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研究課題名（和文）Role of Smad7 in myeloid leukemogenesis

研究課題名（英文）Role of Smad7 in myeloid leukemogenesis

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研究成果の概要（和文）：転写因子MEIS1はHOXA9関連AMLの発症に重要な役割を果たしている。本研究では、TGFbシグナル制御因子Smad7をMEIS1標的遺伝子として同定した。Hoxa9とSmad7を導入した骨髄細胞の移植により白血病が発症したことから、Smad7はMEIS1の機能を代替し、TGFbはAML発症の抑制性サイトカインである可能性が考えられた。しかし、TGFbの機能はin vitroで白血病細胞の増殖を阻害する一方、in vivoでは阻害剤の投与方法に依存し単純ではなかった。また、AMLにおいてSmad7はTGFbシグナルを抑制せず、白血病発症において未知の機構が存在する可能性が示唆された。

研究成果の概要（英文）：In acute myeloid leukemia, the transcription factor MEIS1 is critical for in vivo invasion and propagation of HOXA9-transformed leukemic cells. This project led to the discovery of Smad7 (a TGF-β signaling negative regulator) as a target gene of MEIS1. Inoculation into mice of bone marrow cells engineered to overexpress HOXA9 and Smad7 led to the onset of leukemia, demonstrating the ability for Smad7 to bypass MEIS1 absence and raising the possibility that TGF-β may be employed as a protective cytokine against acute myeloid leukemia. In vitro experiments confirmed the direct anti-proliferative impact of TGF-β treatment on leukemic cells. However, in vivo TGF-β inhibition gave discrepant results depending on the frequency of TGF-β inhibitor injection, demonstrating the high functional complexity for the activity of TGF-β. On the other hand, our Smad7 transgene did not inhibit TGF-β signaling in vitro, suggesting that Smad7 leukemogenecity could be due to another unknown function.

研究分野：　Oncology

キーワード：　がん微小環境　TGF-β　白血病　Meis 1　Smad 7

１．研究開始当初の背景

Acute Myeloid Leukemia (AML) is the most frequent type of leukemia and accounts for ~90% of all acute leukemia in adults. The five-year survival rate of patients with AML is the poorest of all leukemia with only 40%, which highlights the urgent necessity to improve therapy, thereby implying a better comprehension of the molecular mechanisms governing the onset and progression of the disease.

In past years, much effort has been made to characterize the molecular etiology of acute leukemia. It was found that nonrandom chromosomal translocations are hallmark mutations associated with human leukemia. One such translocation involves MLL, a gene that has been shown fused with over 60 different partner genes and which is associated with unfavorable survival. The MLL gene encodes a histone methyltransferase (HMT) that regulates gene transcription. In acute leukemia, the resulting fusion genes encode constitutively active forms of MLL despite of impaired HMT activity. Two genes upregulated directly by MLL were found sufficient for transformation : *Hoxa9* and *Meis1*, as their co-expression is sufficient to transform hematopoietic cells into acute myeloid leukemia.

Hoxa9 is a DNA-binding homeobox protein and Meis1 is a TALE-class homeodomain protein that acts as a DNA-binding cofactor of Hoxa9. Within the hematopoietic system, Hoxa9 and Meis1 are normally expressed in stem cells and immature progenitor compartments, but they are downregulated during myeloid differentiation. In MLL-associated AML downregulation of Hoxa9 and Meis1 does not occur due to transactivation by MLL fusion proteins. This leads to a differentiation block and the subsequent expansion of immature myeloid progenitors unable to generate mature effector cells, thereby leading to AML. Therefore, aberrant overexpression of Hoxa9 and Meis1 is now considered as a hallmark of MLL-rearranged leukemia.

Because Hoxa9 and Meis1 are transcription factors and uneasy to be targeted for therapy, clarification of the critical target genes of Hoxa9 and Meis1 responsible for the onset and progression of AML is important. Interestingly, while overexpression of Hoxa9 is sufficient to induce transformation of cells *in vitro*, Hoxa9-overexpressing cells are unable to induce AML *in vivo*. For *in vivo* propagation, co-expression of Hoxa9 with Meis1 is critically required, demonstrating that **essential target genes are exclusively upregulated in the presence of Meis1**. This observation also suggests that the *in vivo* bone marrow environment does not accept Hoxa9-transformed leukemic cells and that mere transformation of cells does not confer the ability to induce leukemia. Instead, in order to expand *in vivo*, Hoxa9-transformed cells need an additional set of genes that are regulated by Meis1.

２．研究の目的

The aim of this project is therefore to identify the critical Meis1 target genes necessary for *in vivo* expansion of leukemic cells. Adequately, we sought to identify genes exclusively upregulated in the presence of Meis1 through microarray analysis. Our analysis revealed two important genes regulated by Meis1 : ***Sytl1*** and ***Smad7***. The purpose of this project is therefore to investigate the role of *Sytl1* and *Smad7* in AML expansion *in vivo*, and to modulate their function as a strategy for therapy.

Sytl1 is a synaptotagmin-like protein that promotes intracytoplasmic transportation and export of molecules and/or vesicles through interaction with Rab27a/b. Interestingly, we found

that overexpression of Sytl1 in Hoxa9-overexpressing leukemic cells was sufficient to bypass Meis1 absence and restore the ability of leukemic cells to expand *in vivo*. This result suggests that leukemic cells need to communicate with the *in vivo* environment through Sytl1-mediated export of molecules. In this regard, we found that the plasma membrane location of Flt3 and CXCR4 is mediated by Sytl1 and required for the expansion of cells.

Surprisingly, although Hoxa9-Sytl1 overexpressing cells followed the same pattern of expansion than Hoxa9-Meis1 overexpressing cells during the first two weeks after inoculation, Hoxa9-Sytl1 cells gradually disappeared from mice and AML did not occur. This result suggests that Sytl1 is required for the initial engraftment of leukemic cells that allows short term expansion of cells, but not for long term expansion.

Smad7 is an inhibitory Smad that suppresses TGF□ signaling by interacting with the Smad2/3 complex. Overexpression of Smad7 in Hoxa9-overexpressing cells was not sufficient to bypass Meis1 absence, probably due to the inability of these cells to express Sytl1 and to engraft correctly. Nevertheless, a very small number of cells succeeded to engraft and 15 weeks following injection of Hoxa9-Smad7 overexpressing cells, expansion of leukemic cells was found to be prominent and mice developed AML. Therefore, although Smad7 is dispensable for the engraftment and initial expansion of leukemic cells, it is however critical for their long term survival and expansion.

In summary, our preliminary results show that Meis1 controls the expression of two important genes critical for the expansion of cells *in vivo*, *Sytl1* and *Smad7*, but these genes are required at different time period following inoculation. Therefore, their respective temporally regulated role unveiled a previously unknown biphasic mode of expansion where Sytl1 and Smad7 are required at the early and late phases, respectively.

３．研究の方法

1) Is *Smad7* a direct target gene of Meis1 ?

In order to clarify whether *Smad7* is a direct target gene of Meis1 or not, luciferase assay with Smad7 promoter in Meis1 overexpressing cells will be performed. In addition, RT-PCR and western blot analysis of Smad7 in Hoxa9-Meis1-overexpressing cells will be compared to Hoxa9-overexpressing cells, the latter being expected not expressing Smad7.

In case Meis1 does not directly regulate Smad7 expression, other transcription factors will be tested for luciferase assay.

2) Is TGF□ involved in promoting or inhibiting leukemogenesis ?

For *in vitro* experiments, Hoxa9- and Meis1-overexpressing cells will be incubated in the presence of TGF□ and cell number will be investigated. We performed this preliminary experiment and found that 4 days after TGF□ stimulation cell number was 70% lower than control, demonstrating a strong inhibiting capacity for this cytokine. Whether reduced cell number is due to a block in cell proliferation or an increase in cell death remains to be investigated. Therefore, different assays will be performed to assess cell proliferation and cell death at different time. Western blot analysis will also be performed to verify the status of cell cycle proteins and/or caspases in the presence or absence of TGF□.

For *in vivo* experiments, our results with Hoxa9-Sytl1-overexpressing cells demonstrate that Smad7 is dispensable for leukemic cell expansion during the first 2 weeks that follows inoculation, suggesting that TGF□ secretion begins 2 weeks after inoculation of cells in mice.

To validate this hypothesis, TGF□ secretion in bone marrow will be time course monitored following inoculation of Hoxa9-Sytl1-overexpressing cells. Once TGF□ secretion timing will be precisely identified, mice will be inoculated with Hoxa9-Sytl1-overexpressing cells and a neutralizing anti-TGF□ antibody will be inoculated at the time for TGF□ secretion. By neutralizing TGF□, we expect to consequently overcome Smad7 absence and rescue Hoxa9-Sytl1-overexpressing cells from their otherwise inevitable decline and disappearance.

3) Does Smad7 upregulation protect from TGF□ inhibiting ability ?

As a preliminary experiment, Hoxa9-Smad7-overexpressing cells have been subjected to TGF□ treatment. Surprisingly, we found that TGF□ had similar suppressive abilities on all cell lines tested, including Hoxa9-Smad7 cells. Therefore, we concluded that Smad7 does not protect leukemic cells from TGF□-suppressive effects, in these conditions. This result raised 2 possibilities : either *in vitro* conditions are not representative of *in vivo* conditions, or the role of Smad7 in leukemogenesis is independent of TGF□.

We opted for the first possibility and modified *in vitro* conditions by repeating the experiment under hypoxic conditions (3% O_2), since the bone marrow niches are known to be hypoxic. Under hypoxic conditions, Smad7 overexpression indeed afforded protection to leukemic cells from TGF□ suppressive effects. Therefore, for still unidentified reasons, Smad7 inhibits TGF□ signaling only under hypoxic conditions in leukemic cells.

As mentioned earlier, we demonstrated the critical *in vivo* role of Smad7 in leukemogenesis by inoculating intravenously Hoxa9- and Smad7-overexpressing cells into sub-lethally irradiated mice, which showed leukemic cells in blood 15 weeks after inoculation and eventually developed AML. To further demonstrate the critical requirement for Smad7, Smad7 will be overexpressed in Hoxa9-Sytl1-overexpressing cells and the resulting Hoxa9-Sytl1-Smad7-overexpressing cells will be inoculated by intravenous injection to sub-lethally irradiated mice and AML burden will be time course monitored. We expect AML to occur at a similar kinetic than mice inoculated with Hoxa9-Meis1-overexpressing cells. This result will also demonstrate that Sytl1 and Smad7 activities are sufficient to bypass Meis1 absence and restore the full *in vivo* leukemogenicity.

4) Why does Smad7 protect leukemic cells from TGF□ suppressive effects specifically under hypoxia ?

In order to answer this question, leukemic cells will be stimulated with TGF□ in normoxic and hypoxic conditions and subjected to western blot analysis for TGF□ signaling pathway proteins to identify the crucial step inhibited by Smad7 specifically under hypoxia. In particular, it has been shown that Smad4 is able to bind and sequester Hoxa9 in the cytoplasm. Therefore, immunoprecipitation assays to study the interaction between Smad4 and Hoxa9 will be performed. We hypothesize that Smad7 disrupts Hoxa9-Smad4 interaction under hypoxia.

In addition, since hypoxia is well known to stabilize HIF1□, we suggest that, in order to be able to inhibit TGF□ signaling, Smad7 requires the expression of genes regulated by HIF1. Therefore, the expression of HIF1 target genes will be analyzed by RT-PCR and/or Western blot. Gene profile through microarray analysis might also be performed.

Furthermore, these results might suggest that hypoxia (and HIF1) promote AML development, which may lead to a therapeutic strategy by augmenting oxygenation of patients with AML potentially combined with TGF□ inoculation.

5) What are the TGF□ producing cells ?

If TGF□ has the ability to suppress leukemic cells propagation *in vivo*, it is important to identify the cells in bone marrow that produce TGF□ in response to leukemic cells detection. Indeed, driving the TGF□ producing cells to produce high amount of TGF□ could be an alternative therapeutic strategy to exogenous TGF□ inoculation. *In vitro* experiments will be performed by incubating leukemic cells with bone marrow stromal cells (OP9 cell line). The supernatant will be collected and TGF□ production will be analyzed by ELISA. If stromal cells indeed secrete TGF□ when into contact with leukemic cells, frozen sections of bone marrows from mice inoculated with leukemic cells will be performed and intracellular TGF□ will be visualized by immunohistochemistry with an anti-TGF□ antibody. If *in vitro* experiments show that stromal cells do not secrete TGF□, other cells such as immune cells able to produce TGF□ (i.e. Tregs) will be investigated. Alternatively, it is possible that leukemic cells are the actual source of TGF□, at least in part, as a means to downmodulate the immune system. Identifying the TGF□ producing cells might also help to explain why TGF□ secretion begins 2 weeks after inoculation of leukemic cells in mice.

４．研究成果

In order to clarify whether or not *Smad7* is a direct target gene of Meis1, we performed ChIP seq analysis which revealed that Meis1 indeed binds directly to *Smad7* promoter. However, luciferase assays gave discrepant results since co-transfection of Meis1 and a Smad7 reporter into 32D cells did not necessarily result in upregulated luciferase activity. The nature of this discrepancy is unknown. It is therefore still unclear whether Meis1 regulates *Smad7* expression directly or indirectly.

Whatever the regulating process, inoculation into mice of bone marrow cells stably overexpressing Hoxa9 and Smad7 led to the development of leukemia. In addition, the same observation could be made when using *in vitro* transformed leukemic cells, although at a very slow onset.

Smad7 overexpression therefore favors the onset of leukemia. Since Smad7 is implicitly related to the negative regulation of TGF□ signaling, this suggests that TGF□ may inhibit leukemia onset and Smad7 would protect leukemic cells from this TGF□ inhibition.

In order to clarify the effect of TGF□ on leukemic cells, we performed *in vitro* experiments by incubating leukemic cells with different concentrations of TGF□ followed by proliferation assay. We found that TGF□ inhibits cell proliferation of leukemic cells at all doses tested. However, when leukemic cells were co-incubated with OP9 cells, TGF□ instead promoted cell proliferation of leukemic cells.

In vivo inhibition of TGF□ also showed discrepant results. When a TGF□ inhibitor was injected every 2-3 days into Hoxa9-Meis1 inoculated mice, the onset of leukemia occurred earlier, suggesting that TGF□ inhibits leukemic cells *in vivo*. However, when the TGF□ inhibitor was injected every day, leukemia occurred at later time, suggesting that TGF□ is actually required for the onset of leukemia. It therefore seems that TGF□ can either promote or inhibit the onset of leukemia, presumably depending on the *in vivo* concentration of the cytokine. The bivalent role of TGF□ during leukemogenesis is consequently presently unclear.

Of note, we attempted to create leukemic cells knocked down for TGF□ receptor I or II to determine whether these cells are affected positively or negatively for the onset of leukemia. However, knocked down of TGF□ receptors was unsuccessful and could not be achieved, although multiple tries were performed.

In addition and very surprisingly, following *in vitro* TGF□ stimulation of leukemic cells, Smad7 overexpression did not inhibit Smad2 phosphorylation. Indeed, *in vitro* experiments showed that phosphorylation of Smad2 was identical between Hoxa9 and Hoxa9-Smad7 overexpressing cells. This result suggests that Smad7 may not inhibit TGF□ signaling in leukemic cells.

We initially thought that this unexpected result was specific to leukemic cells. However, when Smad7 was overexpressed in other cell lines, such as HeLa cells, U2OS cells and NIH3T3 cells, either transiently or stably overexpressed, Smad7 could not inhibit phosphorylation of Smad2 in all of the cell lines tested. Moreover, Smad7 did not interfere with Smad2 translocation into the nucleus as viewed by immunofluorescence studies.

We therefore sequenced the Smad7 transgene but found no mutation. Therefore, for an unidentified reason, Smad7 does not inhibit TGF□ signaling in our hands, although the protein is clearly overexpressed as detected by immunoblotting.

Nevertheless, we found that Smad7 overexpressing cells are protected from the anti-proliferative effect of TGF□ under hypoxia. However, in these conditions, phosphorylation of Smad2 was not affected by Smad7 overexpression, similarly to normoxic conditions

These unexpected results therefore raised the possibility that the leukemogenicity of Smad7 is not due to its TGF□ signaling inhibition activity. Instead, Smad7 seems to favor leukemia onset through other functions which remain to be identified. Instead of overexpressing Smad7, we then attempted a different strategy by knocking down Smad7 with shRNA in leukemic cells. However, we were unable to obtain Smad7 knocked down cells.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　1 件）

Yokoyama T, Nakatake M, Kuwata T, Couzinet A, Goitsuka R, Tsutsumi S, Aburatani H, Valk PJM, Delwel R, Nakamura T. MEIS1-mediated transactivation of synaptotagmin like 1 promotes CXCL12/CXCR4 signaling and leukemogenesis. J Clin Invest, 126:1664-1678, 2016.

〔学会発表〕（計　1 件）

Oral presentation :
Smad7 confers AML onset capability to HOXA9-transformed HSPCs. Arnaud COUZINET, Takashi YOKOYAMA, Mayuka NAKATAKE, Takuro NAKAMURA.
The 73rd Annual Meeting of the Japanese Cancer Association (JCA) – Sept 2014 (English oral session)

〔図書〕（計　0 件）

〔産業財産権〕
○出願状況（計　0 件）

○取得状況（計　0 件）

〔その他〕
ホームページ等
http://www.jfcr.or.jp/english/laboratory/department/carcinogenesis/index.html

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