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Functional analysis of β1 integrin and its *N*-glycosylation on cell proliferation and migration

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Integrins comprise a group of transmembrane heterodimeric proteins consisting of α and β subunits that drive most of the interactions between cells and the extracellular matrix (ECM). β1 integrin, which constitutes the largest subgroup of integrins, contributes to diverse malignant phenotypes. Due to its multiple important roles in cancer, a better understanding of β1 is critical for the development of efficacious treatments for cancer. Therefore, we firstly compared cell behaviors between normal and deletion of β1 gene MDA-MB-231 cells. As shown in part 1, we found that β1 exhibited opposite effects on cell proliferation dependent on cell densities, and β1 was essential for the abilities of cell survival and migration.

As an *N*-glycosylated protein, the *N*-glycosylation of integrin β1 is involved in diverse biological functions including cell adhesion, cell migration and trans-membrane signaling. However, most studies have focused mainly on the entire alteration in *N*-glycosylation and little is known about the function of individual *N*-glycans on integrin β1. Given the fact that β1 contains 12 potential *N*-glycosylation sites, during which our laboratory have previously identified that three potential *N*-glycosylation sites on the I-like domain of β1, No. 4, 5, and 6, are essential for the α5β1 heterodimer formation, cell surface expression and cellular function. So we hypothesized that the other *N*-glycosylation sites on β1 subunit may also have unique functions. As shown in part 2, we found that activation of β1, β1-mediated complex formation and cellular signaling were strictly regulated by *N*-glycosylation on the membrane-proximal region of β1. In addition, the structures of *N*-glycans were also important for the functions, since the silence of α2,6-sialylation significantly decreased the β1 activation and cell membrane complex formation.

Part 1. Distinct effects of β1 integrin on cell proliferation and cellular signaling in MDA-MB-231 breast cancer cells

β1 integrin is aberrantly expressed in human breast carcinoma and contributes to a multitude of malignant phenotypes, including epithelial-to-mesenchymal transition (EMT), metastasis, and angiogenesis. Although ample evidence has demonstrated that β1

plays critical roles in breast cancer, the targeting of β1 by using a monotherapy approach has not shown much benefit. Some possible mechanisms are involved in this phenomenon, such as the activation of intracellular protein kinase signaling pathways and cross-talk between β1 and receptor tyrosine kinases (RTKs). These mechanisms provide evidence that the biological events mediated by β1 are not limited to one signaling pathway, which highlights the fact that these signaling networks act dynamically and intersect with each other to control the physiological and pathological responses. These signal integrations can also be achieved even when other signaling pathways are constitutively deregulated. However, the roles of β1 in these processes remain unclear.

To explore the functional significance of β1 integrin, we established β1-knockout (KO) MDA-MB-231 cells via a CRISPR/Cas9-based approach, then investigated the biological functions of β1 in wild-type (WT) and KO cells. As expected, knockout of the β1 integrin gene altered cell morphology and decreased cell migration. Immunofluorescent staining showed that KO cells exhibited cortical actin around the cell surface and decreased the formation of filopodia and lamellipodia. Furthermore, the phosphorylation levels of focal adhesion kinase (FAK), a focal adhesion marker, were significantly decreased, while β4 integrin, a hemi-desmosome marker, showed an increase in the KO cells compared with the results seen in WT cells. These results indicated that β1-KO cells induced a disruption of focal adhesions and instead enhanced the cell-cell adherens junctions, which inhibited cell mobility.

We further investigated the effect that the deletion of β1 on cell proliferation, and found when cells were cultured at a lower density level in the first 24 h of incubation, the KO cells showed a significant decrease in cell proliferation, by comparison with the WT cells. However, once the KO cells reached a certain density, their cell proliferation ability was significantly increased, compared with the WT cells. As the effects of β1 on cell proliferation were dependent on cell densities, we then examined the signaling events triggered by the cell-ECM interactions of WT and KO cells under different cell density conditions. By comparison with WT cells, the phosphorylation levels of ERK in KO cells were consistently suppressed under sparse culture conditions, but consistently up-regulated under dense culture conditions. The phosphorylation levels of epidermal growth factor receptor (EGFR) were increased in the KO cells. By contrast, the phosphorylation levels of AKT were decreased in the KO cells. These data also suggested that cell survival signaling from β1 integrin might play an important role in cell proliferation when cells are under strict conditions. To confirm this notion, we performed a clonogenic and soft agar assay, the result showed that the deletion of β1 dramatically suppressed cell survival. And the xenograft tumor formation in the KO group was also significantly suppressed,

compared with the WT group, which indicated that β1 also played important roles in tumorigenesis in vivo.

Importantly, these phenotypes in the KO cells were restored in the β1-rescue cells. Furthermore, the inhibitory effectiveness of AG1478, an EGFR inhibitor, for cell proliferation was greatly increased in the KO cells as well as in the WT cells pretreated with anti-β1 antibody.

Overall, this study clearly showed that integrin β1 dynamically regulates cell proliferation and cellular signaling. Basically, the expression of β1 negatively regulates EGFR activation. Considering the importance of β1 in cell survival and cell migration, a combination treatment with anti-EGFR and anti-β1 drugs is recommended as a useful therapy for breast cancer.

Part 2. The importance of membrane-proximal *N*-glycosylation on integrin β1 in its activation and complex formation

In part 1, we have compared cell behaviors between normal and β1-KO MDA-MB-231 cells and found loss of β1 resulted in cell-cell adhesion, decreased cell migration. Considering the fact that our group have previously identified three potential *N*-glycosylation sites on the I-like domain of β1 are essential for the α5β1 function, so we hypothesized that if the importance of other *N*-glycosylation sites can be identified, this will facilitate an understanding of the underlying molecular mechanisms as to how and why *N*-glycans alter integrin functions. Therefore, the purpose of the part 2 is to elucidate the mechanisms by which *N*-glycans regulate the integrin functions.

Firstly, we reconstituted with wild-type (WT), and a *N*-glycosylation mutant on the β1 I-like domain (S4–6), which represents the minimal *N*-glycosylation required for α5β1 function, in β1-KO MDA-MB-231 cells. We found that the expression level of integrin α5β1 on the cell surface in S4-6 cells was comparable to that in the WT cells, but the cell spreading and migration ability were significantly decreased in S4-6 cells. Furthermore, β1-mediated intracellular signaling was also attenuated in the S4-6 cells compared with WT cells, which indicated that *N*-glycosylation on the β1 subunit might affect the status of integrin activation. As expected, loss of *N*-glycosylation resulted in a significant decrease in the expression level of active β1 both in the intracellular domain and on the cell surface. These results suggest that *N*-glycosylation on other domains, rather than the I-like domain of the β1 subunit may be involved in the regulation of its various cellular functions.

Next, we established several rescue mutant cells, S4–6+1-3, S4–6+7-8 and S4–6+9-12, expressing β1 with the restoration of rest *N*-glycosylation sites on different domains. We

found that integrin mediated cellular signaling and cell migration could be rescued only in S4–6+S9-12 cells, compared with other *N*-glycosylation mutant cells. Unexpectedly, the expression levels of active β1 were also rescued in the S4–6+7-8 cells, although the cell migration could not be rescued as described above. Considering the fact that β-galactoside α2,6-sialyltranferase 1 (ST6GAL1) -mediated sialylation plays vital roles in β1 function, we checked the α2,6-sialylation levels in these mutants. The results showed the levels of the sialylated β1 and α5 were clearly higher in the S4–6+7-8 and S4–6+9-12 mutant cells than that in other mutant cells. These results demonstrated that α2,6-sialylated *N*-glycans are crucial for integrin β1 activation.

The question regarding why only the *N*-glycosylation on membrane-proximal domain (S9-12) could rescue both the cell migration and cellular signaling. Considering that integrin-mediated cellular events are thought to be regulated by not only a conformational change caused by integrin activation, but also by coordination between integrin and other membrane proteins, we further investigated β1-mediated complex formation. Here, we chose two representative membrane proteins, syndecan-4 and EGFR, which have a vital influence on integrin-mediated cellular functions. The results showed the interaction between integrin β1 with EGFR or syndecan-4 can be only rescued in S4–6+9-12 cells, compared with other mutant cells, even S4–6+7-8 cells. Moreover, the terminal α2,6-sialic acids on the membrane-proximal *N*-glycans was also required for its cooperation with EGFR and syndecan-4. Taken together, these results strongly suggest that the *N*-glycosylation of β1 on the membrane-proximal domain regulates cell migration and cellular signaling by promoting β1 activation and complex formation.

References

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