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Non-genetic cell-surface modification with a self-assembling molecular glue†

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This report describes the development of a non-genetic cell-surface modification method, in which a self-assembling small molecule is combined with Halo-tag proteins. Cell-surface functionalization with cancer-linked extracellular proteins led to enhanced cell motility, angiogenesis, and immune shielding of the cells, paving the way for translational opportunities for cell therapy.

Cell-based therapies are gaining promise in treating a wide variety of disease states, such as neurodegenerative and cardiovascular diseases.[1–3] However, the desired therapeutic effects are limited primarily due to low survival rates and host immune rejection of the engrafted cells.[4] One approach to overcoming these drawbacks is genetic modification of the cells,[5] in which exogenous genes are integrated into the host genome for increasing cell survival,[6–8] angiogenesis,[9] and immune evasion.[10] A major concern of the genetic modification is potential malignant transformation of the transplanted cells.[11] Such cells would not be eliminated through normal immune mechanisms.

A representative alternative to deal with the risk is non-genetic cell-surface modifications, in which cell surfaces are functionalized through covalent conjugation of synthetic macromolecules to native membrane proteins,[12–15] metabolic labelling of cell-surface glycoproteins with synthetic sugars,[16–18] and non-covalent hydrophobic insertion of macromolecule into the cell membrane.[19–21] These powerful means have been employed to impart unique functionalities for basic science purposes, yet only a few examples have been documented in the literature for the purpose of improving cell engraftment: cell–polymer conjugation has enhanced immune evasion in the islets of Langerhans[22] and directed migration of mesenchymal stem cells to damaged heart tissues.[23]

To complement and potentially synergize with these cell-surface modifications, we envisioned that a fundamentally different mechanism to impart multiple functionalities to the cells. Our approach takes advantage of a synthetic small molecule, adhesamine, and its derivatives, which increase survival of suspended cells by self-assembling with the heparan sulfate of syndecan, a key cell-surface protein in cell survival signals.[24–27] An optimized version of adhesamine, called adhesamine 2.0 (adh2.0), forms submicrometer particles with heparan sulfate and syndecan on the cell surface to sustain survival of the cells for extended time periods.[25] Coupling of adh2.0 with an alkylchloride handle provided adh3.0, which covalently reacts with a haloalkane dehalogenase mutant (Halo-tag). The conjugation of adh3.0 with a Halo-tag fusion of matrix metalloproteinase-2 (MMP-2) allowed projection of MMP-2 on the cell surface to impart cell invasion activity to the cells.

The present study extends our previous effort to engineer the cell surface with the adh3.0-Halo-tag system. The hallmarks of metastatic cancer are cell survival, invasion, angiogenesis, and immune evasion[28,29] (Fig. S1, ESI†). Non-genetic implementation of these properties into transplanted cells would increase the efficacy of cell engraftment. Toward this goal, we chose three cell-surface or extracellular proteins of metastatic cancers and prepared their Halo-tag fusion proteins (Fig. 1): programmed death ligand-1 (PD-L1), angiopoietin-2 (Ang-2), and vascular endothelial growth factor (VEGF-A). PD-L1 is required for the immune evasion of malignant cancer cells,[30] while Ang-2 and VEGF-A play essential roles in cancer cell migration and angiogenesis.[31,32] The design and evaluation of these fusion proteins are shown in Fig. S2–S5 (ESI†). Given that PD-L1 is the membrane-inserted protein, we used secreted form of PD-L1 (secPD-L1),[33] which is known to suppress immune responses through the interaction with PD-1.[34] First, we evaluated the conjugation of these three Halo-tag fusion

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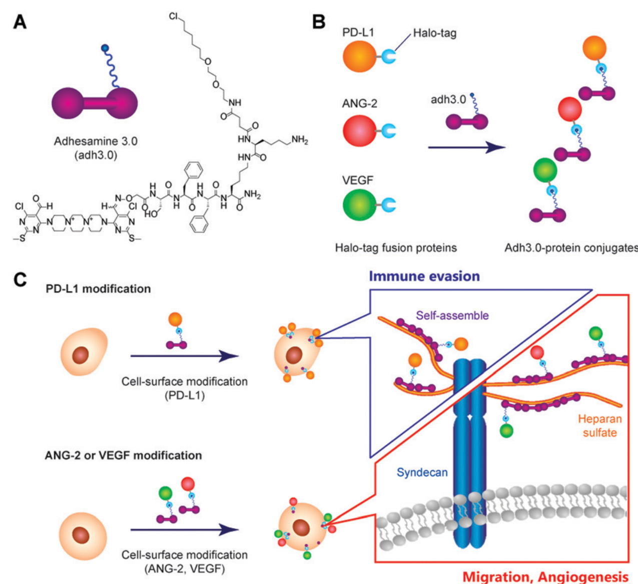

Fig. 1 Conceptual illustration of Cell-surface modification with adh3.0 and Halo-tag fusion proteins. (A) Structure of adh3.0. (B) Conjugation of Halo-tag fusion proteins with adh3.0. (C) Functionalization of cells with the cell-surface modification through adh3.0-Halo-tag fusion proteins. PD-L1 modification leads the cells to facilitate the immune evasion, whereas ANG-2 or VEGF modification induce migration and angiogenesis. Cell-surface modification with multiple proteins synergistically enhance cell migration and tube formation.

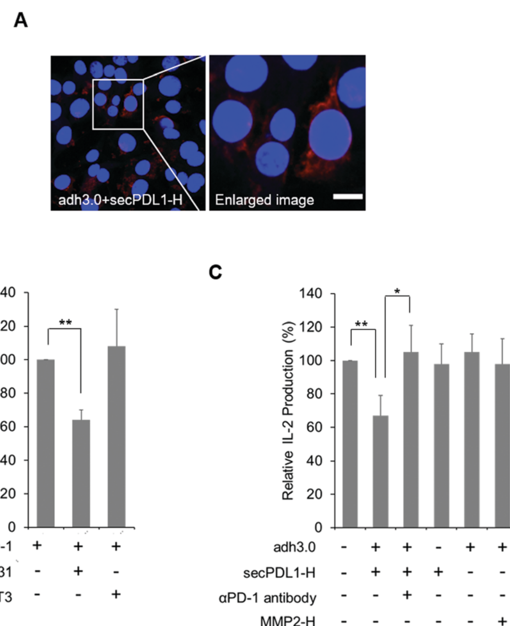

Fig. 2 Cell surface modified with adh3.0 and secPDL1-H. (A) Confocal images of NIH3T3 cells treated with adh3.0 and secPDL1-H in serum-free DMEM. The secPDL1-H was immunostained with αHaloTag antibody as shown in red. Nuclei stained with DAPI is shown in blue. Scale bar, 10 μm. (B) hPD-1-expressing Jurkat T cells stimulated with PHA/PMA (PHA 3 μg mL^{-1}, PMA 2 ng mL^{-1} final) were co-cultured with MDA-MB-231 cells or NIH3T3 cells. (C) hPD-1-expressing Jurkat T cells stimulated with PHA/PMA (PHA 1 μg mL^{-1}, PMA 2 ng mL^{-1} final) were co-cultured with NIH3T3 cells whose cell surface had been pre-treated with adh3.0 alone, secPDL1-H alone, or the mixture of both. (B and C) The IL-2 cytokine amount secreted from the T cells was measured after 48 hours co-culture. Data represent mean ± SD. n = 3. Significance was determined using an unpaired two-tailed Student's t-test. * $p < 0.05$, ** $p < 0.01$.

proteins and adh3.0 *in vitro*. Each Halo-tag fusion protein (7.5 μM) was incubated with HaloTag TMR Ligand (5 μM), a fluorescent alkylchloride ligand specific for Halo-tag proteins. Twenty-min incubation resulted in fluorescent labeling of the Halo-tag proteins (Fig. S2–S5, ESI†). Of note, the observed labelling rates were lower than we expected, potentially due to low purity and degradation of Halo-tag fusion proteins (Fig. S6, ESI†). Nevertheless, co-incubation with increasing amounts of adh3.0 (5–250 μM) competed off the labeling, suggesting the covalent conjugation between the Halo-tag fusion proteins and adh3.0 (Fig. S2–S5, ESI†).

To investigate the ability of the resulting protein-adh3.0 conjugates to modify the cell surface, we used NIH3T3 fibroblasts, non-cancerous cells devoid of anoikis resistance, immune evasion, and angiogenesis. The cells were incubated with adh3.0 (50 μM) and each Halo-tag fusion protein (7.5 μM) at 37 °C for 20 min in a serum-free medium. Immunostaining with a Halo-tag antibody showed that the conjugates indeed induce the formation of dot-like assemblies of the Halo-tag fusion proteins on the cell surface (Fig. S7–S11, ESI†). In contrast, adh3.0 or each Halo-tag protein alone failed to display such cell-surface particles, indicating that the conjugation with adh3.0 is required for the presentation of the Halo-tag proteins.

We set out to examine whether these cell-surface modifications impart expected functions to the cells. The first barrier for transplanted cells to overcome is the immunosurveillance known as allorecognition, such as by the T cells.[4] Cancer-cell-like immune evasion might be achieved by the cell surface modification with adh3.0 and secPD-L1-Halo fusion protein (secPDL1-H). To test the hypothesis, we employed human PD-1(hPD-1)-expressing Jurkat T cells as a T-cell model and monitored its activation by measuring production of interleukin-2 (IL-2), an essential cytokine for T cell activation. Treatment of the cells with phorbol myristate acetate (PMA) and phytohemagglutinin (PHA) induced the production of IL-2 from the Jurkat T cells.[35] The IL-2 production was significantly reduced by coculturing with MDA-MB-231 cells, human breast cancer cells that highly express PD-L1 (hPD-L1). In contrast, co-culture with NIH3T3 cells, which poorly express PD-L1,[36] exhibited no detectable effects (Fig. 2B). As expected, when the Jurkat T cells were co-cultured with the NIH3T3 cells whose cell surface had been pre-modified with adh3.0 and secPDL1-H, the IL-2 production was reduced to the level as low as that of the coculture with MDA-MB-231 cells. Treatment with adh3.0 alone, secPDL1-H alone, or a combination of adh3.0 and MMP2-Halo fusion protein had no detectable effects (Fig. 2C). The IL-2 production was restored by adding an anti-PD-1 antibody, indicating that the observed immunosuppressive activity attributes to the PD-1/PD-L1 interaction.

Cell migration also enhances cell engraftment. VEGFs and angiopoietins are known to play pivotal roles in cell migration during cancer metastasis.[37] Cell surface modifications with

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VEGF-Halo (VEGF-H) and Ang2-Halo (ANG2-H) fusion proteins might enhance migration.

To evaluate their effects on cell migration, we performed wound-healing assays. After the wounding, NIH3T3 cells were treated with adh3.0 and VEGF-H/ANG2-H at 37 °C for 20 min, followed by microscopic observation at 0, 4, and 6 h. As expected, treatment with adh3.0 and VEGF-H/ANG2-H significantly accelerated the cell motility, whereas adh3.0 or VEGF-H/ANG2-H alone had little effects (Fig. 3A, B, and Fig. S12–S14, ESI†). Cell-surface modification with a combination of adh3.0 and PDL1-H had no detectable effects (Fig. S15, ESI†), indicating that the observed migration-accelerating activity is selective for VEGF-H/ANG2-H. VEGF and Ang-2 are known to play complementary and coordinated roles in vascular development processes.[37] Their functional synergism prompted us to test the effects of the simultaneous cell-surface modification on the cell motility. Indeed, a mixture of adh3.0 conjugates with VEGF-H and ANG2-H (a total concentration of 0.5 μM) accelerated the cell migration activity more than that of each protein (0.25 μM) (Fig. 3C). To validate the simultaneous modification, the cells treated with a mixture of adh3.0 conjugates with VEGF-H and ANG2-H were immunostained with anti-VEGF and anti-Ang2 antibodies. Confocal microscopic images of the stained cells showed that both of the fusion proteins were tailored on the cell surface (Fig. S16, ESI†). Taken together, these results demonstrate that our technology allows simultaneous cell-surface modifications with multiple proteins to exert synergistic activity.

We also evaluated the ability of these conjugates to induce angiogenesis, another beneficial factor for cell engraftment expected for VEGF and Ang2.[37,38] Tube formation of HUVECs, human endothelial cells were monitored on ECMatrix™ gel-coated plates, on which the cells rapidly align and form hollow tube-like structures. When the cells were treated with adh3.0 and VEGF-H/ANG2-H, the number of tube nodes and junctions were doubled (Fig. 4A, B and Fig. S17, S18, ESI†). In contrast, treatment with adh3.0 or each Halo-tag protein alone failed to induce such an increase. These results indicate that the cell-surface modification with adh3.0 and VEGF-H/ANG2-H enhance tube formation activity of HUVECs. The synergy of VEGF-H and ANG2-H was similarly accessed. As expected, a mixture of adh3.0 conjugates with VEGF-H and ANG2-H (a total concentration of 5 μM) increased the number of nodes more than that of each protein (2.5 μM) (Fig. 4C).

In conclusion, we have developed a modular, versatile, and efficient strategy for cell-surface functionalization, in which a self-assembling small molecule is combined with recombinant Halo-tag fusion proteins. This non-genetic cell surface engineering permitted cell-surface modifications with multiple

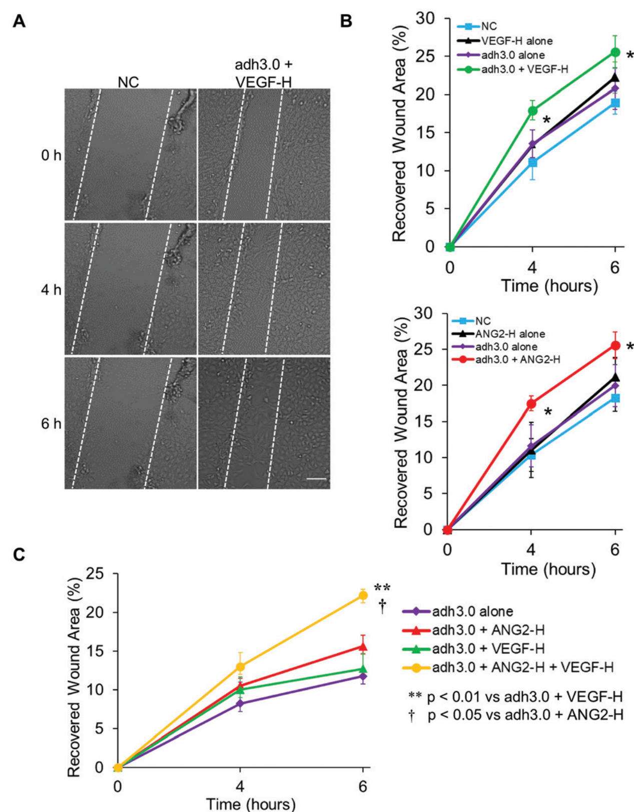

Fig. 3 Wound healing cell migration assay. (A) Bright-field images of NIH3T3 cells for 0, 4, and 6 hours after the treatment. Scale bars represent 100 μm. (B and C) The wound areas were recorded over time (14 hours). (B) Recovered wound area (%) of NIH 3T3 cells after the treatment of adh3.0 (50 μM) and each Halo-tag fusion protein (7.5 μM) at 37 °C for 20 min. (C) Recovered wound area (%) of NIH3T3 cells after the treatment of adh3.0 (50 μM) and each or both of Halo-tag fusion protein (0.25 μM) at 37 °C for 20 min. Images were quantified by NIH ImageJ, using Wound Healing Size Tool. All experiments were replicated at least three times. Data represent mean ± SEM. Significance was determined using an unpaired two-tailed Student's *t*-test. * $p < 0.05$.

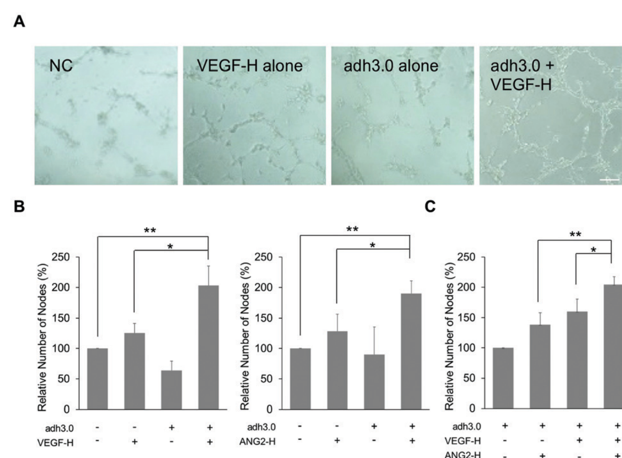

Fig. 4 Tube formation assay. (A) Bright-field images of HUVECs of each treatment. Scale bar represents 100 μm. (B and C) Relative Number of nodes (%) after 5 hours of incubation. (B) Cells were treated with adh3.0 (50 μM) and each Halo-tag fusion protein (7.5 μM) at 37 °C for 20 min followed by the incubation. (C) Cells were treated with adh3.0 (50 μM) and each or both of Halo-tag fusion protein (2.5 μM) at 37 °C for 20 min followed by the incubation. Acquired images were analysed with ImageJ (angiogenesis analyzer) for quantification. Experiments were replicated at least in duplicate. n = 3. Data represent mean ± SD. Significance was determined using an unpaired two-tailed Student's *t*-test. * $p < 0.05$, ** $p < 0.01$.

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proteins to facilitate immune shielding, cellular motility, and angiogenesis. Clinical application of this strategy requires further efficacy and safety evaluation including animal studies. Nonetheless, these findings may ultimately result in new translational opportunities for cell therapy.

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Conflicts of interest

There are no conflicts to declare.

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