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科学研究費助成事業　　研究成果報告書

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研究課題名（和文）膵がん幹細胞と微小環境の相互作用を担うMiTを断ち切る新規治療法の開発

研究課題名（英文）Targeting MiT transcription factors as a novel therapeutic approach to disru pt the adaptive response to microenvironmental stresses that gives rise to d ormant and cancer stem cell

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交付決定額（研究期間全体）：（直接経費）　　3,600,000円

研究成果の概要（和文）：最初の部分では、一般的なストレスに対する細胞の適応応答に関与するタンパク質が、癌細胞の生存と代謝および治療ストレス下での繁殖能力に重要な役割を果たすという最初の仮説を検証しました。ゲノミクスアプローチ(トランスクリプトミクスとゲノムワイド転写因子結合部位プロファイリング)を用いて、上記のタンパク質の機能に関連する分子経路の解明に着手しました。確立された同質遺伝子培養細胞株内に転写休眠細胞が存在することが初めて実証されました。これは、細胞の可塑性の概念を支持し、休眠細胞を標的にして患者を治療することの有効性を強調しています。

研究成果の学術的意義や社会的意義
Should this drug resistant dormant population that is responsible for relapse can be effectively targeted, we can improve overall survival, life quality of the patients (as they will need fewer rounds of chemotherapies) and reduce overall economic burden to the patient and society as a whole.

研究成果の概要（英文）：In the first part, I have verified the initial hypothesis that proteins involve in cellular adaptive response to prevailing stress plays crucial role in cancer cells survival and the ability to thrive under metabolic and therapeutic stress. Through the use of genomics approaches (transcriptomics and genome-wide transcription factors binding site profiling) I have begun to elucidate the underlying molecular pathways associated with the function of the proteins mentioned above.
The existence of transcriptionally dormant cells within established isogenic culture cell lines had been demonstrated for the first time. This support the concept of cellular plasticity and highlight the validity of targeting dormant cells to cure patient.

研究分野：Tumour biology

キーワード：TFEB　TFE3　PDAC　ISR　OSCAR　Transcription quescence　Dormancy

１．研究開始当初の背景

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most deadly of all cancers, having inadequate tools for early detection as well as **absence of effective novel therapeutics. Chemotherapies remain the standard of care for 80-90% of patients**, but relapse is almost always inevitable irrespective of the therapeutic scheme **owing in large part to the inherent cellular plasticity** and consequent phenotypic heterogeneity of PDAC cells. **As chemotherapeutic agents are mostly designed to preferentially target dividing cancer cells, these drugs are ineffective against the dormant or stem-like cancer cells**, phenotypic subpopulations believed to be responsible for relapse, sometimes decades after apparent successful therapeutic interventions. Little is known about the mechanism(s) of their generation, what triggers them to re-populate the tumour and what would be appropriate biomarkers for their detection. Our published and ongoing work in melanoma highlight the importance of cellular adaptive response to stress (metabolic and therapeutic) through activation of transcription factors TFE3/B and induction of integrated stress response (ISR) which lead to the generation of highly metastatic and drug resistance population. Given the highly desmoplastic nature of PDAC that is poorly vascularized, it stand to reason that PDAC tumours are highly reliant on TFE3/B-ISR response to adaptively adopt appropriate phenotypic state including the drug resistance dormant state to ensue overall survival of the cancer.

２．研究の目的

To understand if and how TFE3/B contribute to adaptive responses leading to generation of dormant and quiescence cells in PDAC.

To assess validity of the proposed novel therapeutic strategies against PDAC through targeting of TFE3/B function.

３．研究の方法

I applied a combination of molecular techniques including **siRNA**, **shRNA** knock-down (KD) and **CRISPR/Cas9** knock-out (KO) of TFE3/B in a number of PDAC cell lines with very different phenotype and performed metabolic, proliferation studies and sensitivity to current standard chemotherapies. Through a combination of **RNA-seq** and **ChIP-seq** I have profiled TFE3/B contribution to microenvironmental stress over time. Using **OSCAR reporter**, I demonstrated phenotypic heterogeneity and the presence of transcription quiescence cells (a hallmark of dormancy) even among isogenic population (Cell lines) and demonstrated that, at least in vitro, standard chemotherapy leads to an increased in this dormant population

4．研究成果

Constitute KD of TFE3 reduce expression of pro-proliferative genes.

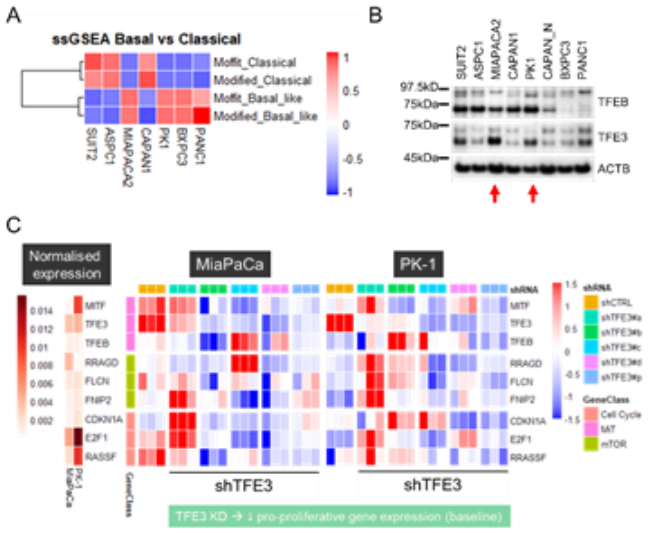

Figure 1. A. ssGSEA classification of commonly used PDAC cell lines. **B.** Western-blot of cell line panels in **A.** showing differential expression of TFE3 and TFEB. Equal number of cells were loaded in each lane. Red arrow highlight cell lines used to generate stable KD for further studies **C.** RT-qPCR of MIAPACA2 and PK-1 with TFE3 stable KD using shRNA

We and others have previously shown that panel of cell lines {Louphrasitthiphol, 2019 #2940}{Tsoi, 2018 #2753} can effectively be used to recapitulate distinct phenotypic state that constitute the phenotypic heterogeneity found in the tumour as cell line establishment and the culturing condition which they are maintained artificially fixed each cell line in a semi-stable cellular state. Using single-sample geneset enrichment (ssGSEA) we could clearly define the cell lines, based on their transcriptomes, as either Classical or the more aggressive Basal-like subtype {Moffitt, 2015 #5667} (Figure 1A). Western-blotting of transcription factor of the MiT family, TFEB and TFE3 of extract from equal number of cells (15k) reveal differential expression patterns (Figure 1B). To assess the biological role of TFE3/B, MIAPACA2 and PK-1 were subjected Lentiviral transduction of shRNA against TFE3, TFEB or both to generate cell line with stable KD of TFE3, TFEB or both. The KD cell lines were analysed using RT-qPCR to assess potential effect on key pro-proliferative genes of the mTOR and cell cycle, exemplified in Figure 1C. As expected based on our previous studies in melanoma {Louphrasitthiphol, 2020 #4439}, stable KD of TFE3 leads to reduction in expression of genes associated with cell proliferation only in cell lines where TFE3/B were effectively KD.

TFE3 constitutive KD impair cell cycle progression and confer increased resistance towards chemotherapies.

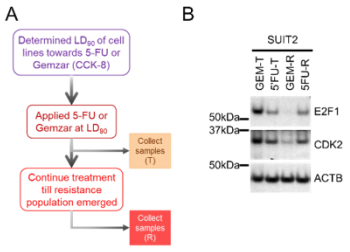

Figure 2. A. Schematic of generation of drug resistance cell state. **B.** Western-blotting of cell extract as depicted in **A.**

Our previous work in melanoma revealed phenotypic plasticity driven through altered expression of the MiT family member, MITF in response to metabolic stress {Vivas-García, 2020 #2944}. Slow cell cycling and reduced metabolic demand are some of the hallmark of drug resistance state. As proposed in this KAKENHI proposal, we expected PDAC that have acquired resistance towards antimetabolite chemotherapeutic compounds to have reduce cell cycling capacity. To validate this, we subject PDAC cell lines towards two commonly used chemotherapies at LD_{90} dose and collect protein sample for western-blotting analyses of cell cycle regulator as outlined in Figure 2A. As expected, acquired resistance towards Gemzar (Figure 2B, GEM-R) is associated with reduction in CDK2 and E2F1. 5-FU appeared to exert it effect even while on-treatment.

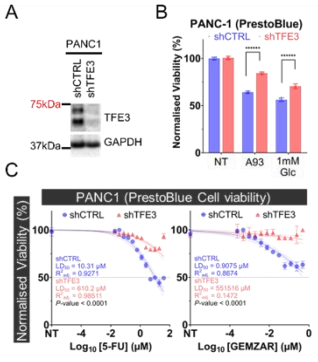

Figure 3. A. Western-blot of PANC1 transduced with indicated shRNA. **B.** PrestoBlue cell viability assay when subjected to additional metabolic stresses. C. Sensitivity towards 5-FU and Gemzar of the TFE3 KD PANC1 vs control.

Having demonstrated TFE3/B dependent reductions of pro-proliferative genes expression, I went on to assess how these might translate into changes in drug sensitivity. PANC1 cell lines with stable TFE3 KD (Figure 3A) were assess for changes in sensitivity towards metabolic stress expected to be relevant in poorly vascularized PDAC tumour (Figure 3B). As predicted, TFE3 KD PANC1 cells demonstrated enhance fitness when compare to shCTRL PANC1 cells. Similar results were observed with chemotherapeutic drugs 5-FU and Gemzar (Figure 3C).

Transient TFE3/B KD suppress activation of genesets associated with stress induced cell death

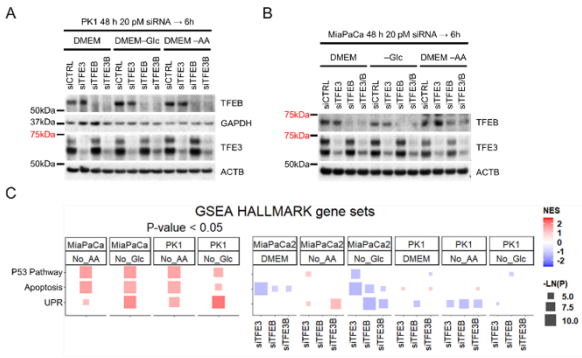

Figure 4. Western-blot validation of RNA-seq samples for PK-1 (**A**) and MIAPACA2 (**B**). **C.**GSEA of MIAPACA2 and PK-1 subjected to Glucose or amino-acid starvation ±siTFE3, siTFEB or both.

To understand how TFE3/B KD confers enhance resistance towards metabolic and therapeutic stresses, I subjected PK1 and MIAPACA2 cell lines ±siTFE3/B or both to glucose or amino-acid starvation (Figure 4A, B) and performed transcriptomics analyses (funded by John Fell Fund, Application no. 0010987) which revealed KD of TFE3/B leads to suppression of key stressed induced pathways (TP53 pathway, apoptosis and unfolded protein response (UPR)) which can induce cell death.

TFE3 and TFEB ChIP-seq reveals binding to ATF4 establishing direct regulation of the master regulator of ISR.

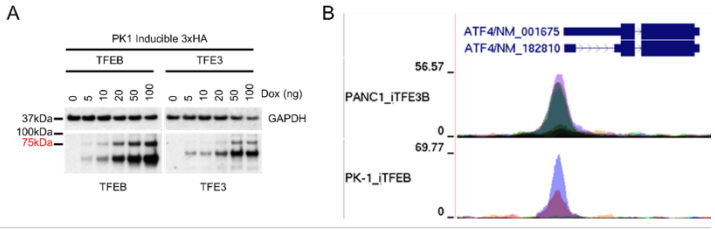

Figure 5. A. Western-blot validation of inducible TFEB and TFE3 PDAC cell lines. **B.** UCSC genome browser screenshot showing strong binding of TFE3 and TFEB to the promoter of ATF4.

To gain a comprehensive view of PDAC relevant regulatory network of TFE3 and TFEB, I generated cell line stably expressing doxycycline inducible 3xHA-tagged TFE3 and TFEB (Figure 5A) which we have previously used to successfully generated series of very high quality ChIP-seq dataset as well as having extensively characterised genome wide non-specific binding {Louphrasitthiphol, 2020 #2946}{Lu, 2021 #5647} and performed ChIP-seq experiments in 3 PDAC cell lines (Funded by Goodger and Schorstein Scholarship, Application no. 0011933). The data revealed direct binding to the promoter of ATF4, a key transcription factors that orchestrates the integrated stress response (ISR). Given that I have also shown that PDAC are sensitive to induction of ISR mediated cell death in response to induced proteotoxic stress (data not shown), these results render support for therapeutic targeting of TFEB/3 as a mean to sensitise PDAC to ISR induction, a prolonged of which induced cell death.

Ratiometric fluorescent reporter of dormancy, OSCAR reveal a stable presence of transcriptionally quiescence dormant cell even among isogenic cells grown under standard tissue culture.

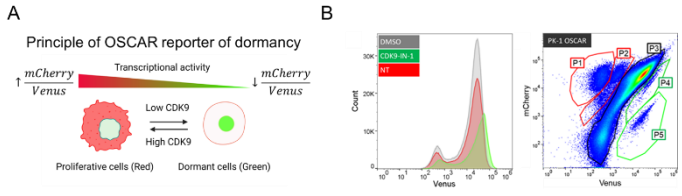

Figure 6 A. Schematic of the OSCAR ratiometric fluorescent reporter of cell phenotype. **B, left panel.** Flow-cytometry profile of the PK-1-OSCAR cell line treated with CDK9-IN-1 for 16 h or a DMSO control. A. Histogram of the Venus signal showing a right shift towards higher Venus fluorescence when treated with CDK9-IN-1. **B, right panel.** Venus-mCherry fluorescence profile, 5 potential populations are marked with polygons.

The laboratory of Prof. Goding recently published the establishment of an Optical Stem Cell Activity Reporter (OSCAR) that can serve as a generic fluorometric read-out of dormancy by reporting the activity of CDK9, a key kinase required for transcription elongation that is low in dormant cells {Freter, 2021 #5646}. The reporter is designed to express a 1:1 ratio of mCherry:Venus proteins. Phosphorylation of the inserted CDK9 phosphorylation motif on Venus, greatly diminishes Venus fluorescence and consequently high CDK9 (transcriptionally active) cells appear red. Conversely, low CDK9 (dormant) cells appear green due to slower turnover of nuclear-localised Venus (Figure 6A).

Using this concept, I delivered the OSCAR reporter into a number of PDAC cell lines and flow-cytometry analysis confirmed that reporter correctly reflects CDK9 activity and showed an increased in Venus fluorescence in the presence of a highly specific CDK9 inhibitor (CDK9-IN-1) (Figure 2A). Analysis of the fluorescence profile using flow-cytometry revealed rare side populations: high CDK9 red cells (P1 and P2) and low

CDK9 green cells (P4 and P5) in addition to the bulk of the population with equal green and red fluorescence (P3) (Figure 6B, left panel) that can be observed in variable ratios in different cell lines, with PK-1 having the most prominent side population. The observed heterogeneity is consistent with the long-standing concept of cancer cells dynamic heterogeneity where rare sub-populations are stochastically generated until equilibrium is established. This results will be used for further KAKENHI applications for comprehensive single cell characterisation using the LUTHOR 3' mRNA-Seq library prep kit, chosen as it is reported to yield comparable depth of information as bulk sequencing, far superior to the mainstream 10X pipeline, albeit at a higher cost per cell.

TFE3, TFEB and TFE3/B KO PDAC cell lines with integrated luciferase reporter of *in vivo* orthotopic model has been generated.

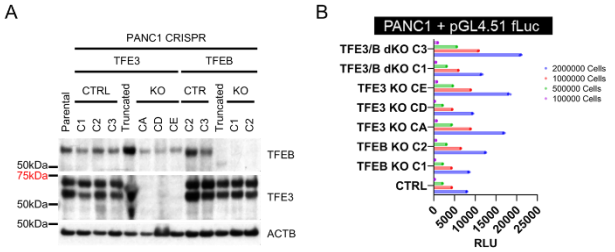

Figure 7. A. Western blot validation of CRISPR/Cas9 KO cell lines. **B.** Validation of luciferase reporter cell lines derived from **A.**

The result so far strongly support out initial hypothesis that targeting TFE3/B may provide a novel therapeutic avenue against dormant and cancer stem like subpopulation that give rise to relapse after apparently successful treatment. To further explore the feasibility of this I have generated TFE3, TFEB and TFE3/B double KOs (Figure 7A) engineered to stably express firefly luciferase (Figure 7B) where the luminescence signal directly correlate to cell number for future KAKENHI application for *in vivo* bioluminescence tracking of tumour growth as well as response to therapies.

５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関