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HB-EGF function in cardiac valve development requires interaction with heparan sulfate proteoglycans

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SUMMARY

HB-EGF, a member of the EGF family of growth factors, plays an important role in cardiac valve development by suppressing mesenchymal cell proliferation. Here, we show that HB-EGF must interact with heparan sulfate proteoglycans (HSPGs) to properly function in this process. In developing valves, HB-EGF is synthesized in endocardial cells but accumulates in the mesenchyme by interacting with HSPGs. Disrupting the interaction between HB-EGF and HSPGs in an ex vivo model of endocardial cushion explants resulted in increased mesenchymal cell proliferation. Moreover, homozygous knock-in mice ($HB^{\Delta hb/\Delta hb}$) expressing a mutant HB-EGF that cannot bind to HSPGs developed enlarged cardiac valves with hyperproliferation of mesenchymal cells; this resulted in a phenotype that resembled that of *Hbegf*-null mice. Interestingly, although *Hbegf*-null mice had abnormal heart chambers and lung alveoli, $HB^{\Delta hb/\Delta hb}$ mice did not exhibit these defects. These results indicate that interactions with HSPGs are essential for the function of HB-EGF, especially in cardiac valve development, in which HB-EGF suppresses mesenchymal cell proliferation.

KEY WORDS: HB-EGF (HBEGF), HSPGs, Valvulogenesis, Mouse

INTRODUCTION

Heparan sulfate (HS)-glycosaminoglycan (GAG) chains are linear polysaccharides composed of alternating N-acetylated or N-sulfated glucosamine units (N-acetylglucosamine or N-sulfoglucosamine) and uronic acids (glucuronic acid or iduronic acid). HS proteoglycans (HSPGs), which consist of HS-GAG chains covalently attached to a core protein, reside on the plasma membrane of all animal cells. HSPGs have been implicated in a variety of cell signaling pathways (Bernfield et al., 1999; Perrimon and Bernfield, 2000). Several ligands are known to bind to heparin or HS, including the fibroblast growth factor (FGF) family of growth factors (Rapraeger, 1995), the transforming growth factor β (TGFβ) family of growth factors (Lyon et al., 1997), vascular endothelial growth factor (VEGF) (Ferrara et al., 1991), interleukin 3 (IL3) (Roberts et al., 1988), granulocyte-macrophage colony-stimulating factor (CSF2) (Roberts et al., 1988), interferon-γ (Lortat-Jacob et al., 1991), hedgehog (Lin and Perrimon, 2002) and Wnt (Lin and Perrimon, 2002). The binding of ligands to cell-surface HS is thought to result in a high local ligand concentration that activates signaling receptors. Studies with FGFs and their receptor tyrosine kinases indicate that HSPGs function as co-receptors, and these data suggest that HS promotes ligand dimerization, leading to receptor dimerization and the stimulation of kinase activity (Bernfield et al., 1999).

Heparin-binding EGF-like growth factor (HB-EGF; HBEGF) is a member of the EGF family of growth factors and has a high affinity for heparin and HS (Higashiyama et al., 1991; Higashiyama et al., 1993; Mekada and Iwamoto, 2008). HB-EGF is synthesized as a type I transmembrane protein (proHB-EGF) composed of propeptide, heparin-binding, EGF-like, juxtamembrane, transmembrane and cytoplasmic domains (Higashiyama et al., 1992). ProHB-EGF is cleaved within the juxtamembrane domain on the cell surface, resulting in the release of soluble HB-EGF (sHB-EGF) (Goishi et al., 1995), which acts as a mitogenic signal through the EGF receptor (EGFR) (Higashiyama et al., 1991). sHB-EGF is a potent mitogen and chemoattractant for many cell types (Raab and Klagsbrun, 1997). ProHB-EGF is also biologically active as a juxtacrine growth factor that signals to neighboring cells in a non-diffusible manner (Higashiyama et al., 1995; Iwamoto et al., 1999; Iwamoto and Mekada, 2000). Importantly, recent analyses of *Hbegf*-null mice have implicated a role in several physiological and pathological processes (Kimura et al., 2005; Mine et al., 2005; Shirakata et al., 2005; Xie et al., 2007; Mekada and Iwamoto, 2008; Minami et al., 2008), including cardiac valve development (Iwamoto et al., 2003; Jackson et al., 2003; Yamazaki et al., 2003; Iwamoto and Mekada, 2006).

Cardiac valve development (valvulogenesis) occurs in two consecutive steps: cardiac cushion formation and valve remodeling (Armstrong and Bischoff, 2004; Schroeder et al., 2003). During mid-gestation and after cardiac looping, cardiac cushions are formed from localized expansions of the extracellular matrix (cardiac jelly) in the atrioventricular (A-V) boundary as well as in the distal portion of the outflow tract (OFT). Upon receiving signals from the myocardium, the endocardium undergoes an endothelial-to-mesenchymal transition (EMT) and begins to secrete soluble factors that promote further differentiation of the cardiac cushions. After migrating into the cardiac jelly, the mesenchymal cells proliferate to form cushions that subsequently give rise to cardiac valves and the septa of the four-chambered heart (Eisenberg and Markwald, 1995; Lamers et al., 1995). In mice, endocardial cushion formation is complete by embryonic day (E) 12.5 (Lakkis and Epstein, 1998) and is followed by remodeling of the cushions to form thin valve leaflets. Although much is known about the

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signaling pathways involved in cushion formation, the molecular mechanisms underlying valve remodeling are still poorly understood. EGFR signaling appears to function in valvulogenesis. Normal OFT (aortic and pulmonary) valve development requires EGFR tyrosine kinase signaling (Chen et al., 2000), and *Egfr*-deficient mice exhibit global abnormalities in both the A-V and OFT valves (Jackson et al., 2003).

Abnormal valvulogenesis in *Hbegf*-null mice and knock-in mice expressing an uncleavable proHB-EGF have provided new insight into the physiological functions of HB-EGF. These mutant mice exhibit enlarged cardiac valves in which mesenchymal cells have hyperproliferated (Iwamoto et al., 2003; Jackson et al., 2003; Yamazaki et al., 2003). These studies suggest that the soluble form of HB-EGF functions as a 'growth-inhibitory' factor for mesenchymal cells. However, the precise molecular mechanism by which HB-EGF transduces growth-inhibitory signals to mesenchymal cells is still unclear.

Previous studies have reported that HSPGs modulate HB-EGF activities (Raab and Klagsbrun, 1997; Mekada and Iwamoto, 2008). However, a mutant form of HB-EGF that cannot bind heparin possesses a higher full activity than wild-type HB-EGF (Takazaki et al., 2004), suggesting that the ability to bind to HSPGs is not essential for HB-EGF function. Therefore, the physiological significance of the interaction between HB-EGF and HSPGs, especially in vivo, has remained unclear.

During cardiac valve remodeling, HB-EGF is expressed in the endocardium, whereas cellular abnormality (hyperproliferation) occurs in the mesenchyme of valves when *Hbegf* is deleted (Iwamoto et al., 2003; Jackson et al., 2003). Moreover, secretion of sHB-EGF is required for normal valvulogenesis (Yamazaki et al., 2003). These findings strongly suggest that sHB-EGF secreted from the endocardium acts on mesenchymal cells in a paracrine manner (Iwamoto and Mekada, 2006). However, the mechanism by which HB-EGF accumulates in the mesenchyme is unknown. Although the role of HSPGs in valvulogenesis is unclear, HSPGs are candidates in this mechanism, as HB-EGF has HS-binding activity. To examine this possibility, we performed ex vivo studies using cardiac cushion explants and mutant mice ($HB^{\Delta hb}$) that express a truncated form of HB-EGF lacking the heparin-binding domain (HB-ΔHB). Our results demonstrate that interactions with HSPGs are essential for the function of HB-EGF, especially in valvulogenesis, in which HB-EGF suppresses mesenchymal cell proliferation.

MATERIALS AND METHODS

Mice

The generation of *Hbegf*-null mice (HB^{del}) and of knock-in mice expressing uncleavable (uc) proHB-EGF (HB^{uc}) have been described previously (Iwamoto et al., 2003; Yamazaki et al., 2003). These mice were back-crossed for more than seven generations onto a C57BL/6J background. All experimental procedures in this study were approved by the institutional Animal Care and Use Committee of Osaka University.

Histological analysis

Hematoxylin-Eosin staining, Masson's trichrome staining and *lacZ* staining were performed as previously described (Iwamoto et al., 2003). The BrdU-incorporation assay was performed as described (Mine et al., 2005). TUNEL analysis was performed on heart sections using the Dead End Colorimetric TUNEL System as directed by the manufacturer (Promega). For immunohistochemistry of HS, embryos were fixed with 4% paraformaldehyde (PFA), dehydrated and embedded in paraffin. Sections (5 μm) were pretreated with heparitinase I (Seikagaku), incubated with anti-ΔHS mouse monoclonal antibody 3G10 (Seikagaku), and then incubated with Alexa Fluor 488-conjugated streptavidin (Molecular Probes).

To bind HB-EGF to valve sections, serial frozen sections (8 μm) were either left untreated or treated with heparitinase I for 1 hour at 37°C and then incubated with 200 ng/ml Myc- and His-tagged recombinant human HB-EGF or HB-ΔHB (Takazaki et al., 2004) for 4 hours at 4°C, in the presence or absence of 100 μg/ml heparin. Bound HB-EGF was detected with anti-Myc monoclonal antibody 9E10 (Calbiochem) and an Alexa Fluor 546-conjugated anti-mouse IgG antibody. Digestion of HS with heparitinase was evaluated by HS staining with the anti-HS mouse monoclonal antibody 10E4 (Seikagaku) or with 3G10.

Endocardial cushion explant cultures

The OFT and A-V canal ('explant') were dissected from E10.5 wild-type (WT) and mutant embryonic mouse hearts. The explant was placed on a type I collagen gel (Cellmatrix type I-A, Nitta Gelatin) containing 0.5 mg/ml hyaluronic acid (HA-Col; 0.5 ml/well of a 24-well multi-plate) such that one side of the endocardium was in contact with the gel surface. The explant was grown in 0.5 ml Medium 199 supplemented with 1% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.1% each of insulin, transferrin and selenium (Gibco-BRL) at 37°C in 5% CO_2 for 7 days. During this culture period, endocardial cells from the endocardium of the cushion explants (termed 'ON-gel' cells) proliferate and spread on the surface of the HA-Col, whereas differentiated mesenchymal cells from the endocardium of the explants (termed 'IN-gel' cells) invade the HA-Col and proliferate. ON-gel (endocardial) and IN-gel (mesenchymal) cells were discriminated based on whether their focal plane was on the gel surface or in the gel and by their cellular morphology. After 7 days of culture, the proliferation of ON-gel and IN-gel cells was measured by BrdU incorporation. Briefly, 3 μg/ml BrdU was added to the culture medium and incubated for 1 hour. The HA-Col gel and explant were fixed with 4% PFA, permeabilized with 0.5% Triton X-100, prestained with 0.3 μg/ml propidium iodide, post-fixed with 4% PFA, denatured with 2 M HCl and 1% Triton X-100, and blocked with blocking buffer (PBS containing 2% goat serum, 1% BSA, 0.05% Tween 20). The HA-Col gel and explants were then stained with anti-BrdU monoclonal antibody (Abcam) and Alexa Fluor 488-conjugated anti-rat IgG secondary antibody. An image of the BrdU-stained explant was captured and analyzed using a confocal laser-scanning microscope system (LSM5 PASCAL, Carl Zeiss).

lacZ staining was performed as follows. The HA-Col gel and explant were fixed with 1% formalin, 0.2% glutaraldehyde, 0.02% Triton X-100, permeabilized with 1% Triton X-100, and stained with an X-Gal solution. Finally, the HA-Col gel and explant were counterstained with Nuclear Fast Red. Endothelial and mesenchymal markers were stained with anti-CD31 (BD Pharmingen) monoclonal antibody or with anti-SMA (Sigma).

To inhibit EGFR activity in WT explants, the indicated concentrations of ZD1839 (AstraZeneka) were added 3 days after starting the culture, and then further cultured for 4 days. To inhibit the HB-EGF–HS interaction in WT explants, heparin (1 or 10 μg/ml), heparitinase I (3.4 mU/ml), or sodium chlorate (30 mM) was added 3 days after starting the culture, and then further cultured for 4 days. To rescue the knockout explants with WT sHB-EGF and sHB-ΔHB, each recombinant protein was added 1 day after starting the culture, and then further cultured for 6 days.

Cell lines, culture and transfection

DER cells, which are 32D cells that stably express human EGFR, and the CHO mutant cell line 677 were maintained as previously described (Iwamoto et al., 1999; Shishido et al., 1995). Mouse sHB-EGF and sΔHB were prepared as previously described (Takazaki et al., 2004). Vero cells expressing an ecotropic retrovirus receptor (Vero-ecoR) were prepared and maintained as previously described (Wang et al., 2006). To obtain transfectants expressing mouse proHB-EGF, proHB-UC and proHB-ΔHB, Vero-ecoR cells were transduced with retrovirus encoding each cDNA (Wang et al., 2006).

Characterization of HB-ΔHB

Immunoblotting of HB-EGF, heparin-Sepharose chromatography and the mitogenic assay were performed as described previously (Takazaki et al., 2004). The shedding assay was performed as described previously (Wang et al., 2006).

Generation of $HB^{\Delta hb}$ mice

The targeting vector for $HB^{\Delta hb}$ is depicted in Fig. S5B in the supplementary material. The cDNA encoding mouse $HB^{\Delta hb}$ (Met1-Gly92/Asp106-His208), which also contains a 162 bp *Not*I fragment in the 5′ non-coding region, was generated by PCR. The targeting vector is similar to that for HB^{uc} (Yamazaki et al., 2003), but contains the cDNA encoding $HB^{\Delta hb}$. The targeting vector was transfected into the TT2 ES cell line (Yagi et al., 1993). G418-resistant colonies were isolated and screened for homologous recombination by Southern blot analysis of *Hin*dIII-digested DNA with probes A and B (see Fig. S5C in the supplementary material). Positive clones were injected into ICR blastocysts. Two independent ES cell clones generated chimeric mice carrying the $HB^{\Delta hb}$ allele. The chimeric mice were mated with C57BL/6J female mice. The resulting mice were genotyped by PCR amplification of tail DNA samples (see Fig. S5D in the supplementary material), as described previously (Yamazaki et al., 2003). $HB^{\Delta hb}$ mice were back-crossed for more than eight generations onto a C57BL/6J background.

Northern blotting and immunoblotting of tissue samples were performed as described previously (Yamazaki et al., 2003).

Data analysis

Statistical significance was assessed by Student's *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

HB-EGF secreted from the endocardium suppresses mesenchymal cell proliferation during cardiac valve remodeling

We examined the expression pattern of *Hbegf* mRNA during valvulogenesis using a targeting vector containing the *lacZ* reporter gene to monitor *Hbegf* expression in $HB^{del/+}$ mice (Iwamoto et al., 2003). Chronological analysis of *Hbegf* expression revealed that, especially during the remodeling stage after E13.5, expression was exclusively throughout the endocardium, but not in mesenchymal cells in the cardiac jelly (see Fig. S1 in the supplementary material). Coincident with the onset of *Hbegf* expression, increased OFT and A-V valve thickness in both $HB^{del/del}$ and $HB^{uc/uc}$ (see Materials and methods) embryos appeared during the remodeling stage (see Fig. S2 in the supplementary material), indicating that sHB-EGF functions during valve remodeling rather than during the cushion formation/EMT stage. Moreover, this valve enlargement was caused by increased proliferation of mesenchymal, but not endocardial, cells (see Fig. S3A-C in the supplementary material). The number of apoptotic cells in the endocardium and mesenchyme was similar in $HB^{del/+}$ and $HB^{del/del}$ valves (see Fig. S3D,E in the supplementary material). Collectively, these results indicate that sHB-EGF, secreted from the endocardium, inhibits the proliferation of mesenchymal cells during valve remodeling.

HB-EGF accumulates in the mesenchyme of developing cardiac valves through interactions with HS

We found that HS was abundantly expressed in the mesenchyme of developing valves of E15.5 embryos (Fig. 1A). The extent of HS expression was similar in WT and *Hbegf* mutant ($HB^{del/del}$ and $HB^{uc/uc}$) valves. Magnification of the staining showed that HS was predominantly expressed in the mesenchyme and basement membrane, but not the endocardium (Fig. 1Ab).

Hbegf mRNA was exclusively expressed in endocardium, and not in the mesenchyme (see Fig. S1 in the supplementary material). However, mesenchymal cells, rather than endocardial cells, abnormally hyperproliferated upon deletion of *Hbegf* (see Fig. S3 in the supplementary material). Secretion of sHB-EGF was required for normal valvulogenesis (see Fig. S2D in the supplementary material). Moreover, mesenchyme of the developing valves abundantly expressed HS (Fig. 1A). These findings suggest that sHB-EGF, secreted from the endocardium, accumulates in the mesenchyme of developing valves in association with HSPGs. To verify this hypothesis, we examined the localization of HB-EGF protein in developing valves. Despite extensive efforts, we were unable to analyze the localization of endogenous HB-EGF in mouse valves by immunohistochemistry

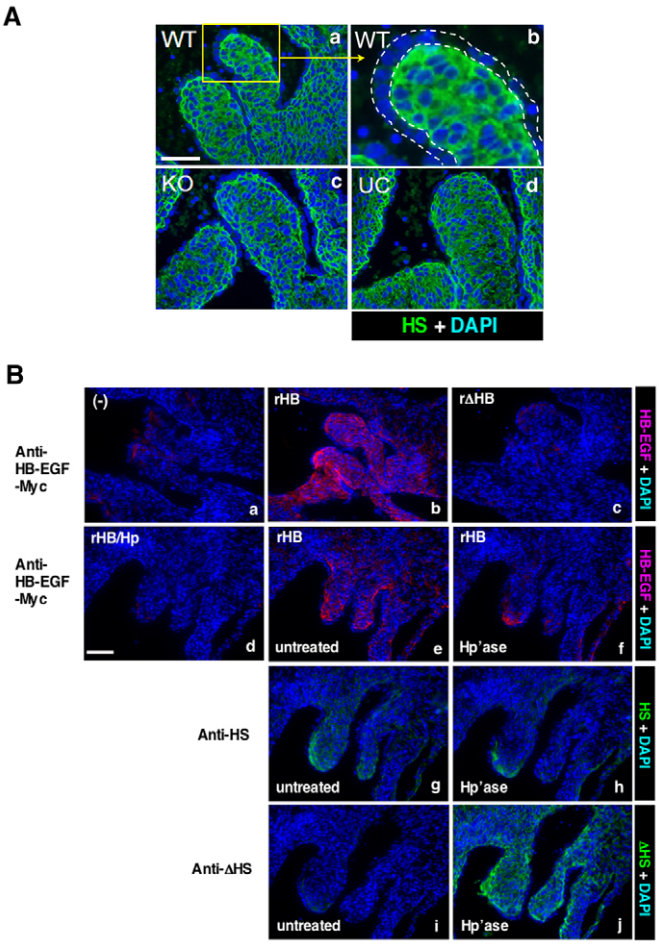

Fig. 1. Accumulation of HB-EGF in the mesenchyme of developing valves through an interaction with HS. (**Aa-d**) Heparan sulfate (HS) is abundantly expressed in the mesenchyme of developing valves. HS in cardiac valve sections from E15.5 wild-type (WT, a), $HB^{del/del}$ (KO, c) and $HB^{uc/uc}$ (UC, d) mice was digested with heparitinase and then stained with anti-ΔHS antibody (green); nuclei were stained with DAPI (blue). (b) High magnification of the boxed area in a. Note that there is no HS staining in the endocardium (between the dashed lines). (**B**) (**a-d**) HB-EGF binding to developing valve sections. E16.5 WT valve serial sections were either left untreated (–, a) or treated with Myc-tagged recombinant WT HB-EGF (rHB, b,d) or mutant HB-ΔHB (rΔHB, c) in the absence (a-c) or presence (rHB/Hp, d) of heparin. Bound HB-EGF was detected with an anti-Myc tag monoclonal antibody (red, anti-HB-EGF-Myc); nuclei were stained with DAPI (blue). (**e-j**) Binding of HB-EGF to developing valve sections is mediated by an interaction with HS. E16.5 WT valve serial sections were either left undigested (e,g,i) or were digested (f,h,j) with heparitinase I and then incubated with Myc-tagged recombinant WT HB-EGF (e,f). Bound HB-EGF was detected with an anti-Myc tag monoclonal antibody (red, anti-HB-EGF-Myc). Successful digestion was determined by immunostaining for HS (green, anti-HS, g,h) or for an anti-neo-epitope digested with heparitinase (green, anti-ΔHS, i,j). Scale bars: 50 μm.

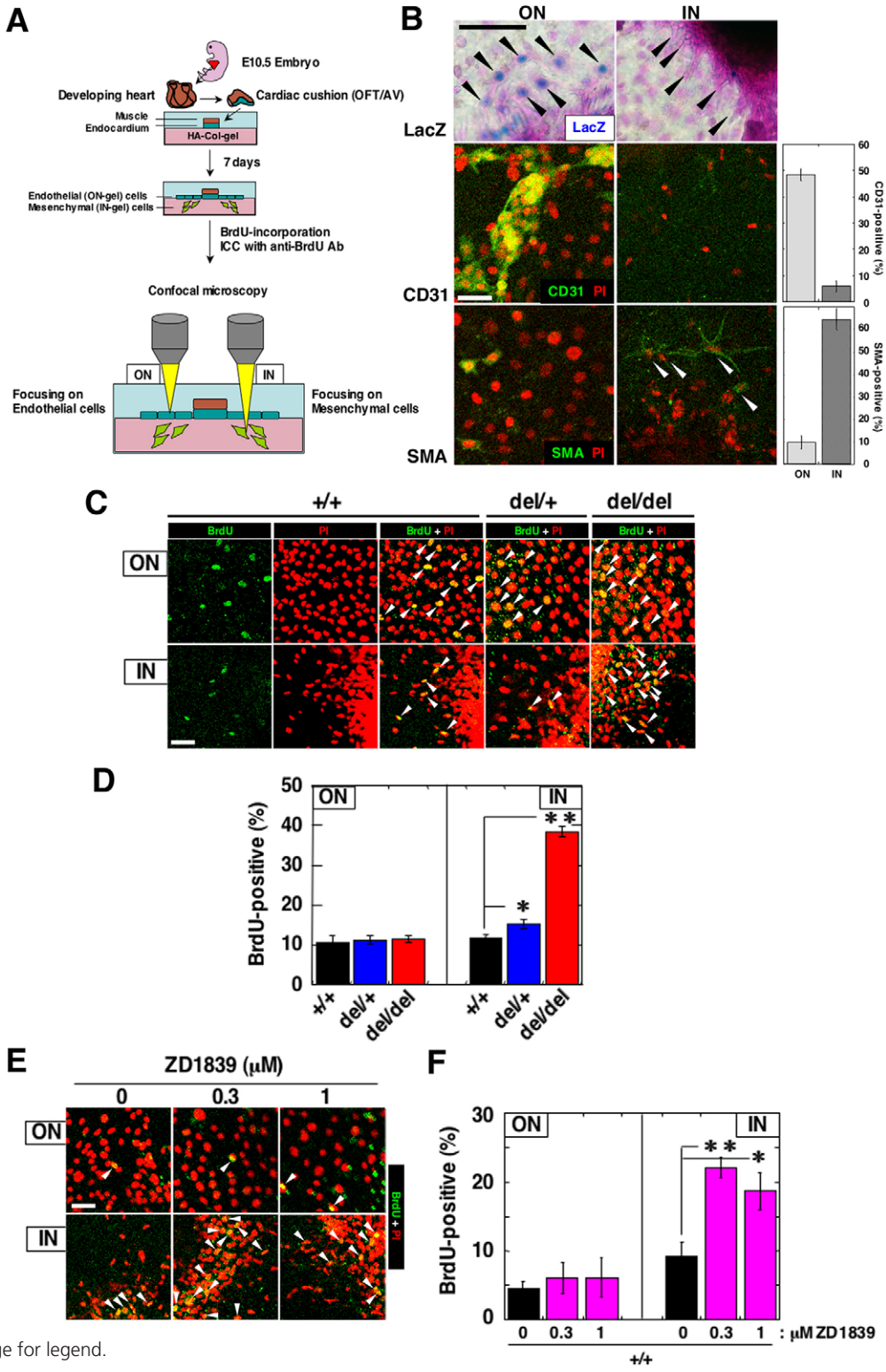

Fig. 2. See next page for legend.

because there are no appropriate antibodies to detect endogenously expressed mouse HB-EGF. Therefore, we took an alternative approach in which we examined the ability of recombinant HB-EGF proteins to bind to sections of WT valves. Serial frozen sections were incubated with Myc- and His-tagged recombinant HB-EGF, and bound HB-EGF was detected with an anti-Myc monoclonal antibody. Recombinant full-length HB-EGF, but not the truncation mutant lacking the heparin-binding domain (HB-ΔHB) (Takazaki et al., 2004), specifically bound to the mesenchyme of valves. Moreover, this binding was competitively inhibited in the presence of heparin (Fig. 1Ba-d) and reduced when the valve sections were digested with heparitinase, which digests HS-GAGs (Fig. 1Be-j). These results indicate that the ability of HB-EGF to bind to valve sections is dependent on intact HS, suggesting that HB-EGF accumulates in the valve mesenchyme through interactions with HSPGs.

Fig. 2. Cushion explant culture system. (**A**) The cushion explant culture system. (**B**) Confirmation of ON-gel cells and IN-gel cells as endocardial and mesenchymal, respectively. ON-gel (ON) or IN-gel (IN) cells of $HB^{del/+}$ explants were (top) stained for β-galactosidase (*lacZ*) to detect *Hbegf* expression (blue) and counterstained with Nuclear Fast Red (pink, nuclei) or (middle and bottom) immunostained for CD31 (green) as an endocardial marker or for SMA (green) as a mesenchymal marker and with propidium iodide (PI, red, nuclei). *lacZ*-positive ON-gel cells, *lacZ*-negative IN-gel cells, and SMA-positive IN-gel cells are indicated with arrowheads. Scoring of CD31-positive cells and SMA-positive cells among ON-gel and IN-gel cells is shown in the bar charts. Not all ON-gel cells expressed CD31 and some of these cells also expressed SMA, suggesting that some ON-gel cells have committed to EMT under these culture conditions, although they have not yet undergone morphological changes. (**C**,**D**) Mesenchymal and endocardial cell proliferation in explants from $HB^{del/del}$ cushions. (C) Mesenchymal (IN) and endocardial (ON) cells of explants from WT (+/+), $HB^{del/+}$ (del/+) and $HB^{del/del}$ (del/del) mice. Cells were stained with PI (red, nuclei) and an anti-BrdU antibody (green), and the images were merged. Yellow cells are BrdU positive (arrowheads). (D) Scoring of BrdU-positive mesenchymal and endocardial cells of explants based on data shown in C. Data represent the mean ± s.e. of results obtained from at least six individual explants; n=12, 17 and 6 for +/+, del/+ and del/del, respectively. *P<0.05; **P<0.01. (**E**,**F**) Effects of an EGFR kinase inhibitor on mesenchymal cell proliferation in explants from WT cushions. (E) Mesenchymal (IN) and endocardial (ON) cells of cushion explants from WT embryos treated with the indicated concentrations of ZD1839. Cells were stained with PI (red, nuclei) and an anti-BrdU antibody (green), and the images were merged. Yellow cells are BrdU positive (arrowheads). (F) Scoring of BrdU-positive mesenchymal and endocardial cells of cushion explants based on the data shown in E. Data represent the mean values ± s.e. of results obtained from three individual explants for all points. *P<0.05; **P<0.01. Scale bars: in B, 100 μm for *lacZ* and 50 μm for CD31 and SMA; 50 μm in C,E.

Inhibiting the interaction between HB-EGF and HS enhances the proliferation of mesenchymal cells

To examine the role of the interaction with HSPGs in HB-EGF-mediated valvulogenesis, we used an ex vivo system that employs cultures of endocardial cushion explants (Camenisch et al., 2002). As this system was originally established for studying the EMT process in cushion formation, we slightly modified it to examine the proliferation of cells from the explants (Fig. 2A). *Hbegf* was exclusively expressed in ON-gel cells (see Materials and methods), but not in IN-gel cells (Fig. 2B, upper panels), which is consistent with our in vivo observations (see Fig. S1 in the supplementary material). ON-gel cells expressed CD31 (PECAM1 – Mouse Genome Informatics), an endocardial marker, and IN-gel cells expressed α-smooth muscle actin (SMA; ACTA2 – Mouse Genome Informatics), a mesenchymal marker (Fig. 2B, middle and lower panels). There were no differences in the extent of proliferation between explants used for OFT or A-V cushions (data not shown).

To verify this culture system, we compared the proliferation of both explant cell types from $HB^{+/+}$ (WT) and $HB^{del/del}$ knockout (KO) cushions. IN-gel cells from KO explants had a significantly greater proliferative capacity than those from WT explants (Fig. 2C,D). By contrast, ON-gel cells from WT and KO cushions proliferated to comparable levels. These results were consistent with the in vivo observations in $HB^{del/del}$ valves (see Fig. S3 in the supplementary material). When WT explants were treated with ZD1839, a kinase inhibitor for EGFR, IN-gel cells, but not ON-gel cells, underwent increased proliferation (Fig. 2E,F), confirming that EGFR signaling is required to suppress mesenchymal cell proliferation.

To determine whether the interaction between HB-EGF and HSPGs is involved in the ability of HB-EGF to inhibit the growth of mesenchymal cells, we treated WT cushion explants with three reagents that inhibit HS: heparin, heparitinase and sodium chlorate (an inhibitor of HS-GAG synthesis). WT explants treated with these inhibitory reagents, especially heparin or heparitinase, showed a significant increase in IN-gel, but not ON-gel, cell proliferation (Fig. 3A,B). Heparitinase and sodium chlorate were confirmed to digest HS and decrease HS-GAG synthesis, respectively, by both HS staining and ΔHS (the digested form of HS) staining (Fig. 3C). These results indicate that the interaction between HB-EGF and HS is required to suppress mesenchymal cell proliferation.

Generation of mice expressing an HB-EGF truncation mutant that lacks the heparin-binding domain

To directly examine the importance of the association between HB-EGF and HSPGs in valvulogenesis, we generated $HB^{\Delta hb}$ knock-in mice that express a truncated form of HB-EGF (HB-ΔHB) that lacks the heparin-binding domain (see Fig. S5A in the supplementary material). Prior to generating the $HB^{\Delta hb}$ mice, we examined the molecular characteristics of the HB-ΔHB mutant protein. Similar to previous findings with the comparable human HB-EGF truncation mutant (Takazaki et al., 2004), mouse sHB-ΔHB (see Fig. S4A,B in the supplementary material) lacked heparin-binding activity (see Fig. S4C in the supplementary material) and possessed higher mitogenic activity than WT sHB-EGF in DER cells, which are EGFR-expressing 32D cells (Iwamoto et al., 1999) (see Fig. S4D in the supplementary material). The susceptibility of proHB-ΔHB (see Fig. S4E in the supplementary material) to ectodomain shedding in response to various shedding-inducing stimuli was comparable to that of WT proHB-EGF (see Fig. S4F in the supplementary material). These results confirmed that HB-ΔHB activity is similar to that of WT HB-EGF but lacks heparin-binding activity and exhibits higher mitogenic activity.

To generate mutant $HB^{\Delta hb}$ knock-in mice that express HB-ΔHB instead of HB-EGF, we used targeted gene replacement to replace the *Hbegf* gene with $HB^{\Delta hb}$ cDNA (see Fig. S5A,B in the supplementary material). Homozygous mutant mice ($HB^{\Delta hb/\Delta hb}$) were identified by Southern blotting (see Fig. S5C in the supplementary material) and PCR (see Fig. S5D in the supplementary material). Two independent ES cell lines carrying the $HB^{\Delta hb}$ allele were used to generate chimeric mice, which transmitted the mutant alleles to their progeny. Both lines of mice showed similar results. Homozygous mice ($HB^{\Delta hb/\Delta hb}$) were born and subsequently matured (see Fig. S5E in the supplementary material). Northern blotting of transcripts obtained from adult mice indicated that the WT HB^{lox} allele, which is the WT *Hbegf* cDNA knock-in allele used as a control for the cDNA knock-in (Iwamoto et al., 2003), and the $HB^{\Delta hb}$ allele were comparably expressed in the heart, lung and kidney (see Fig. S5F in the supplementary material). Furthermore, immunoblotting of WT HB-EGF and HB-ΔHB obtained from the all-trans retinoic acid-treated back skin of WT and $HB^{\Delta hb/\Delta hb}$ mice, respectively, showed comparable protein induction (see Fig. S5G in the supplementary material), similar to that of previously described knock-in mice expressing an uncleavable proHB-EGF mutant ($HB^{uc/uc}$ mice) (Yamazaki et al., 2003; Kimura et al., 2005).

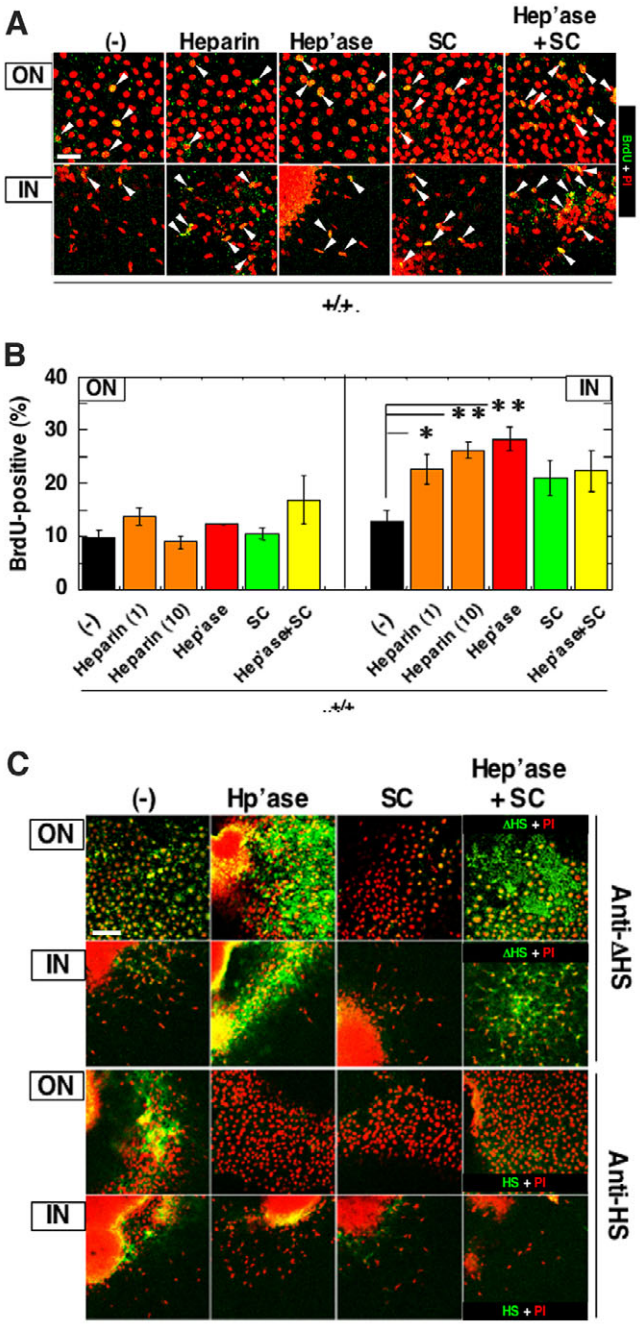

Fig. 3. Inhibition of the interaction between HB-EGF and HS in WT explants. (**A**) Mesenchymal (IN) and endocardial (ON) cells of cushion explants from WT mouse embryos that were untreated (–) or treated with 10 μg/ml heparin, heparitinase I (Hep'ase), sodium chlorate (SC), or both heparitinase I and sodium chlorate (Hep'ase + SC). Cells were stained with PI (red, nuclei) and an anti-BrdU antibody (green) and the images merged. Yellow cells are BrdU positive (arrowheads). (**B**) Scoring of BrdU-positive mesenchymal and endocardial cells of WT cushion explants treated with each inhibitor based on the data in A. Data represent the mean ± s.e. of results obtained from at least three individual explants; n=6, 5, 5, 3, 3 and 3 for (–), heparin (1 μg/ml), heparin (10 μg/ml), Hep'ase, SC and Hep'ase + SC, respectively. *P<0.05; **P<0.01. (**C**) HS staining of mesenchymal (IN) and endocardial (ON) cells of cushion explants from WT embryos that were left untreated or treated with heparitinase I, sodium chlorate or both. Cells were stained with PI (red, nuclei) and either an anti-HS or anti-ΔHS antibody (both green), and the images merged. Scale bars: 50 μm in A; 100 μm in C.

Truncating the heparin-binding domain of HB-EGF causes cardiac valve enlargement with increased mesenchymal cell proliferation

Similar to the $HB^{del/del}$ and $HB^{uc/uc}$ embryos, both the OFT and A-V heart valves were enlarged in $HB^{\Delta hb/\Delta hb}$ embryos after E15.5, during the remodeling process (Fig. 4A,B). This valve enlargement was accompanied by an increased number of mesenchymal, rather than endocardial, cells (Fig. 4C). BrdU incorporation in E15.5 valves demonstrated that this valve enlargement was caused by increased proliferation of mesenchymal cells (Fig. 5A,B) and not decreased apoptosis (Fig. 5C,D). Whereas endocardial cells in $HB^{del/del}$ and WT valves proliferated to comparable levels (see Fig. S3B in the supplementary material), endocardial cells in $HB^{\Delta hb/+}$ and $HB^{\Delta hb/\Delta hb}$ valves proliferated to a greater extent than endocardial cells in WT valves (Fig. 5B). These results are in part consistent with our finding that $HB^{\Delta hb/\Delta hb}$ valves had a slightly, but significantly, increased number of endocardial cells compared with WT valves at E17.5 (Fig. 4C). The fact that $HB^{\Delta hb/\Delta hb}$, $HB^{del/del}$ and $HB^{uc/uc}$ embryos displayed similar developmental valve abnormalities with mesenchymal cell hyperproliferation strongly suggests that the heparin-binding domain, and therefore HS-binding activity, is necessary for HB-EGF to function normally in valve development by inhibiting mesenchymal cell proliferation.

Interestingly, although *Hbegf*-null mice show severe defects in heart chamber formation and distal lung development (Iwamoto et al., 2003; Minami et al., 2008), we found that $HB^{\Delta hb/\Delta hb}$ mice did not exhibit these abnormalities (see Fig. S5H,I in the supplementary material). This suggests that heparin-binding activity is not essential for HB-EGF to function normally in heart chamber formation and distal lung development, and that valvulogenesis is the particular process in which HB-EGF requires HS-binding activity for proper function. Moreover, these findings exclude the possibility that the HB-ΔHB expressed in $HB^{\Delta hb/\Delta hb}$ mice is non-functional, resulting in abnormal valvulogenesis, and suggest that HB-ΔHB functions normally in heart chamber formation and distal lung development.

HB-ΔHB is functionally weaker than WT HB-EGF in the suppression of mesenchymal cell proliferation

To examine the $HB^{\Delta hb/\Delta hb}$ valve phenotype in a cushion explant culture system, we compared the proliferation of both cell types in WT and $HB^{\Delta hb/\Delta hb}$ (ΔHB) explants. Both IN-gel and ON-gel cells from $HB^{\Delta hb/\Delta hb}$ explants proliferated to a significantly greater extent than those from WT explants (Fig. 6A,B). These results are consistent with the in vivo observations in ΔHB valves (Fig. 5A,B).

Finally, we performed an experiment that compared the ability of WT HB-EGF and HB-ΔHB to rescue the hyperproliferation of IN-gel cells of KO explants (Fig. 6C,D). Although, at higher concentrations (20 ng/ml), HB-ΔHB was able to suppress the hyperproliferation of IN-gel cells of KO explants to a comparable degree as WT HB-EGF, at lower concentrations (0.1 ng/ml) the suppressive activity of HB-ΔHB was significantly lower than that of WT HB-EGF. By contrast, both WT HB-EGF and HB-ΔHB slightly increased the proliferation of ON-gel cells, although there was no significant difference in their activities. These results indicate that the ability of HB-EGF to associate with HS is important for suppressing mesenchymal cell proliferation, although HB-ΔHB does possesses weak suppressive activity.

DISCUSSION

HSPGs regulate the molecular and physiological functions of several growth factors and cytokines with heparin-binding properties. The physiological significance of these functions of HSPGs, especially in

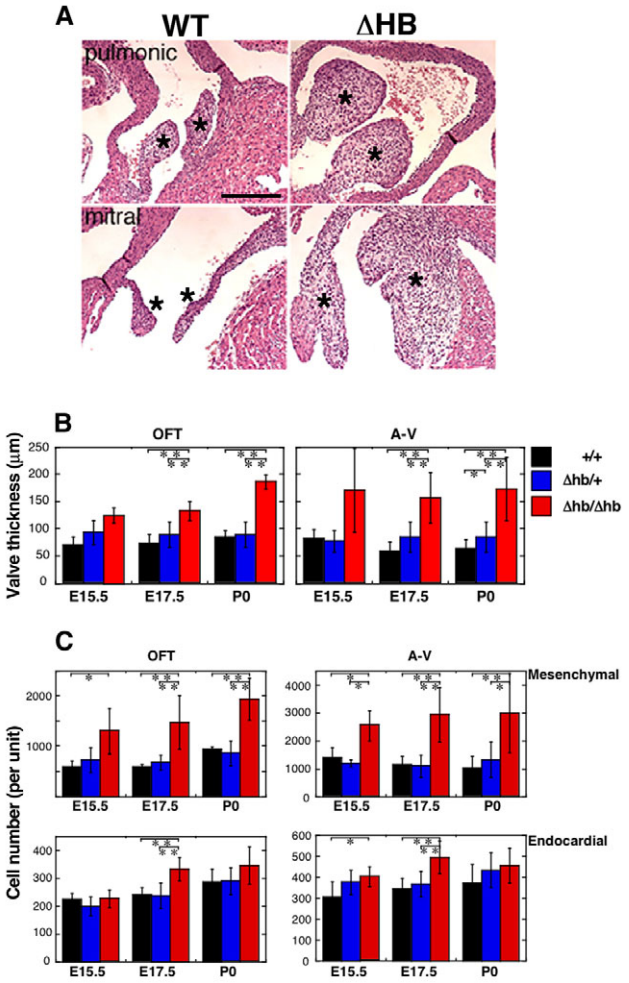

Fig. 4. Cardiac valve defects in $HB^{\Delta hb/\Delta hb}$ embryos. (**A**) Histological analysis of cardiac valves. Hematoxylin-Eosin-stained longitudinal sections of E17.5 embryo hearts from WT and $HB^{\Delta hb/\Delta hb}$ (ΔHB) mice are shown. Pulmonic and mitral valves are indicated by asterisks. Scale bar: 150 μm. (**B**) Comparison of outflow tract (OFT, left) and atrioventricular (A-V, right) valve thickness between $HB^{+/+}$ (black), $HB^{\Delta hb/+}$ (blue) and $HB^{\Delta hb/\Delta hb}$ (red) embryos at E15.5 to P0. The largest diameter of each valve in the serial sections was measured. The valve sizes were calculated as the mean ± s.e. of results obtained from at least five individual embryos; *n*=10, 6 and 5 for +/+, Δhb/+ and Δhb/Δhb, respectively, in OFT; and *n*=10, 6 and 6 for +/+, Δhb/+ and Δhb/Δhb, respectively, in A-V. **P*<0.05; ***P*<0.01. (**C**) Comparison of the number of mesenchymal (top) and endocardial (bottom) cells in OFT (left) and A-V (right) valves in $HB^{+/+}$ (black), $HB^{\Delta hb/+}$ (blue) and $HB^{\Delta hb/\Delta hb}$ (red) embryos at E15.5 to P0. Data represent the mean ± s.e. of results obtained from at least five individual embryos; *n*=10, 6 and 5 for +/+, Δhb/+ and Δhb/Δhb, respectively, in OFT; and *n*=10, 6 and 6 for +/+, Δhb/+ and Δhb/Δhb, respectively, in A-V. **P*<0.05; ***P*<0.01.

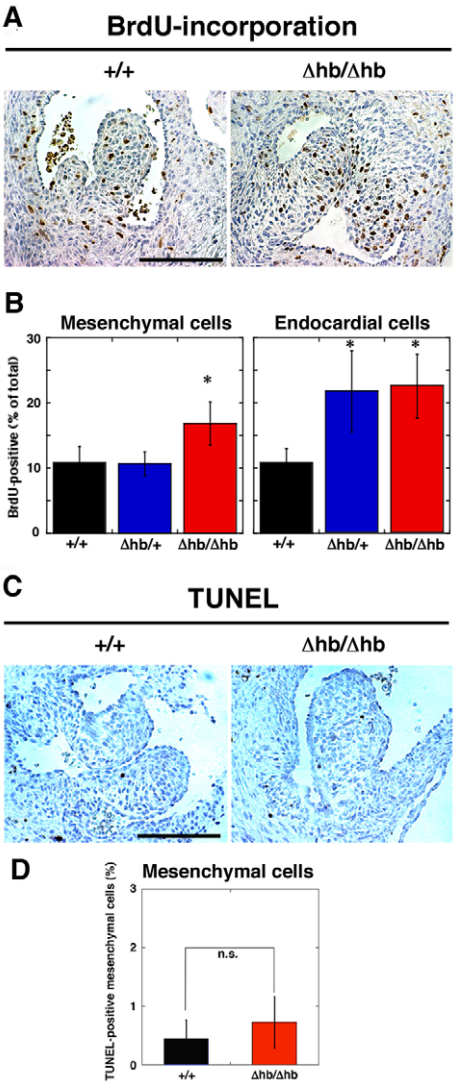

Fig. 5. Increase in mesenchymal cell proliferation in $HB^{\Delta hb/\Delta hb}$ valves. (**A**,**B**) BrdU incorporation in developing valves. (A) BrdU incorporation in OFT valves of $HB^{+/+}$ (+/+) and $HB^{\Delta hb/\Delta hb}$ (Δhb/Δhb) E15.5 mouse embryos. BrdU-positive cells are visualized as brown nuclear spots. (B) Scoring of BrdU-positive mesenchymal (left) and endocardial (right) cells in OFT valves based on the data shown in A. Data represent the mean ± s.e. of results obtained from at least eight individual embryos; *n*=8, 12 and 25 for +/+, Δhb/+ and Δhb/Δhb, respectively. **P*<0.05 for WT (+/+). (**C**,**D**) TUNEL staining in developing valves. (C) TUNEL staining in OFT valves of $HB^{+/+}$ (+/+) and $HB^{\Delta hb/\Delta hb}$ (Δhb/Δhb) E15.5 embryos. A few TUNEL-positive cells can be seen (brown nuclear spots). (D) Scoring of TUNEL-positive mesenchymal cells in OFT valves based on the data shown in C. Data represent the mean ± s.e. of results obtained from at least four individual embryos; *n*=4 and 6 for +/+ and Δhb/Δhb, respectively. n.s., not significant. Scale bars: 100 μm.

developmental processes, has been documented in numerous studies (Bishop et al., 2007; Perrimon and Bernfield, 2000). Most of these studies examined a loss of function of HS synthesis and/or HSPG core proteins. However, in such genetic approaches it can be difficult to identify the particular signaling factor that interacts with HSPG and is involved in the affected processes. To resolve this issue for HB-EGF, which is a heparin-binding growth factor, we adopted an alternative genetic approach by generating knock-in mice that express a mutant form of HB-EGF that cannot bind to HSPGs. Using combined analyses of ex vivo cushion explant cultures and $HB^{\Delta hb}$ mutant mice, we determined the physiological significance of the interaction between HB-EGF and HSPGs for the function of HB-EGF in valvulogenesis. This is the first demonstration that the interaction between HB-EGF and HSPGs is physiologically significant in vivo.

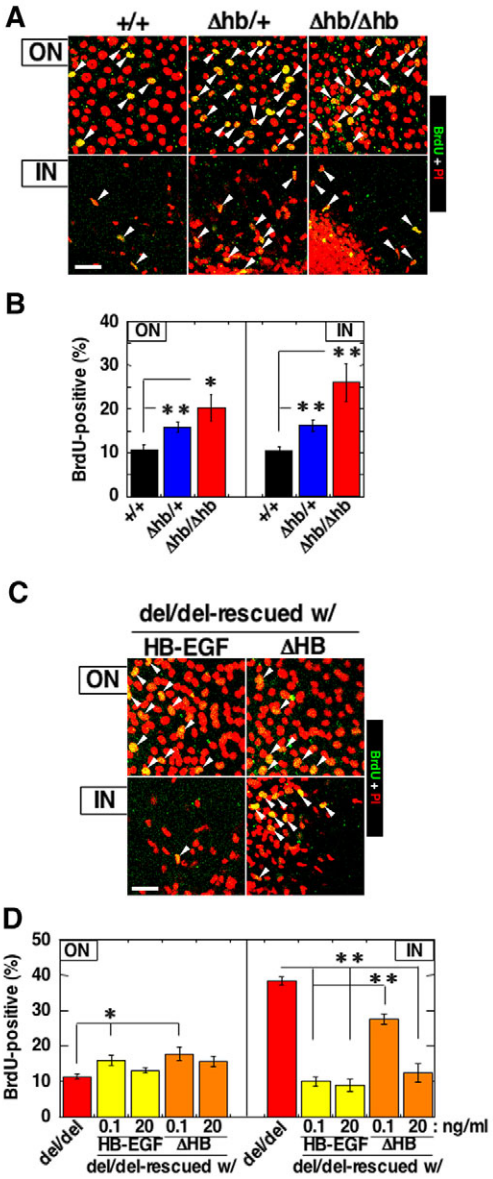

Fig. 6. Inability of HB-ΔHB to suppress mesenchymal cell proliferation. (**A**,**B**) Increase in mesenchymal and endocardial cell proliferation in explants from $HB^{\Delta hb/\Delta hb}$ cushions. (A) Mesenchymal (IN) and endocardial (ON) cells of cushion explants from $HB^{+/+}$ (+/+), $HB^{\Delta hb/+}$ (Δhb/+) and $HB^{\Delta hb/\Delta hb}$ (Δhb/Δhb) mouse embryos. Cells were stained with PI (red, nuclei) and an anti-BrdU antibody (green). Yellow cells in the merged picture are BrdU positive (arrowheads). (B) Scoring of BrdU-positive mesenchymal and endocardial cells of explants from $HB^{\Delta hb}$ embryos based on the data shown in A. Data represent the mean ± s.e. of results obtained from at least ten individual explants; *n*=13, 21 and 10 for +/+, Δhb/+ and Δhb/Δhb, respectively. **P*<0.05; ***P*<0.01. (**C**,**D**) Comparison of the ability of WT HB-EGF and mutant HB-ΔHB to rescue knockout explants. (C) Mesenchymal (IN) and endocardial (ON) cells of explants from $HB^{del/del}$ that were treated with 0.1 ng/ml WT HB-EGF (HB-EGF) or mutant HB-ΔHB (ΔHB). Cells were stained with PI (red, nuclei) and an anti-BrdU antibody (green), and then the images merged. Yellow cells are BrdU positive (arrowheads). (D) Scoring of BrdU-positive mesenchymal and endocardial cells of explants based on the data shown in C. Data represent the mean ± s.e. of results obtained from at least four individual explants; *n*=6, 4, 4, 4 and 4 for del/del, del/del-treated with 0.1 ng/ml HB-EGF, del/del-treated with 20 ng/ml HB-EGF, del/del-treated with 0.1 ng/ml ΔHB, and del/del-treated with 20 ng/ml ΔHB, respectively. The scores of del/del for IN-gel cells and ON-gel cells are identical to those indicated in Fig. 2D. **P*<0.05; ***P*<0.01. Scale bars: 50 μm.

Ex vivo studies using endocardial cushion explants confirmed that the proliferation of mesenchymal cells of both $HB^{del/del}$ and $HB^{\Delta hb/\Delta hb}$ explants was much higher than that of WT explants (Figs 2, 6). Moreover, in this culture system, the ability of mutant HB-EGF (HB-ΔHB) to rescue (inhibit) the hyperproliferation of mesenchymal cells of $HB^{del/del}$ explants was much lower than that of WT HB-EGF (Fig. 6). These results indicated that this ex vivo experimental system reproduces in vivo data and showed a functional relationship between HB-EGF and mesenchymal cell proliferation. The mutant HB-ΔHB possesses higher mitogenic activity than WT HB-EGF on DER cells (see Fig. S4 in the supplementary material) but is unable to bind to heparin (see Fig. S4 in the supplementary material), as was previously reported for the comparable human mutant protein (Takazaki et al., 2004). Thus, the reduced ability of HB-ΔHB to rescue the hyperproliferation phenotype might not be because of the lower activity of this mutant, but because of its inability to interact with HS. The ex vivo study also demonstrated that inhibiting the association between HB-EGF and HS by heparin, heparitinase or sodium chlorate increased mesenchymal cell proliferation in WT explants (Fig. 3). These results strongly suggest that the interaction between HB-EGF and HSPGs in the mesenchyme of developing valves is necessary for HB-EGF-mediated growth inhibition of these cells.

Moreover, we demonstrated that $HB^{\Delta hb/\Delta hb}$ embryos also developed enlarged cardiac valves (Fig. 4), with abnormal hyperproliferation of the mesenchymal cells during valve remodeling (Fig. 5), a phenotype that is similar to that of $HB^{del/del}$ and $HB^{uc/uc}$ embryos. These results indicate that the heparin-binding domain of HB-EGF, and therefore the ability to interact with the HS chain of HSPGs, is essential for the physiological function of HB-EGF and the suppression of mesenchymal cell proliferation during the later remodeling process in normal valvulogenesis. Analyses of the valve phenotypes of recently generated heparanase knockout (Zcharia et al., 2009) and transgenic (Zcharia et al., 2004) mice could reveal whether mesenchymal cell proliferation is also affected when the degradation of HSPGs is dysregulated.

Unlike mesenchymal cells, the proliferation of endocardial cells in $HB^{del/del}$ valves was normal (see Fig. S3 in the supplementary material), whereas these cells exhibited higher proliferation in $HB^{\Delta hb/\Delta hb}$ and heterozygous valves (Fig. 5). These dominant mitogenic effects of HB-ΔHB on endocardial cells were also observed in ex vivo experiments using cushion explants (Fig. 6). Together with the observation that HB-ΔHB has higher mitogenic activity than WT HB-EGF (see Fig. S4 in the supplementary material), these findings suggest that HB-ΔHB acts as a mitogenic factor on endocardial cells in $HB^{\Delta hb/\Delta hb}$ valves.

Although HSPGs are abundantly expressed, especially in the mesenchyme of developing valves (Fig. 1A), the importance of HSPGs in valvulogenesis is largely unknown. The HSPG family currently comprises at least 16 different types of core protein, including syndecan 1-4, glypican 1-6, CD44v3, agrin, betaglycan (TGFβR3), serglycin, perlecan (HSPG2) and collagen type XVIII (COL18A1) (Bishop et al., 2007). It has not been determined whether developing valves contain HSPG species that are specific

for HB-EGF, and, if so, which of these HSPGs specifically associate and function with HB-EGF. Our RT-PCR analysis of mesenchymal tissue sections of developing valves prepared by laser micro-dissection has identified at least nine types of HSPGs, including perlecan and collagen type XVIII (R.I., unpublished). To our knowledge, these two species are the only HSPGs that have been associated with valvulogenesis. perlecan-null mice have abnormally enlarged cardiac valves (Costell et al., 2002), resembling the phenotype of *Hbegf* mutant mice. However, according to the report by Costell et al., this abnormality is caused by defects in the migration of neural crest cells to the cardiac cushion during the cushion formation process, resulting in immature valve development. The enlargement of valves in *Hbegf* mutants is caused by hyperproliferation during the later remodeling process. Thus, valve enlargement in *Hbegf*-null and perlecan-null mice might be the result of different processes. *Col18a1*-null mice also have mild valve defects, in which the basement membrane in valves is poorly formed (Utriainen et al., 2004). Thus, to date, there have been no reports of HSPG-deficient mice with valve defects that are similar to those of *Hbegf* mutant mice, including the $HB^{\Delta hb/\Delta hb}$ mice examined in this study. Further studies are necessary to determine whether HB-EGF-specific HSPGs are involved in valvulogenesis.

Together with the analyses of valve defects in $HB^{del/del}$ and $HB^{uc/uc}$ embryos, our findings in $HB^{\Delta hb/\Delta hb}$ embryos and cells reveal a potentially novel HB-EGF function that acts in a matricrine manner (Fig. 7). In the later remodeling process of valvulogenesis, *Hbegf* is expressed in endocardial cells, in which it is processed. ADAM17 might be responsible for this HB-EGF processing because *Adam17*-null embryos have valve abnormalities that resemble those of HB-EGF mutants (Jackson et al., 2003). When HB-EGF is secreted into the mesenchyme, it associates with the HS chain of HSPG(s), which may result in the effective presentation of HB-EGF to EGFR on the surface of mesenchymal cells. Upon activation by the HB-EGF–HSPG complex, EGFR transduces downstream signals that inhibit cell proliferation.

The mechanisms involved in the hyperproliferation of mesenchymal cells in *Hbegf* mutant mice are unclear. *Egfr*-null embryos and *Hbegf* mutant embryos exhibit similar valve abnormalities (Jackson et al., 2003). An ex vivo study using ZD1839, an EGFR kinase inhibitor, showed that inhibiting EGFR activity increased mesenchymal cell proliferation in WT explants (Fig. 2). These findings strongly suggest that loss of EGFR activity results in hyperproliferation. However, which signaling cascades downstream of EGFR govern the negative regulation of cell proliferation and which factors and signals stimulate cell proliferation in the absence of HB-EGF remain unknown. Future studies are necessary to clarify these issues.

The $HB^{\Delta hb/\Delta hb}$ mice examined in this study did not show any defects in longevity, whereas *Hbegf*-null mice show postnatal lethality (Iwamoto et al., 2003; Minami et al., 2008). *Hbegf*-null mice develop not only cardiac valve defects, but also heart chamber defects, with symptoms that resemble human cardiomyopathy and result in early postnatal lethality (Iwamoto et al., 2003; Jackson et al., 2003). We found that $HB^{\Delta hb/\Delta hb}$ mice did not show heart chamber defects (see Fig. S5H in the supplementary material) and progressed normally to adulthood (see Fig. S5E in the supplementary material). These findings suggest that the HB-EGF–HSPG interaction is not essential for cardiac muscle homeostasis, and that when HB-EGF is defective in its interaction with HSPGs, this does not affect HB-EGF signaling in this process. *Hbegf*-null mice also have abnormal perinatal distal lung development (Jackson et al., 2003; Minami et al., 2008). However, we could not detect any abnormalities in $HB^{\Delta hb/\Delta hb}$ lung development (see Fig. S5I in the supplementary material), suggesting that the HB-EGF–HSPG interaction is not essential for this process. Such phenotypic differences between *Hbegf*-null and $HB^{\Delta hb/\Delta hb}$ mice suggest that the interaction with HSPGs is not a common mode of action for HB-EGF in vivo. Rather, the interaction between HB-EGF and HSPGs appears to be important for valvulogenesis, in which HB-EGF acts in a matricrine manner.

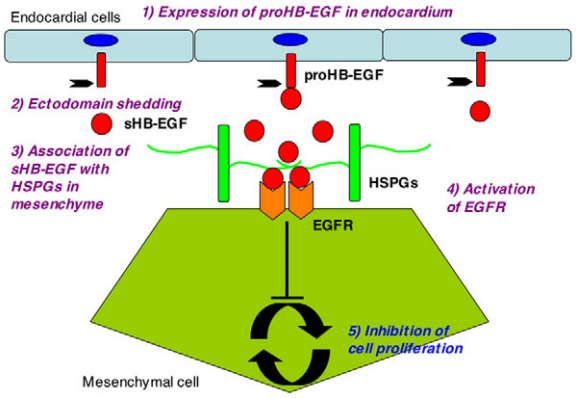

Fig. 7. Model of the matricrine action of HB-EGF in cardiac valve remodeling. In the valve remodeling process, HB-EGF is expressed (1) and secreted (2) from endocardial cells. Secreted HB-EGF associates with HSPGs in the valve mesenchyme (3) and is presented to EGFR on the surface of mesenchymal cells. When EGFR is activated by the HB-EGF–HSPG complex (4), this receptor transduces signals that inhibit mesenchymal cell proliferation (5).

The finding of normal heart chambers in $HB^{\Delta hb/\Delta hb}$ mice provides new insight into the pathophysiological relationship between cardiac valve abnormalities and heart chamber defects in *Hbegf*-null mice. *Hbegf*-null mice also have abnormally enlarged cardiac valves after birth, which cause aortic stenosis, resulting in obstruction of blood flow. Blood flow insufficiency is a burden on the heart and this induces heart chamber defects. Thus, it is possible that the heart chamber defects in *Hbegf*-null mice might be the result of primary valve defects. However, our results suggest that this is not the case because $HB^{\Delta hb/\Delta hb}$ mice only developed valve defects and not heart chamber defects, even in adulthood (see Fig. S5H in the supplementary material). Therefore, heart chamber defects in *Hbegf*-null mice might occur through dysfunction of the cardiac muscles, in which HB-EGF is normally expressed (Iwamoto et al., 2003), and independently of any valve defects.

In conclusion, the association of HB-EGF with HSPGs in the developing valve mesenchyme is necessary for valvulogenesis, which is mediated by this growth factor. Further studies will be necessary to determine how the HB-EGF–HSPG interaction modulates HB-EGF activity to suppress cell growth and how EGFR, activated by the HB-EGF–HSPG complex, transduces inhibitory signals.

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Competing interests statement
The authors declare no competing financial interests.

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