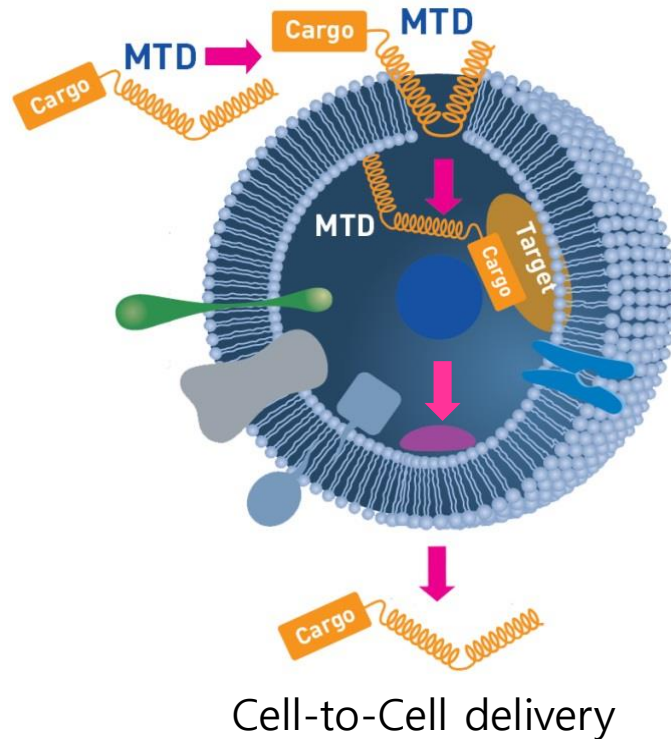


ProCell Therapeutics, Inc.

PII-SKIN SCI Journal

2017. 05. 10

Platform Technology : MITT™



Cargo (Functional Material)

Therapeutic antibodies

Recombinant Proteins

Peptides

siRNA and DNAs

Low MW molecule(chemical)

Nano-particle or liposome

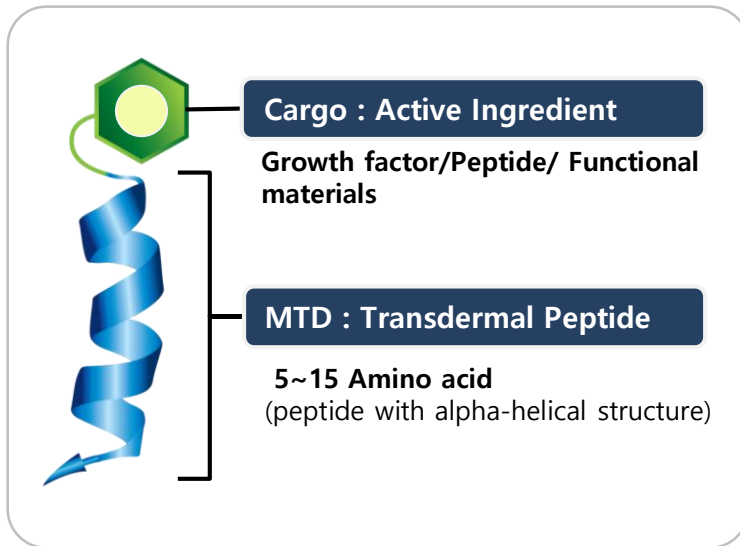
◆ MTD (Macromolecule Transduction Domain)

Consist of 5~15 amino acids

Intracellular Cargo delivery

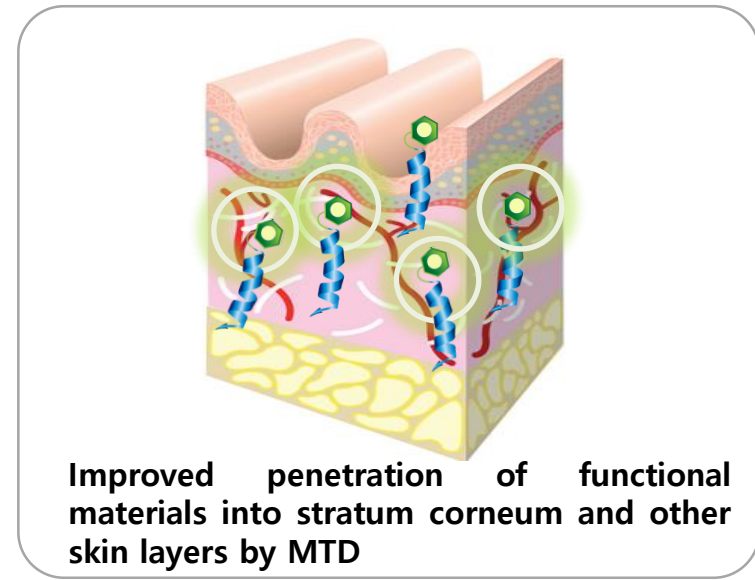
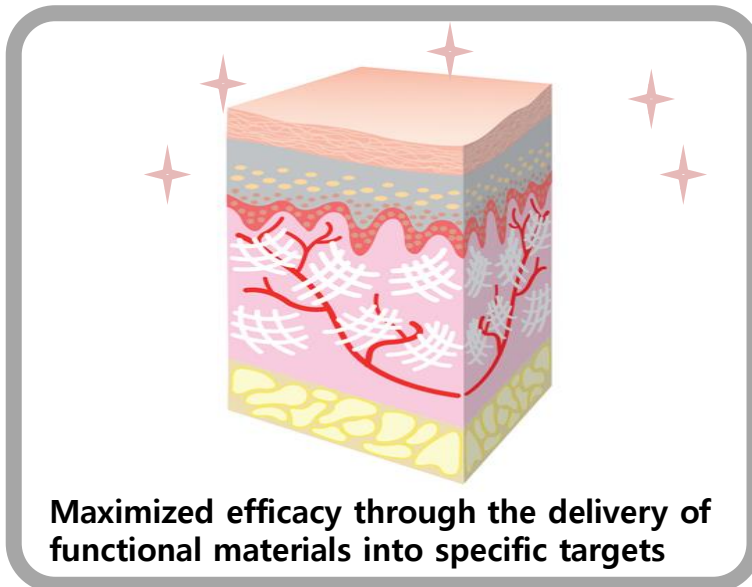
- MITT - Macromolecule Intracellular Transduction Technology
- MTD - Macromolecule Transduction Domain
- PII-SKIN - “Penetrating to Skin”

PII-SKIN Platform technology



- PII-SKIN Platform technology to improve transdermal delivery of functional materials

: Anti-aging/wrinkle, Hair growth, Brightening, etc.



SC Journal of ProCell Therapeutics

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RESEARCH ARTICLE

Epigenetic regulation of gene structure and function with a cell-permeable Cre recombinase

Daewoong Jo¹, Abdui Nashabi¹, Christie Doweke¹, Qing Lin¹, Derya Unlutamiz¹, Jin Chen¹, and H. Earl Raley¹*

Studies of mammalian gene function are hampered by temporal limitations in which phenotypes occurring at one stage of development interact with analysis at later stages. Moreover, phenotypes resulting from altered gene activity include both direct and indirect effects that may be difficult to distinguish. In the present study, recombinant fusion proteins bearing the 12 amino acid membrane translocation sequence (MTS) from the Kapozi fibroblast growth factor (FGF-4) were used to transduce enzymatically active Cre proteins directly into mammalian cells. High levels of recombination were observed in a variety of cultured cell types and in all tissues examined in mice following intraperitoneal administration. This represents the first use of protein transduction to induce the enzymatic conversion of a substrate in living cells and animals and provides a rapid and efficient means to manipulate mammalian gene structure and function.

The Cre recombinase from bacteriophage P1 has been widely used to induce DNA sequence-specific recombination in mammalian cells¹. LoxP sites, which are a target of Cre-mediated recombination in the P1 genome, also function as recombination substrate in mammalian cells. Application involving Cre-*loxP* recombination have included conditional mutagenesis^{2,3}, gene replacement⁴ and chromosome engineering^{5,6} in mice, and conditional gene expression^{7,8}. However, the use of site-specific recombination in genetic studies is often hampered by difficulties expressing the recombinase enzyme in cells of the appropriate type and developmental stage. Moreover, even conditional mutants induced by tissue-specific Cre expression may interfere with tissue development, thus precluding later studies in terminally differentiated cells.

To address this issue, we have isolated whether a protein domain with membrane-translocating activity could be used to deliver enzymatically active Cre recombinase into cultured cells and mice. The approach exploits the fact that leader sequences from Kapozi FGF-4 can promote the translocation of heterologous peptides across the plasma membrane and into the cytoplasm of cultured cells⁹. Protein transduction exploits biophysical properties common to mammalian cell membranes, and consequently, most cell and cell types were expected to acquire the enzyme. In addition to its potential utility in studies of mammalian gene function, a cell-permeable Cre could provide a convenient and stable method of protein transduction in cells containing an appropriately modified reporter gene. This would permit a quantitative assessment of protein trafficking and uptake in both cells and animals.

Results and discussion

Recombinant cell permeable Cre proteins. Purified recombinant fusion protein bearing the 12 amino acid MTS from Kapozi FGF-4 (ref. 10) were used to deliver enzymatically active Cre proteins directly into mammalian cells. Of the four recombinant proteins tested, His-NLS-Cre-MTS (Fig. 1A), which also contains a nuclear localization sequence (NLS) from simian virus 40 (SV40 large-T

antigen and an N-terminal His affinity tag displayed the best combination of yield, solubility, in vitro specific activity, nuclear localization, and activity in cells. Protein with either affinity tag or lacking an NLS performed less well. For example, addition of the NLS to GST-Cre-MTS (tagged with glutathione S-transferase) enhanced the biological activity of the protein in cells (see below) but also reduced the yield and solubility of the fusion protein. Replacement of the GST tag with the Zelenkita cell matrix-binding protein (MBP) domain improved solubility but impaired enzymatic activity.

Localization of transduced protein. Uptake of recombinant Cre protein into mammalian NIH3T3 cells was monitored by confocal fluorescence microscopy (Fig. 1B–D). All of the Cre fusion proteins were efficiently transduced, with 100% of treated cells showing intense staining. GST-Cre-MTS was localized predominantly in the cytoplasm (Fig. 1C) and MBP-NLS-Cre-MTS localized to both cytoplasm and nucleus (Fig. 1D), while His-NLS-Cre-MTS was predominantly nuclear (Fig. 2E). Treatment of cells with increasing concentrations of cell permeable protein resulted in increasing protein uptake as assessed by indirect immunofluorescence (data not shown).

Site-specific recombination in cultured cells. Cells containing individual genes modified with loxP sites were used to assess whether introduced Cre proteins could elicit recombination. The loxP-EGFP is a 13-bp loxP site in which Cre-mediated recombination activates the expression of a green fluorescent protein (GFP) reporter gene. The cells were exposed to His-NLS-Cre-MTS, washed extensively, and cultured for 24 h, providing time for GFP expression. Treatment with 4 μM His-NLS-Cre-MTS for 4 h induced recombination in 30% of cells, this percentage increased to 60% following exposure to 10 μM Cre (Fig. 2A). Recombination was also observed in 30% of cells exposed for 30 min to 10 μM His-NLS-Cre-MTS, and this percentage increased to 75% after 2 h (Fig. 2B) and to 87% following three consecutive 2 h treatments with 10 μM enzyme (Fig. 2C). Southern blot analysis confirmed that expression of the GFP reporter accurately reflected the extent

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LETTERS

nature medicine

Intracellular protein therapy with SOCS3 inhibits inflammation and apoptosis

Daewoong Jo¹, Danya Liu¹, Shan Yao¹, Robert D Collins² & Jack Hwang¹*

Suppressor of cytokine signaling (SOCS) 3 attenuates janinomyeloid signaling by the signal transducer and activator of transcription (STAT) family of proteins. But acute inflammation can occur after exposure to pathogen-derived inducers, staphylococcal enterotoxin B (SEB) and lipopolysaccharide (LPS), or the lectin concanavalin A (ConA), suggesting that physiologic levels of SOCS3 are insufficient to stem proinflammatory signaling under pathogenic circumstances. To test this hypothesis, we developed recombinant cell-penetrating forms of SOCS3 (CP-SOCS3) for intracellular delivery to counteract SEB-, LPS- and ConA-induced inflammation. We found that CP-SOCS3 was distributed in multiple organs within 2 h and persisted for at least 8 h in leukocytes and lymphocytes. CP-SOCS3 protected animals from lethal effects of SEB and LPS by reducing production of inflammatory cytokines and attenuating liver apoptosis and hemorrhagic necrosis. It also reduced ConA-induced liver apoptosis. Thus, replenishing the intracellular stores of SOCS3 with CP-SOCS3 effectively suppresses the devastating effects of acute inflammation.

Inflammation represents a fundamental response to microbial, chemical and physical injury. The production of inflammatory mediators depends on tightly regulated intracellular signaling by stress-responsive transcription factors as positive activators of the proinflammatory gene program¹. Concurrently, the genome can respond physiologically by eliciting a set of responses that extinguish inflammation. SOCS3 and SOCS3 are rapidly induced and then degraded². They block phosphorylation-dependent activation of STAT3 in response to interferon (IFN)-γ or STAT3 phosphorylation in response to interleukin (IL)-6, and target the IFN-γ receptor and/or IL-6 receptor signaling complex for proteasomal degradation³. Paradoxically, despite the presence of physiologic regulators such as SOCS3, the host defense systems can pathologically perpetuate inflammation by overproducing host mediators that cause collateral damage to multiple organs. In the well-established animal models of acute organ injury resulting from pathogen-derived inducers SEB and LPS or lectin ConA, inflammatory mediators that cause collateral damage to multiple organs. In the well-established animal models of acute organ injury resulting from pathogen-derived inducers SEB and LPS or lectin ConA, inflammatory mediators that cause collateral damage to multiple organs. In the well-established animal models of acute organ injury resulting from pathogen-derived inducers SEB and LPS or lectin ConA, inflammatory mediators that cause collateral damage to multiple organs.

To confirm that the CP-SOCS3 protein could penetrate cells we tested their effect on intracellular STAT3 phosphorylation. Inducible tyrosine phosphatase SOCS3 and SOCS3 are known to block STAT3 phosphorylation by Janus kinase (JAK) 1 and 2, a key step in intracellular signaling induced by IFN-γ. IFN-γ-induced phosphorylation of STAT3 was readily detected in cells exposed to H5N1, which lacks the M2M1 motif required for membrane penetration (Fig. 1B). In contrast, both forms of CP-SOCS3, H5M1 and H5M2, suppressed STAT3 phosphorylation in a dose-dependent manner, with a concentration causing 50% inhibition (IC₅₀) of 2 μM (Fig. 1C). We confirmed the inhibitory effect of

We designed and developed recombinant mouse CP-SOCS3 protein (Fig. 1A). A membrane-translocating motif (MTM) composed of 12 amino acids from a hydrophobic signal sequence from fibroblast growth factor 4 (ref. 11) was attached to either the N-terminal (H5M1) or C-terminal (H5M2) ends to mediate uptake into cells. We also constructed a control protein (His-SOCS3-H53) lacking the MTM. Purity and yields of the recombinant SOCS3 proteins were comparable (Fig. 1C).

We detected the intracellular delivery of recombinant SOCS3 proteins in mouse macrophage RAW cells by confocal laser scanning microscopy. Fluorescent isothiocyanate (FITC)-labeled SOCS3 lacking MTM was not detectable in RAW cells. In contrast, the two MTM-bearing CP-SOCS3 proteins, His-SOCS3-MTM1 (H5M1) and His-MTM1-SOCS3 (H5M2), were abundantly present in the cytoplasm of RAW cells (Fig. 1C). The cells were not dead and the broad-range protease proteasome K was used after pulsing cells with FITC-labeled proteins to prevent background fluorescence from cell surface-adsorbed SOCS3 proteins. Thus, the protease-resistant fluorescence indicated that only MTM-bearing SOCS3 proteins were able to penetrate cells.

To confirm that the CP-SOCS3 protein could penetrate cells we tested their effect on intracellular STAT3 phosphorylation. Inducible tyrosine phosphatase SOCS3 and SOCS3 are known to block STAT3 phosphorylation by Janus kinase (JAK) 1 and 2, a key step in intracellular signaling induced by IFN-γ. IFN-γ-induced phosphorylation of STAT3 was readily detected in cells exposed to H5N1, which lacks the M2M1 motif required for membrane penetration (Fig. 1B). In contrast, both forms of CP-SOCS3, H5M1 and H5M2, suppressed STAT3 phosphorylation in a dose-dependent manner, with a concentration causing 50% inhibition (IC₅₀) of 2 μM (Fig. 1C). We confirmed the inhibitory effect of

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ARTICLES

nature neuroscience

Observational fear learning involves affective pain system and Ca_v1.2 Ca²⁺ channels in ACC

Daehjong Jeon^{1,6}, Sangwoo Kim^{1,2}, Mattu Chetani¹, Daewoong Jo¹, H. Earl Raley¹, Shih-Yao Lin³, Daniil Rabah¹, Jean-Pierre Kinet⁴ & Hee-Sup Shin^{1,5}*

Fear is developed vicariously through social observation of others suffering from aversive stimuli. We found that mice (observers) developed freezing behavior by observing other mice (demonstrators) receive repetitive foot shocks. Observers had higher fear responses when demonstrators were socially related to themselves, such as siblings or mating partners. Inactivation of anterior cingulate cortex (ACC) and parafascicular or mediodorsal thalamic nuclei, which comprise the medial pain system representing pain affection, substantially impaired this observational fear learning, whereas inactivation of sensory thalamic nuclei had no effect. The ACC neuronal activities were increased and synchronized with those of the lateral amygdala at the theta rhythm frequency during this learning. Furthermore, an ACC-limited deletion of Ca_v1.2 Ca²⁺ channels in mice impaired observational fear learning and reduced behavioral pain responses. These results demonstrate the functional involvement of the affective pain system and Ca_v1.2 channels of the ACC in observational social fear.

Fear is a biological response to dangerous, threatening situations or stimuli. Fear can be acquired and expressed in a variety of ways¹. First, fear can be learned from direct experience of an aversive situation (for example, an unconditioned stimulus in classical Pavlovian fear conditioning). It is a classical conditioning experiment, pairing of a neutral, conditioned stimulus (for example, a tone) with an aversive, unconditioned stimulus (for example, a foot shock) causes an animal to express fear behaviors when the animal is later exposed to the conditioned stimulus in the absence of the unconditioned stimulus. The neural mechanism and circuitry of this fear has been well studied across species, including rodents^{2,3}. Fear can be socially acquired from instruction by verbal information (instructed fear) or from a vicarious observation of a conspecific's distress (observational fear) in primates, including humans^{4,5}. For example, a higher primate can recognize fear by observing a conspecific's distressed face or a conspecific suffering from a disease^{6,7}. Previous studies using a fear-prone protocol found that rats using a distressed conspecific (by electric shock) display fearful behavioral responses, such as crouching or motoric avoidance^{8,9}. A recent study found that C57BL/6 mice that observed unfamiliar mice experiencing classical fear conditioning displayed freezing behaviors when they were later exposed to the conditioned stimulus alone¹⁰. These findings demonstrate social transfer of fear in rodents. Unlike classical conditioning, however, the neural substrate and mechanism underlying observational social fear has not been well defined.

ACC is known to receive sensory signals from the somatosensory cortex and other cortical areas, including the anterior insular cortex¹¹. We hypothesized that the medial pain system, such as the ACC, representing affective or emotional components of pain might

activity of the ACC and the amygdala change during observation of others experiencing fear or others' fearful facial expressions^{12,13}. In addition, animal studies have suggested that the ACC is involved in pain affection or emotion behavior, as well as pain sensation^{14–17}. Thus, the ACC is considered to be an important brain region for the convergence of sensory and emotional information and may mediate affective or emotional responses to noxious stimuli. However, the functional involvement of the ACC in observational social fear learning in animals remains unknown.

Brain-imaging studies in humans have shown that some brain regions related to the affective or emotional dimension of pain are activated during observation of distress in individuals suffering from noxious stimuli^{18–20}. There are at least two pathways for pain processing in the thalamus in the CNS^{21,22}: One is the lateral pain system, which expresses the sensory or discriminative dimension of pain and involves the perception of intensity, location and quality of pain stimuli. This pathway projects from the spinal cord dorsal horn to the ventral posterior nucleus (VPL) and posterior lateral (VPM) thalamic nuclei, which relay nociceptive information to the somatosensory cortex. The other is the medial pain system, which represents the affective or emotional dimension of pain and involves perception of the unpleasantness of pain. In this pathway, the spinal nociceptive inputs project to the midline and intralaminar thalamic nuclei (MTN), including parafascicular and mediodorsal thalamic nuclei, and then proceed to limbic cortical areas, including the ACC.

We hypothesized that the medial pain system, such as the ACC, representing affective or emotional components of pain might

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Nature Biotechnology Nature Medicine Nature Neuroscience

- Nature Biotechnology : MTD development history - Personal paper of founder
- Nature Medicine: Study of MTD applied to immunotherapy proteins - Personal paper of founder
- Nature Neuroscience: Study of MTD applied to brain therapeutic proteins
 - KIST & Procell jointly published paper.
 - Provided MTD for KIST research.
 - An example of proven MTD technology from other organizations.

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original article

Antitumor Activity of Cell-permeable p18^{INK4c} With Enhanced Membrane and Tissue Penetration

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Practical methods to deliver proteins systemically in animals have been hampered by poor tissue penetration and inefficient cytoplasmic localization of intranasal proteins. We therefore pursued the development of improved macromolecule transduction domains (MTDs) and tested their ability to deliver therapeutically active p18^{INK4c}. MTD103 was identified from a screen of 1,500 signal peptides, tested for the ability to promote protein uptake by cells and tissues, and analyzed with regard to the mechanism of protein uptake and the delivery of biologically active p18^{INK4c} into cancer cells. The therapeutic potential of cell-permeable MTD₁₀₃-p18^{INK4c} (CP-p18^{INK4c}) was tested in the HCT116 tumor xenograft model. MTD₁₀₃-p18^{INK4c} appeared to traverse plasma membranes directly, was transferred from cell-to-cell and was therapeutically effective against cancer xenografts, inhibiting tumor growth by 55–98% after 3 weeks ($P < 0.05$). The therapeutic responses to CP-p18^{INK4c} were accompanied by high levels of apoptosis in tumor cells. In addition to enhancing systemic delivery of CP-p18^{INK4c} to normal tissues and cancer xenografts, the MTD103 sequence delayed protein clearance from the blood, liver, and spleen. These results demonstrate that macromolecule intracellular transduction technology (MITT) enabled by MTDs may provide novel protein therapies against cancer and other diseases.

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INTRODUCTION

The mammalian cell cycle is regulated by the sequential activation of cyclin-dependent kinases (CDKs) whose activities are restrained by physical interactions with CDK inhibitors (CKIs).^{1–3} These controls integrate the cell cycle with extracellular signals required for cell growth, differentiation, and survival and provide check-points to guard against unsheduled cell proliferation and maintain genome integrity. For example, transition through the first gap phase (G₁) and entry into S phase is coordinated initially by CDK2 and 6

in association with their activating subunits, the E type cyclins, and negatively regulated by members of the inhibitor of kinase 4 (INK4) family, p15^{INK4}, p16^{INK4}, and p18^{INK4} CKIs proteins and subsequently by CDK2-cyclin E, restrained by the Cip/Kip CKIs (p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}). In addition, several of the Cip/Kip CKIs stimulate the assembly and activity of the Cyclin D-CDK4/6 complexes with which they associate; these interactions then divert the Cip/Kip proteins from suppressing Cyclin D-CDK2 activity.⁴

Although not required for mammalian cell proliferation, the G₁ CKIs play critical roles in the development and maintenance of specialized cell types, including erythroid progenitors, cardiomyocytes and pancreatic β cells.⁵ Moreover, the activities of the G₁ CKIs are frequently elevated in human tumors by a variety of mechanisms, including Cyclin D overexpression, activating mutations in CDK4, and loss-of-function mutations involving INK4 and Cip/Kip CKIs. The latter rank among the most common tumor suppressor gene mutations in human cancer. Finally, in some settings, CDK2 activity is required for malignant transformation^{6,7} and allows tumor cells to tolerate the otherwise lethal effects of an activated oncogene.⁸ Such considerations establish the G₁ CKIs as potential targets for anticancer drug development⁹ and provide the rationale for ongoing clinical trials of several CDK4/6 inhibitors.¹⁰

A common problem with small-molecule kinase inhibitors is the potential for off-target drug interactions.¹¹ To address this issue, we have investigated the use of intracellular transduction technologies (MITTs) to deliver biologically active CKIs, specifically p18^{INK4c}, into cultured tumor cells and animal tissues. In principle, cell-permeable p18^{INK4c} proteins provide a means to inhibit CDK4 and 6 specifically, establishing if a protein-based cancer therapy, there is a reference agent to evaluate candidate small-molecule CDK inhibitors. Moreover, by modulating the differentiation and/or renewal of stem cells with specific interactions for CKIs and 4-family (e.g., erythroid progenitors, cardiomyocytes, and pancreatic β cells), cell-permeable p18^{INK4c} could have therapeutic applications in such areas as stem cell optimization and regenerative medicine.

Intracellular macromolecule transduction exploits the ability of specific basic, amphiphilic, and hydrophobic lipophilic

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Cancer Research

Therapeutics, Targets, and Chemical Biology

Cell-Permeable NM23 Blocks the Maintenance and Progression of Established Pulmonary Metastasis

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Abstract

Overt metastasis is a major cause of cancer mortality, even among patients undergoing curative resection. Therefore, practical strategies to target the growth and persistence of already established metastases would provide an important advance in cancer treatment. Here, we assessed the potential of protein therapy using a cell-permeable NM23-like metastasis suppressor protein. Hydrophobic transduction domains derived from a screen of 1,500 signaling peptide sequences enhanced the uptake of the NM23 protein by cultured cells and systemic delivery to animal tissues. The cell-permeable (CP)-NM23 inhibited metastasis-associated phenotypes in tumor cell lines, blocked the establishment of lung metastases, and cleared already established pulmonary metastases, significantly prolonging the survival of tumor-bearing animals. Therefore, these results establish the potential use of cell-permeable metastasis suppressors as adjuvant therapy against disseminated cancers. *Cancer Res.* 71(23):7216–7226, 2011. AACR.

Introduction

Metastasis is an acquired and separately evolving phenotype that enables cancer cells to disseminate and grow at locations distant from the primary tumor site. For many tumors, the molecular changes responsible for initiating metastasis spread have already occurred by the time of initial diagnosis, and are ultimately responsible for most cancer death. (1, 2). Effective strategies to target disseminated tumors are therefore limited to immunomodulatory therapeutic but not to drug treatments.

In principle, antimetastatic therapies could either block activities required for the growth of normal or disseminated cancer cells or restore the expression and/or activity of proteins that function to suppress metastasis. The latter includes more than 20 metastasis suppressor proteins that selectively inhibit the seeding, growth, or persistence of metastasis but which having only limited effects on primary tumors (3). NM23, the first reported metastasis suppressor gene, was initially characterized as a nucleotide diphosphate kinase (NDK), an enzyme required to maintain cellular pools of nucleoside triphosphates. Interest in NDK as a metastasis suppressor (alternatively named NM23H1 or N23H) was generated by studies describing inverse correlations between N23 expression and metastatic potential. (4). In metastatic cells (1) and later in other types of tumors (5), subsequent gene transfer experiments documented the ability of NM23 to suppress metastasis-associated phenotypes both in cultured cells and in animal metastasis models (6–10). The precise mechanism by which NM23 influences metastasis is not understood, in part, because the protein possesses multiple enzymatic activities that directly or indirectly suppress metastasis-associated protein kinases (MAPK) signaling (11, 12) regulate small G-protein functions important in cell motility, cytoskeletal reorganization, and cell adhesion (13–15) and influence genome maintenance (16, 17). Nevertheless, clinical trials based on hormonal activation of endogenous N23 expression are currently in progress (18).

In the present study, we described an antimetastatic therapy based on the systemic delivery of a cell-permeable NM23-like protein. For this experiment, we developed novel macromolecule transduction domains (MTD) modified after hydrophobic signal peptides previously shown to promote protein uptake by cultured cells and animal tissues (19). The MTD-NM23 inhibited metastasis-associated phenotypes in tumor cell lines and not only prevented the establishment of lung metastases but also cleared previously established metastases, significantly prolonging the survival of animals harboring disseminated tumor cells.

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Clinical Cancer Research

Cancer Therapy: Preclinical

Antitumor Activity of Cell-Permeable RUNX3 Protein in Gastric Cancer Cells

Junghye Lim^{1,2}, Tam Duong³, Nga Do⁴, Phuong Do⁵, Jaetaek Kim⁶, Hyuncheol Kim⁶, Wael El-Rifai⁷, H. Earl Rulley⁸, and Daesoo Jung^{1,2}

Abstract

Purpose: Gastric cancer is a leading cause of cancer death worldwide. Limited therapeutic options highlight the need to understand the molecular changes responsible for the disease and to develop therapies based on this understanding. The goal of this study was to develop cell-permeable (CP-) forms of the RUNX3-related transcription factor 3, RUNX3, a candidate tumor suppressor implicated in gastric and other epithelial cancers to study the therapeutic potential of RUNX3 in the treatment of gastric cancer.

Experimental Design: We developed novel macromolecule transduction domains (MTDs) which were tested for the ability to promote protein uptake by mammalian cells and tissues and used to deliver of biologically active RUNX3 into human gastric cancer cells. The therapeutic potential of CP-RUNX3 was tested in the NCI-N87 human stomach xenograft animal model.

Results: RUNX3 fusion proteins, HMs₁B and HMs₂B, containing hydrophobic MTDs enter gastric cancer cells and suppress cell phenotypes (e.g., cell cycle progression, wounded monolayer healing, and survival) and induce changes in biomarker expression (e.g., p21^{Cip1} and VEGF) consistent with previously described effects of RUNX3 on TCF- β signaling. CP-RUNX3 also suppressed the growth of subcutaneous human gastric tumor xenografts. The therapeutic response was comparable with studies augmenting RUNX3 gene expression in tumor cell lines; however, the protein was most active when administered locally, rather than systemically (i.e., intravenously).

Conclusions: These results provide further evidence that RUNX3 can function as a tumor suppressor and suggest that practical methods to augment RUNX3 function could be useful in treating of some types of gastric cancer. *Clin Cancer Res.* 19(12):406–416, 2012. AACR.

Introduction

Gastric cancer is the most common cancer in Asian countries (e.g., Korea and Japan) and a leading cause of cancer death worldwide, providing considerable effort to understand the pathogenesis of the disease and to develop improved methods for diagnosis and treatment (1, 2). Gastric tumors arise by multiple etiologies, including an intestinal type that emerges through a stepwise dysplasia-carcinoma sequence in which inflammatory responses

to *Helicobacter pylori* infection play an initiating role and a diffuse type that arise without clearly defined precursor lesions or etiologies. Therapeutic options are limited for gastric cancer cases not staged by surgical resection, and overall 5-year survival rates are in the range of 30% (1). As a consequence, there is considerable interest in characterizing the molecular changes responsible for tumor type and grade to better predict disease outcome and possibly to inform individualized therapies (2).

RUNX3-related transcription factor 3 (RUNX3) has been implicated as a tumor suppressor gene in gastric cancers (3) as well as a variety of malignancies (4). RUNX3 knockout mice develop gastric hyperplasia and tumors, associated with reduced levels of apoptosis, altered cellular responses to TCF- β (5) and changes in the cyclin-dependent kinase inhibitor p21^{Cip1} and VEGF expression consistent with enhanced proliferation and angiogenesis, respectively (6, 7). Reductions in RUNX3 expression have been attributed to promoter hypermethylation (8), G1, and protein mislocalization (9) and correlate with poor prognosis (10–13). Conversely, enhanced RUNX3 expression suppresses the proliferation and tumorigenicity of gastric cancer cell lines (13, 17).

However, other studies have challenged the concept that RUNX3 functions directly as a tumor suppressor in gastric

Molecular Biology

Cancer Research

Clinical Cancer Research

■ Molecular Biology & Cancer Research & Clinical Cancer Research & Biomaterials

: Study of MITT applied to development of anticancer drug

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Biomaterials

Journal homepage: www.elsevier.com/locate/biomaterials

The effect of intracellular protein delivery on the anti-tumor activity of recombinant human endostatin

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ABSTRACT

Endostatin (ES), a 20 kDa protein derived from the carboxy-terminus of collagen XVIII is a potent angiogenesis inhibitor, but clinical development has been hindered by poor clinical efficacy and toxicity. Recent functional information from which to design agents with improved activity. The present study investigated protein uptake by cells as a determinant of ES activity. We developed a cell-permeable ES protein (HMES) with enhanced capacity to enter cells by adding a macromolecule transduction domain (MTD). HMES exhibited angiogenesis-associated phenotypes in cultured endothelial cells (as assessed by tube formation, wound-healing, cell proliferation and survival assays). These effects were accompanied by reductions in MMP expression (ESK phosphatidylase), and in EC markers, eNOS, SREB, and VEGF protein expression. The cell-permeable ES displayed greater tissue penetration in mice and improved the growth of human tumor xenografts to a significantly greater extent than ES protein without the MTD segment. Our results suggest that anti-angiogenic activity of native ES are limited by the level of protein uptake and/or subcellular localization, and that much of the activity of ES against tumors depends on one or more intracellular functions. This study will inform future efforts to understand ES function(s) and suggest strategies for improving ES-based cancer therapeutics.

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1. Introduction

Angiogenesis, the process of blood vessel formation, is essential for tumor growth and therefore provides an attractive target for anti-cancer therapies. Toward this end, a number of agents targeting angiogenesis have entered clinical trials [1,2]. These trials confirm that anti-angiogenic agents can produce measurable clinical benefits (primarily progression-free interval) when used in combination with other therapies, and consequently, the most effective have been approved for use against selected tumor types [1,2]. However, with limited impact on overall survival and toxicity profiles that limit application [3], the therapeutic benefits of targeting angiogenesis have fallen short of expectations.

Endostatin (ES), a 20 kDa protein derived from carboxy-terminus of collagen XVIII, was among the first endogenous proteins identified with anti-angiogenic activity as assessed by *in vitro* effects on endothelial cell proliferation, survival and tube formation [4]. Interest in Endostatin, and interest in angiogenesis as a therapeutic target, was spurred by a report describing tumor inhibition in mice treated with high doses of recombinant endostatin [5]. ES moved quickly into clinical trials; however, clinical development ended in the US in 2003 due to limited efficacy and problems with protein formulation and application [1]. Clinical development later resumed in China using a recombinant histidine-tagged protein (Endostar[®]) with improved solubility and activity [1,6]. However, current clinical development of ES continues to be hampered by insufficient understanding about the mechanism(s) by which the protein inhibits angiogenesis, despite an extensive literature on

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Partial Somatic to Stem Cell Transformations Induced By Cell-Permeable Reprogramming Factors

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ABSTRACT

The production of pluripotent stem cells (iPSCs) for therapeutic applications will require practical methods to achieve tight temporal and quantitative control of reprogramming factor (RF) expression, while avoiding the mutagenic potential of gene transfer. Toward this end, we have developed cell permeable RF proteins (CP-RFs) incorporating newly developed macromolecule transduction domains (MTDs). Treatment of human dental fibroblasts (hDFs) with combinations of cell-permeable OCT4, SOX2, KLF4, CMYC and either NANOG or LIN28 proteins induced the outgrowth of stem cell-like colonies (SCCs). SCs colonizing spheres with CP-RFs resembled embryonic stem cells with regard to morphology, biomarker expression, and extended capacity for self-renewal, but failed to expand as iPSC on ES cell lines. Partial reprogramming appeared to be a common response to protein-based delivery of reprogramming factors into somatic cells.

INTRODUCTION

Emotionally differentiated somatic cells can be reprogrammed to become induced pluripotent stem cells (iPSCs) by induced expression of reprogramming factors (RFs) that promote self-renewal and render cells pluripotent with regard to cellular differentiation¹. RFs include proteins individually required to maintain embryonic stem (ES) cells in a pluripotent state (stably octamer-binding transcription factor 4 (OCT4), sex determining region Y box 2 (SOX2), and NANOG^{2,3}), as well as proteins (e.g. CMYC, Kraspp-like factor 4 (KLF4) and LIN28) that promote self-renewal and suppress cellular differentiation^{4,5}. Combinations of RFs with highest iPSC activity (e.g. OCT4 and SOX2 plus either KLF4 and CMYC or alternatively, NANOG and LIN28) were discovered from libraries of candidate RFs by combinatorial screens^{6,7}.

In principle, patient-derived iPSCs could be used for autologous stem cell therapies without the ethical and graft rejection problems associated with using embryo-derived stem cells. In practice, the application of iPSCs to human regenerative medicine will require efficient methods to introduce RFs into somatic cells combined with ways to guard against dysregulated RF activity and vector-induced mutations. Unfortunately, somatic cell reprogramming is relatively inefficient and thus requires relatively efficient methods to regulate RF activity. Although retroviruses can achieve sufficient gene transfer efficiencies, any vector that integrates into the genome is potentially mutagenic. Moreover, RFs that enhance ES formation may undermine subsequent clinical applications, as illustrated by CMYC, which collaborates with other RFs to enhance iPSC formation but induces tumors in iPSC-derived tissues⁸. Similarly, ectopic OCT4 and KLF4 promote epithelial dysplasias^{9,10}. For these reasons, various strategies have been developed to produce transient free iPSCs, including: *in situ* induced vectors¹¹, excisable transposons¹², adenovirus¹³ and Sendai virus¹⁴ vectors and non-integrating episomal vectors¹⁵. Other approaches have avoided completely away from DNA-based expression vectors, including synthetic modified RNA's enzymatic regulation by chemical compounds (see¹⁶ for a review) and direct epigenetic control of gene reprogramming.

In the present report, we investigated the use of macromolecule intracellular transduction technology (MITT) to deliver biologically active RFs into human skin fibroblasts. MITT was used previously to deliver peptides and proteins to a variety of tissues (notably liver, lung, pancreas and lymphoid tissues), resulting in dramatic protection against lethal inflammatory diseases^{17,18}, suppression of pulmonary metastases¹⁹ and inhibition of subcutaneous tumor xenografts²⁰. The technology exploits the ability of hydrophobic, macromolecule transduction domains (MTDs) to promote bidirectional transfer of peptides and proteins across the plasma

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Cell-Permeable Parkin Proteins Suppress Parkinson Disease-Associated Phenotypes in Cultured Cells and Animals

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder of complex etiology characterized by the selective loss of dopaminergic neurons, particularly in the substantia nigra. Parkin, a tightly regulated E3 ubiquitin ligase, promotes the survival of dopaminergic neurons in both PD and Parkinsonian syndromes induced by acute exposure to neurotoxic agents. The present study assessed the potential of cell-permeable parkin (CP-Parkin) as a neuroprotective agent. Cellular uptake and tissue penetration of recombinant, enzymically active parkin was markedly enhanced by the addition of a hydrophobic macromolecule transduction domain (MTD). The resulting CP-Parkin proteins (P9M1) and P9M2 suppressed dopaminergic neuronal toxicity in cells and mice exposed to 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). These included enhanced survival and dopamine expression in cultured CA1Ha and SH-SY5Y neuronal cells, and protection against MPTP-induced damage in mice, notably preservation of parkin hydrophobic-positive cells with enhanced dopamine expression in the striatum and midbrain, and preservation of gross motor function. These results demonstrate that CP-Parkin proteins can compensate for intrinsic limitations in the parkin response and provide a therapeutic strategy to augment parkin activity *in vivo*.

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra. These selective clinical features have formed efforts to understand the mechanisms responsible for neuronal death and reasons why dopaminergic neurons are differentially affected. An extensive literature implicates oxidative stress, mitochondrial dysfunction and protein misfolding in disease etiology [1,2], as illustrated by loss-of-function mutations in genes such as alpha-SYNUKIN, PINK1, DJ-1 and LRRK2, and by the action of toxic agents that induce Parkinsonian disease in both animals and man.

The parkin protein, function as an E3 ubiquitin ligase and catalyzes K48 and K112 linked mono- and poly-ubiquitination involved in protein turnover and trafficking [3]. Parkin substrate include proteins known to accumulate in the striatum of parkin knockout mice, although NMDA-stimulation suggests parkin function normal beyond protein degradation. The PINK1-induced lesion [1] DNK1 is activated by mitochondrial depolarization and

infilters parkin recruitment to damaged mitochondria and their subsequent removal by autophagy. DJ-1, although associated with diverse functions, appears to play a parallel protective role to that of parkin/PINK1 in oxidative stress response. Agens capable of inducing subtle Parkinson-like responses include chemical neurotoxins, notably 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone and 6-hydroxydopamine (6-OHDA) and α-synuclein, a protein that accumulates in Lewy bodies, a clinical signature of human PD [4]. These agents promote neuronal degeneration/dysfunction through a combination of oxidative stress and mitochondrial regulatory impairment.

Despite the complexity of PD etiology, parkin appears to play a broadly protective role in maintaining neuronal function and viability. These protective effects extend to a variety of neurotoxic, mitochondrial proteins and modified proteins including dopamine [5], rotenone and carbonyl cyanide 3-hydroxyphenylhydrazone [6], 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), melatonin kinase acid [7], unfolded protein stress response [8], Bax/Bcl-2 proapoptotic protein [9], heat shock

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PLOS One

- Scientific Report: Study of MITT applied to development of induced differentiated stem cells - A paper published by Procell
- PLOS One: Study of MITT applied to development of Parkinson's remedy - A paper published by Procell

Summary

- **Journals of Procell is focused on the study of "Development of Cell-permeable Protein Therapeutics for New Drug Development" applying MITT, the Source technology, and MTD (Macromolecule transduction domain), the basis of technology.**
- **PII-SKIN is a technique of MITT applied to skin, as one branch of MITT, and works by MTD, permeating skin cells, is applied to various substances (eg, growth factor, peptide).**
- **This is included in the technology of MITT & MTD, which allows substances to function by imparting cell permeability. And each study can be used as a resource to confirm the technology.**