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Heparanase cleavage of perlecan heparan sulfate modulates FGF10 activity during ex vivo submandibular gland branching morphogenesis

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Heparan sulfate proteoglycans are essential for biological processes regulated by fibroblast growth factors (FGFs). Heparan sulfate (HS) regulates the activity of FGFs by acting as a coreceptor at the cell surface, enhancing FGF-FGFR affinity, and being a storage reservoir for FGFs in the extracellular matrix (ECM). Here we demonstrate a critical role for heparanase during mouse submandibular gland (SMG) branching morphogenesis. Heparanase, an endoglycosidase, colocalized with perlecan in the basement membrane and in epithelial clefts of SMGs. Inhibition of heparanase activity in organ culture decreased branching morphogenesis, and this inhibition was rescued specifically by FGF10 and not by other FGFs. By contrast, exogenous heparanase increased SMG branching and MAPK signaling and, surprisingly, when isolated epithelia were cultured in a three-dimensional ECM with FGF10, it increased the number of lateral branches and end buds. In a solid-phase binding assay, an FGF10-FGFR2b complex was released from the ECM by heparanase. In addition, surface plasmon resonance (SPR) analysis showed that FGF10 and the FGF10-FGFR2b complex bound to purified perlecan HS and could be released by heparanase. We used the FGF10-FGFR2b complex as a probe for HS in SMGs, and it colocalized with perlecan in the basement membrane and partly colocalized with syndecan 1 in the epithelium, and binding was reduced by treatment with heparanase. In summary, our results show heparanase releases FGF10 from perlecan HS in the basement membrane, increasing MAPK signaling, epithelial clefting, and lateral branch formation, which results in increased branching morphogenesis.

KEY WORDS: Heparanase, FGF10, Heparan sulfate, Perlecan, Salivary gland development

INTRODUCTION

Heparan sulfate proteoglycans (HSPGs) are transmembrane, GPI-linked, or secreted into the extracellular matrix (ECM) and are involved in many developmental processes (Bishop et al., 2007; Iozzo, 2005). Their broad biological activities primarily result from the diversity of sulfation patterns on their heparan sulfate (HS) side chains, which bind a wide range of growth factors, are critical for growth factor signaling, and act as a storage reservoir in the ECM (Vlodavsky et al., 1996; Whitelock and Iozzo, 2005). Additionally, different HS structures are spatially and temporally expressed in cell- and tissue-specific patterns throughout development (Allen and Rapraeger, 2003). Understanding how growth factor storage and release from HSPGs occurs is fundamental to understanding how organogenesis is regulated. The activities of HSPGs are modulated by three main mechanisms: sheddases, which release the extracellular domains from the cell surface; extracellular 6-O-sulfatases, which modify the HS sulfation patterns; and cleavage of the HS chains by endoglycosidases such as heparanase (Sanderson et al., 2005).

Heparanase, an endoglycosidase that requires proteolytic activation, has been extensively studied for its role in angiogenesis and tumor metastasis (Bame, 2001; Elkin et al., 2001; Sanderson et al., 2004; Vlodavsky et al., 2002; Zcharia et al., 2005b). Heparanase releases HS fragments that are more bioactive than the native HS chains from which they are derived (Kato et al., 1998; Sanderson et al., 2005; Sanderson et al., 2004; Vlodavsky and Friedmann, 2001). Heparanase also cleaves perlecan HS in the basement membrane and releases FGF2, making it available for growth factor-dependent signaling during angiogenesis, wound healing and tumor formation (Elkin et al., 2001; Ishai-Michaeli et al., 1990; Reiland et al., 2006; Vlodavsky et al., 2001; Whitelock et al., 1996). The bioactivity of an FGF may be modulated by its release from the ECM as a complex with a fragment of HS (Bame, 2001; Reiland et al., 2006), and the high-affinity activation of FGF receptors (FGFRs) and FGFs requires the formation of a ternary complex with HS (Kan et al., 1999; Pantoliano et al., 1994). The role of heparanase during early embryo development and particularly FGF10-dependent organogenesis is unknown.

FGF10-FGFR2b signaling is critical for mouse submandibular gland (SMG) development, and embryos lacking either FGFR2b or FGF10 have salivary gland agenesis, as well as other severe developmental problems (De Moerlooze et al., 2000; Ohuchi, 2000; Sekine et al., 1999). However, mice heterozygous for either *Fgf10* or *Fgfr2b* only have hypoplastic salivary and lacrimal glands, whereas other major organ systems develop normally (Entesarian et al., 2005; Jaskoll et al., 2005). In humans, mutations in either *FGF10* or *FGFR2b* cause aplasia of lacrimal and salivary glands (ALSG) (Entesarian et al., 2005) and lacrimo-auriculo-dento-digital (LADD) syndrome, characterized by aplasia or hypoplasia of the salivary and lacrimal systems (Entesarian et al., 2007; Milunsky et al., 2006). Therefore, salivary gland development in both humans and mice is particularly sensitive to levels of FGF10-FGFR2b signaling, and defining how the biological activity of FGF10 is regulated is necessary to understand SMG development.

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Branching morphogenesis of embryonic mouse SMGs in organ culture is particularly sensitive to levels of FGF10 (Hoffman et al., 2002; Steinberg et al., 2005) and is thus a useful model to investigate the regulation of FGF10 bioactivity. FGF10-FGFR2b signaling activates the MAPK cascade, regulating epithelial FGFR gene expression as well as the expression of ECM proteins in the basement membrane (Rebustini et al., 2007). HS is critical for SMG branching morphogenesis, as the addition of b-D-xyloside, bacterial heparitinase or exogenous heparin, inhibits ex vivo SMG branching (Mori, 1994; Nakanishi, 1993; Thompson and Spooner, 1982; Thompson and Spooner, 1983). Importantly, HS biosynthesis and accumulation in the basement membrane is coordinated with epithelial cell proliferation and end bud expansion. Pulse-chase experiments with radiolabeled sulfate showed that newly synthesized BM HSPGs initially accumulated at the tip of the end bud, associated with areas of epithelial proliferation. However, the basement membrane HSPG involved in epithelial proliferation was not identified.

We hypothesized that the activity of FGF10 was influenced by its binding to, and release from, an HSPG in the ECM or on the cell surface. FGF10 secreted by the mesenchyme must traverse the basement membrane to bind FGFR2b, its epithelial receptor. Therefore, modifications of HS chains that affect FGF10 binding, storage and release from the ECM are likely to be important during SMG development. Here, we show that heparanase colocalized with perlecan in the SMG epithelial basement membrane and that exogenous heparanase increased SMG branching, while inhibiting heparanase decreased branching. This inhibition was rescued specifically by exogenous FGF10. Furthermore, we demonstrate that heparanase released the FGF10-FGFR2b complex from purified perlecan as well as from perlecan in the endogenous SMG basement membrane. Our results show that heparanase plays an important role during SMG development by cleaving perlecan HS and releasing FGF10 from the basement membrane to modulate FGFR2b-dependent branching morphogenesis.

MATERIALS AND METHODS

Ex vivo SMG organ culture

SMGs from either E12 or E13 ICR mice were cultured on Nuclepore filters (VWR, IL, USA), as previously described (Hoffman et al., 2002; Steinberg et al., 2005). The filters were floated on 200 μl of serum-free DMEM-F12 in 50-mm glass-bottom microwell dishes (MatTek, MA, USA). The medium contained 100 U/ml penicillin, 100 μg/ml streptomycin, 150 μg/ml vitamin C and 50 μg/ml transferrin. SMGs were cultured at 37°C in 5% CO_2 and photographed at 2, 24 and 48 hours, and the epithelial buds were counted using MetaMorph software (Universal Imaging, PA, USA). Experiments were repeated at least three times.

Mesenchyme-free epithelial rudiments were cultured as described previously (Steinberg et al., 2005). SMGs were incubated in 1.6 U/ml of Dispase (Roche, IN, USA) in medium at 37°C for 20 minutes. Epithelia were separated from the mesenchyme in medium containing 10% BSA and then washed in medium. The epithelia were placed in 15 μl of laminin-111 (Trevigen, MD, USA) on top of a filter, as described above. FGF10 (200 ng/ml) or FGF7 (200 ng/ml; R&D Systems, MN, USA) was added to the media.

Heparanase reagents

Function-blocking rabbit anti-heparanase Ab733, an affinity purified IgG fraction of this antiserum IgG733 (1 mg/ml), and anti-heparanase Ab1453 have been previously described (Schubert et al., 2004; Zetser et al., 2004). Preimmune rabbit serum was used as a control. Laminaran sulfate (LMS) (Miao et al., 1999) and unsulfated laminarin (Sigma, MO, USA) were added at the beginning of the experiment (10 or 50 μg/ml). In control experiments the LMS or Ab733 were washed out at 24 hours and the SMGs cultured for a further 24 hours to show that neither were toxic, since the SMG resumed branching (not shown). E12 SMGs were cultured for 48 hours with 1 μl of Ab733 and a range of concentrations of exogenous recombinant FGFs and heparin-binding EGF-like growth factor (HB-EGF: all from R&D Systems, MN, USA). FGF1 and FGF2 were added at 1, 10, 20, 100 and 200 ng/ml (FGF1=0.065, 0.65, 1.3, 6.5 and 13 nM; FGF2=0.058, 0.58, 1.2, 5.8 and 12 nM); higher concentrations inhibit branching (not shown). FGF7 and FGF10 were added at 10, 50, 100, 200, 750 and 1000 ng/ml (FGF7 and FGF10=0.053, 1.1, 5.3, 10.5, 39.8 and 53 nM); higher concentrations are without additional effects (not shown). HB-EGF was added 1, 10, 20 and 200 ng/ml (0.083, 0.83, 1.7 and 17 nM). The preparation of active, inactive, and unprocessed forms of the human recombinant heparanase enzymes has been described previously (Abboud-Jarrous et al., 2005; Vlodavsky et al., 1999). The enzymes were added to the culture media at the beginning of an experiment at doses of 2.5, 5, 10, 20 and 30 μg/ml.

Immunofluorescence analysis

SMGs were fixed with either 4% PFA-PBS for 1 hour or with ice-cold methanol:acetone (1:1) for 10 minutes and washed with PBS. They were blocked overnight at 4°C in 10% heat-inactivated donkey serum, 1% BSA, MOM IgG blocking reagent (Vector Laboratories, CA, USA), and 0.1% Tween-20-PBS, then incubated for 3 hours with anti-heparanase Ab1453 (1:200), SYBR-green (Molecular Probes; 1:5000), and anti-perlecan mAb1948 (Chemicon, CA, USA), washed, and incubated with Cy3 and Cy5-conjugated secondary Fab fragment antibodies (Jackson Laboratories, PA, USA).

Perlecan ELISA assay

Purified perlecan was isolated as described previously (Knox et al., 2002) from human arterial endothelial cell (HUAEC)-conditioned medium by DEAE-Sepharose chromatography with elution in 1 M NaCl, and purified using an anti-perlecan affinity column. Perlecan was monitored in column fractions using antibodies to the protein core or to HS in an ELISA.

The three-dimensional (3D) laminin-111 ECM (Cultrex Laminin-1, Trevigen, MD, USA), is purified from EHS tumor extract and is >90% pure by SDS-PAGE. Round-bottom 96-well plates were coated overnight at 4°C with Laminin-111, collagen type IV, or growth factor reduced-Matrigel (Trevigen, MD, USA). The wells were blocked with 5% BSA for 1 hour at 37°C, incubated with anti-perlecan mAb 1948 (1:5000), washed three times and then incubated with anti-rat IgG-HRP (1:10000). The HRP binding was detected with TMB substrate (Antigenix America, NY), and stopped with 1 M sulfuric acid. Absorbance was read at 445 nm.

Solid-phase assay of FGF10-FGFR2 complex binding and release from ECM

Laminin-111 ECM (0.05 μg/ml) was used to coat round-bottom, 96-well plates overnight at 4°C. The wells were incubated with 0.5 μg/ml recombinant heparanase, 10 mU/ml heparitinase, 10 mU/ml chondroitinase ABC, 0.1 μg/ml heparin, or a carrier control (0.01% BSA in PBS) for 1 hour at 37°C. Wells were blocked with 5% BSA for 1 hour at room temperature, incubated with 1 nM recombinant mouse FGFR2b-human Fc chimera (referred to as FGFR2b) with or without 1 nM recombinant FGF10 or FGF1 diluted in 0.05% Tween-20-PBS (wash buffer) for 1 hour at 37°C, washed four times, incubated with 0.15 μg/ml biotinylated anti-human-Fc antibody (Jackson Laboratories) for 1 hour at 37°C, washed four times, incubated for 1 hour at 37°C with streptavidin-HRP, and then developed with TMB substrate solution as described above.

Ligand and carbohydrate engagement (LACE) assay

A modification of the LACE assay (Allen and Rapraeger, 2003) was performed using a recombinant mouse FGFR2b-human Fc chimera (referred to as FGFR2b) and FGF10 (all from R&D Systems, MN, USA). SMG cultures were treated with 100 μg/ml heparin for 30 minutes at 37°C, washed, fixed with 4% PFA-PBS for 15 minutes at RT, washed, and permeabilized with 0.5% Triton X-100-PBS for 10 minutes. Some glands were treated for 3 hours with 10 μg/ml active heparanase or 0.005 U/ml heparitinase I (Seikagaku, USA) at 37°C and then washed with PBST. The glands were blocked overnight at 4°C with 10% BSA in 0.1% Tween-20-PBS (PBST), incubated for 3 hours with either 50 nM of FGFR2b, with or without 50 nM FGF10, anti-heparanase Ab1453, anti-perlecan (rat

mAb1948; Chemicon, CA, USA), or anti-syndecan 1 (mouse mAb281-2; Pharmingen, CA, USA). After washing, Cy dye-conjugated secondary antibodies were added for 1.5 hours. The FGFR2b was detected with anti-human Fc using a Zeiss LSM 510 microscope.

Preparation of RNA and cDNA and real-time PCR

RNA and cDNA were prepared from SMGs using the RNAqueousTM-4PCR kit with DNase treatment (Ambion, TX, USA) and reverse transcription reagents (Bio-Rad, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed as previously described (Rebustini et al., 2007) using primers designed with similar properties using Beacon Designer software (sequences available on request), SYBR-green PCR Master Mix, and a MyIQ real-time PCR thermocycler (all Bio-Rad). Each cDNA (0.5-1.0 ng) was amplified with 40 cycles of 95°C for 10 seconds and 62°C for 30 seconds. Gene expression was normalized to the housekeeping gene, *29S*. Melt-curve analysis confirmed a single product was amplified. The reactions were run in triplicate and the experiment repeated three times.

Surface plasmon resonance (SPR) analysis

Perlecan was immunopurified as described above and analyzed using a BIAcore 2000 (Amersham Biosciences). Biotinylated perlecan (10 μg/ml) in PBS was coupled to each flow cell of a streptavidin-derivatized sensor chip at a flow rate of 5 μl/min (ΔRU ~1000). HS chains of immobilized perlecan in one flow cell were treated with heparanase (5 μg/ml in PBS) for 10 minutes at 5 μl/minute at 25°C and the surface washed with three 1-minute pulses of 2 M NaCl. An untreated flow cell was used as a reference. Binding experiments were performed at a flow rate of 30 μl/minute at 25°C. The injected volume was 50 or 55 μl, and the kinject function was used with a programmed dissociation time of 150 seconds. All growth factors and growth factor-receptor complexes were diluted in HBS-P [0.01 M Hepes, 0.15 M NaCl, 0.005% (v/v) polysorbate 20, pH 7.4; BIAcore] running buffer to a final concentration of 50 nM with or without 0.1 μg/ml heparin (Celsus, OH, USA). FGFR2b was incubated with FGF10 for 5 minutes prior to injection. In some experiments, 50 nM FGF-FGFR2b was injected at 30 μl/minute, the flow rate was changed to 5 μl/minute, and heparanase (5 μg/ml in PBS) was injected. The perlecan surface was regenerated with a 30 second pulse of 2 M NaCl or 100 μg/ml heparin. Sensograms were analyzed using the BIAcore 2000 Evaluation Software 3.0.

Statistical analysis

Values are reported as means and standard errors for each group from two or more experiments. The data were analyzed by one-way ANOVA.

RESULTS

Heparanase is expressed throughout SMG development and is colocalized with perlecan in the basement membrane of epithelial clefts

Heparanase was expressed throughout SMG development, suggesting it may be involved in branching morphogenesis and gland homeostasis (Fig. 1A). The expression of *Fgf10* and perlecan increased during the early stages of SMG development (E12-E14) when branching morphogenesis begins (Fig. 1A). E13 SMGs were also separated into epithelium and mesenchyme and analyzed by RT-PCR to determine the relative level of expression in the mesenchyme compared to the epithelium (Fig. 1B). *Fgf10* and E-cadherin were used as mesenchyme and epithelium markers, respectively, to show the purity of the cDNA pools following the dissections. Heparanase was more abundant in the mesenchyme, whereas perlecan was present at similar levels in the epithelium and mesenchyme (Fig. 1B). Whole-mount immunostaining of E13 SMGs also showed that heparanase was mainly present in the mesenchyme and accumulated around the epithelial buds in the basement membrane (Fig. 1C, green). Perlecan also localized to the basement membrane, and SYBR-green stained the nuclei (Fig. 1C). Heparanase colocalized with perlecan in the basement membrane,

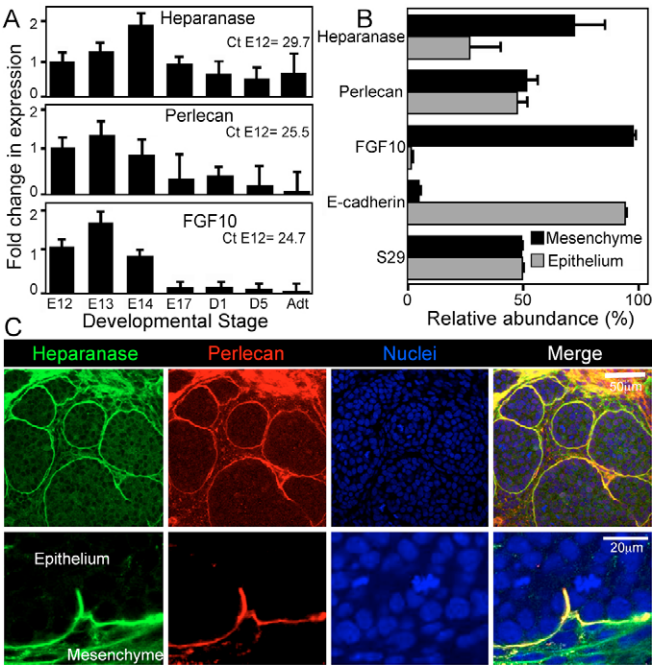

Fig. 1. Heparanase is expressed in mouse SMGs throughout development, mainly in the mesenchyme, and colocalizes with perlecan in the epithelial basement membrane. (**A**) RT-PCR analysis of heparanase, *Fgf10*, and perlecan were compared at various developmental stages. Gene expression was normalized to *29S* and is expressed relative to gene expression at E12. Data were obtained from triplicate experiments, repeated three times, and are mean±s.d. (**B**) Relative abundance of gene expression comparing E13 epithelium with mesenchyme. cDNA were prepared from E13 epithelium separated from mesenchyme. Gene expression was normalized to *29S*. (**C**) Immunolocalization of heparanase and perlecan in E13 SMGs cultured for 36 hours. Heparanase (green) localizes in the mesenchyme and colocalizes with perlecan (red) in the basement membrane. The images are of a single 2 μm confocal section. Nuclei stained with SYBR-green (blue).

and at higher magnification was clearly present in epithelial clefts (Fig. 1C). Heparanase cleaves perlecan HS (Hirata and Nakamura, 2006; Reiland et al., 2004; Whitelock et al., 1996), but the striking colocalization in the clefting regions of SMG epithelium suggested it might influence epithelial clefting and branching.

Inhibition of heparanase function decreases SMG branching and is rescued by exogenous FGF10

Heparanase function was decreased using laminaran sulfate (Fig. 2A), a potent inhibitor of heparanase that binds and inhibits its hydrolytic activity (Miao et al., 1999), a function-blocking heparanase antiserum (Fig. 2B) (Schubert et al., 2004; Zetser et al., 2004). Both treatments significantly inhibited branching in a dose-dependent manner, (Fig. 2) compared to laminarin, an unsulfated polysaccharide control, or control rabbit serum. Additionally, an affinity purified IgG fraction of Ab733 (25 μg/ml) also inhibited branching by ~50%, in a similar manner to 1 μl of Ab733 antiserum (data not shown). SMGs were then treated with a dose of Ab733 that decreased the number of buds by ~50% (Fig. 3), and a range of concentrations of exogenous FGFs or HB-EGF were added (see Materials and methods) to the culture medium. FGF10, above 100 ng/ml, was able to rescue the glands from the inhibitory effects of Ab733 (Fig. 3A,B). However, FGF1, FGF2 (both 10 ng/ml shown),

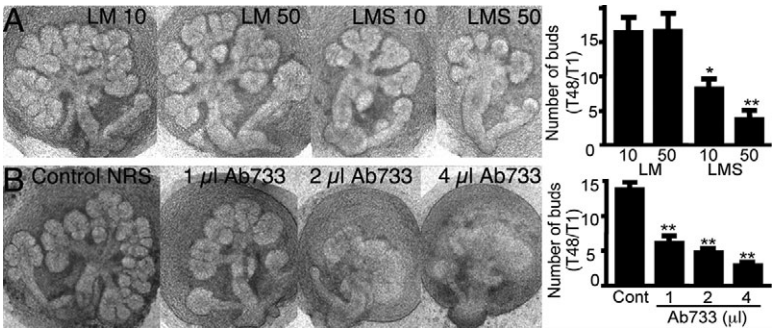

Fig. 2. Inhibition of heparanase function decreases branching morphogenesis of mouse SMGs. Laminaran sulfate (LMS), which inhibits heparanase activity, decreases branching morphogenesis (**A**), whereas the unsulfated laminarin control (LM) does not. In addition, a function-blocking anti-heparanase antiserum (**B**) decreases branching morphogenesis in a dose-dependent manner, whereas the control serum does not. E12 SMGs were cultured in the presence of unsulfated laminarin (LM; 10 or 50 μg/ml) or LMS (10 and 50 μg/ml), a heparanase function-blocking antiserum (Ab733; 1, 2, 4 μl/well), or normal rabbit serum (4 μl/well shown) for 48 hours. The number of buds was expressed as a ratio of the number of buds at 48 hours/number of buds at 1 hour (T48/T1). At least five SMGs/condition were used and the experiment repeated at least three times. ANOVA; *P<0.05; **P<0.01.

FGF7 (100 ng/ml shown) or HB-EGF (20 ng/ml, data not shown) were unable to rescue the SMGs. Higher concentrations (>200 ng/ml) of FGF1 and FGF2 inhibit SMG morphogenesis and higher concentrations of FGF7 or HB-EGF were without additional effect. The morphogenesis of SMGs treated with Ab733 and FGF10 resembled that of the control glands, whereas those treated with other FGFs, did not continue to cleft and branch, but did increase in size. We hypothesized that heparanase functions to release the endogenous HS-bound FGF10, which is required for branching.

Exogenous heparanase increases branching morphogenesis and MAPK activation of intact SMGs, and increases end bud clefting and lateral branch formation in isolated epithelium cultured with FGF10

To determine whether cleavage of SMG HS by heparanase increased endogenous growth factor activity, we added an active, an unprocessed, and an inactive form of heparanase to SMGs. The unprocessed form requires proteolytic cleavage to become active (Levy-Adam et al., 2005; Vlodavsky et al., 1999), whereas the inactive form has two point mutations at the proteolytic cleavage sites, located at Glu^{109}-Ser^{110} and Gln^{157}-Lys^{158}, which inhibit its activation (Abboud-Jarrous et al., 2005). The active and unprocessed forms increased SMG branching morphogenesis (Fig. 4A,B), whereas, the inactive form had no effect. These data suggest that heparanase releases endogenous HS-binding growth factors that stimulate branching morphogenesis. Since FGF10-FGFR2b signaling involves downstream MAPK activation, we measured the phosphorylation of ERK1/2 (also known as MAPK3/1) after heparanase treatment (Fig. 4C). Phosphorylation of ERK1/2 was increased over control levels by both the active (~3-fold increase) and unprocessed (~2-fold increase) forms of heparanase. Thus, heparanase releases endogenous growth factors from the ECM reservoir that activate MAPK signaling.

We added recombinant heparanase isoforms to isolated salivary epithelium cultured in a 3D laminin ECM supplemented with FGF10. Exogenous active heparanase has a dramatic effect on epithelial morphogenesis in FGF10-cultured epithelia, increasing lateral branching, duct elongation and end bud clefting (Fig. 4D). We added less FGF10 (200 ng/ml) to these assays than previously described (Steinberg et al., 2005) to avoid maximal FGF10 stimulation. Unprocessed heparanase also increased branching compared to the epithelial rudiments treated with the inactive enzyme (Fig. 4D), implying that the epithelium activates heparanase, although we have no direct evidence that this occurs in the intracellular or extracellular compartment. The inactive enzyme had no effect on morphogenesis and appears the same as the carrier control (not shown). The increased lateral branching and end bud clefting was not simply due to heparanase releasing FGF10 and increasing its bioavailability, because when we added more FGF10 (500 ng/ml; Fig. 4D) without heparanase, we observed increased duct elongation, similar to what we previously reported (Steinberg et al., 2005), but not increased lateral branching. These results indicate that the salivary epithelium can process heparanase to its

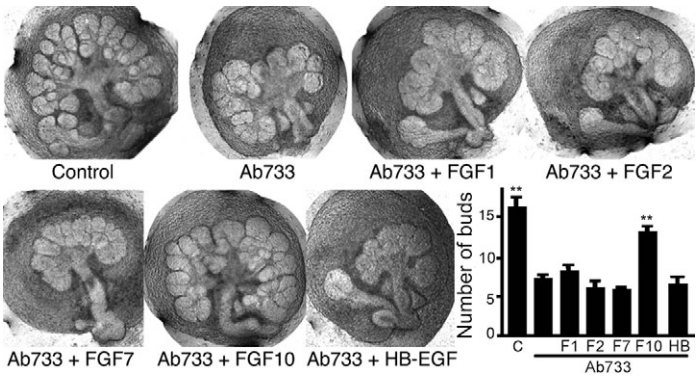

Fig. 3. Inhibition of heparanase function by Ab733 is specifically rescued by FGF10. SMGs were cultured for 48 hours with 1 μl of function-blocking Ab733 (~IC_{50} number of end buds) and increasing doses (see Materials and methods for concentrations) of exogenous FGF1 (10 ng/ml), FGF2 (10 ng/ml), FGF7 (100 ng/ml), FGF10 (100 ng/ml) and HB-EGF (20 ng/ml) were added. The number of buds was expressed as a ratio of the number of buds at 48 hours/number of buds at 1 hour (T48/T1). At least five SMGs per condition were used and the experiment was repeated at least three times. ANOVA compared with the Ab733 alone, **P<0.01.

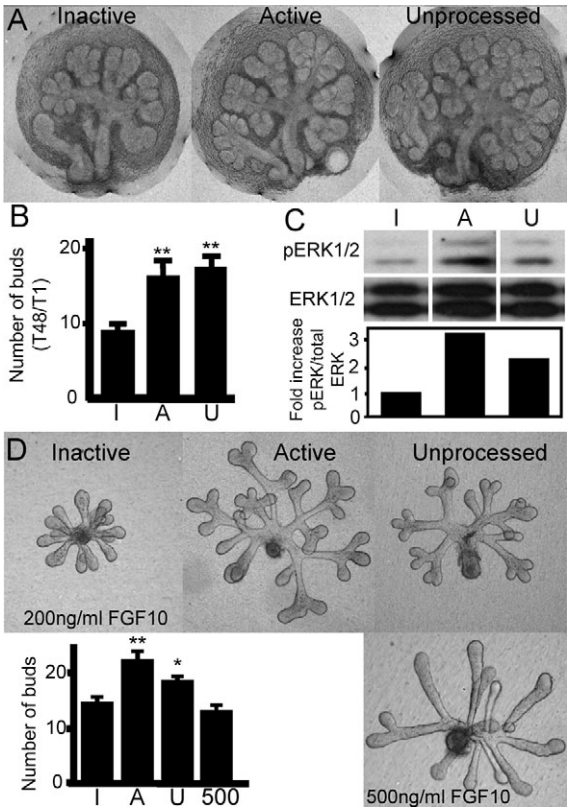

Fig. 4. Recombinant heparanase increases branching morphogenesis of the intact SMG, increases phosphorylation of ERK1/2, and when added to isolated epithelium cultured in a 3D ECM, increases lateral branching, end bud clefting and duct elongation. (**A**) E12 SMGs were cultured with 5 μg/ml of either inactive (I), active (A), or unprocessed (U) forms of heparanase for 48 hours. (**B**) The number of buds was expressed as a ratio of the number of buds at 48 hours/number of buds at 1 hour (T48/T1). (**C**) Western blot analysis of phospho-ERK1/2 and total ERK1/2 after 48 hours of treatment with either inactive, active, or unprocessed heparanase resulted in an ~3-fold increase in pERK1/2 with active and an ~2.3-fold increase with unprocessed heparanase. (**D**) Isolated SMG epithelia were cultured with 200 ng/ml of FGF10 (a sub-optimal dose for growth) and treated with 5 μg/ml of either inactive (which appeared similar to a carrier control, not shown), active or unprocessed heparanase and compared after 48 hours with epithelia cultured with 500 ng/ml of FGF10. The total number of end buds was counted from at least five glands/condition and the experiments repeated twice. ANOVA compared with inactive heparanase; **P*<0.05; ***P*<0.01.

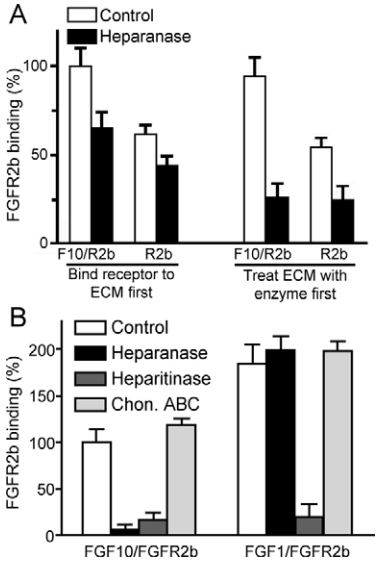

Fig. 5. Heparanase releases an FGF10-FGFR2b complex bound to ECM in a solid-phase assay, and pretreatment of the ECM by heparanase decreases FGF10-FGFR2b but not FGF1-FGFR2b binding. (**A**) FGF10-FGFR2b or FGFR2b alone, was incubated in a 96-well plate precoated with a laminin-111 ECM that contains 2% perlecan by ELISA (data not shown). Either inactive (control) or active heparanase was added to release the bound complex, and FGFR2b was detected by ELISA. Pretreatment of the ECM with heparanase resulted in a larger decrease in binding of both the complex and the receptor alone. (**B**) The binding of FGF10-FGFR2b was decreased by both heparanase and heparitinase treatment; however, FGF1-FGFR2b could still bind the remaining HS after heparanase but not heparitinase treatment. ECM-coated wells were incubated with or without inactive heparanase (control), active heparanase, bacterial heparitinase, or chondroitinase for 1 hour, followed by incubation with FGF10-FGFR2b or FGF1-FGFR2b (1 nM each) for 1 hour. ELISA assays were performed in triplicate and repeated at least three times.

active form, but importantly suggest that heparanase cleavage of ECM HS increases FGF10 bioactivity to induce lateral branching and end bud clefting.

Heparanase releases FGF10-FGFR2b complex bound to the HS on perlecan in the 3D laminin ECM

The 3D ECM used in the epithelial morphogenesis assay contains mainly laminin-111 from murine EHS tumor; however, HSPGs such as perlecan are copurified with laminin, which has numerous HS binding domains. A perlecan ELISA, which is not strictly quantitative, was used to confirm that perlecan is present in the 3D laminin-111 ECM. Purified endothelial cell perlecan (0.625-80 ng/well) was used to generate a standard curve. Using 300 ng/well of 3D laminin-111 ECM we detected ~2% perlecan by weight (data not shown). In addition, basement membrane Matrigel contained ~4% perlecan, and purified collagen IV had undetectable perlecan, as determined by ELISA (data not shown).

We developed a solid-phase binding assay using laminin ECM-coated plates to measure FGF10-FGFR2b binding to, and heparanase-mediated release from, the ECM using an anti-human-Fc-biotinylated antibody and streptavidin-HRP to detect the FGFR2b-Fc. As expected, the combination of FGF10-FGFR2b showed increased binding to the ECM compared with that of FGFR2b alone (Fig. 5A), supporting previous biochemical data that the ternary complex of FGF10-FGFR2b and HS has greater affinity than either FGFR or FGF alone (Ibrahimi et al., 2004; Mohammadi et al., 2005a). FGF10-FGFR2b complex binding to the ECM after inactive heparanase treatment (control) was reported as 100% binding (Fig. 5A). The addition of active heparanase after FGF10-FGFR2b complex binding to the ECM resulted in release of about 40% of the complex from the ECM (Fig. 5A, first set of bars). FGFR2b also has a heparin-binding site, independent of its ligand binding site, but binds to the ECM ~44% less than the FGF10-FGFR2b complex (Fig. 5A, second set of bars), and FGFR2b could also be partly released from the ECM by treatment with heparanase. Alternatively, the ECM was pretreated with either inactive (control) or active heparanase before adding the FGF10-FGFR2b complex or

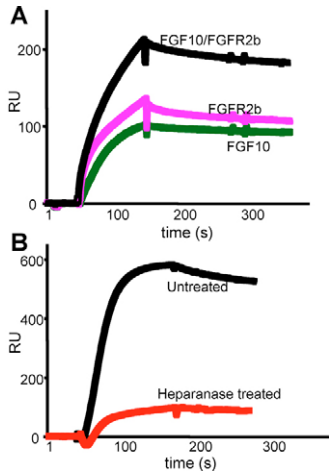

Fig. 6. The FGF10-FGFR2b complex shows greater binding to purified intact HUAEC perlecan than FGF10 or FGFR2b alone, and binding of the complex is reduced by heparanase treatment. (**A**) SPR analysis of FGF10, FGFR2b, and FGF10-FGFR2b complex binding to intact perlecan. Proteins were diluted in HBS-P containing 0.1 μg/ml heparin. (**B**) SPR analysis of FGF10-FGFR2b binding to perlecan before and after heparanase treatment. Heparanase (5 μg/ml at 5 μl/minute) was applied to the chip surface. RU, response units.

the receptor alone (Fig. 5A, third and fourth set of bars). There was a decrease of ~75% in FGF10-FGFR2b complex binding to the ECM when it was pretreated with heparanase and ~50% decrease in FGFR2b binding to the ECM. These results show that FGF10-FGFR2b binding to the ECM is mediated by HS and can be released by heparanase. Pretreatment of the ECM with heparitinase, but not chondroitinase ABC, also decreased the binding of the FGF10-FGFR2b complex (Fig. 5B); as did co-incubation of the complex with heparin (data not shown), further demonstrating that the complex binding is HS dependent. We also compared FGF1-FGFR2b binding to the ECM; interestingly, binding increased ~150% compared with that of the FGF10-FGFR2b complex, suggesting the FGF1 complex binds to more HS epitopes than the FGF10 complex. Surprisingly, treatment of the ECM with heparanase did not decrease FGF1-FGFR2b complex binding, in contrast to both heparitinase (Fig. 5B) and heparin (data not shown), suggesting that although interaction of FGF1 with FGFR2b is HS dependent, there are differences in the HS bound by the FGF1-FGFR2b compared with the FGF10-FGFR2b.

Heparanase releases the FGF10-FGFR2b complex from purified perlecan

We used SPR analysis to provide additional biochemical evidence that heparanase releases the FGF10-FGFR2b from perlecan. We compared the binding of FGF10 and FGFR2b alone and in a complex to purified endothelial cell perlecan (Fig. 6A). However, the purified FGF10 binds nonspecifically to the chip surface when not complexed to FGFR2b, therefore 0.1 μg/ml of heparin was added to samples with FGF10 (Fig. 6A) to reduce nonspecific binding, but it also reduced overall binding by a factor of 2. Additionally, 1.0 μg/ml heparin completely abolished binding (data not shown), suggesting that FGF10 binding was HS dependent. The flow cell was treated with heparanase to determine whether FGF10-FGFR2b binding was via perlecan HS chains. Heparanase pretreatment greatly reduced binding of FGF10-FGFR2b to perlecan (Fig. 6B). This loss of binding was also observed after heparitinase treatment of perlecan (data not shown). Heparanase was also able to release bound FGF10-FGFR2b complexes under flow (data not shown). Importantly, these data show that FGF10 and FGF10-FGFR2b bind to perlecan HS and that heparanase treatment decreases the complex binding to the HS side-chain of a purified, intact proteoglycan.

Heparanase treatment decreases binding of FGF10-FGFR2b to endogenous SMG HSPGs

The biochemical data suggested that the FGF10-FGFR2b complex bound to perlecan HS and was released by heparanase. Therefore, we confirmed that this also occurs with endogenous SMG HSPGs, using a whole-mount, modified ligand and carbohydrate engagement (LACE) assay. SMGs were treated with heparitinase or heparanase, then probed with the FGFR2b alone (Fig. 7A) or with an FGF10-FGFR2b complex (Fig. 7B,C), which was detected with anti-human-Fc antibodies and also immunostained for perlecan and syndecan 1. Single confocal sections through an epithelial bud reveal that FGFR2b bound to the basement membrane, where it colocalized with perlecan, and also bound to epithelial cell membranes. This suggests that HS on perlecan as well as other cell surface HSPGs bind FGFR2b. Increased binding was detected in both the basement membrane and the epithelial cell membranes when glands were incubated with the FGF10-FGFR2b complex. In addition, the complex colocalized with perlecan in the basement membrane (Fig. 7B), and partly colocalized with syndecan 1 in the epithelium (Fig. 7C). Importantly, heparanase pretreatment decreased the binding of FGFR2b and the FGF10-FGFR2b complex, indicating that both the receptor and the complex required heparanase-sensitive HS for binding (Fig. 7A,B), and that they both colocalized with perlecan in the basement membrane. Additionally, there was more syndecan 1 in the mesenchyme than in the epithelium that did not colocalize with FGF10-FGFR2b, suggesting that syndecan 1 in the mesenchyme may have different HS chains. Staining with the syndecan antibody, which recognizes the syndecan core protein, was not decreased by pretreatment with either heparanase or heparitinase (data not shown). The partial colocalization in the epithelium also suggests that other epithelial HSPGs bind the FGF10-FGFR2b complex.

DISCUSSION

Although HSPGs have well-known roles in embryonic development, including acting as a reservoir for heparin-binding growth factors, less is known about the mechanisms by which growth factor activity is influenced by cleavage of HS. Here, we provide evidence that heparanase cleaves perlecan HS in the SMG epithelial basement membrane and releases FGF10, which increases MAPK signaling, lateral branching and end bud clefting, all of which increase branching morphogenesis.

Genetic knockouts of heparanase have not been reported, but transgenic mice overexpressing heparanase are viable and fertile but have abnormal mammary, hair, kidney and implantation phenotypes. In particular, the mammary gland has excess branching, widening of the ducts, increased neovascularization and disruption of the epithelial basement membrane (Zcharia et al., 2001; Zcharia et al., 2005a). Thus, we investigated the role of heparanase in SMGs where it is expressed throughout development (Fig. 1A). The localization of heparanase in the basement membrane and epithelial clefts (Fig. 1C), and its colocalization with perlecan, suggested that it might be involved in ECM regulation of morphogenesis. In support of this hypothesis, branching morphogenesis of SMGs was blocked by a function-blocking heparanase antiserum. Importantly, only FGF10

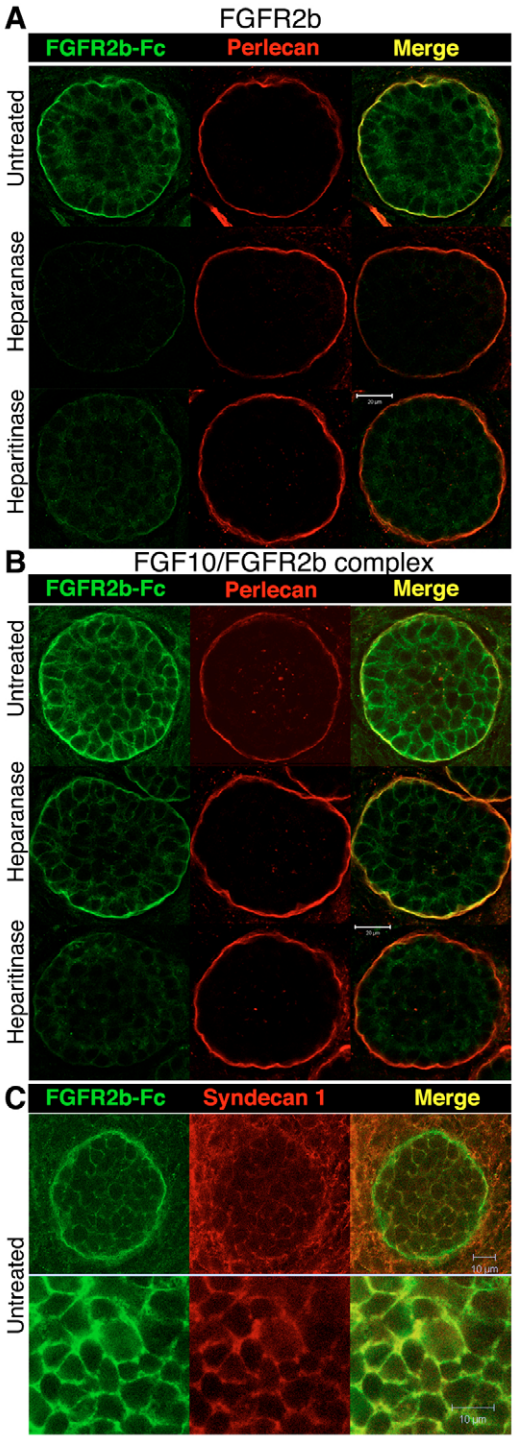

Fig. 7. FGFR2b and the FGF10-FGFR2b complex colocalize with endogenous SMG perlecan HS, and the binding is decreased by heparanase treatment. A whole-mount ligand and carbohydrate engagement (LACE) assay using E13 SMGs shows that FGFR2b (**A**) and the FGF10-FGFR2b complex (**B**) colocalize with perlecan in the basement membrane. Increased binding was detected with the complex compared to the receptor alone. There was decreased binding of both FGFR2b and the FGF10-FGFR2b complex after heparanase or heparitinase treatment. In addition, the FGF10-FGFR2b complex also colocalizes with syndecan 1 in the epithelium (**C**), but also binds other epithelial HSPGs, and the staining was not decreased by pretreatment with either heparanase or heparitinase (data not shown). Images are single confocal sections. Scale bar: 20 μm in A and B; 10 μm in C.

was able to rescue the morphogenesis (Fig. 3), suggesting that FGF10-FGFR2b signaling was specifically required during epithelial morphogenesis. Surprisingly, FGF1 and FGF7, which bind to FGFR2b, were unable to rescue branching. The level of FGF10-FGFR2b signaling is critical for SMG development, and simply stimulating FGFR2b does not restore normal branching morphogenesis, suggesting that endogenous HS specifies FGF10 function and that the release of FGF10 is required for branching morphogenesis. It is probable that heparanase releases other heparin binding growth factors that promote branching morphogenesis, such as HB-EGF. However, while HB-EGF stimulates SMG proliferation (Umeda et al., 2001), it was unable to rescue the growth of Ab733-treated glands (Fig. 3), demonstrating specific modulation of FGF10 function in SMGs by heparanase.

Exogenous, active heparanase promoted branching of intact glands, increased MAPK signaling as measured by ERK1/2 phosphorylation, and promoted lateral budding, duct elongation, and end bud clefting in isolated epithelium (Fig. 4). However, unprocessed heparanase was also active in assays with both the intact gland and the isolated epithelium. Processing of heparanase occurs in the lysosomal/endosomal compartment, and cathepsin-L can activate heparanase (Abboud-Jarrous et al., 2005; Cohen et al., 2005), although other proteases may be involved. Extracellular heparanase binds low-density lipoprotein receptor-related proteins, mannose 6-phosphate receptors and HSPGs, and is proteolytically activated after being internalized by endocytosis (Gingis-Velitski et al., 2004; Nadav et al., 2002; Vreys et al., 2005). Our experiments with unprocessed heparanase (Fig. 4D) suggest that the SMG epithelium could either activate heparanase by endocytosis and intracellular proteolysis, or by an extracellular protease in the extracellular compartment. FGF10-mediated SMG epithelial morphogenesis is matrix metalloproteinase (MMP) dependent (Steinberg et al., 2005). However, we have no direct evidence to suggest that unprocessed heparanase is activated in the intracellular or extracellular compartment.

In contrast to heparanase, both exogenous heparitinase and heparin inhibit branching [our data, not shown, which reproduces a previous report (Nakanishi et al., 1993)]. Heparitinase cleaves the HS chains into di- and tetrasaccharides that are too small to facilitate biological activity, such as growth factor binding (Reiland et al., 2004). Heparanase, however, cleaves the glycosidic bonds of HS chains at only a few sites, producing fragments that are reported to be 10-20 sugar residues long, or ~5-7 kDa, and there is evidence that these fragments are more biologically active than the native HS chain from which they are derived (Elkin et al., 2001; Kato et al., 1998; Sanderson et al., 2004; Vlodavsky and Friedmann, 2001). Additionally, the spacing of the glycosaminoglycan S-domains (regions of HS rich in N- and O-sulfate groups and iduronate residues) is critical for the cleavage and for the substrate specificity of the resulting fragments (Bame, 2001). Our data suggest that the heparanase-derived HS fragments increase the bioactivity of FGF10. Firstly, the addition of heparanase allowed us to use less FGF10 in our assays (200 versus 1500 ng/ml, Fig. 4D) compared to our previous work (Steinberg et al., 2005). However, the increased bioactivity of FGF10 was not simply due to increased bioavailability; if we added more FGF10, the epithelium grew larger with longer ducts, and there was no increase in end bud number (see 500 ng/ml FGF10, Fig. 4D). Secondly, the FGF10 and HS fragments initiate lateral bud formation along the duct; however, there does not appear to be increased localization of FGFR2b at potential sites of lateral branching along the duct; our previous immunostaining and in situ analysis showed that FGFR2b is expressed evenly along the

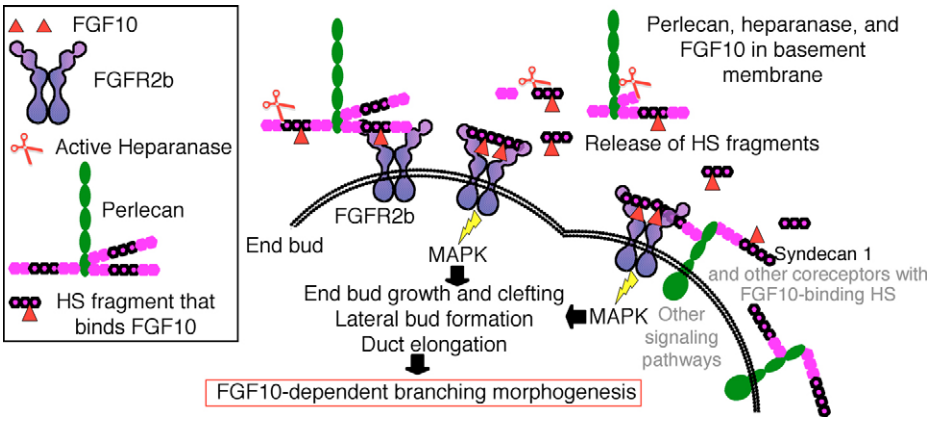

Fig. 8. Model shows release of FGF10-bound HS fragments from perlecan by heparanase in the basement membrane. HS fragments modulate the biological activity of FGF10 by increasing FGF10-FGFR2b complex formation to promote MAPK phosphorylation, end bud growth and clefting, and lateral branch formation. Syndecan 1 in the epithelium binds FGF10-FGFR2b, and we speculate (grey text) that other unidentified epithelial HSPG may specify the location of lateral buds or end bud clefting. Epithelial HSPGs may form a signaling complex with FGFR2b, in combination with HS fragments released by heparanase, and increase MAPK or other intracellular signaling.

ducts (Patel et al., 2006; Steinberg et al., 2005). This suggests that a cofactor on the ductal epithelium may localize the FGF10 or the FGF10-HS fragment to a specific region to initiate FGFR2b signaling, leading to lateral bud formation. Our data suggest that heparanase-derived HS fragments influence the location and specificity of an FGF10-FGFR2b-HS signaling complex, possibly with other unidentified cofactors (see model in Fig. 8). Future studies will explore the hypothesis that defined HS structures in specific regions of the epithelium influence FGF10-dependent epithelial morphogenesis.

An important question is whether the HS in the FGF-FGFR signaling complex is attached to an HSPG on the same cell (in cis), is localized on another cell or in the ECM (in trans), or is an HS cleavage product of HSPGs. The crystal structure of FGFR with FGF and a heparin fragment demonstrates that HS is required for FGFR activation (Mohammadi et al., 2005b). Dimerization of the FGFR signaling complex involves HS-mediated synergistic binding of an FGF to the FGFR, as well as the FGFRs to each other. Therefore, FGF-FGFR ternary complex formation may be influenced by changes in cell- and tissue-specific sulfation patterns on HSPGs during development, which could modulate FGF signaling. We also speculate that there may be two different sources of HS within a dimerized FGF-FGFR-HS signaling complex. HS, in trans, potentiates VEGFR-mediated angiogenesis, which may provide a mechanism for crosstalk between adjacent cell types (Jakobsson et al., 2006). Further work is required to resolve this issue in regards to FGFR signaling.

During embryonic development, tissue-specific differences in HS regulate different FGF-FGFR interactions, suggesting that developmental changes in HS specifically modulate FGF signaling (Allen et al., 2001; Allen and Rapraeger, 2003). Little is known about how HSPGs modulate FGF10 biological activity during development, although HS-FGF10 interactions are important for lung morphogenesis, and influenced by developmentally regulated regional patterns of HS sulfation (Izvolsky et al., 2003a; Izvolsky et al., 2003b). The enzymes that synthesize and modify HS have unique spatiotemporal expression patterns during development and are likely to be important for FGF10 function during SMG morphogenesis. We have used the FGF10-FGFR2b complex in solid-phase binding assays with the 3D laminin ECM to demonstrate that heparanase cleaves the HS that binds the FGF10-FGFR2b complex (Fig. 5A). Using this assay to compare binding of FGF1-FGFR2b with FGF10-FGFR2b, we demonstrated that heparanase cleavage of HS leaves an HS stub that can still bind FGF1-FGFR2b, but does not bind FGF10-FGFR2b (Fig. 5B). In addition, heparitinase cleavage of the HS showed that FGF1-FGFR2b binding was HS dependent. These data provide evidence that different FGFs binding the same receptor bind different HS structures, to potentially mediate different signaling responses.

We used SPR analysis to show that heparanase released FGF10-FGFR2b from purified, intact, HUAEC perlecan HS. Previously it was shown that FGF7-FGFR2b binds to endothelial cell-derived perlecan HS chains (Knox and Whitelock, 2006) and that perlecan HS purified from adenocarcinoma WiDr cells was degraded by heparanase (Reiland et al., 2004). Specific HS structures on perlecan, at different stages of development and in different tissues, may affect the localization of specific FGFs in the local cell environment, controlling processes such as cell differentiation and proliferation during tissue morphogenesis (Knox et al., 2002; Knox and Whitelock, 2006; Melrose et al., 2006). Perlecan binds FGF1, FGF2 and the FGFR1-FGF2 complex via HS chains (Aviezer et al., 1994; Knox et al., 2002). We confirmed the previous SPR findings that FGFR2b binds to perlecan (Knox and Whitelock, 2006; Mongiat et al., 2000) and, for the first time, report that heparanase releases FGF10 bound to purified perlecan HS chains (see model in Fig. 8). Interestingly, perlecan null mice have major developmental anomalies, involving cartilage and heart development, problems with basement membrane integrity under mechanical stress, and as a result up to 40% of embryos dies before SMG organogenesis occurs (Arikawa-Hirasawa et al., 1999; Costell et al., 1999). Additionally, those that survive have not been reported to have defects with branching morphogenesis, however, expression levels of other HSPGs or analysis of heparanase function were not reported. We speculate that other HSPGs in the basement membrane functionally compensate in the absence of perlecan, but this requires further investigation.

The LACE assay showed that FGF10-FGFR2b-binding HS is present on HSPGs in both the epithelial basement membrane and cell surface. Surprisingly, there was very little binding to mesenchymal cells, suggesting that epithelial basement membrane

and epithelial HS is different from the mesenchymal HS. The FGF10-FGFR2b complex colocalized with perlecan in the BM, and partly colocalized with syndecan-1 in the epithelium. Syndecans are transmembrane HSPGs that act as coreceptors for multiple growth factor receptors (Iozzo, 2001). Heparanase cleavage of syndecan 1 HS (Reiland et al., 2004) also increases FGF2 activity (Kato et al., 1998) and increases syndecan 1 expression and shedding (Yang et al., 2007). Epithelial HSPGs may specify the location of lateral buds or end bud clefting, syndecan 1 binds FGF10-FGFR2b, and we speculate that other epithelial HSPGs are involved in FGF10-FGFR2b signaling (Fig. 8).

In summary, we have identified a role for heparanase during normal development, specifically during SMG branching morphogenesis. Heparanase releases FGF10 from perlecan HS chains in the basement membrane and modulates the biological activity of FGF10 by increasing MAPK phosphorylation, end bud clefting, and lateral branch formation. We conclude that a heparanase-derived HS fragment released from perlecan influences not only the bioavailability but also the bioactivity of FGF10 during SMG branching morphogenesis. Identifying the HS structures required for FGF10 bioactivity will further our understanding of the regulation of growth factor activity during development.

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