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Recombinant soluble glypican 3 protein inhibits the growth of hepatocellular carcinoma *in vitro*

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Dear Editor

We read with great interest an article by Zittermann *et al*.[1] showing that hepatocellular carcinoma (HCC) cells infected with lentivirus for soluble glypican 3 (sGPC3) expression had a lower proliferation rate. The authors indicated that the growth inhibition might be due to the sGPC3 protein secreted by infected HCC cells. Here we provide direct evidence showing that the sGPC3 protein does in fact inhibit the growth of HCC cells *in vitro*.

Our experiments are summarized in Figure 1. Consisting of 580 amino acids, GPC3 (also called MXR7, OCI-5 or GTR2-2) is a heparan sulfate proteoglycan that is attached to the cell surface via a glycosyl-phosphatidylinositol (GPI) anchor. We constructed an expression plasmid (called pMH133) for producing sGPC3 (GPC3ΔGPI, amino acid residues Q25-H559) that lacks the COOH-terminal hydrophobic GPI-anchoring domain[2] (Fig. 1A). The recombinant sGPC3 protein with a His tag at the COOH-terminal was purified on a 1-ml Ni Sepharose column (HisTrap FF; GE Healthcare, Piscataway, NJ) from the serum-free media of human HEK293F cells (Invitrogen, Carlsbad, CA) transfected with pMH133. Fractions of the single dominant peak were collected using the ÄKTA explorer FPLC system (GE Healthcare), run on a SDS-PAGE gradient gel (4–20%) (Invitrogen), pooled and concentrated. Final protein concentration was measured using Coomassie Plus Protein Assay Reagent (Thermo Scientific/Pierce, Rockford, IL). The recombinant sGPC3 protein detected by the 1G12 antibody specific for the COOH-terminal of GPC3 (Santa Cruz Biotechnology, Santa Cruz, CA) was heavily glycosylated and had a high molecular weight consistent with those of native GPC3 proteins found in HCC cells[1–5] (Fig. 1B & C). To examine whether or not recombinant sGPC3 protein can inhibit the growth of HCC cells, we screened 6 HCC lines (SK-Hep-1, HepG2, Hep3B, Huh-1 Huh-4 and Huh-7) by flow cytometry and found that the HepG2 cell line had the highest protein expression level of GPC3 on the cell surface (Fig. 1D). We then incubated HepG2 cells with various concentrations (1 –100 μg/mL) of sGPC3. Fig. 1E shows a dose-dependent inhibition of cell proliferation measured by WST-8 cell proliferation assays (Dojindo, Rockville, MD). Using the Prism software (GraphPad Software, San Diego, CA), we estimated EC_{50} was about 15 μg/mL (0.2 μM). We then treated HepG2 cells with sGPC3 and evaluated the cells daily in the course of 6 days by live cell counting using Countess Automated Cell Counter (Invitrogen) (Fig. 1F) and WST-8 cell progression assays (Fig. 1G). Cell proliferation was significantly inhibited as early as 72 h (day 3) after treatment (Fig. 1F).

In summary, our data shows that recombinant sGPC3 protein can inhibit the proliferation of HCC *in vitro*. The precise mechanisms require further investigation.

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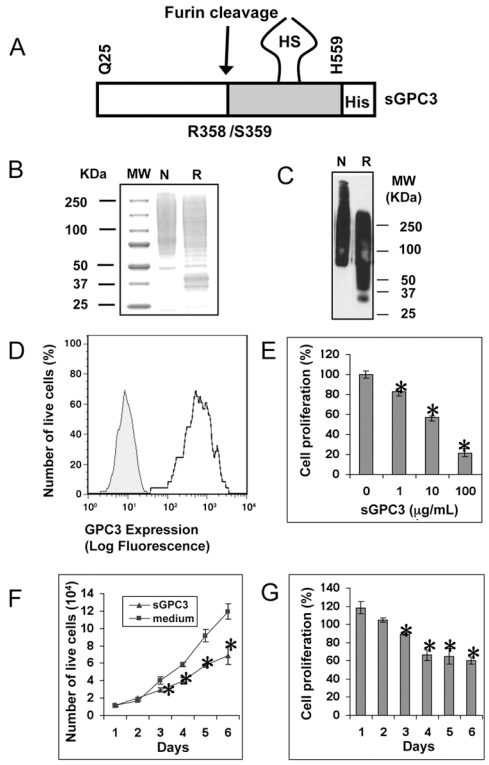

Fig. 1.

A. Schematic diagram of the primary structure of His-tagged sGPC3. His, six-histidine tag; arrow, furin-cleavage site; HS, heparan sulfate glycosaminoglican. **B**. SDS-PAGE analysis of purified recombinant sGPC3 (10 μg/lane). N, non-reducing; R, reducing; MW, molecular weight. **C**. Western blot analysis of purified recombinant sGPC3 (5 μg/lane) was performed using 1G12 (1 μg/mL). **D**. Flow cytometric analysis. HepG2 HCC cells were incubated with 1G12 (1 μg/mL). The binding was visualized with a goat anti-mouse IgG FITC-conjugated secondary antibody (Invitrogen) by flow cytometry (gray line). Gray shaded plot, secondary antibody only. **E**. Growth inhibition of HepG2 cells by sGPC3. Cells (1×10^4 per well) were cultured in a 24-well plate with increasing concentrations (1 – 100 μg/mL) of sGPC3 in growth medium (DMEM with 10% FBS). Cell growth was measured by WST-8 assays. The end point values at day 9 were shown. **F** and **G**. Proliferation time course of HepG2 cells in the presence of sGPC3 (50 μg/mL). Cell viability was assessed by the Trypan blue exclusion assay; viable cells (non-stained with Trypan blue) were counted using Countess Automated Cell Counter (Invitrogen) (**F**). Cell proliferation was measured by WST-8 assays (**G**). The data obtained was entered in Prism (GraphPad Software, San Diego, CA) for statistical analysis comparing treated groups with the untreated groups. Raw data was analyzed by analysis of variance (ANOVA) with Dunnett's and Newman-Keuls multiple comparison post tests. * p values < 0.01 (compared to the untreated group) were considered statistically significant. Each point shows mean ± S.E. of a representative one of two experiments done in triplicate.