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科研費
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研究課題名（和文）Physiological role of primary cilium-derived extracellular vesicles in fine-tuning signal transduction in target cells.

研究課題名（英文）Physiological role of primary cilium-derived extracellular vesicles in fine-tuning signal transduction in target cells.

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交付決定額（研究期間全体）：（直接経費）　　2,200,000円

研究成果の概要（和文）：我々は、一次繊毛由来細胞外小胞（pcEVs）が、標的細胞での効率的なシグナル伝達のための高濃度カーゴのキャリアーとして働くことを提唱している。その結果、細胞外小胞に暴露された細胞では、細胞増殖率や遊走率が上昇することが確認された。さらに、pcEVs曝露後の標的細胞のプロテオーム解析では、細胞増殖や代謝に関連するタンパク質が増加し、標的細胞の移動速度が増加するというこれまでの知見と一致することが示された。
さらに、pcEVs がどのような分子カーゴを運び、細胞の移動と増殖にどのような役割を果たすかを知るために、pcEVs と他の細胞外小胞を分離するフィルター精製の最適化も行った。

研究成果の学術的意義や社会的意義
These findings will provide a rationale to translate this knowledge for therapeutic strategy for pcEVs relevant cellular mechanisms such as chronic wound healing. Also, the pcEVs purification method will help overcome the challenge of separating the pcEVs and identify biomarkers for ciliopathies.

研究成果の概要（英文）：We propose that primary cilium-derived extracellular vesicles (pcEVs) act as carriers of a high concentration of cargo for efficient signal transduction in target cells. We observed an increase in cell proliferation and migration rates in cells exposed to pcEVs. Further more, proteomic analysis of the the target cells after exposure to pcEVs showed an increase in proteins related to cell migration which were in line with our findings of increased rate of migration in target cells.
Furthermore, in order to know the type of molecular cargo pcEVs carry and their role in cell migration and proliferation, we also optimized a filter purification to separate the pcEVs from the other extracellular vesicles. In addition stable lines expressing fluorescently-tagged pcEVs were established. Using these knock-in cell lines, we were able to visualize the uptake of pcEVs by target cells.

研究分野：Cell Biology

キーワード：Primary Cilium　Extracellular Vesicles　Wound Healing

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１．研究開始当初の背景

Extracellular vesicles (EVs) are the membrane vesicles released from the outer surface of the cells into their surrounding environment. These extracellular vesicles have been shown to carry a range of biological effectors such as functional proteins, DNA and RNA and have functions in many patho-physiological conditions. EVs composition and quantity changes under different physiological and pathological conditions, therefore understanding the fundamental processes about EVs production, cargo and target cell interaction holds promise for diagnosis and therapeutics.
Primary cilium has also been observed to release a part of its membrane into extracellular space refer to as primary cilium-derived extracellular vesicle (pcEV).Various ciliary and other signaling proteins are present in the pcEVs that are released into the extracellular space. What are the functions of ciliary proteins and other cargo in the pcEVs?

２．研究の目的

Recently it has been shown that mammalian pcEVs contain components of Sonic Hedgehog (Shh) pathway including Gli3 and Sufu proteins. We believe that pcEVs are the key determinants that fine-tune the signaling pathways such as Shh in acceptor cells. pcEVs act as vessels carrying signaling molecules for regulating cellular responses through robust signal transduction. Therefore, we will examine the novel role of pcEVs as signaling entities.

３．研究の方法

Specific Aim1: The novel role of pcEVs as carriers of signal transduction cargo
To examine whether pcEVs can change the Shh signaling in acceptor cells, we checked the expression of *Gli (Gli1 and Gli3) genes*. Following exposure of Kif3A-KO (lacks primary cilium; inability to produce pcEVs) cells to wild-type pcEVs or *Kif3A*-KO EVs, resulted cells total RNA was extracted and *Gli* genes expression was checked by RT-PCR.

Specific Aim2: Identification of pathways affected by pcEVs uptake in acceptor cells.
We also screened for broader changes in gene expression and cellular pathways in response to pcEVs. The NIH/3T3-*Kif3A*-KO cells were exposed to pcEVs derived from wild-type cells. Following exposure to pcEVs, total proteins were extracted for proteomics analysis.

Specific Aim3: Uptake of pcEVs and mechanism of signal transduction in acceptor cells
To compare the proteins composition of pcEVs accurately, a filter-based pcEVs isolation and purification method was established. In addition, stable cell lines expressing fluorescently labeled pcEV marker protein were generated using a novel 5'UTR knock in approach (Ijaz and Ikegami, 2021). Subsequently, the acceptor cells were exposed to the labeled pcEVs and the cellular uptake of labeled pcEVs was observed through florescence microscopy.

４．研究成果

1. In order to screen for the novel role of pcEVs as carriers of signal transduction cargo first we checked the activation/inactivation of Shh signaling pathway in response to pcEVs in target cells by. The results of RT-PCR demonstrated that there was no significant difference in the expression of Shh pathway genes Gli1 and Gli3 between pcEVs (WT-EVs) treated cells and EVs from primary cilium-deficient Kif3a-KO cells. (KO-EVs) treated cells (Fig.1). These results suggests that pcEVs does not induce hedgehog signaling in target cells.

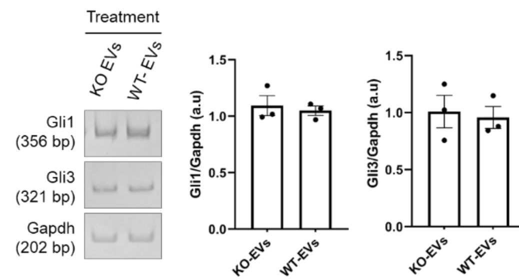

Fig.1: Expression of Shh pathway genes in response to pcEVs treatment.

Next, to explore the potential role of pcEVs in signal transduction other than the hedgehog pathway, wound healing and cell proliferation bioassays were performed where rate of cell migration and proliferation was determined respectively after treatment of target cells with pcEVs. The NIH/3T3 fibroblasts exposed to pcEVs derived from NIH/3T3 wild type cells (WT-EVs) showed enhanced migration than cells exposed to EVs from primary cilium-deficient Kif3a-KO cells (KO-EVs) (Fig. 2A and 2B). Similar results were obtained when the target cells were treated with pcEVs derived from mouse epithelial cell line IMCD3. In contrast, cell proliferation rate was decreased in cells exposed to pcEVs (WT-EVs) than cells exposed to EVs from primary cilium-deficient Kif3a-KO cells (KO-EVs) (Fig. 2C).

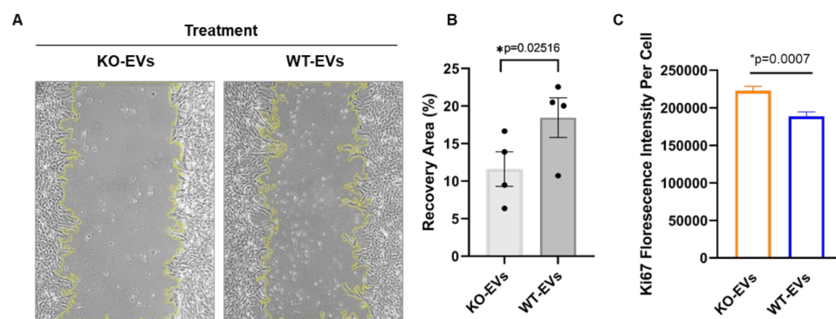

Fig.2: pcEVs regulate cell migration and proliferation in target cells (A) Images showing the area recovered by target cells during EVs treatment after scratch wound was made (B) Quantitative analysis of scratch wound assay in A (C) Quantitative analysis of cell proliferation assay.

2. To screen for broader changes in gene expression and cellular pathways in response to pcEVs, proteomics analysis of the target cells after treatment with pcEVs was done. Proteins related to cell adhesion, protein metabolism, stress response and transport were upregulated while proteins related with cell cycle and proliferation, cell organization were down regulated in the cells treated with pcEVs (WT-EVs) (Fig.3). The upregulation of cell adhesion proteins were consistent with the enhanced cell migration rates as a result of exposure to pcEVs as cell adhesion is involved in regulation of cell migration. Similarly, the downregulation of cell proliferation genes were consistent with the results of cell proliferation assays where cell proliferation decreased in cells treated with WT-EVs. The proteomics data was confirmed by checking the expression of selected proteins in target cells treated with pcEVs by western blotting.

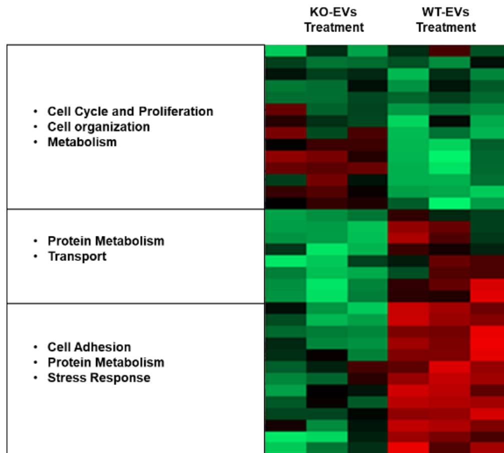

Fig.3: Heat Map of proteins expression changes in target cells after pcEVs treatment. Red color indicates proteins which are upregulated. Green color indicates proteins that are down regulated.

3. Next, for accurately determining the protein compositions and cargo molecules of pcEVs, we devised a method to separate pcEVs from other EVs fractions such as exosomes. With filtration-based pcEVs purification, CD81 and Alix positive exosomes were successfully separated from the pcEVs as shown in Fig.4. Arl13b was used as a marker for pcEVs detection. Arl13b is a primary cilium marker and is also present on pcEVs.

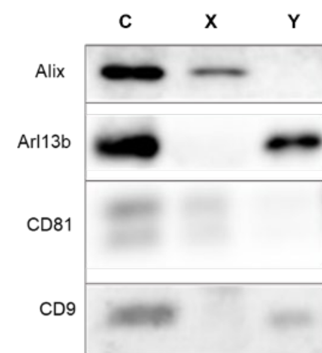

Fig.4: Blot showing the separation of pcEVs after filter purification. C: Total EV lysate, X: Fraction X, Y: Fraction Y

Furthermore, to visualize the uptake of pcEVs by target cells, stable cell lines expressing fluorescently labeled Arl13b-Venus were generated (Fig. 5). Subsequently, fluorescent pcEVs were collected from stable cell lines expressing Arl13b-Venus and target cells were exposed to these pcEVs. Fluorescent microscopy images showed that target cells can uptake pcEVs as shown in Fig.6.

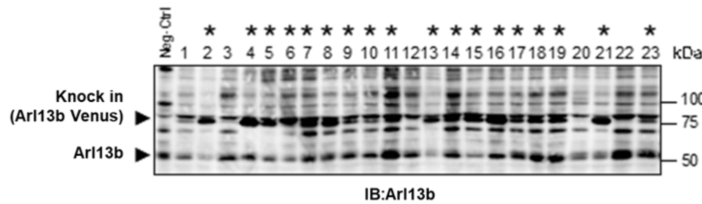

Fig.5: Blot confirming the knock-in of Arl13b-Venus into NIH/3T3 cells. Asterisk * indicates positive clones.

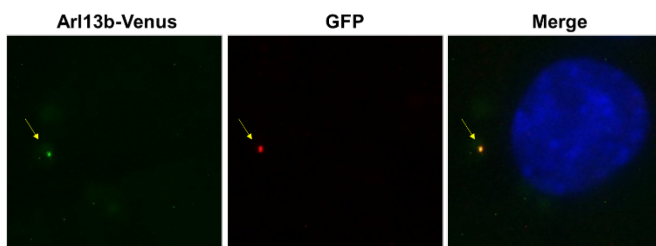

Fig.6: Uptake of fluorescently labeled pcEVs by target cells.

In summary, we show that pcEVs acts as signal transducer to enhance cell migration in target cells possibly via cell adhesion signaling pathways. In addition, pcEVs isolation method was devised which will be useful in accurately determining the proteome of pcEVs independent of exosomes. We also established a knock-in method to fluorescently tag pcEV and visualized their up by target cells. Future work will entail to elucidate the pcEVs uptake mechanisms in target cells.

５．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　1件／うち国際共著　1件／うちオープンアクセス　1件）

１．著者名	４．巻
Faryal Ijaz、 Koji Ikegami	46
２．論文標題	**５．発行年**
Knock-in of Labeled Proteins into 5'UTR Enables Highly Efficient Generation of Stable Cell Lines	2021年
３．雑誌名	**６．最初と最後の頁**
Cell Structure and Function	21～35
掲載論文のDOI（デジタルオブジェクト識別子）	**査読の有無**
10.1247/csf.21002	有
オープンアクセス	**国際共著**
オープンアクセスとしている（また、その予定である）	該当する

〔学会発表〕　計1件（うち招待講演　0件／うち国際学会　1件）

１．発表者名
Faryal Ijaz
２．発表標題
Knock-in of labeled proteins into 5'UTR enables highly efficient generation of stable cell lines
３．学会等名
第73回日本細胞生物学会大会（国際学会）
４．発表年
2021年～2022年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

The following article has been published as a preprint and is under review in a peer-review journal.

A pair of primers facing at the double-strand break site enables to detect NHEJ-mediated indel mutations at a 1-bp resolution
Faryal Ijaz, Ryota Nakazato, Mitsutoshi Setou, Koji Ikegami
bioRxiv 2022.02.07.479376; doi: https://doi.org/10.1101/2022.02.07.479376

６．研究組織

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研究協力者	池上　浩司 (Ikegami Koji) (20399687)		

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