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論文題目	Programmed cell-immobilization of living cells by independent molecular interaction （細胞膜へのクリック反応性官能基修飾の生細胞配置固定への応用 ）		

（論文内容の要旨）

Therapeutic devices incorporating living cells have been intensively investigated for applications in tissue engineering and regenerative medicine. As many biological processes are governed by spatially dependent signals, mapping of multi cell types on substrates serves as a potent tool for investigating tissue-related biological processes Therefore, I developed the mythology of programmed immobilization of living cells with following requirements. Firstly, introduction of independent pairs on living cells is necessary. Secondly, the independent pairs themselves must be capable of interacting effectively within the dynamic environment of living cells. Thirdly, the techniques employed should be versatile enough to be effectively applied to a wide variety of cell types, ensuring broad utility in diverse biological contexts. Furthermore, cell modification method is preferable to exert negligible influence on cellular physiology to ensure the cell's functional integrity. Based on those requirements, I designed two non-genetic surface modification approaches, namely metabolic labeling facilitated by click chemistry tools and the introduction of antigen-antibody interaction, which might meet the demand for programmed immobilization of living cells.

Chapter 1 Programed immobilization of living cells modified with click reactive functional groups

Chemical molecules exhibit remarkable reaction selectivity and robust covalent bond formation, making them potentially valuable for achieving programmed immobilization of living cells through chemical reactions. However, many chemical reactions are unsuitable for living cells due to their inability to react effectively within a cellular environment. Nevertheless, click chemistry pairs stand out as highly bio-compatible and bio-orthogonal. To exploit this, I introduced click chemistry substrates onto cell membranes and cover glasses, with the goal of programmed anchoring living cells onto the glass via covalent bonds. Initially, Azide group (Az)-labeled living cells were prepared by metabolic labeling with Ac4ManNAz for 48 h. Following the introduction of Az, TCO (trans-cyclooctene) was metabolically labeled into the living cells by reacting with TCO-PEG_{12}-DBCO (dibenzo-cyclooctyne). Az and TCO in the cells were detected using DBCO-FAM (fluorescein)and Tetrazine-Cy3, respectively. The cover glasses were cleansed using piranha solution, followed by a silanization process to attach thiol ligands. Subsequently, they were treated with maleimide-PEG-amino or maleimide-PEG-methyl (MW=2k) to add amino or methyl groups. Lastly, the glasses with amino groups were further modified with DBCO or Tetrazine molecules through reactions with DBCO-COOH or Tetrazine-PEG5-NHS, respectively. The mixture of Az-labeled green fluorescent protein HeLa cells (GFP-cells) and TCO-labeled red fluorescent protein HeLa cells (RFP cells) was reacted in a culture dish in which three different cover glasses, DBCO-, Tetrazine-, or methyl-coated, were added. Az- or TCO-labeled cells could be immobilized in a functional group-dependent manner.

Chapter 2 Programed immobilization of living cells via antigen – VHH antibody interaction

Antigen-antibody recognition offers robust independence and numerous pairs. Thus, I considered it’s potential of introducing antigen-antibody interactions as an alternative for click pairs. In this strategy, site-specific conjugation of antibody, without interfering the antibody's functional integrity, is crucial for following cell immobilization. In this chapter, firstly my efforts were directed towards the conjugation of aligned VHH (variable domain of the heavy chain of heavy-chain) antibodies. Secondly, I explored the possibility of conjugating VHH onto cell membranes, with the goal of facilitating cell-cell stacking.

Section 1 Development of site-specific conjugation of VHH-antibody for effective recognition of antigen on living cells

Aligned antibody immobilization leads to effective antigen recognition. Site incorporating Azido-phenylalanine into antibody is helpful to achieve site specific conjugation, which reveals aligned antibody immobilization Initially, azido-phenylalanine (Az) was introduced to fluorescent protein mKO2. The mKO2-Az was immobilized on the DBCO-coated glass within 30 minutes of reaction, while mKO2 without Az modification remained unbound. These findings demonstrate DBCO on the substrate enables immobilization Az tagged proteins. Anti-mCherry VHH and Anti-EmGFP VHH, with Az introduced at the C-terminus, distal to its antigen-binding site for the mCherry and EmGFP, were expreesed and purified in Escherichia coli. Through a GST-tag, mCherry was bound to resin, and VHH was then added. The bound and unbound fractions obtained after the addition of glutathione were analyzed using SDS-PAGE. Bands for both mCherry and VHH were observed in the bound fraction but not in the unbound fraction, confirming the binding of VHH to mCherry. And Anti-EmGFP VHH yields similar results. Following the conjugation of both VHH-Az to the DBCO substrate, a mixture of mCherry and EmGFP proteins was applied to the surface. Each protein selectively adhered to its complementary VHH. Finally, HeLa cells expressing mCherry on their surfaces (mCherry-coated cells) were incubated with an anti-mCherry VHH-coated substrate for 1 hour. This resulted in increased immobilization of mCherry-coated cells on the VHH substrate, while normal HeLa cells did not adhere.

Section 2 Effect of peptide spacer between VHH antibody and cell membrane on programmed cell-cell interaction

In the pursuit of establishing cell-cell interaction, introducing aligned low molecular weight antibodies (VHH) into the cell membrane to enhance intercellular adhesion with specific cells proves beneficial for programmed cell-cell stacking. In particular, analogous to the spacer section that augments antigen recognition in chimeric antigen receptor T cells, I believed it was helpful to improve cell-cell stacking by incorporating peptide spacers between VHH and cells. Initially, Anti-mCherry VHH with Az was prepared for the selective cell adhesion. After introducing alkyne group on GFP-HeLa cells, VHH connected GFP-cells via click reaction. I proceeded to generate VHH constructs incorporating two distinct peptide spacers between VHH and Az: a flexible three-repeat of glycine- glycine-glycine-glycine-serine (GGGGS) and a more rigid three-repeat of glutamic acid-alanine-alanine-alanine-lysine (EAAAK). I harvested mCherry coated cells on plates as the first cell layer. VHH conjugated or unmodified GFP-cells were incubated over the first cell layer, mCherry-coated cells. More VHH-cells remained on the first cell layer than unmodified cells. And both peptide spacer enhanced the cell adhesion on the first cell layer compared with non-spacer VHH. VHH with peptide linker is helpful for programmed cell immobilization to the target cells.

In conclusion, I successfully employed independent click pairs and interaction of antigen-aligned -VHH-antibodies to selectively manage cell adhesion, achieving patterned cell organization on various surfaces and layers.

（続紙 ２ ）

（論文審査の結果の要旨）

生細胞をパターン化して配置固定できる技術は、センサーを利用した細胞機能の評価や、3D培養技術の向上において、今後重要になってくることが予想される。

申請者は、基板上あるいは平面培養された細胞上に生細胞を配置固定する方法の開発に取り組んだ。まず、生細胞をパターン化して配置固定するための選択的結合素子として、クリック反応で共有結合を形成する官能基のペアとして知られる、DBCO とアジド基、あるいは、TCO とテトラジンを生細胞あるいはガラス基板上にそれぞれ固定した。アジド基あるいは TCO を細胞膜の表面に修飾することで、ガラス基板上に固定化された官能基特異的に細胞を配置固定することができた。さらに、選択的結合素子のペアの組み合わせを拡張するために、抗原抗体の相互作用を利用した生細胞の配置固定法の開発へと展開した。VHH 抗体に遺伝子工学的手法でアジド基を有するアジドフェニルアラニンを１つ挿入し、前述の生細胞修飾技術を利用して修飾した細胞膜上の DBCO と反応させることで、遺伝子導入を介さずに共有結合で抗体を修飾する方法を確立した。この方法により、VHH 抗体の特定の箇所において細胞膜への配向性を揃えた修飾が可能になり、抗原への認識結合を向上させることが期待できる。さらに、天然に存在する抗原を標的とすることで細胞同士の選択的結合を可能にした。最後に、クリック反応を利用した部位特異的な共有結合において、ペプチドスペーサーの導入により細胞間相互作用の効果を向上させた。本研究で開発された手法を利用して精密設計して実装することにより、センサー上への複数の種類の細胞の配置固定や、細胞の積層・組織化における細胞の配置の正確な制御が可能になる。このアプローチは、組織工学や再生医療の応用において新たな方向性を提供し、細胞の挙動や組織形成を操作するための新たな手法となり得る。

本論文は博士（薬科学）の学位論文として価値あるものと認める。また、2024 年 2 月 16 日に論文内容とそれに関連した事項について試問を行った結果、合格と認めた。

要旨公表可能日：　　年　　月　　日以降