and liver transplantation give the best curative chances (5-year survival rates of up to 70%), while molecular therapies are only palliative in nature. Sorafenib, a broad RTK inhibitor, prolongs survival of non-operable HCC patients for a mere 3 months when compared to placebo.[4] Nonetheless, sorafenib is the current standard of care for systemic treatment of HCC. New therapies are therefore urgently needed to face the challenges of liver cancer.

Recent breakthroughs in cancer immunotherapy highlight the significance of the tumor microenvironment.[5] It contains a plethora of cellular subtypes, of which immune cells and CAF are in majority.[6] While tumor-infiltrating immune cells are well established as a heterogeneous population with both cancer suppressing and promoting functions, CAF have been, and still largely are, regarded as genuine cancer cell collaborators.[7] In a static picture of a tumor, CAF engage in a multitude of tumor supporting processes.[8] This accounts for numerous attempts to target and deplete CAF in tumors in the hope of achieving a therapeutic benefit. However, beyond animal studies, where these strategies have indeed shown to be successful,[9, 10] we have yet to clinically prove the expected benefit in humans.[11] Current data clearly show that indiscriminate CAF targeting accelerates tumor progression.[12,13] Similar to the concept of immunity, fibroblasts are naturally programmed to suppress rather than to support cancer growth.[14-16] Carcinogenesis is a dynamic process, thus fibroblasts, along with other stromal cells, have to undergo programming steps in order to shift the balance from tumor protection to promotion.[17,18]

In line with the thought that not all CAF are the same, recent studies have demonstrated the co-existence of several CAF subtypes within the same tumor. Some of the subtypes are characterized with immunosuppressive and chemoresistant phenotypes.[19-21] However, we have had very little insight into the population of CAF that have tumor antagonizing properties. This is probably due to the fact that this cell population is in minority in advanced tumors. On the other hand, tumors that do not develop are presumably rich in such CAF and are obviously difficult to study. In this regard, only a handful of studies have characterized molecular markers of tumor-antagonizing CAF.[22-24] Recently, we uncovered asporin as a CAF-derived tumor suppressor protein in triple negative breast cancer that inhibits TGF-β1 signaling.[25]

In this study, we use for the first time a combination of innovative proteomics approach with single cell sequencing data analysis to better characterize the CAF in human HCC. Among numerous stromal proteins already mainly characterized as tumor promoters, a newly identified cancer-related protein, prolargin (PRELP), was found to be specifically expressed by portal-derived CAF. Here, we examine the molecular function of PRELP by highlighting its novel ability to antagonize tumor progression (*in vitro* and *in vivo*) and propose its significance for conceiving future HCC treatments.

Materials and Methods

Full description of Experimental Procedures is found in the Supplemental Material section.

Patient information and clinical samples

The study was conducted in compliance with the Declaration of Helsinki. The use of human material was approved by the institutional ethical committees of the University Hospital of Liège and Gunma University Graduate School of Medicine. Clinical information concerning the patients used in the present study are outlined in the Supplemental Data, **Table S1**.

Single Cell RNA-seq Analysis

We reanalysed previously published single cell RNA-seq analysis involving 9 HCC patients.[26] Processed 10X Genomics (Pleasanton, CA, USA) data were downloaded from GEO repository (GSE125449). They were imported into R computational environment (4.0) and then processed using *Seurat 3.1* package and default parameters.[27] CAF identification/origin (stellate or portal fibroblast) was inferred by comparing the present data set to the single cell study of Dobie et al.[28] Ligand-receptor analysis was conducted using *SingleCellSignalR* package.[29]

Immunohistochemistry (IHC) & Immunofluorescence (IF)

Formalin-fixed paraffin-embedded (FFPE) tissue sections were prepared from primary hepatocellular carcinoma lesions and from xenografted tumors. Tissue samples were sliced from paraffin blocks (5-µm sections), deparaffinated three times in xylene for 5 min and hydrated in a methanol gradient (100%, 95%, 70%, and 50%). Blocking of unspecific peroxidase activity was performed for 30 min with 3% H_2O_2 and 90% methanol. Diluted ImmunoSaver solution (1:200 in DDW, Nisshin EM, Tokyo, Japan) was used for antigen retrieval. Tissues were treated with Protein Block Serum-Free solution (Protein Block Serum-Free Ready-to-Use, catalog no. X0909, Dako, Glostrup, Denmark) for 30 min at room temperature, preceding the primary antibody

incubation. Primary antibodies used for IHC and IF are described in the Supplemental Materials section. They were detected with corresponding secondary antibody: i) donkey anti-sheep IgG H&L (HRP polymer) (catalog no. ab214884, Abcam) or ii) M-Histofine Simple Stain MAX PO (Multi) (catalog no. 414152F, Nichirei Biosciences Inc., Tokyo, Japan). For IHC signals were detected using 3,3'-diaminobenzidine tetrachlorhydrate dihydrate (DAB) in 5% H_2O_2. The slides were counterstained with hematoxylin and dehydrated with ethanol and xylene for mounting. For multiplexed immunohistochemistry signals were detected using Opal™ 4-Plex Kit according to the manufacturer's protocol (catalog no. NEL810001KT; Perkin Elmer, Waltham, MA, USA). For more details see Supplemental Materials section.

Cell culture

Human hepatocellular carcinoma (HCC) cell lines HepG2, HUH7, Alexander (PLC/PRF/5), HLE and HLF were obtained from Japan Collection of Research Bioresources Cell Bank (Osaka, Japan). H-6019 human primary liver fibroblast cell line (NLF) was obtained from Cell Biologics (Chicago, IL, US). Both cancer cells and liver fibroblasts were maintained in DMEM supplemented with 10% heat inactivated FBS and 1% penicillin/streptomycin (all from Gibco, Thermo Fisher Sci., Waltham, MA, USA).

HLF cancer cells were silenced for MMP1 and MMP3 expression using following siRNA (Horizon Discovery, Waterbeach, UK): human anti-*MMP1* (siGENOME Human MMP1, catalog no. M-005951-01-0005), anti-*MMP3* (siGENOME Human MMP3, catalog no. M-005968-03-0005), scramble siRNA (ON-TARGETplus NonTargeting Control Pool, catalog no. D-001810-10-05). The cells were transfected with 20 nM of each siRNA using Lipofectamine (Lipofectamine 2000 reagent, catalog no. 11668-019, Life Technologies, Carlsbad, CA, USA).

Western blot analysis

Total proteins from both tissues and cells were extracted using 1% SDS buffer [40mM Tris-HCl (pH 7.6), 1% sodium dodecyl sulfate (SDS) and protease/phosphatase inhibitor cocktails (catalog no. 16829900; Sigma-Aldrich). CM samples were concentrated 10-fold using Amicon Ultra-0.5 3kDa filters (catalog no. UFC500324; Millipore), while the cell culture medium was exchanged with the lysis buffer (same as described above). The protein content of all samples was determined using the Pierce BCA Protein Assay Kit (catalog no. 23225; Thermo Scientific). Twenty micrograms of proteins were supplemented with Laemmli buffer (0.1% 2-mercaptoethanol, 0.0005% bromophenol blue, 10% glycerol, 2% SDS in 63 mM Tris-HCl (pH

6.8)), were separated on 10% polyacrylamide denaturing gel and transferred to nitrocellulose membranes for Western blotting.

Murine in vivo models

All experimental procedures used in the current work were performed in accordance with the ARRIVE ethical guidelines.[30] They were reviewed and approved by the Ethical Committees of the Bioresource Center in Gunma University (Japan) and the French National Committee of animal experimentation. To evaluate the impact of fibroblast-secreted PRELP on tumor development, HUH7-RFP-Luc cells ($0.5x10^6$ cells) and NLF-shNT or -shPRELP ($0.5x10^6$ cells) were suspended in 20 µl of cell culture medium and growth-factor-reduced Matrigel (catalog no. 356230; BD Biosciences) 1:1 and co-injected into the liver of six-week old athymic nude mice (n=5 per condition).

Gene expression analysis

Total RNA was isolated with the Nucleospin RNA Isolation Kit (catalog no. MN740955; Macherey-Nagel, Dueren, Germany). According to the manufacturer's instructions, one microgram of RNA was reverse-transcribed using the SuperScript III Reverse Transcriptase (catalog no. 18080; Invitrogen, Carlsbad, CA, USA). Twenty nanograms of cDNA were used for respective PCR reactions.

Surface plasmon resonance analysis

Surface plasmon resonance (SPR) measurements were performed on a BIAcore X100 instrument (GE-Healthcare, Chicago, IL, USA).

Results

Prolargin is a novel CAF-derived protein of human HCC. We have previously developed a proteomic method to characterize extracellular and membrane-bound proteins in fresh human tumors.[31,32] Here we employed this methodology for the first time on fresh human HCC and non-tumoral liver specimens from 6 individual patients (**Figure S1**). We identified 144 significantly modulated proteins in at least 3 out of 6 patients (**Figure S1a**). The proteins in question mainly participate in extracellular matrix organization (**Figure S1b**). Pathway analysis (KEGG) revealed alterations in ECM-receptor interaction, focal adhesion and notably the PI3K-Akt signalling pathway (**Figure S1b**). These intriguing findings led us to perform further network analysis using STRING software. The most prominent network was observed around a strong collagen

cluster, complemented by known collagen-interactors such as fibronectin (FN1), periostin (POSTN) and tenascin (TNC) (**Figure S1c**). We also observed asporin (ASPN), a small leucine-rich proteoglycan (SLRP) that was previously described by our group as a fibroblast-derived tumor suppressor in triple negative breast cancer. The analysis highlighted another SLRP protein; prolargin (PRELP), which to the best of our knowledge has not yet been functionally described in cancer. We thus sought to better understand the significance of prolargin modulation in the context of human HCC. To this end, we first aimed to delineate the cell population responsible for prolargin expression. We have re-analysed recently published single cell RNAseq data in human HCC.[26] As outlined in **Figure 1a**, 16 cell populations were identified using tSNE analysis, including 3 distinct CAF cell populations. Comparative analysis with recently published data on liver fibrosis,[28] clearly identified 2 of these clusters as hepatic stellate cell-derived CAF, while one cluster was derived from portal fibroblasts. Based on relevant markers permitting to distinguish these two cell types (**Figure S2a)**, prolargin mRNA was exclusively found in portal fibroblasts-derived CAF (**Figure 1b**). Examination of PRELP protein expression and localisation in normal liver confirmed its localisation in portal areas and not in the liver sinusoids (**Figure 1c**). PRELP was found expressed in the tumor (α-SMA$^+$/PRELP$^+$ cells), however not all CAF were positive for PRELP (α-SMA$^+$/PRELP$^-$), emphasizing the expected heterogeneity from single cell analysis (**Figure 1d**).

Based on single cell data, portal fibroblast-derived CAF represented only a minority population of all CAF in HCC, which were mainly stellate cell-derived (**Figure 1a**). Thus, we next sought to estimate the importance of the individual CAF populations with respect to their communication with other stromal and cancer cells in HCC. For this purpose we examined ligand-receptor expression on individual populations and used inference of intercellular networks to quantify and score these interactions.[29] Surprisingly, and as shown in the **Figure S2b**, portal fibroblast-derived CAF although in minority had 3-times more interactions with other HCC cell populations compared to stellate cell-derived CAF.

Having identified the cells that express prolargin, we next sought to better understand its function in HCC. To this end, we performed IHC analysis on HCC patients with the objective to examine its relationship with patient survival (clinical details are outlined in **Table S1**). Our findings showed that prolargin levels positively correlated with good overall survival (HR = 0.37, p = 0.01, **Figure 1e**).

Extracellular prolargin levels are controlled by MMP-mediated degradation. In order to functionally study prolargin we established a cell line model. This model was based on a set of five HCC cell lines that vary in their aggressiveness, as well as one primary cell line of normal

hepatic fibroblasts (denoted NLF). In clonogenicity assay, HLE, HLF and Alexander cells formed large and dense colonies, while HepG2 and HUH7 in this regard were less aggressive (**Figure 2a**). In accordance with their aggressiveness, on the molecular level HepG2 and HUH7 cells had a pronounced epithelial phenotype ($CDH1^{high}/VIM^{low}$), while Alexander, HLE and HLF were mesenchymal-like ($CDH1^{low}/VIM^{high}$) (**Figure S3a**). Proteomic analysis of conditioned media (CM) derived from all the five cancer cell lines suggested that HepG2 and HUH7 cells were particularly well differentiated, with certain secretory aspects similar to hepatocytes (e.g. secretion of albumin) (**Figure S3b**). In contrast to this, we found increasing levels of several proteases in CM from HLE, HLF and Alexander compared to HepG2 and HuH7 cells (**Figure 2b**).

In line with the evidence from single cell analysis, prolargin expression was not detectable in any of the cancer cells, while it was positive in NLF (**Figure S4a**). Next, we assessed the response of NLF following 48h treatment with CM from the individual cancer cell lines. As prolargin is a secreted protein, we sought to verify the modulation of prolargin levels in the medium of CM-treated fibroblasts (intracellular prolargin levels were unaffected, **Figure S4a**). Extracellular levels revealed that prolargin was gradually degraded upon NLF treatment with media of cancer cells with increasing aggressiveness potential HepG2 < HUH7 < Alexander < HLE < HLF (**Figure 2c**). To delineate the mechanism of prolargin degradation we incubated CM from HUH7 and HLF cells with recombinant prolargin and evaluated the respective protein levels at different time points. In remarkable contrast to HUH7 cells, CM obtained from HLF cells was able to rapidly degrade exogenously added prolargin (**Figure 2d**). To further narrow down which proteases are involved we tested two MMP inhibitors, batimastat and GM6001, as well as amiloride HCL (inhibitor of urokinase-type plasminogen activator (uPA)), and a broad protease inhibitor cocktail (Roche Complete). As demonstrated in **Figure S4b**, both batimastat and GM6001 were able to inhibit prolargin degradation in the CM from HLF cells, suggesting that MMP play a major role. We next sought to determine the identity of the MMP involved, and thus performed a pull-down of recombinant prolargin incubated with different cancer cell-CM. Mass spectrometry analysis revealed MMP1 and MMP3 proteins to be the potential degradation enzymes (**Figure S4c**). The selective silencing of MMP1 and MMP3 (as well as the combination of both, **Figure S4d**) confirmed MMP3 as the main protease involved in the cancer cell-mediated degradation of secreted prolargin (**Figure 2e** and **Figure S4e**). Based on these findings we next examined human HCC for MMP3 and prolargin expression on a limited cohort of patients (N=40). The analysis showed a significant inverse relationship between the levels of the

two proteins, suggesting that the mechanism described *in vitro* might also apply *in vivo* (**Figure 2f**).

Prolargin is a CAF-derived tumor suppressor with antiangiogenic features. Based on our observations made in both patients and *in vitro*, we assumed that prolargin might function as a fibroblast-derived tumor suppressor. In order to verify this, we grafted human HCC cells with fibroblasts expressing different levels of prolargin in the liver of nude mice. Human NLF were silenced for prolargin expression using shRNA (**Figure S5a/b**). These cells, or their non-silenced counterparts, were then grafted with HUH7 cancer cells. A luciferase expression vector in HUH7 cells permitted a regular monitoring of tumor development *in vivo*. Our findings show that HUH7 cells, when co-injected with prolargin-depleted fibroblasts, developed faster growing HCC in comparison to the same cells co-injected with fibroblasts that were not silenced for prolargin (**Figure 3a**). To further evaluate the mechanism behind this tumor suppressive behaviour of prolargin, we analysed the tumors recovered from HUH7-NLF orthotopic co-injection. Initial histological inspection revealed clear signs of necrosis in the prolargin proficient tumors. Prolargin deficient tumors, were compact with no sign of necrosis. These observations suggested that prolargin proficient tumors were less oxygenated. We further explored this by evaluating the density of vasculature/endothelial cells (CD31) in both tumors (**Figure 3b**). In contrast to those tumors where prolargin was silenced, the vasculature in wild-type tumors presented higher number of smaller vessels typically found during neo-vascularization. These findings collectively prompted us to put forward the idea that CAF-derived prolargin could be interfering in the crosstalk between cancer and endothelial cells, leading to the suppression of tumor angiogenesis. To explore this, we used ligand-receptor analysis on the single cell data. We specifically charted the interaction of portal fibroblast-derived CAF which express prolargin with different sub-clusters of endothelial cells identified in the t-SNE analysis (see **Figure 1a**). Portal fibroblast-derived CAF engaged in numerous strong interactions with all endothelial cell clusters (**Figure 3c** and **Figure S5c**), notably via collagen binding to vascular endothelial growth factor receptors 2 and 3 (VEGFR2/3), decorin interaction with c-MET and angiopoietin-like 4 (ANGPTL4) interaction with tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (TIE1). To further test the relationship between prolargin, tumor angiogenesis and endothelial cells we performed proliferation and spheroid sprouting assays using human umbilical vein endothelial cells (HUVEC). CM obtained from Alexander or HLE cells when mixed with recombinant prolargin were significantly less potent in inducing both HUVEC proliferation and sprouting (**Figure 3d**). Together these data suggested that prolargin exerts its activity through a possible interaction with pro-angiogenic growth factors in the extracellular space.

Prolargin binds several key growth factors and inhibits their activity. In order to investigate the mechanism behind the prolargin anti-tumor function, we performed surface plasmon resonance. Prolargin was immobilized on the chip and 15 pro-angiogenic growth factors were screened for binding (**Figure 4a**). Of the 15 growth factors, prolargin demonstrated binding affinity towards 7 molecules, most notably FGF1, FGF2, HGF and TGF-β1. Interestingly, no binding was detected for VEGFA or VEGFC, a prominent angiogenic/lymphangiogenic factors. We next sought to understand how prolargin could interact with these growth factors on molecular level. To do so, we studied the ligand-receptor interactions using protein docking methods. In absence of crystal structure, we constructed a homology model of PRELP (**Figure S6a/b**) and used it to dock with known structures of several growth factors. As shown in the **Figure 6a/b**, PRELP model adopted a solenoid (horseshoe) folding. The most obvious structural feature of PRELP that could affect ligand binding is shape complementarity (Sc).[33] The computed 'Sc' ratio was highest between PRELP and HGF (Sc~0.7) and lowest for the FGF1 ligand (Sc~0.6). Further, each of the high-affinity ligands (FGF1/2 and HGF) in this study docked within the curve of the solenoid, while TNFA and VEGFA did not (**Figure 4b**). In support of this, previous studies using other leucine-rich repeat (LRR) containing proteins have demonstrated similar docking behaviour when the interaction between LRR and growth factors takes place.[34] We thus concluded that high-affinity binding in these growth factors likely favour productive binding that maximizes surface area exposure between receptor and ligand, as well as maximizing H-bond interactions between PRELP and the various growth factors. Lower affinity growth factor interactions, for example between PRELP and FGF1, are predicted to bind with a smaller footprint on the inner surface of PRELP.

Next, we sought to examine if prolargin binding neutralizes or enhances the activity of the respective growth factors. We thus incubated recombinant prolargin with FGF1, FGF2, HGF, TGF-β1 and VEGF respectively, and then tested growth factor activity on HUVEC as target cells (sprouting and migration assays) (**Figure 4c/d**). Prolargin inhibited the function of all the tested growth factors, with exception of VEGF, whereas prolargin denaturation by high temperature suppressed its functional activity (shown for HGF in **Figure 4d**). Among the different growth factors, HGF is particularly relevant in the context of HCC. The latter is known for its key ability to promote angiogenesis, fuel therapy resistance and enhance metastasis. Incubation of prolargin with HGF inhibited the activation of c-MET in both Alexander and HLE cells, as well as HUVEC (**Figure 4e** and **Figure S6c**). This inhibition functionally resulted in reduced scattering and migratory capacity of HLE cancer cells (**Figure 4f/g**). Together the data suggested that

prolargin's ability to bind and inhibit a broad range of growth factors has multiple paracrine effects, both on cancer and endothelial cells.

Inhibition of prolargin degradation and VEGFR targeting is a meaningful therapeutic combination. Considering that prolargin binds to multiple growth factors and inhibits angiogenesis we aimed to test if stabilization of prolargin using MMP inhibitors (such as batimastat) could be therapeutically exploited. Knowing however that prolargin has no effect on VEGFA/VEGFC, and hence the activity of their receptors, we sought to combine prolargin stabilization with another treatment that should act mainly on VEGFR-1/-2/-3. To assess this we selected sorafenib as this inhibitor acts mainly on VEGFR-1, VEGFR-2, VEGFR-3 and PDGFR-β.[35] Additionally, sorafenib is already used as first-line systemic treatment for HCC, despite having shown only partial tumor regression effects in HCC-bearing animals.[36] To reduce the number of animals used in subsequent experiments we examined the impact of individual drugs and combination on HLF tumors in chick chorioallantoic membrane model (CAM). We chose HLF cells because they were the most aggressive in our panel and we have added to them NLF cells as natural source of prolargin. CAM HCC tumors were treated with placebo, sorafenib, batimastat or a combination of sorafenib and batimastat. Both drugs were used in the nM range, known to be sufficient to inhibit only MMP and VEGFR activity, without causing toxicity to cancer cells. As shown in **Figure 5a**, within the 7 days of tumor development we could not observe a significant effect with either drug alone. However, the combination of sorafenib and batimastat significantly reduced the tumor volume (50% reduction) compared to placebo or either treatments alone. Western blot quantification of prolargin expression in CAM tumors confirmed higher levels following batimastat treatment (**Figure 5b**). Based on these data we proceeded to the *in vivo* study of mice bearing orthotopically transplanted HLF cells that were treated with two lines of therapy: i) sorafenib (benchmark for HCC) and ii) sorafenib and batimastat combination treatment. As shown in **Figure 5c**, HLF tumors started to develop at day 38 despite the on-going treatment with sorafenib, suggesting that *in vivo* these cells can rapidly develop tolerance to the dose used. Remarkably, the combination of sorafenib and batimastat successfully controlled tumor growth, with no significant tumor growth until the end of the experiment (day 52).

Discussion

Cancer-associated fibroblasts have traditionally been regarded as a supportive part of a tumor microenvironment. Contrary to this view, here we describe prolargin, a novel protein characterizing a tumor-antagonizing subtype of CAF in human hepatocellular carcinoma. For the

first time, we identify a CAF-derived protein with a pleiotropic capacity to bind and inhibit the function of many crucial growth factors. This in turn leads to a significant decrease in HCC development *in vivo*, owing at least in part to the inhibition of tumor angiogenesis. Whether alterations of prolargin expression are also causative for HCC development still remains to be determined. Our data however suggest that prolargin probably belongs to a larger group of stromal proteins that need to be lost or their levels diminished for successful progression of HCC. Until recently, tumor-suppressive functions of CAF were not widely acknowledged, and thus the observations of both tumor promoting and suppressive roles were regarded as contrasting and controversial.[9,12,13,37] This apparent dichotomy could be reconciled by understanding that CAF are a heterogeneous population. One direct reason for CAF heterogeneity is their respective cells of origin. Just like normal fibroblasts, CAF can be derived from multiple cellular sources.[38] In the HCC, there are at least two different source of CAF: stellate cells and portal fibroblasts. In the current study, we confirmed this and showed based on single cell RNAseq data those two populations of CAF in a collection HCC cases. Importantly here, prolargin was solely expressed by portal fibroblast-derived CAF, and it was this population (despite being in minority) that had the most numerous ligand-receptor interactions with other cell types in HCC. CAF heterogeneity has been recently shown in numerus types of tumors. Interestingly, in a recent study, Öhlund and collaborators[19] identified two CAF populations in pancreatic ductal carcinoma (PDAC), one with high α-SMA and the other with low α-SMA expression. The α-SMA^{high} CAF population was found in close vicinity of the tumoral cells (termed myCAF), while the α-SMA^{low} CAF population was located at a distance from cancer cells (named iCAF). The authors placed both CAF populations in the tumor-supporting category. However, this classification was not done based on function, but solely on transcriptomic profiles. The latter suggests that the α-SMA^{high} myCAF population is mainly involved in desmoplastic reaction. Conversely, α-SMA^{low} iCAF secrete immunosuppressive cytokines. We previously identified prolargin as being modulated in human PDAC using proteomic analysis of accessible proteins.[31] A detailed analysis of data from Öhlund et al.[19] indicates that prolargin is overexpressed 35-fold in iCAF when compared to myCAF (see dataset GSE93313). This suggests a stronger prolargin expression away from the cancer cells, which is understandable knowing the tumor suppressive function of prolargin at least in HCC. In yet another study, Costa et al.[20] have shown the existence of four CAF subtypes in breast cancer and Li et al.[39] identified two distinct CAF subtypes in colorectal cancer. While the functional significance of different CAF population is not always clear, a few studies succeeded in identifying certain CAF subpopulations that bear particular tumor promoting functions. For example, Su et al.[21] identified

$CD10^+GPR77^+$ CAF as a relevant population promoting chemoresistance and stemness in lung cancer. Costa et al. identified $CD29^+FAP^+$ CAF as key to recruiting $CD4^+CD25^+$ immunosuppressive T cells in breast cancer. Interestingly, Su et al. showed that the percentage of $CD10^+GPR77^+$ CAF in both breast and lung tumors is prognostic of disease-free survival. Conversely, findings from a study by Costa et al. lacked a similar correlation concerning $CD29^+FAP^+$ CAF. This raises a pertinent question if these newly identified CAF subpopulations do bear clinical significance. One evident explanation could be the discrepancy between gene and protein expression and the difficulty to predict functions from gene expression data (such as single cell RNAseq). To make things more complex, and as the present case of prolargin shows, for secreted proteins intra- and extra-cellular levels can additionally vary due to other regulation mechanisms such as degradation. Our present data based on protein expression clearly shows that the levels of prolargin secreting fibroblasts are positively correlated with good clinical outcome in HCC patients (overall survival). In our previous study on breast cancer, we reported asporin as a marker of tumor-antagonizing CAF, while also demonstrating its strong correlation with patient outcome.[25] Admittedly, the patient cohorts used in both the present study and our previous study[25] are not representative of all HCC or breast cancer patients. This is because they include specimens exclusively from operated cases (30% were also transplanted), and hence these patients already have better survival than non-operable patients. Nevertheless, the ability of both asporin and prolargin to distinguish patients with better overall survival in breast cancer and HCC cohorts respectively, even among patients with better outcomes, highlights the strength of these as predictive markers.

In the future, successful cancer treatment will involve targeting of the microenvironment/stroma and at least some aspects of personalized medicine. Thus, for every new molecular treatment, clinicians will require markers that enable the use of the right drug. One of the most important findings of our study is the identification of prolargin as a putative biomarker. Indeed, we show that MMP inhibitors prevent prolargin degradation, and thus impede aggressive cancer cells to eliminate this natural growth factor inhibitor. While MMP inhibition as a treatment concept has suffered from clinical failure because of toxicity, lessons learned suggest that the choice of the right patient and correct timing may have been critical.[40] Based on the present data, it is not unreasonable to suggest that the right patients for MMP treatment in HCC are those that have low prolargin expression. The drug dose could be individually adjusted by monitoring circulating prolargin levels, which in turn may limit toxicity. Here, further studies are needed to examine if serum levels of prolagin are sufficient to detect changes in its overall secretion/ degradation. The present *in vitro* and *in vivo* findings suggest that sorafenib might be

usefully combined with MMP-inhibitors given that prolargin does not affect VEGFR activity. Here, we had at our disposal only commercially available drugs that have new generation compounds with better solubility and activity. Even so, our *in viv*o data showed a remarkable control of tumor growth when the two drugs were combined together. These results call for further studies aimed at developing treatments that will augment prolargin levels in HCC. Additionally, research should also focus on determining the effectiveness of prolargin testing for disease follow-up, prediction and prognosis.

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Figures

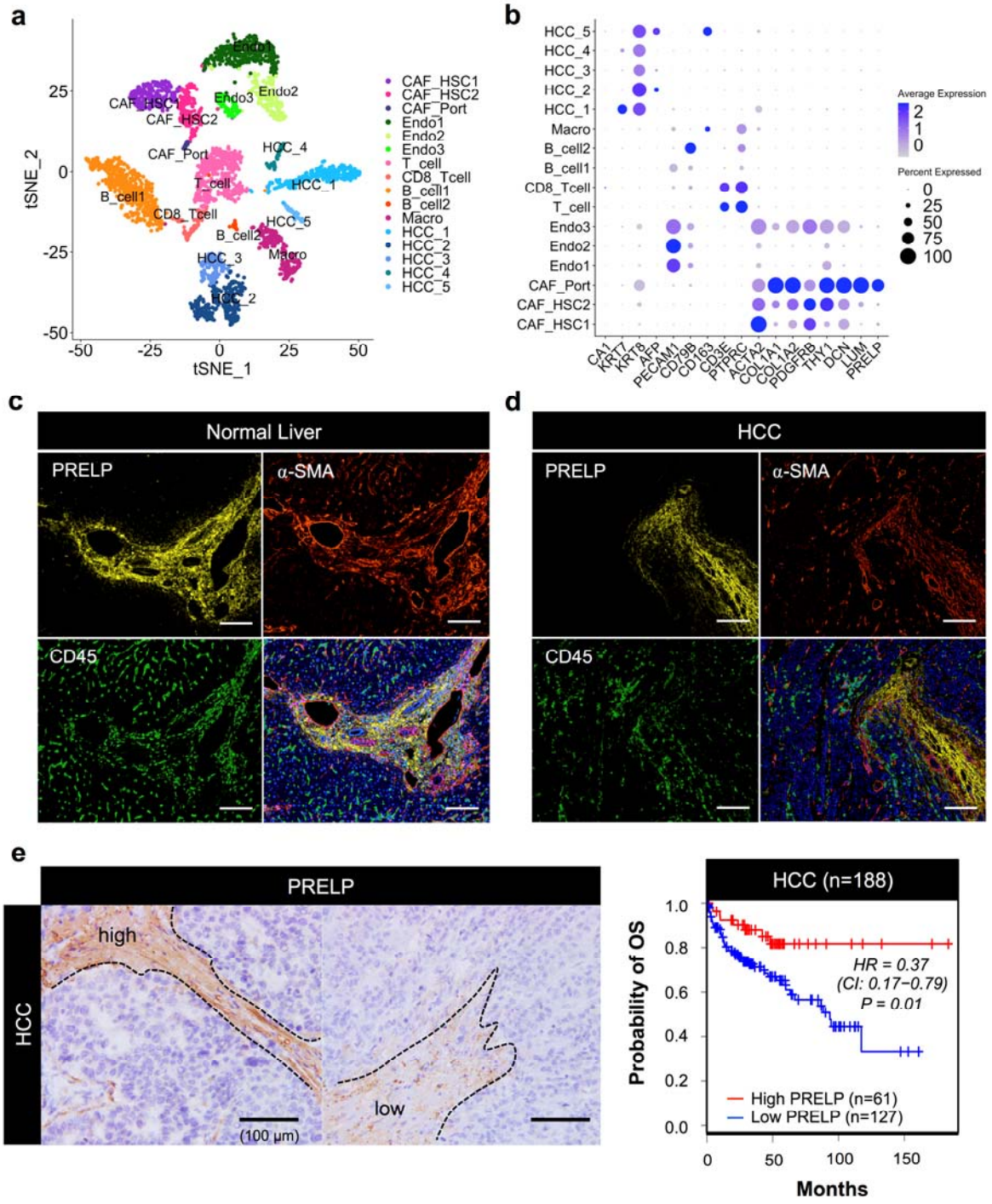

Figure 1: Prolargin is expressed by CAF deriving from portal fibroblasts. **a.** t-SNE plot of different cell populations found in the HCC from 9 patients. **b.** PRELP expression in individual cell populations. Panels **a** and **b**: single cell RNAseq analysis of previously published dataset GSE125449. **c./d.** IF analysis of PRELP expression in human normal liver (**c**) and liver cancer (HCC) PRELP is co-stained with αSMA (stellate/fibroblast cells) and CD45 (immune cells). Shown are representative images of 20 individual cases. **e.** IHC analysis of PRELP expression in human HCC (N=188 cases). Shown are representative cases with high/low PRELP expression in the HCC lesion. High PRELP levels correlate with good clinical outcome in human HCC. Kaplan-Meier survival curve indicates the probability of overall survival (OS).

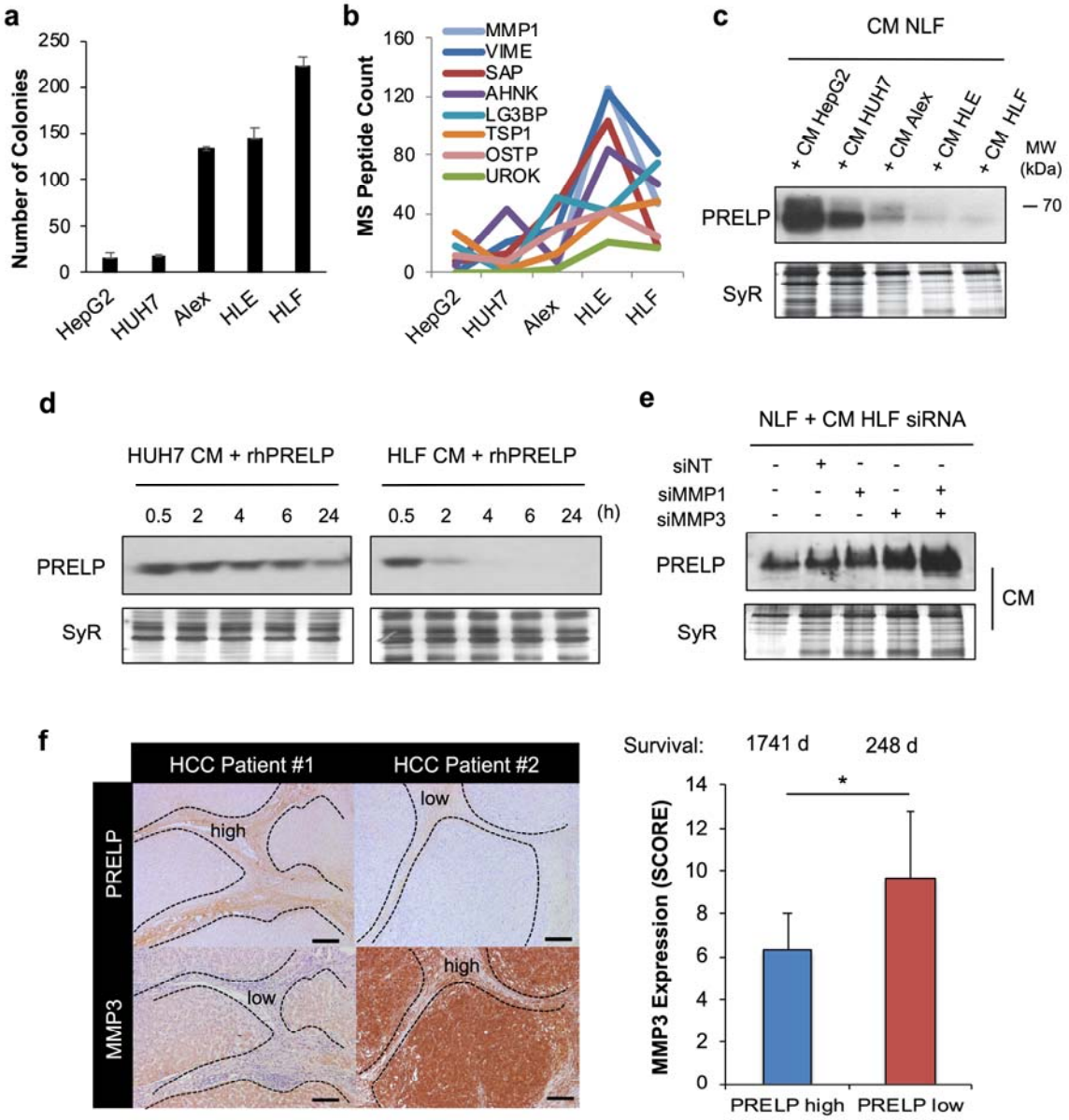

Figure 2: Extracellular prolargin levels depend on cancer cell MMP1 and MMP3 expression. **a.** Colony formation ability of 5-cell HCC panel. **b.** Proteomic analysis of the secretome obtained different HCC cells. Displayed are the proteins that increase in expression along with increasing aggressiveness of HCC cells lines. **c.** Levels of extracellular NLF-derived PRELP following the challenge of NLF with conditioned media (CM) from cancer cells. **d.** CM from HUH7 and HLF cells incubated with recombinant human PRELP at 37°C. **e.** Treatment of NLF with CM from HLF cells obtained following their silencing for MMP1 and MMP3. Panels **c**, **d** and **e**: Total protein stain with SYPRO Ruby (SyR) were used for normalization. **f.** Expression of MMP3 in a selection of PRELP high- and low-expressing HCC cases (20 patients each). Stroma is delineated with dashed lines; shown are serial sections. Average survivals are indicated in days (top of the graph). *denotes statistical significance with $p<0.05$, error bars are standard deviations of means.

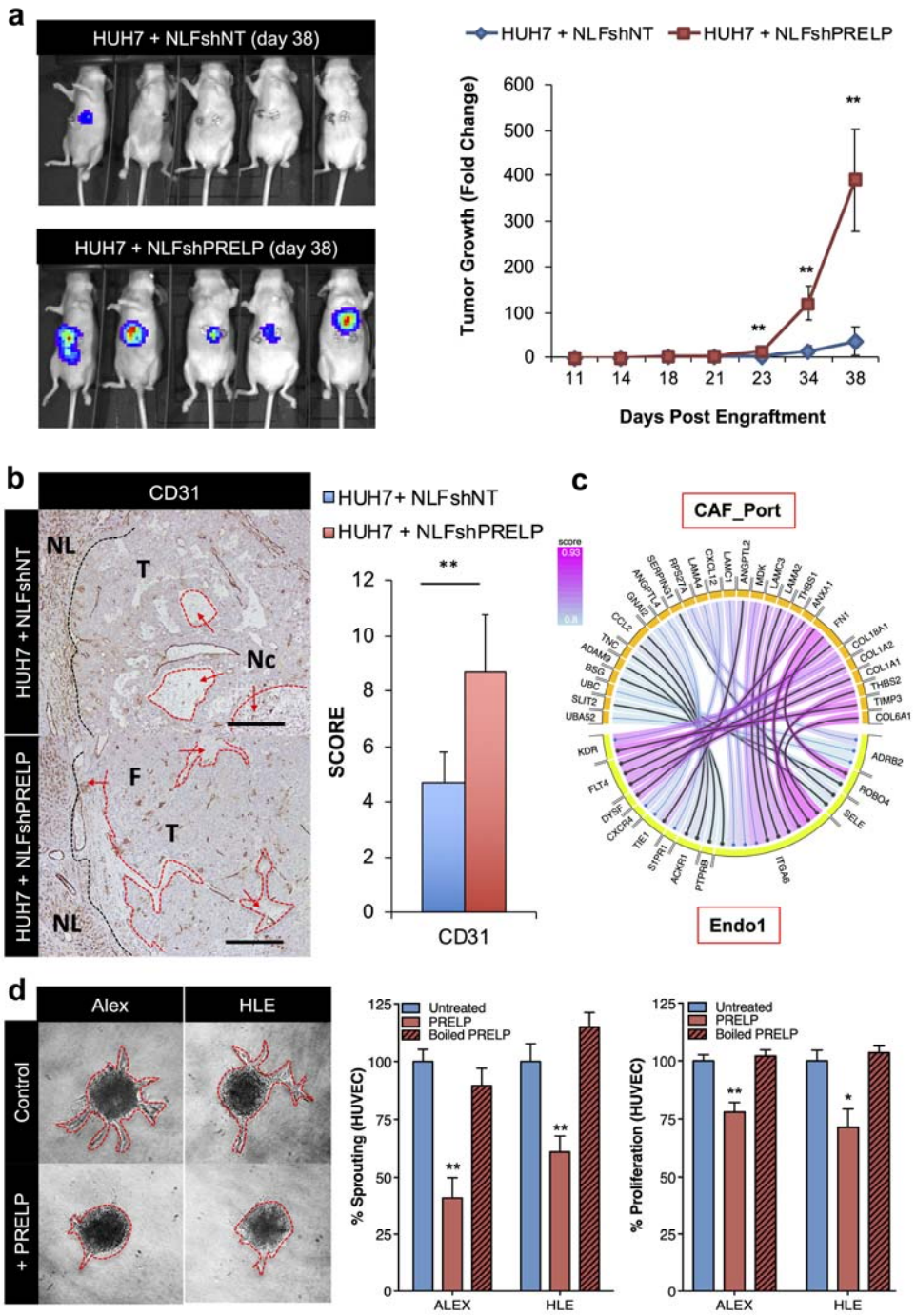

Figure 3: Prolargin inhibits tumor growth by suppressing angiogenesis. **a.** (*left panel*) Bioluminescence imaging of control and prolargin silenced orthotopic xenografts. Red color indicates the highest and blue the lowest fluorescent intensity. (*right panel*) Fold-changes of average radiance in liver tumors (compared to day 7 post engraftment). The data are presented as mean ± standard error of means (N=5 for each group). Statistical significance was calculated using Student's *t*-test (*: 0.05<p **: p<0.01). **b.** Histological analysis of control and PRELP silenced tumors, showing the distribution/quantification of murine vasculature/endothelial cells (CD31). Labelled are: normal liver (NL), tumor region (T), fibrosis (F) and necrosis (Nc). **c.** Ligand-receptor analysis based on single cell RNAseq data. Shown is the interaction between portal-fibroblast derived CAF and endothelial cell cluster 1 (see **Figure 1** and **Figure S5**). **d.** Proliferation and sprouting assay using HUVEC treated with conditioned media of Alexander and HLE cells supplemented with recombinant PRELP. **b** and **d**: */**denote statistical significance with p<0.05 and p<0.01 respectively, error bars are standard deviations of means.

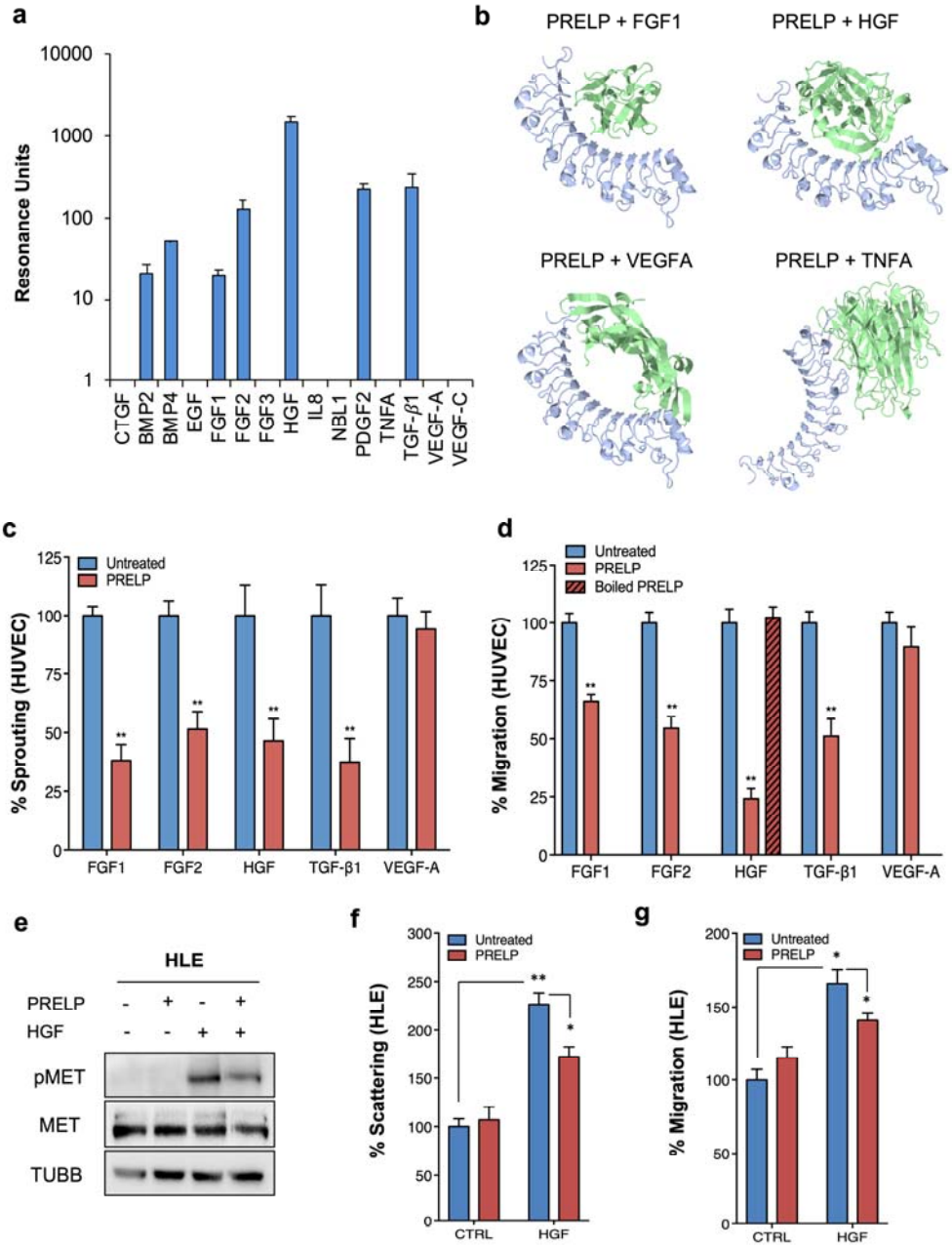

Figure 4: Prolargin binds pro-angiogenic growth factors and inhibits their activity. **a.** SPR profiling of growth factor binding to recombinant PRELP. **b.** Highest scoring docking solutions between PRELP and FGF1, HGF, VEGFA and TNFA. **c.** Sprouting assay using HUVEC treated with various growth factors or growth factors pre-incubated with recombinant prolargin. **d.** Migration assay with HUVEC; same conditions as panel (**c**). **e.** Western blot analysis of MET receptor activation following the treatment of HLE cells with PRELP (P), HFG (H) or a mix of PRELP and HGF (H+P). TUBB is used as loading control. **f.** Scattering assay with HLE cells treated with HGF or HGF pre-incubated with prolargin. **g.** Migration assay with HLE cells. Panels **a, c, d, f** and **g** */** denote statistical significance with $p<0.05$ and $p<0.01$ respectively, error bars are standard deviations of means.

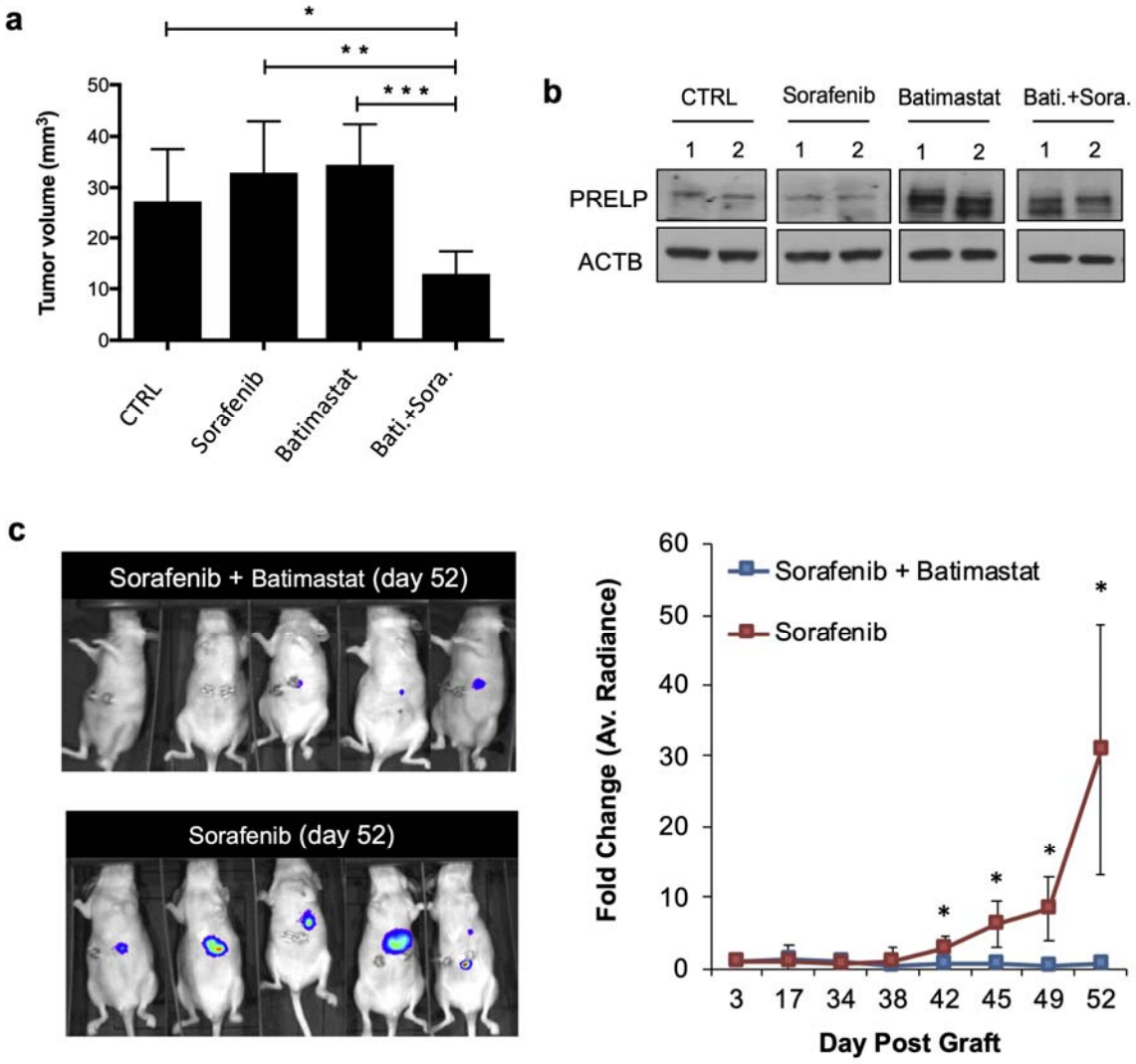

Figure 5: Combination of MMP inhibitor batimastat with sorafenib results in strong tumor control. **a.** Combined treatment with batimastat and sorafenib, reduces tumor growth on CAM. HLF cells were co-injected with NLF and then treated with placebo, sorafenib and batimastat alone or in combination. After 7 days, tumors were collected and tumor volume was measured. */**/ *** denote statistical significance with $p<0.05$, $p<0.01$ and $p<0.001$ respectively, error bars are standard error of means; N=5 for each group. **b.** WB analysis of PRELP levels in CAM-derived tumors following their treatment with batimastat/sorafenib. **c.** (*left panel*) Bioluminescence imaging post orthotopic engraftment of HLF tumor cells. Red color indicates the highest and blue the lowest fluorescent intensity. (*right panel*) Fold-change of average radiance in liver tumors (compared to day 3 post engraftment). The data are presented as mean ± standard error of means (N=5 for each group). Statistical significance was calculated using Student's *t*-test (*: $0.05<p$).

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