RESEARCH ARTICLE

GPR48 Promotes Multiple Cancer Cell Proliferation via Activation of Wnt Signaling

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Abstract

The key signaling networks regulating cancer cell proliferation remain to be defined. The leucine-rich repeat containing G-protein coupled receptor 48 (GPR48) plays an important role in multiple organ development. In the present study, we investigated whether GPR48 functions in cancer cells using MCF-7, HepG2, NCI-N87 and PC-3 cells. We found that GPR48 overexpression promotes while its knockdown using small interfering RNA oligos inhibits cell proliferation. In addition, Wnt/β-catenin signaling was activated in cells overexpressing GPR48. Therefore, our results indicated that GPR48 activates Wnt/β-catenin signaling to regulate cancer cell proliferation.

Keywords: GPR48 - Wnt/β-catenin - cancer cell - cell proliferation

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Introduction

G-protein-coupled receptors (GPCRs) containing seven transmembrane domains functions a diverse group of membrane-bound signaling molecules (Venkatakrishnan et al., 2013). Being the largest family of cell surface proteins critically involved in the regulation of signal transduction, it is now accepted that GPCRs plays important roles in tumorigenesis (Lin, 2012; Venkatakrishnan et al., 2013). GPCRs could be considered as potential oncogenes. Indeed, some gain-of-function mutations of certain GPCRs could directly result in tumor formation and progression (Lappano et al., 2011; Wu et al., 2012). For instance, activating mutation of luteinizing hormone receptors leads to familiar male precocious puberty, and mutation of thyroid-stimulating hormone causes thyroid adenoma (Parma et al., 1993; Shenker et al., 1993). Therefore, understanding of the function of GPCRs in cancer cells may provide a potential therapeutic strategy during the treatment of tumorigenesis (Ho et al., 2009; Lappano et al., 2011).

G protein-coupled receptor 48 (GPR48) is a newly identified receptor, with multiple functions in the developmental events (Mendive et al., 2006). GPR48 deficiency causes developmental defects in several organs, including male reproductive tracts, gallbladder, cystic duct and bone (Luo et al., 2009; Yamashita et al., 2009; Li et al., 2010). In addition, recent studies demonstrate that proteins of R-spondin family could be endogenous ligands for GPR48, directly linking this receptor to the Wnt/β-catenin signaling (de Lau et al., 2011; Glinka et al., 2011). In this study, we will investigate the functions of GPR48 in cancer cell proliferation using MCF-7, HepG2, NCI-N87 and PC-3 cells.

Materials and Methods

Cell culture and tissue samples

MCF-7, HepG2, NCI-N87 and PC-3 cells were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum.

siRNA, RNA extraction and Real-time Analysis

Small interfering RNA oligos (siRNA) targeting GPR48 or negative controls were obtained from Dharmacon (USA). Cells were seeded on to 6-well plates then transfected with 50nM siRNA oligos. Total RNAs were isolated from cells by TRIzol reagent, and reverse transcriptions were performed by Takara RNA PCR kit (Takara, Dalian, China) following the manufacturer's instructions. In order to quantify the transcripts of the interest genes, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Japan) on Light Cycler 480 (Roche, Switzerland).

BrdU Assays

A cell proliferation enzyme-linked immunosorbent assay (BrdU kit; Beyotime) was used to analyze the incorporation of BrdU during DNA synthesis following the manufacturer's protocols. All experiments were performed in triplicate. Absorbance was measured at 450 nm in the Spectra Max 190 ELISA reader (Molecular Devices, Sunnyvale, CA).

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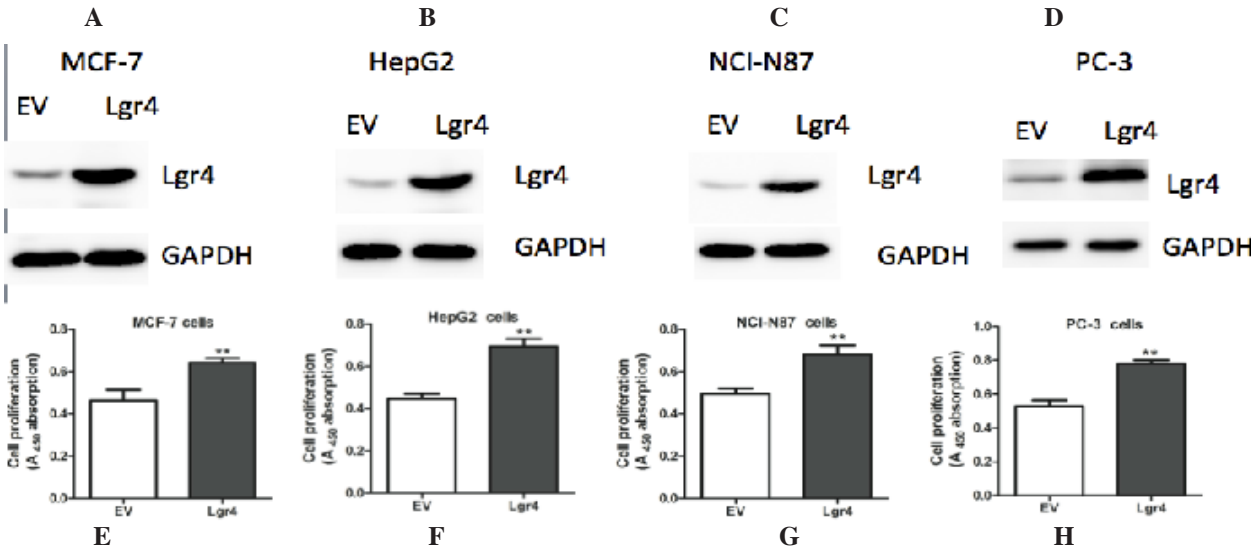

Figure 1. Overexpression of GPR48 Promotes Cancer Cell Proliferation. (A-D) GPR48 expression was determined by western blot in MCF-7, HepG2, NCI-N87 and PC-3 cells transfected with plasmids containing empty vector (EV) or GPR48. (E-H) The cell proliferative potential (BrdU) was determined in MCF-7, HepG2, NCI-N87 and PC-3 cells

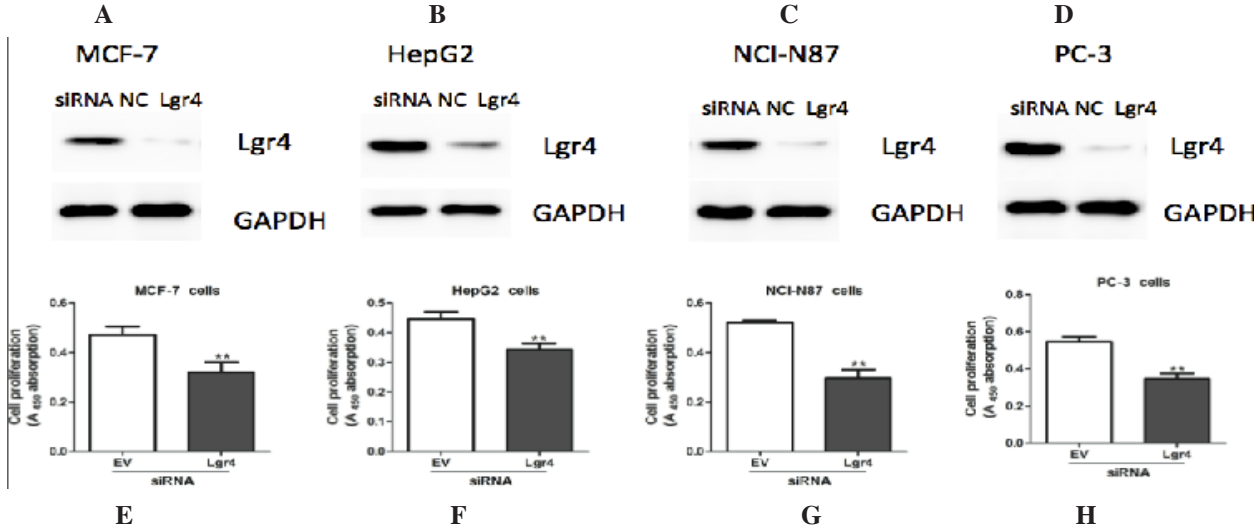

Figure 2. GPR48 Knockdown Reduces the Proliferation of Cancer Cells. (A-D) GPR48 expression was determined by western blot in MCF-7, HepG2, NCI-N87 and PC-3 cells transfected with siRNA oligos targeting GPR48 or negative controls (NC). (E-H) The cell proliferative potential (BrdU) was determined in MCF-7, HepG2, NCI-N87 and PC-3 cells

Western blot

Cells were harvested and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 6.8, 100 mM 2-ME, 2% w/v SDS, 10% glycerol). After centrifugation at 20000× g for 10 min at 4 °C, proteins in the supernatants were quantified and separated by 10% SDS PAGE, transferred to NC membrane (Amersham Bioscience, Buckinghamshire, U.K.). After blocking with 10% nonfat milk in PBS, membranes were immunoblotted with antibodies as indicated, followed by HRP-linked secondary antibodies (Cell Signaling). The signals were detected by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL) according to manufacturer's instructions. Anti-GPR48 and β-catenin antibodies were purchased from Abcam Company (USA). Protein levels were normalized to lamin B (Abcam, USA) or GAPDH (Santa Cruz, USA).

Statistical analysis

Data are expressed as the mean±SEM from at least three separate experiments. Differences between groups were analyzed using Student's t-test. A value of $p< 0.05$ was considered statistically significant.

Results

The effects of GPR48 overexpression on cell growth

In order to assess the effects of GPR48 on cancer cell growth, four cells were used in our study. MCF-7 cells are derived from breast cancer. HepG2 cells are from hepatocellular carcinoma. NCI-N87 cells are from gastric cancer and PC-3 cells are from prostate cancer. We transfected GPR48 expression plasmid into these cells and examined cell growth by BrdU incorporation assays (Figure 1A-1D). As a result, forced expression of GPR48 significantly promoted proliferation in these cells (Figure 1E-1H).

Inhibition of GPR48 blocks cancer cell proliferation

Next, these cells were transfected with small interfering

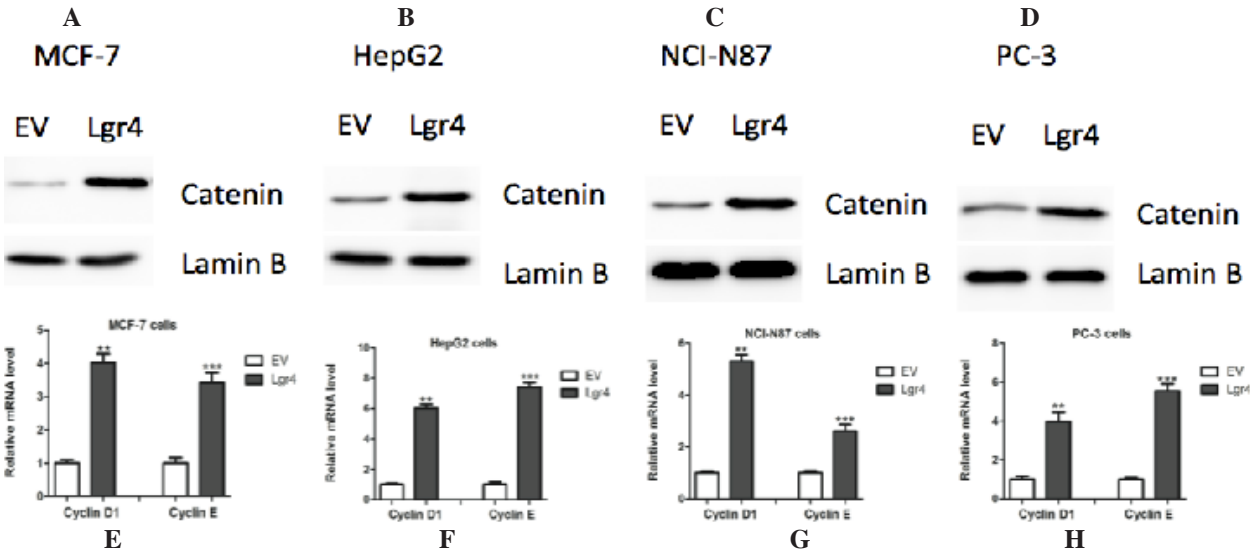

Figure 3. GPR48 Positively Regulates Wnt Signaling Activation in Cancer Cells. (A-D) Nuclear β-catenin expression was determined by western blot in MCF-7, HepG2, NCI-N87 and PC-3 cells transfected with plasmids containing empty vector (EV) or GPR48. Lamin B levels were measured as a loading control. (E-H) mRNA levels of Cyclin E and Cyclin D1 were examined by real-time PCR in MCF-7, HepG2, NCI-N87 and PC-3 cells overexpressing empty vector (EV) or GPR48

RNA oligos (siRNA) targeting GPR48 or negative controls (NC). As shown in Figure 2A-2D, siRNA treatment led to a dramatic reduction of GPR48 proteins. Knockdown of GPR48 expression reduced the growth of four cells, compared to NC-transfected cells (Figure 2E-2H).

GPR48 activates Wnt signaling in cancer cells

GPR48 has recently been reported to activate Wnt signaling after binding to its ligand R-spondin (de Lau et al., 2011; Glinka et al., 2011). To test whether the Wnt pathway mediates GPR48 in cancer cells, abundance of nuclear β-catenin, the hallmarker of Wnt signaling activation, was examined. As shown in Figure 3A -3D, the protein contents of nuclear β-catenin was increased in cells overexpressing GPR48. Besides, Cyclin D1 and Cyclin E, two downstream target genes of β-catenin, were also upregulated by GPR48 overexpression (Figure 3E-3H).

Discussion

In this study, we report that upregulation of GPR48 promoted cell proliferation while inhibition of GPR48 inhibited cell proliferation in four cancer cells. Our findings suggest that GPR48 overexpression may promote the initiation and progression of cancer cells through activation of Wnt signaling pathway. Previous studies have shown that GPR48 expression is up-regulated in colon cancer cells and associated with lymph node metastasis of tumors. Forced over-expression of GPR48 promotes the invasive and metastasis potential of colon cancer cells (Gao et al., 2006), suggesting that dys-regulation of GPR48 contributes to tumor invasiveness and metastasis.

Gpr48 is widely expressed in mouse and human tissues, suggesting its important roles in the development. Several groups have demonstrated that GPR48 regulates embryonic growth (Mendive et al., 2006), postnatal male reproductive duct system development (Li et al., 2010), gallbladder and bone formation (Luo et al., 2009; Yamashita et al., 2009), hair follicle development (Mohri et al., 2008), early eye open (Weng et al., 2008; Wang et al., 2010), midgestational erythropoiesis (Song et al., 2008), Mammary Gland Development and Stem Cell Activity (Oyama et al., 2011). Initially, Gpr48 functions by activating heterotrimeric Gα proteins to activate cyclin AMP/protein kinase A signaling pathway (Weng et al., 2008; Li et al., 2010). Subsequent studies indicate that GPR48 could also activate Wnt/β-catenin pathway upon binding with R-spondins (de Lau et al., 2011; Glinka et al., 2011). Activation of Wnt/β-catenin pathway is the common hallmark in various human caner tissues (Moon et al., 2004; Angers et al., 2009). For instance, in human breast cancer, cytoplasmic and nuclear localization of b-catenin were shown to predict a poor outcome (Deng et al., 2002). In human medulloblastomas, nuclear immunoreactivity of b-catenin is established as a favorable-risk indicator (Baryawno et al., 2010). Therefore, Wnt signaling pathway has become a therapeutic target for the control of tumor progression (Niehrs et al., 2012).

In summary, we found that GPR48 can promote the proliferation of cancer cell lines by activation of Wnt/β-catenin pathway. Understanding the precise role played by GPR48 progression will not only advance our knowledge of cancer biology, but also will help determine if GPR48 has potential as a novel therapeutic target for the treatment of certain cancers.

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