様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

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研究課題名（和文）CRISPR/Cas9 mediated identification of novel gene targets for XPA-associated skin cancer using in vitro reconstituted skin from iPS cells

研究課題名（英文）CRISPR/Cas9 mediated identification of novel gene targets for XPA-associated skin cancer using in vitro reconstituted skin from iPS cells

研究代表者

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研究成果の概要（和文）：皮膚がんは、組織の幹細胞に突然変異が蓄積されることで発症するが、その進行の理解は未だ不十分であり、治療法の検討を難しいものにしている。そこで本研究では、紫外線誘発突然変異の新規ターゲットを同定するために、iPS細胞と遺伝子編集技術を利用した。XP患者由来のiPS細胞を入手し、XPA変異を修復して同等の遺伝的背景を持つ対照細胞を作製した。次いで、前述の細胞をCRISPR/Cas9を用いて腫瘍抑制遺伝子を削除できるように遺伝子操作した。この細胞より分化した皮膚ケラチノサイトに紫外線による突然変異誘発を行うことで、がん化が誘導され、がんの原因となる突然変異をNGSにより同定できる可能性がある。

研究成果の学術的意義や社会的意義
The aim of this work is to generate a model of skin cancer in vitro. The model permits to emulate the acquisition of cancer driver mutations and may allow design custom-made individual patient therapies, compound screening and drug development thus reducing the use of animals for experimentation.

研究成果の概要（英文）：Skin cancer develops as a result of the accumulation of mutations in stem cells of the tissue. Exposure to ultraviolet light leads to accumulation of mutations in the skin; however, the understanding of how skin malignancy progresses is poor and imposes a bottleneck to designing efficient treatments to overcome the disease.
In order to identify novel oncogenes and tumor suppressor genes as targets of UV-induced mutagenesis, I took advantage of iPSCs and gene editing technologies. Xeroderma pigmentosum (XP) patient-derived iPSCs were obtained. The XPA mutation was repaired in order to generate isogenic control cells. Then, I engineered genetically the aforementioned cells to be able to delete tumor suppressor genes using CRISPR/Cas9 nuclease. Application of UV-induced mutagenesis in conjunction with CRISPR/Cas9 in iPSC differentiated skin keratinocytes may allow to induce cell transformation and identify cancer causative mutations using massive parallel sequencing.

研究分野：Skin differentiation, gene editing

キーワード：Human iPSCs　CRISPR/Cas9　Squamous Cell Carcinoma　Skin differentiation　Xeroderma pigmentosum

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

Xeroderma pigmentosum (XP) is an autosomal recessive disorder caused by mutations in components of the Nucleotide Excision Repair (NER) pathway, which detects and restores UV light-induced mutations in the genome. XP has an elevated frequency in Japan of about 1:22,000, being the *XPA* gene predominantly mutated in 50% of the cases. Patients carrying mutations in the *XPA* gene are prone to develop basal cell carcinomas (BCCs) or cutaneous squamous cell carcinomas (cSCCs) by the simple exposure to sunlight.

XP-associated cSCC is a prototypical model of cancer caused by somatic mutations in the epidermal stem cells. Approximately, 90% of cSCC patients accumulate mutations in the tumor suppressor *TP53*. However, loss of function in other tumor suppressors such as *NOTCH*, *PTEN* and *CDKN2A* and gain of function in oncogenes such as *RAS, c-Myc*, *FYN* and *EGFR* are also involved in skin cancer development. Additional acquisition of mutations promotes malignant progression, thereby generating metastatic cancers, however, driver genes involved in malignant progression are largely unknown. Therefore, it is important to establish a model for XP-associated cSCC using human keratinocytes to analyze genotype-phenotype causal relations.

２．研究の目的

Xeroderma pigmentosum (XP) is an inheritable disease with high incidence in the Japanese population. XP patients are sensitive to sunlight with high propensity to develop skin cancer. However, genes involved in skin cancer progression are largely unknown. I will use keratinocytes differentiated from XPA patient-derived iPS cells to develop a model of skin carcinogenesis in vitro. Our experimental model will help identify novel driver mutations generated by UV-induced mutagenesis and allow the functional validation of genes/pathways that underlie skin transformation thus helping to find cures for this disease.

３．研究の方法

To elucidate genes/pathways required for the progression of skin cancer, I plan to establish an *in vitro* three-dimensional skin reconstitution model, which recapitulates a stratified squamous architecture using patient-derived XPA iPSCs. Using this model, I will perform UV-induced mutagenesis screening that will identify novel genes/pathways required for the skin cancer progression. My study will establish genetically engineered iPSCs that express Cas9 constitutively in order to ablate tumor suppressor proteins. Furthermore, the Cas9 expressing iPSCs will provide us with the readiness to validate driver genes that halt the progression of malignancy using single-guide RNAs (sgRNA) for genes identified in the mutagenesis screen.

Aims:

1. Establish XPA-patient derived iPSCs and their isogenic control cells that express CRISPR/Cas9 constitutively.
2. Generate and validate vectors containing sgRNAs to knock out tumor suppressor genes (*TP53, PTEN, NOTCH, CDKN2A*).
3. Differentiate and prepare 3D-reconstructed epidermis using the cells mentioned in Aim 1. Apply CRISPR/Cas9 to knock out tumor suppressor genes followed by UV-irradiation to induce mutagenesis.
4. Identify, analyze and validate functionally the mutations that conferred malignancy in the screen.

４．研究成果

XPA iPSCs were reprogramed from XPA-patient derived fibroblasts and were facilitated by Professor Kenji Kabashima, a collaborator in this project. The *XPA* IVS3AS G-C is a recessive mutation that leads to miss-splicing and loss of functional XPA protein, rendering cells unable to activate the DNA nucleotide excision repair pathway (NER). A key step in this project was the derivation of isogenic cell lines of the XPA patient derived iPSCs. XPA corrected isogenic cell lines provide with a genomic reference to compare the XPA genotype within the context of the same cellular background. Genome engineering allows to perform precise correction or creation of pathogenic mutations using the programmable endonuclease CRISPR/Cas9. The *XPA* IVS3AS G-C mutation was repaired using CRISPR/Cas9 and single stranded oligonucleotides (ssODN), thus generating isogenic control cells. *XPA* IVS3AS G-C mutation shows an autosomal recessive inheritance pattern, therefore, restoration of a single *XPA* allele was expected to revert the XP phenotype. The genotypes of the newly generated cell lines were verified

both by Sanger sequencing and by Southern blotting and confirmed the correction of the *XPA* locus. Two different XPA iPSCs lines obtained from distinct donors were targeted and successfully corrected. The restored expression of the XPA protein was confirmed by Western blot analysis. In order to test the NER function, cells were subjected to UV irradiation. UVC treatment (1 J.m-2) of normal, XPA and gene-corrected iPSCs generated DNA mutations that were identified using anti-cyclobutane pyrimidine dimers antibodies. Both normal and isogenic control iPSCs but not XPA-derived iPSCs were able to grow and form colonies upon UV-irradiation, thus confirming that DNA repair had being restored in XPA-corrected iPSCs. Nevertheless, XPA-derived iPSCs showed persistent colonies when treated with subacute UV-doses (Figure 1).

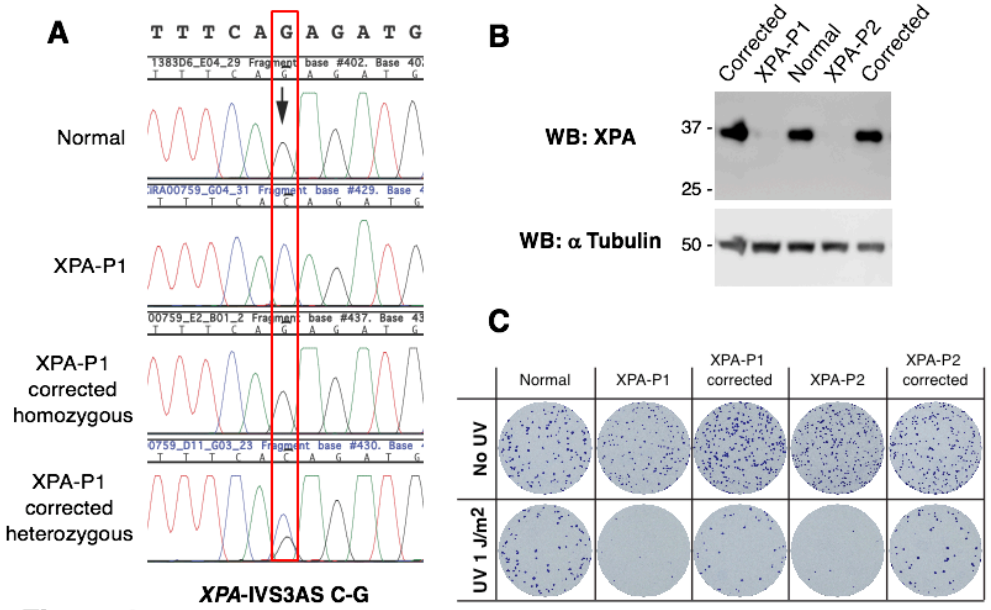

Figure 1

The *XPA* IVIS3AS G-C mutation, renders cells highly sensitive to UV-light exposure resulting in enhanced mutation rates. To take advantage of this phenomenon, I proceeded to create the pathogenic mutation IVIS3AS G-C in the *XPA* gene in normal 409B2 iPSCs. I used a combination of CRISPR/Cas9 nuclease with engineering of the microhomology-mediated end joining (MMEJ) repair pathway, a technique pioneered in our laboratory (Kim, Matsumoto, *et al.*, *Nat. Communications*. 2018). Importantly, the 409B2 cell line with de novo-created XPA mutation should retain a genomic background without accumulated mutations that serves as reference in NGS analyses when comparing UV-irradiated genomes obtained from patient and isogenic control iPSCs. UVC-exposure of iPSCs (1 J.m-2), both, in normal or XPA-created iPSCs, showed that XPA mutant cells were not able to grow after UV-irradiation in a colony formation assay, thus confirming that the NER function is impaired in these cells (Figure 2).

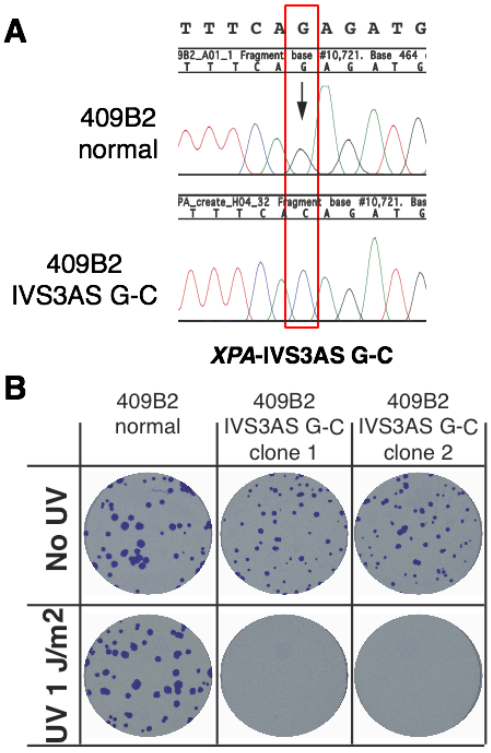

Figure 2

During the initiating stages of cancer evolution, acquisition of driver mutations that lead to clonal expansion and eventually confer to malignancy differ from passenger mutations that have no effect on cell survival. Nevertheless, the order of mutation acquisition is an important feature to understand the exact function of individual mutations during the development of cancer. In order to knock-out the expression of tumor suppressor genes and to validate driver genes that halt the

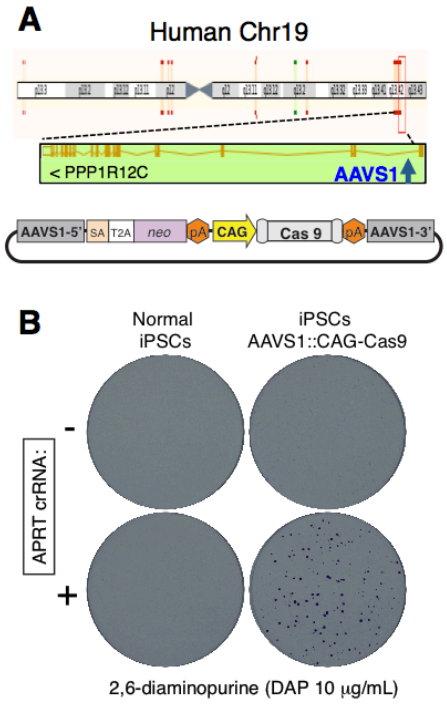

Figure 3

progression of malignancy in a systematic manner, I engineered XPA-patient derived iPSCs and their isogenic control cells to express Cas9 constitutively from the AAVS1 locus, according with the methodology described in my previous report (Oceguera-Yanez *et al.*, *Method*s. 2016). XPA and isogenic corrected iPS cell lines were targeted and the expression of Cas9 was verified. As proof of principle, I transfected AAVS1-CAG::Cas9 201B7 iPSCs with sgRNAs complementary to the *APRT* gene and observed the ability of *APRT* knocked-out cells to grow and form colonies in the presence of 2,6-diaminopurine (DAP), thus verifying the expression of Cas9 from the AAVS1 locus (Figure 3). I prepared vectors that drive the expression of sgRNAs complementary to the genomic regions encoding for the tumor suppressor genes *TP53*, *CDKN1A* (P21) and *CDKN2A* (P16INK4A). Transfection of these vectors into AAVS1-CAG::Cas9 normal or XPA iPSCs will be used to generate indels that halt the expression of tumor suppressors while UV-induced mutagenesis may allow for transformation in these cells.

In summary, during the term of this project I made use of iPS cell technology and utilized genome engineering to establish a complete line-up of patient-derived iPS cell lines and their respective genetically corrected isogenic control cells, and the tools and methods that will allow us to identify cancer causative mutations generated by UV-induced mutagenesis in skin equivalents prepared with keratinocytes differentiated from XPA iPSCs. The model will permit to design custom-made individual patient therapies, compound screening and drug development while allowing to reduce the need to use animals for experimentation.

５．主な発表論文等

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〔産業財産権〕

〔その他〕

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6．研究組織

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