論文の内容の要旨

論文題目　**Biological Characterization of Cells Expressing Muc21/epiglycanin**

Muc21/epiglycanin 発現細胞の生物学的な特性

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[Introduction]

Mucins are a group of glycoproteins characterized by tandem repeat domains consisting of high proportion of serines and threonines, through which *O*-glycosylation occurs. More than 20 mucin genes have been identified. They are largely classified by two groups; secreted mucins and transmembrane mucins. Mucins have been known to be involved in various biological processes. Under normal conditions, they are expressed on the epithelial cell surfaces of respiratory, gastrointestinal or reproductive tracts and function as a protective barrier. Some malignant cells express mucins aberrantly or with abnormal glycosylation patterns, which possibly causes several cellular behavioral changes including altered responses to external signals or modified interactions with other cells. Particularly, several transmembrane mucins were proved to be involved in pathogenesis such as cancers, and further their usefulness as diagnostic or prognostic markers, or therapeutic targets was presented.

As one of transmembrane mucins, Muc21 was recently identified as a molecular entity of epiglycanin, which has long been known for its unique roles on TA3-Ha mouse mammary adenocarcinoma cells. Having a molecular weight of 500 KDa and a length of 500 nm from cell surfaces, epiglycanin has been hypothesized to enable TA3-Ha cells to grow immunologically incompatible hosts and to provide anti-adhesive properties and enhanced malignancies to the cells. However, definite evidence was not obtained because the gene was not identified.

In the present work, we show for the first time the functions of Muc21 in cells by overexpressing the identified Muc21 cDNA. Expression of Muc21 caused striking morphologic changes of the cells through its anti-adhesive property. The underlining mechanism was studied using transient transfection system in HEK293T human embryonic kidney cells. Moreover, the effect of Muc21 expression on cellular behaviors of tumor cells was determined using B16-F1 cells.

[Results and Discussion]

1. Characterization of anti-adhesive property of Muc21

Expression of Muc21 in cells using an artificially constructed cDNA and morphologic changes of the cells

Under the circumstance that Muc21 cDNA was not fully cloned yet, an artificial cDNA was constructed by connecting three fragments; the N-terminal signal sequence, the tandem repeat domain consisting of 84 tandem repeats of 15 amino acids, and the C-terminal cytoplasmic domain. The artificial cDNA was further incorporated into an IRES-Venus vector. When the cDNA was transiently transfected into 293T cells, Muc21 was expressed at the cell surfaces as detected by Muc21 mAb 1A4-1 along with Venus expression (Fig. 1A). By western blotting and lectin blotting, Muc21 was shown to be a large highly glycosylated molecules (Data not shown). Markedly, Venus-positive cells lost their cellular extensions and become round and more than half of the Venus-positive cells became floating (Fig. 1B). In contrast, 293T cells transiently expressing human MUC1 did not show similar morphologic changes indicating that the effect is unique for Muc21. The floating cells were TUNEL negative indicating that the floating phenotypes were not due to undergoing apoptosis (data not shown).

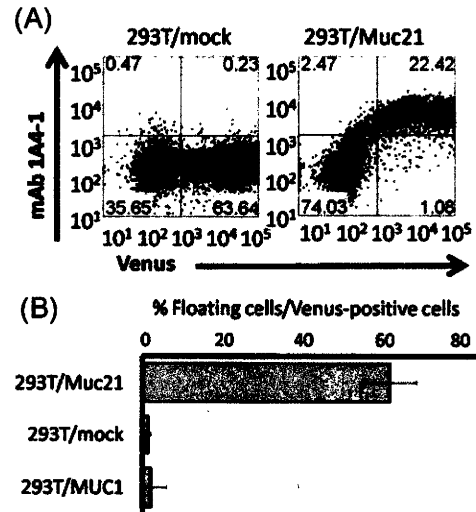

Fig. 1. Non-adhesive phenotypes induced by Muc21 expression. The Muc21-IRES-Venus vector was transfected into 293T cells and Muc21 expression was detected with mAb1A4-1 (A). The floating cells were collected from culture media and counted (B).

Examination of molecular domains of Muc21 responsible for the non-adherent phenotypes

The structural basis of anti-adhesive properties of Muc21 was investigated. In its cytoplasmic tail, Muc21 has several functional motifs, which might be involved in cellular signaling events leading to the morphologic changes. Thus, cytoplasmic domain-deficient mutants were constructed with an N-terminal flag tag and transfected into 293T cells. The floating phenotype was maintained, although the proportion of the floating cells was reduced compared to Muc21 transfectants having the intact cytoplasmic tail (Fig. 2A). Since the cytoplasmic domain of Muc21 seemed not to be fully responsible for the anti-adhesiveness, the contribution of the extracellular domain of Muc21 was determined. When various Muc21 mutants having different numbers of tandem repeats or no tandem repeats were constructed with an N-terminal Flag-tag and transiently

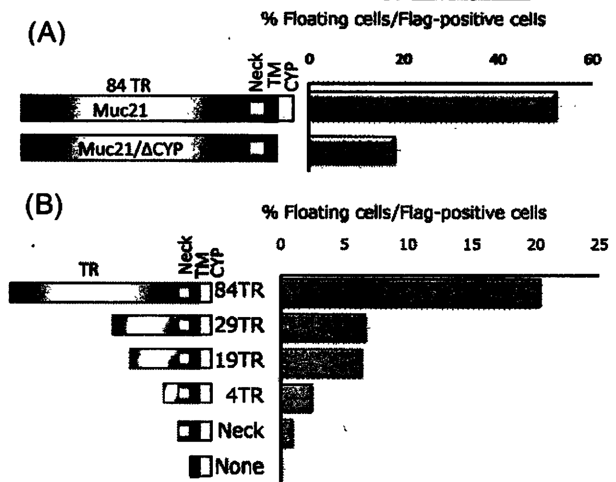

Fig. 2. Tandem repeat dependency of non-adhesive phenotypes. Cytoplasmic domain deficient mutants were constructed with an N-terminal Flag-tag and transfected into 293T cells (A). Extracellular domain mutants were constructed and transfected into 293T cells (B). Note that the mutants in B were constructed using a different vector system from mutants in A.

transfected into 293T cells, the percentages of floating cells among the Flag-positive cells closely correlated to the number of tandem repeats (Fig. 2B). Therefore, the large highly glycosylated tandem repeat domain of Muc21 is crucial for inducing the anti-adhesiveness.

<u>*Investigation of the mechanism of the anti-adhesive effect induced by Muc21*</u>

The mechanism by which the large tandem repeat domain of Muc21 induces anti-adhesive property was explored. To see whether Muc21 interferes cell-cell adhesion or cell-extracellular matrix (ECM) adhesion, over 90% Venus positive cells were collected by cell sorting after transient transfection of the Muc21-IRES-Venus vectors and subjected to homotypic aggregation assay and adhesion assay. Muc21 transfectants having 84 tandem repeats completely prevented cell-cell adhesion (Fig. 3A) as well as cell-ECM components adhesion (Fig. 3B). Muc21 transfectants having 4 tandem repeats partially inhibited homotypic cell adhesion and showed no inhibitory effect on cell-ECM interaction. Because the large tandem repeat domain of Muc21 prevented integrin-dependent cell-ECM interaction, but not integrin-independent cell to poly-lysine interaction, cell surface accessibility of integrin was assessed by the binding of antibody specific for β1 chain of integrin. The antibody binding was significantly reduced by the expression of Muc21 (Fig. 3C), without affecting the protein levels of β1 integrin in whole cell lysates (data not shown) indicating that interference of integrin function might play an important role in the anti-adhesive effect. Sialic acids on the tandem repeats of Muc21 provide negative charges which might induce anti-adhesive effect by charge repulsion. The contribution of sialic acids was determined by performing homotypic aggregation assay and adhesion assay after the treatment of sialidase. Sialidase treated cells and untreated cells showed similar anti-adhesive property (Fig. 3A and 3B) showing negligible contribution of sialic acids. Collectively, these results indicate that Muc21 induce anti-adhesive effect through its large tandem repeat domain possibly by preventing the interaction of cell adhesion-related molecules with its counterpart.

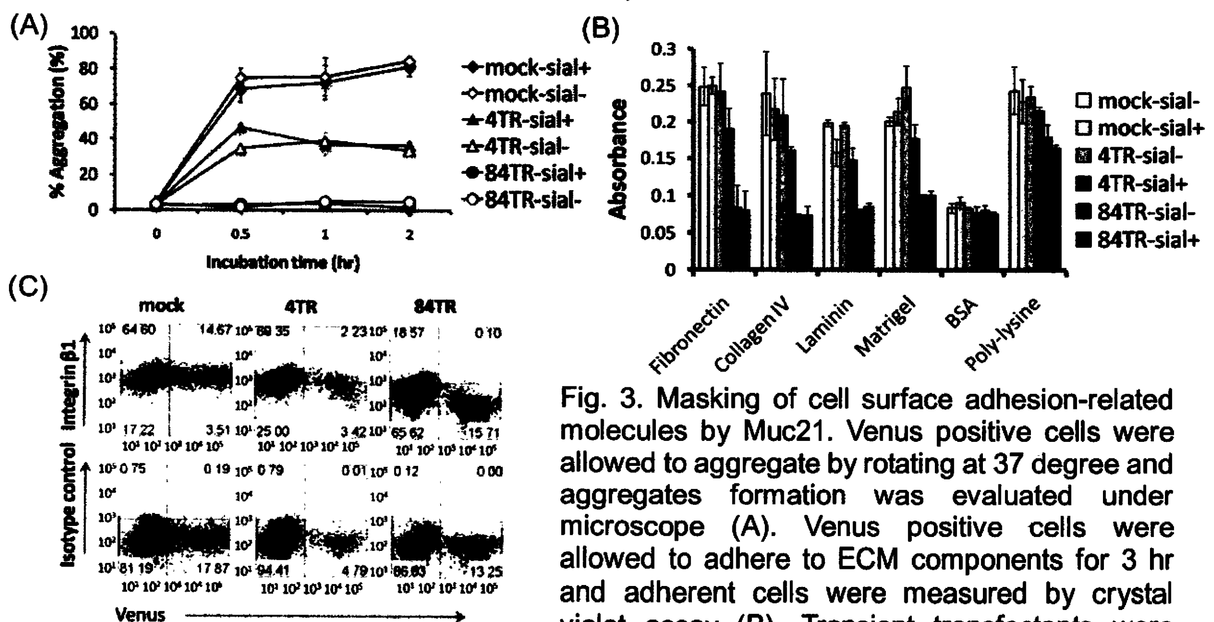

Fig. 3. Masking of cell surface adhesion-related molecules by Muc21. Venus positive cells were allowed to aggregate by rotating at 37 degree and aggregates formation was evaluated under microscope (A). Venus positive cells were allowed to adhere to ECM components for 3 hr and adherent cells were measured by crystal violet assay (B). Transient transfectants were assayed by flow cytometry using integrin β1 antibody (C). Sial+; sialidase treated cells, Sial-; sialidase untreated cells

2. Examination of tumor cell behavioral changes induced by Muc21 expression *in vitro* and *in vivo*

<u>*In vitro cellular behavioral changes induced by Muc21 expression*</u>

To determine how Muc21 contributes to malignant cell behaviors, B16-F1 melanoma cells were transfected with the Muc21-IRES-Venus vectors and stable transfectants were obtained by Venus positive-cell sorting and cloning. The clones with high expression of Muc21 showed higher percentages of floating cells similar to the 293T/Muc21 transfectants. To examine whether Muc21 expression provide survival or growth advantages in these cells, poly(2-hydroxyethylmethacrylate) (poly-Hema) coated plates were used as non-adherent surfaces. B16-F1/Muc21-84TR transfectants showed increased cell growth compared to B16-F1/mock transfectants (Fig. 4A). In the B16-F1/mock cells, cell viability was reduced as incubation time increased on the poly-Hema coated plates whereas the viability of B16-F1/Muc21 transfectants was not affected even after prolonged incubation under the same condition (Fig.4B). These results suggest that Muc21 expression not only rendered cells to be non-adherent but also contributed to enhanced survival under non-adherent conditions.

The masking role of Muc21 on the tumor cell surfaces could provide escape mechanisms by inhibiting tumor cell interactions with cells in the immune system. To prove or disprove this hypothesis, cytotoxic effects by IL-2 activated splenocytes on B16-F1/Muc21 transfectants were examined. B16-F1/Muc21 transfectants showed substantially reduced sensitivity to cytotoxic effects than mock transfectants. The reduction was apparently dependent on the size of tandem repeats (Fig. 4C). Strong anti-adhesive property of Muc21 might prevent tumor cells from interaction with endothelial cells. Using SvBCE bovine corneal endothelial cells, B16-F1/Muc21 transfectants showed notably reduced adhesion to the endothelial cells (Fig. 4D).

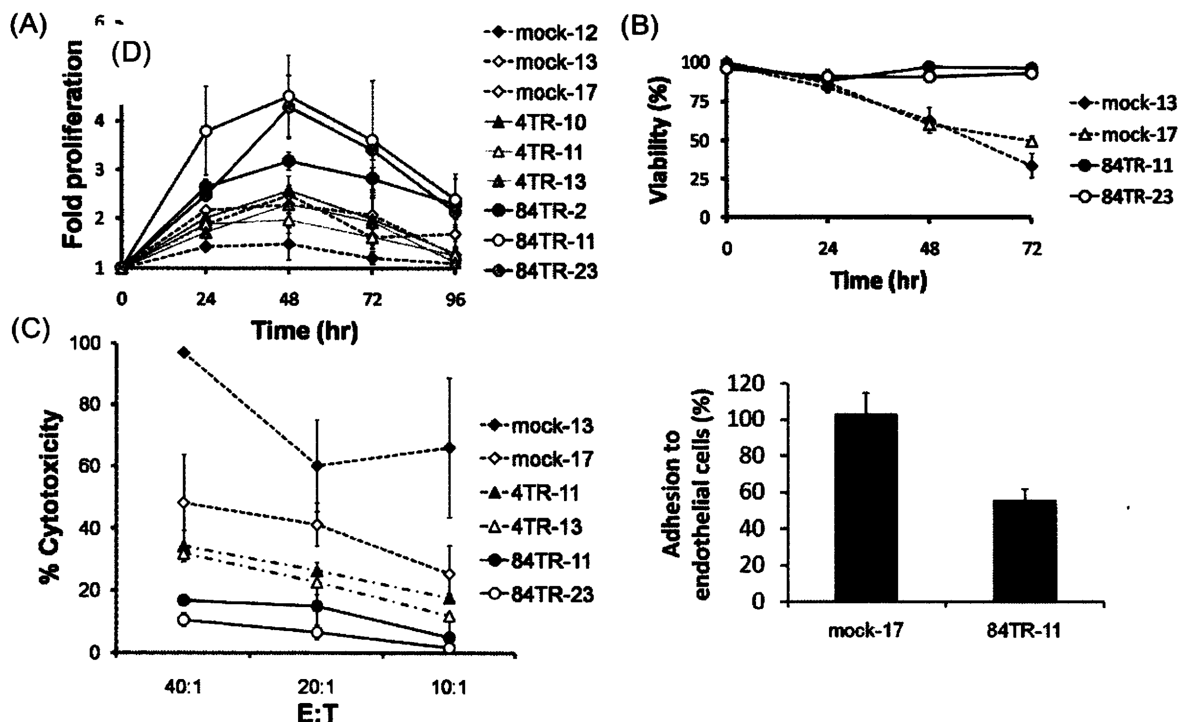

Fig. 4. *In vitro* tumor cells behavioral changes induced by Muc21. Stable transfectant clones in B16-F1 cells were seeded on the poly-Hema coated plates and cell growth was measured by a cell counting kit, CCK-8 (A). Viability of transfectants grown on the poly-Hema coated plates was measured by trypan blue exclusion assay (B). Ligand-labeled transfectants were incubated with IL-2 activated splenocytes for 2 hr and ligand release was detected by fluorescence (C). Transfectants were allowed to adhere to endothelial cells for 40 min and adherent cells were evaluated with flow cytometry by detecting Venus-positive cells (D).

<u>In vivo cellular behavioral changes induced by Muc21 expression</u>

Two well known *in vivo* experimental models for B16-F1 melanoma cells were applied to examine whether Muc21 expression could change tumor cell behaviors *in vivo*. In intravenous lung metastasis model which has been known to strongly reflect adhesive or invasive properties of tumor cells, Muc21 expression showed a negative effect on metastasis formation. Three weeks after i.v. injection of transfectants, the numbers of lung metastases from B16-F1/Muc21 transfectants were smaller than those from mock transfectants (Fig. 5A). These results suggest that reduced tumor cell adhesion to endothelial cells by Muc21 expression probably led to diminished metastasis formation. In subcutaneous model, Muc21 expression did not affect tumor growth (Fig. 5B) indicating a possible correlation with *in vitro* results of a comparable anchorage-dependent growth between mock and Muc21 transfectants (Data not shown).

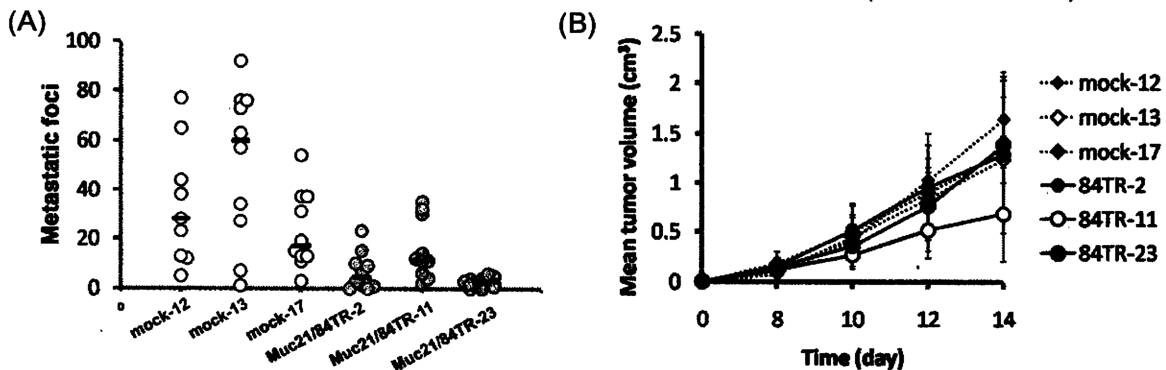

Fig. 5. *In vivo* tumor cell behavioral changes induced by Muc21. Transfectants were injected into tail vein of syngeneic mice and lung metastatic foci were counted after 3 weeks (A) (n=10). Transfectants were injected into the right flanks of syngeneic mice and tumor size were measured ever other day (B) (n=5).

3. Production of Muc21 gene deficient mice and verification of their Muc21 gene expression (Data not shown)

In a means to investigate the function of Muc21 under normal and pathological states, the construction of Muc21 gene deficient mice was attempted. Exon I including the transcription initiation region was targeted to be replaced by a neomycin gene and the correctly targeted ES cells were used for the production of chimeric mice. The disruption of exon I was confirmed by PCR or RT-PCR in homozygous Muc21 gene deficient mice. Unexpectedly, Muc21 transcripts were identified in C-terminal region of exon II and exon III. Using 5'-RACE and subsequent PCR, transcripts encoding more than 15 tandem repeats close to the C-terminal region were recognized. By immunohistochemistry, mAb 1A4-1 stained esophagi and vagina from the Muc21 gene deficient mice after sialidase treatment. Considering the fact that the specificity of mAb 1A4-1 to the Muc21 protein is not clearly defined yet, extinction or existence of the Muc21 protein in the Muc21 gene deficient mice should be further elucidated.

[Conclusion]

Muc21 expression substantially changed cellular behaviors *in vitro* and *in vivo*. The observed strong anti-adhesive property of Muc21 suggest that Muc21 expression might affect various biological processes including prevention of epithelial cells from interaction with pathogens or detachment of cancer cells from surrounding cells or ECM to initiate metastasis. The *in vitro* results using B16-F1/Muc21 transfectant cells further suggest that Muc21 might be involved in enhanced tumor cells survival. Further research to correlate the *in vitro* behaviors of Muc21 transfectants with *in vivo* behaviors should be done. This is the first report on the functions of a novel transmembrane mucin Muc21. We believe this work will provide the basis for the further research on the functions of Muc21 in immunology and cancer biology.