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Knockdown of FAM3B triggers cell apoptosis through p53-dependent pathway

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ABSTRACT

FAM3B, also named PANDER, is a cytokine-like protein identified in 2002. Previous studies showed that FAM3B regulates glucose and lipid metabolism through interaction with liver and endocrine pancreas. FAM3B is also expressed by other tissues but its basic function is unclear. In this study, we found that FAM3B was expressed in mouse colon, intestine, liver and lung tissues and multiple types of cell lines, including murine pancreatic β-cell (Min6), microglia (N9) and muscle cell (C2C12); human colon cancer cells (HCT8, HCT116, HT29), hepatocyte (HL-7702), hepatocellular carcinoma cell (SMMC-7721) and lung carcinoma cell (A549). Inhibition of FAM3B expression by RNA interference induced apoptotic cell death of HCT8, HCT116, A549, N9, C2C12 and Min6 cells and decreased cell viability of HL-7702 and murine primary hepatocytes. Further studies with HCT8 cells showed that knockdown of FAM3B increased the protein levels of membrane-bound Fas and Bax, reduced the expression of Bcl-2, promoted the cleavage of caspases-8, -3, -9 and PARP, and the nuclear translocation of cleaved PARP. These results suggest that FAM3B silencing activates both extrinsic and intrinsic apoptotic pathways. Mechanistic studies showed that neutralizing antibody against Fas or silencing Fas-associated death domain had no effect on, while caspase inhibitors could significantly reverse FAM3B knockdown induced apoptosis, suggesting Fas and death receptor mediated extrinsic apoptotic pathway is not involved in FAM3B silencing induced apoptosis. Further studies showed that p53 was significantly upregulated after FAM3B knockdown. Silencing p53 could almost completely reverse FAM3B knockdown induced upregulation of Bax, downregulation of Bcl-2, cleavage of caspases-8, -9, -3, and apoptotic cell death, suggesting p53-dependent pathway plays critical roles in FAM3B silencing induced apoptosis. Studies with HCT116 cells confirmed that inhibition of FAM3B expression induced apoptosis through p53-dependent pathway. Furthermore, knockdown of FAM3B reduced the protein level of Mdm2 and promoted p53 phosphorylation. Taken together, our studies demonstrated that silencing FAM3B promoted p53 phosphorylation and induced p53 accumulation by decreasing Mdm2 expression, which resulted in apoptotic cell death.

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1. Introduction

Family with sequence similarity 3 (FAM3) is a cytokine-like gene family identified in 2002 through computational genomic searching for novel cytokines using structure-based homology methods (Zhu et al., 2002). There are four members in FAM3 family (FAM3A, FAM3B, FAM3C and FAM3D), which encode proteins consist of 224–235 amino acids with a predicted secondary structure of four-helix bundle existing in many other cytokines (Zhu et al., 2002). The members among FAM3 family share 31.6–53.3% homology at amino acid level. The human and mouse FAM3B both have 235 amino acids, sharing 78% identity at amino acid level with 4 conserved cysteines (Zhu et al., 2002). FAM3B is also named pancreatic-derived factor (PANDER) due to its expression in pancreatic α- and β-cells (Cao et al., 2003; Carnegie et al., 2010). Human FAM3B mRNA is expressed highly in pancreas, and to a lesser extent in small intestine and prostate, as examined by Northern blot (Zhu et al., 2002). Western blot, immunocytochemistry, or immunohistochemistry analysis showed that mouse FAM3B protein is expressed by pancreatic α- and β-cells, hepatocytes, Purkinje cells in the cerebellum, neurons in the brainstem, and spermatids in testis (Zhu et al., 2002; Carnegie et al., 2010; Li et al., 2011). In vitro studies demonstrate that FAM3B expression is upregulated by glucose, fatty acids, CCL2, IFNγ alone or combined with IL-1β and TNFα in β-cells (Xu et al.,

Abbreviations: Bak, Bcl-2 antagonist/killer; Bax, Bcl-2-associated X protein; FADD, Fas-associated death domain; FAM3B, family with sequence similarity 3, member B; LDH, lactate dehydrogenase; MTT, 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Nec-1, necrostatin-1; PANDER, pancreatic-derived factor; PARP, poly (ADP-ribose) polymerase-1; PI, propidium iodide; p53AIP-1, p53-apoptosis inducing protein-1; RNAi, RNA interference.

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2005; Wang et al., 2008; Chen et al., 2011; Hou et al., 2011) and by insulin and arginine in α-cells (Carnegie et al., 2010). Initial in vitro studies revealed a potential role of FAM3B in pancreatic islet apoptosis. However, animal studies indicate that FAM3B is involved in the regulation of glucose metabolism and lipogenesis by interaction with endocrine pancrease and liver (Yang et al., 2009; Robert-Cooperman et al., 2005, 2010; Wilson et al., 2010, 2011; Li et al., 2011; Wang et al., 2012). A recent study showed that mouse FAM3B mRNA is not only expressed in tissues reported before, but also in a broad range of other tissues, including muscle, white and brown fat tissues, small and large intestines, stomach, kidney and heart (Li et al., 2011), suggesting that FAM3B may play a general role in maintaining cell function.

In this study, we found FAM3B protein was expressed by multiple mouse tissues and different types of cell lines derived from human and mouse. Knocking down FAM3B by RNA interference (RNAi) decreased cell viability and triggered cell apoptosis. Further study showed that silencing FAM3B activated both the extrinsic and intrinsic apoptotic pathways. p53-dependent apoptotic pathway plays a critical role in FAM3B knockdown induced apoptotic cell death.

2. Materials and methods

2.1. Reagents

MTT (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and necrostatin-1 were purchased from Sigma–Aldrich. Lactate dehydrogenase (LDH) activity assay kit was from Biovision (Milpitas, CA, USA). Propidium iodide (PI) was purchased from Invitrogen (Carlsbad, CA, USA). FITC labeled Annexin V and allophycocyanin (APC) conjugated anti-CD95 (Fas) antibody were obtained from BD pharmingen (San Diego, CA, USA). Mdm2 antibody and caspase inhibitors z-VAD-fmk, z-DEVD-fmk, and z-IETD-fmk were from Calbiochem (La Jolla, CA, USA). Antibodies against phosphorylated p53 (Ser46), β-tubulin, cleaved PARP, FADD, cleaved caspase-8, cleaved caspase-3, and Bax were purchased from Cell signaling (Beverly, MA, USA). Anti-cleaved caspase-9 and anti-p53 antibodies (DO-1) were from Santa Cruz. Fas neutralizing antibody (anti-CD95-ZB4) and Bcl-2 antibody were purchased from Milliopore. Antibodies against human and mouse FAM3B were purchased from Abgent (San Diego, CA, USA) and R&D (Minneapolis, MN, USA), respectively.

2.2. Cell culture

HT29, HCT116 and C2C12 cells were cultured in DMEM (Gibco BRL, Burlinton, ON, Canada) supplemented with 10% FBS. HCT8, HL-7702, SMMC-7721 and A549 cells were cultured in RPMI 1640 (Gibco) supplemented with 10% FBS. N9 cells were maintained in IMDM (Gibco) supplemented with 5% FBS. All cells were cultured at 37 °C under an atmosphere of 5% CO_2. Mouse hepatocytes were isolated by two-step collagenase (Roche) perfusion followed by Percoll (Sigma) density gradient centrifugation as described previously (Li et al., 2010), and cultured in M199 (Invitrogen) containing 10% heat-inactivated FBS.

2.3. RNA interference

Cells were transfected with siRNA targeting human FAM3B, p53, or FADD (Genepharma, Shanghai, China), or stealth siRNA against murine FAM3B (Invitrogen) using lipofectamine 2000 (Invitrogen) according to the manufactures' instructions. Target sequences of siRNA are as follows: human FAM3B siRNA: 1# GGAAATGTTGCCAGAGGAA, 2# GCTCTTCATGGTGACCTAT; human p53 siRNA: GTAATCTACTGGGACGGAA; human FADD siRNA: GATTGGAGAAGGCTGGCTC. Target sequence of stealth siRNA against murine FAM3B is: TCCAGCACTCTCTACAACATCCGAA.

2.4. Cell viability and apoptosis analysis

Cells were transfected with siRNA targeting FAM3B or nonspecific siRNA and cell viability was examined with MTT assay (Wang et al., 2008) 24 and 48 h later; the supernatant were collected and measured LDH activity 48 h later. Apoptosis was analyzed by propidium iodide (PI) and Annexin V staining using flow cytometry.

2.5. Immunofluorescence

HCT8 cells were transfected with siRNA targeting FAM3B or nonspecific siRNA. After 36 h, the cells were fixed with 4% formaldehyde for 15 min at room temperature, washed, and blocked with 5% BSA. Then the cells were incubated with primary antibody against cleaved PARP (Cell signaling, Beverly, MA, USA) at 4 °C overnight, washed and incubated with FITC conjugated secondary antibody. The localization of cleaved PARP was detected by confocal microscopy.

2.6. Immunohistochemistry

Mouse colon tissue sections were fixed in 4% formaldehyde for 15 min at room temperature, washed with PBS and incubated with 3% H_2O_2 in methanol. After washing with PBS, the slides were incubated with blocking buffer (5% BSA in PBS/T) for 1 h at room temperature. Primary antibody against FAM3B (R&D) (1:50) or control IgG was applied to the slides and incubated overnight at 4 °C. The slides were washed and incubated with secondary antibody conjugated with HPR (1:500) for 1 h at room temperature. FAM3B expression signals were detected by DAB detection kit (Dako, Carpinteria, CA, USA) according to the manufacture's instructions.

2.7. Western blotting

Cells were lysed in ice-cold lysis buffer (20 mM Tris–HCl (pH7.4), 1 mM EDTA, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 1 mM PMSF) and centrifuged. The supernatants were collected and protein concentration was determined using the Bradford's method. Equal amounts of proteins were separated by 12% SDS-PAGE and transferred onto a PVDF membrane (Millipore). Primary antibodies specific for FAM3B, cleaved caspase-3, -8, -9, PARP, FADD, p53, Ser46 phosphorylated p53, Bax, Bcl-2, Mdm2, or β-tubulin (1:1000) were used to probe target proteins. Horseradish peroxidase-conjugated secondary antibodies (Abmart, Shanghai, China) and Super Signal Reagents (Pierce, Rockford, IL, USA) were used to detect bound immunocomplexes. Immunoblot results were quantified by using Gel-Pro Analyzer software (Media Cybernetics Inc., Silver Spring, MD, USA).

2.8. Statistics

All experiments were performed at least three times. The data shown represent the mean ± SD for triplicates. Statistical differences between groups were analyzed by Student's *t*-test or ANOVA. $P < 0.05$ was considered statistically significant.

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