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α1,6-Fucosyltransferase Is Required for Liver Regeneration and Chemical Induced Hepatocarcinogenesis

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N-Linked glycosylation is a common type of glycosidic bond. It is thought to be important for folding, stability, and vast degree of biological functions of glycoproteins. These different effects on glycoproteins mainly result from the different *N*-linked glycan structures determined by various glycosyltransferases. Among these, α1,6-fucosyltransferase (Fut8) is the only enzyme that catalyzes the transfer of a fucose from GDP-fucose to the innermost GlcNAc residue via α1,6-linkage to form core fucosylation in mammals. The enzymatic products, core fucosylated *N*-glycans, are widely distributed in a variety of glycoproteins and have been shown to play important roles in cell signaling. As examples, we previously showed that core fucosylation is crucial for the ligand binding affinity of transforming growth factor (TGF)-β1 receptor, epidermal growth factor (EGF) receptor, and integrin α3β1. Lacking the core fucose of these receptors led to a marked reduction in their ligand-binding ability and downstream signaling. Recently, our group found that a loss of core fucose on activin receptors resulted in an enhancement of the formation of activin receptor complexes, which constitutively activated intracellular signaling. These studies indicate that core fucosylation is able to negatively or positively affect signaling pathways through regulation of receptor binding ability, which could be important for appropriate signaling *in vivo*.

Part 1. Loss of α1,6-fucosyltransferase suppressed liver regeneration: implication of core fucose in the regulation of growth factor receptor-mediated cellular signaling

Liver regeneration after partial hepatectomy (PH) is a complicated process. At the cellular level, it has been generally accepted that the restoration of liver volume depends mainly on the proliferation of hepatocytes. Molecularly, PH triggers multiple intracellular signaling cascades, leading to great changes in the expression of genes associated with cell proliferation. The convergence of these signaling pathways has been reportedly mediated via EGFR and hepatocyte growth factor receptor (c-Met). Blocking the EGFR- or c-Met-mediated signaling pathway could cause a severe delay of liver regeneration in mice. In addition to the expression level of EGFR and c-Met proteins, it has been shown that the post-translational modification of these receptors such as ubiquitination, phosphorylation, and glycosylation also plays a crucial role in the

regulation of these signaling pathways.

Recently it was reported that core fucosylation on some glycoproteins, such as vitronectin, increased during liver regeneration after PH. However, the role of Fut8 in liver regeneration remains poorly understood. Firstly, we found that the Fut8 activities were increased in the first 4 days after operation, and returned to normal levels after liver mass is restored. These data indicated that the induction of Fut8 expression might be required for liver regeneration. To testify the hypothesis above, we performed a 70% PH on both $Fut8^{+/+}$ and $Fut8^{-/-}$ mice. Interestingly, the regeneration index calculated as an increase in liver-to-body weight ratio was significantly lower in $Fut8^{-/-}$ mice than that in $Fut8^{+/+}$ mice. Furthermore, a decrease in liver regeneration was also observed in the $Fut8^{+/-}$ mice during the first 2 days.

Liver regeneration was achieved by the coordinated proliferation of all types of mature hepatic cells. Consistent with the results above, quantitative assessment of Ki67 by immunostaining revealed little difference between $Fut8^{-/-}$ and $Fut8^{+/+}$ mice without PH, while, the percentage of Ki67 positive versus TO-PRO-3 iodide positive cells in the livers of $Fut8^{-/-}$ mice were markedly less than that in $Fut8^{+/+}$ mice at day 2 after PH. These differences in cell proliferation were further reflected by the cell proliferation signaling. Overall, these data indicated that the delayed liver recovery in $Fut8^{-/-}$ mice resulted from the lower cell proliferation.

It is known that two pathways for the synthesis of GDP-fucose in mammalian cells, the GDP-mannose-dependent *de novo* pathway and the free fucose-dependent salvage pathway. And what is more, administration of oral L-fucose, an enhancement of the salvage pathway, has been proven useful for correction of fucosylation defects in leukocyte adhesion deficiency type II patients. To determine whether enhancing GDP-fucose salvage pathway could complement the delayed liver regeneration of the $Fut8^{+/-}$ mice as described above, we checked the effects of L-fucose supplementation in the $Fut8^{+/-}$ mice. Interestingly, an oral administration of L-fucose significantly accelerated liver regeneration of the $Fut8^{+/-}$ mice, but did not affect sham mice. Consistently, in contrast to the little difference in the case of livers without 70% PH, immunostaining with Ki67 showed the ratio of $Ki67^{+}$ to TO-PRO-3 $iodide^{+}$ cells in the livers treated by PH were clearly increased after L-fucose administration. These results further suggest that Fut8 and its products are important for cell proliferation in liver regeneration.

To determine whether the delayed liver recovery in the $Fut8^{-/-}$ mice is due to the impaired EGFR and/or c-Met signaling, we tested the expression levels of the key effectors in these signaling pathways. Although c-Met and EGFR associated signaling pathways were activated in both $Fut8^{+/+}$ and $Fut8^{-/-}$ mice 2 days post PH, the levels of phosphorylated c-Met and EGFR in $Fut8^{-/-}$

mice were obviously lower than that in Fut8$^{+/+}$ mice. These results indicated that loss of Fut8 impaired EGFR and c-Met associated signaling during liver regeneration. To further corroborate the results above *in vitro*, we examined the downstream signaling cascades of EGF or HGF using the primary hepatocytes isolated from Fut8$^{+/+}$ and Fut8$^{-/-}$ mice. Consistently, the treatments with EGF or HGF significantly increased the expression levels of phosphorylated ERK and AKT in the Fut8$^{+/+}$ cells. However, these increases were greatly suppressed in the Fut8$^{-/-}$ cells. The results above clearly demonstrated that the impaired regeneration in Fut8$^{-/-}$ livers was due, at least mainly, to the down-regulated EGFR- and c-Met-mediated signaling in hepatocytes.

Overall, this study marks the first clear demonstration of the biological functions of Fut8 in the liver, suggesting that core fucosylation plays important roles in liver regenerating progression.

Part 2. Loss of α1,6-fucosyltransferase inhibits chemical induced hepatocellular carcinoma and tumorigenesis by down-regulating several cell signaling pathways

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortality worldwide, and hepatocarcinogenesis is a complicated process associated with the accumulation of pathological changes during the initiation, promotion, and progression of the disease. Identifying these changes may provide an avenue to develop a new generation of potential biomarkers, as well as therapeutic targets for HCC. It has been reported that the up-regulation of core fucosylation catalyzed by Fut8 has been observed in pathological conditions such as HCC, and the fucosylated AFP (AFP-L3) is a reliable marker that can be used to distinguish patients with HCC from those with chronic hepatitis and liver cirrhosis. The HCC cases with high Fut8 expression is associated with the poor prognosis. These information prompted us to wonder what the pathological role of high Fut8 expression is in HCC progression and whether it could serve as a potential therapeutic target for liver cancer.

To explore the effects of high Fut8 expression and their molecular mechanisms in hepatocarcinogensis, here, the Fut8$^{+/+}$, Fut8$^{+/-}$ and Fut8$^{-/-}$ mice were used to establish the chemical induced HCC models by diethylnitrosamine (DEN) and pentobarbital (PB). The induction of HCC was significantly suppressed in Fut8$^{-/-}$ mice, meanwhile, the expression of Fut8 was greatly increased in the liver tissues of Fut8$^{+/+}$ mice during the process. Consistently, liver functions were destroyed in the Fut8$^{+/+}$ mice and Fut8$^{+/-}$ mice, but not Fut8$^{-/-}$ mice.

DEN exerts carcinogenicity after being bioactivated by cytochrome P450 (CYP) enzymes in the liver. To check the early effects of DEN in Fut8$^{+/+}$ and Fut8$^{-/-}$ mice, PCR analysis of mRNAs encoding the CYP enzyme and Mgmt genes was performed at 2 h after DEN injection. There was no significant difference in the transcriptional levels of either CYP genes or DNA repair

gene between $Fut8^{+/+}$ and $Fut8^{-/-}$ mice. DEN-induced tumor formation was further associated with substantial and marked induction of proinflammatory chemokines within the livers of mice. Quantitative PCR analysis of livers after DEN injection revealed increase in IL-6 and TNFα expression levels in both $Fut8^{+/+}$ and $Fut8^{-/-}$ mice, but there was no significant difference between the them. Moreover, TUNEL assay for the livers showed the similar results. These data demonstrate that the expression of Fut8 may not affect the acute phase response to DEN.

Cell proliferation plays important roles in HCC process. In order to find the possible mechanisms for the differences in tumorigenesis described above, we carried out Ki67 immunostaining for the frozen liver tissues of $Fut8^{+/+}$ and $Fut8^{-/-}$ mice. After chemical induction, the $Fut8^{+/+}$ mice showed a significant increase by more than ~6.5-fold in positive immunostaining as compared with the untreated control. However, only a 2-fold increase was observed in $Fut8^{-/-}$ mice. The expression levels of cyclin mRNAs, including Cyclin B1, Cyclin D1 and Cyclin E2, were also up-regulated in $Fut8^{+/+}$ mice after DEN/PB treatment, and the elevations in gene expression were attenuated by the ablation of Fut8. These results indicate that Fut8 may influence the progress of DEN/PB induced HCC by affecting the cell proliferation.

The effects of Fut8 expression on tumorigenesis were further confirmed by xenograft tumors formed by HepG2 cells in NOD/SCID mice. Knockout of Fut8 gene in the cells completely inhibited tumor formation. The HepG2 cell line was also used to reveal possible mechanisms of Fut8 for the regulatory roles. The responses to EGF and HGF were attenuated in the Fut8 knockout cells, although the expression levels of their receptors on the cell surface were similar. Considering also our previously reported that core fucosylation was required for the binding of the EGF to EGFR, it is reasonable to conclude that lacking the core fucosylation on both receptors may decrease their biological functions *in vitro* or *in vivo.*

Taken together, it could be postulated that a loss of the Fut8 gene may affect the biological functions of some target membrane proteins and their subsequent downstream signaling, thereby inhibiting the hepatocarcinogenesis. These results suggest that the levels of core fucosylation are not only biomarkers, but also functional modulators in the liver. Thus, Fut8 might be a novel therapeutic target for HCC.

References

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