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HuR controls lung branching morphogenesis and mesenchymal FGF networks

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ABSTRACT

Lung development is controlled by regulatory networks governing mesenchymal–epithelial interactions. Transcription factors and signaling molecules are known to participate in this process, yet little is known about the post-transcriptional regulation of these networks. Here we demonstrate that the RNA-binding protein (RBP) HuR is an essential regulator of mesenchymal responses during lung branching. Its epiblast-induced deletion blocked the morphogenesis of distal bronchial branches at the initiation of the pseudoglandular stage. The phenotype originated from defective mesenchymal responses since the conditional restriction of HuR deletion in epithelial progenitors did not affect distal branching or the completion of lung maturation. The loss of HuR resulted in the reduction of the key inducer of bud outgrowth and endodermal branching, FGF10 and one of its putative transcriptional regulators, Tbx4. Furthermore, exogenous FGF10 could rescue the branching defect of affected lung buds. HuR was found to bind and control the *Fgf10* and *Tbx4* mRNAs; as a result its deletion abolished their inducible post-transcriptional regulation by the mesenchymal regulator FGF9. Our data reveals HuR as the first RBP identified to play a dominant role in lung development and as a key post-transcriptional regulator of networks guiding tissue remodeling during branching morphogenesis.

Introduction

Lungs develop through a series of delicate morphogenetic steps. The initiating event is the endodermal projection of the primitive foregut which first invades the adjacent mesoderm, and then elongates to form the primary lung buds. Later, the endodermal component of these buds undergoes dichotomous subdivisions and differentiates over a rapidly proliferating mesenchyme to form the complex bronchial branches of the airways and then the alveoli of the respiratory tree. Induced mutations in the mouse revealed that this process is coordinated by reciprocal paracrine and/or autocrine signals among the developing endoderm and the adjacent mesenchyme (Cardoso and Lu, 2006). The secretion of FGF10 from the mesenchyme is central to these interactions and drives the outgrowth of the lung bud as well as attracting epithelia distally during branching. FGF10 binds to the FGfR2 receptor expressed by the developing endodermal epithelium, setting off a series of complex cross-talk signals involving FGFs, TGFβ/Bmp, Shh and Wnt to form the mesenchymal scaffold that assists epithelial outgrowth and differentiation (Cardoso and Lu, 2006;Maeda et al., 2007).

The array of reciprocal interactions involved in lung branching relies on the proper expression of the genes encoding the appropriate signaling molecules to drive the process. Recent studies have reported the emerging role of microRNAs as regulators of lung branching morphogenesis (Carraro et al., 2009; Dong et al., n.d.; Harris et al., 2006), suggesting that post-transcriptional mechanisms could be involved in the process. Post-transcriptional regulators include RNA-binding proteins (RBPs) that determine mRNA maturation, localization, stability and translation (Keene, 2007). Within the large family of RBPs, HuR has emerged as a pleiotropic modulator of mRNA utilization, particularly for mRNAs possessing of U- and AU-rich elements. HuR contains RNA recognition motifs (RRMs) with affinity for a U-rich motif and it is the only ubiquitously expressed member of the otherwise neuronal Elavl/Hu family (Keene, 1999). HuR binds to its target RNAs and shuttles between the nucleus and the cytoplasm via interactions with nuclear export/import adaptor proteins and several signaling modules (Gallouzi and Steitz, 2001). In the cytoplasm, HuR can affect mRNA stability and/or translation through complex interplay with ribonucleoprotein structures containing members of the hnRNP, TTP and TIA families as well as micro-RNAs (Katsanou et al., 2006; van der Giessen and Gallouzi, 2007).

HuR expression is known to vary throughout extraembryonic and embryonic development (Gouble and Morello, 2000;Katsanou et al., 2009). Loss and gain-of function experiments suggested that HuR is involved in the control of the cell cycle, differentiation programs and stress responses of numerous cell types (Abdelmohsen et al., 2007; Gorospe, 2003; Keene, 1999;Levadoux-Martin et al., 2003;van der Giessen and Gallouzi, 2007). In vivo, deletion of HuR in the mouse blocks the morphogenesis of the extraembryonic placenta, leading to embryonic lethality (Katsanou et al., 2009). Tissue-specific ablation

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revealed that HuR can control several processes including limb patterning and spleen development (Katsanou et al., 2009). A common denominator in these phenotypes is their dependency on cross talk between mesenchyme and endodermal epithelium. However, the role of HuR in this process has not been elucidated.

Here we dissect the function of HuR in the cellular compartments of the developing lung bud. We demonstrate that HuR controls intercellular interactions that shape the lung as a branched structure.

Materials and methods

Mice

The generation of *Elavl1*$^{fl/fl}$ and epiblast-specific (*Sox2Cre*$^{+}$*Elavl1*$^{fl/-}$; *referred to as Elavl1*$^{-/-}$) HuR mutants has been previously described (Katsanou et al., 2009). *Sftpc-Cre* mice (Okubo et al., 2005) were kindly provided by B. L. Hogan (USA). Mice were backcrossed to C57Bl6/J and were maintained in the animal facilities of the Biomedical Sciences Research Center Alexander (Al.) Fleming under specific-pathogen free conditions. Experiments on live animals were approved by the Hellenic Ministry of Rural Development-(Directorate of Veterinary Services) and by the Animal Research and Ethics Committee of the BSRC Al. Fleming to comply with FELASA regulations.

Histology

For general histology, embryos from timed-pregnant mice were isolated at the times indicated and embryonic lungs were removed by standard dissection protocols in phosphate-buffered saline (PBS). Embryonic lungs were fixed in neutral buffered formalin (BDH) overnight at 4 °C. Fixation was followed by dehydration in an ethanol series prior to embedding in paraffin and sectioning. Sections of 4–7 μm were stained with hematoxylin and eosin for general histology according to standard techniques or used for immunohistochemistry. Alternatively, specimens were directly embedded onto OCT cryopreservative (TissueTec) and mounted via freezing for cryostat sections.

Lung bud and organ cultures

Isolation and culture of wild type and mutant distal lung buds were performed as previously described (Bellusci et al., 1997). Briefly, lung primordia derived from E11.5 and E12.0 embryos were microdissected with tungsten needles under sterile conditions to separate distal lung bud tips – for use in FGF complementation assays – from the rest of lung buds (amputated buds) that were used for FGF9 induced FGF10 expression and proliferation as shown in Supplementary Fig. 5. Both distal lung buds and amputated lungs were cultured in Matrigel diluted 1:1 with DMEM/F12. Lung bud cultures were treated with the following recombinant FGFs (Peprotech EC): hFGF10 (250–500 ng/mL), hFGF1 (200 ng/mL) and mFGF9 (200 ng/mL) for a period of up to 96 h; media were replaced every 24 h. Amputated lung explants were used for proliferation assays, immunohistochemistry or RNA analyses.

Cell culture

Primary mouse embryonic fibroblasts (MEFs) were derived from E13.5 embryos and were cultured in complete DMEM + 10% FBS (Biochrom AG) for three to five passages prior to experimentation. For stimulation, cultures were synchronized in DMEM media supplemented with 0.2% FBS and then treated with recombinant mFGF9 (200 ng/mL-Peprotech) for 6 h. Primary lung mesenchymal cells were isolated from E13.5 fetal lungs as previously described (Lebeche et al., 1999) and cultured in DMEM/F12 media supplemented with 0.5% FBS in presence or absence of the abovementioned stimuli.

Cellular proliferation and apoptosis

For cell death determination, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assays were performed on paraffin-embedded sections using an in situ cell death detection kit (cat. no. 11 684 795 910; Roche) according to manufacturer's instructions. For proliferation assays, pulse BrdU experiments were performed by the intraperitoneal injection of pregnant females 3 h prior to sacrifice. Additionally, amputated lung explants derived from E11.5 embryos were cultured as described above in Matrigel diluted 1:1 with DMEM/F12. The explants were treated with FGF9 (200 ng/ml) for 24 h before labeling for 3 h with BrdU (10 μM in culture). For detection, cryostat sections (7 μm) were permeabilized with proteinase K, treated with 2 N HCl and borate buffer (pH 7.6), blocked and incubated with anti-BrdU antibody (M0744; Dako-Cytomation). Peroxidase-conjugated anti-mouse IgG1 (Southern Biotech) or Alexa555 anti-mouse IgG secondary antibodies (1:1000) were used. Sections were visualized either with DAB and counterstained with haematoxylin or by fluorescence microscopy. Quantitation was performed in ×200 magnification images, either using the ImageJ software (NIH; for BrdU) or manually (for TUNEL).

Immunoblotting, and immunohistochemistry

For western blots, total or nuclear lysates were analyzed on SDS-PAGE according to standard protocols. Nuclear lysates were prepared with NE-PER reagent (Pierce) according to the manufacturer's instructions. For immunohistochemistry/immunofluorescence cryosections were used immediately, whereas paraffin sections were deparaffinized and treated with microwaves in citrate buffer for epitope unmasking. Primary antibodies included: HuR (3A2 or 19F12), actin (C11), Tbx4 (N-19) and FGF-10 (H121) for blots from Santa Cruz; FGF-10 (ab9988) from Abcam for immunocytochemistry; SMA from Sigma; and Ki67 (ab15580). Detection was performed using horseradish peroxidase-conjugated, alkaline phosphatase-conjugated or Alexa-488/-555 (Southern Biotech) secondary antibodies. Blots were visualized by enhanced chemiluminescence (ECL+; Amersham). Sections were developed using either DAB or Fast Blue Substrates (Sigma). Nuclear counterstaining was carried out with haematoxylin, Nuclear Fast Red (Vector) or DAPI.

RNA analysis

Total RNA was derived from whole embryonic lungs or primary MEFs using Trizol™ according to the manufacturer's protocol. For cDNA synthesis, 2–5 μg of total RNA was used for cDNA synthesis with MMLV-RT (Promega). Quantitative-Real Time PCR (qRT-PCR) was performed using EvaGreen SsoFast Qpcr mix (Biorad) on a RotorGene 6000 machine (Corbett Research). HuR-RNA immunoprecipitation (IP) experiments, analysis of polysome-coupled RNA, and mRNA stability assays were performed as previously described (Katsanou et al., 2009). PCR primers are listed in Supplementary methods.

Results

HuR is required for distal lung branching

We previously used a *Sox2-Cre* mediated strategy to induce the recombination of a loxP-flanked *Elavl1* allele (*Elavl1fl*) in the epiblast and bypass the placental defects that hamper the analysis of HuR function beyond gastrulation (Katsanou et al., 2009). Examination of the developing lungs in these HuR-null embryos revealed that the formation of primary lung buds, the specification of the trachea and the initial branching of the stem bronchi occurred normally suggesting that HuR is not required for the progression from the embryonic to the pseudoglandular stage (E9.0–E12.0) of lung development (Fig. 1A;

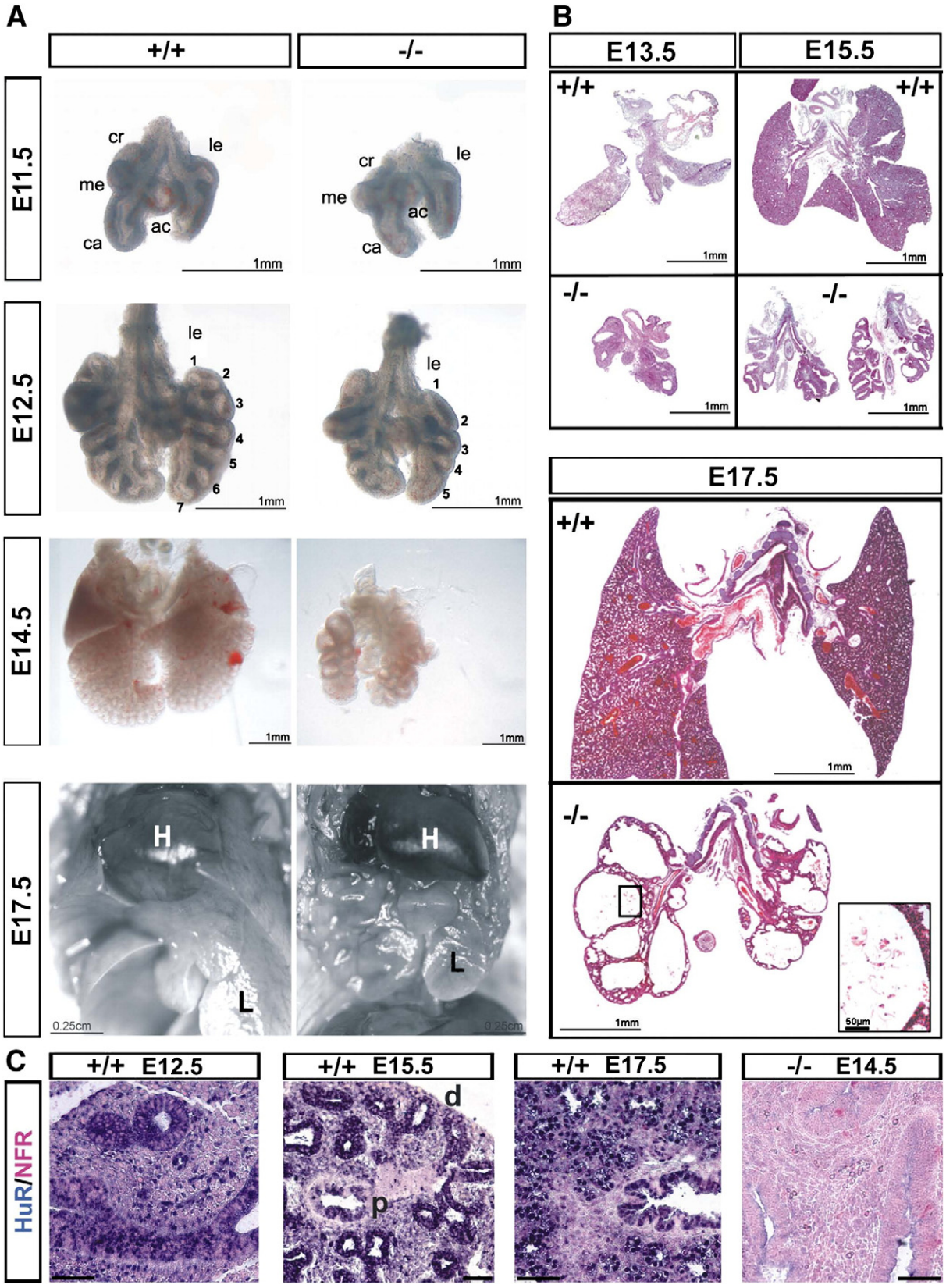


Fig. 1. Impaired lung branching in fetal lungs lacking HuR. (A) Macroscopic comparisons of control (+/+) and mutant (−/−) lungs in the staged progression of lung development. The left (le) and right cranial (cr), middle (me), caudal (ca) and accessory (ac) lobes are indicated. Numericals correspond to the numbers of epithelial branches observed in the left lobes. (B) Comparative histology at embryonic days 13.5, 15.5 and 17.5. Stain: Haematoxylin/eosin. Note the expelled epithelial cells at E17.5 (inset) (C) Immunohistochemical detection of HuR protein (blue) from the pseudoglandular to the saccular stage of lung development counterstained with Nuclear Fast Red (NFR). HuR-deficient lungs at E14.5 are used as negative controls. Size bars correspond to 100 μm.

see also Supplementary Fig. 1 for the staging of lung development). However, a block in the subsequent branching of most bronchi was observed during the pseudoglandular stage (E12.0–E15.0). Starting at E12.5, the mutant primitive lobes contained a lesser number of epithelial branches than the control lobes. At E14.5, where control lungs possessed an expanding network of distal branches, mutant lungs contained dilated, sac-like structures and lost their lobular architecture. These deformities persisted through the canalicular and saccular stages of development i.e. till E17.5 (e.g. Fig. 1A). Histological assessment revealed that a few rounds of branching to bronchioles occurred in the mutant lungs, particularly in some of the developing upper right lobes (Fig. 1B and Supplementary Fig. 3); however, distal branches were absent and the dilated structures appearing from E13.5 in the mutant lungs were characterized by the absence of terminal alveolar saccules and the presence of a few wide-bore bronchiolar tubules connecting to large fluid and debris-filled sacs (Fig. 1B). Thus, the loss of HuR blocked the secondary branching of the major bronchi to distal bronchioles and primitive alveoli that occurs between the pseudoglandular and the canalicular stages of murine lung development. A close examination of HuR protein during the progression of these stages in wild-type lung buds revealed that its expression changed during branching (Fig. 1C and Supplementary Fig. 2). At the initiation of the pseudoglandular stage (E11–12.5), HuR was detected in all epithelial and mesenchymal compartments, but its cellular and subcellular distribution variegated. As branching progressed (i.e. E15.5), HuR increased in distal epithelial cells and the surrounding mesenchyme; toward the saccular stage (e.g. E17.5), HuR acquired a widespread expression pattern that was maintained after birth. Taken together, the expression of HuR in the developing lung reflected its control over the morphogenesis of distal lung branches.

The loss of HuR from the developing endoderm does not block epithelial differentiation or branching

The branching of the developing lung bud depends on the proliferation of all compartments and the differentiation of endodermal progenitor cells. To assess whether the loss of HuR affected these responses, we administered BrdU and looked for changes in the mutant lung buds. At E13.5, extensive proliferation was observed in the epithelia and the mesenchyme of control buds. At the same age, the mutant epithelia of proximal conducting airways contained comparable numbers of proliferating cells; in contrast, the distal developing epithelia and their mesenchymal support showed reductions in the number of proliferating cells (Fig. 2A, C). At this age TUNEL assays revealed also an increase in the number of apoptotic cells in the epithelium and the mesenchyme of HuR-deficient lungs (Fig. 2B, D).

Immunohistochemical examination of E17.5 mutant lungs with markers of differentiated epithelia (Supplementary Fig. 3) revealed that regions maintaining a rudimentary architecture – particularly in the upper right lobes – possessed cells expressing secretoglobin1α1 (*Scgb1α1* or *CC10*) which marks Clara cells; and (b) epithelial cells expressing the type I (squamous) and type II (cuboid) cells markers Glycoprotein 38 (Gp38/*T1α*) and surfactant protein C (*SPC*), as well as cells co-expressing CC10 and SPC which are considered bronchioalveolar progenitor/stem cells (BASCs) and normally reside in the bronchioalveolar duct junctions (BADJ) (Kim et al., 2005;Nolen-Walston et al., 2008;Reynolds et al., 2008). We noted that in the mutant lungs, regions with incomplete branching maintained some "BADJ" like structures that contained $CC10^{+}SPC^{+}$ cells; interestingly, some of these double positive cells acquired both the typical squamous phenotype of control BASCs as well as an "atypical" columnar phenotype (Supplementary Fig. 4A). This phenomenon could reflect a block in the transition to columnar, $CC10^{+}$ epithelia which were absent in distal parts of the mutant lungs, confirming the loss of distal airways. Furthermore, mutant epithelia were separated by thin layers of disorganized mesenchyme expressing smooth muscle actin (Sma; Supplementary Fig. 4B) beyond the layers encircling the endoderm of proximal airways and the developing blood vessels. Thus, endoderm differentiation can occur in the absence of HuR but the lack in the formation of the distal structures could relate to changes in mesenchymal organization.

To dissect further the function of HuR, we made use of the conditional *Elavl1*^{fl} allele that was crossed to *Sftpc-Cre* transgenic mice (Okubo et al., 2005), to delete HuR in the epithelial compartment of the lung endoderm prior to E10.5. *Sftpc-cre*$^{+}$*Elavl1*$^{fl/fl}$ embryos developed to term and were breathing at birth. The recombination of the *Elavl1* locus was restricted to the lungs of the *Sftpc-cre Elavl1*$^{fl/fl}$ embryos (Fig. 3A). Similarly, the deletion of HuR protein was confined to proximal and distal epithelial layers (Fig. 3 E–H). Examination of lungs at E18.5 (Fig. 3 B–D) revealed the proper development of the bronchial tree and the completion of all stages in lung development. Thus, epithelial HuR is not required for lung branching morphogenesis and does not affect the reciprocal communication with HuR-proficient mesenchyme. As a result, the branching defect of mutant buds relates to the loss of mesenchymal HuR functions.

The loss of HuR induces a deficiency in FGF10

In an effort to identify networks affected by the loss of HuR, we isolated total RNA from control and mutant lungs at E11.5 to E12.5 and screened them for mRNAs involved in this process by quantitative real-time PCR (qRT-PCR) (Fig. 4A). We detected mRNAs of pleiotropic morphogens (e.g. retinoic acid receptor-*RAR, and BMP4*), or mRNAs expressed in the mesenchyme (e.g. *Fgf10, FgfR1c, Gli1, Tbx4, and Wnt2a*) or developing epithelia (*Fgf9, FgfR2b, GATA6, Lef1, Shh, Sox2, Spry1, Spry 2, and Ttf1*). The most striking reductions (≤50%) were observed in mRNAs encoding: FGF10, a major mesenchymal determinant of primary lung bud formation and branching morphogenesis (Cardoso and Lu, 2006;Maeda et al., 2007); TBX4, a T-box transcription factor involved in endodermal branching and the transcriptional regulation of the *Fgf10* gene (Cardoso and Lu, 2006;Cebra-Thomas et al., 2003; Sakiyama et al., 2003); and the epithelial receptor of FGF10, FGFR2b, the growth inhibitor SPRY2 and the epithelial transcription factor TTF1, all of which are induced by FGF10 in distal epithelia (Cardoso, 2001;Cardoso and Lu, 2006;Maeda et al., 2007). Significant increases were detected in mRNAs encoding: FGF9, an epithelia-derived inducer of mesenchymal/epithelial proliferation and FGF10 expression (Colvin et al., 2001;del Moral et al., 2006); BMP4 and WNT2a, induced by FGF9 and regulators of growth factor signals; and SOX2 which is blocked by FGF10 to aid epithelial progenitor differentiation during branching (Gontan et al., 2008). Given that besides the *Fgf10* and *Tbx4* mRNAs, other mesenchymal mRNAs *(FgfR1c, Gli1 and Wnt2a)* showed minimal or positive changes in their expression, we hypothesized that HuR-deficient buds may not lack a properly expanding mesenchyme but rather lack sufficient FGF10 or cell autonomous Tbx4 transcription which are needed for branching. Indeed, the levels of FGF10 and Tbx4 proteins were consistently less than 50% in mutant lung extracts at E12.5 (Fig. 4B). By exploiting an antibody applicable for the detection of FGF10 protein in tissues, we further detected diffused mesenchymal and parabroncial FGF10 signals (Mailleux et al., 2005), in control sections that were almost undetectable in mutant sections (Fig. 4C). Most importantly, the reduction of *Tbx4 and Fgf10* mRNAs – but not of other mesenchymal mRNAs like *Gli1* or *Bmp4* mRNAs – was also apparent in cultured lung mesenchymal cells (LMCs) isolated from mutant E13.5 lungs and passaged for – at least – two generations (Fig. 4D); this suggested that the observed decreases of these mRNAs in the developing buds were not due to changes in mesenchymal cellularity but rather in their expression. Thus, the lung branching defect of HuR-deficient embryos correlates with a deficiency in Tbx4 and FGF10.

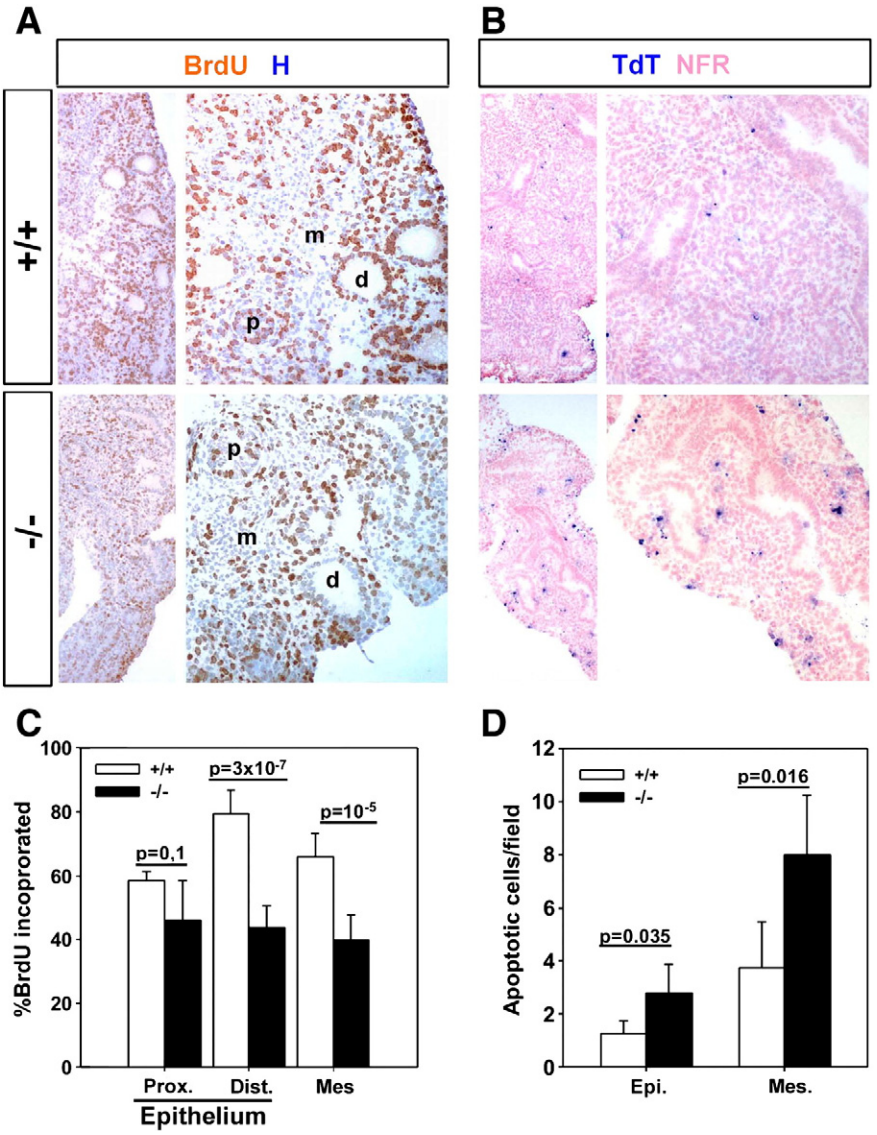


Fig. 2. Abnormal proliferation and apoptosis of fetal lungs lacking HuR. (A) Immunohistochemical detection of BrdU incorporation (brown) in sections from control (+/+) and mutant (−/−) lungs at E13.5, at a ×100 and ×200 magnification. *m*:mesenchyme; *p*: proximal epithelium; *d*: distal epithelium. *H*, Hematoxylin counterstain. (B) TUNEL detection of apoptotic cells (TdT; blue) in control and mutant lungs at E13.5, at ×100 and ×200 magnification. *NFR*, Nuclear Fast Red counterstain. (C) Bar graphs and corresponding p values derived from the comparison of the percentages of $BrdU^{+ve}$ cells (±SEM) in control and mutant lungs at E13.5. Data derived from high power field measurements of 2703 ± 244 mesenchymal cells and 553 ± 46 epithelial cells per lung and from 3 control lungs; and 1835 ± 283 mesenchymal cells and 442 ± 37 epithelial cells per lung from 4 mutant lungs. (D) Bar graphs and corresponding p values derived from the comparison of the number of TUNEL positive cells per high power field (±SEM) in the epithelium and mesenchyme of control (n = 3) and mutant (n = 3) lungs. *H*: haematoxylin.

FGF10 rescues the branching defect of HuR-deficient buds

To verify that mutant lungs suffered from a loss in FGF10 signals, we dissected distal lung buds (endoderm plus mesenchyme) from E11.5 embryos and cultured them in Matrigel supports (Supplementary Fig. 5). As shown in Fig. 5A, control buds divided internally after 48 h in culture suggesting that the limited mesenchyme or FGF present in the bud forced the adjacent endoderm to slowly branch. In striking contrast, a limited division was observed in the mutant buds and they failed to form defined branches under the same culture conditions. FGF1, which can bind to FGFR2 (Bellusci et al., 1997), could induce endodermal budding in the mutant cultures suggesting that the mutation affected the presence of FGF10 and not FGFR2 signaling. On the other hand, the addition of FGF10 rescued the branching defect of HuR-null buds and induced endodermal swelling and rapid budding, as in control buds, which could be observed even at 48 h in culture. Thus, FGF10 is crucial to overcome the branching impairment in HuR-null lung buds (Fig. 5B).

To discriminate between HuR-related defects in the proliferation of the mesenchyme from an effect of HuR on FGF10 biosynthesis, we analyzed the response of HuR-null lung buds to FGF9. FGF9 is an essential epithelia-derived regulator of lung mesenchyme and deficient animals possess hypoplastic and poorly branched lungs that show reduced mesenchyme and *Fgf10* mRNA expression (Colvin et al., 2001). Subsequent studies demonstrated that FGF9 derived from the mesothelium and the distal epithelium can induce at least three processes supporting bud extension in an independent fashion : (a) the expansion of the adjacent mesenchyme and the repression of its differentiation to smooth muscle cells; (b) the induction of mesenchymal FGF10, to act on the developing epithelium, and (c) the partial – yet direct – induction of epithelial proliferation through binding to epithelial FGFR2b (del Moral et al., 2006;White

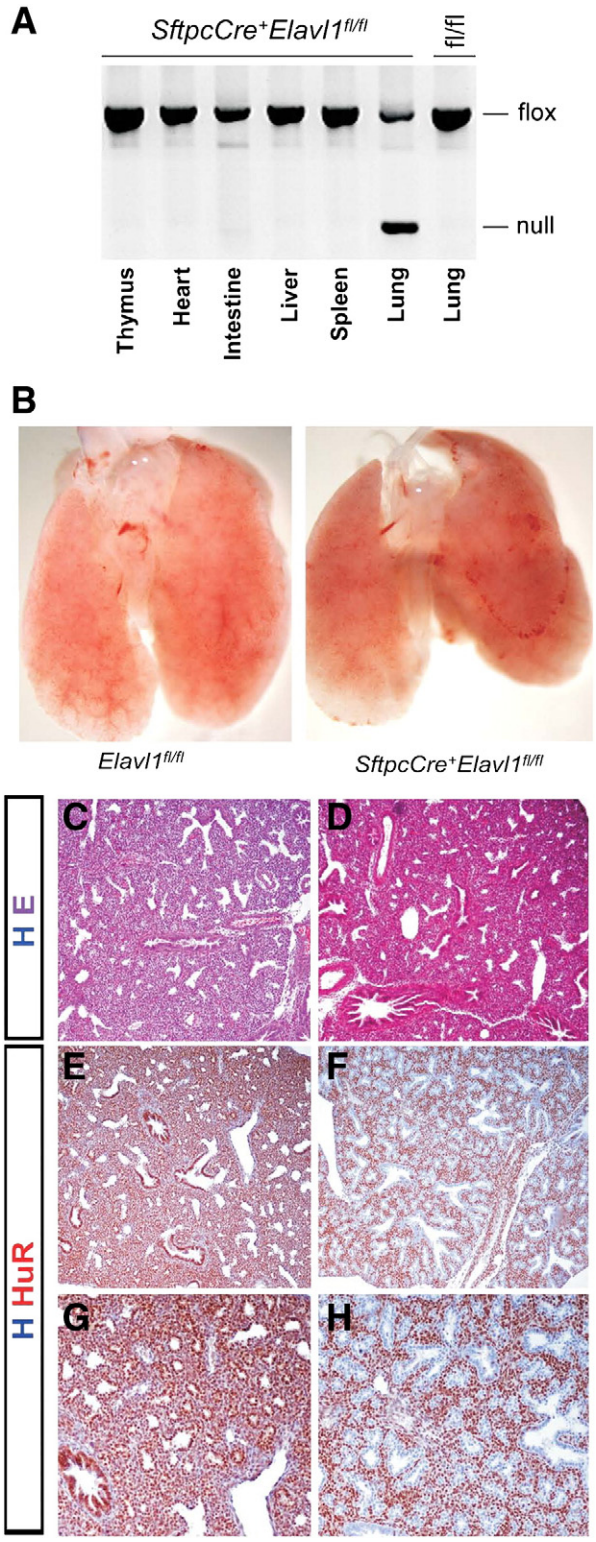


Fig. 3. The loss of epithelial HuR does not impair lung development. (A) PCR-based detection of targeted (fl) and Cre-recombinant (null) loci in DNA extracts from tissues of *Sftpc-cre*$^{+}$*Elavl1*$^{fl/fl}$ E18.5 embryos. Extracts from *Elavl1*$^{fl/fl}$ lungs are shown as controls. (B–D) Macroscopic and histological analysis of *Sftpc-cre*$^{+}$*Elavl1*$^{fl/fl}$ and *Elavl1*$^{fl/fl}$ lungs at E18.5. Low (E, F) and high power (G, H) immunohistochemical detection of HuR (brown) in sections from E18.5 *Elavl1*$^{fl/fl}$ and *Sftpc-cre*$^{+}$*Elavl1*$^{fl/fl}$ lungs indicating the epithelial deletion in the latter. Scale bar: 100 μm.

et al., 2006). As can be seen in Fig. 5D, recombinant FGF9 was able to induce the proper dilatation of the epithelium in control buds; a lesser yet detectable response was also observed in mutant buds suggesting that FGF9 could also partially overcome the branching defect induced by the loss of HuR. To understand the target of FGF9 functions on the mutant buds, we compared its effects on amputated lung explants that possess a lower level of FGF10 than the distal tips (Bellusci et al., 1997) and thus changes in FGF10 expression versus proliferation could be more readily observed. Analysis of BrdU incorporation using these FGF9-treated explants, showed a clear increase in the epithelial and mesenchymal proliferation in both the control and the mutant explants (Fig. 6A, B). Given that FGF9 can also repress the differentiation of the lung mesenchyme to smooth muscle to enforce a proliferative state (del Moral et al., 2006), we also examined the response of LMCs to this factor. As demonstrated by the Ki67 and SMA signals, FGF9 could induce the proliferation and the reduction in SMA expression in HuR-null LMCs as in control LMCs (Fig. 6D). In contrast, and although FGF10 protein was locally enhanced in FGF9-treated control explants, the mutant explants lacked FGF10 protein expression (Fig. 6A). qRT-PCR analyses of explant-derived RNA (Fig. 6C) verified that FGF9 could induce a two-fold increase in *Fgf10* mRNA in HuR-proficient explants, whereas the basal levels of this mRNA were reduced in HuR-deficient explants and not altered by the presence of FGF9. We also observed a dramatic increase in *Tbx4* mRNA in FGF9 treated HuR-proficient explants which was in compliance to previous observations suggesting a relationship between the domains of expression *Fgf10* and *Tbx4* mRNAs in embryonic lung mesenchyme (del Moral et al., 2006); in contrast and although the *Tbx4* mRNA could be induced in HuR-deficient explants, both its basal and inducible levels were significantly reduced relative to control values. The augmented responses of *Fgf10* and *Tbx4* mRNAs in FGF9-treated, HuR-proficient explants also correlated to an increase in the levels of *Elavl1*/*HuR* mRNA (Fig. 6C). Collectively, our data demonstrate that HuR is not required for the transmission of FGF9 signals promoting the proliferation of mesenchymal and epithelial cells but is required for the inducible biosynthesis of FGF10 in the lung mesenchyme.

HuR controls the synthesis and translation of Fgf10 mRNA in mesenchymal cells

A previous high-throughput screen in mouse embryonic fibroblasts (MEFs) had identified the *Fgf10* and the *Tbx4* mRNAs as potential HuR targets (Katsanou et al., 2009). In the same study, HuR-null MEFs showed a reduction in the basal accumulation and transcription of the *Fgf10* gene that correlated with a reduction in the basal accumulation and stability of *Tbx4* mRNA; conversely, the overexpression of HuR in these MEFs could increase *Tbx4* mRNA stability and *Fgf10* mRNA abundance. However, the study failed to identify any evidence for the post-transcriptional regulation of *Fgf10* mRNA. Our current findings suggested that HuR functions could extent to the inducible regulation of the *Tbx4* and *Fgf10* mRNAs. We therefore examined their expression in cultured LMCs, in the absence or presence of induction by FGF9 (del Moral et al., 2006;Jean et al., 2008;White et al., 2006). The *Fgf10* and *Tbx4* mRNAs were readily detectable in resting HuR^{+} LMCs; moreover, the stimulation of these cells with FGF9 led to 1.8 fold and 2-fold increases in *Fgf10* and *Tbx4* mRNAs respectively (Fig. 7A). In the same cells, FGF9 induced a substantial increase in *Elavl1*/*HuR* mRNA confirming that FGF9 targets HuR's biosynthesis. In contrast, the basal levels of *Fgf10* and *Tbx4* mRNAs were significantly reduced in HuR^{-} LMCs. Moreover, the addition of FGF9 had no effect on the levels of these mRNAs in HuR^{-} LMCs, suggesting that HuR is required for their basal and inducible regulation.

The next series of experiments necessitated a large number of cells ($>10^{8}$ per condition and per assay) and thus the reduced number of primary LMCs derived from E13.5 embryos could not meet this requirement. Thus, we switched to MEFs which allowed more detailed analyses due to their easy maintenance and expansion in culture. The basal and inducible accumulation of *Fgf10*, *Tbx4* and *Elavl1*/*HuR* mRNAs in HuR^{+} and HuR^{-} MEFs was similar to that in the corresponding LMCs

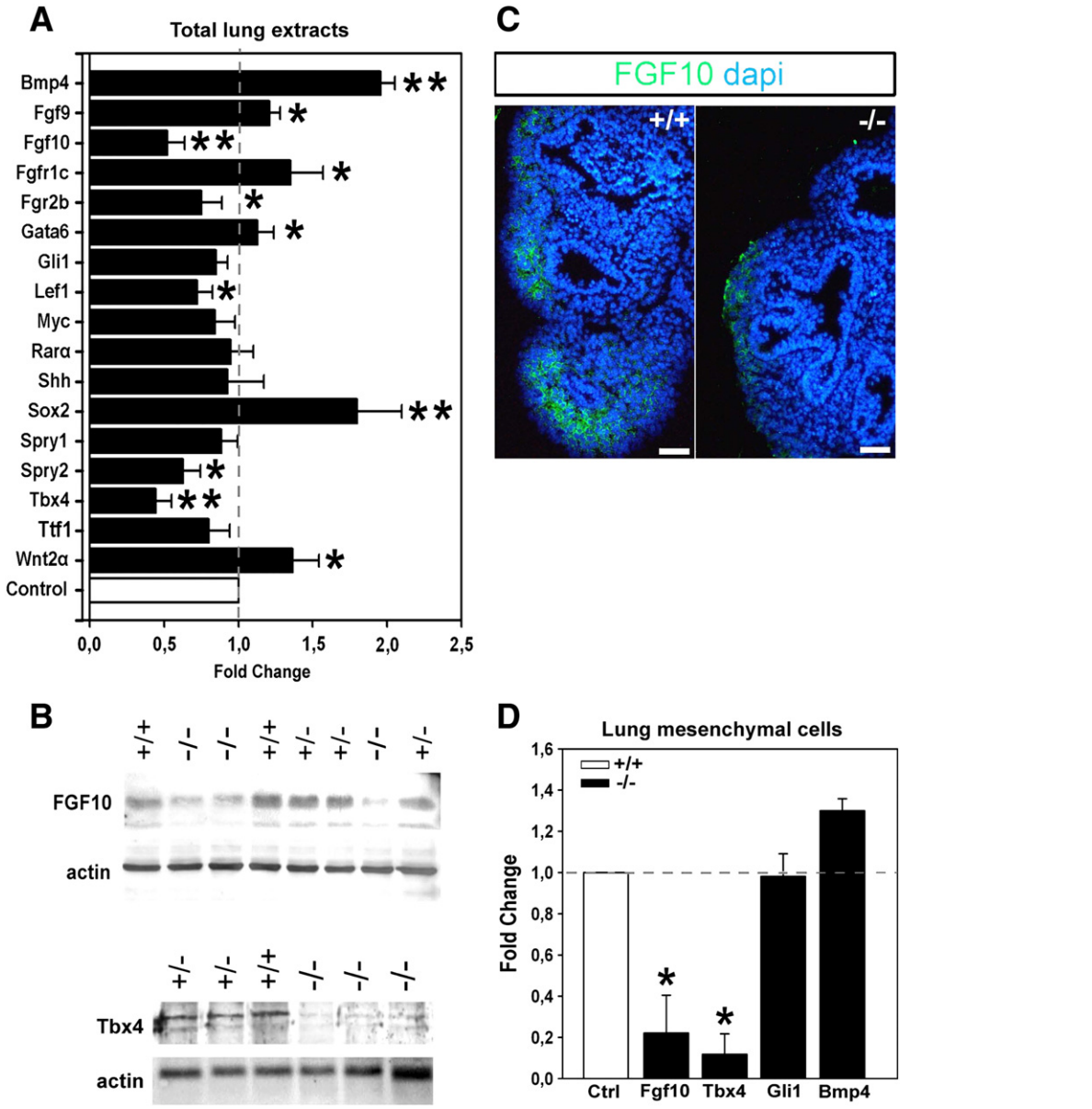


Fig. 4. Reduced FGF10 in fetal lungs lacking HuR. (A) Bar graph depicting the differential (fold change) levels of morphogenic mRNAs in extracts from fetal lungs at the early pseudoglandular stage. Data (arbitrary units ± SEM) derived from qRT-PCR experiments performed in RNAs from >5 lungs per genotype. The dotted line represents the control value of 1. * and ** denote minimal and maximal statistical differences with $p<0.01$, respectively. (B) Detection of FGF10 and Tbx4 proteins in immunoblots from control and mutant lung extracts at E12.5. (C) Immunohistochemical detection of FGF10 (green) in control and mutant sections from E12.5 lungs displaying reduced FGF10 detection in mesenchyme adjacent to developing epithelial tips of mutant lungs. (D) qRT-PCR analysis in isolated lung mesenchymal cells showing reduced levels of *Fgf10* and *Tbx4* mRNAs but not for *Gli1* nor *Bmp4* mRNAs in HuR-deficient cells. Scale bar: 100 μm.

(Fig. 7A). In accordance to the RNA measurements, the FGF10 protein increased in total protein extracts from FGF9-treated HuR^+ MEFs, whereas it was reduced in resting and stimulated HuR^- MEFs (Fig. 7B). The Tbx4 protein was clearly detectable in nuclear extracts from HuR^+ MEFs; interestingly FGF9 induced an increase in Tbx4 protein levels as well as a shift in size (Fig. 7C), suggesting that FGF9 affects both the expression and the post-translational modification of Tbx4. In HuR^- MEFs, FGF9 could still cause a change in the size of Tbx4 protein, but both its basal and inducible levels were dramatically reduced relative to its levels in HuR^+ MEFs. Finally, the examination of HuR protein in HuR^+ MEFs revealed that FGF9 induced an increase in the levels of HuR protein but not in its nucleo-cytoplasmic distribution (Fig. 7B and Supplementary Fig. 7).

Next, we used HuR:RNA immunoprecipitation assays (R-IPs) from HuR^+ MEFs to detect whether HuR's associations to the *Fgf10* and *Tbx4* mRNAs varied following stimulation. The specificity of associations was controlled by (a) HuR IP assays from HuR^- MEF extracts and (b) isotype mIgG1 IP assays from HuR^+ MEF extracts (Fig. 7D), whereas the quality of the IP reactions was monitored via immunoblotting (Fig. 7E). In each IP, the presence of each mRNA was tested by qRT-PCR and positive associations were considered if mRNAs were enriched, compared to control IPs. Given the fluctuations in HuR levels, values were normalized to levels of *GAPDH* mRNA, an abundant mRNA that is not a specific target of HuR but is present as a low-level contaminant in all HuR-IP samples and relates to the quantity of protein in the IP material. As shown in Fig. 7D, the *Fgf10 mRNA* associated with HuR under all conditions tested and to a similar extend. In contrast, the *Tbx4 mRNA* associated with HuR in the absence of stimulation, but this association increased 2-fold in the presence of FGF9-stimulation.

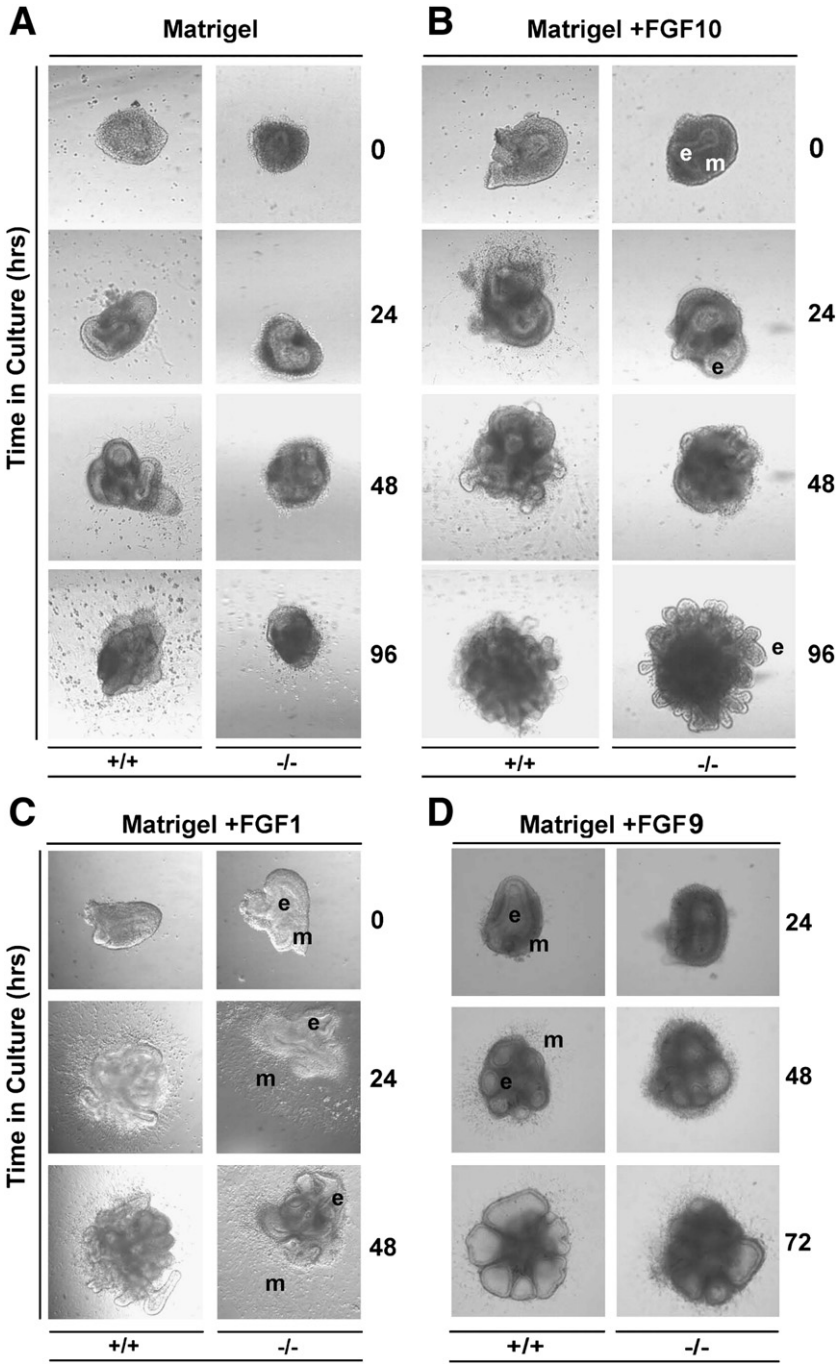


Fig. 5. Effect of recombinant FGFs on the branching of HuR-deficient distal buds. (A) Isolated E11.5 distal lung bud tips cultured in a Matrigel support without FGFs. Limited branching of the endoderm surrounded by mesoderm is observed in the control (+/+) but not in the mutant (−/−) lung bud tips after 48 h. Shown are representative images from 11 explants derived from 6 to 7 lungs per genotype (B) Effect of recombinant FGF10 on E11.5 lung buds. Note that both genotypes exhibit extending budding between 48 and 96 h. Shown are representative images from 8 explants derived from 4 lungs per genotype (C) Response of lung buds to the presence of recombinant FGF1. Shown are representative images from 4 explants derived from 2 lungs per genotype. (D) Response of lung buds to the presence of recombinant FGF9. Shown are representative images from 8 explants derived from 5 lungs per genotype. In some micrographs, the endodermal (e) and mesenchymal (m) layers are indicated.

To highlight the effects of HuR on *Tbx4* and *Fgf10* mRNAs, we analyzed their post-transcriptional regulation in HuR^{+} and HuR^{-} MEFs. To assess whether the loss of HuR affected the basal and/or the inducible stabilization of the *Tbx4* and *Fgf10* mRNAs, we blocked their transcription in MEFs using actinomycin D and measured their temporal disappearance (decay) via qRT-PCR. The remnant levels of *Tbx4* and *Fgf10* mRNAs were then used for the estimation of the corresponding mRNA half lives in the absence or presence of FGF9 (Fig. 7F and Supplementary Fig. 8). The *Fgf10* mRNA appeared as relatively unstable in HuR^{+} MEF and its half life increased in the presence of FGF9; interestingly the half lives of the *Fgf10* mRNA in untreated and FGF9-treated HuR^{-} MEF were comparable to control values. Thus, HuR is not required for the stabilization of the *Fgf10* mRNA and the observed changes in its abundance in HuR^{-} MEFs are probably due to effects on *Fgf10* mRNA transcription or maturation. The half-life of the *Tbx4* mRNA was also significantly augmented by FGF9 treatment in HuR^{+} MEFs. In sharp contrast however, the decay of *Tbx4* mRNA in HuR^{-} MEFs was persistently reduced under basal

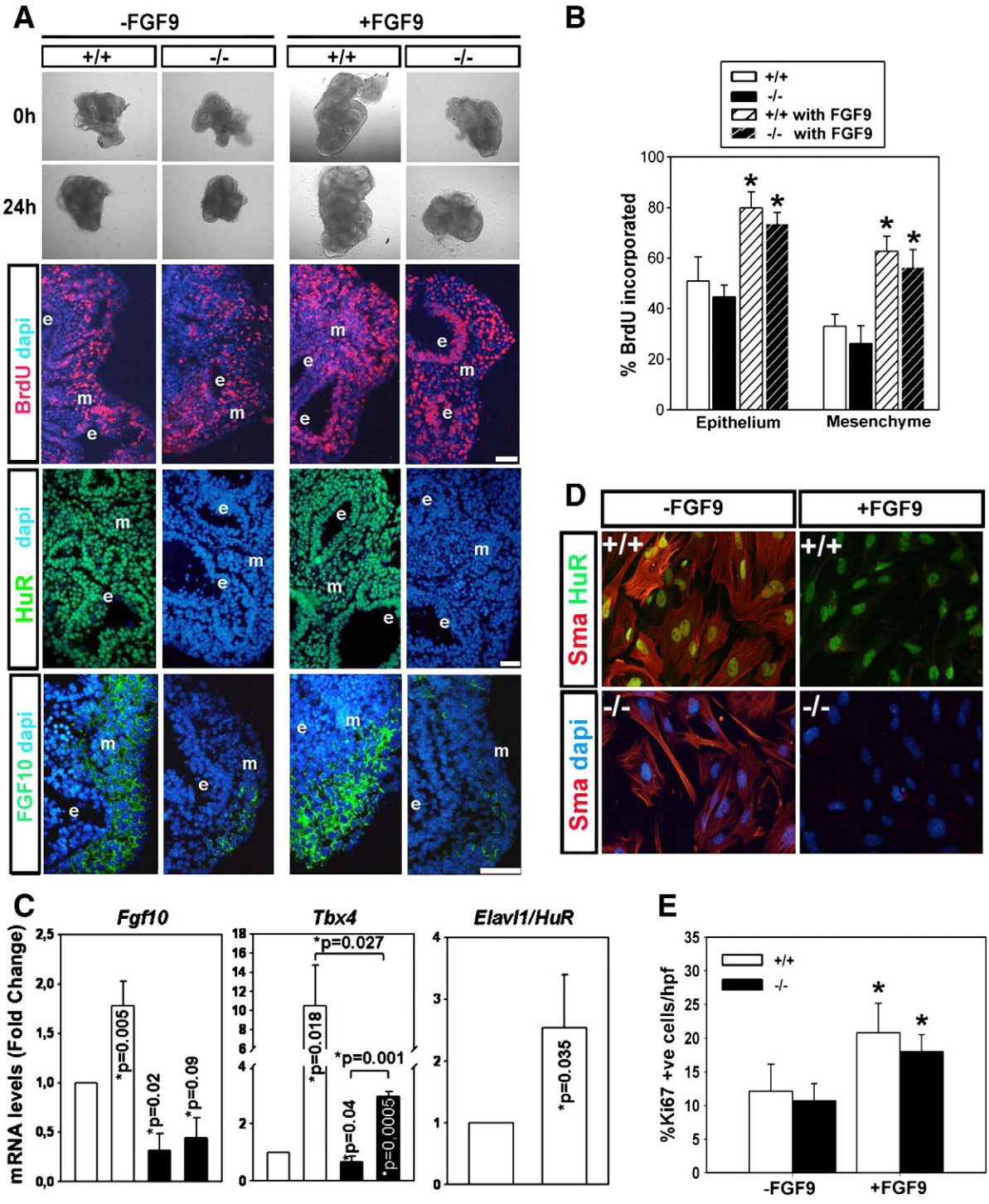
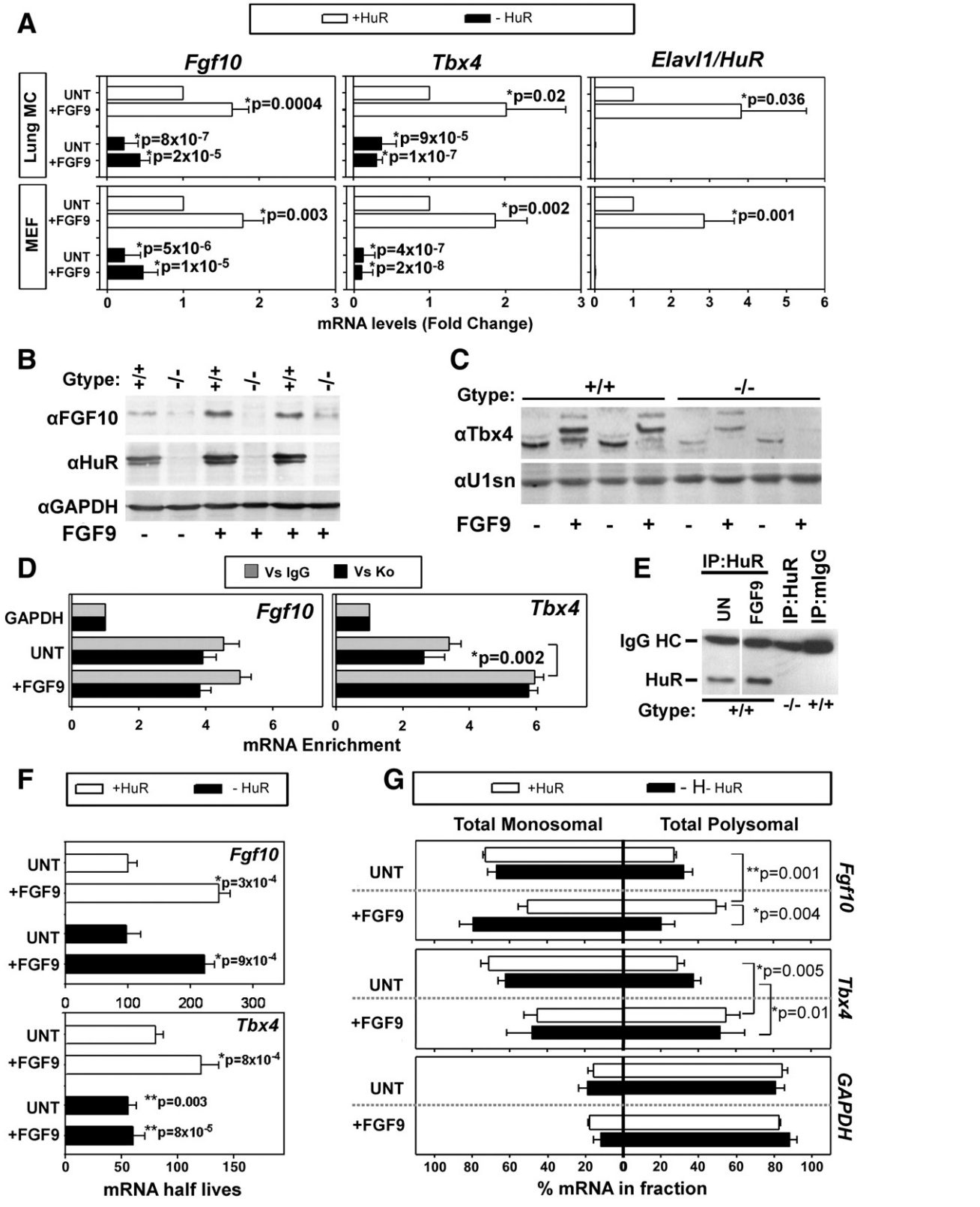


Fig. 6. Effect of FGF9 on HuR-deficient amputated lungs. (A) Isolated E11.5–E12.0 amputated lung explants cultured in Matrigel in presence or absence of recombinant FGF9 for 6 h. Shown are photomicrographs and immunofluorescent stains for BrdU, HuR and FGF10 and counterstained by DAPI. FGF9 can induce proliferation of mesenchyme (m) and epithelium (e) both in control and mutant specimens as shown by the detection of BrdU positive cells on sectioned tissue. On the other hand, a lack of FGF10 is detected in the mesenchyme adjacent to developing epithelial tips of mutant lungs, which becomes locally enhanced in FGF9 treated control lungs. (B) Bar graphs depicting the percentage of BrdU incorporation per high power field, in the epithelium and the mesenchyme of control and mutant lung explants. Data derived from high power field measurements of 1716 ± 103 mesenchymal cells and 220 ± 33 epithelial cells from 4 control explants; and 1335 ± 83 mesenchymal cells and 257 ± 27 epithelial cells from 4 mutant explants. (*) denote statistical differences to untreated explants with $p<0.05$. (C) qRT-PCR detection of *Fgf10, Tbx4* and *Elavl1/HuR* mRNAs in extracts from control and mutant lung explants treated with FGF9 (200 ng/ml) for 6 h. *HuR mRNA* measurements in the mutants were omitted since they were below detection. Values ($\pm$SEM) presented as fold changes to the values of untreated control explants. Vertical p-values denote statistical differences to unstimulated control explants; horizontal p-values denote differences between stimulated control and mutant explants or between untreated and treated mutant explants. (D) Immunofluorescent detection of SMA (red) and HuR (green) on control (+/+) and HuR deficient (−/−) cultured LMCs, in the absence or presence of FGF9 for 48 h. (E) Bar graph showing the percentage of proliferating-Ki67 positive-LMCs in response to FGF9. Data collected from immunofluorescent stainings of cells with anti-Ki67 (see also Supplementary Fig. 6). *$p<0.05$ relative to the unstimulated conditions.

conditions and was not altered by the addition of FGF9. Thus, changes in the abundance of *Tbx4* mRNA in HuR^{-} cells correlate to its defective stabilization.

To assess whether HuR could affect the translation of its target mRNAs, we fractionated monosomes (i.e. containing non-translated mRNAs) and polysomes (i.e. containing mRNAs engaged in translation) from the cytoplasm of MEFs using sucrose gradients (Supplementary Fig. 9); extracted RNAs were subsequently analyzed via qRT-PCR for the detection mRNAs in each fraction (Fig. 7G and Supplementary Fig. 9). The monosomal/polysomal fractions and the distribution of the *GAPDH* mRNA in these fractions from control and mutant MEFs were comparable under all tested conditions, indicating



that the mutation did not affect the translational machinery. The polysomal distribution of the *Tbx4* mRNA in HuR$^+$ MEF was augmented by FGF9, but this response was also invariably observed in HuR$^-$ MEF; thus, HuR is not required for the translational control of this mRNA. In sharp contrast, and although the polysomal distribution of the *Fgf10* mRNA was comparable between control and mutant MEFs under basal conditions, a detectable increase in polysomal *Fgf10* mRNA that was induced by FGF9 in HuR$^+$ MEF was not observed in HuR$^-$MEF; thus HuR is required for the inducible translation of the *Fgf10* mRNA.

Taken together, our data demonstrate that HuR: (a) responds to signals targeting the lung mesenchyme at the levels of its biosynthesis and binding to its targets; (b) regulates *Fgf10* mRNA both directly – at the level of its inducible translation – and indirectly at the level of its abundance; (b) regulates the *Tbx4* mRNA at the level of its inducible stabilization. All these effects correlate with the critical role of HuR in lung branching morphogenesis.

Discussion

In this study we unraveled the role of post-transcriptional regulation by HuR in lung development. Our data demonstrate that HuR is a determinant of tissue remodeling interactions that govern branching morphogenesis. Although the embryonic ablation of HuR did not block the generation of primary branches, the majority of these branches failed to divide further and to form bronchioli and primitive alveoli. Several epithelial mutations are known to block lung branching morphogenesis, including mutations in molecules whose mRNAs are targeted by HuR-like members of the Wnt/β-catenin pathway (Briata et al., 2003;Katsanou et al., 2009;Leandersson et al., 2006). HuR has also been considered as a positive regulator of epithelial proliferation, differentiation and survival (Ghosh et al., 2009). However, the presence of differentiating epithelia in HuR-deficient lungs and their proper branching in mutants lacking epithelial HuR demonstrate that HuR is neither required for intrinsic programs guiding endodermal responses, nor for the expression of derived autocrine/paracrine signalers. Similarly, we have reported previously that the lack of HuR from developing endothelia does not hinder any aspects of embryonic development, including vascular morphogenesis of the lung (Katsanou et al., 2009 and data not shown). Thus, and by exclusion, we suggest herein that the arrested secondary branching of the HuR$^-$ endoderm stems from a defect on the surrounding mesenchyme. In the current study, we have not used transgenic systems to restrict the deletion of HuR in the developing lung mesenchyme, and thus we cannot exclude any additional defects than the ones reported herein; still the data derived from the examination of explant cultures, LMCs and MEFs support our hypothesis that a dominant mesenchymal defect in HuR-deficient lungs stems by the loss of HuR's control over the biosynthesis of Tbx4 and FGF10.

The key role of FGF10 in lung branching outgrowth and the effects of its binding to FGFR2 in the nearby epithelium are well documented. *Fgf10* mRNA is present in discreetly localized sites in the mesenchyme from the earliest stages of lung formation and subsequently undergoes a dynamic shift to sites of prospective bud or branch formation (Bellusci et al., 1997). As previously described and also demonstrated here, exogenous FGF10 can induce the expansion and budding of distal epithelial tips (Bellusci et al., 1997), and denuded epithelia proliferate and migrate toward localized sources of FGF10 (Weaver et al., 2000). The most convincing evidence for the key role of FGF10 is found in the block of epithelial branching by mesenchymal deletion of FGF10 or epithelial deletion of FGFR2 in the mouse (Abler et al., 2009). HuR deficient lungs phenocopy the FGF10-deficient state or states of FGF10 suppression that occur due to the epithelial overexpression of Sox2 and Bmp4 (Bellusci et al., 1996;Gontan et al., 2008). This conclusion is clearly supported by the rescue of HuR-deficient distal buds by the exogenous provision of FGF10. Our data also supports a positive relationship between Tbx4 and FGF10. Ectopic expression of Tbx4 induces lung bud outgrowth (Sakiyama et al., 2003) and activates FGF10 expression whereas blocking Tbx4 functions attenuates FGF10 synthesis and branching (Cebra-Thomas et al., 2003). Similarly, the positive effect of FGF9 on mesenchymal FGF10 expression has also been hypothesized to depend – at least in part – upon Tbx4 transcription (del Moral et al., 2006). As demonstrated herein, HuR appears to be in the epicenter of the Tbx4:FGF10 axis since it can target both of their corresponding mRNAs.

Is HuR required solely for the regulation of mesenchymal Tbx4 and FGF10? HuR could also be required for the proliferation and survival of mesenchymal cells, especially since the mutant mesenchyme showed symptoms of early degeneration. However several pieces of evidence argue against a cellular defect in mesenchymal responses as the cause of defective lung branching in HuR-deficient buds. Firstly, the foregut outgrowth and the initial branching of mutant lung buds occur normally. Secondly, HuR-deficient mesenchyme can respond to expand ex vivo in the presence of growth stimulating signals such as FGF9. Similarly, we did not observe any symptoms of defective proliferation of cell death in HuR$^-$ LMCs which also responded to FGF9 signals. Considering our expression data, the apoptotic damage in the lung mesenchyme could be a secondary effect due to the loss of mesenchymal:epithelial FGF cross talk and the expression of epithelial regulators counteracted by FGF10 signals such as Shh, Sox2 or Bmp4 (Bellusci et al., 1997;Cardoso and Lu, 2006;Gontan et al., 2008). This is also supported by the excessive number of cells acquiring a smooth muscle phenotype in the mutant lungs, a phenotype which is known to be enforced by excessive Bmp4 signaling (Jeffery et al., 2005). Thus, it is most likely that FGF10 deprivation is a key cause for the defective branching of HuR-deficient buds.

While we demonstrate a relationship between HuR and FGF10 expression, we cannot provide an answer to why other FGF10 dependant processes – like the primary outgrowth of the lung buds, the branching of the stem bronchi and some secondary branching in the upper lobes – do occur in HuR-deficient lungs. A possible explanation could be that lower FGF10 levels, or lower signal thresholds, are required for these primary events than the ones needed for secondary branching. This could also relate to the small size or the proximity of primary and septated lobes to the trachea and the requirement a more limited diffusion of FGFs as opposed to the

Fig. 7. HuR regulates the Tbx4 and FGF10 at multiple levels. (A) Bar graphs depicting the differential (fold change) response of *Fgf10, Tbx4 and Elavl1/HuR* mRNA in extracts from HuR$^+$ and HuR$^-$ LMCs and MEFs in the presence or absence (UNT) of FGF9. Data (arbitrary units ± SEM) derived from qRT-PCR experiments performed in RNAs from at least three cultures per genotype. (*) and p = values denote differences from untreated controls. (B) Detection of FGF10 and HuR proteins via immunoblots of total lysates from control and mutant MEFs under basal or FGF9-treated conditions. GAPDH is shown as a loading control. (C) Detection of Tbx4 protein via immunoblots of nuclear extracts from control and mutant MEFs under basal or FGF9-treated conditions. U1 snRNP is shown as a loading control. (D) qRT-PCR detection of *Fgf10* and *Tbx4* mRNAs immunoprecipitating with HuR in HuR$^+$ MEF extracts untreated (UNT) or treated FGF9. Data (biological triplicates ± SEM) are represented as fold enrichment of the mRNA in HuR IP samples compared to its abundance in IgG1 IPs (Vs IgG) or HuR IPs from HuR$^-$ MEF (Vs Ko). Enrichment levels were normalized to the levels of *Gapdh* mRNA. (*) p = values indicate statistical differences between stimulated and unstimulated conditions. (E) Representative anti-HuR immunoblot of the anti-HuR IP or mIgG1 IP material derived from HuR-proficient and deficient MEFs after treatment with FGF9. All lanes are from the same blot. The signals for HuR and IgG heavy chains are indicated. (F) Evaluation of *Fgf10* and *Tbx4* mRNA stability in actinomycin D-treated MEFs. Data are half-lives in minutes (± SD) derived from decay plots from three independent experiments. (*) denote statistical differences between treated and untreated conditions whereas (**) denote statistical differences between control and mutant values. (G) qRT-PCR detection of *Fgf10* mRNA in monosomal/polysomal fractions from untreated (UNT) or FGF9 treated MEFs. Data are derived from measurements in individual fractions and presented as total monosomal or polysomal percentages (± SD) of cytoplasmic mRNA. *p values denote statistical differences in polysomal fractions.

large area of the developing distal structures. Another possibility, could be that different spatial/feedback mechanisms – and hence HuR requirements – are needed to confine FGF10 expression domains to proximal than distal branches. Finally, we cannot exclude a partial replacement of FGF10 functions by other FGFs that could be affected positively by the loss of HuR and remain to be identified.

On a cellular level, our data clearly demonstrate that HuR responds to signals regulating mesenchymal:epithelial interactions – like FGF9 – to control the biosynthesis of Tbx4 and FGF10 in mesenchymal cells, albeit at different levels as proposed in our model in Fig. 8. In the current study, we have not examined whether the domains of *HuR* mRNA expression overlap with the expression domains of the *Tbx4* and *Fgf10* mRNAs during distal branching in vivo; still our studies on MEFs, MLCs and explants show that the biosynthesis of HuR responds to branching signals and correlates closely to the responses of its target mRNAs.

Our analyses demonstrated that HuR affects the accumulation of the *Fgf10* mRNA indirectly, since the turnover of this mRNA was not affected in HuR^{-} MEFs. Based on our previous observation on the reduced basal transcription of the *Fgf10* gene in these cells (Katsanou et al., 2009), we hypothesize that HuR controls the biosynthesis of transcription factors targeting the *Fgf10* gene. Although not directly addressed, we speculate that Tbx4 is one such transcription factor. Tbx4 and Tbx5 share expression domains with *Fgf10* and can synergize with the zinc-finger transcription factor Sall4 toward the activation of the *Fgf10* promoter in mesoderm-derived cells (Koshiba-Takeuchi et al., 2006). We have previously shown that the loss of HuR did not affect the basal transcription of the *Tbx4* gene whereas the re-introduction of HuR increased *Tbx4* mRNA accumulation in a fashion similar to the accumulation of the *Fgf10* mRNA. Although the loss of HuR may have also affected the inducible transcription of the *Tbx4* gene, the fact that HuR associates directly with the *Tbx4* mRNA and that its loss increases its degradation are in support for a direct role of HuR toward the *Tbx4* mRNA stabilization.

We also revealed that HuR can affect the inducible protein-synthesis of FGF10 by affecting *Fgf10* mRNA translation. Our data shows that FGF9 increases the translation of the *Fgf10* mRNA to FGF10 protein in mesenchymal cells and that this effect does not occur in the absence of HuR. However, the fact that HuR binding to the *Fgf10* mRNA – presumably via its 3′ AU-rich element (Katsanou et al., 2009) – remained unaltered in the presence of FGF9 is puzzling. One possibility could be that HuR cooperates or antagonizes other inducible co-factors to regulate the inducible translation of the *Fgf10* mRNA. For example,

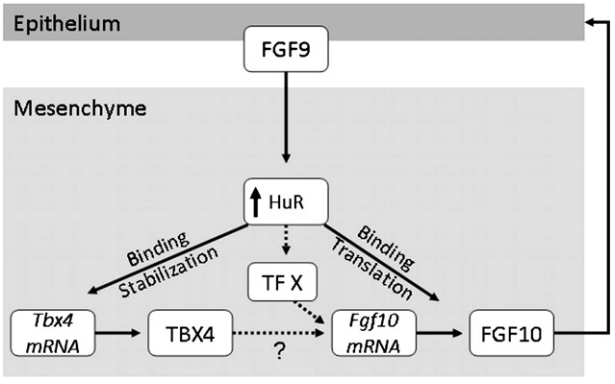


Fig. 8. Proposed model for the regulation of Tbx4 and FGF10 biosynthesis in mesenchymal cells during lung branching. Positive inducers derived from developing epithelia, such as FGF9, regulate HuR levels and its associations with the *Tbx4* and *Fgf10* mRNAs. Through these associations HuR regulates the Tbx4 protein by the inducible stabilization/destabilization of the *Tbx4* mRNA to act on its target promoters which may include the *Fgf10* promoter. This or additional transcription factors that require HuR for their expression (TFX) can facilitate the inducible transcription of the *Fgf10* gene. At the same time the association of HuR with the *Fgf10* mRNA controls the translational output of FGF10 protein which feeds back to act on the developing epithelium.

this effect could involve the interaction of HuR with micro RNAs (miRNAs) (Bhattacharyya and Filipowicz, 2007;Kim et al., 2009) that control translational regulation. As suggested by the analysis of *Dicer*-deficient mutants, mi- or si- RNAs plays a role in the regulation of *Fgf10* mRNA translation during lung branching (Harris et al., 2006), and considering our findings, it seems likely that biosynthesis of FGF10 is controlled by a combination of HuR and miRNAs. In addition, and as indicated for the *Fgf10* mRNA, HuR may affect multiple stages in the biosynthesis of its target mRNAs in the lung mesenchyme in an indirect fashion and in response to numerous signals that remain to be indentified. Future studies will reveal the actual domains required for the post-transcriptional regulation of the *Tbx4* and *Fgf10* mRNAs, the effect of HuR on associated transcription and maturation factors, its response to positive as well as negative regulators of branching and the involvement of small RNA populations in these processes.

In conclusion, the identification of HuR as a determinant of lung branching supports the central role of post-transcriptional control in morphogenetic programs. Most importantly, the requirement of HuR for lung branching highlight its role as an organizer of mesenchyme-driven epithelial remodeling and implicates it in tissue regeneration.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.04.003.

Disclosure statement

The authors declare that they have no actual, financial or potential conflicts of interest.

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