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審査研究科	人間総合科学研究科
学位論文題目	Role of THG-1-mediated regulation of HIF-1α stability in squamous cell carcinoma （THG-1 による HIF-1α 安定性制御の扁平上皮癌における役割）
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論文の内容の要旨
Abstract of thesis

（目的　Purpose）

Squamous cell carcinoma (SCC) is an epithelial malignancy and shows a poor prognosis despite the advances of treatment. Previous results demonstrated that THG-1 (also called TSC22D4) is diffusely and highly expressed in SCCs. Knockdown of THG-1 in esophageal SCC cell line TE13 cells reduces their cell proliferation, invasion, and tumor formation compared with parental TE13 cells. However, the molecular mechanism of THG-1 functions in tumors remains to be determined. To investigate the molecular functions of THG-1, the applicant has analyzed the interacting proteins of THG-1 by proteomics approach and identified prolyl hydroxylase domain-containing protein 2 (PHD2). PHD2 is a negative regulator of hypoxia-inducible factor-1α (HIF-1α). The applicant has investigated the roles of the THG-1-PHD2 interaction in HIF-1α regulation and tumorigenesis.

（対象と方法　Materials and Methods）

1) The interaction between THG-1 and PHD2 was examined by immunoprecipitation in HEK293T and TE13 cells.
2) The localization of THG-1 and PHD2 was examined by immunofluorescence staining in HaCaT cells.
3) HIF-1α stabilization in the presence of THG-1 and PHD2 was examined by western blotting in HEK293T cells.
4) HIF-1α stability in TE13 cells was examined by western blotting in 2D and 3D culture conditions.
5) Expressions of the HIF-1α target genes were examined by quantitative RT-PCR in 2D and 3D culture

conditions.

6) HIF-1α expression in tissues was detected by immunohistochemical staining.

（結果 Results）

1) THG-1 physically interacts with PHD2

To determine the molecular functions of THG-1, the applicant attempted to identify the component(s) that interact with THG-1. Subsequent liquid chromatography-tandem mass spectrometry analyses identified PHD2 as a THG-1-binding factor. The applicant confirmed the interaction between THG-1 and PHD2 in HEK293T cells by immunoprecipitation. THG-1 interacted with PHD2 and this interaction was increased by co-transfection with a constitutively active form of HRas (G12V). The applicant also examined the subcellular localization of THG-1 and PHD2 by immunofluorescence. THG-1 and PHD2 were colocalized in the cytosol. The applicant also examined the endogenous THG-1 and PHD2 interaction in TE13 cells which have endogenous THG-1 expression. THG-1 interacts with PHD2 in the presence of EGF.

2) THG-1 regulates HIF-1α by binding with PHD2

The applicant identified the domains required for interacting PHD2 in THG-1. The N-terminal 111 amino acids of THG-1 were required for interaction with PHD2. To determine the effect of the THG-1 and PHD2 interaction on the PHD2-mediated degradation of HIF-1α, the applicant examined the stability of HIF-1α in the presence of PHD2, THG-1 (WT and ΔN0 lacking N-terminal 111 amino acids), and constitutively active form of HRas. HIF-1α expression was reduced by co-transfection with PHD2 WT, but not by PHD2 R383A which cannot induce prolyl hydroxylation of HIF-1α, suggesting that the reduction of HIF-1α by PHD2 requires the enzymatic activity of PHD2. HIF-1α expression was recovered by co-transfection with THG-1 WT and constitutively active form of HRas compared with THG-1 ΔN0 and HRas(G12V) co-transfection. Thus, the N-terminal 111 amino acids of THG-1 is required to inhibit the PHD2-mediated degradation of HIF-1α.

3) Identification of THG-1-PHD2 binding site in THG-1

The applicant constructed the point mutations of THG-1 between 71-111 amino acids, and found that Leu81 and Thr91 of THG-1 were important for binding to PHD2. Then the applicant determined the effect of L81A and T91A mutants on the PHD2-mediated HIF-1α regulation. HIF-1α expression was decreased in the cells expressing either of the mutants THG-1 (L81A) or THG-1 (T91A) compared with those expressing THG-1 WT. Thus, the residues Leu81 and Thr91 of THG-1 are important for the THG-1-PHD2 interaction and the PHD2-mediated HIF-1α stability.

4) THG-1 regulates HIF-1α stability in TE13 cells

The applicant next investigated the effect of THG-1 on HIF-1α stabilization in the esophageal SCC cell line TE13 cells. The TE13 cells were treated with $CoCl_2$ (to mimic hypoxic condition), with or without EGF induction in 2D culture condition, and HIF-1α expression was examined. HIF-1α expression was increased by $CoCl_2$ treatment, and the expression level was higher in parental TE13 cells compared with that in the THG-1 knockdown TE13 cells in the presence of EGF. The THG-1 knockdown TE13 cells had reduced HIF-1α expression both in cytosol and nucleus. The sphere forming assay using TE13 cells revealed that TE13 control spheres had reduced HIF-1α expression compared with the THG-1 knockdown TE13 spheres. Thus, THG-1 regulates HIF-1α stability in TE13 cells in both 2D and 3D culture conditions.

5) THG-1 increases HIF-1α transcriptional activities

Since THG-1 can induce the stabilization of HIF-1α, the applicant assessed the HIF-1α target genes including VEGF-A, HK2, PKM2 and GLUT-1. Quantitative RT-PCR analysis showed that VEGF-A mRNA expression was significantly decreased by THG-1 knockdown under $CoCl_2$ treatment in 2D culture condition. In 3D culture condition, not only VEGF-A, but also other HIF-1α target genes PKM2, HK2, and GLUT1 mRNA were

significantly decreased in the THG-1 knockdown spheres. HIF-1α mRNA was not affected by THG-1 knockdown. Thus, THG-1 enhances HIF-1α transcriptional activities in both 2D and 3D culture conditions.

6) Effect of THG-1 knockdown on HIF-1α expression in 3D culture and xenograft

The applicant determined HIF-1α expression by immunohistochemistry. In xenograft tumors with HRas (G12V) expression in HaCaT cells, it has been previously shown that THG-1 WT enhances the Ras-mediated tumor formation. Nuclear HIF-1α positive cells were detected in THG-1 WT expressing tumor. In 3D culture invasion assay, the invasive front of TE13 cells highly expressed HIF-1α compared with the THG-1 knockdown cells. Thus, HIF-1α stabilization was also observed in xenograft tumors and 3D culture invasion model.

7) THG-1 is involved in tumor angiogenesis

Since VEGF-A is a major inducer of tumor angiogenesis, the applicant examined intratumoral vessel density in xenograft tumors from TE13 cells and HaCaT-Ras (G12V) cells by podocalyxin staining. Podocalyxin positive v*ascular* endothelial density was significantly reduced in the THG-1 knockdown tumors. In HRas(G12V) mediated xenograft tumors, podocalyxin positive v*ascular* endothelial cells were strongly observed in HRas(G12V) plus THG-1 WT expressing tumors. Thus, THG-1 is involved in tumor angiogenesis.

（考察　Discussion）

Oxygen-dependent hydroxylation of HIF-1α by PHD2 is a well-known degradation pathway regulating HIF-1α stability. The applicant's data in this study suggested that THG-1 interacts with PHD2 to protect HIF-1α protein from the PHD2-mediated HIF-1α degradation. THG-1 increases the HIF-1α protein expression level and transcriptional activities. The HIF-1α stabilization confers upregulation of HIF-1α target genes involved in cancer progression, invasion, cell survival, glucose metabolism, and angiogenesis. Thus, THG-1 may promote tumor growth and angiogenesis by regulating HIF-1α stability.

審査の結果の要旨
Abstract of assessment result

（批評　General Comments）

In the present study, the applicant has demonstrated that the THG-1-PHD2 interaction plays a pivotal role in the HIF-1α stabilization and angiogenesis in development of squamous cell carcinoma. Thus, findings in this study will surely have impact in the field and will benefit for understanding of the molecular mechanisms of THG-1 functions in tumor and for development of a new clinical cancer therapy for squamous cell carcinoma.

（最終試験の結果　Assessment）

The final examination committee conducted a meeting as a final examination on December 25, 2017. The applicant provided an overview of dissertation, addressed questions and comments raised during Q&A session. All of the committee members reached a final decision that the applicant has passed the final examination.

（結論　Conclusion）

Therefore, the final examination committee approved that the applicant is qualified to be awarded Doctor of Philosophy in Medical Sciences.