THE NAGAI FOUNDATION TOKYO
THE NAGAI AWARD THAILAND FOR
PHARMACEUTICAL SCIENCE RESEARCH 2015

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SLUG is Required for SOX9 Stabilization and Functions to Promote Cancer Stem Cells and Metastasis in Human Lung Carcinoma

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Lung cancer is a leading cause of cancer death worldwide, attributable to the late-stage diagnosis with local or advanced metastasis at distant organs [1,2]. The presence of cancer stem cells (CSCs), a subpopulation of tumor initiating cells, in primary tumors is strongly correlated with an increased incidence of metastasis and poor survival of patients [3−5], suggesting them to be a prime target for cancer therapy. The present study aimed to investigate the regulatory mechanisms of CSCs that determine their metastatic behavior. CSCs were isolated based on their side population phenotype from various human non-small cell lung cancer (NSCLC) cell lines, including H460 and A549 cells, and verified for their (i) self-renewal capacity using tumor sphere assay, (ii) aggressive cancer phenotypes that are a prerequisite for tumor metastasis, including cell invasion and chemoresistance, and (iii) tumor formation *in vivo* in comparison their non-CSC counterpart. To identify the molecular signatures of CSCs, we first focused on epithelial-mesenchymal transition (EMT), a trans-differentiation process by which cells undergo a morphological change into a more mesenchymal phenotype that commonly occurs in metastatic tumors [6,7]. Protein profiling revealed that CSCs highly expressed the EMT transcription factor SLUG as compared to non-CSCs. However, we further observed that SLUG is not required for the EMT activation of NSCLC cells, pointing out that pathways other than EMT may contribute to their aggressive phenotypes. As CSCs and normal stem cells share many common characteristics, we hypothesized that SOX9, a major player in normal lung development [8] and a cooperating factor of SLUG in mammary stem cells [9], may be involved in lung CSC regulation. Expression analysis of SLUG and SOX9 in human clinical specimens demonstrated a high level of both proteins in tumor tissues from advanced stage (stage III) lung cancer in comparison to their matched normal lung tissues. Likewise, an elevated expression of SLUG and SOX9 was observed in lung CSCs as compared to non-CSCs. To our knowledge, this is the first demonstration of SLUG and SOX9 co-upregulation in advanced stage human lung cancer and CSCs. To study the impact of such upregulation, we used gene manipulation strategies to selectively knockdown and overexpress SLUG and SOX9 in the tested lung cells using short hairpin RNA interference and ectopic gene expression. We found that both SLUG

and SOX9 are essential to the development of lung CSCs and experimental lung metastasis in a xenograft mouse model. Mechanistic studies further revealed that knockdown of SLUG resulted in a parallel decrease in SOX9 expression in lung cancer cells as compared to control cells. In contrast, knockdown of SOX9 had minimal effect on SLUG expression, suggesting that SLUG is an upstream regulator of SOX9. Immunofluorescence and in-cell co-immunoprecipitation studies further showed that SLUG directly binds to SOX9, which subsequently interferes with its stability. Mechanistically, SOX9 expression is regulated primarily through ubiquitin-mediated proteasomal degradation, and SLUG binding inhibits this process. The half-life of SOX9 protein is significantly shortened in the absence of SLUG and prolonged in its presence. Functionally, SLUG is critical for SOX9-mediated lung CSCs and metastasis *in vivo*, as demonstrated by the inability of SOX9 to promote lung CSCs and experimental metastasis in the absence of SLUG. It is likely that the high-level co-expression of SLUG and SOX9 in clinical samples of aggressive lung carcinoma is a result of their binding interaction, although other mechanisms may be involved. Together, our study demonstrated a novel mechanism of CSC regulation and metastasis via the SLUG-SOX9 regulatory axis, which represents a promising new target for CSC therapy that could overcome cancer chemoresistance, relapse, and metastasis.

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