様　式　Ｃ－１９、Ｆ－１９、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

平成　２７　年　　６　月　　６　日現在

機関番号：　１４３０１
研究種目：　若手研究(B)
研究期間：　2013～2014
課題番号：　２５８７０３６５

研究課題名（和文）Small Bioactive Molecules for Orchestrating Embryonic Stem Cell Differentiation

研究課題名（英文）Small Bioactive Molecules for Orchestrating Embryonic Stem Cell Differentiation

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交付決定額（研究期間全体）：（直接経費）　　3,200,000円

研究成果の概要（和文）：a)インドール構造をもつ1800個の化合物からHes1の発現を変調する3つのヒット化合物を発見。D8C化合物がHes1によるレポーター遺伝子の制御を解除することを立証。176個の誘導体を合成しD8Cの最適化を検証。D8Cに比べ10倍の活性をもつJI051を発見した。
b)未分節中胚葉をJI051で処理するとHes7遺伝子の発現振動が減少。
c)ケミカルバイオロジー的な検証がJI051の標的はTLE1ではない可能性を示唆。Gro/TLEコリプレッサーに関与するキナーゼがJI051と考えたがこれらの活性を変調しなかった。マイクロアレイ解析によると低酸素状態での複数遺伝子の発現がJI051により上昇。

研究成果の概要（英文）：a) Screening and SAR studies: Using a transcriptional assay, we isolated 3 hits from an indole-containing chemical library of 1800 small molecules. D8C was validated as a Hes1 modulator as it was able to inhibit Hes1-mediated repression. 176 derivatives were synthetized and tested to optimize D8C. JI051 showed a 10-fold improvement as compared to D8C.
b) Effect on Oscillations: Presomitic mesoderm preparations showed a decrease in Hes7 oscillations upon treatment with JI051.
c) Target Validation Procedure: Pull-down assays revealed a band corresponding to Gro/TLE. However, competition with 10-fold excess of JI051 was partially successful, suggesting that TLE1 may not be the sole target. 211 kinases were tested to investigate if kinases involved in Gro/TLE corepressor activity, could be modulated by JI051. No inhibition or stimulation higher than 50% was shown. Microarray analysis to identify other potential targets revealed an up-regulation of several genes involved in hypoxia.

研究分野：　small organic molecules

キーワード：　SAR Studies　Hes1　Regenerative Medicine　Stem Cells　Oscillations　Microarray　Transcription　Gro/TLE

１．研究開始当初の背景

(1) Embryonic stem (ES) cells represent a powerful tool for regenerative medicine but ES cells tend to asynchronously differentiate into diverse cell types, requiring additional purification steps to collect homogenous cell populations.

(2) Oscillations in the expression of the Notch effector Hes1 have been shown to play a key role in ES cell fate preference upon differentiation induction showing heterogeneous responses depending on Hes1 expression levels[1].

(3) Hes1 expression oscillates through transcriptional feedback repression wherein the transcription factor Hes1 recruits the transcriptional corepressor Groucho/Transducing-Like Enhancer of split (Gro/TLE) to its own promoter (**Fig. 1**).

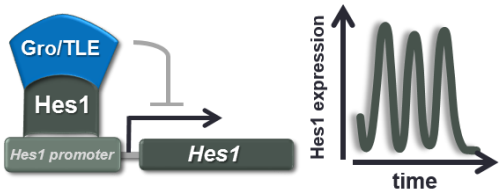

Figure 1: Oscillatory Expression of Hes1

２．研究の目的

(1) We are aiming at developing small bioactive molecules to regulate Hes1 expression in order to block Hes1 oscillations, leading to sustained levels of expression.

(2) As the interaction of Hes1 with Gro/TLE depends on the WRPW motif of Hes1, we intend to develop small bioactive molecules based around this tetrapeptide motif to competitively block Gro/TLE binding to Hes1 (**Fig. 2**).

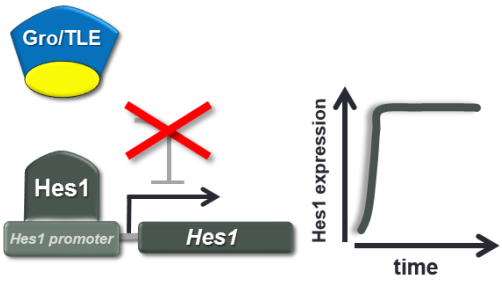

Figure 2: Blockade of Gro/TLE repression of Hes1

(3) Such small organic molecules could be ultimately used to synchronize ES cell differentiation and provide homogenous transplants for regenerative medicine.

３．研究の方法

(1) *Compound Screening Procedure:* Since the WRPW motif of the transcriptional repressor Hes1 is constituted of 2 tryptophan residues which each of them comprise an indole group, a chemical library of 1800 small organic molecules containing indole moieties was used for the high throughput screening procedure. The compounds were screened in 96-well plates based on a transcriptional assay using a luciferase reporter gene. Hek293 cells were cotransfected with a luciferase reporter gene under the control of Hes1 promoter together with Hes1 gene under the control of a constitutive promoter (CMV) to repress the transcription of the reporter gene. The hit compounds were selected according to their ability to block Hes1-mediated repression of the reporter gene as reported as an increase in the luciferase signal.

(2) *Hit Compound Validation:* Hit compounds isolated through the screening procedure were validated using an EGFP-based reporter assay for visualizing the effect of the compounds on Hes1 oscillations in single Hek293 cells by confocal fluorescence microscopy. The EGFP reporter gene was expressed under the control of Hes1 promoter (as for the luciferase reporter gene) but also comprised a PEST amino acid sequence to target the protein for degradation. This PEST sequence was added for a rapid turnover of the protein to allow the monitoring of the expression dynamics of the protein.

(3) *Structure-Activity Relationship (SAR) Studies:* Commonalities among the different chemical groups were analyzed and further optimization was carried out using organic chemistry to synthetize additional compounds related to the hit compounds. Compound derivatives were then tested in Hek293 cells according to the EGFP reporter gene assay mentioned above. 120 of the derivatives were also tested for turbidimetric aqueous solubility and 17 of them were further tested for metabolic stability by the UK preclinical contractor Cyprotex.

(4) *Oscillations in the Presomitic Mesoderm (PSM):* Hes7 is a transcription factor with a conserved WRPW motif at the carboxy-terminal end, like Hes1, that is known to play a major role in somitogenesis due to its oscillatory pattern of expression[2]. To investigate whether JI051 is affecting Hes7 oscillations in the presomitic mesoderm (PSM), we have examined the effect of JI051 on Hes7 oscillations in PSM preparations from mouse embryos expressing luciferase reporter gene under the control of Hes7 promoter (collaboration with Prof. Kageyama from Kyoto University).

(5) *Target Validation Procedure:* A photoaffinity probe based on our hit compound comprising a photoactive diazirine moiety together with a biotin moiety was synthetized for pull-down experiments with streptavidin labelled beads using cell lysates from transfected Hek293 cells transfected with a Flag-tag version of Gro/TLE. The lysates were then analyzed through a Western blotting procedure using a Flag antibody. Competition experiments with an excess of JI051 were also carried out to confirm the specificity of the labeling.

(6) *Comprehensive Kinase Profile:* A panel of 211 kinases was tested with a radiometric protein kinase assay by Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) to investigate whether kinases involved in Gro-TLE corepressor activity could be modulated directly by our hit compound.

(7) *Effect to the Compounds on Gene Expression:* Microarray analysis with the GeneChip® mouse gene array (Affymetrix) was carried out to identify pathways and potential targets involved in the response mediated by our hit compound. A total of 40,000 genes were investigated.

４．研究成果

(1) The compound screening procedure using a luciferase-based transcriptional assay developed in the laboratory allowed us to isolate 37 hit compounds out of 1800 indole-containing small molecules with a Z score (distance from the mean) above 2 (**Fig. 3**).

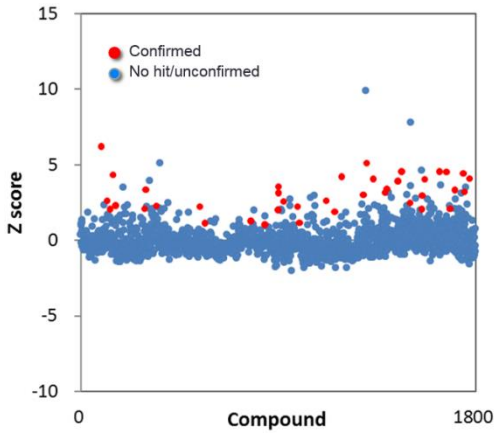

Figure 3: Screening for Hes1 Modulators

(2) 3 compounds (L4F, V7D and D8C) were further validated using an EGFP-based reporter assay. Among them, D8C showed the highest response on Hes1-mediated gene repression with a half-maximal response (EC_{50}) at 2.5 μM and a 5-time increase in fluorescence as compared to cells treated with the vehicle (DMSO). Furthermore, confocal fluorescence microscopy revealed that the typical oscillation pattern of Hes1 expression was also affected in D8C treated cells with a significant decrease in oscillation amplitude (**Fig.4**).

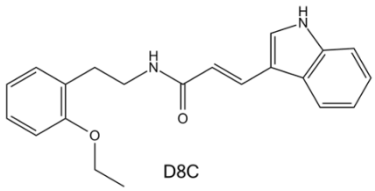

Figure 4: Chemical Structure of D8C

(3) 176 derivatives were synthetized and tested for optimization of our hit compound (D8C). SAR studies indicated that the addition of a methyl group on the indole ring (JI021) increased the potency of the compound. The potency was further increased after a substitution with a methoxy group at the same position (JI051). Modifications at other positions (e.g. double bound, amide, phenethyl moiety) were however not tolerated. JI051 showed a 10-fold improvement as compared to D8C (EC_{50} = 0.25 μM) .

(4) Oscillations in PSM preparations were also affected upon incubation with JI051, as revealed by a decrease in the amplitude and oscillation number in mouse embryos expressing a luciferase reporter gene under the control of Hes7 promoter.

(5) We have then carried out a series of target validation experiments with a photoaffinity probe based on JI051 comprising both a UV reactive and biotin moieties. Although pull-down assays revealed a band corresponding to Gro/TLE, competitive displacement with a 10-fold excess of JI051 was only partially successful, suggesting that Gro/TLE may not be the sole target for JI051.

(6) A comprehensive kinase profile containing a total of 211 kinases was also carried out to investigate whether kinases such as CK2, a serine threonine kinase involved in Gro/TLE corepressor activity, could be directly modulated by JI051. However, the results did not show any inhibition or stimulation higher than 50%, suggesting that JI051 does not significantly affect any of the kinases tested.

(7) Microarray analysis revealed an up-regulation of several genes involved in hypoxia, suggesting that HIF-1 may be involved in JI051 response. Further studies are however needed to confirm this hypothesis.

(8) Targeting the Notch pathway represents an attractive approach for regulating the differentiation competency of ES cells. To our knowledge, there are no known therapies that could modulate Hes1 expression levels apart from the potential use of gamma-secretase inhibitors[3]. Small bioactive molecules mimicking the WRPW

motif of Hes1 would have the advantage of specifically targeting Hes1 itself, rather than upstream components of the signaling pathway. Such small bioactive molecules will provide pure populations of defined cell types that will increase the success rate of cell transplants.

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③ Sang L, Roberts JM, Coller HA. Hijacking Hes1: how tumors co-opt the anti-differentiation strategies of quiescent cells. *Trends Mol Med* **16**: 17-26, 2009.

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）

〔学会発表〕（計　2　件）
① Perron A, Iwata J, Kobayashi T, Kageyama R, and Uesugi M. Small molecule Hes1 modulators for synchronizing embryonic stem cell differentiation. Poster presentation at the 6th Annual *iCeMS Retreat*, Kyoto, Amanohashidate, 2014.

②Perron A, Kawazoe Y, Shimogawa H, Kobayashi T, Kageyama R, and Uesugi M. Small molecule Hes1 modulators for orchestring embryonic stem cell differentiation. Poster presentation at the 5th Annual *iCeMS Retreat*, Shiga, Hikone, 2013. *Poster Award

６．研究組織

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