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Roles of the Hippo pathway in lung development and tumorigenesis

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Lung cancer is the most commonly diagnosed cancer and accounts for one fifth of all cancer deaths worldwide. Although significant progress has been made toward our understanding of the causes of lung cancer, the 5-year survival is still lower than 15%. Therefore, there is an urgent need for novel lung cancer biomarkers and drug targets. The Hippo signaling pathway is an emerging signaling pathway that regulates various biological processes. Recently, increasing evidence suggests that the Hippo pathway may play important roles in not only lung development but also lung tumorigenesis. In this review article, we will summarize the most recent advances and predict future directions on this new cancer research field.

Mini Review

According to the World Health Organization (2014), lung cancer is the most common cancer worldwide, killing 1.6 million people annually. Surprisingly, despite the advancement of surgical, radio- and chemotherapies over the years, the 5-year survival rate of lung cancer remains <15%.[1] While many tumor suppressor genes (*p53*, *Rb*, *etc.*), oncogenes (*K-ras*, *EGFR*, *HER2*, *etc.*) or signaling pathways (MAPK, PI3K, *etc.*) have been identified to be important for the development and progression of lung cancer, only a few genes (*e.g.*, *EGFR* and *HER2*) have successfully been targeted for therapy of lung cancer.[2,3] Therefore, there is an urgent need to identify new therapeutic targets and drugs in order to successfully treat lung cancer. Recently, a novel signaling pathway called the Hippo pathway, which plays important roles in organ size control, tumorigenesis and stem cell renewal, [4–15] has been shown to play important roles in both lung development and tumorigenesis. Therefore, we will summarize these new findings in this review.

The Hippo Pathway

The Hippo pathway, which was originally identified in *Drosophila* from genetic screen, is an emerging signaling pathway that plays important roles in regulating cell proliferation, apoptosis, tumorigenesis, organ size, mechanotransduction, drug resistance, stem cell renewal and differentiation in mammals.[5,7,16–25] The core components of this pathway are composed of two serine/threonine (S/T) kinases *Mst1* and its homolog *Mst2* (mammalian homolog of *Drosophila Hippo*) and *LATS1* and its homolog *LATS2*, two adaptor/scaffold proteins *hMOB1* and *WW45* and two WW domain-containing transcriptional co-activators *TAZ* and its paralog *YAP* (Fig. 1). Upon activation by upstream stimuli such as increased cell–cell contact, the Mst1/2 kinases are activated which subsequently cause phosphorylation of hMOB1, WW45 and LATS1/2, resulting in full activation of LATS1/2 kinases (Fig. 1). Activated LATS1/2 will subsequently phosphorylate and inactivate WW-domain containing transcriptional co-activators TAZ and YAP by promoting either their cytoplasmic retention or degradation, preventing them from transactivating downstream genes (*CTGF*, *Cy61*, *BMP4, etc.*) in the nucleus through transcription factor TEAD or Smad (Fig. 1; please note that AMOT can both increase and decrease Hippo signaling).[26–33] Inactivation/activation of the Hippo pathway by cell–cell contact or certain stimuli such as changes of extracellular matrix (ECM) stiffness or stimulation of serum factors [lysophosphatidic acid (LPA) and sphingosine 1-phosphoreceptor (S1P)] will cause activation or inactivation of LATS1/2 or TAZ/YAP and its downstream genes in Hippo (Mst)-dependent or Hippo (Mst)-independent manners, leading to increased cell proliferation, survival and tumorigenesis (Fig. 1).[10,34,35]

Hippo Pathway in Lung Development

The first evidence for the importance of the Hippo signaling pathway in lung development came from analysis of *Taz* knockout mice, which in addition to forming renal cysts, exhibited defects in alveolarization.[36,37] In the absence of TAZ, adult mice had severely enlarged air spaces in the alveolar, which resembles human pulmonary emphysema, a disease whose pathogenesis remains poorly understood.[36,37] While a previous study found TAZ to interact and activate TTF1/Nkx2.1, a transcription factor that plays a critical role

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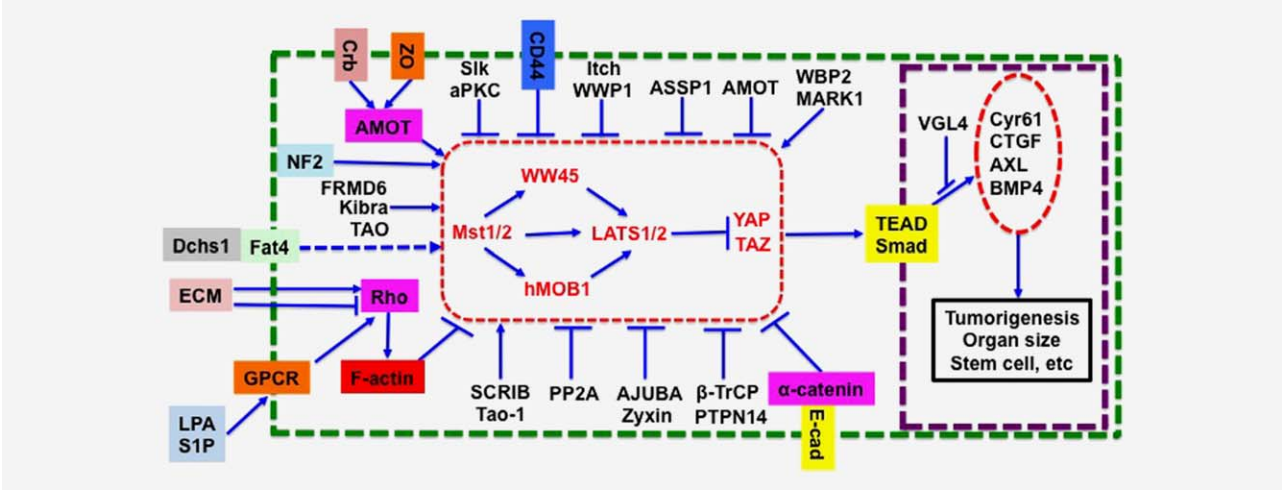

Figure 1. The mammalian Hippo signaling pathway.

in branching morphogenesis, expression levels of TTF1 did not differ in *Taz*-knockout lungs.[37] Interestingly, *Taz*-heterozygous mice were found to be resistant to bleomycin-induced lung fibrosis, a major side effect in bleomycin-treated patients for Hodgkin's lymphoma and germ cell cancers.[37]

Since the initial studies of *TAZ*, other labs have explored the pleotropic roles of other Hippo pathway components in lung development. Recent reports of *YAP*, a *TAZ* paralog, show that it plays crucial roles in regulating lung embryonic and adult stem cell differentiation.[38–40] YAP was found to mark the boundary between progenitors that eventually generated airways (YAP phosphorylated and localized in the cytoplasm) and alveoli (nuclear YAP). It enables initiation of the progenitor program required for branching morphogenesis by controlling Sox2 *mRNA* expression and sensitizing cells to TGF-β signaling.[39] Without YAP, adult basal stem cells were greatly reduced due to uncontrolled differentiation.[38] In contrast, overexpression of YAP in the same cells promotes stem cell proliferation, resulting in epithelial hyperplasia and stratification.[38] Loss of another two components of the Hippo pathway, *Dchs1* or *Fat4,* in mice caused a reduction in lung size.[41] This was not simply due overall decreased body size since other organs were not significantly smaller in *Dchs1* and *Fat4* mutants. Moreover, conditional knockout of *Mst1/2* from the respiratory epithelium impaired peripheral lung differentiation and maturation, reduced surfactant proteins, and ultimately causes perinatal lethality.[40,42] Whether these phenotypes occur *via* the canonical Hippo pathway is unclear as one group reported decreased YAP/TAZ levels upon loss of Mst1/2. Rather, these changes were attributed to Foxa2 transcription factor dysregulation.[42] It is possible these discrepancies may be due to varying promoter-driven Cre as *Sonic hedgehog* (*Shh*) and *TTF1* have differing roles in distal lung fate. Regardless, downstream YAP transcriptional targets *Ctgf* and *Birc5/survivin* were increased following Mst1/2 deletion, supporting the involvement of Hippo signaling to some degree.[40] Taken together, it is clear that the Hippo pathway is intimately involved in numerous processes of lung morphogenesis. Further study is needed to elucidate the potential roles of other Hippo components in both developing and mature lung.

Hippo Pathway in Lung Tumorigenesis

There are two main types of lung cancer: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC).[43,44] The Hippo pathway has been reported to be involved in the development of NSCLC (see below for discussion), which accounts for over 80% of lung cancers.

Core components

LATS1/2. Yang *et al.* provided primary biological evidence that the core component of the Hippo pathway, LATS1, is a tumor suppressor in lung cancer. We showed that overexpression of *LATS1* suppresses NSCLC cell proliferation, anchorage-independent growth, and tumor formation in nude mice.[45] Later study also showed that knockdown of LATS1 by small interference RNA (siRNA) in NSCLC cells increases both cell proliferation and cell migration.[46] Moreover, LATS1 homolog LATS2 is down-regulated in NSCLC cell lines and overexpression of LATS2 also suppresses lung cancer cell growth and migration, whereas siRNA-mediated suppression of LATS2 expression resulted in increased cell proliferation due to augmentation of extracellular signal-regulated kinases (ERK) phosphorylation in epithelial growth factor receptor (EGFR) wild-type rather than EGFR mutant lung adenocarcinoma cell lines.[47] Clinically, *LATS1* is down-regulated in 60% of NSCLC cancer and its levels are significantly correlated with pathological tumor-node-metastasis (p-TNM), lymph node metastasis and patient survival.[46] On the other hand, *LATS2* mRNA levels were found to be a significant independent predictor for survival status.[47] *LATS2* is down-regulated in all NSCLC and mutated in less than 10% of NSCLC.[48,49] Interestingly, tumors with *LATS2* mutation often harbor a *p53* but not *K-ras* gene mutation and were

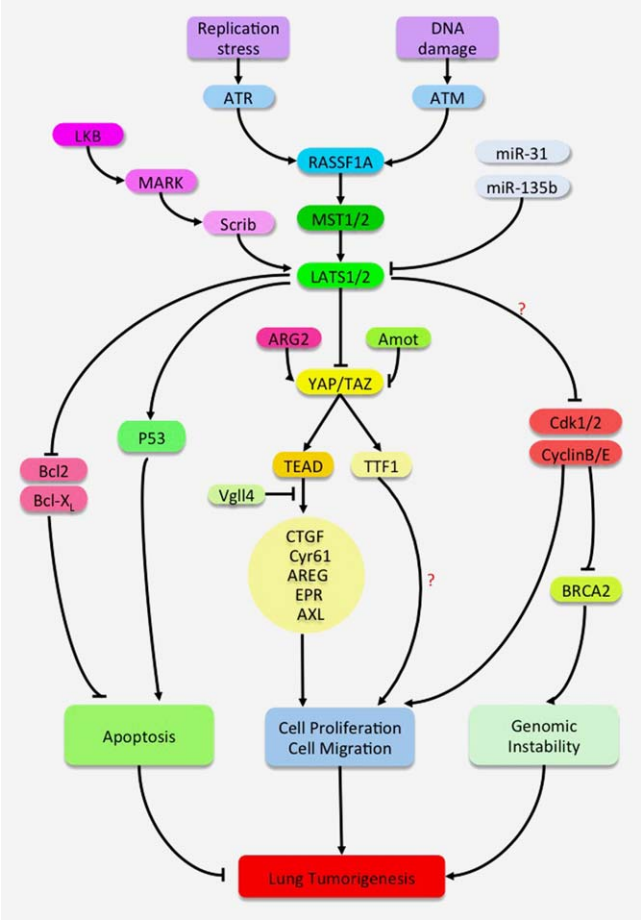

Figure 2. A model for roles of the Hippo pathway in lung tumorigenesis.

mostly in an advanced stage of development, with regional lymph node involvement.

Mst1 and hMOB1. Beside LATS1/2, Mst1 has also been shown as a tumor suppressor in lung cancer and overexpression of Mst1 inhibits cell proliferation and survival in NSCLC cells by inhibiting YAP,[50] suggesting that Mst1 may suppress lung cancer cell growth through the Hippo signaling pathway (Figs. 1 and 2). In addition, another Hippo core component *hMOB1* mRNA was significantly decreased in 63% NSCLC patients.[51] However, whether this down-regulation of hMOB1 contributes to lung tumorigenesis is unknown.

TAZ and YAP. Our recent studies provide the first evidence that *TAZ* is an oncogene in lung cancer. We have shown that *TAZ* is overexpressed in 70% of NSCLC cell lines examined and overexpression of *TAZ* causes transformation of non-tumorigenic lung epithelial cells, whereas knockdown of TAZ by short hairpin RNA (shRNA) inhibits NSCLC cell growth, anchorage-independent growth *in vitro* and tumor formation in *vivo*.[52] Similar results were obtained for TAZ paralog YAP.[53] Significantly, constitutively activated YAP, YAP-S127A (A, alanine), which lacks phosphorylation and inhibition by LATS1/2, was sufficient to drive lung tumor progression in mice *in vivo*.[54] Moreover, YAP and TAZ are also identified as driver genes for lung cancer metastasis.[54] It was shown that TAZ and YAP are enriched in metastatic tumor cells and knockdown of YAP or TAZ decreased cell migration *in vitro* and tumor metastasis *in vivo*. In conclusion, these *in vitro* cell line and *in vivo* mice studies strongly suggest that activation of *TAZ* and *YAP* oncogenes is important for lung tumorigenesis and metastasis.

Most significantly, clinical lung cancer patient studies also confirm *TAZ* and *YAP* as oncogenes in lung cancer. It has been reported that TAZ is overexpressed in over 60% of NSCLC and its expression is significantly associated with adenocarcinoma (ADC), poor differentiation, metastasis and poor prognosis and survival.[55,56] In addition, YAP is found to be a direct oncogenic target of the chromosome 11q22 amplicon and YAP amplification is detected in 23% of human cancers including lung cancer.[57] Moreover, YAP is overexpressed in 60–70% of NSCLC patients and significantly correlated with p-TNM stage, lymph node metastasis, poor prognosis and shorter survival.[53,58,59] Interestingly, YAP levels were significantly higher in ADC than in squamous cell carcinoma (SCC) patients, supporting the experimental data that YAP is activated by LKB1 in lung ADC and YAP inhibits squamous transdifferentiation (Fig. 2).[60]

Together, these studies strongly suggest that almost all of the core components of the Hippo pathway including LATS1/2, hMOB1, TAZ and YAP function as tumor suppressors or oncogenes and play important roles in lung tumorigenesis.

Downstream signaling

LATS1/2 downstream targets. Conflicting results were reported regarding downstream targets mediating *LATS1/2*-induced suppression of lung cancer cell proliferation. LATS1/LATS2 were shown to suppress lung cancer cell growth through inhibition of cell-cycle regulators Cdk1/Cyclin B and Cdk2/Cyclin E complex, respectively (Fig. 2).[45,61] In contrast, while LATS2 was also shown to negatively inhibit cell proliferation through suppression of NF-κB signaling,[62] overexpression of LATS1 inhibits NSCLC cell growth by negatively regulating YAP (Fig. 2).[46] Therefore, it is still not completely understood how LATS1/2 inhibit cell proliferation in lung cancer cells. In addition, LATS1/2 induce lung cancer cell apoptosis by either up-regulation of tumor suppressor and pro-apoptotic gene *p53* or down-regulation of anti-apoptotic protein Bcl2 and Bcl-X_L, respectively (Fig. 2).[45,63] However, the mechanism of how LATS1/2 regulate *p53*, Bcl2 and Bcl-X_L is still unknown. Moreover, it has recently been shown that LATS1 suppresses genomic instability by suppressing Cdk2-induced inhibition of BRCA2 (Fig. 2).[64]

TAZ/YAP downstream targets. *Thyroid transcription factor 1/NKX2-1.* Thyroid transcription factor 1 (TTF1), also known as NKX2-1, is a homeobox-containing transcriptional

factor import for the development of lung and thyroid.[65] Similar to *TAZ* and *YAP*, *TTF1* also functions as an oncogene and is overexpressed in lung ADC.[66,67] Significantly, TAZ was shown to directly bind to TTF-1 and trans-activates TTF-1's ability to activate downstream targets such as surfactant protein C (*SP-C*) in lung epithelial cells (Fig. 2).[68] These studies strongly suggest that TAZ/YAP may interact with transcription factor TTF1 in transcriptionally regulating downstream targets during lung tumorigenesis.

Transcriptional targets. Several TAZ/YAP transcriptional targets including *AXL, Cyr61, amphiregulin (AREG)* and *epiregulin (EPR)* have been identified to be involved in TAZ/YAP-induced lung tumorigenesis and metastasis (Fig. 2).[56,69–71] However, no single downstream gene seems fully responsible for TAZ/YAP-induced tumorigenic phenotypes. In addition, TAZ/YAP may activate different downstream targets in different cell types.[56,69–72]

Upstream regulators

LKB1. LKB1 is a S/T kinase and tumor suppressor that is frequently mutated and inactivated in NSCLC.[73,74] Loss of LKB1 was shown to promote lung cancer progression and metastasis by regulating energy metabolism, cell polarity and cell growth via AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) signaling.[74] Recently, LKB1 was shown to be a novel upstream regulator of the core Hippo component YAP.[75] It has been shown that LKB1 suppresses lung cancer cell growth by activating the core Hippo pathway via MARK-Scrib signaling, which results in inactivation of YAP oncoprotein (Fig. 2).[75] Most significantly, knockdown of YAP by RNAi inhibits loss-of-LKB1-induced tumorigenesis.[75] Later studies showed that YAP is specifically activated by LKB1 loss to induce lung ADC and inhibits SCC.[60] Since *LKB1* is frequently inactivated in lung cancer,[73] identification of YAP as a critical mediator of LKB1 may provide a novel strategy to treat *LKB1*-associated lung cancers by targeting YAP.

RASSF1A (Ras-associated domain family 1A). RASSF1A is a member of RASSF family scaffold proteins that function as tumor suppressors by regulating cell cycle progression and apoptosis.[76–78] *RASSF1A* is frequently inactivated in a variety of human cancers by promoter hypermethylation and subsequent down-regulation of transcription. Epigenetic inactivation and reduced expression of *RASSF1A* are detected in 30–50% of NSCLC.[79–81] *RASSF1A* methylation is also an independent prognostic factor for poor survival in surgically treated NSCLC.[81] Recently, RASSF1A has been identified as a critical upstream regulator of the core Hippo pathway.[78] It has been shown that RASSF1A can be activated by Ataxia telangiectasia mutated upon DNA damage and subsequently induces tumor cell death by activating the mammalian Hippo homolog Mst2 (Fig. 2).[82,83] Significantly, under DNA replication stress, RASSF1A can be activated by ATR kinase, which can activate LATS1-Cdk2-BRCA2 signaling to maintain genomic stability. It has been shown that perturbation of the ATR-RASSF1A-Mst2-LATS1 signaling axis leads to genomic defects that contribute to genomic instability and tumorigenesis in lung cancer cells (Fig. 2).[64] These studies strongly suggested that regulation of the Hippo pathway by RASSF1A may be important in regulating apoptosis and genomic instability during lung tumorigenesis.

microRNAs. microRNAs (miRNAs) are small (19–22 nucleotides) non-coding RNAs that bind to the 3′ untranslated region (3′-UTR) of mRNAs, resulting in reduced protein expression of target genes. Previous studies suggest that miRNAs can function as either oncogenes or tumor suppressor genes and are involved in lung tumorigenesis and metastasis.[84] In a screen for miRNAs that are overexpressed in lung cancers, miR-31 was identified as an oncogene in lung cancer. It has been shown that miR-31 is overexpressed in lung cancer and knockdown of miR-31 repressed lung cancer cell growth and tumorigenicity.[85] Further studies showed that miR-31 caused lung tumorigenesis by inhibiting translation of Hippo component *LATS2* (Fig. 2). Significantly, enhanced levels of miR-31 in lung cancer are correlated with reduced expression of LATS2 in mouse and human lung cancer tissues. In another screen for miRNAs important for lung metastasis, miR-135b was found activated in highly invasive NSCLC and promotes lung tumor cell migration and metastasis. Further studies showed that miR-135b suppresses Hippo core components LATS2 and hMOB1 but enhances TAZ (Fig. 2).[86] Knockdown of TAZ in miR-135b-overexpressing NSCLC cells dramatically reduced cancer cell invasive and colony-forming abilities. Examination of NSCLC tumor samples further shows that increased levels of miR-135b are correlated with reduced levels of LATS2, nuclear TAZ levels and poor overall survival in patients.[86] Together, these studies indicate that up-regulation of miR-31 and miR-135b promotes lung tumorigenesis and metastasis by regulating the Hippo signaling pathway.

YAP regulators. *VGLL4.* Recently, VGLL4 has been identified as a novel negative regulator of YAP-TEAD transcriptional complex (Fig. 2). It has been shown that *VGLL4* is frequently down-regulated in lung cancer and ectopic expression of VGLL4 significantly suppresses lung cancer growth *in vitro* and *in vivo* by inhibiting YAP-induced activation of TEAD.[87]

Others. AMOT (angiomotin) was originally identified as an angiostatin-binding protein that enhanced endothelial cell motility by reducing tight junctions and altering actin dynamics.[88] Recently, AMOT was identified as a major regulator of YAP (Fig. 2). It was shown that AMOT was significantly down-regulated in clinical lung cancer specimen and overexpression of AMOT suppresses lung cancer progression by sequestering YAP/TAZ in the cytoplasm.[70] On the other hand, it has been shown that overexpression of ADC-specific oncogene AGR2 induces lung ADC by activating YAP.[71]

Conclusion and Future Directions

Based on the previous studies described above, we have proposed a model describing the roles of the Hippo pathway in

lung tumorigenesis (Fig. 2). Since many components of the Hippo pathway (Fig. 1) have not been studied in lung cancer, it will be more interesting to test whether these Hippo components are also involved in lung development and tumorigenesis. In addition, we and others have shown that the Hippo pathway is involved in many aspects of cancer progression and therapy such as drug resistance.[18,20,89–92] Therefore, a new direction to explore is how the Hippo pathway is involved in the response of lung cancers to current chemotherapy (*e.g.*, Taxol, cisplatin, *etc*) or target therapy [*e.g.*, EGFR inhibitors (erlotinib, gefitinib, *etc*)] and whether the Hippo components can be used as prognostic or diagnostic biomarkers for drug resistance in lung cancer therapy. In addition, since the Hippo pathway has been shown to interact with many other pathways such as the Wnt pathway, which is critical for lung homeostasis,[93] it will be very interesting to further explore how the Hippo interacts with these known Hippo-interacting signaling pathways or identify novel signaling pathways interacting with the Hippo signaling in regulating lung development, tumorigenesis and metastasis. Moreover, since the Hippo pathway plays important roles in the formation of cancer stem cells such as breast cancer stem cells[16,90,94,95] and lung cancer stem cells play critical roles in lung tumorigenesis and therapy,[96–98] it will be very important to examine whether and how the Hippo pathway is involved in lung cancer stem cell development. Most recently, a transcriptome meta-analysis of lung cancer reveals that high numbers of Hippo pathway component (*NF2*, *LATS1*, *PTPN14*, *YAP1*, *TAZ*, *TAOK* and *FAT1*) gene fusions were observed in lung cancer and are independent prognostic factors for poor survival in lung cancer.[99] It will be very interesting to study how these Hippo gene fusions contribute to lung tumorigenesis. Finally, since there is no Hippo-targeted therapeutic drug for lung cancer, development of small molecule or antibody drugs targeting the Hippo components such as YAP and TAZ will provide new therapeutic strategies for future successful treatment of lung cancer.

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