様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

平成　３０　年　　６　月　２４　日現在

機関番号：　１４３０１
研究種目：　挑戦的萌芽研究
研究期間：　2016～2017
課題番号：　１６Ｋ１２８９６

研究課題名（和文）ナノ粒子ベース人工転写因子を用いた弓矢リプログラミング法による心筋細胞の誘導

研究課題名（英文）Induction of cardiomyocyte differenciation using a nano-particle based artificial transcription factor

研究代表者

ナマシヴァヤム　パンディアン（Namasivayam, Pandian）

京都大学・高等研究院・助教

研究者番号：２０６２５４４６

交付決定額（研究期間全体）：（直接経費）　　2,700,000円

研究成果の概要（和文）：我々の目的は人工転写因子により遺伝子発現を制御し、弓矢リプログラミング法により臨床応用可能な心筋細胞の分化誘導を行うことである。SOX2の結合阻害を狙ったピロールイミダゾールポリアミド(PIP)はiPS細胞において中胚葉分化を促進し、さらにWntシグナル阻害剤との併用により拍動心筋細胞が得られた。また心筋細胞には多くのミトコンドリアが含まれていることから、ミトコンドリア標的PIPを開発した。さらに特定のタンパクドメインに結合できる化合物をPIPに導入することで、塩基配列依存的な遺伝転写活性化剤を報告した。これらはナノ粒子を用いた人工転写因子に応用することで、更なる機能向上が期待される。

研究成果の概要（英文）：Harnessing the chemical biology of nucleic acids, our aim was to successfully carry out bow-arrow reprogramming to achieve clinically useful cell types like cardiomyocytes (CMs) by switching ON and OFF the key transcription factors (TFs) using artificial TFs. We have successfully achieved the first-ever synthetic DNA-binding inhibitor of SOX2, which not only suppressed the pluripotency program in the hiPSCs but also activated the cardiac mesoderm program to generate functional spontaneously contracting CMs when supplemented with a Wnt-signalling inhibitor. Considering the presence of numerous mitochondria in CMs, we also created a ligand for mitochondrial gene modulation. Also, we programmed synthetic molecular codes capable of inducing targeted transcriptional activation of therapeutically important developmental genes in human somatic cells. Current studies with NANO-TFs, the nanoparticle version of synthetic-TFs and distinct bioactive peptides suggest enhanced bioefficacy.

研究分野：　ケミカルバイオロジー

キーワード：　ピロールイミダゾールポリアミド　人工転写因子　金ナノ粒子　分化誘導

１．研究開始当初の背景

Cardiovascular disease is a leading cause of morbidity and mortality worldwide. Cardiomyocytes (CM) are the critical component that makeup the atria and ventricle of the heart. Damaged CMs are difficult to repair as they lose their capacity for proliferation and regeneration shortly after birth, making it unavailable later in life. Until now, heart transplantation using donor organs is the only definitive therapeutic option. Cellular reprogramming strategies like the use of induced pluripotent stem (iPS) cells overcome the ethical problems associated with embryonic stem cells, however, several barriers including the epigenetic memory and variable cell quality hinder the clinical translation. The adult CMs possesses numerous mitochondria occupying at least 30% of their cell volume, which are prone with population variability and are associated with the heart failure (*Nat. Rev. Cardiol.* 2017. *14*, 412). The modern sequencing techniques have been revealing the CM-associated transcription factors (TFs). However, the current genetic and trans-gene free strategies to generate CMs through modulation of specific TFs are yet to have clinical utility because of several drawbacks including the reprogramming efficiency, time and tumorigenic risk. Therefore, there is a growing demand to develop a clinically useful synthetic strategy for controlled reprogramming and differentiation.

２．研究の目的

In nature, TFs have DNA-binding domain (DBD) that selectively recognizes specific DNA sequences and multi-functional components for modulating several factors including the epigenetic enzymes. As a novel chemical approach to achieve selective gene induction through site-specific chromatin modifications, we developed a novel class of small molecule called SAHA-PIP by conjugating the epigenetic modifier like SAHA with sequence-specific pyrrole-imidazole polyamide (PIP) [*Angew. Chem. Int. Ed.* 2013, *52*, 13410). However, lack of step-wise modulation was a major concern. Targeted transcriptional regulation using PIP-based TF-mimics is not straightforward because of the epigenome and the requirement of several artificial effectors such as small molecules for concomitant ON and OFF of the multiple genes at the right place and time. Encouraged with the initial success with the SAHA-PIPs to trigger targeted transcriptional activation, we proposed to harness the chemical biology of nucleic acids and construct next generation TF mimics called NANO-TFs. NANO-TFs are expected to mimic the structure and function of the natural TFs to generate clinically useful cell types like the CMs (***Fig. 1***).

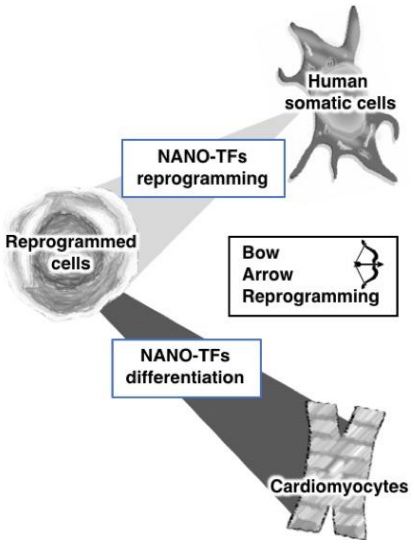


Figure 1. *Illustration of proposed plan to achieve Bow and Arrow reprogramming by constructing next-generation synthetic TFs (NANO-TFs) for reprogramming and differentiation.*

３．研究の方法

There is an exponential increase in the knowledge of TFs like *SOX2* and *GATA4* whose timely modulation could generate cardiomyocytes (CM). Our approach is to construct DNA-based programmable synthetic molecular codes that could localize and alter therapeutically important TFs inside the living cells. To this end, we created distinct PIPs and their conjugates having variable bioactivity. We have previously shown that the conjugation of PIP 'I' with SAHA and CTB could notably activate the pluripotency gene network (*Angew. Chem. Int. Ed.* 2015, *54*, 8700). To explore the potential as the pluripotency inhibiting NANO-TF, we created a new type of pluripotency inhibiting C646-PIP-I by conjugating the known histone acetyl transferase (HAT) inhibitor C646 (***Fig. 2A***). Likewise, we prepared the distinct PIP 'L' (***Fig. 2B***) and conjugated them with the epigenetic modulators, histone deacetylase (HDAC) inhibitor SAHA or HAT activator CTB.

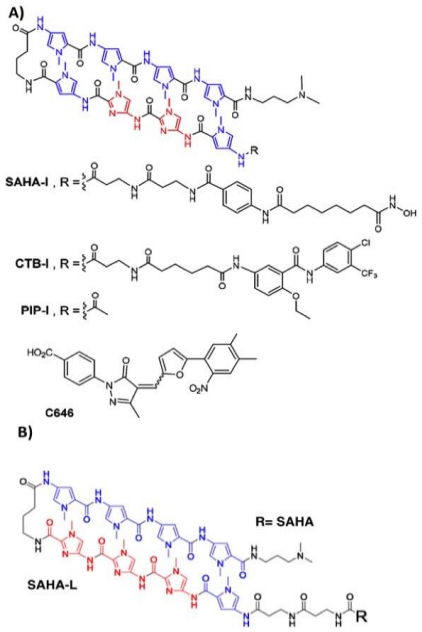


Figure 2. A) *Chemical structures of the HDAC inhibitors SAHA and SAHA-I, HAT activators CTB and CTB-I, and HAT inhibitor C646.* ***B)*** *Structure of PIP 'L'.*

We treated the PIPs in respective optimal conditions and isolated RNA using RNeasy MINI Kit. Micro array studies were carried out using either SurePrint G3 Human GE v2 8x60K Microarray (Agilent Technologies, USA) or Human Gene 2.1 ST Array (Affymetrix, USA). Functional analysis of the effector-modulated genes was performed using ingenuity pathway analysis (IPA™). Fischer's exact test was employed to measure the p-value. Quantitative RT-PCR analysis was used to validate the bioactivity of the PIPs and to further optimize the treatment conditions. Surface Plasmon Resonance assay was used to evaluate the binding affinity of the effectors towards promoter sequence. Chromatin immunoprecipitation sequencing (ChIP-Seq) analysis was performed with the specific antibody. Enriched libraries were sequence with Ion PGM sequencer using Ion PGMTM template 200 kit v2 and 316/318 chip. *In vitro* ChIP-qPCR assay was performed using HeLa nuclear extract. Live cell imaging was performed using CellLight Mitochondria-GFP, BacMam 2.0 (ThermoFisher Scientific), imaged by the FV1200 Laser Scanning Microscope and analyzed using FV10-ASW (Olympus).

Harnessing the accumulated information about the key TFs associated with cardiac differentiation, we also created a designer ligand 'PIP-S2' targeting the 5'-CTTTGTT-3' sequence to inhibit the key TF i.e., *SOX2* (***Fig. 3A***). As indicated in our proposal, we also expanded our study by creating another synthetic DNA binding inhibitor, 'PIP-RBJ-1' targeting the neurogenesis-associated notch signaling TF i.e., *HES1* (***Fig. 3B***).

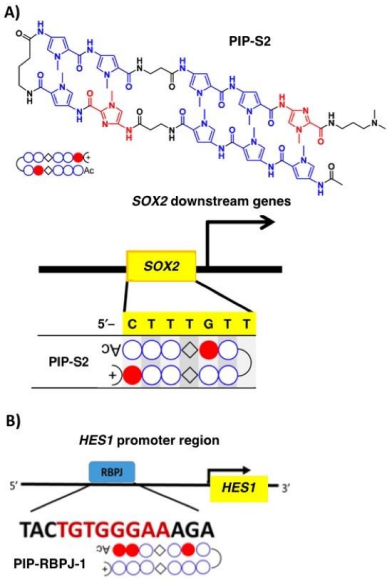


Figure 3. A) *PIP-S2 targeting the consensus sequence of a SOX2 binding site and* ***B)*** *PIP-RBJ-1 targeting the RBPJ-binding site in the promoter region of HES1.*

４．研究成果

Next-generation TFs for reprogramming

To achieve the precise orchestration observed in the natural cellular environment, there is a need to target multiple factors. But, for clinical translation, synthetic small molecules and the employment of fewer factors are preferred. Until now, no single small molecule has been known to trigger the transcriptional activation of multiple genes associated with autism spectrum disorders. While using SAHA-L as the control for pluripotency PIP, we identified SAHA-L as the first-ever multi-target small molecule capable of inducing transcription programs associated with the human neural system. Ingenuity pathway analysis showed that SAHA-L exerts a similar bioactivity of in a somatic and a pluripotent cell type and activated the critical genes like REELIN that are key components of autism spectrum disorders. *(Published in ChemistryOpen,* 2016, *5*, 517). We also evaluated the reactivity of synthetic small molecules in nucleosome architecture by conducting *in vitro* studies on nucleosomes with core and linker DNA using sequencing gel electrophoresis *(Published in Chem. Eur. J.*

2016, *22*, 8756). Our predicted working model about dual functionality of the sequence-specific HAT inhibitor C646-PIP-I, i.e., sequence-specific DNA binding and HAT inhibitory activity got substantiated using thermal stability assay and HAT activity assay, respectively (Published in *Eur. J. Med. Chem.* 2017, *138*, 320).

Considering the recent surge in the evidence linking mitochondrial genome and heart failure, there is an increasing demand to devise new strategies to modulate mitochondrial gene transcription. We designed first-ever mitochondrial gene switch termed MITO- PIPs to target the known binding site of a mitochondrial transcription factor TFAM in LSP (light stand promoter) and HSP1 (heavy strand promoter-1). Biological evaluation studies in HeLa cells showed that MITO-PIP-LSP triggered targeted suppression of mitochondrial encoded NADH dehydrogenase 6 (*ND6*) that is located downstream of LSP (***Fig. 4A***). Live cell-imaging studies using MITO-PIP labeled with TAMRA showed the efficient localization only in the mitochondria and not the nuclei (***Fig. 4B***). *(Published in J. Am. Chem. Soc.* 2017, *139, 8444 as FRONT COVER and JACS SPOTLIGHT ARTICLE and got highlighted in > 30 Science Portals including ACS Chemical and Engineering News, `Designer molecule silences mitochondrial genes` Vol.95 Issue 30 / p. 7 / July 24, 2017*

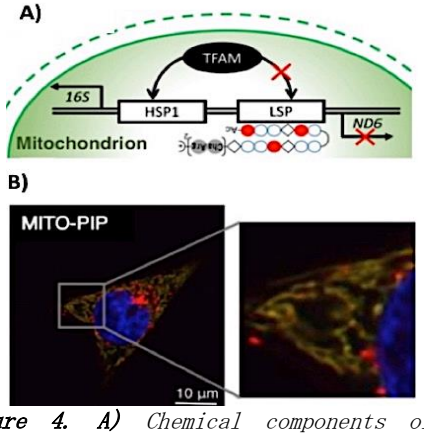


Figure 4. A) *Chemical components of the mitochondrial gene switch termed MITO-PIP and their localization inside mitochondria to target LSP.* ***B)*** *Live cell imaging fluorescent agent TAMRA-labeled MITO-PIP.*

Recently, we developed a unique strategy to introduce acetylation by constructing a bifunctional molecule designated Bi-PIP. Bi-PIP has a P300/CBP-selective bromodomain inhibitor (Bi) as a P300/CBP recruiter. Biochemical assays verified that Bi-PIPs recruit P300 to the nucleosomes having their target DNA sequences and extensively accelerate acetylation. (Published in *J. Am. Chem. Soc.* DOI: 10.1021/jacs.8b01518*).*

<u>Next-generation TFs for differentiation</u>

Despite the availability of several chemical inhibitors targeting protein-protein interactions, no synthetic DNA-binding inhibitors targeting key TFs like *SOX2*, are not available. Genome-wide gene expression analyses and Q-RT-PCR analysis revealed that our designer ligand PIP-S2 inhibited the pluripotency genes and ensued the induction of mesoderm markers including GATA4 by targeted alterations in SOX2-associated gene regulatory networks (***Fig. 5A***). Also, employment of PIP-S2 along with a Wnt/β-catenin inhibitor successfully generated spontaneously contracting cardiomyocytes (***Fig. 5B***) and the efficiency got increased with culturing on the Matrigel (***Fig. 5C***).

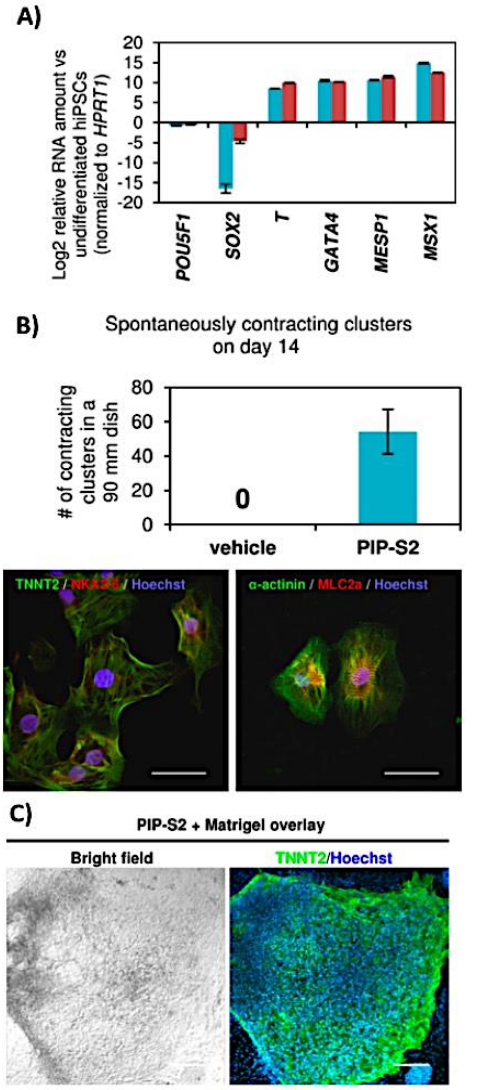
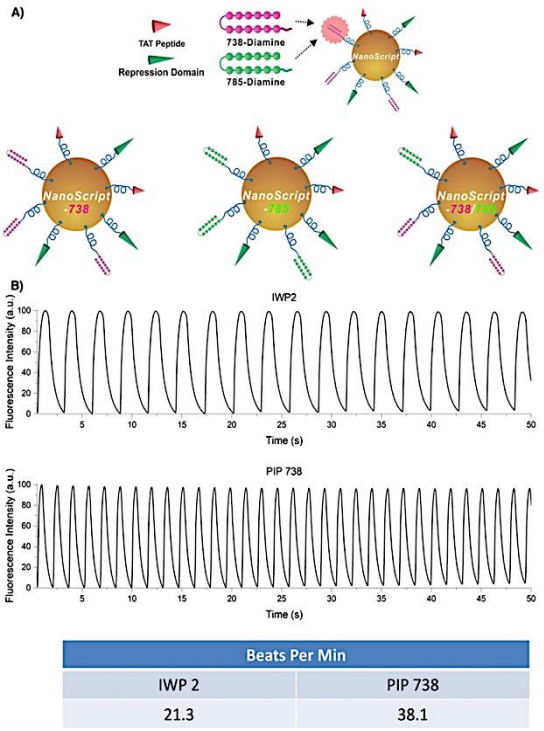


Figure 5. A) *RT-qPCR analysis of 201B7-hiPSCs treated with PIP-S2. Relative RNA amount (vs undifferentiated 201B7-hiPSCs) is shown in log. scale.* ***B)*** *Spontaneously contracting clusters counted on day 14. The mean ± SEM of three 90 mm culture dishes is indicated. Immunohistochemistry of cardiomyocyte markers in differentiated 201B7-hiPSCs with PIP-S2. On day 16, spontaneously contracting clusters were picked up, dissociated, and plated on cover glasses. Staining was performed on the next day. Scale bars; 50 μm.* ***C)*** *Immunocytochemistry after the treatment of PIP-S2 + Matrigel overlay (day 20).*

While in our proposal, we expected to have two different designer NANO-TFs to achieve CMs. Our data suggested that a single DNA-binding inhibitor could drive the directed differentiation of hiPSCs (Published in *Nucleic Acids Res.* 2017, *45*, 9219) and got Media coverage > 20 Science Portals including a Highlight in Asian Scientist, `Making stem cells to switch to muscle`. Encouraged with the success of synthetic DNA-binding inhibitor in cardiac differentiation, we expanded our work and created the first-ever synthetic DNA-binding inhibitor to gain chemical control over neurogenesis. This inhibitor called PIP-RBPJ-1 was designed by harnessing sequence information to target the binding site of a transcription factor called RBPJ is located in the promoter region of the critical gene *HES1*. PIP-RBPJ-1 successfully generated Tuj1 active neurons with longer neurite outgrowth with an efficiency that was comparable to that of the conventional protocol, suggesting the potential of this DNA- binding inhibitor to induce targeted differentiation (Published in *ACS Omega,* 2018, *3*, 3608)

The critical bottleneck in achieving the clinically useful CMs is the maturation stage. To this end, we created designer PIPs 738 and 785 targeting the key motif associated with the beta-Catenin/TCF. Encouraged with the results attained with synthetic DNA-binding inhibitor, we sequentially assembled the distinct PIPs as DNA-binding domains (DBDs) along with the transcription recruiting TAT peptide and repressor domain (WRPW peptide) on to the magnetic core-shell nanoparticles (MCNPs) (***Fig. 6A***). Biological evaluation studies including the calcium imaging studies and the FESEM (Field Emission Scanning Electron Microscope) studies suggested enhanced bioefficacy. In particular, PIP-738-treated cells showed enhanced maturation than that compared with those attained with the control IWP-2 treatment (***Fig. 6B***). Taken together, our studies suggested the potential of our synthetic strategy to generate clinically useful CMs, which will be validated with the ongoing studies.



Beats Per Min	
IWP 2	PIP 738
21.3	38.1

Fig. 6. A) *Schematic illustration of the nanoparticle-based TF constructed with the PIP 738 and 785 targeting the key motif associated with the beta catenin and TCF,* ***B)*** *Calcium transient analysis and representative recording of beats per minute in the PIP-738 treated cells and control IWP2 treated cells.*

５．主な発表論文等

〔雑誌論文〕（計 ８ 件）

(1) J. Taniguchi, Ganesh N. Pandian, T. Bando, H. Sugiyama *et al.*, Biomimetic artificial epigenetic code for targeted acetylation of histones, *J. Am. Chem. Soc.*, REFEREED 2018. DOI: 10.1021/jacs.8b01518

(2) Y. Wei, Ganesh N. Pandian, T. Bando, H. Sugiyama *et al.*, Synthetic DNA-binding inhibitor of HES1 alters the Notch signalling pathway and induces neuronal differentiation, *ACS Omega.* REFEREED 3, 2018, 3608-3616. DOI: 10.1021/acsomega.8b00220

(3) J. Taniguchi, Ganesh N. Pandian, T. Bando, H. Sugiyama *et al.*, A synthetic DNA-binding inhibitor of SOX2 guides human induced pluripotent stem cells to differentiate into cardiac mesoderm,

Nucleic Acid. Res., REFEREED 45, 2017, 9219-9228. DOI: 10.1093/nar/gkx693.

(4) T. Hidaka, Ganesh N. Pandian, T. Bando, H. Sugiyama *et al.*, Creation of a Synthetic Ligand for Mitochondrial DNA Sequence Recognition and Promoter-specific Transcription Suppression, *J. Am. Chem. Soc.* REFEREED 139, 2017, 8444-8447. DOI: 10.1021/jacs.7b05230

(5) Z.Yu, J.Taniguchi,Y. Wei, Ganesh N. Pandian, T. Bando, H. Sugiyama, *et al.*, Antiproliferative and apoptotic activities of sequence-specific histone acetyl transferase inhibitor., *Eur. J. Med. Chem.* REFEREED 138, 2017, 320-327. DOI: 10.1002/cbic.201402497

(6) Ganesh N. Pandian, H. Sugiyama, Nature-inspired design of smart biomaterials using the chemical biology of nucleic acids, *Bull.Chem.Soc.J,* REFEREED 89, 2016, 843-868. DOI: 10.1246/bcsj.20160062

(7) Y. Wei, Ganesh N. Pandian, T. Bando, H. Sugiyama et al., A multi-target small molecule for targeted transcriptional activation of therapeutically significant nervous system genes, *ChemistryOpen,* REFEREED 5, 2016, 517-521. DOI: 10.1002/open.201600125

(8) T.Zou, S. Kizaki, Ganesh N Pandian, H. Sugiyama *et al.*, Nucleosome assembly alters the accessibility of the antitumor agent duocarmycin B2 to duplex DNA, *Chem.Eur.J.*, REFEREED 22, 2016, 8756-8758. DOI: 10.1002/chem.201600950

〔学会発表〕(計 1 件)

(1) Y. Wei. Targeted stem cell differentiation, November 10 - 12, The International Society for Stem Cell Research (ISSCR)-Guangzhou 2017, China.

〔その他〕

Invited Talks (Selected)

(1) Ganesh N. Pandian, Smart genetic switches for therapeutic gene modulation, Spring 2018 Colloquium Series at Rutgers University, NJ, USA, January 16, 2018.

(2) Ganesh N. Pandian, Creation of a designer molecule to target and silence mitochondrial gene transcription, 8th World Congress on Targeting Mitochondria, Berlin, Germany, October 23-24, 2017.

(3) Ganesh N. Pandian, Smart transcription factors, AO Research Institute-Lecture Series, Switzerland, August 9, 2017.

(4) Ganesh N. Pandian, Nature-inspired smart genetic switches, 7th Indian JSPS Alumni Association (IJAA) International Conference on Science and Technology: Future Challenges and Solutions (STFCS-2016), Mysore, India, August 08-10. 2016.

プレスリリース

特定の場所の遺伝子を活性化できる新しい分子を開発
http://www.kyoto-u.ac.jp/ja/research/research_results/2018/180525_1.html

ミトコンドリア内の遺伝子発現を制御 -遺伝性疾患に治療の可能性拓ける-
http://www.kyoto-u.ac.jp/ja/research/research_results/2017/170616_1.html

遺伝子を直接制御する合成分子で組織再生の道が開ける http://www.kyoto-u.ac.jp/ja/research/research_results/2017/170731_2.html

Designer molecule silences mitochondrial genes
https://cen.acs.org/content/cen/articles/95/i30/Designer-molecule-silences-mitochondrial-genes.html

Making Stem Cells Switch To Muscle
https://www.asianscientist.com/2017/10/in-the-lab/synthetic-molecule-tissue-regeneration/

京大、特定の場所の遺伝子を活性化できる新しい分子を開発－遺伝子活性異常が引き起こす病気の研究に応用
https://www.nikkei.com/article/DGXLRSP480703_V20C18A5000000/

６．研究組織

(1)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 高等研究院 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