科学研究費助成事業　　研究成果報告書

平成　２６　年　　６　月　２５　日現在

機関番号：　１４３０１
研究種目：　若手研究(B)
研究期間：　2012～2013
課題番号：　２４７１０２６１

研究課題名（和文）多能性に関する転写機構をオンにするプログラム可能な小分子の開発

研究課題名（英文）Development of programmable small molecules capable of switching ON the transcriptional machinery of pluripotency genes.

研究代表者
ナマシヴァヤム　パンディアン（NAMASIVAYAM, Ganesh Pandian）

京都大学・物質-細胞統合システム拠点・特定拠点助教

研究者番号：２０６２５４４６

交付決定額（研究期間全体）：（直接経費）　　3,600,000円、（間接経費）　　1,080,000円

研究成果の概要（和文）：我々は化合物によって特定の遺伝子の転写を活性化することを目指し、DNA結合部位と活性部位を併せ持つSAHA-PIPという小分子化合物を開発してきた。本研究では様々な結合DNA配列をもつSAHA-PIPライブラリを合成・スクリーニングすることで、マウス繊維芽細胞で多能性遺伝子を発現上昇させるSAHA-PIPを同定した。またヒト繊維芽細胞では生殖細胞関連遺伝子を上昇させるSAHA-PIPなど、それぞれのSAHA-PIPが特徴的な組織に関連する遺伝子群を上昇させることを見出した。この研究成果により、化合物による細胞の運命の制御法の開発が示唆された。

研究成果の概要（英文）：Artificial transcriptional activators with epigenetic activity can retain the capability of their natural equivalents to rewire transcriptional machinery within a cell. During this tenure, we have demonstrated the potential of our novel small molecules for genome engineering termed `SAHA-PIP` containing sequence-specific pyrrole-imidazole polyamides (PIPs) and SAHA, a histone deacetylase inhibitor. We tailored SAHA-PIPs to distinctively activate the pluripotency genes by triggering transcriptionally permissive chromatin in mouse fibroblasts. We have generated PIP conjugates with fluorescent dyes and those targeting different epigenetic enzymes. In human fibroblasts, a SAHA-PIP got characterized as germ cell gene switch and genome-wide gene analysis revealed the capability of thirty-two distinct small molecules to trigger the transcriptional activation of exclusive developmental genes. Our results contribute to the development of novel chemical approach for controlling cell fate.

研究分野：　複合新領域

科研費の分科・細目：　生体分子科学・ケミカルバイオロジー

キーワード：　人工遺伝子スイッチ　細胞リプログラミング　小分子　合成生物学　iPS細胞

１．研究開始当初の背景

Cellular reprogramming involves profound alterations in genome-wide gene expression that is precisely orchestrated by coordinated chromatin modifications. Transcriptional activators switch “ON” and “OFF” the appropriate genes at the right place and time and have the ability to manipulate the cell fate specification. Accordingly, artificial induction of pluripotency in somatic cells through enforced transcriptional activation of the four factors was achieved to offer new modes of therapy (*Cell*, 2006, 126, 663). Notwithstanding the recent promising breakthroughs, several hurdles, including the retention of epigenetic memory, need to be overcome before the possible therapeutic use of induced pluripotent stem (iPS) cells.

Since the epigenome is inherently flexible, it could be modulated through pharmacological interventions. Accordingly, several small molecules targeting the epigenetic enzymes or key transcription factors were shown to enhance the somatic cell reprogramming. However, these effectors artificially alter the epigenome in a sequence independent manner. Taking cues from nature, it is evident that complementing selectivity to chromatin modifying small molecules could enhance their efficacy.

２．研究の目的

Recent developments in bioinformatics and techniques such as diversity-oriented synthesis suggest that it is possible to design small molecules to achieve transcription factor-based reprogramming to convert the somatic cells into pluripotent stem cells. Deng and Colleagues finally accomplished this complex feat and using seven small molecules they successfully generated chemical induced iPS cells or CiPS cells from mouse somatic cells with an induction efficiency of about 0.2% (*Science*, 2013, 341, 651).

Optimization feasibility of complete chemical reprogramming facilitates the chance of improvement in current protocol. However, requirement of several small molecules and time taken to achieve completely reprogrammed cell line are the major concern. Since cellular reprogramming is multi-factorial in nature, one way to achieve this complex feat is to develop versatile small molecules capable of modulating the complicated gene networks associated with pluripotency.

３．研究の方法

Hairpin pyrrole - imidazole polyamides (PIPs) are programmable synthetic molecules that can bind to the minor groove of DNA with an affinity that is similar to that of natural transcription factors (*Biotechnol. J.*, 2012, 7, 798). PIPs could be developed as versatile small molecules with many applications as they have flexible sites for covalent attachment to molecules, such as fluorescent dyes and/or some enzyme inhibitors (*ChemBioChem*, 2012, 13, 2170). As a novel chemical approach to control cell fate, we have designed and synthesized a new type of small molecule by conjugating the selective DNA-binding PIPs with the potent HDAC inhibitor, SAHA to form SAHA-PIP.

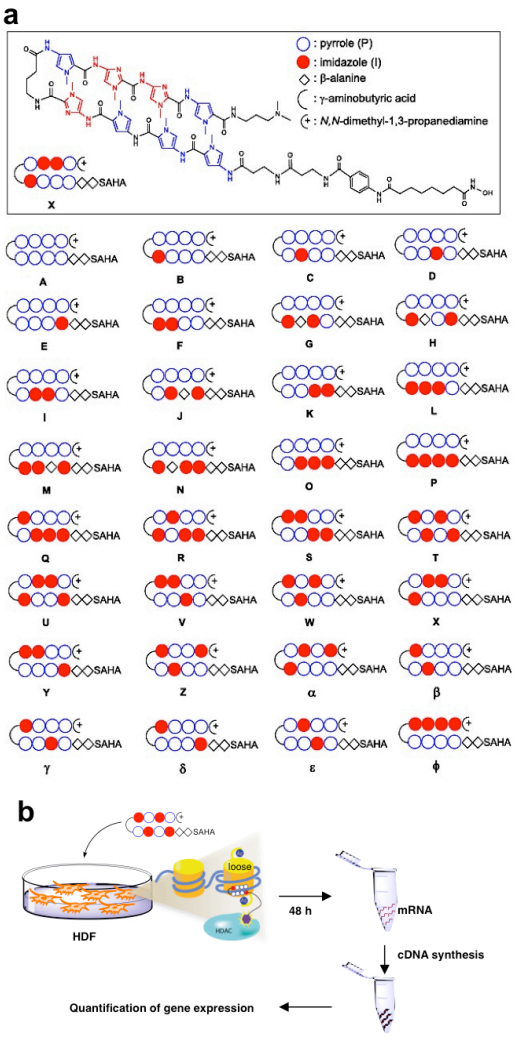

Figure 1. *(a) Chemical structures of the synthetic SAHA - pyrrole-imidazole polyamide conjugates (PIPs)* ***A - ϕ****. PIPs were designed by placing imidazole at various positions in the top and bottom. (b) Workflow of microarray analysis using individual SAHA-PIP treated cells.*

A library of thirty-two different SAHA-PIPs (**A** to **ϕ**) (Figure 1a) to specifically target a 6-base-pair sequence was synthesized by conjugating the SAHA moiety to the N-tail with a double β-alanine linker. We have also attached a HDAC8 enzyme modulating small molecules called JAHA. All the SAHA-PIPs were used after HPLC purification. Mouse embryonic fibroblasts (MEFs) and human dermal fibroblasts (HDFs) with in the passage 6 were trypsinized for 5 min at 37 ° C, and were resuspended in the fresh DMEM medium to a concentration of 2 x 10^5 cells/mL in a 35 mm plate. Incubation time and the concentration of the SAHA-PIPs were standardized based on the optimization experiments with individual SAHA-PIPs. Quantification of gene expression was done using real-time PCR and microarray analysis (Figure 1b).

４．研究成果

Initial screening studies carried out to evaluate the effect of SAHA-PIPs (**A** to **P**) on iPSC factors (*Oct-3/4, Nanog, Sox2, Klf4* and *c-Myc*) in MEFs indicated that certain SAHA-PIPs could distinctively activate the iPSC factors by triggering epigenetic marks that are associated with transcriptionally permissive chromatin (*ChemBioChem*, 2011, 12, 2822). However the expression levels in SAHA-PIP treated MEFs were very low when compared to that observed in the embryonic stem cells. To improve the induction of pluripotency factors, we have designed and synthesized the derivatives of a hit SAHA-PIP called **E**. Screening studies in MEFs indicated that a notable increase in the expression of pluripotency genes could be observed with the modification in the chemical architecture of PIPs (*Bioorg. Med. Chem.*, 2012, 20, 2656).

The scope of improving SAHA-PIPs as efficient genetic switches was further substantiated with the generation of a second generation of SAHA-PIPs (**Q** to **ϕ**). We have placed the imidazole at different positions with the assumption that the selective inducing ability of SAHA-PIPs will be superior owing to the improved recognition of GC rich sequences. Consistent with our hypothesis, we identified a novel SAHA-PIP, termed **δ** that could rapidly induce multiple pluripotency genes. Interestingly, δ -OMe, the non-functional SAHA-PIP did not activate any of the pluripotency genes and this result substantiated SAHA as the functional moiety in **δ** (Figure 2a). Analysis of the set of genes that were induced and suppressed in **δ** -treated MEFs suggested that in just 24 h, **δ** could rapidly overcome the mesenchymal epithelial transition stage, an important rate-limiting step during dedifferentiation of the somatic genome (Figure 2b). Microarray analysis suggested that **δ** -treated MEFs up-regulated about twice the number of genes than SAHA treated MEFs, and down-regulated only 200 genes in comparison with 400 genes, which were down-regulated in SAHA treated MEFs.

About 33% of the **δ** -induced genes belonged to the core pluripotency gene network that comprises of about 345 inter-twined genes, which are governed by *Oct-3/4*, *Sox2* and *Nanog*. Hence, it is reasonable to suggest that the PIP in **δ** directs SAHA to the core pluripotency gene network. This manuscript (*Sci. Rep.*, 2012, 2, e544) was highlighted in stem cells portal as an important study that is destined to make an impact on stem cell research and clinical studies.

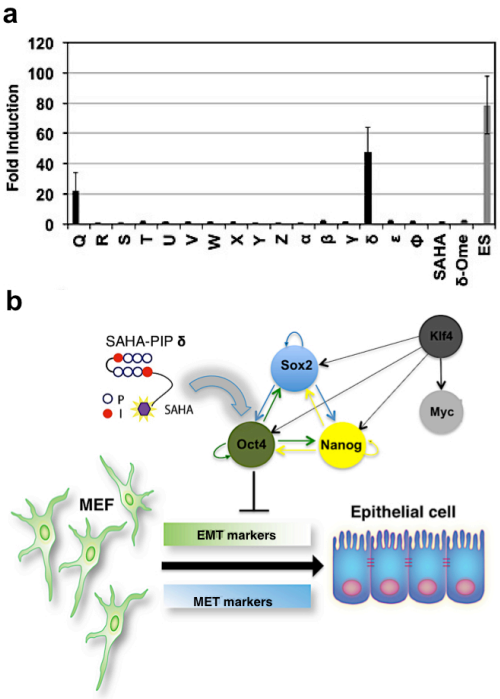

Figure 2. *(a) Expression profile of the Nanog with 100 nM of 16 individual SAHA-PIPs (**Q-Φ**). Light gray bar represent control samples (ES cells as positive control, SAHA and **δ** having methyl ester in the functional group of SAHA (δ -OMe) as a negative control. Mean ± SD from 18 well plates. (b) SAHA-PIP **δ** triggers the core pluripotency gene network, but not Klf4, to initiate cellular reprogramming by down regulating the mesenchymal(M)) markers and the up regulating the epithelial transition (ET) markers.*

We have also developed the PIPs containing

pyrene fluorophore with a β-alanine linker at the γ-turn NH_2 position for versatile applications in biological and physicochemical studies (*Bioorg. Med. Chem.*, 2013, 21, 852). We developed a small molecule called JAHA-PIP δ or J δ lacking the surface-recognition domain of SAHA. Through enzymatic activity assay and biological studies, it was demonstrated that the chemical modification of functional SAHA in SAHA-PIP could alter its activity against HDAC8 but not against HDAC1. An additive effect was observed with the combination of HDAC8-specific J δ with δ. Rapid activation of skull morphogenesis associated HDAC8 regulated *Otx2* and *Lhx1* with J δ opened up the opportunities in regenerative medicine.

As SAHA-PIPs selectively activate a set of pluripotency genes in mouse fibroblasts, we assumed that they might have a similar effect in human dermal fibroblasts (HDFs) to modulate the typically conserved gene network. Like pluripotency, gametogenesis is a silent biological process in somatic cells. Meiosis is a highly specialized cell-division process in multicellular eukaryotes that is specific to germ cells and not somatic cell. Aberrations in the orderly meiotic process are a prominent cause of human infertility and a recent study revealed that the epigenetic disruption of the PIWI pathway could be associated with spermatogenic disorders in infertile male human patients (*PloS One* 2012, 7, e47892). Screening studies indicated that the SAHA-PIP **K** and not the control **O** distinctively activated the meiosis controlling PIWI pathway genes.

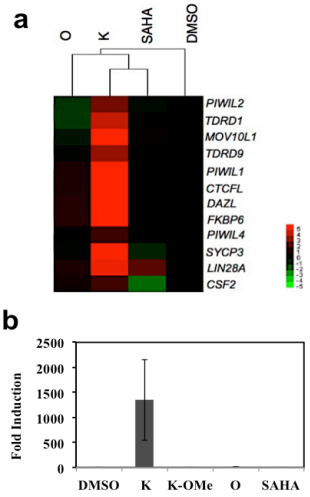

Figure 3 *(a) Heat map based on unsupervised hierarchical clustering analysis of germ cell gene expression data from fibroblasts treated with DMSO (set as the control), SAHA,* ***K****, and* ***O****. (b) Expression profile of PIWIL 1 in HDF treated with* ***K****, SAHA,* ***O****, and K-OMe. Mean ± SD from 24 wells.*

The heat map of the summarized expression profile of the gametogenesis-associated genes suggested that these usually conserved genes are overexpressed only in **K**-treated HDFs and not in SAHA-, **O**-, or DMSO-treated HDFs (Figure 3a). q-RT-PCR studies substantiated that **K** triggered a significant enhancement (approx. 500-fold) of the endogenous expression of both *MOV10L1* and *PIWIL1* in HDFs (Figure 2c). This first ever report on a germ cell gene switch was selected as a `Hot paper` by the editor for the importance of this work in a rapidly evolving field of high current interest (*Angew. Chem. Int. Ed.* 2013, 52, 13410). Based on the above-mentioned result, we assumed that the distinct DNA binding PIPs could be directing SAHA to a set of silent genes and activate them. To clarify this notion, we treated a library of thirty-two SAHA-PIPs (**A** to **Φ** renamed as **1** to **32**) to HDFs and evaluated their effect on the genome-wide gene expression. Through extensive analyses with independent lines of evidence, we revealed the remarkable ability of unique SAHA-PIPs to trigger transcriptional activation of exclusive developmental genes (Figure 4).

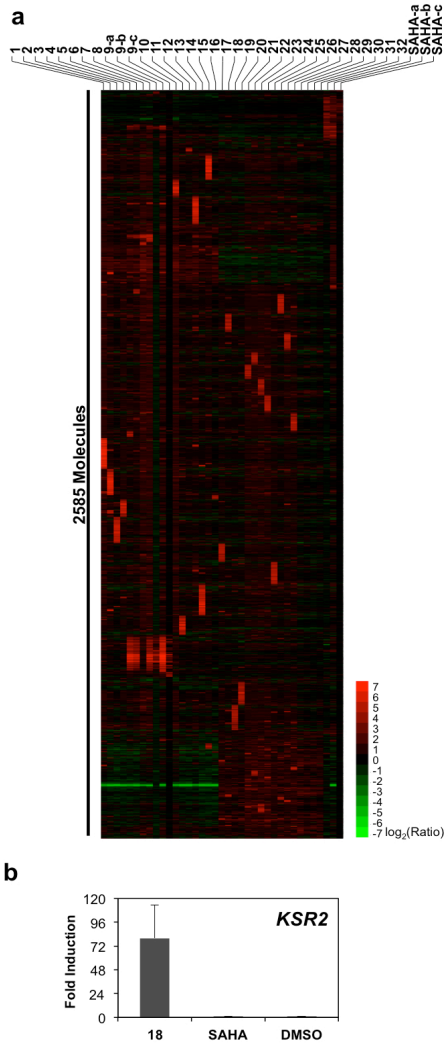

Figure 4. *(a) An unsupervised hierarchical clustering analysis of top 100 up-regulated genes in SAHA-PIP 1-32 treated fibroblasts suggests that each SAHA-PIP activate a unique cluster of genes. (b) Induction of KSR2 by* ***18****. Mean ± SD from 6 wells.*

Furthermore, these targeted transcriptional activators also activate a different set of noncoding RNAs and suppress an identical set. QRT-PCR studies validated the pattern observed with microarray analysis and some SAHA-PIPs activated the therapeutically important genes including the recently identified *KSR2*, the obesity gene and *SEMA6A*, the retinal 'ON' circuit factor. These DNA-based epigenetic switches could be developed to have the ability of modulating the transcription of therapeutically important genes and non-coding RNAs in a precise manner. Our proof-of-concept study demonstrates the possibility to develop this kind of DNA-based epigenetic switches for controlling the transcription of silent genes associated with cell fate and/or the genes of therapeutic importance. This paper got highlighted in various web portals including one in GEN News titled, `Install Epigenetic Switches to Give Silent Genes a Voice`.

Taken together, the reported results indicated that the PIP conjugates could be tailored to bind predetermined DNA sequences. Hence, strategies to expand them could create an epoch-making approach in cellular reprogramming as they may precisely coax the somatic cells into pluripotent stem cells and/or a totally new type of cells. Since targeted treatments ensure better drug efficacy and fewer long-term side effects, these targeting molecules could de developed to modulate the silent genes of therapeutic importance in a diseased cell.

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 11 件）

(1) Ganesh N. Pandian *et al.*, Distinct DNA-based epigenetic switches trigger transcriptional activation of silent genes in human dermal fibroblasts, *Sci. Rep.* (Nature publishing group), REFEREED 4, 2014, e3843.

(2) Ganesh N. Pandian *et al.*, Cellular reprogramming for pancreatic beta cell regeneration: clinical potential of small molecule control, *Clin. Transl. Med.* REFEREED 3, 2014, 6.

(3) L. Han, Ganesh N. Pandian *et al.*, A synthetic small molecule for targeted transcriptional activation of germ cell genes in a human somatic cell, *Angew. Chem. Int. Ed.,* **HOT PAPER**, REFEREED 125, 2013, 13652-13655.

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(6) T. Vaijayanthi, T. Bando, K. Hashiya, Ganesh N. Pandian, *et al.*, Design of a new fluorescent probe: Pyrrole/Imidazole hairpin polyamides with pyrene conjugation at their γ-turn, *Bioorg. Med. Chem.,* REFEREED 21, 2013, 852-855.

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(10) T. Vaijayanthi, T. Bando, Ganesh N. Pandian *et al.*, Progress and prospects of pyrrole-imidazole polyamide-fluorophore conjugate as sequence-selective DNA probes, *ChemBioChem.,* REFEREED 13, 2012, 2170-2185.

(11) Ganesh N. Pandian *et al.*, Development of programmable small DNA-binding molecules with epigenetic activity for induction of core pluripotency genes, *Bioorg. Med. Chem.,* REFEREED 20, 2012, 2656-2660.

〔学会発表〕（計 6 件）

(1) Ganesh N. Pandian *et al.*, Synthetic small molecules to distinctively trigger transcriptional activation of pluripotency and germ-cell genes in a somatic cell, January 16 - 18, Poster presentation in iPS cells in drug

discovery and development/ CiRA international symposium, 2014, Osaka, Japan.

(2) Ganesh N. Pandian *et al.*, DNA-based Epigenetic Switches to Control Cell Fate, November 13-15, Poster presentation in 40th International Symposium on Nucleic Acid Chemistry, 2013, Yokohama, Japan

(3) Ganesh N. Pandian *et al.*, Novel chemical approach for cellular reprograming, June 19 - 21, Oral presentation in 8th Annual Meeting of the Japanese Society for Chemical Biology, 2013, Tokyo, Japan.

(4) Ganesh N. Pandian *et al.*, Targeting small molecules as transcriptional activators for selective induction of pluripotency genes in somatic fibroblasts. **Won Biomaterials Science Poster Award (First Prize)** in RSC-iCeMS Joint International Symposium: Cell-Material Integration and Biomaterials Science, March 18-19, 2013, Kyoto, Japan.

(5) Ganesh N. Pandian *et al.*, Artificial epigenetic switches to selectively reprogram the transcriptional machinery conferring to cell fate. World Stem Cell Summit-2012, December 1-3, 2012, Palm Beach, FL, USA.

(6) Ganesh N. Pandian *et al.*, Synthetic genetic switches for selective induction of pluripotency in mouse and human fibroblasts. **SPOTLIGHT TALK and won CROSS-DISCIPLINARY RESEARCH PRIZE** at 4th annual symposium of Institute for integrated cell-material sciences (iCeMS). August 31-September 1, 2012, Osaka, Japan.

〔図書〕(計 1 件)

(1) Ganesh N Pandian and H. Sugiyama, Targeted editing of therapeutic genes using DNA-based transcriptional activators: Scope and challenges in Chemical Biology of Nucleic Acids: Fundamentals and Clinical Applications ed. by Volker A. Erdmann, Wojciech T. Markiewicz, and Jan Barciszewski REFEREED 2014, 19, 347-365.

〔その他〕

Invited Talks

(1) Ganesh N. Pandian, Induced pluripotent stem cells and Genetic switches, Stem Cell Research Laboratory, Government Stanley Hospital, Tamil Nadu, India, 21 Sep. 2013.

(2) Ganesh N. Pandian, Cellular reprogramming - A chemical approach, Madurai Kamraj University, Tamil Nadu, India, 18 Sep. 2013.

(3) Ganesh N. Pandian, Cellular time machine: Back to the future, Kalasalingam University, Tamil Nadu, India, 17 Sep. 2013.

(4) Ganesh N. Pandian, Genetic switches to control cellular time machine, Lady Doak College, Tamil Nadu, India, 13 Sep. 2013.

ホームページ等

- 日刊工業新聞「京大、“人工スイッチ”開発−細胞の遺伝子発現を制御」(2014 年 2 月 3 日 17 面)
- GEN News「遺伝子発現スイッチ、眠った DNA の目覚ましに」(2014 年 1 月 31 日 [ウェブ])
- マイナビニュース | Yahoo!Japan ニュース「京大、狙った DNA に結合する化合物で細胞の遺伝子発現の制御に成功」(2014 年 1 月 28 日)
- nano.com「iCeMS 研究者ら、DNA に基づく化合物を作製、細胞の遺伝子発現コントロール」(2014 年 1 月 27 日 [ウェブ])
- 朝日新聞「遺伝子のスイッチ、直接入れることに成功 京大グループ」(2014 年 1 月 25 日 8 面)
- 中日新聞「遺伝子使わず iPS か、京大、化合物加え似た細胞」(2014 年 1 月 25 日 3 面)
- 京都新聞「狙った遺伝子 制御、京大グループ成功」(2014 年 1 月 25 日 28 面)
- 日本経済新聞「眠れる遺伝子働かせる、京大が化合物、ｉＰＳ応用も。」(2014 年 1 月 25 日 42 面)
- 産経新聞「皮膚に化合物加えｉＰＳに似た細胞 京大チーム作製」(2014 年 1 月 25 日 28 面)
- 読売新聞「特定遺伝子 スイッチ ON、化合物で活性」(2014 年 1 月 25 日 34 面)
- 47 News「皮膚に化合物加えｉＰＳに似た細胞 京大チーム作製」(2014 年 1 月 24 日)

６．研究組織

(1)研究代表者

ナマシヴァヤム パンディア (NAMASIVAYAM Ganesh Pandian)

京都大学 物質－細胞統合システム拠点 (iCeMS) 特定助教

研究者番号：20625446

(2)研究分担者

杉山 弘 (SUGIYAMA Hiroshi)

京都大学 理学研究科 教授

研究者番号：50183843

板東俊和 (BANDO Toshikazu)

京都大学 理学研究科 准教授

研究者番号：20345284