BBA - Gene Regulatory Mechanisms xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

BBA - Gene Regulatory Mechanisms

journal homepage: www.elsevier.com/locate/bbagrm

p63 and p73 repress CXCR5 chemokine receptor gene expression in p53-deficient MCF-7 breast cancer cells during genotoxic stress

Nikita A. Mitkin[a,1], Alisa M. Muratova[a,b,1], George V. Sharonov[c,d], Kirill V. Korneev[a,b], Ekaterina N. Sviriaeva[a], Dmitriy Mazurov[e], Anton M. Schwartz[a], Dmitry V. Kuprash[a,b,*]

[a] Laboratory of Intracellular Signaling in Health and Disease, Engelhardt Institute of Molecular Biology Russian Academy of Sciences, Vavilov str. 32, 119991 Moscow, Russia
[b] Department of Immunology, Lomonosov Moscow State University, Leninskye gory 1, 119234 Moscow, Russia
[c] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Ul. Miklukho-Maklaya 16/10, 117997 Moscow, Russia
[d] Faculty of Medicine, Lomonosov Moscow State University, Leninskye gory 1, 119234 Moscow, Russia
[e] Institute of Gene Biology, Russian Academy of Sciences, 34/5 Vavilov St., Moscow 119334, Russia

ARTICLE INFO

Keywords:
p53
p63
p73
CXCR5
Chemokine receptors
NFkB

ABSTRACT

Many types of chemotherapeutic agents induce of DNA-damage that is accompanied by activation of p53 tumor suppressor, a key regulator of tumor development and progression. In our previous study we demonstrated that p53 could repress CXCR5 chemokine receptor gene in MCF-7 breast cancer cells via attenuation of NFkB activity. In this work we aimed to determine individual roles of p53 family members in the regulation of *CXCR5* gene expression under genotoxic stress. DNA-alkylating agent methyl methanesulfonate caused a reduction in CXCR5 expression not only in parental MCF-7 cells but also in MCF-7-p53off cells with CRISPR/Cas9-mediated inactivation of the p53 gene. Since p53 knockout was associated with elevated expression of its p63 and p73 homologues, we knocked out p63 using CRISPR/Cas9 system and knocked down p73 using specific siRNA. The *CXCR5* promoter activity, CXCR5 expression and CXCL13-directed migration in MCF-7 cells with inactivation of all three p53 family genes were completely insensitive to genotoxic stress, while pairwise p53 + p63 or p53 + p73 inactivation resulted in partial effects. Using deletion analysis and site-directed mutagenesis, we demonstrated that effects of NFkB on the *CXCR5* promoter inversely correlated with p63 and p73 levels. Thus, all three p53 family members mediate the effects of genotoxic stress on the *CXCR5* promoter using the same mechanism associated with attenuation of NFkB activity. Understanding of this mechanism could facilitate prognosis of tumor responses to chemotherapy.

1. Introduction

p53 is a tumor suppressor protein which induces apoptosis, cell cycle arrest and DNA-repair in response to DNA-damage [1]. Functional p53 represses local inflammation [2] while p53 inactivation stimulates expression of pro-inflammatory genes such as IL1, IL6, IL11 and Ptgs2 [3,4]. However, in some cases positive cooperation between p53 and NFkB can lead to activation of a specific subset of pro-inflammatory genes in response to genotoxic stress [5]. Such cooperative behavior has been shown to involve chromatin-bound complexes between gain-of-function mutant p53 and p65/RelA NFkB subunit [6]. In epithelial cancer cells this effect was observed during standard chemotherapy only in the presence of active signaling by inflammatory cytokines, such as TNFα [7]. About 50% of all cancers carry mutations in p53 gene which are often associated with increased metastatic potential and elevated expression of a number of chemokines and their receptors [8]. In most instances, p53 acts on chemokine and chemokine receptor genes indirectly through repression of pro-oncogenic transcription factors. For example, p53 attenuates expression of CXCR4 chemokine receptor gene in breast cancer by inhibiting AP-1 transcription factor [9] and reduces the expression of CCL20, CXCL1, CXCL2, CXCL3 and CXCL8 chemokines in ovarian cancer by suppressing NFkB activity, presumably through stabilization of IkB proteins [10]. Elevated p53 was also reported to inhibit nuclear content and activity of NFkB protein p65/RelA in immortalized mouse striatal cells, with subsequent repression of a p65-dependent CCL5 pro-inflammatory chemokine gene

* Corresponding author at: Laboratory of Intracellular Signaling in Health and Disease, Engelhardt Institute of Molecular Biology Russian Academy of Sciences, Vavilov str. 32, 119991 Moscow, Russia.
E-mail address: kuprash@eimb.ru (D.V. Kuprash).
[1] Equal contribution.

http://dx.doi.org/10.1016/j.bbagrm.2017.10.003
Received 7 July 2017; Received in revised form 2 October 2017; Accepted 24 October 2017

[11]. Recently we have shown that suppression of p53 in human breast cancer cell lines leads to elevated expression of CXCR5 chemokine receptor gene due to repression of NFkB activity [12].

Both p63 and p73, homologues of p53, have isoforms expressed from alternative promoters and carrying opposite functions [13]. While TA isoforms of p63 and p73 containing trans-activating domain (TAD) are able to regulate cell cycle arrest, genome stability, apoptosis and cell migration potential in a p53-like manner, ΔN-isoforms of both proteins stimulate cell proliferation and survival and act as oncogenes [14]. ΔN-isoforms are also known to act as dominant-negative inhibitors of TA-isoforms and p53 [15]. Despite the fact that p63 and p73 genes are rarely mutated in tumors [16,17], their knockouts are associated with increased levels of spontaneous and inducible tumorigenesis [18] and elevated metastasis [19].

DNA-damage is associated with stabilization of TA-isoforms of both p63 and p73 and their accumulation in the cell [20]. Elevated TA-p73 induces a number of genes responsible for cell cycle arrest (p21) and apoptosis (Bax, Puma) [17]. Genotoxic stress increases the level of TA-p63 in most types of cancer, leading to activation of a number of p53-dependent genes such as p21, 14-3-3-σ, GADD45 and PIG3 [21]. Genotoxic stress also strengthens p73 and p63 anti-tumor effect by enhancing proteasome-mediated degradation of their ΔN isoforms through ubiquitin-dependent mechanisms [22,23,24,25]. All these processes enhance tumor-suppressive functions of TP63 and TP73 genes [26] and may explain the response of cancer cells to chemotherapeutic agents that act through induction of genotoxic stress [27].

In this work we demonstrate that response to genotoxic stress in breast cancer cells includes repression of CXCR5 chemokine receptor gene. CXCR5 repression is mediated by all three members of the p53 family that attenuate NFkB activity in a redundant manner.

2. Materials and methods

2.1. Cell lines

MCF-7 breast cancer cell line was kindly provided by late Dr. E. Zabarovsky from Karolinska Institute (Stockholm, Sweden). The local stock was maintained by Dr. V.S. Prasolov and was verified by transcriptome profiling as described [12]. All variants of MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Life technologies, Carlsbad, USA) supplemented with 10% fetal bovine serum and 0.01% human insulin. For transfection by electroporation, cells were grown to no > 50% confluency.

2.2. Ethical approval

According to the decision of Scientific Council of the Engelhardt Institute of Molecular Biology, all the studies were performed using commercially available cell lines and did not require any specific approval.

2.3. Inactivation of p53 and p63 genes in MCF-7 cell line using CRISPR/Cas9 system

Sequences for crRNA (see Supplementary Table) were designed using nickase service of MIT CRISPR Design Tool [28], commercially synthesized (Syntol, Moscow, Russia), annealed as described [28] and cloned into pSpCas9n(BB)-2A-GFP(PX461) vector containing Cas9n, GFP and tracrRNA genes (a gift from Feng Zhang, Addgene plasmid # 48140) using *Bbs*I restriction site. We tested 3 variants of crRNA pairs for each p53 and p63 genes (sequences are represented in Supplementary Table). A GFP- or RFP-expressing cassette was integrated into double strand break location utilizing homology directed repair (HDR) for simplified detection of MCF-7 cells with damaged p53 or p63 gene, respectively. p53 and p63 left and right homology arms were amplified using genomic MCF-7 DNA as a template and specific primers containing *Apa*I/*Xho*I restriction sites for left arms and *Eco*RI/*Sac*I for right arms. GFP- and RFP-expressing cassettes were amplified from peGFP-n3 and peRFP-n3 plasmids (Promega, Madison, USA), respectively using primers containing *Xho*I/*Eco*RI restriction sites. All primer sequences are represented in Supplementary Table. Donor constructs were assembled by integration of homology arms and GFP/RFP-expressing cassettes into Bluescript II KS + plasmid (Stratagene, USA) using respective restriction sites.

Cells were transfected with the mix of 2 μg px461(sgRNA1), 2 μg px461(sgRNA2) and 4 μg donor construct using Neon electroporation system (Thermo Scientific, Waltham, MA, USA) (see below). MCF-7 cells with constitutive GFP- or/and RFP-fluorescence were sorted using BioRad S3e cell sorter (BioRad, USA). The sorting gate included approximately 10 brightest percent of the GFP- or RFP-positive single-cell population (Supplementary Fig. S1). Cells were grown for 7 days, sorted again and plated at single-cell density into 96-well plates and cultivated for 14 more days. PCR analysis and Sanger sequencing (primers are represented in Supplementary Table) was used to select clones with homozygous inactivation of p53 and/or p63. Based on flow cytometry, the efficiency of transfection was between 20% and 30%, and the frequency of clones with homologous recombination in both alleles was between 1% and 2%.

2.4. p73 knockdown in MCF-7 cells using siRNA

We tested three previously published [29] pairs of siRNAs targeting p73 gene (targeting all p73 isoforms), one of which (sequence is represented in Supplementary Table) demonstrated the most stable effect and was chosen for subsequent experiments. Single-stranded RNAs were commercially synthesized (Syntol, Moscow, Russia), siRNA duplexes were prepared as described earlier [30] and used for MCF-7 cells electroporation (500 pmol per 1 million cells) 24 h prior to electroporation with luciferase constructs.

2.5. RNA extraction and real-time quantitative RT-PCR

Total RNA isolation from MCF-7 cells was performed using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. First strand cDNA was synthesized using M-MULV reverse transcriptase and oligo-dT primer from First strand cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA). For quantitative real-time PCR we used Applied Biosystems 7500 PCR system, PCR reaction mix with SYBR Green (Evrogen, Moscow, Russia) and intron-spanning primers specific for human CXCR5, p53, p63 and p73 cDNAs (sequences are represented in Supplementary Table). We also used the primers for specific detection of total TAp63, ΔNp63, TAp73, ΔNp73 isoforms and separate detection of TAp63α, TAp63β and TAp63γ isoforms (sequences are represented in Supplementary Table). The PCR program consisted of preheating stage (95 °C, 10 min) and 40 cycles of amplification at 95 °C for 15 s, 62 °C for 20 s, 72 °C for 20 s. Melting curve analysis was performed to control the quality of amplification products. mRNA levels in all samples were determined using the ΔΔCt approach and normalized to β-actin.

2.6. Evaluation of CXCL13-directed chemotactic activity of MCF-7 cells

Chemotactic potential of MCF-7 cells was estimated using commercially available 8 μm pore ThinCert cell culture inserts (Greiner Bio-One, Frickenhausen, Germany) according to manufacturer's protocol. We used purified recombinant CXCL13 (Thermo Scientific, Waltham, MA, USA) as chemoattractant. The quantity of migrated cells was estimated with Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma, St. Louis, USA) in MTT test (incubation with MTT for 24 h with subsequent incubation with solubilizing solution for 3 h) according to manufacturer's protocol.