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研究課題名（和文）SynNotch EV cells-flexible ”CAR T”-cells

研究課題名（英文）SynNotch EV cells-flexible CAR T-cells

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研究成果の概要（和文）：キメラ抗原受容体T細胞（CAR T）の治療が注目されている。最近、Synthetic Notch (Syn-Notch)受容体が活性化されたときに標的遺伝子の直接転写制御を可能にする新しい手法が開発された。Syn-Notch受容体の認識ドメインは、標的認識を可能にするために、単鎖可変フラグメント（scFv）またはペプチドに改変されている。本研究では、Syn-Notch活性化後に、Syn-Notch受容体細胞が、細胞外分泌小胞（EV)を介して、タンパク質およびRNAのような治療に用いる高分子を標的細胞の細胞質に送達し得ることを示した。

研究成果の学術的意義や社会的意義
細胞外分泌小胞（EV）は細胞間で高分子を移動できることが示されており、これは治療に応用されている。EVの利用により、Syn-Notch細胞が、タンパク質およびRNAのような治療に用いる高分子を受容体細胞の細胞質に送達することを可能にし、EV-およびCAR T細胞に基づく治療の制限を同時に克服する。EVはまた、多種多様のタンパク質およびRNAを送達できると考えられるため、薬物の標的を増加させうる。

研究成果の概要（英文）：Chimeric antigen receptor T-cell (CAR T) therapies have gained momentum. However, CAR T cells have limitations; they can only respond with a T cell response, they are affected by the tumour microenvironment and can easily be exhausted. Recently, a new concept has been developed with Synthetic Notch (Syn-Notch) receptors, which allow direct transcriptional control of genes of interest when the receptor is activated. The recognition domain has further been altered to a single-chain variable fragment (scFv) or peptide to allow for target recognition. Up to date, the Syn- Notch cells have merely been capable of secreting therapeutic proteins into the extracellular space, such as antibodies and cytokines. Here, we utilised engineered extracellular vesicles (EVs) to deliver macromolecular drugs into recipient cells after Syn-Notch activation. EVs have been shown to be able to transfer macromolecules between cells, which has been utilised in therapeutic settings.

研究分野：免疫学

キーワード：キメラ抗原受容体T細胞　Synthetic Notch受容体　細胞外分泌小胞

様　式　C－１９、F－１９－１、Z－１９（共通）

１．研究開始当初の背景

Survey of the field

CAR T cells rise to the 1st approved gene therapy by the Food and Drug Administration

CAR T cells are cells with a chimeric antigen receptor, recognising antigens similar to a B-cell receptor, but with the response of a T-cell. This enhances the targeting capabilities of the T-cells and eliminates HLA-matching issues. **In several clinical trails there have been deaths due to severe side effects** of the treatments. The most common side effect – a cytokine storm, resembling sepsis in the patients.[1] Nevertheless the potential of CAR T cell treatments have been shown with CD19 targeting CAR T cells, which eradicate large tumours and **even brain metastasis have been reported to be amenable to treatment**.[3] However, for solid tumours the treatment has not been such a great success. Two main hurdles prevent the treatment to be efficacious in solid tumours; firstly a lack of exclusive antigens found on solid tumours and secondly immune inhibitory mechanisms that down regulate the CAR T cells.[2] However, CAR T cells for haematological malignancies have gone from an experimental treatment in a few phase I studies to become an FDA approved treatment in less than a decade and the technology is still advancing. Nonetheless **CAR T cells have an inbuilt shortcoming and that is that they can only use the T-cell machinery** to mediate cancer eradication.

The Versatile Synthetic-Notch receptor

To overcome the disadvantage that CAR T cells only generate a T-cell response after encountering an antigen, Syn-Notch receptors have recently been developed in Wendell Lim's lab, which take **engineered cells and their response beyond existing CAR T cells**. See **Fig. 1** for a schematic representation of a Notch receptor and a Syn-Notch receptor. When Notch receptors bind a ligand the confirmation of the receptor changes and this exposes several cleavage sites for proteases that cleave the protein backbone. This subsequently releases a transcription factor (TF) on the cytoplasmic side, which transcends to the nucleus and start transcription of target genes.[5] The Lim lab exchanged the ligand binding part of the receptor to a scFv, nanobody or a peptide so the receptor in theory can recognise any cell surface target. Furthermore, the TF part of the receptor was engineered to include artificially derived TFs instead of the normal domain. The cell was further equipped with a sensing element responding to the artificial TF released upon antigen recognition. Hence, the Lim lab developed **a cell, which can respond with designed transcriptional programs when recognising a user defined antigen.** They have in several publications showed the potential of the technique with cells that respond to specific antigens and secrete anti-inflammatory molecules, pro-inflammatory molecules and immune check point inhibitors, both *in vitro* and *in vivo*.[4, 5] They furthermore demonstrated that **the activation is both spatially and temporally limited** if the antigen is no longer present. However, one issue that remains unsolved is how to make the cell secrete therapeutically active molecules that can penetrate the nearby cells and affect proteins and/or RNA in the cytoplasm or nucleus of the recipient cells and **thereby increase the drugable targets immensely** for the Syn-Notch platform.

Engineered Extracellular Vesicles as messengers for Synthetic-Notch cells

Most cells, if not all, release EVs, which are lipid enclosed vesicles 40-1000 nm in diamter, that influence neighbouring cells or cells at a distance. EVs have been isolated from most body fluids and it is increasingly **evident that they play a key role** not only in the regulation of normal physiological processes but also in the pathology underlying a range of diseases, particularly linked to tumourigenesis and neurological diseases (reviewed in 6 and 7). EVs exert their biological effects in a pleiotropic manner; directly activating cell surface receptors on recipient cells via protein and bioactive lipid ligands or delivering effectors including proteins and RNAs (e.g. microRNAs (miRNAs) and mRNA).[7] Such wide ranging biological functions suggest that EVs may have innate therapeutic potential, for example in the fields of regenerative medicine and malignant diseases. In addition to the innate therapeutic capacity of EVs, increasing attention has been drawn to **their ability to naturally convey RNAs and proteins into cells,** potentially making them ideal non-viral drug delivery vehicles (reviewed in 8). Indeed, numerous studies have today implicated the potential of EVs for delivery of miRNAs and other exogenous macromolecular drugs. For example have we harnessed the RNA-transporting capacity of exosomes and exploited it for delivery of therapeutic siRNAs.[9] Others

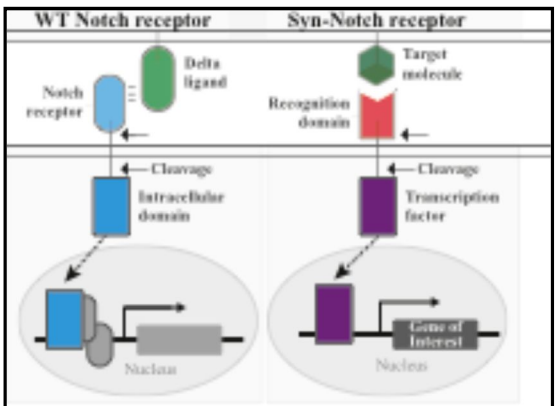

Figure 1. Schematic of wild type- and Synthetic Notch (Syn- Notch) receptors. Syn-Notch receptor recognition domain can be a single chain antibody, peptide or any other binding partner and the transcription factor (TF) domain is normally an artificial TF, such as Gal4-VP64.

[1. Research Objectives, Research Method, etc. (continued from the previous page)]

have shown therapeutic potential of EVs with endogenous loading techniques and **I have furthermore developed highly efficient endogenous loading techniques** for both therapeutic proteins as well as RNAs (see below for details). However a caveat with EVs are the **short circulation time** that has recently been discovered by us and others, with EV half-lives in circulation after intravenous injection of less than a couple of minutes. The majority of the injected EVs are rapidly taken up by the phagocytic cells residing in liver, lung and spleen.[10] Hence the need for high doses and high risk of immune responses towards the therapeutic EVs exist. Nevertheless we and others have observed that EVs are highly capable of transferring cargo from producer cells to recipient cells when the cells are co-cultured *in vitro*. The amount of functional transfer of cargo is normally **increased in co-culture** experiments compared to adding purified EVs to the cell culture.[11] When purifying EVs there is always a risk that certain subpopulations of vesicles are removed during the purification process. This can explain why the effects of EVs are increased in co-culture experiments. Furthermore is the purification and handling of EVs very cumbersome, work intensive and **it is possible that purification techniques remove physiologically important subpopulations of EVs**. Together with the low half-life in circulation, this hampers the therapeutic effect *in vivo* of a treatment with tremendous potential. Therefore I suggest in this proposal to **combine the techniques of Syn-Notch receptors and designer EVs to generate a cell that secretes designer EVs upon stimulation** by a user defined antigen, SynNotch EV cells (see **Fig. 2** for schematic of a SynNotch EV cell). This would allow us to deliver macromolecular drugs directly into the cytoplasm of recipient cells in a specific microenvironment. As a result, this leads to several advantages over existing cell therapies:

1. **The treatment will be highly specific,** since the activation and subsequent secretion of therapeutic EVs will only occur in the presence of a user defined antigen. Additionally, specificity will be further achieved from the therapeutic cargo that is loaded into the EV, such as a miRNA, which can be designed to be highly specific for a target existing only in diseased cells.
2. The specificity will abolish the unspecific side effects seen with previous cell treatments.
3. Antigens that cannot be utilised for CAR T cell treatments, due to severe side effects, can now be exploited because of point 1 and 2.

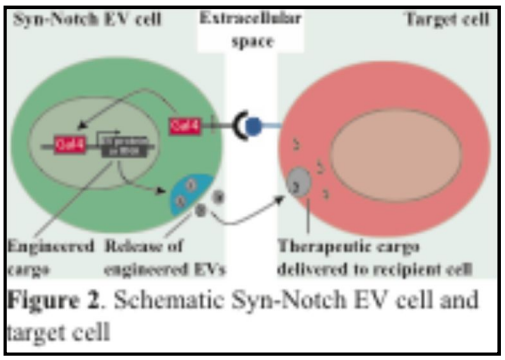

Figure 2. Schematic Syn-Notch EV cell and target cell

4. The system will **make undrugable targets drugable**. In theory any gene and/or non-coding RNA can be targeted.
5. Different cell types can be manipulated and used as active therapeutic cells.

The system will be very adaptable, since the therapeutic cargo molecule and/or antigen recognised by the receptor are easily interchanged.

２．研究の目的

Chimeric antigen receptor T-cell (CAR T) therapies have gained momentum after the **approval of Novartis CAR T cell drug Kymriah** by the Food and Drug Administration in August 2017. However, CAR T cells have limitations; they can only respond with a T-cell response, they are affected by the tumour microenvironment and can easily be exhausted. Recently, a new concept has been developed with **Synthetic Notch (Syn-Notch) receptors**, which allow direct transcriptional control of genes of interest when the receptor is activated. The recognition domain has further been altered to a single-chain variable fragment (scFv) or peptide to allow for user-defined target recognition. Up to date, the Syn- Notch cells have merely been capable of secreting therapeutic proteins into the extracellular space, such as antibodies and cytokines. **The aim of this project** is to **utilise engineered extracellular vesicles (EVs) to deliver macromolecular drugs** into recipient cells after Syn-Notch activation. EVs have been shown to be able to transfer macromolecules between cells, which has been utilised in therapeutic settings. This would permit the Syn-Notch cells to deliver therapeutically active macromolecules such as proteins and RNAs to the cytoplasm of recipient cells **and overcome limitations of EV- and CAR T cell based therapies simultaneously**. This would increase the drugable targets, since all proteins and RNAs are possible targets. The aims of the project are as follows:

① To create cells that secrete designer EVs (Syn-Notch EV cells) upon stimulation from a defined antigen.
② To evaluate the Syn-Notch EV cells in therapeutic cancer models *in vitro* and *in vivo*.

３．研究の方法

The first part of the work plan involves the cloning of Syn-Notch vectors and the generation of stable cells to be able to examine the efficiency of the Syn-Notch EV cell technology. The Lim lab has generated Syn-Notch applicable plasmids, which are available through Addgene. Hence for an efficient start of the project, the Lim lab's plasmids will be utilised and either CD19 or GFP will be used as the antigen molecule on target cells. Firstly reporter proteins will be cloned into the vector to evaluate the efficiency of the system in an EV context. See **Fig.**

[1. Research Objectives, Research Method, etc. (continued from the previous page)]

3 for a schematic picture of the preliminary time lines. I will utilise the fact that I have loading constructs already optimised fused with both eGFP as well as luciferase species that allow for the detection of EV release in the culture media and also the percentage of loaded EVs. To examine the amount of released engineered EVs the luciferase level can be analysed directly in the medium, with remarkable sensitivity. The engineered vesicles will be further analysed by flow cytometer techniques, electron microscopy and western blot to further validate the system. When the system has been optimised regarding spatial and temporal release of EVs, the next step will be to investigate the system in *in vitro* co-culture and transwell uptake assays. Here both a Cre-lox reporter as well as an uptake assay will be utilised. The Cre-lox reporter requires successful uptake of EVs and release of the loaded Cre for a positive read out. The system is very sensitive, requires functional delivery of Cre and is therefore an ideal system for this type of assay. With both assays the amount of target molecules per cell and ratio between receiver and sender cells can be optimised before therapeutically relevant targets are evaluated. Since CAR-T and previous SynNotch cells have been used to treat cancer I will firstly evaluate the efficiency of the SynNotch EV cells on cancer models. Here PANC-1 pancreas cancer cells will be used as model cells for cancer treatments. miRNA against mutated $KRAS^{G12D}$ will be delivered to PANC-1 cells with or without CD19 expression. The effect will be examined through mRNA and protein levels of the target as well as proliferation and apoptosis of the cells. Furthermore the treatment will be evaluated with BxPC-3 (*KRAS* wild type) and Capan-1 ($KRAS^{G12V}$) cells to show that the treatment is specific for the $KRAS^{G12D}$ mutation. At the same time p53-protein replacement therapy in p53 sensitive cancer cell lines will be evaluated hence both protein and RNA delivery will be evaluated. Thereafter the treatment will be evaluated *in vivo* by utilizing an orthotopic PANC-1 tumour model and treatment with Syn-Notch EV cells. If the treatment is successful against PANC-1 cells the next step will be to evaluate the treatment for a non-malignant disease, such as Duchenne muscular dystrofi (DMD). Here Cas9 targeting the dystrophin gene and splice switching U7 RNAs will be loaded into the EVs upon stimulation by a muscle specific antigen. The cells will only release EVs with Cas9-RNPs and U7 splicing RNA in the muscle microenvironment and hence the side effects of the treatment will be very limited.

４．研究成果

Chimeric antigen receptor T-cell (CAR T) therapies have gained momentum. However, CAR T cells have limitations; they can only respond with a T cell response, they are affected by the tumour microenvironment and can easily be exhausted. Recently, a new concept has been developed with Synthetic Notch (Syn-Notch) receptors, which allow direct transcriptional control of genes of interest when the receptor is activated. The recognition domain has further been altered to a single-chain variable fragment (scFv) or peptide to allow for target recognition. Up to date, the Syn- Notch cells have merely been capable of secreting therapeutic proteins into the extracellular space, such as antibodies and cytokines. Here, we utilised engineered extracellular vesicles (EVs) to deliver macromolecular drugs into recipient cells after Syn-Notch activation. EVs have been shown to be able to transfer macromolecules between cells, which has been utilised in therapeutic settings. This would permit the Syn-Notch cells to deliver therapeutically active macromolecules such as proteins and RNAs to the cytoplasm of recipient cells and overcome the limitations of EV- and CAR T cell-based therapies simultaneously. This would also increase the druggable targets since all proteins and RNAs are possible targets.

キメラ抗原受容体 T 細胞 (CAR T) の治療が注目されている。しかしながら、CAR T 細胞には限界がある;T 細胞応答のみで応答し、腫瘍微小環境の影響を受け、容易に消耗する。最近、Synthetic Notch (Syn-Notch)受容体が活性化されたときに標的遺伝子の直接転写制御を可能にする新しい手法が開発された。Syn-Notch 受容体の認識ドメインは、標的認識を可能にするために、単鎖可変フラグメント (scFv) またはペプチドに改変されている。現在まで、Syn-Notch 細胞は、治療に用いる抗体およびサイトカインなどを細胞外に分泌できるだけであった。本研究では、Syn-Notch 活性化後に、Syn-Notch 受容体細胞が、細胞外分泌小胞 (EV)を介して、タンパク質および RNA のような治療に用いる高分子を標的細胞の細胞質に送達し得ることを示した。

細胞外分泌小胞 (EV) は細胞間で高分子を移動できることが示されており、これは治療に応用されている。EV の利用により、Syn-Notch 細胞が、タンパク質および RNA のような治療に用いる高分子を受容体細胞の細胞質に送達することを可能にし、EV-および CAR T 細胞に基づく治療の制限を同時に克服する。EV はまた、多種多様のタンパク質および RNA を送達できると考えられるため、薬物の標的を増加させうる。

５．主な発表論文等

〔雑誌論文〕　計2件（うち査読付論文　2件／うち国際共著　2件／うちオープンアクセス　2件）

１．著者名 Nordin Joel Z、Aoki Yoshitsugu	４．巻 249
２．論文標題 Autoimmune response and its long‐term consequences after exon‐skipping therapy in a Duchenne muscular dystrophy mouse model	５．発行年 2019年
３．雑誌名 The Journal of Pathology	６．最初と最後の頁 271～273
掲載論文のDOI（デジタルオブジェクト識別子） 10.1002/path.5327	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

１．著者名 Kuhn Jasmin、Klein Philipp M.、Al Danaf Nader、Nordin Joel Z.、Reinhard S?ren、Loy Dominik M.、H?hn Miriam、El Andaloussi Samir、Lamb Don C.、Wagner Ernst、Aoki Yoshitsugu、Lehto Taavi、L?chelt Ulrich	４．巻 29
２．論文標題 Supramolecular Assembly of Aminoethylene‐Lipopeptide PMO Conjugates into RNA Splice‐Switching Nanomicelles	５．発行年 2019年
３．雑誌名 Advanced Functional Materials	６．最初と最後の頁 1906432～1906432
掲載論文のDOI（デジタルオブジェクト識別子） 10.1002/adfm.201906432	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔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
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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

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