Reviewer's report

**Title:** Decreased expression of the mannose 6-phosphate/insulin-like growth factor-II receptor promotes growth of human breast cancer cells

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**Reviewer:** Douglas Yee

**Level of interest:** A paper whose findings are important to those with closely related research interests

**Advice on publication:** Unable to decide on acceptance or rejection until the authors have responded to the compulsory revisions

Decreased Expression of the Mannose 6-Phosphate/Insulin-like Growth Factor-II Receptor Promotes Growth of Human Breast Cancer Cells

This study sought to investigate the potential role of the mannose 6-phosphate/insulin-like growth factor 2 receptor as a tumor suppressor by using adenoviral expression of a ribozyme targeted against M6P/IGF2R mRNA. While the authors address an important question regarding the role of the M6P/IGF2R in cancer development and progression, several weaknesses in the study must be addressed:

1. Demonstrate specificity of the ribozyme. The observed enhancement of proliferation and reduced susceptibility to apoptosis may be independent of M6P/IGF2R expression. To confirm specificity, a control ribozyme must be included in all experiments. This control could either be an enzymatically inactive mutant ribozyme or a ribozyme designed with the same sequence as the M6P/IGF2R-targeted RNA.

2. Confirm the ability of the ribozyme to decrease M6P/IGF2R mRNA and protein levels. The RT-PCR data is not fully convincing as to the ability of the ribozyme to suppress M6P/IGF2R expression. For example, the B-actin control used does not fully address the potential for unequal loading. This data would be more believable if accompanied by Northern blot or RNase protection assay. Similar concerns about the demonstration of reduced M6P/IGF2R protein levels exist. Changes in internalization (as shown in Figure 4) are not a good way to estimate expression levels of a receptor. A binding assay, such as Scatchard analysis, would be more convincing. Alternatively, an immunoblot for M6P/IGF2R would be a better quantitative assay.

3. Include control cells for both IGF-II internalization and cell growth data. Experiments must be performed using cells with no adenoviral infection to verify the observed effects are independent of infection. To further verify the effect of ribozyme expression on the functional activity
of M6P/IGF2R, it might be interesting to look at other established functions of the M6P/IGF2R, such as activation of TGF-B and lysosomal enzymes.

4. Demonstrate mechanism responsible for enhanced IGF-II-induced proliferation upon decreased expression of M6P/IGF2R.

The authors claim the enhanced proliferation may be due to an increased bioavailability of IGF-II to the IGF-I receptor. There are several problems with the proliferation data. First, these cells do not proliferate in response to IGF-II as shown in Figure 5B. This is contrary to the published literature, and it is perhaps due to the culture conditions. Were these experiments done in full serum? If so, then the growth effects of Rz could be due to other factors in the serum and not specifically attributed to IGF-II. Second, a control for IGF mediated growth would be useful. In this instance, IGF-I would provide an excellent control for the authors’ claim that enhanced IGF1R signaling accounts for the growth effects. Finally, analysis of IGF1R signaling would be informative for these experiments to support the idea that more IGF-II is available to IGF1R after Rz treatment.

5. Perform additional apoptotic assays to verify M6P/IGF2R ribozyme-infected cells are protected from apoptosis.

Data shown in Figure 6 are not convincing. The reproduction quality is poor, so other methods to measure apoptotic cell death need to be performed. Again, the culture conditions for this experiment needs to be clarified. Flow cytometry would be useful to measure proliferative fraction and sub-G1 peak. While the Rz seemed to affect apoptosis and viability, it is not clear if IGF-II was present in the culture conditions. In the Discussion, the authors suggest that IGF-II independent mechanisms may be responsible for the effects, but this is not supported by the experiment. The work would be more easily interpreted if all of the growth/death experiments were done in defined culture conditions. Certainly, many investigators have examined growth factor responses in MCF-7 cells under serum-free conditions.

6. Include statistical methods with the materials and methods section.

7. Verify written MTT assay method section

The Materials and Methods section states that the cells are incubated overnight following addition of the solubilizing solution (Page 4, line 6). Is this correct? In addition, it would be beneficial to see the MTT readings of cells prior to induction of apoptosis to verify equal plating.

Competing interests:

None declared.