Research Article

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Explore the activation efficiency of different ligand carriers on synNotch-based contact-dependent activation system

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Abstract

Objectives: synNotch receptors can get different inputs about the outside environment into cells; however, synNotch system doesn't work for soluble ligands. This study aims to explore the activation efficiency of different ligand carriers on synNotch system.

Methods: SynNotch-based contact-dependent activation system was constructed in 293T cells (293T-synNotch), then the synNotch receptor ligands eGFP proteins were provided by three different carriers: 400 nm Ni magnetic beads (Mag Ni-eGFP), 300 nm carboxyl modified Fe₃O₄ magnetic beads (Mag COOH-deGFP), and 4T1 cell (4T1-mGFP). Three eGFP carriers were incubated with 293T-synNotch, the activation efficiencies of these three eGFP carriers were evaluated by fluorescence microscopy.

Results: The 293T-synNotch cell activated by Mag Ni-eGFP shows no observable red fluorescence; when Mag COOH-deGFP are incubated with 293T-synNotch, about 50% cells with red fluorescence appeared, also eGFP proteins have not dropped off from the Mag COOH-deGFP beads; 4T1-mGFP cells show the highest activation efficiency, about 21% synNotch cells are activated, when 4T1-mGFP and 293T-synNotch are incubated at high cell density.

Conclusion: The 4T1-mGFP cells are the most effective ligands for synNotch receptor activation.

Introduction

It is important to trace the migration of specific cell populations in some *in vivo* studies; however, we are short of reliable markers for tracking cells. For example, studying the migration of immunoedited suppressive cells from tumor microenvironment to distal immune organs/tissues is vital for tumor immunology research [1]; however, the lack of specific cell surface markers makes it difficult to obtain reliable results for cell tracking. Taking myeloid-derived suppressor cells (MDSCs) as an example, the phenotype of MDSCs in mice is CD11b⁺Gr-1⁺, which can be divided into two subtypes: granulocyte-like G-MDSCs (CD11b⁺Ly6C⁺Ly6G⁺) and monocyte-like M-MDSCs (CD11b⁺Ly6C⁺Ly6G⁻); however, the phenotype of CD11b⁺Ly6G⁺Ly6C^{low} G-MDSCs was identical to neutrophils, and the phenotype of CD11b⁺Ly6G⁺Ly6C^{high} M-MDSCs was identical with those inflammatory monocytes [2]. Therefore, it is impossible to trace the migration of immune cells in tumor tissues with these markers. In addition, CD45.1 mice and CD45.2 mice, which are often used to study the origin, differentiation and migration of cell lineages [3], also can't provide the information whether a specific immune cell population migrates from tumor tissues to distal immune organs or tissues. The binding of Notch receptor to Delta family proteins presented by a neighboring cell leads to the intramembrane proteolysis, then the Notch intracellular domain (NICD) travels to the nucleus. There, the NICD associates with a DNA binding protein to assemble a transcription complex that activates endogenous downstream transcription [4–6]. Based on these characteristics of Notch receptor, modified Notch receptor or synthetic Notch receptors (synNotch) have been widely used in synthetic biology research to realize the input and output of signals in cells [7], or construct various signal circuits in cells [8, 9], synNotch receptor could function in diverse cells, including epidermal cells, fibroblasts, HEK293 cells, immune cells and neurons [10, 11]. We

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changed the extracellular segment of wild Notch receptor to VHH antibody sequence against eGFP, and the intracellular segment to the Gal4-VP64, which contains both the DNA binding domain Gal4 and transcriptional activation domain VP64, to form the synNotch receptor. When synNotch receptor-expressing immune cells get into tumor tissues and contact with eGFP that presented on tumor cells' outer membranes, the intracellular Gal4-VP64 domain of synNotch receptor dissociates and enters the nucleus to activate the expression of mCherry-Nup133 reporter gene that is under control of Gal4 UAS promoter. In this way, we can realize the tracking of immune cells migrating from tumor microenvironment to other organs or tissues. In this study, we successfully constructed 293T cells expressing synNotch receptors, and the reporter gene (293T-synNotch) eGFP protein was provided by three different carriers: 400 nm Ni magnetic beads, 300 nm carboxyl modified Fe₃O₄ magnetic beads and 4T1 cells. Three eGFP carriers were incubated with 293T-synNotch, the activation efficiencies were compared at different time points, these results paved the way for our further using this synNotch-based contact-dependent activation system both *in vitro* and *in vivo*.

Materials and methods

Reagents

RPMI 1640 and the Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco, Thermo Fisher, Shanghai, China. Fetal bovine serum was from Biological Industries (BI), Israel. Puromycin was from Solarbio, Beijing, China and 30% PAGE pre-solution (Cat: #A1010, Lot: NO20181218) was from Solarbio, Beijing, China. Trypsin 0.25% (Lot:1904160107) was from GENOM, Hangzhou, China. Stbl3 competent cell (Cat: BC108, Lot: 746164AA) was from Biomed, Beijing, China. Polybrene (Cat: 40804ES76, Lot: P48080) was from Yeasen Biotech, Shanghai, China. FITC labeled goat anti-mouse IgG (A0568) and calcium phosphate cell transfection kit (C0508) were from Beyotime, Haimen, China. Endo-free plasmid maxi kit was from OMEGA, USA. Immobilon Western Chemiluminescent HRP substrate (Cat: WBKLS0100, Lot: NO1819701) was from Millipore. FITC-labeled goat anti-Llama IgG H&L (Cat: ab112785, Lot: GR326044-3, the dilution ratio was 1:100) was from Abcam, Shanghai, China. Anti-eGFP (M048-3) was from MBL. Denatured eGFP protein (Cat: P20190618322, Lot: RPD025Ge01) was from Cloud-Clone Corp. Wuhan, China. Also 400 nm Mag Ni-eGFP magnetic beads (Cat: PMSi099) were from PuriMag Biotech, Xiamen, China and 300 nm carboxyl modified Fe₃O₄ magnetic beads (70106-5) were from BEAVER, Suzhou, China.

Plasmid

synNotch gene and response element together with the reporter gene were directly synthesized and subcloned to pCDH-CMV-MCS-EF1-Puro plasmid (gene synthesis conducted in GenScript, Nanjing, China).

Cell culture

The 4T1 (GDC0294) and HEK293T (GDC0187) cell lines were obtained from China Center for Type Culture Collection (Wuhan, China) and cultured according to the manufacturer's instructions. DMEM medium was supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (mycoplasma contamination check was carried out by our group). 293T-synNotch and 4T1-mGFP were initially selected on 5 µg/mL puromycin (Invivogen) and subsequently grown in the presence of 1 µg/mL puromycin.

Transfections and lentiviral infections

Transfection of HEK293T was performed by using calcium phosphate cell transfection kit (Cat No.: C0508, Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's instructions. Use transfer vector: viral packaging (psPAX2); viral envelope (pMD2G) at 4:3:1 ratio. Lentivirus containing supernatant was harvested at both 48 h and 72 h after transfection and was filtered using 0.45 µm filter. Acquired lentivirus was mixed with PEG solution (5% PEG8000 and 0.5 M NaCl) overnight and concentrated by centrifugation at 3,000 g for 30 min in 4 °C. Dilution of lentivirus in DMEM complete +10 µg/mL polybrene was used for infections.

Flow cytometry analysis

For analysis of surface antigens expression, cells were harvested at the indicated times and washed with ice-cold phosphate-buffered saline (PBS) and suspended at a concentration of 10⁶ mL⁻¹ in 100 µL buffer (0.1% BSA in PBS), and then were stained with FITC-labeled goat anti-Llama IgG H&L antibody (1:100) for 30 min on ice and then washed three times with PBS. The cells were analyzed on a fluorescence-activated cell sorting (FACS) Calibur cytometer using Cellquest software (Becton Dickinson) and FlowJo software (V. 7.6.4, TreeStar). For detection, the binding of denatured eGFP to 293T-synNotch, firstly, 293T-synNotch was incubated with denatured eGFP for 1 h, then anti-eGFP primary antibody (1:100) and FITC labeled goat anti-mouse IgG (H + L) (1:500) were used. The statistics presented are based on at least 10,000 events gated on the population of interest.

Western blot

Two micrograms of purified BSA and denatured eGFP were added to SDS loading buffer. The samples were boiled for 5 min and loaded immediately onto 10% SDS-PAGE gels, then run at 90 V/140 V for a total of 1 h. Proteins were transferred for 40 min onto PVDF membranes (Bio-Rad) using a Trans-Blot semi-dry transfer apparatus (Bio-Rad). Membranes were blocked in 5% skim milk for >1 h, then exposed to diluted primary antibodies (Anti-eGFP, 1:1,000) in 5% skim milk overnight at 4 °C, washed in TBST five times, and exposed to HRP labeled goat anti-mouse secondary antibodies (1:2,000) for 1 h at room temperature. The Immobilon Western Chemiluminescent HRP Substrate System (Thermo) was used for protein detection. Membranes were then imaged on Amersham Imager 600 (GE Healthcare, USA).

Preparation of 300 nm Mag COOH-deGFP magnetic beads

Magnetic beads carboxyl groups activation: take 100 µL Mag COOH magnetic beads to 1.5 mL centrifuge tube, remove supernatant by

magnetic separation, wash it twice with 200 μ L MEST solution (100 mM MES, pH 5.0, 0.05% Tween 20), then remove supernatant, add 100 μ L EDC solution (10 mg/mL, dissolved in MEST solution) and 100 μ L NHS (10 mg/mL, dissolved in MEST solution) immediately, the magnetic beads are incubated at 25 $^{\circ}$ C for 30 min on a rotary mixer. After the above steps, the carboxyl groups on the surface of magnetic beads have been activated. It can be covalently coupled with biological ligands with primary amino group.

Covalent coupling magnetic beads with denatured eGFP

Removal of supernatant by magnetic separation, add 200 μ g denatured eGFP protein (pH=8.0, 0.05% Tween 20), gentle mixing; coupling at 25 $^{\circ}$ C for 2 h on a rotary mixer; remove supernatant by magnetic separation, add 200 μ L PBST solution (pH 7.2, containing 1% BSA), and the residual activated carboxyl groups were sealed by reaction at 25 $^{\circ}$ C for 1 h on a rotary mixer. The supernatant was removed by magnetic separation, wash three times with 200 μ L PBS, it was resuspended in PBS solution and preserved at 4 $^{\circ}$ C. A small amount of magnetic beads were incubated with 293T-synNotch cells and observed under fluorescence microscope once a day.

Results

Construction of 293T-synNotch cell line

Firstly, we obtained the anti-GFP nanobody sequence (anti-GFP VHH) LaG16_2 from the other researchers' work [12]. Then we replaced the extracellular ligand binding domain of wild Notch receptor with the VHH and linked it

with notch core by EGF repeat linker. The intracellular domain of wild Notch receptor was replaced with Gal4-VP64, thus forming a synthetic Notch receptor (synNotch) (as shown in Figure 1A). The reporter of synNotch receptor is mCherry-Nup133 fusion protein under control of a 5 $\times$ Gal4 UAS promoter [11]. A total length of 7407 bp gene fragment including synNotch and its reporter was synthesized and inserted into lentivirus vector (Figure 1B). Lentiviruses were then prepared and transfected into 293T cells to construct 293T-synNotch cell line. Since the synNotch receptor expressed on 293T-synNotch cell surface contains nanobody sequence from alpaca, we used FITC labeled goat anti-Llama IgG H&L antibody to detect synNotch expression by flow cytometry. The results showed that 95.7% cells were synNotch receptor positive (Figure 1C).

Activation of 293T-synNotch by 400 nm Mag Ni-eGFP beads

Mag Ni-eGFP magnetic beads are silicon-based superparamagnetic nanoparticles. The surfaces of Mag Ni-eGFP beads are modified with nickel ions by different chelating groups and bind to 6 $\times$ his-labeled eGFP in a non-covalent manner. PBS-washed Mag Ni-eGFP beads were incubated with 293T-synNotch and observed under fluorescence microscope once a day. After 48 h of incubation, eGFP proteins dropped off from the Ni magnetic beads (black particles

Figure 1: Construction of 293T-synNotch. (A) Schematic of 293T-synNotch system. 293T-synNotch co-cultured or incubated with GFP-presenting sender cells or GFP carriers induces mCherry-Nup133 expression. (B) Structure of modified lentivirus vector that express the synNotch system. (C) Flow cytometry analysis of synNotch expression on 293T cells.

Figure 2: The activation of 293T-synNotch by 400 nm Mag Ni-eGFP. (A–C) 400 nm Mag Ni-eGFP beads were incubated with 293T-synNotch for 48 h, then cells were observed under bright field (A), GFP channel (B) and mCherry channel (C).

in Figure 2A), and the eGFP proteins combined with 293T-synNotch cells, resulting in the green fluorescence of 293T-synNotch cells (Figure 2B). The expression of mCherry was almost invisible. We also found that green fluorescence interferes greatly with mCherry fluorescence (Figure 2C).

Activation of 293T-synNotch by 300 nm Mag COOH-deGFP

When we use 400 nm Mag Ni-eGFP beads to activate 293T-synNotch cells, we found two problems: eGFP proteins were easy to drop off from the Ni magnetic beads and the strong eGFP fluorescence interferes greatly with mCherry detection. In order to solve these two problems, we covalently linked denatured eGFP (deGFP) proteins to 300 nm carboxyl modified Fe_3O_4 magnetic beads (designated as Mag COOH-deGFP beads), we could not observe green fluorescence of the deGFP protein under fluorescence microscope, but deGFP still retained the binding ability with 293T-synNotch cells. We also verified this characteristic by western blotting and flow cytometry. The results of western blotting showed that anti-eGFP antibody well recognized deGFP, and clearly showed bands at 37 KDa, but not BSA control protein at 66 KDa (Figure 3A). Flow cytometry also confirmed that deGFP still had good binding ability to 293T-synNotch cells (Figure 3B). Incubating 300 nm Mag COOH-deGFP beads with 293T-synNotch cells for 48 h, we found that about 50% red fluorescent cells appeared (Figure 3C) and also deGFP protein haven't dropped off from the Mag COOH-deGFP beads. The results showed that 300 nm Mag-COOH-deGFP beads activates 293T-synNotch cells more efficiently than 400 nm Mag-Ni-eGFP beads.

Activation of 293T-synNotch cells by 4T1-mGFP cells

To construct 4T1-mGFP cell line, we obtained the amino acid sequence that expresses outer membrane anchored eGFP (mGFP) from previous literature [11], then we constructed 4T1-mGFP cell line through lentivirus transfection

Figure 3: The activation of 293T-synNotch by 300 nm Mag COOH-deGFP. (A) The confirmation of deGFP protein by Western Blotting. (B) flow cytometry analysis of the binding ability of deGFP to 293T-synNotch cells. (C) Incubate 300 nm Mag COOH-deGFP and 293T-synNotch cells for 48 h, and then mCherry positive cells were observed under microscopy.

Figure 4: The activation of 293T-synNotch by 4T1-mGFP. (A) Outer membrane (mGFP) or cytoplasmic (cGFP) expressed eGFP. (B–D) 293T-synNotch and 4T1-mGFP were cocultured for 48 h at lower cell density (15×10^5 cells in total) or higher cell density (30×10^5 cells in total), then mCherry was observed by fluorescence microscope (B), or analyzed by flow cytometry (C, D).

and subsequent screening. At the same time we also constructed 4T1 cell line that express cytoplasm located GFP (cGFP). Compared with the 4T1 cells expressing cGFP, we found that the green fluorescence of 4T1-mGFP was mainly expressed on the cell membrane (Figure 4A).

5×10^5 293T-synNotch cells and 10×10^5 4T1-mGFP cells (15×10^5 cells in total) or 10×10^5 293T-synNotch cells and 20×10^5 4T1-mGFP cells (30×10^5 cells in total) were cocultured in 100 mm cell culture dish, mCherry expression of 293T-synNotch cells was observed during coculture. mCherry appeared when cocultured for 48 h, we found that, there are much more mCherry positive cells when 293T-synNotch cells and 4T1-mGFP cells were cocultured at higher cell density (Figure 4B). Flow cytometry was used to detect mCherry expression in 293T-synNotch cells, 4T1-mGFP cells, mixture of 293T-synNotch and 4T1-mGFP cells after coculture for 48 h. The results show that, when cells were cocultured at lower density, mCherry expression increased by 0.33% after coculture (Figure 4C), since 2/3 of the cocultured cells were 4T1-mGFP, so mCherry expression increased by 10‰ in fact, however, when cells were cocultured at higher density, mCherry positive 293T-synNotch cells increased about 21% (Figure 4D). The results showed that 4T1-mGFP activates 293T-synNotch cells more efficiently than 400 nm Mag-Ni-eGFP beads or 300 nm Mag-COOH-deGFP beads.

Discussion

synNotch receptors can get different inputs about the outside environment into cells, outputs can also be defined by using the synNotch architecture. synNotch system could be hugely useful for cancer immunotherapy, smart cell design or *in vivo* cell tracking. However, synNotch doesn't work for soluble ligands. Also, the system didn't work well with weak inputs, since there is no signal amplification. So the ligands carriers optimization is of great significance to its activation efficiency.

Many studies suggest that a mechanical force is involved in Notch activation. This conformational change exposes two sites in Notch for cleavage by ADAM metalloproteinases and γ -secretase. After cleavage Notch intracellular domain (NICD) releases from membrane, then enters the nucleus where it associates with the DNA-binding protein CSL (CBF1/RBPjk/Su(H)/Lag-1) and activates downstream genes expression. Ligand endocytosis is also thought to generate mechanical force to promote a conformational change in the bound Notch receptor [13–15]. Four hundred nanometer Mag-Ni-eGFP magnetic beads generated the lowest activation efficiency among these three eGFP carriers, this might be due to the exfoliation of eGFP, free

eGFP protein binds to the extracellular domain of synNotch receptor, which makes the magnetic beads couldn't access to 293T-synNotch cells and thus unable to provide sufficient mechanical force to activate synNotch receptor. Due to covalent link, Mag-COOH-deGFP beads can fully contact with cells, thus showing a better activation effect. Although the activation efficiency of Mag-COOH-deGFP beads has been improved, the efficiency is still very low in terms of the number of activated cells, we speculate that the low activation efficiency of these beads to 293T-synNotch is due to these motionless beads hardly provide sufficient mechanical force to activate synNotch receptors. So further optimization both of the synNotch receptor and ligands carriers is still needed to improve the activation efficiency.

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Author contributions: ZYN and WLZ designed this study and wrote the paper. ZYN and GXC performed the experiments. The other authors conducted the experiments and contributed to the discussion. ZYN and WLZ supervised the project. All authors critically reviewed the article and approved the final manuscript.

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Informed consent: Informed consent was obtained from all individuals included in this study.

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