Endostatin regulates branching morphogenesis of renal epithelial cells and ureteric bud

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Endostatin (ES) inhibits endothelial cell migration and has been found to bind to glypicans (Gpcs) on both endothelial cells and renal epithelial cells. We examined the possibility that ES might regulate epithelial cell morphogenesis. The addition of ES to cultured epithelial cells causes an inhibition of both hepatocyte growth factor- and epidermal growth factor-dependent process formation and migration. In contrast, ES does not inhibit epidermal growth factor-dependent morphogenesis in renal epithelial cells derived from Gpc-3 −/mice, whereas expression of Gpc-1 in these cells reconstitutes ES responsiveness. Gpc-3 −/mice have been shown to display enhanced ureteric bud (UB) branching early in development, and cultured UB cells release ES into the media, suggesting that ES binding to Gpcs may regulate UB branching. The addition of ES inhibits branching of the explanted UB, whereas a neutralizing Ab to ES enhances UB outgrowth and branching. Thus, local expression of ES at the tips of the UB may play a role in the regulation of UB arborization.

Endostatin (ES) is a recently described carboxyl-terminal proteolytic cleavage product of collagen XVIII that has been shown to significantly inhibit endothelial cell proliferation and migration and also prevent new vessel growth (1–4). Collagen XVIII consists of a central interrupted triple-helical domain flanked at the C terminus by a large NC1 domain (1, 5–7) that can be cleaved to release the carboxyl-terminal 18–22-kDa ES domain (8). Regulation of the proteolysis of the NC1 domain to release active ES is not yet fully understood, although this function has been ascribed to elastase activity and cathepsin L, along with other proteases (9–11). Collagen XVIII is expressed in the basement membranes of vessels in the intestinal villi, choroid plexus, skin, liver, and kidney (12).

Two observations have led us to hypothesize that ES may play a role in renal epithelial cell biology. First, we have found recently that renal tubular epithelial cells bind ES, displaying both high and low affinity binding similar to that seen in endothelial cells, and glypicans (Gpcs) serve as the low-affinity receptor (13). Secondly, Lin *et al.* (14) recently have demonstrated staining for collagen XVIII in the kidney around the developing rat ureteric bud (UB), whereas Halfter *et al.* (15) have demonstrated the presence of collagen XVIII in the chick kidney tubular basement membrane. Because ES causes inhibition of endothelial cell migration and vessel formation, we hypothesized that the local generation of ES at sites of basement membrane degradation might play a similar role in regulating renal epithelial cell branching morphogenesis and UB branching.

Materials and Methods

Cell Culture and Reagents. Two immortalized cell lines of UB origin, mIMCD-3 [mouse inner medullary collecting duct (16)] cells and UBs [rat UBs (17, 18)], were grown in DMEM-F12 media supplemented with 10% FCS. Madine–Darby canine kidney (MDCK) cells were maintained under similar conditions. Gpc-3 −/cells were isolated from the IMCDs of Gpc-3 −/embryonic day (E)18.5 mouse embryos as described (19). Cells were cultured in DMEM-F12 containing 5% FCS at 32°C.

Hepatocyte growth factor (HGF), glial-derived neurotrophic factor, and fibroblast growth factor-7 were obtained from R & D Systems. Epidermal growth factor (EGF) was obtained from Upstate Biotechnology (Lake Placid, NY).

ES Production and Anti-ES Neutralizing Ab. Recombinant murine ES (mES) was expressed in yeast as has been characterized by our laboratory (20). Polyclonal rabbit antiserum to mouse ES was raised as described and shown to specifically neutralize the activity of mES (20).

Branching Morphogenesis Assay. Branching morphogenesis was examined in cells suspended in rat tail type I collagen as described (21) in the presence or absence of growth factor ± appropriate mES concentration. After a 24-h period of incubation at 37°C, an average of 50 individual cells were scored for the presence or absence of branching processes in each well, and the average number of processes per cell was calculated. Each condition was set up in triplicates with each well representing an *n* of 1. Experiments were repeated on at least three separate occasions. Significance was determined by using the Student's *t* test. Cells were photographed through a ×20 objective by using a Nikon Eclipse inverted microscope with Hoffman modulation, using a Spot RT digital camera.

ES Binding Assay. Binding assays were performed by using varying amounts of alkaline phosphatase-tagged ES (AP-ES) for 2 h at 4°C in the presence or absence of 500-fold excess of mES, as described (13). For visualization of ES binding, day E13 rat embryonic kidneys were cultured for 4 days on Transwell filters (22), fixed in 2% para-formaldehyde, incubated with either AP-ES or an ES mutant that does not bind Gpc (AR-ES3.1), and ES binding was visualized as described (13).

Retrovirus Production and Transduction. pMSCV and pMSCV-G1 (carrying the rat Gpc-1 cDNA) retroviral vectors were obtained from M. Simons (23). Retrovirus was produced as described by Ory *et al.* (24). Target cells were infected with concentrated retrovirus and after 48 h, more than 90% of the cells expressed the target gene as determined by *lacZ* expression. Expression of Gpc-1 was confirmed by Northern blot analysis and reverse transcription (RT)-PCR.

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Abbreviations: ES, endostatin; HGF, hepatocyte growth factor; EGF, epidermal growth factor; mES, recombinant murine ES; Gpc, glypican; mIMCD-3, murine inner medullary collecting duct; MDCK, Madine–Darby canine kidney; UB, ureteric bud; AP-ES, alkaline phosphatase-tagged ES; E*n*, embryonic day *n*.

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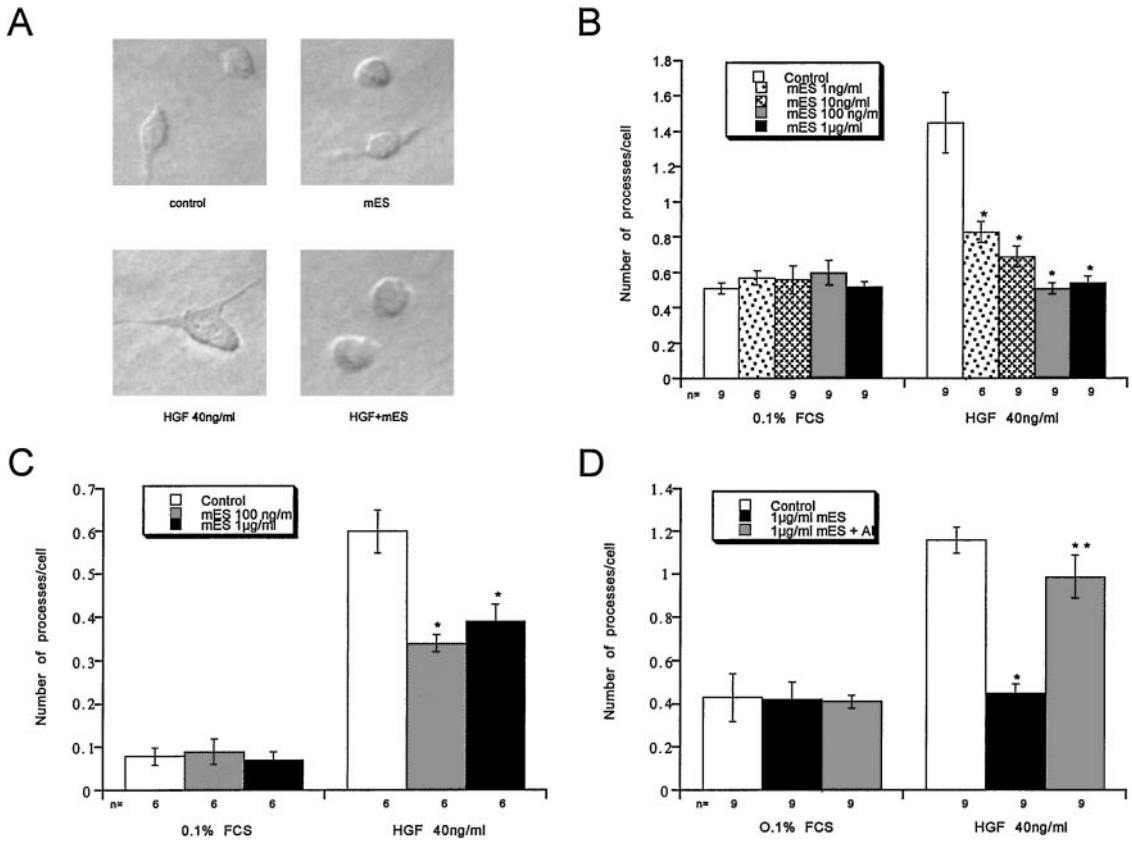

Fig. 1. mES inhibits renal epithelial cell branching morphogenesis. (*A*) mIMCD-3 cells were cultured for 24 h in a three-dimensional matrix of type 1 collagen in DMEM-F12 media supplemented with 0.1% FCS ± HGF (40 ng/ml) ± mES (1 μg/ml). Representative fields were photographed at ×20. (*B*) Quantitation of the average number of processes per cell was performed in the presence of increasing concentrations of mES. *, $P < 0.001$ as compared with HGF alone. (*C*) Quantitation of HGF-stimulated processes per cell in MDCK cells was performed ± mES. *, $P < 0.001$ as compared with HGF alone. (*D*) mES was preincubated overnight with either a 1:1 molar ratio of neutralizing Ab or preimmune serum, followed by addition to the branching morphogenesis assay as previously described. Quantitation of the average number of process per cell was performed ± 1 μg/ml of mES as compared with ± 1 μg/ml of mES + neutralizing Ab. *, $P < 0.001$ compared with HGF alone. **, ns compared with HGF alone and $P < 0.001$ compared with HGF + mES.

Protein Analysis. Appropriate cells were cultured in serum-free DMEM-F12 media for 48 h. Then the media was centrifuged (5,000 × *g*). The resultant supernatant was concentrated, and a 30-μl aliquot was separated by means of 12% SDS/PAGE, transferred to Immobilon (Millipore), and blotted with anti-ES Ab (20). Purified mES generated in yeast was run as a positive control.

Isolation and *ex Vivo* Culture of UB. UBs were cultured in a three-dimensional matrix of Matrigel (BD Biosciences, Bedford, MA) by using a modification of the method of Qiao *et al.* (25). UBs isolated from timed-pregnant Sprague–Dawley rats at gestation day 13 were suspended between 2 layers of Matrigel on polycarbonate membrane inserts in a 12-well plate with at least 10 UBs in each well. The growth medium consisted of DMEM-F12, 10% FBS, and 75 ng/ml glial-derived neurotrophic factor, HGF, and fibroblast growth factor-7. Wells were supplemented with 50 μg of either neutralizing Ab to ES or preimmune serum, or 10 μg of purified mES. Because newly synthesized matrix by the growing UB might absorb the added Ab, we replaced Ab and mES on alternate days. Cultures were maintained for 17 days, and UBs were scored for the number of branches per bud on days 3, 7, 10, and 17. Experiments were repeated on at least three separate occasions. Whole kidney cultures were performed ± mES by using E13 rat kidneys as described (22, 26).

Results and Discussion

ES Inhibits Growth Factor-Induced Branching Morphogenesis of mIMCD-3 Cells and MDCK Cells. To study the effects of ES in epithelial morphogenesis, we used mIMCD-3 cells that respond to HGF and EGF by exhibiting marked increases in migration and branching-process formation (27–29). These early events in epithelial cell morphogenesis ultimately result in the formation of branching tubules by these cells *in vitro* (ref. 27 and C.H.N., unpublished observations). HGF stimulated branching-process formation of mIMCD-3 cells suspended in type 1 collagen by 3-fold as compared with vehicle control, an effect that was inhibited by recombinant mES in a dose-dependent fashion (Fig. 1 *A* and *B*). In addition to its effects in mIMCD-3 cells, mES caused significant inhibition of HGF-stimulated process formation in MDCK cells (Fig. 1*C*). Compared with mIMCD-3 cells, mES was less effective in inhibiting MDCK cell process formation, possibly because of species differences between the purified mES (mouse) and the cells (canine).

The specificity of these effects of mES was confirmed by using ES-specific neutralizing Ab (20). Cells were pretreated with 1 μg/ml of mES or mES + neutralizing Ab for 2 h at 37°C and were scored for branching-process formation after 24 h in matrix culture ± HGF, as before. mES (1 μg/ml) again inhibited HGF-induced process formation (Fig. 1*D*). In contrast, mES that had been incubated with neutralizing Ab failed to evoke a significant inhibitory response. Taken together, these results demonstrate that ES can inhibit epithelial cell morphogenesis in a manner similar to its effects on endothelial cell angiogenesis.

ES Inhibits Migration of mIMCD-3 Cells Toward HGF and EGF. The ability of mES to modulate growth factor-induced cell migration

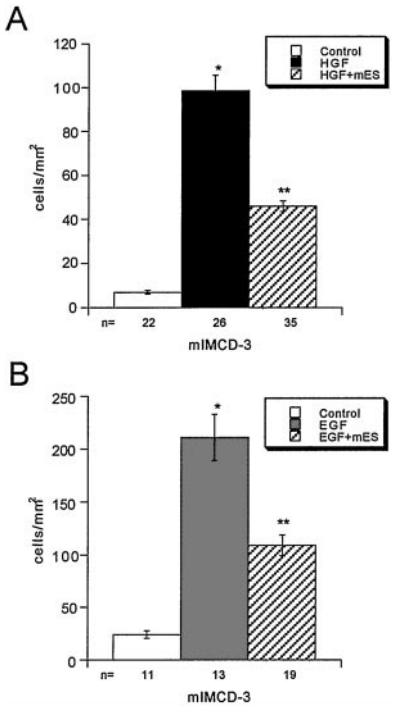

Fig. 2. Inhibition of HGF- and EGF-mediated epithelial cell migration by mES. mIMCD-3 cell migration was determined by using a blind-well Boyden chamber assay: (*A*) ±HGF ± 1 μg/ml of mES; (*B*) ±EGF ± 1 μg/ml of mES. *, $P < 0.001$ compared with control; **, $P < 0.001$ compared with growth factor-directed migration.

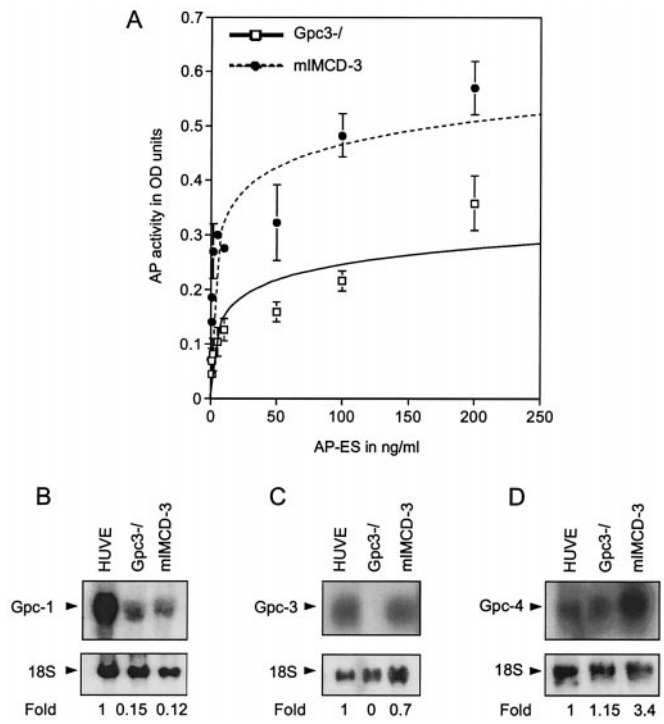

Fig. 3. ES binding and Gpc expression in Gpc-3 −/renal epithelial cells. (*A*) mIMCD-3 cells and Gpc-3 −/IMCD cells were incubated with AP-ES, and binding was determined by quantifying AP activity in membrane lysates. (*B–D*) Northern analysis for expression of Gpc-1 (*B*), Gpc-3 (*C*), and Gpc-4 (*D*) in human umbilical vein endothelial cells, Gpc-3 −/renal tubular cells, and mIMCD-3 renal tubular cells is shown. Gpc mRNA expression compared with that seen in HUVE cells (arbitrarily assigned a value of 1) was calculated by densitometry, using 18S as the normalization control.

was examined by using mIMCD-3 cell migration toward a gradient of HGF or EGF in a modified Boyden chamber assay. Pretreatment of mIMCD-3 cells with 1 μg/ml of mES had no effect on basal cell migration (data not shown). However, pretreatment with mES for 2 h resulted in a more than 50% decrease in migration toward both HGF and EGF (Fig. 2 *A* and *B*). Of note, pretreatment of these cells with mES did not affect HGF or EGF receptor phosphorylation, or downstream activation of mitogen-activated protein kinase (MAPK) or Akt. Thus, similar to the documented effects of ES on endothelial cell migration in response to vascular endothelial growth factor (VEGF), mES inhibits the migratory response of cultured renal epithelial cells to HGF and EGF.

Gpc Expression and ES Binding in Renal Tubular Cells. A major advance in understanding how ES regulates cell events has been provided by our recent demonstration of Gpc as a low-affinity cell surface receptor that is critical for the action of ES in endothelial and epithelial cells (13). Gpcs comprise a family of proteoglycans with extracellular heparin sulfate proteoglycan side chains and a peptide core attached to the membrane by a glycosyl-phosphatidylinositol (GPI) linkage (30–32). During development of the kidney tubule, the expression of Gpcs is differentially regulated. Gpc-3 seems to be expressed most heavily during embryogenesis [including high levels of expression in the UB (33)] and then down-regulated in the adult kidney (34, 35), whereas Gpc-4 expression is low during early tubule formation (E13) followed by up-regulation when mature tubules with lumens form (E16; ref. 32).

The recent generation of a Gpc-3 −/mouse (an X-linked gene where the −/male fails to express the protein) and the subsequent isolation of IMCD cell lines from Gpc-3 −/mouse embryos (33) allowed us to determine whether Gpc-3 expression might be a critical determinant in modulating ES activity in the developing kidney tubule. When compared with mIMCD-3 cells [which demonstrate ES binding similar to that seen in endothelial cells (13)], total ES binding to the Gpc-3 −/cells was reduced by ≈50% (Fig. 3*A*). Northern analysis for Gpcs-1, -3, and -4 demonstrates that mIMCD-3 cells express primarily Gpc-4 with lower levels of Gpcs-1 and -3 (Fig. 3 *B–D*). As expected, Gpc-3 −/cells fail to express Gpc-3 and also demonstrate 65% less Gpc-4 expression than mIMCD-3 cells. Thus, Gpc-3 −/collecting duct cells demonstrate a marked reduction in ES binding correlating with a loss of Gpc-3 and reduction in Gpc-4 mRNA levels.

Gpc Expression Is Critical for ES Action in Tubular Epithelial Cells. Because HGF and other heparin-binding growth factors require the expression of anchored cell surface heparin sulfate proteoglycans (such as Gpcs) to bind and normally activate their high affinity receptors (36), we compared the ability of a non-heparin-binding growth factor, EGF, to induce branching-process formation in Gpc-3 −/and mIMCD-3 cells. EGF induced process formation in both mIMCD-3 and Gpc-3 −/cells, but mES significantly inhibited this outcome only in mIMCD-3 cells (Fig. 4*A*). In addition, mES failed to inhibit EGF-dependent migration in Gpc-3 −/cells as compared with mIMCD-3 cells (Gpc-3 −/control = 11 ± 1.07; +EGF = 39.83 ± 6.08; +EGF + mES = 51.68 ± 1.08 cells per mm^2; P = not significant vs. EGF alone). The loss of mES action in these cells, despite detectable levels of Gpc-4 mRNA, suggests that the absolute amount of Gpc expression on the cell surface may be critical in determining the action of these Gpc-binding proteins. This idea is supported by results in *Drosophila*, where the Gpc homolog dally has been found to be haplolethal, suggesting that simply reducing the level

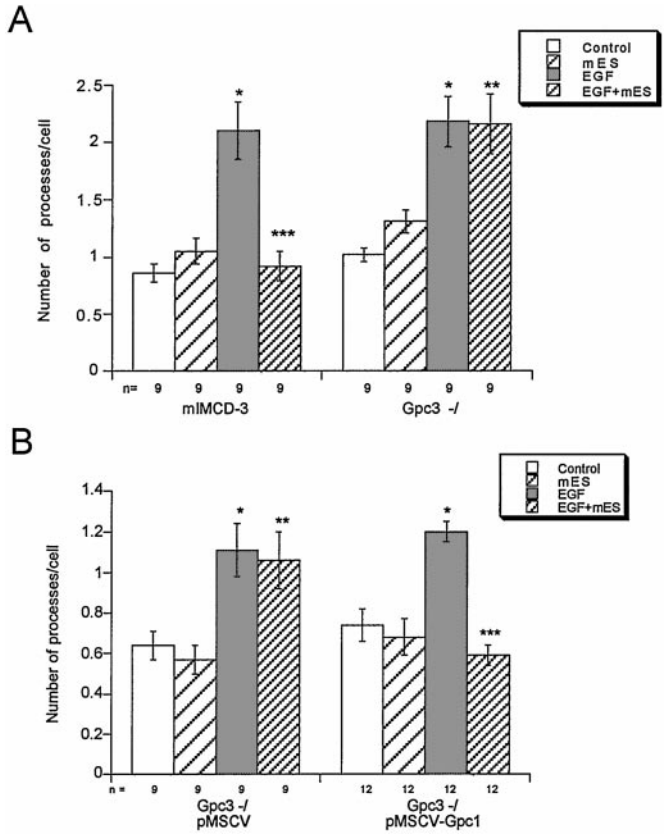

Fig. 4. Gpc expression regulates ES responsiveness. (*A*) EGF-dependent branching morphogenesis was compared in mIMCD-3 cells and Gpc-3 −/cells. *, $P < 0.001$ compared with control; **, ns compared with EGF alone; ***, $P < 0.001$ compared with EGF alone. (*B*) Gpc-3 −/cells were infected with either a control retrovirus (pMSCV) or the retrovirus encoding the cDNA for Gpc-1 and then assayed for EGF-dependent branching morphogenesis. *, $P < 0.001$ compared with control; **, ns compared with EGF alone; ***, $P < 0.001$ compared with EGF alone.

of functional Gpc expression is sufficient to prevent normal embryonic development (37–39).

To determine whether the lack of inhibitory effect of mES in Gpc-3 −/cells is specific for the loss of Gpc expression, we expressed Gpc-1 in Gpc-3 −/collecting duct cells and again examined the effect of mES on EGF-mediated branching morphogenesis. In cells transfected with control vector (pMSCV), mES had no effect, whereas in Gpc-1-expressing cells, mES significantly inhibited EGF-induced branching morphogenesis (Fig. 4*B*). Thus, overexpression of Gpc-1 in the Gpc-3 −/collecting duct cells reconstitutes ES responsiveness.

UBs Secrete ES. Mutational inactivation of Gpc-3 in the mouse causes medullary renal dysplasia (40) similar to that seen in humans with Simpson–Golabi–Behmel Syndrome who have spontaneous mutations in Gpc-3 (41). Interestingly, degeneration of the medullary collecting ducts in the Gpc-3 −/mouse is preceded by hyperproliferation and enhanced branching of the UB (40). Grisaru *et al.* (33) recently demonstrated that Gpc-3 modulates inhibitory and stimulatory signaling downstream of bone morphogenetic proteins (BMPs) and fibroblast growth factor-7, suggesting that Gpc-3 may act in part to modulate binding of these growth factors to the UB cells. Based on our observation that mES can inhibit HGF- and EGF-induced branching morphogenesis in mIMCD-3 cells derived from the UB, we examined the hypothesis that the enhanced UB branching seen in the kidney of the Gpc-3 −/mouse is caused, at least in part, by a diminished ability of ES to bind to Gpc-3 −/epithelial cells and inhibit growth factor-mediated branching morphogenesis.

To postulate that ES may play a role in UB branching requires that ES is actually generated in the vicinity of the UB and binds to cells located at the branch points. As the UB grows into the surrounding metanephros, basement membrane components such as collagen IV (22, 42) and collagen XVIII (14) are detectable along the stem of the UB, but not at the very tip. This phenomenon is believed to be the result of degradation of the basement membrane by either UB or mesenchymally derived metalloproteases (22, 26), potentially resulting in the release of ES. To examine this possibility, UBs were cultured for 48 h in serum-free media, and the supernatant was immunoblotted for ES (Fig. 5*A*). Conditioned media from UBs (lane 3) reveal a band of ≈21 kDa at the same size as purified recombinant mES (lane 1) and a band of ≈35 kDa that likely represents the intact collagen XVIII NC1 domain. The identification of ES from UBs was confirmed further by isolation and sequencing (J.B., unpublished observations). The ability of ES to bind to cells in the developing UB was examined by using AP-ES in explanted embryonic rat kidneys. ES binding was found to be most prominent at the branch points and along the stalks of the UB, and least detectable at the tips (Fig. 5*B*). Mutant ES that does not bind Gpcs (AP-ES3.1) failed to bind to the UB. Thus, ES is produced by UB cells and binds to the UB at branch points and along the stalk.

ES Regulates UB Branching Morphogenesis in an *ex Vivo* Culture Model. The effect of mES then was tested in an *ex vivo* model of embryonic rat UB cultured in a three-dimensional Matrigel matrix as described in *Materials and Methods*. At E13, the rat UB is T-shaped and has only two branches. By day 10 in culture, there were ≈10 branches per UB in the control group (Fig. 5*D Left*). In contrast, in the presence of 10 μg/ml of mES, both UB outgrowth and branching were inhibited (Fig. 5*D Right*). There was a striking reduction in the extension of nascent buds from central segments of the UB, and the branches that did occur appeared widened and their outgrowth inhibited. The buds developed a swollen appearance with loss of normal branching architecture, culminating in fusion of the earlier branches into small lobules by day 17 of culture. This fusion resulted in a decrease in the total number of identifiable independent branches at later time points. Quantitation of these results revealed that mES treatment caused a 32% reduction in the number of branches per UB by day 10 and a 60% reduction by day 17 (Fig. 5*C*; $P < 0.001$). Addition of 2 and 10 μg of mES to whole kidney explants also resulted in a dose-dependent inhibition of UB branching (control = 117 ± 7 branches per UB, $n = 4$; mES 2 μg = 91 ± 3.5, $n = 5$; mES 10 μg = 81 ± 2.8, $n = 6$; $P < 0.001$ compared with the control group).

To determine whether endogenous generation of active ES can affect UB branching, we used the neutralizing Ab to ES in the explanted UB model. UBs cultured in the absence of exogenous ES but in the presence of the neutralizing Ab demonstrated two distinct differences as compared with control UBs, increased outgrowth (length) of branches and increased numbers of branches (Fig. 5*D Center*). Quantification of branch number revealed a 40% increase in the number of branches per UB by day 10 and a more than 75% increase by day 17 (Fig. 5*C*, $P < 0.001$). Neutralizing the action of ES in explanted UBs therefore results in increased UB branching and outgrowth, a phenotype similar to that seen during the early development of the UB in Gpc-3 −/kidneys.

Conclusions

Multiple factors have been found to play a role in the formation and branching of the UB, including matrix metalloproteases, the

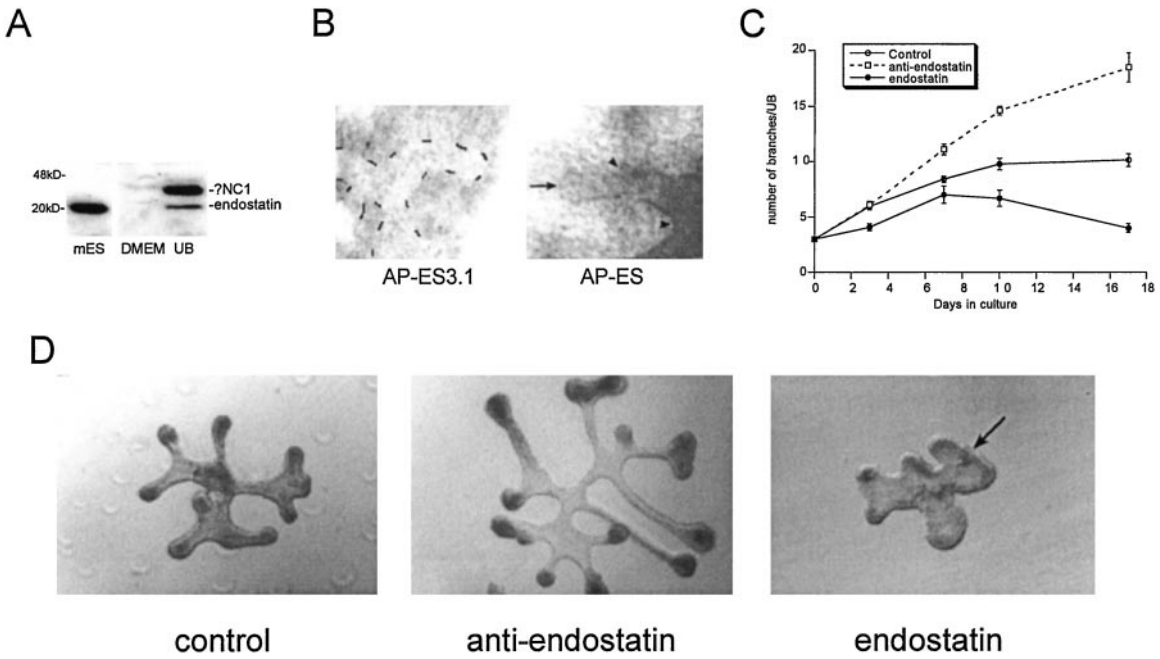

Fig. 5. Role of ES in UB morphogenesis. (*A*) Concentrated supernatant from serum-starved UBs was separated by SDS/PAGE. ES-containing fragments of collagen XVIII were identified by immunoblotting with an Ab against ES. Purified mES was run as a positive control and DMEM media as a negative control. A 21-kDa protein that corresponds in size to recombinant mES, as well as a larger protein of ≈35 kDa which is similar in size to the full-length NC1 domain, was detected. (*B*) ES binding to the UB was determined by incubation of cultured rat embryonic kidneys with AP-ES. ES binding was detected primarily at branch points (*Right*, arrowheads) and along the stalk, with lesser binding at the UB tip (arrow). Incubation with AP-ES3.1 (which fails to bind Gpcs) did not result in detectable staining of the UB (*Left*, outlined with dashed line). (*C*) UBs were cultured *ex vivo* as described. Individual wells containing 10 UBs per well were treated with either preimmune serum (50 μg per well), anti-ES neutralizing Ab (50 μg per well), or mES (10 μg/ml), and quantitation of the number of branches per UB on days 3, 7, 10, and 17 was performed ($n = 10$). (*D*) Photomicrographs taken of representative UBs at day 10. The arrow demonstrates the process of branch fusion in the setting of mES treatment.

extracellular matrix itself, and multiple growth factors (43–48). The observed increase in proliferation and branching of the UB in the Gpc-3 −/mouse suggests that early in development, Gpc-3 may bind a unique factor (or factors) that acts to limit growth factor-mediated UB growth and branching. The recent observation that Gpc-3 may play a role in BMP signaling suggests that one possible candidate for this inhibitory effect is BMP2, which has been shown to inhibit UB growth (49). In the present work, we have identified a second candidate that is capable of binding to Gpcs and regulating UB arborization, ES. Based on these observations, we propose a model in which matrix metalloprotease-induced degradation of the basement membrane components synthesized at the tip of the UB allows migration and branching of the cells in the immediate vicinity, promoting UB arborization. In concert, the specific degradation of collagen XVIII in this region results in the accumulation of ES that then serves as a locally regulated inhibitor which acts to prevent unchecked UB outgrowth and branching.

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