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Mammary Branching Morphogenesis Requires Reciprocal Signaling by Heparanase and MMP-14

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ABSTRACT

The development of the mammary gland involves formation of a branched arboreal structure resulting from the penetration and proliferation of epithelial cells into the fat pad. The mammary cells invade by remodeling their surrounding extracellular matrix (ECM), which are rich in proteins, and glycans such as heparan sulfate proteoglycans (HSPGs). There is increasing literature on how the interaction between signaling by ECM and matrix metalloproteinases (MMPs) is relevant to morphogenetic and physiological contexts. Here we sought to understand how heparanase, the sole mammalian heparan sulfate-degrading endoglycosidase may regulate mammary gland development. We found a robust localization of heparanase within growing end buds during branching *in vivo*. Using three-dimensional (3D) organotypic cultures, we showed that heparanase expression and activity are required for mammary epithelial invasion/branching within dense collagen I gels. Morphometric analysis of glands from both heparanase-overexpressing and knockout mice showed a direct correlation between degree of branching and the heparanase levels, confirming our 3D organotypic culture observations. Finally, we uncovered a reciprocal association between levels of heparanase and MMP14, a membrane-bound MMP, shedding further light on how branching occurs within developing mammary glands. J. Cell. Biochem. 116: 1668–1679, 2015.

KEY WORDS: BRANCHING MORPHOGENESIS; HEPARANASE; MAMMARY GLAND; MMP-14

The anatomy of the mammary gland is characterized by an elaborate network of branched ducts that forms through iterative rounds of epithelial cell proliferation, and invasion into the fat pad. These events of proliferation and invasions take place during puberty and are collectively known as branching morphogenesis [Chuong et al., 2014]. This process is facilitated by the activity of several proteolytic enzymes, such as MMPs that are capable of remodeling the extracellular matrix (ECM) [Alcaraz et al., 2011].

Angélica Maciel Gomes and Ramray Bhat contributed equally to this work.

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Importantly, a large portion of volume and content of the mammary ECM consists of proteoglycans with heparan sulfate (HS) chains [Delehedde et al., 2001], which sequester bioactive molecules and mediate cell interactions with components of the microenvironment [Vlodavsky et al., 1987; Bernfield and Sanderson, 1990]. Enzymatic degradation of heparan sulfate proteoglycans (HSPGs) is thus likely to affect the development and function of the mammary gland. Whereas most of the studies in tissue remodeling have focused on the MMPs, the role of glycosaminoglycan-degrading enzymes in functional and morphogenetic aspects of the mammary gland is yet poorly understood.

Heparanase is the only known HS-specific endoglycosidase, thus its ability to regulate the levels of HS is critical for development and tissue homeostasis, as shown by Patel et al. [2007], in the context of submandibular gland branching. Evidence for the involvement of heparanase in mammary gland morphogenesis has come from Zcharia et al. [2004] on transgenic mice overexpressing human heparanase in all tissues. Mammary glands from these transgenic mice showed increased side-branching and precocious alveolar development in glands from virgin animals, and enhanced lobuloalveologenesis during pregnancy. To our surprise, a brief examination of the mammary glands of heparanase knockout mice (HPSE-KO) also seemed to show increased branching as reported in Zcharia et al. [2009]. The contradiction between the knockout and overexpression phenotypes notwithstanding, it is reasonable to hypothesize that heparanase is involved in modulating the invasive ability of epithelial cells, resulting in a potentially crucial role in branching morphogenesis during mammary gland development.

Here, we demonstrate a critical role for heparanase in branching and invasive behavior of normal mammary epithelia. We observe that during the period of branching in mammary glands of virgin mice, heparanase localizes strongly at the invading edges of the epithelial end buds and terminal end buds (TEBs). Using a 3D culture model, we show that the ability of mammary epithelial cells to invade and branch through dense collagen I (CL-I) gels is directly proportional to their heparanase expression. This led us to perform extensive morphometric analysis of mammary gland whole mounts from heparanase knockout- and overexpressing-mice which confirmed our culture findings. We show that the morphoregulatory function of heparanase could be the result of its mutually reciprocal feedback with MMP-14, a collagenolytic enzyme that promotes epithelial invasion during mammary branching.

MATERIALS AND METHODS

INDIRECT IMMUNOFLUORESCENCE

Freshly dissected mammary glands were fixed with 4% paraformaldehyde for 15 min and with Carnoy's fixative (75% ethanol and 25% glacial acetic acid) overnight. This was followed by serial dehydration and overnight treatment with xylene. Dehydrated tissues were processed for paraffin embedding and sectioning. Deparaffinized sections were subjected to microwave-based antigen retrieval using citrate buffer (pH 6.0) for 10 min and then treated with 20 μg/ml of proteinase K for 5 min. The sections were then blocked with PBS containing 5% normal goat serum, 1% bovine serum albumin (BSA) and 0.05% Tween-20 for 1 h at room temperature followed by incubation with rabbit polyclonal anti-heparanase antibody (Santa Cruz SC-25826), diluted 1:50, overnight at 4°C. The secondary antibody was goat anti-rabbit 488 (Invitrogen, Carlsbad, CA) diluted at 1:300 and incubated for 2 h. Myoepithelial cells were visualized using anti-α-smooth muscle actin-Cy3 antibody (Sigma-Aldrich, St. Louis, MO). Negative controls were obtained by omission of the primary antibody (Fig. S1A). All images were obtained using a laser scanning confocal microscope LSM710 (Zeiss).

EpH4 CELL CULTURE AND PREPARATION OF CELL CLUSTERS

EpH4 cells were originally isolated from the mammary tissue of a mid-pregnant Balb/c mouse [Reichmann et al., 1989] and were kindly provided by E. Reichmann (Institut Suisse de Recherches, Epalinges, Switzerland). Cells were maintained in 1:1 DMEM/F12 (University of California San Francisco Cell Culture Facility), supplemented with 10% fetal bovine serum (Invitrogen) and 50 μg/ml gentamycin. The EpH4 cells were used between passage 10 and 20. EpH4 cells suspended in growth medium were plated in six-well polyhema-coated plates (2×10^5 cells per well) and incubated overnight at 37°C, yielding rounded clusters. Single cells were removed by differential centrifugation, and the final pellet was plated again in six-well plates overnight. Finally, cell clusters were centrifuged and resuspended in the desired amount of the medium described above.

THREE-DIMENSIONAL (3D) COLLAGEN I (CL-1) GEL

For 3D cell cultures, EpH4 cell clusters were embedded in CL-I gels as previously described [Hirai et al., 1998]. Briefly acid-soluble collagen (900 μl of a 5 mg/ml solution), Cellagen^{TM} (AC-5, ICN, Koken, Tokyo, Japan) was gently mixed on ice with 112.5 μl 10× DMEM/F12, followed by 85 μl of 0.1 N NaOH and 375 μl of DMEM/F12. Around 250 clusters were plated in each well (8-well plate—Lab-Tek Chambered Coverglass, borosilicate). Two layers of collagen were poured into each well: a basal layer consisting only of CL-I and an upper layer containing clusters of EpH4 cells. To allow solidification of the basal CL-I layer, 75 μl of the CL-I solution was poured into each well and incubated at 37°C for 15 min. A second layer of 220 μl of CL-I containing 250 EpH4 clusters was added to each well and placed immediately at 37°C. After gelation, 300 μl of chemically defined medium (DMEM/F-12 containing 1% insulin/transferrin/selenium, 1% penicillin/streptomycin) with 9 nM bFGF (Sigma-Aldrich) was added to each well (unless stated otherwise) and replaced every other day. The heparanase inhibitor used was OGT2115 (Tocris) at a concentration of 2.5 μM. Medium was replenished every other day. The cytotoxicity of OGT2115 was assessed using Calcein AM staining. At the concentration used in the assays, no signs of toxicity were observed in EpH4 cells.

ANALYSIS OF BRANCHING MORPHOGENESIS

The branching phenotype of EpH4 clusters embedded in CL-I gel was determined after a 5-day culture period. The branching phenotype was defined as a cell cluster having at least two processes (that were at least half of the diameter of the central body) extending from its central body. Quantification of EpH4 cell branching was carried out by counting the percentage of branching clusters in each well. In addition, we analyzed the number of branches in each cluster. All experiments were repeated at least three times.

IMMUNOSTAINING OF PRIMARY MAMMARY ORGANOIDS EMBEDDED IN COLLAGEN

Primary epithelial organoids were isolated from 8-week-old virgin C57BL/6 mice as previously described by Fata et al. [2007]. After 5 days in culture, the organoids were fixed with 4% paraformaldehyde for 15 min. Serial 100 μm sections of the CL-I gel were generated with a Leica CM3050 S cryostat (Leica Microsystems). After that, immunostaining for heparanase was performed as described before. Antigen retrieval was not used in this experiment.

IMMUNOSTAINING OF MAMMARY WHOLE-MOUNTS

Mammary whole-mount of 5-week-old C57BL/6 mice was used. The mammary tissue was processed as previously described by Mori et al. [2012] with some modifications in order to perform immunostaining. The antigen retrieval was performed as described earlier. Then, the mammary whole-mount was incubated with Triton X-100 (0.1%) for 25 min and blocked (5% normal goat serum + 1% BSA + 0.05% Tween-20) overnight at 4°C. This was followed by incubation with anti-heparanase antibody, diluted at 1:15, overnight at 4°C. The tissue was washed for 24 h. The secondary antibody used was Alexa fluor-633-conjugated goat anti-rabbit (1:100), incubated overnight. Finally, the tissue was washed for 24 h, dehydrated and mounted in Permount (Fisher Scientific, PA). Images were generated using a confocal microscope (LSM710, Zeiss) using 10× and 40× lens. The lambda scan mode in the confocal was utilized to determine the maximum emission of the autofluorescence and from the secondary antibody.

LASER SCANNING-BASED TISSUE AUTOFLUORESCENCE/ FLUORESCENCE IMAGING (LS-TAFI)

In order to analyze the microanatomy of mammary whole-mount from 5-week-old mice (hpa-tg and HPSE-KO) we performed the LS-TAFI technique as described [Mori et al., 2012]. The number of branch points was quantified in the whole gland.

shRNA AND CONSTRUCTS OF EXPRESSION

The lentivirus for silencing heparanase was prepared using five different Mission shRNA constructs (Sigma-Aldrich) with the following sequence: sh1,CGGAGGCAAATAAGTGGAGGATATCTC-GAGATATCCTCCACTTATTTGCCTTTTTTG;sh2,CCGGCGGATGGAT-TACTTTCCAAATCTCGAGATTTGGAAGTAATCCATCCGTTTTT;sh3, CCGGGCCGGATGGATTACTTTCCAACTCGAGTTGGAAAGTAATCC-ATCCGGCTTTTTG;sh4,CCGGCCTTGACTACTGCTCTTCCAACTCGAG-TTGGAAGAGCAGTAGTCAAGGTTTTTG;sh5,CCGGCCTTGACTACT-GCTCTTCCAACTCGAGTTGGAAGAGCAGTAGTCAAGGTTTTTG. To prepare the expression plasmid of human heparanase, the human cDNA heparanase sequence was cloned from the plasmid pOTB7 (Open Biosystems). To generate the heparanase construct, we performed PCR with primers having *Xba*I and *Bst*BI restriction enzyme digestion sites. The forward primer had the following sequence: 5′-GCATATAATTCTAGAATGCTGCTGCGCTCGAAGC-3′ and the reverse primer had the following sequence: 5′-CGGACCTTC-GAATCAGATCTTCTTCAGAAATAAGTTTTTGTTCGATGCAAGCAG-3′. The MYC tag (N-EQKLISEEDL-C) was inserted at the C-terminal end to facilitate the detection of heparanase. After *Xba*I and *Bst*BI digestion, the product was ligated into *Xba*I and *Bst*BI digested pCDH-EF1-MCS-T2A-PURO (System Biosciences). All cDNA constructs were confirmed by DNA sequencing.

LENTIVIRAL PRODUCTION, CONCENTRATION, AND INFECTION OF EpH4 CELLS

Plasmids for lentiviral production as well as those containing shRNA (Mission shRNA; Sigma-Aldrich) against endogenous mouse heparanase, or lentivirus plasmids containing myc-tagged human heparanase were transfected into 293FT cells using FuGene6 (Roche, Basel, Switzerland). Transfected cells were cultured in DMEM containing 10% FBS, 0.1 mM MEM Non-Essential Amino Acids, 1 mM MEM Sodium Pyruvate, 6 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Culture media were replaced after 24 h with fresh media. Forty-eight hours later, lentivirus was concentrated from filtered culture media (0.45 μm filters) using Lenti-X Concentrator (Clontech) and aliquots were stored at −80°C until use. To transduce EpH4 cells, 1.0×10^5 cells were plated in each well of a six-well plate, infected with the lentivirus treated with polybrene and selected by adding 3 μg/ml puromycin to growth medium for 4 days. Lentivirus with scrambled sequence was used as a shRNA control.

WESTERN BLOTTING

To check heparanase knockdown and overexpression, EpH4 cells were cultured in EpH4 media. In order to evaluate MMP-14 levels, Eph4 cells were cultured in CL-I gel (3 mg/ml) for 48 h in serum-free media (DMEM/F-12 containing 1% insulin/transferrin/selenium, 1% penicillin/streptomycin) with 9 nM bFGF (Sigma-Aldrich). For protein isolation, media were removed, the cells were washed with PBS, lysed with a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Tween with protease and phosphatase inhibitor cocktails (EMD Millipore, Billerica). Protein concentration was determined using the BCA Protein Assay kit (Thermo Scientific, Waltham), following the manufacturer's instructions. Protein samples were mixed with Laemmeli sample buffer and heated at 95°C for 5 min. Samples were loaded into a pre-cast 4–20% *tris*-glycine polyacrylamide gel (Invitrogen) using the NOVEX system (Invitrogen). Resolved proteins were transferred to nitrocellulose membrane (Whatman, Maidstone, UK) followed by blocking in TBS, 0.05% Tween-20 with 3% (w/v) BSA for 1 h at room temperature. Membranes were incubated overnight at 4°C in 3% BSA, 0.05% Tween-20 in TBS containing anti-heparanase (Santa Cruz), anti-α-tubulin (Sigma-Aldrich) and anti-MMP-14 antibodies (Abcam). Primary antibodies were detected with the Pierce SuperSignal detection kit (Rockford, IL) and signal was captured with the FluorChem 8900 analysis system (Alpha Innotech, San Leandro, CA). Heparanase overexpression was analyzed using mouse monoclonal anti-Myc antibody (clone4A6, Millipore).

HEPARANASE ACTIVITY ASSAY

Heparanase enzymatic activity was examined using a commercial HS degrading enzyme assay kit (Takara). The kit works on the principle that HS loses its ability to bind to bFGF when degraded by a HS-degrading enzyme. The cell lysate was used to determine the enzymatic activity of heparanase. The assay was performed following the kit instructions.

MICE

The mice strain used in the whole-mount immunostaining was C57BL/6. The background of the transgenic mice overexpressing human heparanase (hpa-tg) is BALB/c [Zcharia et al., 2004] and heparanase knockout mice is C57BL/6 [Zcharia et al., 2009]. C57BL/6 back-crossed mice carrying the *LacZ* gene under the control of the *Mmp-14* promoter (*Mmp-14*^{tm2Ski}; *Mmp-14* (+/−, *lacZ*) heterozygotes) [reported in Yana et al., 2007] were used to gain homozygotes and siblings. Inguinal mammary glands were isolated from each mouse between day 7–day15 after birth. Genotypes were checked by PCR on tail DNA. Animal use protocol was obtained and procedures were followed in strict accordance with guidelines established by the Lawrence Berkeley National Laboratory's Animal Welfare and Research Committee (AWRC).

RESULTS

HEPARANASE IS EXPRESSED AT HIGH LEVELS DURING MAMMARY BRANCHING MORPHOGENESIS

In female mice, the mammary gland develops mainly postnatally and undergoes dramatic phenotypic changes at different stages of pregnancy cycle. We evaluated the presence of heparanase at different developmental stages: 5- and 10-week-old female mice (early and late stages of branching morphogenesis, respectively); mid-pregnancy (characterized by increased secondary and tertiary branching and alveologenesis); lactating (presence of milk-secreting glandular architecture) and at day 7 of involution (characterized by ECM remodeling with restoration of the ductal structure). Immunofluorescence analysis of tissue sections obtained from mammary glands of 5-week-old animals revealed that heparanase is expressed strongly in the epithelial cells and the stroma (Fig. 1A). Heparanase protein levels were moderate within glandular epithelia from 10 week- and midpregnant mice (Fig. 1B,C). During lactation, no heparanase staining was observed in any of the cells of the mammary gland (Fig. 1D). In involuting mammary glands, heparanase levels were restored to that observed in the glands from 10-week virgin animals (Fig. 1E). These data indicate that the levels of heparanase are upregulated when the mammary epithelial cells remodel their ECM-rich microenvironment in order to form—and in the case of involution, restore—the tree-like ductal structure of the mammary gland.

HEPARANASE SHOWS STRONG LOCALIZATION AT THE GROWING TIPS OF MAMMARY EPITHELIAL BRANCHING STRUCTURES *IN VIVO* AND IN 3D CULTURES

During mammary branching morphogenesis, the distal growing tips of the primary and secondary branches form bulbous multilayered epithelial structures known as TEBs and end buds, respectively. The end buds form an interface directly with the stromal ECM, sculpting it to allow the mammary tree to fill the fat pad. By co-assaying heparanase immunofluorescence with autofluorescence imaging to identify the tissue microanatomy [based on Mori et al., 2012], we observed strong localization for heparanase at the invasive end of the end buds and TEBs, relative to the ductal and stromal cells (Fig. 2A and Fig. S1B).

The processes of mammary epithelial cell invasion during branching morphogenesis can be mimicked by embedding primary mammary organoids in dense CL-I gels (3 mg/ml) and culturing them in a defined serum-free, growth factor-supplemented medium [as reported in Lo et al., 2012]. In this 3D gel heparanase localization was similar to that observed *in vivo*: it was present prominently at the invasive edges of organoid branches (Fig. 2B).

HEPARANASE LEVELS CORRELATE WITH MAMMARY EPITHELIAL BRANCHING IN CULTURE AND *IN VIVO*

We had observed heparanase in both mammary epithelial and stromal cells in 5-week glands. Therefore, to investigate whether the epithelial heparanase levels was sufficient to drive the mammary epithelia to invade into, and branch within, the mammary fat pad, we employed an organotypic 3D cell culture [used before in Hirai et al., 1998; Simian et al., 2001], wherein aggregated cellular clusters of the functionally normal mouse mammary epithelial cell line (EpH4), are embedded in dense CL-I gels (3 mg/ml). We confirmed that EpH4 cells expressed endogenous heparanase (Fig. S2A) and also that branching EpH4 clusters formed a basement membrane (BM) that comprised of one of the cognate substrates of HSPG, that is, perlecan (Fig. S2B). Upon addition of a growth signal (a growth factor such as bFGF) the aggregates invade into the CL-rich microenvironment and form multi-branched structures. To directly show the significance of the endogenous heparanase in branching, we depleted the endogenous heparanase levels using shRNA that selectively targeted its expression (Fig. S2C). Control cells were transduced with lentiviruses carrying non-targeting shRNA (Fig. 3A). In a separate experiment we treated the mammary epithelia with a small-molecule chemical inhibitor of heparanase (OGT2115) [Courtney et al., 2005]. Quantification of the number of branches developed from each structure revealed that the depletion of heparanase and/or its functional inhibition caused a significant reduction in invasion and branching (Fig. 3B). To show the specificity of heparanase silencing in this assay, the branching phenotype was rescued by treatment of EpH4 heparanase-knocked down cells with recombinant heparanase (Fig. S3A).

Our culture findings contradicted what had been observed earlier *in vivo*: that heparanase KO led to increased branching. This led us to re-examine the effect of knockout on mammary gland phenotype through rigorous morphometric analysis. Using LS-TAFI an imaging technique that renders the 3D epithelial arbor visually distinct from its stromal environs, we observed that the branching in whole mounts of mammary glands from 5-week Hpse-KO mice showed a considerable decrease in side (secondary and tertiary) branches and an increase in branch interval compared to control counterparts (Fig. 3C,D). The decreased side-branching resulted in a sparsely populated mammary arbor and confirmed our culture observations.

Conversely, 3D CL-I cultures of human heparanase-overexpressing EpH4 cells showed increased branching (Fig. 4A; heparanase overexpression and activity confirmed in Fig. S3B). The invasion, quantified by the number of branches per structure, was found to significantly exceed control EpH4 cells (Fig. 4B). Likewise, we found that mammary glands from 5-week Hpa-tg mice showed significantly greater mammary side-branching and arboreal density than their control counterparts (Fig. 4C,D). These findings are indicative of the

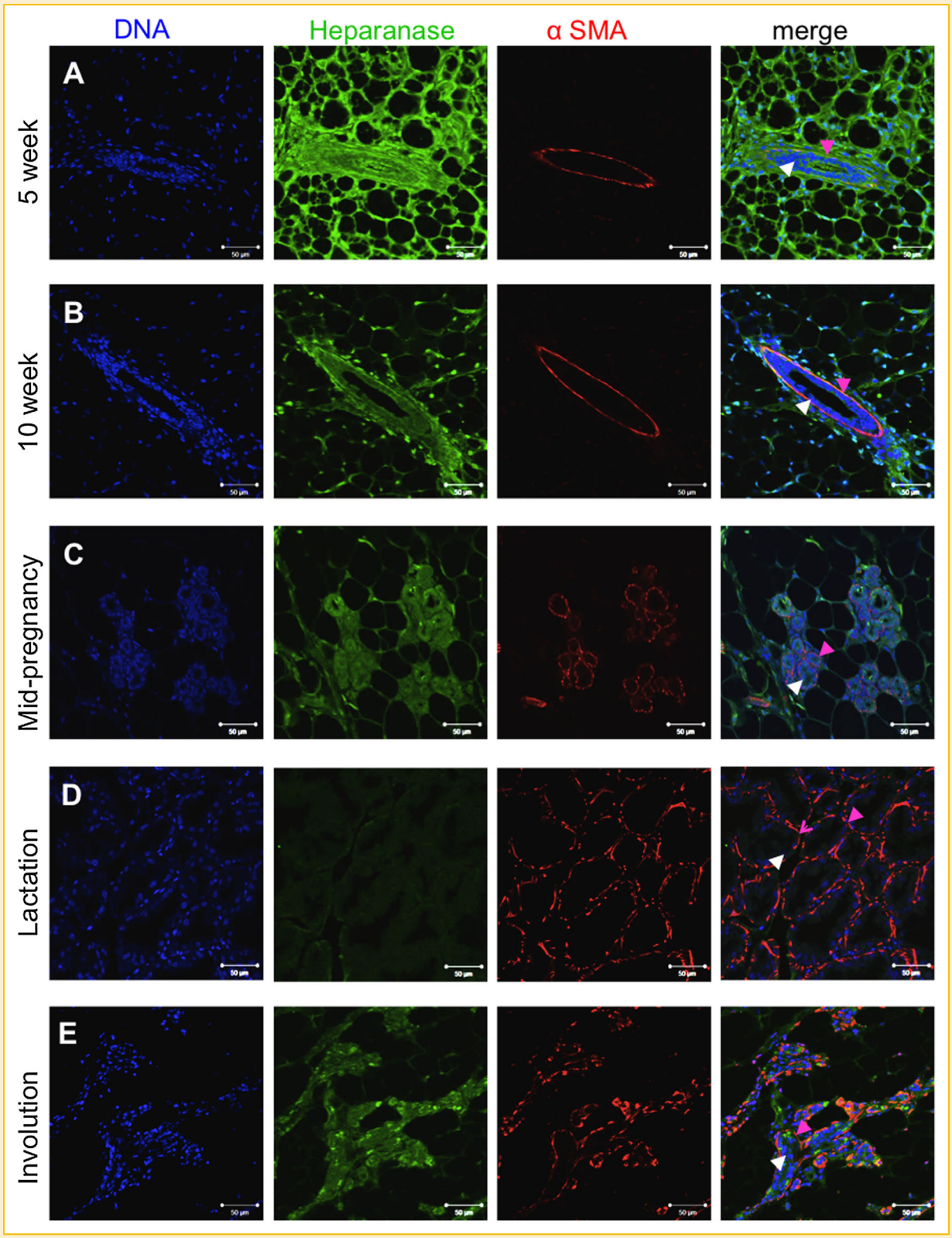

Fig. 1. Heparanase peaks during mammary branching morphogenesis. Representative images of indirect immunofluorescence on paraffin embedded sections of murine mammary glands during distinct stages of development, (A) 5-week branching, (B) 10-week branching, (C) midpregnancy, (D) lactation, (E) involution showing staining for DNA (blue; first column from left), heparanase (green; second column), α-SMA, myoepithelial marker (red; third column), and a merged picture of all three fluorescent signals (fourth column). Heparanase localization within the myoepithelial cells is shown with pink arrowhead, and within luminal epithelial cells is shown with white arrowhead. Heparanase levels are particularly elevated within mammary epithelial cells during branching. There is moderate expression during mid pregnancy and involution with close to no expression during lactation. Scale bar represents 50 μm.

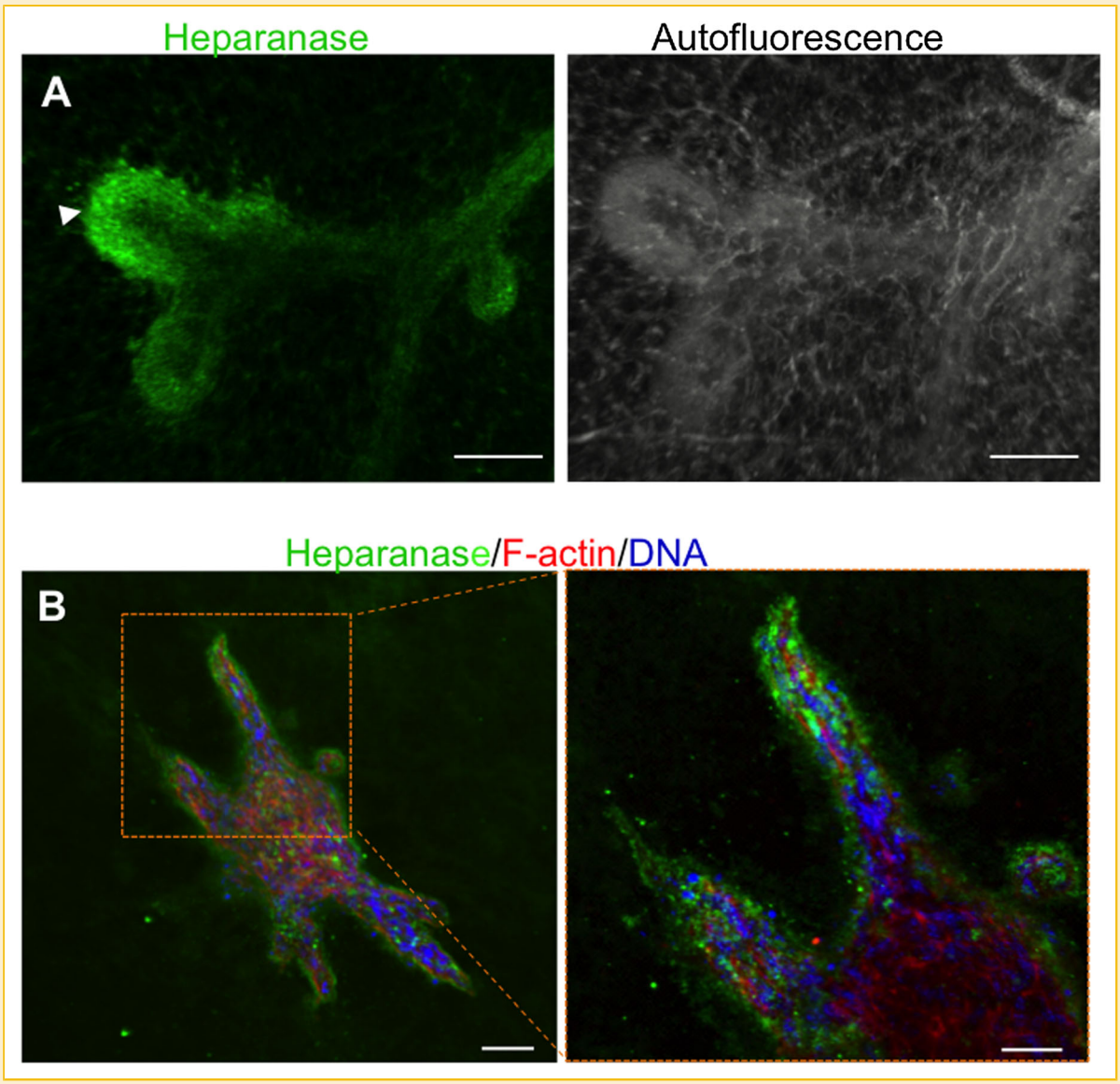

Fig. 2. Heparanase is strongly localized at growing edges of invading and branching mammary epithelia. A: Indirect immunofluorescence on a representative inguinal mammary gland from 5-week C57Bl/6 mouse showing strong staining for heparanase (white arrowhead) in the growing tips of the end buds, relative to ductal staining. In contrast, the fluorophore-free autofluorescent signal from the tissue (right) is uniform through the branched structure. B: Indirect immunofluorescence of a representative murine mammary organoid cultured in collagen I showing higher staining for heparanase (green) at the growing edges of the branches relative to the organoid core. The organoid was costained for actin (red) and DNA (blue). Scale bars for A represent 150 μm and for B represent 70 μm.

involvement of heparanase in regulating the invasive capacity and branching behavior of mammary epithelial cells.

HEPARANASE AND MMP-14 RECIPROCALLY REGULATE EACH OTHER IN MAMMARY EPITHELIAL CELLS

Our 3D culture studies were performed in dense CL-I gels, which impart the microenvironment with mechanical resistance. Cellular invasion under these conditions requires degradation of collagen by proteolytic activity to relieve this resistance. MMP family members exhibit collagenolytic activity because of their catalytic domains as shown in earlier studies [Alcaraz et al., 2011; Mori et al., 2013; reviewed in Page-McCaw et al., 2007]. The membrane-bound MMP-14 is crucial to mammary epithelial cell invasion and is present prominently at the end buds and TEBs *in vivo*, and at invasive fronts of mammary epithelia in 3D cultures [Alcaraz et al., 2011; Mori et al., 2013]. This, in combination with our findings indicating that heparanase is strongly localized at the growing edges of invading epithelia, prompted us to investigate whether the expressions of heparanase and MMP-14 are associated in any way. Indirect immunofluorescence of mammary glands from 5-week heparanase knockout mice showed much lower protein levels of MMP-14 compared to controls, especially at the end buds (Fig. 5A). Conversely,

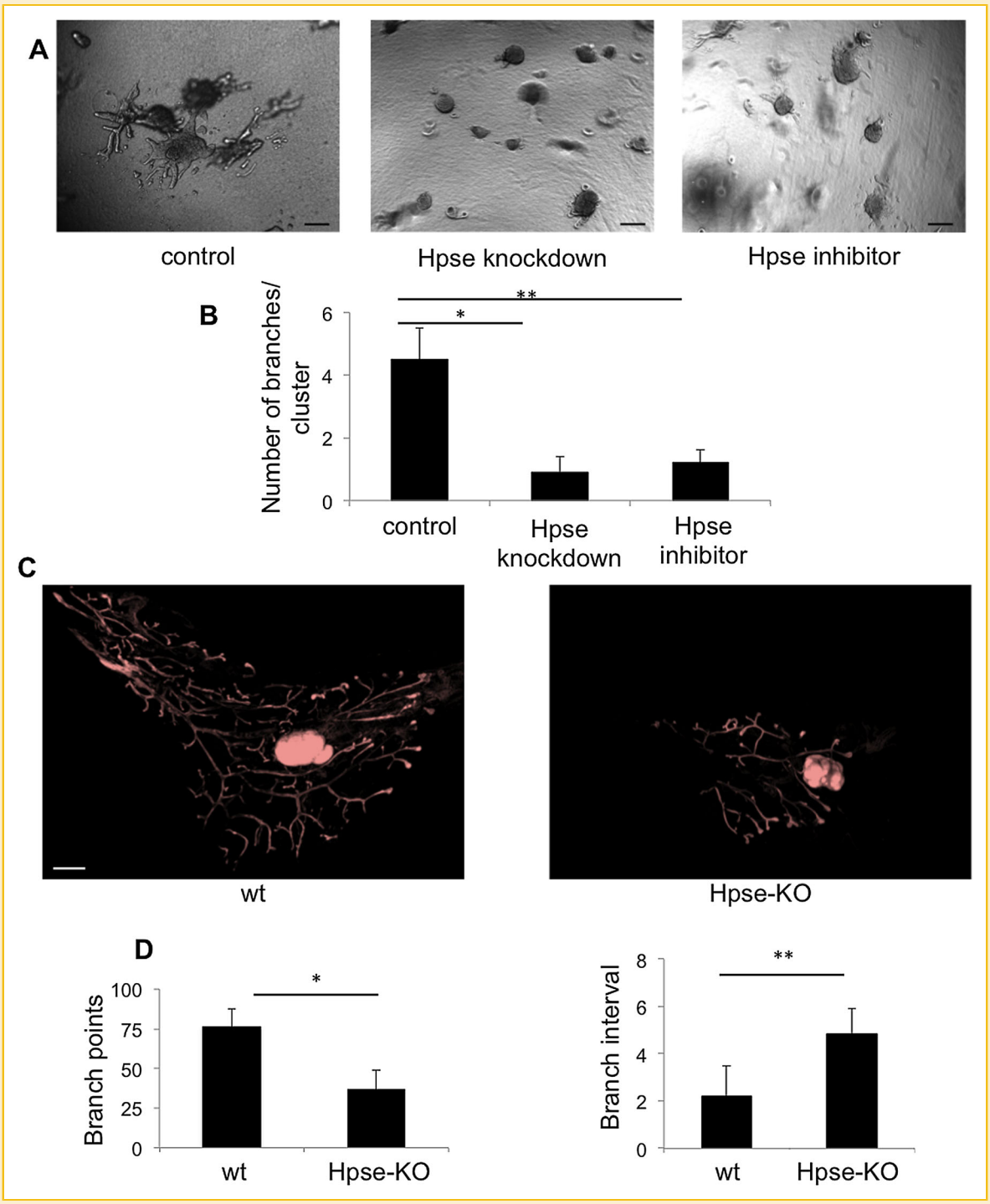

Fig. 3. Heparanase is required for branching and invasion of mammary epithelial cells. A,B: Representative brightfield images of EpH4 cell clusters cultured in the presence of bFGF in dense CL-I. Clusters of control untreated EpH4 cells show branching. Clusters from cells with heparanase knockdown, and from cells treated with OGT2115, an inhibitor of heparanase enzymatic activity, show sparse branching (A). Quantification of the branching per cluster shows a significant decrease when heparanase expression or activity is attenuated (B). C,D: Representative images of mammary glands from 5-week wild-type (wt) and heparanase knockout (Hpse-KO) C57Bl/6 mice with their branching pattern revealed (in pink) through autofluorescent signals from carmine-stained tissue. The Hpse-KO gland shows decreased branching within the mammary fat pad (C). Quantification of branch points and branch intervals as two parameters for determining the extent of branching (D). Scale bars for A represent 200 μm, and for C represent 1 mm. *Statistical significance ($P < 0.05$) as measured by two tailed Student's *t*-test. Error bars are SEM.

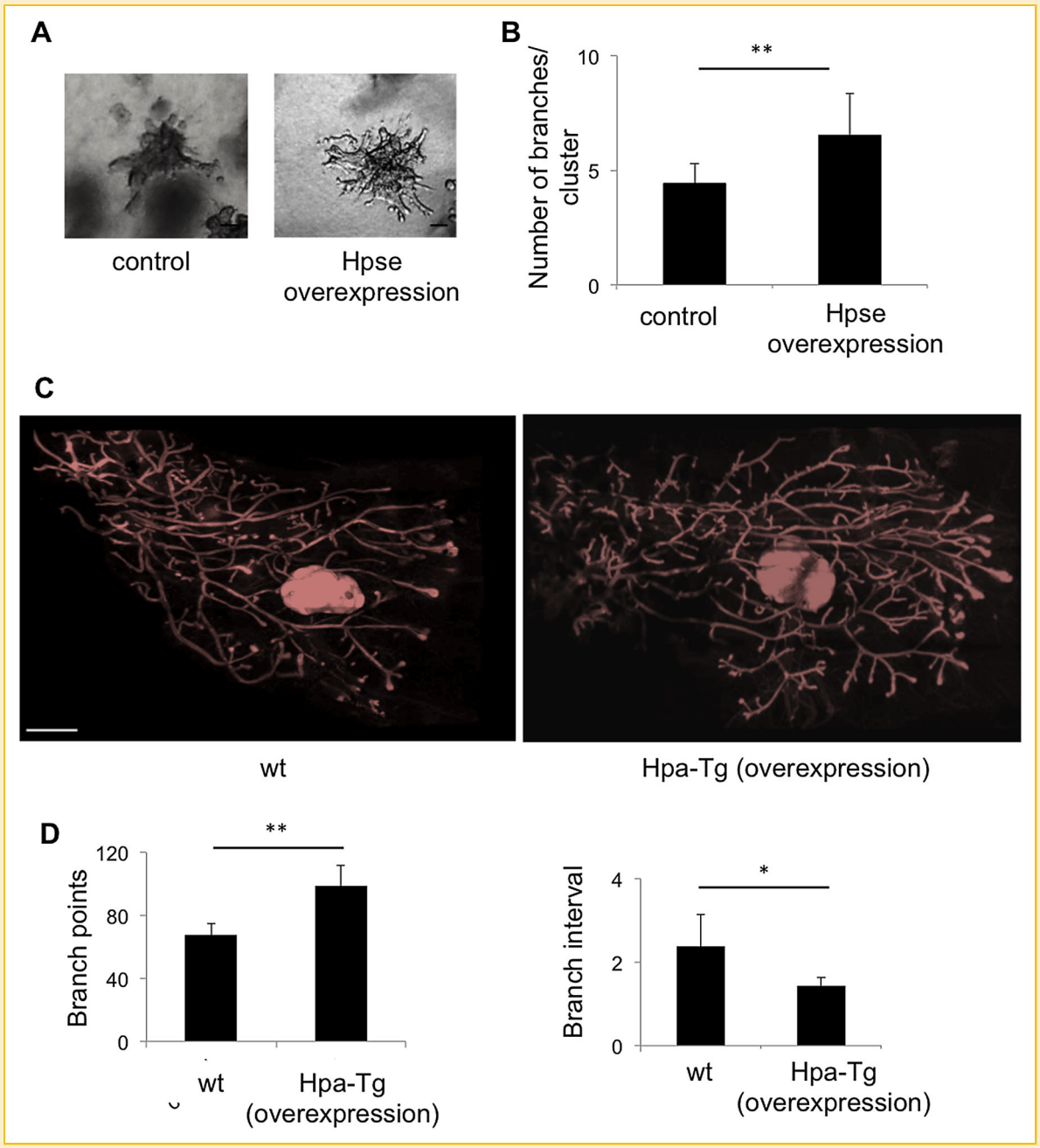

Fig. 4. Heparanase overexpression enhances invasive and branching behavior of mammary epithelia. A,B: Representative brightfield images of EpH4 cell clusters cultured in the presence of bFGF in dense CL-1. Clusters of control untreated EpH4 cells show branching. Clusters from cells with heparanase overexpression show greater branching (A). The quantification of the branches per cluster shows a significant increase upon overexpression of heparanase (B). C,D: Representative images of mammary glands from 5-week wild-type (wt) and heparanase overexpressing (Hpa-Tg) Balb/c mice with their branching pattern revealed through autofluorescent signals from carmine-stained tissue. The Hpa-tg mouse gland shows increased branching (C). Quantification of branch points and branch intervals as two parameters for determining the extent of branching (D). Scale bars for A represent 200 μm and for C represent 1 mm. *Statistical significance ($P < 0.05$) as measured by two tailed Student's *t*-test. Error bars are SEM.

MMP-14 levels were dramatically higher in mammary epithelia from heparanase-overexpressing mice in comparison to their control counterparts (Fig. 5B). In agreement with our *in vivo* results, we found that EpH4 cells overexpressing heparanase cultured in CL-I gels showed a significant upregulation of MMP-14 protein levels (Fig. S4A) and addition of recombinant heparanase to EpH4 cells increased MMP-14 levels as well (Fig. S4B).

We next sought to investigate whether the association between heparanase and MMP-14 was unidirectional or reciprocal. Indirect immunofluorescence of 2-week mammary gland sections from MMP-

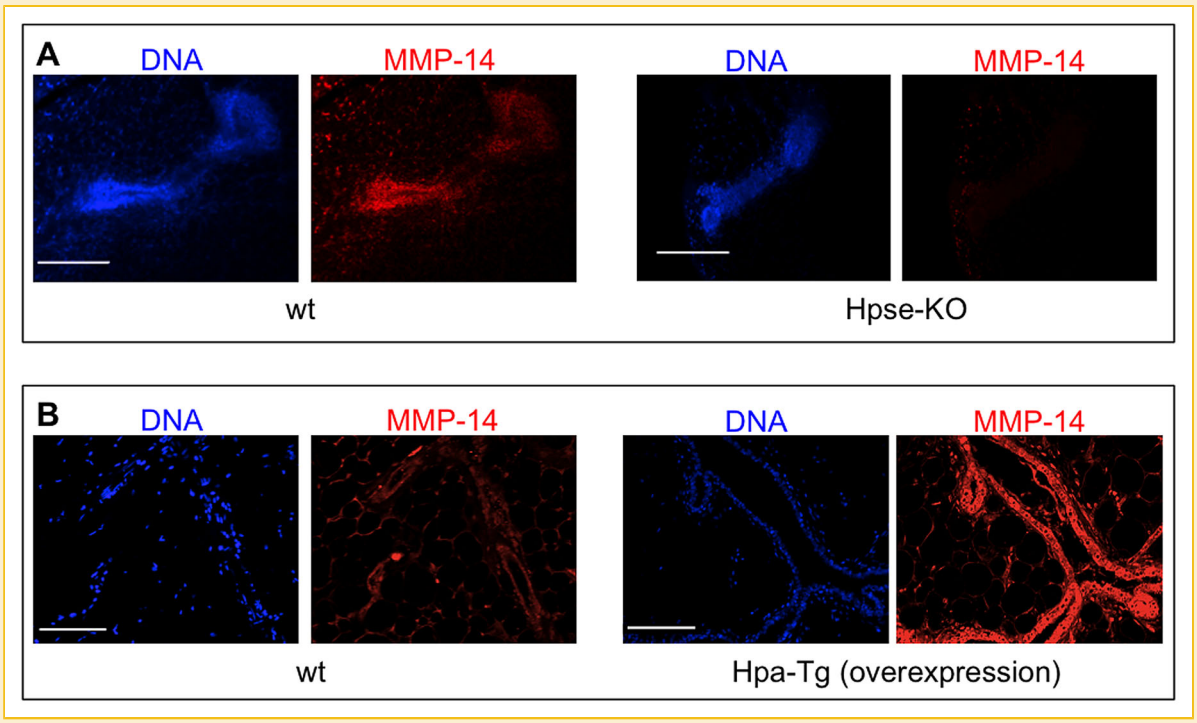

Fig. 5. MMP-14 level varies in direct proportion to endogenous heparanase expression *in vivo*. A: Representative images of indirect immunofluorescence of mammary glands from 5-week wild-type (wt) and heparanase knockout (Hpse-KO) mice showing staining for DNA (blue) and MMP-14 (red). MMP-14 levels in heparanase knockout tissues are lower than in control tissues. B: Representative images of indirect immunofluorescence of paraffin embedded sections of mammary glands from 5-week wild-type (wt) and heparanase overexpressing (Hpa-Tg) mice showing staining for DNA (blue) and MMP-14 (red). MMP-14 levels in heparanase overexpression tissues are higher than in control tissues. Scale bar represents 100 μm.

14 knockout mice (Mmp-14-KO) showed considerably lower protein levels of heparanase than their control wild-type counterparts (Fig. 6).

In summary, our findings define an essentially new role for heparanase in mammary gland branching morphogenesis. In addition, we uncover a reciprocal regulation between MMP-14 and heparanase during mammary epithelial invasion. These findings also provide new impetus for investigation of the role of heparanase in formation and progression of mammary tumors.

DISCUSSION

How the ECM coordinates cellular behavior and tissue morphogenesis is a crucial area of research in developmental biology. A fine balance between ECM deposition and ECM degradation is required for the establishment of normal tissue architecture, and function. Indeed, the ectopic expression of constitutively active MMP-3 in mammary epithelia can induce mammary tumors in mice [Sternlicht et al., 1999], whereas knock-out of MMP-3 or MMP-14 impairs invasion and branching during mammary morphogenesis [Wiseman et al., 2003; Mori et al., 2013]. Although much is known about how MMPs regulate ECM proteins in developing tissues, a lot more remains to be determined about how glycan levels are controlled and how their regulation impacts organ development and physiology. HSPGs are a crucial component of BMs and ECM, and play important roles in morphogenesis as reported in the context of secretory glands [Makarenkova et al., 2009] and mammary glands [Garner et al., 2011]. In fact, Makarenkova et al. [2009] reported that HS chains in the microenvironment of lacrimal and salivary epithelial buds control growth factor gradients, impacting events such as glandular elongation and branching. Moreover, the deletion of the enzyme exostosin glycosyltransferase 1 (which catalyzes the synthesis of HS chain) in mammary glands was also shown to result in severe defects in branching [Garner et al., 2011].

In the present study, we focused on heparanase, the only known glycosidase that cleaves HS chains and its role in branching and invasion. We used immunofluorescence to investigate the expression and localization of heparanase at different stages during the development of murine mammary gland. We found that the enzyme levels are highest in branching glands from virgin mice and is especially expressed in the TEBs and end buds. This expression signature suggested an association between heparanase and the process of mammary epithelial invasion, a process that involves their collective movement through an ECM-rich environment by softening and sculpting the latter [Friedl et al., 1995; Ewald et al., 2008].

In order to test the relationship between heparanase and epithelial invasion, we employed a 3D culture model system where clusters of non-tumorigenic, EpH4 mammary epithelial cells were embedded in

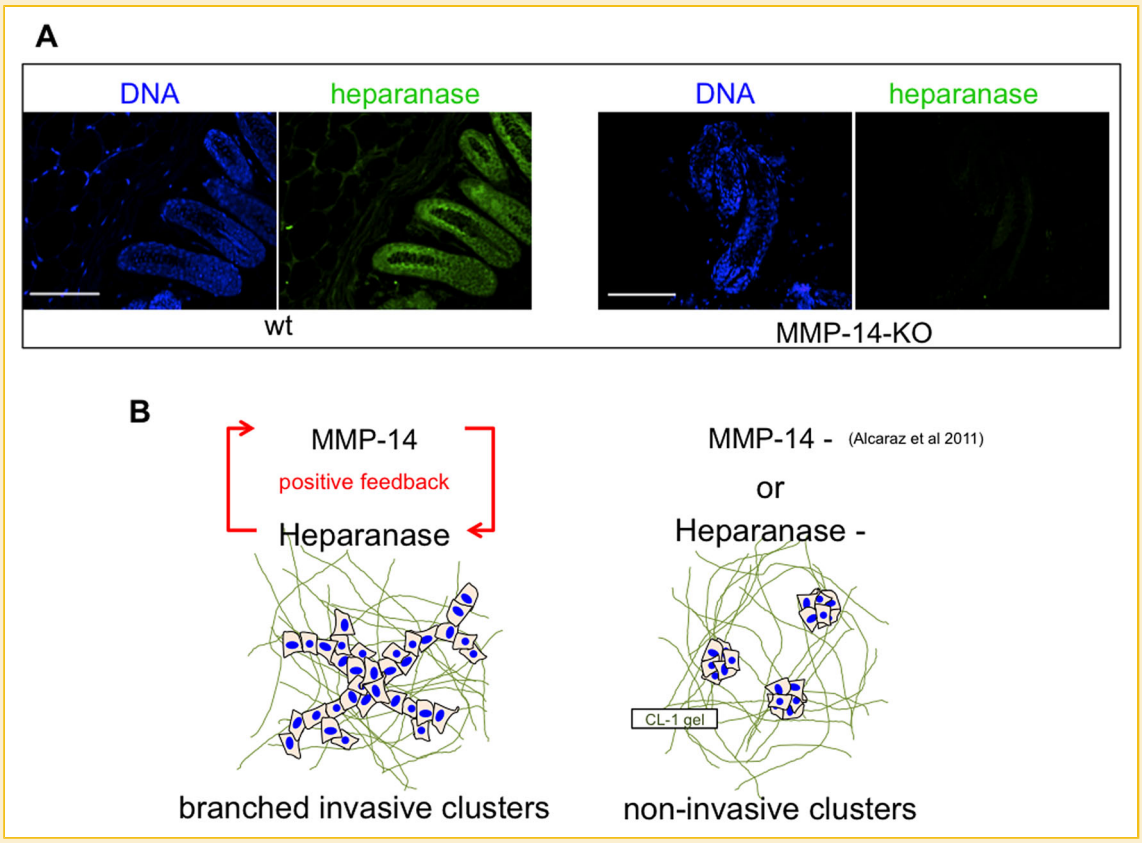

Fig. 6. Mammary epithelia from Mmp-14 KO mice have sparse levels of heparanase. A: Representative images of indirect immunofluorescence of mammary gland from 2-week wild-type (wt) and MMP-14 knockout (Mmp-14-KO) mice, showing staining for DNA (blue) and heparanase (green). Heparanase protein expression in Mmp-14-KO mammary tissue is markedly reduced compared to control tissue. Scale bar represents 100 μm. B: A visual summary of relationship between heparanase, MMP-14 and mammary epithelial branching. When expressed within mammary epithelia, heparanase, and MMP-14 reciprocally reinforce each other's levels and induce the cells to branch and invade within dense CL-I gels (left). When the expression of either MMP-14 or heparanase is knocked down, mammary epithelia fail to branch and form spherical clusters (right).

dense (3 mg/ml) CL-I gels. Our results showed that a stable knockdown of heparanase results in decreased invasive- and branching behaviors of cells within their collagenous microenvironment. The opposite, that is, heparanase overexpression, resulted in greater invasive and branching phenotype in culture. The invasive and branching behavior was associated with the enzymatic activity of heparanase because reducing the enzymatic activity of heparanase, using a small molecule chemical inhibitor of heparanase, also decreased the branching phenotype of cells in 3D cultures.

Our culture results led us to reinvestigate the paradoxical effect of heparanase on mammary branching *in vivo* which we had reported earlier: that mammary glands from mice overexpressing human heparanase as well as mice with heparanase knockout showed increased branching [Zcharia et al., 2004, 2009]. To resolve this paradox, we employed a highly sensitive and quantitative imaging modality recently developed in our laboratory, using laser scanning confocal microscopy to detect autofluorescence from the whole mammary gland followed by the digital reconstruction of the gland anatomy in 3D [Mori et al., 2012]. Our morphometric analysis revealed that mammary glands from heparanase KO mice showed decreased branching (contrary to our earlier observations) and mice with heparanase overexpression showed increased branching (confirming our earlier observations). Therefore, our *in vivo* findings were congruent with our culture data and resolved our earlier obtained paradoxical results. The image analysis and quantification of branching parameters over the whole gland that we presented above, was made with 5-week glands, when the invasion of the fat pad by the branching mammary anlagen is in process, and hence provides a clearer picture of how heparanase impacts the invasion than our previous studies. The estrous cycle of the mouse and its cyclic morphological effects on the mammary gland begin around 5 weeks and is unlikely to confound morphometric assessments at this early stage. Additionally at this stage, we showed intense heparanase localization at the TEB, which are the foci of invasive activity. TEBs can be easily observed within 5-week-old glands; by 12 weeks, the time point of the previous study [Zcharia et al., 2009] these structures already disappear.

Our 3D cultures, within which heparanase levels were found to correlate with branching and invasion, were performed using a high concentration of acid-extracted bovine epidermal CL-I. Such a dense CL-I microenvironment requires softening and lysis of the fibrillar CL-I in order to allow invasion and branching. A protein which has

been shown to be central to mammary epithelial invasion is MMP-14. MMP-14 possesses a gelatinase domain, and it also can activate other metalloproteinases (MMPs) such as MMP-2 and MMP-9. In a dense collagen microenvironment, the collagenolytic activity of MMP-14 has been to shown to be required for the cells to invade. We show that heparanase regulates the levels of MMP-14 protein *in vivo* as well as when mammary epithelial cells are cultured in high density CL-I, in 3D. This finding is consistent with—and builds on—a previous result showing a decrease in mRNA levels of MMP-14 in the mammary glands of heparanase knockout mice [Zcharia et al., 2009]. However this regulation is not unidirectional: in mammary glands from MMP-14 knockout mice, we found significantly lower levels of heparanase. We have therefore uncovered an important cross-regulation that takes place between an enzyme that can degrade CL-I, that is, MMP-14, and an enzyme that can degrade HS, that is, heparanase. Our understanding of how the mammary ECM (of which both HSPGs and fibrillar CL-I are important constituents) is remodeled during epithelial invasion is therefore considerably advanced by elucidation of this cross-regulation.

The level at which the cross-regulation between heparanase and MMP-14 operates (transcriptional, post-transcriptional, or post-translational), remains to be investigated. Purushothaman et al. [2008] and Wang et al. [2012] have shown the regulation of MMPs by heparanase in other tissue-specific contexts. For example, Purushothaman et al. [2008] showed heparanase increases MMP-9 expression in myeloma cells by ERK phosphorylation-mediated mechanism. Therefore we speculate that heparanase could upregulate MMP-14 in a similar way: by releasing growth factors from ECM and allowing them to initiate cell signaling that finally would culminate in MMP-14 expression. In fact, Simian et al. [2001] showed that several growth factors modulate levels of MMP(s) in mammary epithelial cells. Mori et al. [2013] and Correia et al. [2013] have shown that MMPs, such as MMP-3 and MMP-14, can drive epithelial invasion through non-proteolytic mechanisms. Similarly, Gingis-Velitski et al. [2004] and Ben-Zaken et al. [2007] have shown that heparanase by itself can promote phosphorylation of signaling molecules as AKT and Src, independent of its enzymatic activity. Therefore we do not discard the possibility that the regulatory cross-talk between heparanase and MMP-14 may be independent of their individual enzymatic activities. To our knowledge this is the first study describing MMP-14 as a regulator of heparanase protein expression. Future studies will thoroughly investigate the molecular mechanism by which MMP-14 and heparanase regulate each other's protein levels.

One of the cardinal features of the loss of tissue specificity during breast cancer is the destruction of the HSPG-rich BM, suggesting that heparanase may play a role in the etiology of the disease. Indeed heparanase activity and expression has been shown to be correlated with the degree of breast cancer cell invasiveness and aggression; highly invasive and metastatic cell lines, such as MDA-MB-231 shows high heparanase levels and activity, whereas low invasive MCF-7 cells exhibit lower heparanase expression and activity [Vlodavsky et al., 1999]. Higher heparanase protein levels are also associated with metastatic breast cancer [Maxhimer et al., 2002; see also review by Vlodavsky and Friedmann, 2001]. Our results situate these findings within a cell biological context by attributing the induction of mammary epithelial invasion to a reciprocal regulation between heparanase and MMP-14. The concerted action is essential, given that the ECM is rich in both proteins and glycans, both of which need to be degraded to allow cell invasion. Furthermore, this finding could influence specific therapeutic strategies targeting heparanase- and MMP-14-mediated invasive behaviors of breast cancer cells.

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