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DNA damage induced apoptosis suppressor (DDIAS) is upregulated via ERK5/MEF2B signaling and promotes β-catenin-mediated invasion

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ABSTRACT

DNA damage induced apoptosis suppressor (DDIAS) is an anti-apoptotic protein that promotes cancer cell survival. We previously reported that DDIAS is transcriptionally activated by nuclear factor of activated T cells 2 (NFATc1). However, the upstream regulation of DDIAS expression by growth factors has not been studied. Here, we demonstrate that DDIAS expression is induced by extracellular signal-regulated kinase 5 (ERK5) and myocyte enhancer factor 2B (MEF2B) in response to epidermal growth factor (EGF) and that it positively regulates β-catenin signaling in HeLa cells. The genetic or pharmacological inhibition of ERK5 suppressed DDIAS induction following EGF exposure and the overexpression of constitutively active MEK5 (CA-MEK5) enhanced DDIAS expression. In chromatin immunoprecipitation assays, MEF2B, a downstream target of ERK5, exhibited sequence-specific binding to a MEF2 binding site in the DDIAS promoter following treatment with EGF. The overexpression of MEF2B increased the EGF-mediated induction of DDIAS expression, whereas the knockdown of MEF2B impaired this effect. Furthermore, DDIAS promoted invasion by increasing β-catenin expression at the post-translational level in response to EGF, suggesting that DDIAS plays a crucial role in the metastasis of cancer cells by regulating β-catenin expression. It is unlikely that MEF2B and NFATc1 cooperatively regulate DDIAS transcription in response to EGF. Collectively, EGF activates the ERK5/MEF2 pathway, which in turn induces DDIAS expression to promote cancer cell invasion by activating β-catenin target genes.

1. Introduction

Epidermal growth factor (EGF) plays a fundamental role in cell proliferation, survival, migration and development by activating the EGF receptor and downstream signaling. Dysregulation of the EGF/EGFR pathway has been implicated in tumor progression and metastasis of human cancers. The extracellular signal-regulated kinase (ERK) pathway is activated by EGF stimulation [1]. In response to EGF stimulation, ERK2 activates phosphorylation of CK2, which phosphorylates α-catenin to disrupt the association between β-catenin and α-catenin, thereby promoting transcriptional activation of β-catenin for tumor cell invasion [2]. Likewise, ERK5 functions in the regulation of EGF-induced cell proliferation during the G1/S transition [3,4]. ERK5 phosphorylates myocyte enhancer factor-2 (MEF2) family proteins and serum and glucocorticoid-inducible kinase (SGK), which is required for S-phase entry [5,6]. In addition, ERK5 is implicated in tumorigenesis by inducing cyclin D1 and MYCN expression [7] and disrupting the promyelocytic leukemia protein (PML)-MDM2 interaction [8,9]. Moreover, ERK5 contributes to colon cancer progression and metastasis via NF-kB activation [10].

MEF2 family proteins, which include MEF2A, MEF2B, MEF2C and MEF2D, are transcription factors involved in the development of muscle, cardiac, skeletal, vascular, and immune system [11]. MEF2B is the most divergent and least studied of the MEF2 isoforms. Recently, MEF2 family proteins have been reported to be involved in cell differentiation, proliferation, migration and apoptosis [11,12]. Especially, MEF2C promotes VEGF-mediated HCC cell invasion and angiogenesis by regulating β-catenin activity in HCC [11,13]. MEF2 family proteins contain an N-terminal DNA-binding MADS (MCM1-agamous-deficiens-serum response factor) domain, a MEF2 domain and a C-terminal transcriptional activation domain. The MADS domain and the MEF2 domain interact

Abbreviations: DDIAS, DNA damage induced apoptosis suppressor; EGF, epidermal growth factor; MEK5, mitogen-activated protein kinase kinase; ERK5, extracellular signal-regulated kinase 5; MEF2, myocyte enhancer factor 2; hNoxin, human noxin; NFAT, nuclear factor of activated T cells; HCC, hepatocellular carcinoma; siRNA, small interfering RNAs; SRB, sulforhodamine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CsA, cyclosporine A.

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with coactivators or corepressors, including histone deacetylases (HDACs), p300 and Cabin1. Mutations that enhance the transcriptional activity of MEF2B promote BCL6 expression in diffuse large B cell lymphoma (DLBCL) and non-Hodgkin lymphoma [14,15].

The expression of DNA damage induced apoptosis suppressor (DDIAS) is elevated in many types of tumor, particularly lung, colon and liver cancers [16,17]. Overexpression of DDIAS promotes the proliferation, colony formation, migration and in vivo tumorigenicity of hepatocellular carcinoma (HCC) cells, whereas DDIAS knockdown attenuates these effects [17]. Moreover, DDIAS knockdown induces apoptosis of NSCLC in response to DNA damage [16,18]. We recently reported that NFATc1 regulates DDIAS transcription to confer cisplatin resistance in NSCLC [18]. NFATc1 knockdown induces apoptosis and leads to growth inhibition of lung cancer cells, and DDIAS overexpression reverses these effects, supporting a cancer-specific role of *DDIAS* as a target gene of NFATc1. However, the mechanisms and signaling pathways involved in growth factor-mediated regulation of DDIAS expression are poorly understood. Here, we report that DDIAS is upregulated by the EGF-ERK5-MEF2 pathway and activates β-catenin-mediated HeLa cells invasion.

2. Materials and methods

2.1. Reagents

Recombinant human EGF, TNFα, TGF-β, PDGF, VEGF, IGF-1 and XMD8-92 were obtained from R&D Systems (Minneapolis, MN, USA). U0126 and PD98059 were purchased from Millipore (Billerica, MA, USA). Gefitinib, erlotinib, lapatinib and BIX02189 were purchased from Selleckchem (Houston, TX, USA). Cycloheximide, MG132 and DAPI were obtained from Sigma-Aldrich (St. Louis, MO, USA). The following antibodies were used: *anti*-Myc (sc-40), anti-HA (sc-805), *anti*-MEF2B (sc-101097) and *anti*-E-cadherin (sc-7870) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); *anti*-EGFR (2232), *anti*-pEGFR (Y1068, 2236), *anti*-ERK1/2 (4695), *anti*-pERK1/2 (9101), anti-ERK5 (3372), anti-pERK5 (T218/Y220, 3371), *anti*-Snail (3879), anti-vimentin (3390) and anti-β-catenin (9562) from Cell Signaling Technology (Beverly, MA, USA); *anti*-GAPDH (LF-PA0212) from AbFrontier (Seoul, Korea); *anti*-cyclin D1 (554180) from BD Biosciences; *anti*-Flag (F1804, F7425) from Sigma-Aldrich; anti-DDIAS (HPA038540) from Atlas Antibodies; anti-β-catenin (ab2365) and anti-MEF2B (ab33540) from Abcam (Cambridge, MA, USA).

2.2. DNA constructs

Human cDNAs for ERK5 (GenBank accession number NM_139032) and MEF2B (GenBank accession number NM_001145785.1) were provided by the Korea Human Gene Bank, KRIBB, Korea. These clones were amplified by PCR and inserted into pcDNA3.1 with an HA tag or Myc tag. Constitutively active form of MEK5 (CA-MEK5) was curiously given by professor Chang-Hoon Woo at Yeungnam University College of Medicine, Korea [19]. β-catenin was obtained from Addgene (https://www.addgene.org/) (#16,828) and subcloned into pcDNA3.1 with an HA tag. The DDIAS promoter (−1205) in pGL2 [18] was subcloned into pGL4.17 (luc2/neo) (Promega, Madison, WI, USA). A QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used to generate mutations in the MEF2 binding site (MT). The following primers were used for mutagenesis: MT forward, 5′-GCGAAGCTCCAAAACCAAGGGCACGGACTGTACTACTGAT-3′; MT reverse, 5′- ATCAGTAGTACAGTCCGTGCCCTTGGTTTTGGAGCTTCGC-3′. All constructs were confirmed by sequencing analysis.

2.3. Cell culture, transfection and luciferase assay

HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 50 units of penicillin and 50 μg of streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were cultured in an incubator at 37 °C containing 5% CO_2.

To generate cells stably expressing luciferase from the DDIAS promoter, we transfected the pGL4.17-DDIAS promoter (−1205 bp) vector into HeLa cells, followed by selection in medium containing 500 μg/ml G418. Cignal Lenti *Renilla* (hygromycin selection) served as an internal control for normalization. The cells were transiently transfected with the indicated plasmids using Turbofect (ThermoScientific, Rockford, IL), starved for 24 h in serum-free medium, and stimulated with growth factors or cytokines. Firefly luciferase activity and *Renilla* luciferase activity were assayed using a dual-luciferase reporter assay system (Promega). The firefly luciferase activity was normalized to *Renilla* luciferase activity and expressed as relative luciferase units to reflect promoter activity.

2.4. RNA interference

Cells were transfected with 40 nM siRNAs using the Neon electroporation system (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. siRNAs were synthesized by ST Pharm. Co., Ltd. (Kyunggido, Korea) and by Bioneer Corp (Daejeon, Korea). The following siRNAs were used in this study: siScr: 5′-CCUACGCCAC CAAUUUCGU-3′, siERK1: 5′-CUCCCUGACCCGUCUAAUAUA-3′, siERK2: 5′-AAGUUCGAGUAGCUAUCAAGA-3′, siERK5: 5′-CAGACCCACCUUUC AGCCUUA-3′, siMEF2A: 5′-GCCUAGAAAUAUAGAGCAUUA-3′, siMEF2B: 5′-CGGCGACUUUCCUAAGACCUU-3′, siMEF2C: 5′-AUGUAGAUGCUGCU GUUGC-3′, siMEF2D: 5′-CCUCUGAAGAACUGGGCAUUU-3′ siDDIAS: 5′-CUGAAGAGAUCUGCAUGUU-3′.

2.5. RT-PCR and qPCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into cDNA using TOPscript™ RT DryMIX (dT18) (Enzynomics, Daejeon, Korea) according to the manufacturer's instructions. Real-time PCR was performed using a SYBR Green PCR Master Mix kit (Qiagen, Valencia, CA, USA). The cycling conditions were 95 °C for 15 min and 40 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 15 s. The following primers were used: 5′-CTTG CAGCAGTTGTTACGAA-3′ and 5′-GTGACCAAGCACTTCGAGTT-3′ for DDIAS, 5′-TCATGACCACAGTCCATGCC-3′ and 5′-TCCACCACCCTGTTGC TGTA-3′ for GAPDH, 5′-ATCATAAAATCGCACCTGGTC-3′ and 5′-TCTGTA GTGCTCAACATCCC-3′ for MEF2A, 5′-CCAGGAGAGAAGTTTCGGA-3′ and 5′-CTCCCAGGCCATATTCTG-3′ for MEF2B, 5′-AACTCAGACATCGTGGAG AC-3′ and 5′-CGATGTGTTACACCAGGAGA-3′ for MEF2C, 5′-CACAGATT ACCAGTTGACCA-3′ and 5′-AGGTTGCTGAGAGATACAG-3′ for MEF2D, 5′-AACTTGCCACACGTGCAATC-3′ and 5′-AGGTTATGCAAGGTCCCAGC-3′ for CTNNB1, 5′-AAAGGCTGACAGACTCACTG-3′ and 5′-TTAAATTGCC CGGGAAACAG-3′ for Snail, 5′-CAAATGGCGGACACAATTCC-3′ and 5′-CCAGGACCACGTAGACAGAT-3′ for ERK5, 5′-TCAGGGGAGATCATCGGG ACAACTC-3′ and 5′-CAGTTCTAGGGAAGCCAAAGGAGCT-3′ for MMP1, 5′-AAATTGGCCACTCCCTGGGTCTCTT-3′ and 5′-GCTATTTGCTTGGGAA AGCCTGGCT-3′ for MMP3. All reactions were performed in triplicate and normalized to GAPDH as an internal control. The values are presented as the mean ± S.E.M.

2.6. Western blot assay

Cells were lysed with 1 × RIPA buffer (Millipore) containing 1 mM Na_3VO_4, 1 mM sodium fluoride, 1 mM PMSF, and protease inhibitor cocktail (Roche, Basel, Switzerland), and the lysates were quantified with a BCA assay kit (Bio-Rad, Hercules, CA, USA). Lysates were subjected to immunoblotting with specific antibodies. Western blot signals were detected using an enhanced chemiluminescence (ECL) kit (Millipore).