Cathespin H Is an Fgf10 Target Involved in Bmp4 Degradation during Lung Branching Morphogenesis*[

During lung development, signaling by Fgf10 (fibroblast growth factor 10) and its receptor Fgfr2b is critical for induction of a gene network that controls proliferation, differentiation, and branching of the epithelial tubules. The downstream events triggered by Fgf10-Fgfr2b signaling during this process are still poorly understood. In a global screen for transcriptional targets of Fgf10, we identified Ctsh (cathespin H), a gene encoding a lysosomal cysteine protease of the papain family, highly up-regulated in the developing lung epithelium. Here we show that among other cathepsin genes present in the lung, Ctsh is the only family member selectively induced by Fgf10 in the lung epithelium. We provide evidence that, during branching morphogenesis, epithelial expression of Ctsh overlaps temporally and spatially with that of Bmp4 (bone morphogenetic protein 4), another target of Fgf10. Moreover, we show that Ctsh controls the availability of mature Bmp4 protein in the embryonic lung and that inhibiting Ctsh activity leads to a marked accumulation of Bmp4 protein and disruption of branching morphogenesis. Tightly controlled levels of Bmp4 signaling are critical for patterning of the distal lung epithelium. Our study suggests a potentially novel posttranscriptional mechanism in which Ctsh rapidly removes Bmp4 from forming buds to limit Bmp4 action. The presence of both Ctsh and Bmp4 or Bmp4 signaling activity in other developing structures, such as the kidney, yolk sac, and choroid plexus, suggests a possible general role of Ctsh in regulating Bmp4 proteolysis in different morphogenetic events.

Lung organogenesis starts in the mouse at around embryonic day 9.5 (E9.5), when primary buds emerge from the ventrolateral aspect of the foregut endoderm. At E10.5, secondary buds start to form, and from then on the epithelial tubules undergo a series of patterning events that includes budding, clefting, and dichotomous branching to generate the airways and the alveoli. Genetic analysis has implicated a number of signaling molecules, present in the epithelial and mesenchymal layers of growing buds, in controlling cell survival, proliferation, and fate determination during this process. The mechanisms by which expression of these molecules are regulated are complex and include dynamic induction and spatial restriction of expression and negative feedback suppression (reviewed in Ref. 1).

Fibroblast growth factor 10 (Fgf10) is essential for lung formation. No lungs are formed in genetically modified mice in which Fgf10 or its receptor Fgfr2b has been deleted (2–4). Fgf10 is dynamically expressed in the mesenchyme at the presumptive sites of budding. Fgf10 binds to Fgfr2b in the epithelium and activates an intracellular signaling cascade, which leads to the migration and proliferation of lung epithelial progenitor cells in emerging buds (2, 5). The downstream events triggered by Fgf10-Fgfr2b signaling that are essential for lung branching morphogenesis are still poorly understood. In the process of screening for transcriptional targets of Fgf10, we identified Ctsh (cathespin H) (EC 3.4.22.16), which encodes a lysosomal cysteine protease of the papain family, highly up-regulated in the developing lung epithelium (6).

Cathespins represent a heterogeneous group of lysosomal proteases with diverse catalytic mechanisms. Among the 11 members of this family, seven have endopeptidase activity (L, V, S, K, F, B, and H), whereas cathepsin H exhibits mainly aminopeptidase activity (7). There is accumulating evidence that cathespins are critical for tumor invasion and metastasis and for neovascularization (7–9). The distinct developmental pattern of several cathespins suggests that these enzymes play specific functions in the embryo (10). Recent information from cathespin knock-out mouse models has largely confirmed this view and shown that specific cathespin deficiencies have far reaching and discrete consequences on development and homeostasis (11–13). The catalytic events mediated by these enzymes include matrix remodeling by degradation of components of extracellular matrix (14), intracellular processing of the prohormone thyroglobulin by sequential proteolytic events (15), and modulation of hormone action by turnover of nuclear proteins (16).

Ctsh expression has been previously reported in the lung (17, 18). Although in the adult lung, Ctsh is known to be involved in processing of surfactant proteins B and C (19–21), nothing is known about its potential function in the developing lung. Here we investigate this issue and the biological significance of Ctsh as a target of Fgf10 in the epithelium of developing lung buds.

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2 The abbreviations used are: Ex, embryonic day x; PBS, phosphate-buffered saline; RA, retinoic acid; ActD, actinomycin D; MCA, methylcoumarylamide; ctshi, cathepsin H inhibitor; PCNA, proliferating cell nuclear antigen; Bmp4, bone morphogenetic protein 4.

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We provide evidence that, during lung branching morphogenesis, epithelial expression of Ctsd overlaps temporally and spatially with that of Bmp4 (bone morphogenetic protein 4), another target of Fgf10. Moreover, we show that Ctsd controls the availability of mature Bmp4 protein in distal lung buds and that inhibiting Ctsd activity leads to Bmp4 accumulation and disruption of bud formation.

**EXPERIMENTAL PROCEDURES**

**Lung Cultures**—Lungs from CD1 mouse embryos were isolated at E11.5–E12, placed onto MF-Millipore membrane filters on the top of a metal mesh on a tissue culture dish, and cultured for 48–72 h with BGjb medium (20 mg/100 ml of ascorbic acid, 1% of inactivated fetal calf serum, and 50 units of penicillin/streptomycin) (5). In some cultures, heparin beads soaked in either buffer (PBS) or human recombinant FGF10 (100 μg/ml; R&D Systems) were engrafted near distal buds.

For the various experiments, BGjb medium was used with the following reagents: pan-RAR antagonist BMS493 (Bristol Meyers Squibb) or all-trans-RA (Sigma), human recombinant BMP4 or Noggin (a specific inhibitor of BMP4 signaling/receptor binding; R&D Systems) (22), diazomethane derivatives H-Ser(O-Bzl)-CHN2 (cathespin H inhibitor) (23) or benzyloxy-carbonyl-Phe-Tyr(tert-butyl)-CHN2 (cathespin L inhibitor; BACHEM) or pepstatin A (cathespin D inhibitor) (24), and the general transcription inhibitor actinomycin D (Sigma). Lung cultures were collected either for enzyme activity assay, Western blotting, isolation of total RNA for quantitative real time PCR, immunohistochemistry analysis, or in situ hybridization, as previously described (25).

For the bud chemoattraction assay, freshly isolated E11.5 distal lung buds were engrafted in Matrigel (BD Biosciences, Bedford, MA), and FGF10- or PBS-soaked beads were placed near the distal end of explants shortly before Matrigel solidification (26). Matrigel-embedded explants were then cultured for 72 h in BGjb medium supplemented with either 1.0 or 2.0 μM Ctsd inhibitor (Ctsdhi) or Me2SO alone.

**In Situ Hybridization**—RNA probes were generated using the appropriate RNA polymerases (SP6, T7, or T3) and following the manufacturer’s protocol (Ambion). cDNA clones used for probe labeling were obtained from the NIA Mouse 15K cDNA Clone Set, distributed by the Microarray Core Facility of the Tufts University School of Medicine. The accession numbers for the cDNA clones are as follows: Ctsd (BG066250); Ctsl (BG065219); Ctsd (BG074759), and Ctsz (BG064259) (25). Bmp4 and Patched probes (gift from A. McMahon, Harvard University) were labeled as described (27). Isotopic and nonisotopic (digoxigenin) labeling of RNA probes and whole mount in situ hybridization of freshly isolated or cultured embryonic lungs were performed as previously described (6).

**Western Blotting and Immunohistochemistry**—Western blot analysis was carried out in samples of cultured lungs and yolk sacs, as previously described (25). Primary antibodies were as follows: anti-mouse cathepsin H (polyclonal antibody, catalog number AF1013; R&D Systems), anti-Sprouty2 (rabbit polyclonal antibody, Upstate Biotechnology, Inc., catalog number 07-524), anti-α-tubulin (monoclonal antibody; Sigma, catalog number T9206), anti-Bmp4 (mouse monoclonal antibody; Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) catalog number sc-12721). The Immune-Star™ HRP chemiluminescent kit (Bio-Rad) and appropriate secondary antibodies (Bio-Rad) were used for Western blotting detection. Immunohistochemistry was performed in 5-μm paraffin sections using the anti-Ctsh antibody above, the cell and tissue staining kits (CTS Series; R&D Systems), and the proliferating cell nuclear antigen staining kit (Zymed Laboratories Inc.) according to the manufacturer’s protocol.

**Ctsh Activity Assays**—The Ctsh activity assay was performed as described earlier (28, 29). Briefly, embryonic lung cultures were homogenized in a lysis buffer composed of 25 mM MES, adjusted to pH 6.2, and supplemented with 1 mM EDTA-Na2, 50 mM NaCl, 1% (v/v) Triton X-100, and 250 mM sucrose. Complete lysis of the tissues was achieved by three successive, 5–7-s sonication cycles. Clear supernatants were obtained after centrifugation at 4 °C and 10,000 × g for 15 min. The total protein concentration of samples was determined using the micro-BCA kit from Pierce. All enzyme activity measurements were carried out in a calibrated Bio-Tek FLX-800 fluorescence microplate reader, equipped with 355- and 460-nm excitation and emission filters, respectively. Samples (3–10 μl) were preincubated for 20 min at 20 °C in Ctsh assay buffer (50 μM final) in the presence of cathepsin inhibitors or Me2SO (vehicle control). Aminopeptidase activity of Ctsh was assayed in 25 mM MES, at pH 6.8, containing 50 mM NaCl, 5 mM DTT, 1 mM EDTA-Na2, 0.05% (v/v) Triton X-100, 50 μM puromycin, and 250 mM sucrose using H2N-Arg-MCA as substrate. Assay buffer (50 μl) containing H2N-Arg-MCA substrate was added to preincubation mixes, and fluorescence intensity of the reaction was monitored continuously for 30 min at 37 °C.

**Quantitative Real Time PCR**—Total RNA was isolated from cultured lungs, treated with DNA-free DNase (Ambion), and reverse transcribed using Superscript II (Invitrogen). cDNA from reverse transcription reactions were analyzed by quantitative reverse transcription-PCR in an ABI 7000 instrument.
RESULTS AND DISCUSSION

Ctsh Is a Downstream Target of FGF10 in the Developing Lung Epithelium—We have previously characterized the global transcriptional profile of E11.5 mesenchyme-free mouse lung epithelial explants cultured in the presence of recombinant FGF10 (6). Along with the morphological changes associated with initial sealing of the epithelium (0–8 h) and bud initiation (8–24 h), we reported a remarkable induction of Ctsh mRNA expression (4.5- and 36-fold at 8 and 24 h, respectively; \( p = 0.02 \)) (Fig. 1, A and B).

Although other cathepsin genes were identified in our array, none were induced by FGF10 like Ctsh was (Fig. 1). For example, cathepsin L (Ctsl) showed a statistically significant but rather modest increase in expression from 0 to 24 h (Fig. 1). By contrast, cathepsin Z (Ctzz) expression decreased over time. Cathepsin C (Ctsc), cathepsin D (Ctsd), and cathepsin S (Ctss) were detected in lung explants, but expression was not significantly changed in time (Fig. 1; data not shown). Cathepsin B (Ctbb) was undetectable under our experimental conditions (data not shown).

To validate the microarray results, first we localized expression of these cathepsins in the uncultured E11.5–E12 lungs by whole mount in situ hybridization. Although Ctsh expression was strong and clearly restricted to distal epithelial buds, Ctsl, Ctsd, and Ctss transcripts were present mostly in the mesenchyme with some weak signals in the epithelium (Fig. 2, A–D, upper panels). Then we tested the inducibility of cathepsin genes by engrafting heparin beads soaked in recombinant FGF10 or buffer (PBS, control) onto E11.5–12 lung explants, subsequently cultured for 24–48 h. Whole mount in situ hybridization revealed that only Ctsh was consistently induced by FGF10 in our assays (Fig. 2, A–D, bottom panels). High levels of Ctsh were found in epithelial cells surrounding the FGF10 bead but not the PBS bead (Fig. 2, E and F). Induction of Ctsh was restricted to previously reported sites of activation of FGF10-Fgrf2b (2, 5). These results were confirmed by isotopic in situ hybridization and immunohistochemistry (Fig. 2, G and H). Thus, Ctsh showed a unique distribution and responsiveness to FGF10 in the developing lung epithelium.

Ctsh Expression Is Spatially and Temporally Associated with Specific Developmental Events during Organogenesis—We speculated that, in the developing lung, Ctsh could function as a critical mediator of Fgf10-induced morphogenesis. A more
Cathepsin H during Lung Branching Morphogenesis

Detailed survey of the Cts expression pattern in the developing lung confirmed persistent expression in distal epithelial buds throughout branching morphogenesis in vivo and in vitro (Fig. 2, A, G, H, I, and J). However, surprisingly, no Cts expression was detected in the lung epithelium at E9.5–E11, when Fgf10-Fgfr2b signaling is known to be critical for bud induction and growth of the early lung (3, 4). Cts signals were evident in endothelial cells of the pulmonary artery outside the lung, along the trachea, but not in the lung proper (Fig. 2I, arrow). Epithelial signals were promptly detected in nascent lung buds only after secondary buds formed (E12 onward; Fig. 2A). Thus, in the developing lung epithelium, induction of primary and secondary buds occurs in the absence of Cts. In the E14.5 lung or at the equivalent time in culture, Ctsh could be also identified in scattered mesenchymal cells, presumably macrophage precursors, since some of these expressed the macrophage marker F4/80 (30) (Fig. 2K, blue arrowhead; data not shown). In the adult lung, we confirmed Cts expression in type II alveolar epithelial cells, macrophages, and endothelial cells (31).

To learn about the overall distribution of Cts and to gain insights into its potential role in developmental processes, we performed a comprehensive Cts expression analysis in the E7.0–E14.5 mouse embryo. In situ hybridization and immunohistochemical analysis revealed a highly restricted expression pattern, with strong signals in the visceral endoderm/yolk sac membrane, the epithelium of lung, kidney, and choroid plexus, and endothelial cells of a few large blood vessels (Figs. 2 and 3). Together, the data suggested that Cts may mediate specific events in a limited population of epithelial and mesenchymal derived cells in the mouse embryo. In the lung epithelium, Cts does not seem to function until branching morphogenesis.

RA Signaling Suppresses FGF10-induced Expression of Cts in the Lung Epithelium—The lack of Cts expression in the early lung epithelium despite the presence of Fgf10-Fgfr2b signaling was intriguing, particularly because Cts was highly inducible by FGF10 in our assays. We asked whether an epithelial signal active in the primary lung bud but not at subsequent stages could be preventing induction of Cts by FGF10-Fgfr2b. A recent microarray screen for retinoic acid (RA) targets during organogenesis showed high levels of Cts expression in vitamin A-deficient rat embryos (32). Furthermore, we have previously shown that RA signaling is highly active in the epithelium of nascent primary buds, but it is subsequently turned off during branching morphogenesis, coincident with the stage when we first observed epithelial expression of Cts. We reasoned that RA might suppress Fgf10-induced Cts expression in the lung epithelium. To test this hypothesis, we engrafted Fgf10- or PBS-soaked heparin beads onto E11.5 lung explants in which RA signaling was maintained active by treatment with exogenous RA, as previously described (33). Cts expression was determined by immunostaining or Western blotting, and results were compared with lungs cultured under similar conditions in control medium, or in medium containing a pan-RAR antagonist (BMS493) (34). The antagonist was used here as an additional control to ensure that no RA signaling was activated in the whole explant. Western blotting and immunostaining analysis showed that RA treatment markedly reduced the Cts protein levels and prevented FGF10-induced expression of Cts in the lung epithelium, effects not seen in control or BMS493-treated cultures (Figs. 2H and 4, A–C). These results suggest a model in which Fgf10-mediated induction of Cts in the lung epithelium occurs only once endogenous RA signaling has been locally turned off. Although due to technical issues we were unable to properly isolate and culture E9.5–E10 lungs to test this model, we had additional supporting evidence of RA-Cts interaction in a foregut culture system used to study organogenesis in vitro (33). Microarray analysis of E8.5 foregut explants cultured for 24 h in BMS493 revealed significant up-regulation of Cts expression compared with controls (-fold change, 1.57; p = 0.04). Conversely, rescuing RA signaling in retinaldehyde dehydrogenase-2 (Raldh2) null foreguts using exogenous RA resulted in marked up-regulation of Cts (-fold change, 6.07; p = 0.0006). Thus, Cts may be part of a developmental program that is initially suppressed in primary buds by early signals, such as endogenous RA, but is later released during branching morphogenesis.

Inhibition of Cts Activity Using H,N-Ser(O-Bzl)-CHN2—We investigated the role of Cts in the developing lung by inhibiting Cts activity selectively in organ culture systems. Pharmacological inhibitors have been widely used for selective

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3 F. Chen, J. Lu, and W. Cardoso, unpublished observations.
blocking of cathepsin function \emph{in vivo} and \emph{in vitro} (9, 24, 35). Presently, there is only one inhibitor proven to be selective for Ctsh. H$_2$N-Ser(O-Bzl)-CHN$_2$ (referred to hereafter as Ctshi) is a strong and irreversible inhibitor of Ctsh, which shows little or no activity toward two other lysosomal cysteine proteases with exopeptidase activity, Ctsb and Ctsc (23). In addition, Ctshi is a diazomethane derivative that penetrates easily across cell membranes and, thus, is able to block enzyme activity both intracellularly and extracellularly (see below) (37). Since Ctsh has not been studied in organ cultures, first we characterized the effectiveness of Ctsh inhibition in embryonic lung explants. Culturing E11.5–E12 lungs with 1.0 or 2.0 \(\mu\)M Ctshi for 72 h led to a 27 and 69.5% reduction in the total H$_2$N-Arg-MCA-hydrolyzing activity relative to Me$_2$SO vehicle alone (controls). The RA antagonist BMS493 does not interfere with FGF10 induction of Ctsh, because in E12 lungs endogenous RA signaling is already down-regulated in distal buds; thus, Ctsh is expressed at comparable levels in controls and BMS-treated lungs. 8 and C, Ctsh immunostaining confirms that almost no signals are present in the epithelium surrounding the FGF10 bead of RA-treated lungs (C, asterisks), whereas strong expression is seen in BMS-treated (B) and control (Fig. 2H) lungs.

Inhibition of Ctsh activity in cultured embryonic lungs and yolk sac membranes. A, Ctsh activity assay in lungs. The Arg-MCA hydrolyzing activity (pmol of MCA/min/\(\mu\)g of protein) was determined in protein extracts from lung cultures initially treated with 1 or 2 \(\mu\)M Ctshi (H$_2$N-Ser(O-Bzl)-CHN$_2$) or with Me$_2$SO, which were subsequently incubated with Arg-MCA substrate plus Me$_2$SO (blue bars) or with Arg-MCA plus Ctshi at 10 \(\mu\)M (magenta bars). Bars and lines, mean \(\pm\) S.E. Treatment with Ctshi (10 \(\mu\)M) significantly reduced the enzymatic activity of the lungs pretreated with Me$_2$SO (Ctrl 1 and -2; * \(p<0.05\), depicted on the left) but had no further effect in the activity of the lungs pretreated with Ctshi (1 or 2 \(\mu\)M, represented on the right). B, Western blot analysis of Ctsh in the supernatant and tissue homogenates from E12.5 lungs (top) or E12.5 yolk sacs (bottom) cultured in control or Ctshi-containing medium for 48 h. Ctshi treatment leads to an increase in abundance and molecular weight of Ctsh protein (both the 28- and 22-kDa species) in both the lung and yolk sac samples.


**Cathepsin H during Lung Branching Morphogenesis**

**Ctsh Inhibition Disrupts Lung Branching Morphogenesis**—Analysis of Ctshi-treated lungs showed a significant decrease in the number of distal epithelial buds (26 and 35% reduction relative to control lungs at 1.0 or 2.0 μM Ctshi, respectively) (Fig. 6A). Ctshi did not totally prevent, but inhibited lateral epithelial budding and greatly disrupted dichotomous branching. At 2.0 μM Ctshi, epithelial tubules continued to grow and elongate, as indicated by their finger-like appearance and abundant proliferating cell nuclear antigen (PCNA) staining (Fig. 6B). By contrast, treatment of lung explants with selective inhibitors of Ctsl (benzyloxy carbonyl-Phe-Tyr(tert-buty)-CHN₂ at 1.0 or 2.0 μM) or Ctsd (pepstatin A, up to 100 μM) at concentrations previously known to be effective in the lung and other systems (24) resulted in none of the effects described above (Fig. 6C). Thus, neither Ctsd nor Ctsl seems to have a role in lung epithelial branching, which is in agreement with observations reported in Ctsd or Ctsl null mice (41, 42).

We asked whether the reduced branching activity observed in Ctshi-treated lungs could be ascribed to an overall decrease in the expression of endogenous Fgf10. This was not the case, since real time PCR assessment of Fgf10 mRNA levels in lungs treated with Ctshi, Ctsdi, or Ctsli was essentially similar to Me₂SO-treated controls (Fig. 7A). Moreover, we found that the inhibitory effect of Ctshi in lung bud induction occurred even in the presence of high levels of exogenous FGF10. We cultured in Matrigel E11.5 distal lung buds (epithelium and mesenchyme) adjacent to FGF10 or PBS-soaked heparin beads. In this assay, epithelial cells migrate toward the source of FGF10 (beads), leaving mesenchymal cells behind (which do not express Fgfr2b); migration of the embryonic lung epithelium is thus dependent on the FGF10 provided by the beads (26). Applying Ctshi to these cultures resulted in dramatically reduced migration of epithelial cells toward the beads; the overall growth of the explant appeared to be unaffected, since the original gap between the explant and the beads was filled (Fig. 7, B–E). Thus, Ctsh activity was likely to play a role in the response of the lung epithelium to Fgf10.

**Inhibition of Ctsh Selectively Stabilizes Mature Bmp4 in Cultured Lungs**—We hypothesized that proteolysis by Ctsh could be involved in processing or degradation of an epithelial signal key for branching of the distal lung epithelium. We asked which candidate molecules, also present in E11.5–E12 distal epithelial progenitors, could potentially be Ctsh targets. Candidates such as sonic hedgehog (Shh) or Sprouty 2 (Spry2) were less likely to be relevant, since these molecules were already functioning in the lung epithelium since from E9.5–E10, prior to the onset of Ctsh expression. Moreover, analysis of Ctshi-treated lungs did not show obvious changes in levels or distribution of Ptc transcripts, a readout of Shh pathway activation (data not shown).

We reasoned that Bmp4 could be a prime candidate target of Ctsh in the lung for several reasons. Bmp4 is expressed in distal lung buds undergoing branching morphogenesis. Neither Bmp4 nor Ctsh is present in the epithelium of primary buds, and their expression in the lung overlaps temporally and spatially from E11–E12 onward (22). Both Bmp4 and Ctsh are induced by Fgf10 in the distal lung epithelium during branching (6, 26, 27). Furthermore, the migratory activity of the distal epithelium toward an FGF10-soaked bead in vitro is also inhibited by exogenous BMP4 (26), an effect that is similar to what we observed when Ctsh activity is inhibited. Moreover, proper levels of Bmp4 are critical for distal lung development (22, 43, 44).

We assessed Bmp4 protein levels in control and Ctshi-treated lungs, and we asked whether Ctsh could be involved in degradation of endogenous Bmp4 as the distal lung forms. Interestingly, Western blot analysis of Ctshi-treated lungs showed that levels of mature Bmp4 (18 kDa) were markedly increased after 48 h (Fig. 8B). By contrast, levels of the Bmp4 precursor (51 kDa) were comparable with controls (Fig. 8B). This remarkable accumulation of mature Bmp4 protein seemed to occur selectively with Ctshi, since it was not observed by inhibiting the activity of other cathepsins, such as Ctsl or Ctsd, also expressed in the lung (Fig. 8C).

**FIGURE 6.** Inhibition of Ctsh activity disrupts lung branching morphogenesis. A, Ctshi at 1.0 or 2.0 μM significantly reduces the number of terminal buds in E12 lungs cultured for 48 h (26 and 35% reduction relative to control lungs, respectively). Bar and line, mean ± S.E. (*, p < 0.05). B, proliferating cell nuclear antigen (PCNA) staining of control (Me₂SO; DMSO) and Ctshi cultured lungs shows abundant expression in distal epithelium of both groups. C, treatment of E12 lungs with effective concentrations of Ctsi inhibitor (Ctsli) or Ctsd inhibitor (Ctsdi) has no significant effect on lung branching morphogenesis.
Cathepsin H during Lung Branching Morphogenesis

Ctshi stabilized Bmp4 but not other epithelial targets of Fgf10, such as Sprouty2 (Fig. 8C).

The increase in Bmp4 protein could not be ascribed to an increase in Bmp4 transcription by Ctshi. Quantitative real time PCR showed that Bmp4 mRNA levels were actually slightly decreased in Ctshi-treated lungs, compared with control cultures. To prove that Ctshi was exerting its effects in Bmp4 expression via a post-transcriptional mechanism, we cultured control and Ctshi-treated lungs in the presence of actinomycin D (ActD), a known inhibitor of new transcription. Then we assessed expression of Bmp4 mRNA and Bmp4 protein in these samples by real time PCR and Western blot, respectively. As expected, Bmp4 mRNA was down-regulated by ActD in both conditions, with or without Ctshi (Fig. 8D). However, although Bmp4 protein was nearly absent in lungs treated with ActD alone, levels of Bmp4 were greatly increased by treatment with both ActD and Ctshi. These results strongly support the idea that the accumulation of Bmp4 was not due to increased protein synthesis but rather decreased Bmp4 degradation by Ctshi. As shown in Fig. 8D, ActD had minimal effect in Ctshi expression, and also suggested that toxic effects were not present (Fig. 8D).

We tested whether Ctsd degradation of Bmp4 protein could be demonstrated directly in a test tube. For this, we designed an in vitro assay using human recombinant mature BMP4 (R&D System) and human CTSH purified from liver (Athens Research Technology). Conservation of these proteins between humans and mice is high (BMP4, 98%; CTSH, 82%). BMP4 (10 ng) was incubated with different amounts of CTSH (3500, 1750, 875, 438, 219, 109, and 55 ng) in a 20-μl reaction mixture, at different pH values (50 mM sodium acetate, 3 mM cysteine, 1 mM EDTA, pH 6.8 or 5.2) at 37 °C for 24 h (20, 21). Using this approach, we could not demonstrate BMP4 cleavage by CTSH (data not shown). Interestingly, by performing Western blot analysis of homogenates from E14 lung, liver, kidney, heart, and adult lung, we could identify the expected 22 and 28 kDa Ctsh bands; the 22-kDa species, however, was missing in the Ctsh purified from human liver (supplemental Fig. 2A). The 22-kDa Ctsh seemed to have been largely lost during the process of purification. We had evidence that this smaller species may be particularly more susceptible to an autocatalytic process in some systems. For example, we found that in homogenates from cultured MLE15 cells (a mouse lung epithelial cell line), the 22-kDa band is nearly absent. By contrast, inhibiting Ctsd activity in these cells dramatically stabilizes the 22-kDa species and enhances its signal (supplemental Fig. 2B).

Although we do not have functional supporting data, we hypothesize that this smaller Ctsd species is the one responsible for Bmp4 cleavage in vivo. This hypothesis could not be tested in vitro using the enzyme we had available because of the lack of the 22 kDa band. This also could not be tested in the MLE15 cells without preventing autocatalytic degradation, as we discussed above. Alternatively, Bmp4 may not be a direct target of Ctsd in the developing lung in vivo.
Finally, we asked whether Bmp4 could be involved in the induction of CtsH in distal lung buds and found no supporting evidence. Application of either recombinant BMP4 or the Bmp4 antagonist Noggin, alone or in association with FGF10 (in beads), had no effect on CtsH expression (data not shown). This reinforced the idea of Bmp4 as a downstream target of CtsH.

Conclusions—Previous studies have shown that Fgf10-Fgfr2b regulates the transcription of Bmp4 in developing lung buds. Bmp4 expression correlates with Fgf10-Fgfr2b activity in distal epithelial cells, and its expression is quickly down-regulated in the region between two newly formed lung buds (26, 27). There is accumulated evidence that, in the developing lung, tightly regulated levels of Bmp4 signaling are required for epithelial cell proliferation and differentiation and to balance the effects of Fgf10 in bud outgrowth and ensure proper bud morphogenesis (44, 45). It has been proposed that high levels of Bmp4 in the distal lung epithelium act as a lateral inhibitor of budding to ensure extension of a single bud while preventing the appearance of multiple ectopic buds at the tips (26). Here we provide novel evidence that during lung branching morphogenesis, Fgf10 also controls the availability of mature Bmp4 protein in the distal epithelium by locally inducing expression of the cysteine protease CtsH. Our data suggest that CtsH may be one of the regulators of Bmp4 availability produced at the tips. Inhibition of CtsH activity markedly increased Bmp4 expression and resulted in less branched, “finger-like” epithelial structures. The co-localization of both CtsH and Bmp4 or Bmp4 signaling activity in other developing structures, such as the kidney (46), visceral endoderm, yolk sac (47), and choroid plexus (48), suggests a possible general role for CtsH in regulating Bmp4 proteolysis in different morphogenetic events.

Bmp4 is synthesized as a propeptide and is known to be activated by the proprotein convertase endoprotease furin through proteolysis at the multibasic -RSKR- motif (49–52). Several studies have implicated Bmp4 activation by furins in developing structures are less well characterized. We ensure extension of a single bud while preventing the appearance of multiple ectopic buds at the tips (26). Here we provide novel evidence that during lung branching morphogenesis, Fgf10 also controls the availability of mature Bmp4 protein in the distal epithelium by locally inducing expression of the cysteine protease CtsH. Our data suggest that CtsH may be one of the regulators of Bmp4 availability produced at the tips. Inhibition of CtsH activity markedly increased Bmp4 expression and resulted in less branched, “finger-like” epithelial structures. The co-localization of both CtsH and Bmp4 or Bmp4 signaling activity in other developing structures, such as the kidney (46), visceral endoderm, yolk sac (47), and choroid plexus (48), suggests a possible general role for CtsH in regulating Bmp4 proteolysis in different morphogenetic events.

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