Lung development stages

The mammalian lung is a highly branched organ for oxygen exchange with carbon dioxide in the cardiovascular system. Multiple signaling pathways, including the Wnt, bone morphogenetic protein (Bmp), and fibroblast growth factor (Fgf) pathways, are implicated in regulating lung specification, branching and patterning (1).

Lung morphogenesis in mice can be divided into five stages: (I) embryonic stage [embryonic day (E) 9.0–12.5]—formation of the lung buds and major bronchi, division of tracheal-esophageal tube; (II) pseudoglandular stage (E12.5–16.5)—proliferation of bronchial branches, acinar tubules and buds, vasculogenesis and innervation; (III) canalicular stage (E16.5–17.5)—organization of the pulmonary vascular bed, pulmonary acinus, and increasing innervation; (IV) saccular stage [E17.5; postnatal (P) 5]—dilation of peripheral airspaces, differentiation of the respiratory epithelium and increasing vascularity of the saccules, surfactant synthesis; (V) alveolarization stage (P5–28)—growth and septation of the alveoli, maturation of the pulmonary vascular system (2).

Hippo pathway introduction

The Hippo pathway was first discovered in Drosophila functioning in tissue growth (3). In mammalian cells, the core Hippo pathway is composed of Mst1/2 kinases, which together with Sav1 (also called WW45) and Mob1A/B activate the Lats1/2 kinases to promote Yap and Taz phosphorylation (4). The phosphorylated Yap and Taz are restricted to locate in the cytoplasm and inactivated due to degradation. The hypo-phosphorylated Yap and Taz are active, which are translocated into the nucleus and bind TEAD transcription factor inducing expression of genes involving in cell proliferation, differentiation and death. All of these molecules have been implicated as tumor suppressors except Yap and Taz. The wide range of genes regulation by Yap/Taz makes the Hippo pathway a critical player in tissue development, regeneration and tumor development. However, whether the Hippo pathway plays a role in key steps of lung development and physiology are underexplored.

Homzygous-null mutant mice lacking Mst1/2, Sav1, Lats2, or Yap are all embryonic lethal (Table 1) (5-10). However, Taz-null mice showed partial lethality started at the perinatal stage (11). Only 20% of Taz-null mice survived at weaning. Taz-deficient homozygotes showed multiple renal cysts in the kidney and abnormal alveolarization during lung development mimicking emphysema. The early embryonic lethality of most Hippo components precluded study of these mediators in lung development. Conditional knockout and lung specific knockout of Hippo pathway components in mice would provide a more definitive answer to the role of Hippo signaling in the lung and elucidate the molecular mechanisms by which Hippo signaling controls various aspects of lung biology.

This editorial mainly focuses on the function of the Hippo pathway in lung development.

Mst1/2

The expression of Mst1/2 in developing lung has not been...
studied. Mst1/2-null mice lacking both Mst1 and Mst2 genes begin dying at E8.5 (5,6). Lung specific Mst1/2 knockout mice (Mst1^{f/f}; Mst2^{--}; Nkx2.1^{Cre}) die in the perinatal period within the first 15 days after birth and show phenotype mimicking RDS in preterm infants (12). There were no significant differences between the lungs of Mst1/2-dKO and wild-type mice until E17.5. However, at E18.5 and P1, alveoli of Mst1/2-dKO lungs displayed a greatly reduced aerated space compacted with immature AEC2 cells which fail to produce surfactant proteins normally. Chung et al. 2013 demonstrated that Mst1/2 regulated surfactant protein expression and maturation of AEC2 through Foxa2 by phosphorylation and stabilization of Foxa2 and independently of canonical Yap/Taz Hippo signaling pathway (12). The independence of Hippo/Yap was supported by decreased Yap activity in knockout lungs and knockdown Yap level in MLE-12 cells did not affect surfactant protein expression.

However, studies by Lange et al. 2015 utilizing conditional knockout mice in lung tissues found Mst1/2 regulated lung development through conserved Hippo/Yap signaling (13). Lange et al. 2015 constructed Mst1^{f/f}; Mst2^{f/f}; Shh-Cre mouse model (Table 2) to delete Mst1 and Mst2 from respiratory epithelial cell progenitors during lung

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Table 1 Phenotypes of homozygous-null Hippo pathway components mutant mice

<table>
<thead>
<tr>
<th>Gene</th>
<th>Phenotype</th>
<th>References</th>
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<tbody>
<tr>
<td>Mst1/2</td>
<td>Embryonic lethality at E8.5</td>
<td>(5,6)</td>
</tr>
<tr>
<td>Sav1 (WW45)</td>
<td>Embryonic lethality</td>
<td>(7)</td>
</tr>
<tr>
<td>Lats2</td>
<td>Embryonic lethality</td>
<td>(8)</td>
</tr>
<tr>
<td>Mob1a/1b</td>
<td>Embryonic lethality at E8</td>
<td>(9)</td>
</tr>
<tr>
<td>Yap1</td>
<td>Embryonic lethality at E9.5–10.5</td>
<td>(10)</td>
</tr>
<tr>
<td>Taz</td>
<td>Viable, but formed multiple cysts in the kidney and greatly enlarged air space in the lung (resemble human polycystic kidney diseases and pulmonary emphysema respectively)</td>
<td>(11)</td>
</tr>
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Table 2 Mouse model and phenotypes of lung-specific Hippo pathway components knockout mouse

<table>
<thead>
<tr>
<th>Mouse model</th>
<th>Phenotype</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>YAP^{f/f}; Shh^{Cre/+}(Yap^{cnull})</td>
<td>Lethality at birth, lungs from Yap^{cnull} were highly hypoplastic and showed sever disruption in branching morphogenesis at E14.5, resulting in dilated cyst-like structures</td>
<td>(14)</td>
</tr>
<tr>
<td>Mst1^{f/f}; Mst2^{f/f}; Shh-Cre</td>
<td>Lethality at birth, while lobation and lung size were generally unaffected, sacculation was inhibited and lung cellularity was increased in E18.5</td>
<td>(13)</td>
</tr>
<tr>
<td>Scgb1a1-rtTA/tetO-Cre/Mst1^{f/f}; Mst2^{f/f}</td>
<td>Deletion of Mst1/2 in the postnatal bronchial epithelium upon administration of doxycycline induced progressive airway hyperplasia</td>
<td>(13)</td>
</tr>
<tr>
<td>Mst1^{+/-}; Mst2^{f/f}; Nkx2.1^{Cre/+}</td>
<td>Viable and fertile without apparent gross abnormalities</td>
<td>(15)</td>
</tr>
<tr>
<td>Mst1^{+/-}; Mst2^{f/f}; Nkx2.1^{Cre/+}</td>
<td>Postnatal lethality, most died within 3 weeks after birth</td>
<td>(15)</td>
</tr>
<tr>
<td>Mst1^{+/-}; Mst2^{f/f}; Shh^{Cre/+}</td>
<td>Neonatal lethality, 95% died soon after birth because of respiratory failure</td>
<td>(15)</td>
</tr>
<tr>
<td>Mst1^{+/-}; Mst2^{f/f}; Shh^{Cre/+}</td>
<td>Embryonic lethality, causing cyclopia, holoprosencephaly and limb and lung defects due to disruption of the Shh locus</td>
<td>(15)</td>
</tr>
<tr>
<td>Sav^{f/f}; Nkx2.1-Cre</td>
<td>Viable and fertile without apparent gross abnormalities</td>
<td>(12)</td>
</tr>
<tr>
<td>Mst1^{f/f}; Mst2^{+/-}; Nkx2.1-Cre</td>
<td>Postnatal lethality, all mice died within the first 15 days after birth because of respiratory failure, reduced aerated space compacted with immature-looking cuboidal cells at E18.5 and PN1</td>
<td>(12)</td>
</tr>
<tr>
<td>SPC-rtTA/(tetO)-Cre/Mob1A^{f/f}; Mob1B^{--}</td>
<td>Neonatal lethality; ~73% died within 1h of birth. Deletion of Mob1A/1B in the bronchioalveolar epithelium upon administration of doxycycline in utero at E6.5–18.5. Mutant lung at E18.5/PO showed alveolar septal hyperplasia with reduced airspaces</td>
<td>(16)</td>
</tr>
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</table>
formation. The Mst1/2 ablation caused lung abnormalities and resulted in death at birth. The phenotype of Mst1\(^{+/+}\); Mst2\(^{+/+}\); Sbb-Cre mice and Mst1\(^{+/+}\); Mst2\(^{-/-}\); Nkx2.1-Cre are similar and mimicking RDS. The lung in mutant mice started to show apparent abnormalities until E18.5. Reduction of Surfactant proteins expression and mature AEC2 cells were also detected in lungs of Mst1\(^{+/+}\); Mst2\(^{+/+}\); Sbb-Cre mice. In contrast to previous study, Foxa2 expression was unchanged and Yap activity was increased in embryonic lungs of Mst1\(^{+/+}\); Mst2\(^{+/+}\); Sbb-Cre mice. Mst1/2 deletion also increased Ajuba expression in embryonic lungs. Ajuba is a potential regulator of lung epithelial cell proliferation and differentiation through Mst1/2-Yap signaling. Mst1/2-Yap-Ajuba axis was proposed for regulation of respiratory epithelial cell proliferation and differentiation.

Another study by Lin et al. 2015 utilizing a different Mst1/2 conditional knockout mice (Mst1\(^{-/-}\); Mst2\(^{+/+}\); Sbb\(^{Cre/+}\)) model (Table 2) also demonstrated Mst1/2 regulated lung growth through canonical Yap/Hippo signaling (15). Mst1\(^{-/-}\); Mst2\(^{+/+}\); Sbb\(^{Cre/+}\) mice died soon after birth. Lin also carefully compared lung development and phenotypes of different Mst1/2 deleting mice models (Mst1\(^{-/-}\); Mst2\(^{+/+}\); Nkx2.1\(^{Cre/+}\) and Mst1\(^{-/-}\); Mst2\(^{+/+}\); Nkx2.1\(^{Cre/+}\) and Mst1\(^{-/-}\); Mst2\(^{+/+}\); Sbb\(^{Cre/+}\)) which had different Cre-activity. Mst1/2 protein deletion is more compete in which Mst1/2 are removed by Sbb-Cre than those induce by Nkx2.1-Cre. Sbb-Cre seems to exhibit a high efficiency to remove Mst1/2 in the lung epithelium. A severe lung defect causing Mst1\(^{-/-}\); Mst2\(^{+/+}\); Sbb\(^{Cre/+}\) mice neonatal lethality in contrast to a milder lung defect causing Mst1\(^{-/-}\); Mst2\(^{+/+}\); Nkx2.1\(^{Cre/+}\) mice postnatal lethality. A relative elevation of Yap activity was found in Mst1\(^{-/-}\); Mst2\(^{+/+}\); Sbb\(^{Cre/+}\) embryonic lungs and regulation of surfactant proteins expression by Mst1/2 through Yap was confirmed in vitro. This study suggested a conserved Mst1/2-Yap signaling played a role in lung development.

**Mob1/a/b**

Mob1 expression was detected in all stages tested during E14.5 and E18.5 and started to increase at E16.5 (16). Mob1a/b-null mice lacking both Mob1a and Mob1b genes died at E8. Doxycycline (Dox)-inducible, bronchioalveolar epithelium-specific, null mutations of Mob1a/b in mice (SPC-rtTA/(tetO))-Cre/Mob1a\(^{f/f}\)/Mob1b\(^{-/-}\); termed luMob1DKO mice) (Table 2) was constructed to study Mob1 function in lung development (16). Doxycycline was administrated in utero at E6.4 to E18.5. Alveolar hyperplasia with reduced airspaces was found in luMob1DKO lungs at P0. Decreased surfactant protein expression, impaired AEC2 differentiation, unchanged Foxa2 expression and enhanced Yap/Taz activation in luMob1DKO mice lungs. In vitro study using C22 cells with Yap/Taz knockdown and overexpression technique found Yap/Taz formed a complex with Nkx2.1 instead of TEADs to repress expression of Col17a1 and reduction of expression of Col17a1 contributed to promotion of BASC cell detachment in adult lung. The lung histological alterations, decreased SPC and lethality of luMob1DKO (E6.5–18.5) mice were rescued in Mob1-deficient mutants when an additional Yap1 or Taz was mutated. Thus, the phenotypes of luMob1DKO (E6.5–18.5) lungs are strongly dependent on Yap/Taz.

**Taz**

Taz is expressed in respiratory epithelial cells of the developing lung in the mouse (17). Taz mRNA level gradually increased with advancing gestation during the lung development stages E11.5 to E18.0 tested. At E18.0, TAZ mRNA was detected primarily in the respiratory epithelium of peripheral lungs and was not detected in the conducting airways. Lung specification begins around E9.0 by detecting the earliest known marker of the lung epithelial lineage, Nkx2.1, which is a critical transcription factor controlling lung morphogenesis and differentiation of respiratory epithelial cells. Studies demonstrate that Taz physically interacts with Nkx2.1 and acts as a transcriptional co-activator with Nkx2.1 to induce the surfactant protein C (SP-C) transcription (17).

Taz knockout mice (Table 1) are viable but display abnormal alveolarization during lung development starting from P5 which causes airway enlargement, mimicking emphysema in human (11). However, the expression of Sftpe in mutant lungs is not significantly different from wild-type lungs. Instead, connective tissue growth factor (CTGF) was significantly reduced in Taz deficient lungs. The Taz-Nkx2.1-CTGF axis was proposed to be critical for lung alveolarization (18). The lung specific-Taz knockout mice are not been studied.

**Yap**

Yap mRNA expression is detected in mice embryonic lungs through developmental stages E10.5 to E18.5. The strongest distribution of Yap transcripts is in the epithelium of the developing airways and distal buds (14). Nuclear Yap
is co-localized with distal bud marker Sox9. Cytoplasmic Yap is co-expressed with airway marker Sox2. Nuclear Sox2 and cytoplasmic Yap co-expressed cells are committed to an airway fate during branching morphogenesis and later differentiate into specific cellular phenotypes of the conducting airways.

Unlike Taz, systematic Yap knockout led to mice lethality at E9.5 to E10.5 (10) and precluded analysis of phenotypes in the lung. To better study Yap function in lung development, Yap gene was conditionally knocked out from developing lung epithelium (YAP^f/f, Shh^Cre+ termed Yap^null) (Table 2) (14). The Yap^null mice were lethal at birth. Lungs from Yap^null at E12.5 were highly hypoplastic and showed severe disruption in branching morphogenesis, resulting in dilated cyst-like structures. Trachea and primary buds were grossly unaffected.

Yap nucleo-cytoplasmic compartment shift appears critical in regulating proximal-distal patterning of the lung through regulation of Sox2 expression in airway upon TGF-β-induced cues, and a decrease in YAP activity ensures epithelial cells differentiation. A model in which nuclear Yap-Tead complexes cooperate with TGF-β-induced signals to activate a transcriptional program that includes Sox2 to induce airway epithelial cell fate was proposed.

Conclusions

Homozogous deletion of Mst1/2, Sav1, Lats2, Mob1 or Yap in mice resulted in embryonic lethality. Lung-specific knockout Mst1/2 and Mob1 in mice caused lung development abnormalities starting at terminal saccular stage E17.5 to P5 including increased cell proliferation, impaired lung progenitor (AEC2) cell differentiation and reduced surfactant proteins production. The lung development failure causing reduced airspaces in mutant lungs led to mice neonatal death and the mutant mice showed a phenotype mimicking RDS in preterm infants. One study demonstrated Mst1/2 regulated lung development through Foxa2 independently of Yap/Taz signaling (12). However, the other studies indicated that Hippo components Mst1/2 and Mob1 regulated lung development through Hippo effector Yap/Taz, which means a canonical Hippo-Yap signaling pathway was involved (13,15,16). Whether Foxa2 mediates some aspects of Mst1/2 function in the lung is unclear. The discrepancy of Mst1/2 studies of regulatory relationship between Mst1/2 and Yap in the lung may be due to variable efficiencies of the mouse Cre lines to remove Mst1/2 in different mouse models (15).

However, in vitro studies in the same cell line MLE-12 still showed complete different results. Chung 2013 found that depletion of Mst1/2 in MLE-12 cells decreased the level of YAP/TAZ or CTGF. In contrast, Lin 2015 found that depletion of Mst1/2 in MLE-12 increased the level of active YAP and CTGF. It is unclear how this discrepancy has arisen and a repeat study needs to be done.

In canonical Hippo pathway, Mst1/2 interact with Sav1, and Last1/2 bind Mob1a/b. Mst1-Sav1 phosphorylates and activates the Lats1/2-Mob1a/b complex, which in turn phosphorylates Yap and Taz to promote their cytoplasmic localization and targeting for degradation. Thus, Mst1/2 and Mob1a/b may regulate YAP/TAZ in lung development through LATS1/2 (3,4). However, a significant change of Lats activity in Mst1/2 or Mob1 lung specific knockout mice was not found. It is possible that minor changes of LATS activity caused by loss of MST1/2 or MOB1 were masked since LATS from the whole lung instead of purified lung epithelium was analyzed. Lung-specific knockout Lats mice in conjunction with double mutant (Mst1/2 or Mob1 mutant) analysis would shed light on the relationship between Mst1/2, Lats and Yap in the lung. These genetic studies will complement cell-based assays in delineating the interactions of Hippo pathway components in the respiratory system. Interestingly, even though complete deletion of Sav1 in mice leads to embryonic lethality, lung-specific Sav1 knockout mice are viable and fertile without apparent gross abnormalities (12).

In most tissues including lung, Yap plays a more role than Taz for organ development. Yap knockout mice ended up with early embryonic lethality (10). In contrast, one-fifth of Taz knockout mice grow to adulthood (11). Thus, while Taz shares functional redundancy with Yap, Yap is the major player in mediating Hippo signaling in most tissues. Lung-specific Yap knockout mice showed lung abnormalities at pseudoglandular stage starting at E12.0 which is earlier than E18.5 when lung abnormalities became obvious in Mst1/2 and Mob1a/1b lung mutant mice. Yap is required for proximal-distal patterning of the lung through regulation of Sox2 expression in airway. However, in Mst1/2-deleted lungs, Sox2 staining was normally restricted to conducting airway epithelial cells, indicating that proximal/distal patterning of the developing lung epithelium was generally maintained when Mst1/2 was deleted from embryonic lung. The reason for the phenotype differences between Yap mutant lungs and Mst1/2 or Mob1a/1b lungs is unclear.

Respiratory diseases are a major cause of mortality.
and morbidity worldwide. Characterization of the molecular pathways for lung development is important for understanding lung repair, regeneration and tumorigenesis and has an enormous potential impact on prevention and treatment of lung diseases. Even though, the function and interaction of individual Hippo pathway components in the lung development has been studied, the full spectrum of Hippo signaling effects on lung biology and pathology yet to be revealed and also the upstream regulators and downstream effectors of Hippo signaling in the lung are also largely unknown. Further elucidation of Hippo pathway upstream signals, transcriptional targets and crosstalk with other pathways will advance the understanding of lung progenitor cell behavior and improve targeted therapeutic strategies for lung diseases and cancer.

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Footnote
Conflicts of Interest: The authors have no conflicts of interest to declare.

References