RESEARCH ARTICLE

Expression of Glypican-3 in Mouse Embryo Stem Cells and its Derived Hepatic Lineage Cells Treated with Diethylnitrosamine *in vitro*

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Abstract

To clarify the role of stem cells in hepatocarcinogenesis, glypican-3 (GPC-3) and E-cadherin expression was investigated in embryonic cell lineages. Mouse embryonic stem cells (ESCs), hepatic progenitor cells (HPCs) and hepatocyte like cells (HCs), representing 0, 22 and 40 days of differentiation, respectively, were treated *in vitro* with diethylnitrosamine (DEN) at four doses (0, 1, 5 and 15 mM; G1, G2, G3 and G4, respectively) for 24 h and GPC-3 and E-cadherin expression was examined by relative quantitative real-time PCR and immunocytochemistry. GPC-3 mRNA expression was significantly different for G4 at day 0 ($p<0.001$) and for G4 at day 22 ($p<0.01$) compared with the control (G1). E-cadherin mRNA expression was significantly different for G3 and G4 at day 0 ($p<0.05$ and $p<0.001$, respectively), for G2 and G4 ($p<0.05$ and $p<0.001$, respectively) at day 22 and for G2 and G4 ($p<0.01$ and $p<0.001$, respectively) at day 40 compared with G1. Immunofluorescence staining for GPC-3 showed a membranous and/or granular expression in cytoplasm of ESCs and HPCs and granular and/or diffuse expression in cytoplasm of HCs, which were also stained by E-cadherin. DEN treatment increased GPC-3 expression in ESCs, HPCs and HCs, with increase of E-cadherin expression. Taken together, the expression of GPC-3 was altered by DEN treatment. However, its expression pattern was different at the stage of embryo stem cells and its derived hepatic lineage cells. This suggests that GPC-3 expression may be modulated in the progeny of stem cells during their differentiation toward hepatocytes, associated with E-cadherin expression.

Keywords: Mouse embryonic stem cell - hepatic lineage cells - hepatocarcinogenesis - glypican-3 - diethylnitrosamine

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Introduction

In toxicology and pharmacology, ESC-derived hepatocytes have been suggested as an *in vitro* alternative model for toxicity and/or carcinogenicity assay testing (Okura et al., 2009; Greenhough et al., 2010). Hepatic differentiation of mouse ESCs was first developed in a stepwise method by adding specific growth factors after the formation of an embryoid body (Hamazaki et al., 2001). In human, ESC-derived hepatocytes showed similar features of primary hepatocytes (Rambhatla et al., 2003). Recently, functional hepatocytes were generated using chemically defined culture conditions (Touboul et al., 2010). Embryonic stem cells (ESCs)-derived hepatic differentiation is similar to *in vivo* hepatogenesis. Functionality of differentiated hepatocytes has been confirmed by the expression of specific marker proteins, such as cytokeratins (CK), GATA binding proteins (GATA), alpha-fetoprotein (AFP). These indicators are related to hepatic development as well as can be applied to hepatotoxicity testing by estimating their quantity.

It has been reported that diverse solid tumors are hierarchically organized and sustained by a distinct subpopulation of cancer stem cell (Visvader and Lindeman, 2008). Hepatic tumors were originated from liver stem cells and that preneoplastic lesions and nodular changes are adaptive non-oncogenic responses to the toxic effects of carcinogens (Sell and Dunsford, 1989).

Glypican-3 (GPC-3) is a member of the heparin sulfate proteoglycans and binds to the cell membrane through the glycosyl-phosphatidylinositol anchors and plays an important role in cell growth and differentiation (Filmus et al., 1988; Stefaniuk et al., 2010). GPC-3 regulates the growth activity positively or negatively by interacting with several growth factors (Reich-Slotky et al., 1994; Song et al., 1997) and it generally acts as an oncofetal protein and its expression was considered to be a reliable marker for hepatocellular carcinoma (Kandil and Cooper, 2009). And GPC-3 protein was positive in sera of 40 % (16/40) of HCC patients, and negative in sera from subjects with liver cirrhosis (0/13), chronic hepatitis (0/34) and healthy individuals (0/60) (Nakatsura et al., 2003; Sung et al., 2003). As the expression of GPC-3 was not correlated with AFP and at least one of the two markers was elevated in patients with HCC, the simultaneous determination of GPC-3 and AFP may increase the sensitivity for

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diagnosing HCC (Capurro et al., 2003). In clinics, multivariate analysis identified GPC3 expression as an independent prognostic factor for the overall survival and GPC3 expression was correlated with a poor prognosis in HCC patients (Shirakawa et al., 2009b).

Based on the concept that diethylnitrosamine (DEN) carcinogenicity showed different age (Vesselinovitch et al., 1984), we assumed that the expression of GPC-3 would be modulated by the DEN treatment at the differentiating stage of hepatic cells. The assessment was carried out at various developmental stages, from ESCs to hepatic progenitor cells (HPCs) and hepatocyte-like cells (HCs). In the present study, we investigated the expression pattern of GPC-3 from embryonic cell lineages to mature hepatocytes treated with DEN.

Materials and Methods

Culture of mouse ESCs and differentiation of hepatic lineage cells

Mouse ESCs, as NVRQS-11F, were cultured using mitomycin C-treated mouse embryonic fibroblasts as feeder cells on 0.1% gelatin-coated dishes in Dulbecco's modified Eagle's medium (Millipore, Billerica, MA) supplemented with 15 % fetal bovine serum (Invitrogen, Rockville, MD), 2 mML-glutamine (Millipore), 0.1% non-essential amino acids (Invitrogen), 1% penicillin-streptomycin (Millipore), and 10 ng/mL mouse leukemia inhibitory factor (Millipore). To differentiate ESCs into hepatic lineage cells *in vitro*, defined culture media were supplemented with rmHGF (Invitrogen), DMSO and sodium butyrate (Sigma-Aldrich, St. Louis, MO) for HPCs. Subsequently, mEGF (Invitrogen), oncostatin M, dexamethasone, nicotinamide, and ascorbic acid (Sigma-Aldrich) were utilized for differentiating HCs from ESCs (Kang et al., 2013).

DEN treatment of ESCs, HPCs and HCs

ESCs (day 0), HPCs (day 22 of differentiation) and HCs (day 40 of differentiation) were treated with four concentrations of DEN (0, 1, 5 and 15 mM; G1, G2, G3, G4, respectively) for 24 h.

GPC-3 and E-cadherin mRNA expression

RNA was isolated from cultured cells using an RNeasy mini kit (Qiagen, Valencia, CA), dissolved in DEPC-treated distilled water and stored at -80°C until use. RNA concentrations were measured. GPC-3 and GAPDH mRNA expression was determined by relative quantitative real-time PCR in 96-well optical plates using an ABI Steponeplus Real Time PCR System (Applied Biosystems, Foster City, CA).

The expression levels of the target genes were normalized to mouse GAPDH mRNA and were presented as relative expression. The expression of the genes was normalized to GAPDH, using the comparative Ct method. The cycle number at which the fluorescence signal of the target product was detectable (threshold cycle, Ct) was normalized against the Ct of GAPDH, to give $^{\Delta}$Ct. The expression of the genes relative to a reference was calculated as $2^{-\Delta\Delta}$Ct, where $^{\Delta\Delta}$Ct referred to the difference between the $^{\Delta}$Ct values of the test group and the reference.

Immunofluorescence staining of GPC-3 and E-cadherin

Cells were cultured to a low density on cover slips and fixed in 4% paraformaldehyde for 15 min at room temperature. The medium was removed by aspiration, and the cells were washed twice with 1× phosphate buffered saline (PBS; Invitrogen), pH 7.4. The cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 5 min, and then rinsed twice with 1× PBS. After cell permeabilization in 1× PBS and 0.1% Triton X-100 (PT) for 10 min, 4% normal goat serum in PT as a blocking solution was added. After blocking, the appropriate diluents (1:100 dilution) of anti-GPC-3 (abcam, Cambridge, MA) and anti-E-cadherin (abcam) for hepatic lineage cells were applied for 1 h at room temperature (RT). The samples were then incubated with Alexa488-conjugated anti-rabbit IgG and Alexa597-conjugated anti-mouse IgG/IgM (Invitrogen) for 1h at RT. Counterstaining was assessed by Hoechst 33258. After 3 washes with PBS, the cells were mounted in Fluorescence Mounting Medium (DAKO, Glostrup, Denmark). Immunofluorescence was detected under a confocal microscope (LSM-700, Carl Zeiss, New York, NY)

Statistical analysis

Statistical analyses were performed using SPSS software (version 14.0; SPSS Inc., Chicago, IL). All data were analyzed using Student's t-test and are expressed as the mean±SD of at least three independent experiments performed in triplicate or quadruplicate. p values that were <0.05 were considered statistically significant.

Results

GPC-3 and E-cadherin mRNA expression

GPC-3 mRNA expression was significantly different for G4 at day 0 ($p<0.001$) and for G4 at day 22 ($p<0.01$) compared with the control (G1) (Figure 1). There were no differences for G2, G3 and G4 at day 40 compared with G1 at the corresponding time-points.

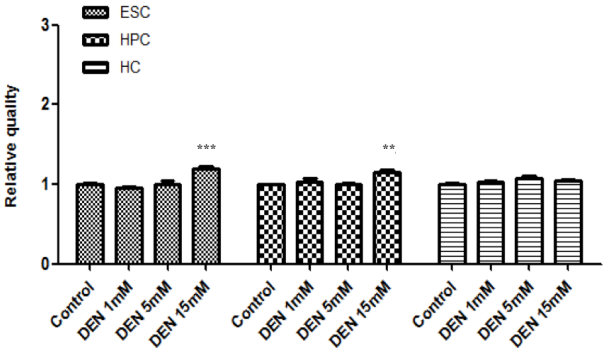

Figure 1. Expression of Glypican-3 (GPC-3) mRNA in Mouse Embryonic Stem Cells (ESCs), Hepatic Progenitor Cells (HPCs) and Hepatocyte-like Cells (HCs). ESCs (day 0), HPCs (day 22) and HCs (day 40) were treated with diethylnitrosamine (DEN) at four doses (0, 1, 5 and 15 mM; G1, G2, G3 and G4, respectively) for 24 h and the expression of GPC-3 mRNA was determined by PCR. **p<0.01, ***p<0.001, n=12 from three independent experiments

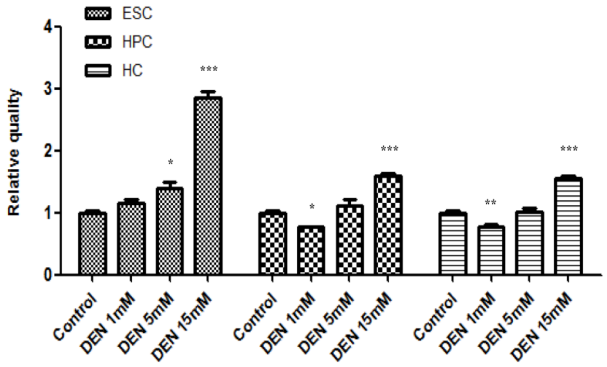

Figure 2. Expression of E-cadherin mRNA in Mouse Embryonic Stem Cells (ESCs), Hepatic Progenitor Cells (HPCs) and Hepatocyte-Like Cells (HCs). ESCs (day 0), HPCs (day 22) and HCs (day 40) were treated with diethylnitrosamine (DEN) at four doses (0, 1, 5 and 15 mM; G1, G2, G3 and G4, respectively) for 24 h and the expression of E-cadherin mRNA was determined by PCR. *p<0.05, **p<0.01, ***p<0.001, n=12, from three independent experiments

E-cadherin mRNA expression was significantly different for G3 and G4 at day 0 (p<0.05 and p<0.001, respectively), for G2 and G4 (p<0.05 and p<0.001, respectively) at day 22 and for G2 and G4 (p<0.01 and p<0.001, respectively) at day 40 compared with G1 (Figure 2).

Immunofluorescence staining of GPC-3 and E-cadherin

Immunofluorescence staining for GPC-3 showed a membranous and/or granular expression in cytoplasm of ESCs and HPCs and granular and/or diffuse expression in cytoplasm of HCs. And these cells were also stained by E-cadherin. Merged images show all cells are positive for both markers.

DEN treatment increased GPC-3 expression in ESCs, HPCs and HCs, with increase of E-cadherin expression (Figure 3). And in ESCs treated with DEN, GPC-3 expression was increased, however, its expression was also localized at cell membrane (Figure 3a). In HPCs and HCs treated with DEN, its expression was strongly observed in cytoplasm (Figure 3b and 3c).

Discussion

GPC-3 expression was down-regulated from ESCs to HCs in our study. Interestingly, GPC-3 expression pattern was changed during differentiation stage. It showed a membranous and/or granular pattern in cytoplasm of ESCs and HPCs and granular and/or diffuse expression in cytoplasm of HCs. And these cells were also stained by E-cadherin and merged images show all cells are positive for both markers, showing all GPC-3 positive cells were of epithelial origin. It was reported that GPC-3 was highly expressed in fetal hepatoblasts from embryonic days 13 through 16 and its expression gradually decreases towards birth and only hepatoblasts expressed GPC-3 and the expression of GPC-3 mRNA and protein was hardly detected in the liver at 3 weeks after birth (Grozdanov et al., 2006). From these data, it seems that GPC-3 expression may be down-regulated according to age development.

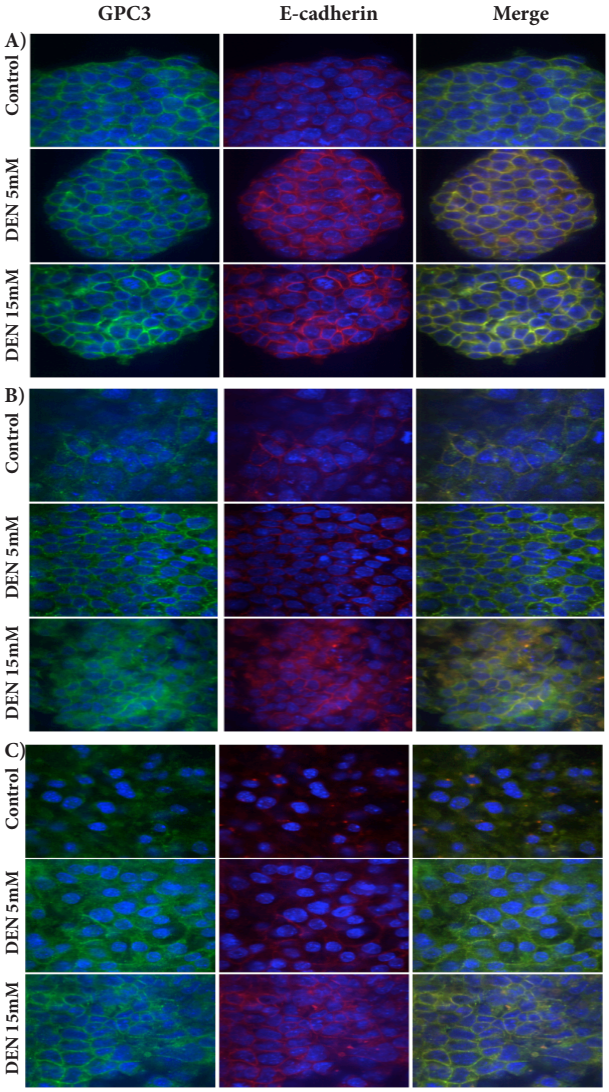

Figure 3. Immunocytochemistry of Glypican-3 (GPC-3) and E-cadherin During the Developing Liver, that in Embryonic Stem Cells (ESCs), Hepatic Progenitor Cells (HPCs) and Hepatocyte-like Cells (HCs) was Modulated by Diethylnitrosamine (DEN) Treatment and Confocal Microscopy for Detection of GPC-3 in (a) ESCs, (b) HPCs and (c) HCs. Immunofluorescence stainings for GPC-3 (green) showed a membranous and/or cytoplasm expression pattern. Epithelial cells were defined by costaining for E-cadherin (red), proving that all GPC-3 positive cells were of epithelial origin merged picture. Nuclei were stained with 40,6-diamidino-2-phenylindol (DAPI) (blue)

In our present study, DEN treatment increased GPC-3 expression in ESCs, HPCs and HCs, with increase of E-cadherin expression. DEN, a well known hepatocarcinogen, is widely used in mouse liver cancer models (Fausto and Campbell, 2010). In ESCs treated with DEN, GPC-3 expression was increased, however, its expression was also localized at cell membrane. In HPCs and HCs treated with DEN, its expression was strongly observed in cytoplasm. And positive GPC-3 expression showed as brown granule-like staining localized in the cytoplasm in a hepatoma model of rats induced with 0.05% 2-fluorenylacetamide (Yao et al., 2011). Actually, immunohistochemical staining of GPC-3 showed its expression was localized diffuse in cytoplasm, granular in

cytoplasm and membranous in hepatocellular carcinoma (Shirakawa et al., 2009a). In D-galactosamine and 2-AAF/PH treated hepatocarcinogenesis model, GPC-3 expression was highly induced in oval cells (Grozdanov et al., 2006). Recent report showed that GPC-3 was markedly elevated in CD90(+) liver cancer stem cells (CSCs) and was highly expressed in human liver tumor tissues but absent in adjacent non-tumorous liver tissues (Ho et al., 2012). These data represented that GPC-3 expression showed various patterns in hepatic tumors and could be different in liver tumor models. As mutations of hepatic stem cells or progenitor cells were associated with cancer, increase of GPC-3 expression and change of its expression pattern in HPCs may have an important meaning in DEN-induced carcinogenesis *in vivo*. On the while, GPC-3 mRNA expression was significantly different for ESCs and HPCs, not for HCs. Further studies will be warranted to explain these discrepancy.

As liver is a major target for toxicity caused by drug effects and, more frequently, a result of the bioactivation process, evaluating potential hepatotoxicity is a critical step in the development of new drugs (Gómez-Lechón et al., 2010). The utilization of cultured hepatocytes is increasing by the pharmaceutical industry for screening the hepatotoxic potential of new molecules because hepatocytes retain critical hepatic functions. Since hepatocytes were proposed as an *in vitro* model for hepatotoxic testing (Maier, 1989), many studies have been conducted to evaluate the toxicity of chemicals using animal and human liver cells (Brambilla and Martelli, 1993; Hammond et al., 1995). Primary hepatocytes from rodent or human have been useful for studying hepatotoxicity, drug metabolism, and liver enzyme induction (Guillouzo et al., 1993; Ratanasavanh et al., 1988; Nussler et al., 2001; Kafert-Kasting et al., 2006). However, primary hepatocytes have several limitations due to their variability and decreasing function over time. Immortalized cell lines also have an inherent disadvantage because they have different metabolic and functional properties from primary hepatocytes and are genetically unstable. ESCs are a useful source of cells for various fields of scientific study due to their unique characteristics: self-renewal and differentiation of all germ layers. Using ESCs and its derived cells, it could be possible to study the role of cancer stem cells in tumors and to evaluate the potential candidates as one of alternative carcinogenicity tests.

Taken together, the expression of GPC-3 was increased in DEN-treated cells. This suggests that GPC-3 expression may be modulated in the progeny of stem cells during their differentiation toward hepatocytes and may be increased during DEN-induced hepatocarcinogenesis.

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