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Epigenetic and functional analysis of IGFBP3 and IGFBPrP1 in cellular immortalization

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Abstract

Carcinogenic transformation of a cell requires bypassing senescence and becoming immortalized. A cellular senescence-like phenotype can be induced in immortal Li–Fraumeni syndrome (LFS) cells by treating them with the DNA methyltransferase inhibitor 5-aza-deoxycytidine. Our microarray-based expression profiling studies of spontaneously immortalized LFS cell lines identified genes that may provide the growth advantage required for the cells to become immortal. Several members of the IGFBP superfamily of genes fit the profile of genes involved in immortalization: silenced during immortalization and reactivated by 5-aza-deoxycytidine. Overexpression of IGFBP3 or IGFBPrP1 in the immortal LFS cell lines suppressed cell growth and inhibited colony formation. Both genes have the expression pattern of an epigenetically regulated gene and contain CpG islands suitable for methylation-dependent silencing. Analysis of how IGFBPs regulate immortalization will lead to a better understanding of this process and may lead to novel methods for the prevention and treatment of cancer.

Keywords: Immortalization; IGFBP3; IGFBP4; IGFBPrP1; DNA methylation; 5-aza-dC; CpG island; Epigenetic silencing; Interferon

The insulin-like growth factor binding protein (IGFBP) superfamily, which includes the IGFBP-related proteins (IGFBPrP), regulates the bioavailability of insulin and insulin-like growth factors. Several reports have shown IGFBPs are capable of inhibiting cell proliferation and inducing apoptosis in cancer cells [1,2]. IGFBP3 has been shown to inhibit cell proliferation in breast, lung, and prostate cancer cells and in some cell lines to reduce tumor growth [3]. Consistent with this observation, p53 and growth inhibitory agents including retinoic acid, transforming growth factor-β (TGF-β) and anti-estrogens have been shown to increase the expression of IGFBP3 [1,2]. In addition, expression of IGFBPrP1 is upregulated upon treatment of cells with retinoic acid or TGF-β [4,5].

The expression of *IGFBP3* and *IGFBPrP1* is frequently decreased in tumors and this decrease is often a consequence of promoter methylation [6,7]. Epigenetic silencing of *IGFBP3* and *IGFBPrP1* by promoter methylation and subsequent suppression of expression is reversible when cells are treated with the DNA methyltransferase inhibitor, 5-aza-deoxycytidine (5-aza-dC). The promoter of *IGFBP3* is hypermethylated in cancers of renal cell, ovarian, liver, gastric, colorectal, breast, and malignant mesothelioma [6,8–10]. *IGFBPrP1* is downregulated in mammary carcinoma and colorectal cancer cell lines [5,7]. 5-aza-dC treatment induced expression of *IGFBP3* in hepatocellular carcinoma cell lines [10] and *IGFBPrP1* in colorectal cancer cell lines [7].

In senescent cells the expression of six IGFBP superfamily gene have been shown to increase, including

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IGFBP2 [11], *IGFBP3* [12,13], *IGFBP5* [11], *IGFBP6* [14], *IGFBPrP1* [4,5], and *IGFBPrP2* [4]. *IGFBP3* is upregulated in senescent and downregulated in immortalized human prostate epithelial cells [13]. Compared to normal human diploid fibroblasts (NDF), *IGFBP3* expression was higher in senescent NDF [12]. IGFBPrP1 was found to be overexpressed during senescence of human mammary and prostate epithelial cells [4,5]. In addition, expression of IGFBPrP1 in MCF-7 breast cancer cells induces a senescence-like state [15]. The increased expression of the IGFBP family during senescence and the decreased expression during immortalization suggest that dysregulation of this gene family may regulate these processes.

We focused on genes, whose expression decreased during immortalization and increased when treated with 5-aza-dC, as candidate epigenetically controlled genes that regulate cellular immortalization [16,17]. Interrogating our microarray database [16] we found that several genes in the IGFBP superfamily, including IGFBP3, IGFBP4, and IGFBPrP1, fit this profile of an immortalization gene. Here, we present functional studies of IGFBPs in immortal LFS cells which suggest that these genes may be regulators of cellular immortalization. The expression of IGFBP3 appears to be inhibited by promoter hypermethylation, and can be activated by IFN-α treatment. This is consistent with our previous studies that the IFN pathway is repressed in immortal cells [16,17].

Materials and methods

Cell culture media and conditions. Four independent, spontaneously immortalized LFS cell lines were used in this study; one immortal cell line from MDAH041 (MDAH041-IM), and three independent immortal cell lines derived from MDAH087 (MDAH087-1, MDAH087-10, and MDAH087-N) [16,18]. Quality control on cell lines was performed as described [16]. Precrisis (MDAH041-PC and MDAH087-PC) and immortal LFS cell lines, and human NDF cell line AFB-10 (established from fresh tissue samples, M.A. Tainsky, unpublished), were cultured as described [16].

5-aza-dC and/or IFN-α treatment, and RNA isolation. Precrisis and immortal MDAH041 and MDAH087 fibroblasts were treated with 5-aza-dC (Sigma–Aldrich, St. Louis, MO) as described [16,17]. For experiments of IFN-α and/or 5-aza-dC combined treatments, cell cultures were treated as described [16,19]. Total RNA was extracted using the Qiagen RNeasy Kit (Qiagen Inc., Valencia, CA).

Affymetrix microarray chip processing, data analysis, and confirmation by Q-RT-PCR. Preparation of cDNA from total RNA, Affymetrix (Santa Clara, CA) HGU95Av2® oligonucleotide microarray processing, data analysis, and clustering analysis were performed as described [16]. Confirmation of gene expression changes by real-time PCR (Q-RT-PCR) was performed on reverse transcribed RNA as described [16]. See Supplemental Table 1 for primers used in Q-RT-PCR.

Plasmid construction. IGFBP3 and IGFBPrP1 were cloned into the mammalian expression vector pIRES puromycin (kindly provided by Dr. P. Christopher Roberts). IGFBP3 was digested out of pUC119-IGFBP3 (kindly provided by Dr. William Wood) with *Eco*RI and cloned into the *Eco*RI site in pIRESpuro. IGFBPrP1 was digested out of pLXSN-IGFBPrP1 (kindly provided by Dr. Karen Swisshelm) with *Bam*HI and cloned into the *Bam*HI site in pIRESpuro. All pIRES constructs were verified by sequencing.

DNA transfection. MDAH041-IM, MDAH087-N, MDAH087-1, and MDAH087-10 were transfected with pIRES, pIRES-IGFBP3, and pIRES-IGFBPrP1 using Lipofectamine™ Transfection Reagent Invitrogen, Carlsbad, CA) in combination with Plus Reagent® (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Transient transfections were harvested 72 h posttransfection. For stable transfections, 48 h posttransfection the cell lines were selected in standard culture media [16] containing 0.5 μg/ml puromyocin (Sigma–Aldrich, St. Louis, MO). Expression of the genes was confirmed by Western blot (data not shown). A modified version of the MTT tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay developed by Mosmann [20] was used to quantify cell growth. Cells were seeded at a density of 750 cells per well in a 12-well plate. To study colony formation 14 days following transfection and selection in 0.5 mg/ml puromycin, cells were seeded at a density of 2500 cells per 100-mm plate. Two weeks later colonies were fixed in MeOH and Giemsa (Sigma–Aldrich, St. Louis, MO) stained.

Protein isolation and Western blot analysis. Western blot analysis was performed as described see Supplemental figures in [16]. The primary antibodies used were anti-IGFBP3, anti-IGFBP4, and anti-IGFBPrP1 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-tubulin (Sigma–Aldrich, St. Louis, MO).

Results and discussion

Previously we identified 14 genes that were epigenetically downregulated after spontaneous immortalization in a series of cells with germline p53 mutations (LFS cells). The objective of this study was to focus on whether *IGFBP3*, a gene silenced in all four immortal LFS cell lines, and *IGFBPrP1*, one of the 14 epigenetically regulated genes in the four immortal LFS cell lines [16], were capable of altering the growth of the immortal LFS cell lines.

Gene expression analysis of the IGFBP family of genes

Expression of four of the 15 IGFBP genes, *IGFBP3*, *IGFBP4*, *IGFBPrP1*, and *IGFBPrP5*, decreased in all four immortal LFS cell lines when compared to their respective precrisis cell line (Fig. 1, Table 1 and Supplemental Table 2). *IGFBP5* decreased in the three immortal MDAH087 cell lines, but not in MDAH041-IM. The microarray results for *IGFBP3*, *IGFBP4*, and *IGFBPrP1* were confirmed by Q-RT-PCR (22 out of 24 these observations were validated by Q-RT-PCR; data not shown).

IGFBP3, *IGFBP4*, and *IGFBPrP1* have CpG islands in their promoters (Supplemental Fig. 1) and could potentially be silenced during immortalization by CpG island promoter methylation. Reexpression of a gene following 5-aza-dC treatment indicated, although not conclusively, that its expression was inhibited by DNA methylation. Following 5-aza-dC treatment *IGFBPrP1* increased in all four of the immortal LFS cells, thus indicating that this gene may be epigenetically regulated in these cell lines.

IGFBP3 expression increased in MDAH041-IM following 5-aza-dC treatment, but its expression remained low in the three MDAH087 immortal cell lines. Therefore, there must be other mechanisms that downregulated *IGFBP3* expression in the MDAH087 cell lines. *IGFBP4* expression