Disruption of Scaffold Attachment Factor B1 Leads to TBX2 Up-regulation, Lack of p19ARF Induction, Lack of Senescence, and Cell Immortalization

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Abstract

Scaffold attachment factor B1 (SAFB1) is a multifunctional protein, which has previously been implicated in breast cancer. Here, we show that genetic deletion of SAFB1 in mouse embryonic fibroblasts (MEF) leads to spontaneous immortalization and altered expression of two proteins involved in immortalization and escape from senescence: low levels of p19ARF and high levels of TBX2. Inactivation of TBX2 using a dominant-negative TBX2 resulted in up-regulation of p19ARF in SAFB1 knockout MEFs. SAFB1 loss also caused lack of contact inhibition, increased foci formation, and increased oncogene-induced anchorage-independent growth. These findings suggest that SAFB1 is a novel player in cellular immortalization and transformation. (Cancer Res 2006; 66(16): 7859-63)

Introduction

Scaffold attachment factor B1 (SAFB1) is a nuclear protein involved in RNA processing, transcriptional regulation, chromatin organization, and stress response (1). The protein contains numerous highly conserved functional domains. SAFB1 can bind RNA via a RNA recognition motif (2) and is found in complexes with RNA processing proteins (3, 4). Because SAFB1 can also interact with RNA polymerase II, it has been suggested to be part of a “transcriptosome” complex (3). The NH2 terminus harbors a SAF-Box, a homeodomain-like DNA-binding motif that interacts with scaffold/matrix attachment regions (S/MAR; ref. 5). In addition, an independent repression domain is located at the COOH terminus (6).

Previous studies suggested that SAFB1 plays an important role in human breast cancer. It functions as an estrogen receptor α (ERα) corepressor by directly binding to ERα and inhibiting its transcriptional activity (7). SAFB1 maps to a chromosomal locus that displays unusually high rates of loss of heterozygosity in invasive breast cancers, and SAFB1 mutations have been identified in highly aggressive breast cancers. SAFB1 has been described previously (9).

We have recently generated SAFB1 knockout mice, which show high preneonatal and neonatal lethality, severe dwarfism associated with low insulin-like growth factor-I levels, and female subfertility and male infertility (9). Here, we show that mouse embryonic fibroblasts (MEF) from SAFB1+/− mice fail to undergo senescence and exhibit spontaneous immortalization, which was associated with a lack of p19ARF induction and high levels of TBX2, a known p19ARF repressor. SAFB1+/− cells proliferate in growth-restricting conditions and show increased anchorage-independent growth in the presence of cooperating oncogenes. These findings place SAFB1 in a unique group of genes that are critical in cellular immortalization and transformation.

Materials and Methods

Cells and tissue culture. MEF pairs were obtained from sibling SAFB1+/+ and SAFB1−/− embryos from the same mother as described previously (9), and experiments were repeated using other MEF pairs from different mothers. Short-term proliferation assays were done by plating 1 × 104 cells per well in 24-well plates in triplicates, and cells were counted daily. For foci formation assays, MEFs were plated at 1 × 105 in a 10-cm plate and cells were transiently transfected using LipofectAMINE (Invitrogen, Carlsbad, CA) with oncogenes. Medium was changed twice weekly, and foci were allowed to form for 2 to 3 weeks. To obtain stably transformed MEFs, cells were transfected and single colonies were expanded. To test anchorage-independent growth, MEFs were suspended in 0.4% SeaPlaque agar (Cambrex, East Rutherford, NJ) in the growth medium and overlayed on 0.8% agar in the same medium. Colonies were stained with X-galactosidase kit (Cell Signaling, Beverly, MA).

Protein and RNA analysis. Immunoblotting was done as described previously (2). The p19ARF antibody was purchased from Abcam (Cambridge, United Kingdom), and the antibody recognizing mouse SAFB1 has been described previously (9).

For the reverse transcription-PCR (RT-PCR) analysis, total cellular RNA was isolated with RNeasy kit (Qiagen, Valencia, CA), RNA was reverse transcribed with SuperScript II RNase H Reverse Transcriptase (Invitrogen), and PCR was done using primers used for SAFB1 (9), p19ARF (10), TBX2 (11), Bmi-1 (5′-TTGGTCTCTGTGGAGGGTA-3′ and 5′-TGTTTGTGTGGAGAGTTCGAGAG-3′), CBX7 (5′-TGTCAACCATAGGCGAGCA-3′ and 5′-AACCTGCTTCTCCGAC-3′), and DMP1 (5′-CTGTAGCTGAAGAGTGTTGA-3′ and 5′-TGTTATCTTTTCCAAACGGGCG-3′). To measure TBX2 and TBX3 mRNA by quantitative PCR using an ABI PRISM 7700 (PE Applied Biosystems, Foster City, CA), RNA was DNase treated and reverse transcribed and cDNA was amplified for 40 cycles of 95°C for 12 seconds and 60°C for 1 minute. The change in expression was calculated by the ΔΔct method. The primers (mouse TBX2, 5′-TGAGCTCTACACGCTAC-3′ and 5′-TTGTGCTCTTCTCTGCATGTG-3′; mouse TBX3, 5′-CCACCTCCAACACACGGTTCT-3′ and 5′-TGGGAAACACGGTGCCGAA-3′; and mouse β-actin, 5′-GGCTGAGTTGGTGCTGCTG-3′ and 5′-GGCTGAGTTGGTGCTGCTG-3′) were purchased from Clontech Laboratories.

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Eurogentec (Philadelphia, PA) or Integrated DNA Technologies, Inc. (Coralville, IA).

**Virus production and MEFs infection.** 293T cells were transfected with ecotropic helper virus DNA (pCL-Eco) and either pBabe-p19ARF, pBabe-rasV12, pBabe-control, or pBabe-dnTBX2 (12). Supernatants containing infectious retrovirus were harvested 24 to 72 hours after transfection, filtered, and stored on ice. MEFs were infected thrice for 3 to 4 hours each with virus-containing supernatant containing 10 μg/mL polybrene (Sigma, St. Louis, MO).

**Results**

SAFB1 deficiency leads to lack of senescence and immortalization. To analyze the effect of SAFB1 loss on tumorigenesis in vitro, we generated pairs of SAFB1+/+ and SAFB1−/− MEFs and confirmed SAFB1 status by RT-PCR and Western blot (Fig. 1A). Growth curves using early passage MEFs revealed slightly slower growth of SAFB1−/− MEFs compared with SAFB1+/+ MEFs (Fig. 1B).

Next, we determined the effect of SAFB1 loss on cellular senescence and immortalization by long-term passaging of MEFs obtained from three independent SAFB1+/+ and SAFB1−/− embryos following the 3T3 protocol. As expected, SAFB1+/+ MEFs showed a decline in their proliferative rate at about passage 12 and ultimately underwent senescence (Fig. 1C). Of three SAFB1+/+ MEFs analyzed, only one immortal population emerged after passage 28. After several attempts, we were also able to generate immortalized populations from the other two SAFB1+/+ MEFs. In contrast, all the SAFB1−/−-independent MEFs did not lose proliferative capacity following serial passaging with increased proliferative potential that emerged at passages 12 to 16. This bypass of cellular senescence was easily observed microscopically. Early passage, exponentially growing SAFB1+/+ and SAFB1−/− MEFs did not differ in cell morphology (Fig. 1D). In contrast, late passage SAFB1+/+ acquired a large, flattened cell morphology, characteristic of senescent cells, whereas SAFB1−/− MEFs remained small and more refractile. This morphology change, together with the biphasic curves for the population doublings (MEF pairs 1 and 3), suggests that there is an outgrowth of a specific subgroup of MEFs. We further confirmed the lack of senescence by staining for SA-β-gal. Whereas SAFB1+/+ MEFs showed high levels of SA-β-gal activity, SAFB1−/− MEFs showed decreased SA-β-gal activity at passages 8 and 15 (Fig. 1E). These data indicate that loss of SAFB1 results in immortalization by allowing MEFs to bypass cellular senescence.

**Immortalization of SAFB1−/− MEFs is associated with lack of p19ARF induction and increased TBX2 levels.** Next, we measured levels of p19ARF, a known inducer of senescence in MEFs...
As expected, p19ARF levels were induced with increased passaging of SAFB1+/+ MEFs. In contrast, we found unusually low p19ARF levels in SAFB1−/− MEFs; pairs 1 and 2 showed very weak p19ARF induction and no p19ARF protein was detected in pair 3 even after prolonged exposure. Reintroduction of p19ARF into SAFB1−/− cells by retroviral infection resulted in growth arrest, whereas cells infected with empty virus maintained constant growth and did not undergo senescence (Fig. 2B). These findings strongly suggest that the lack of senescence in SAFB1−/− cells was due to lack of p19ARF induction and that other downstream effectors of p19ARF were not inactivated.

To determine whether p19ARF protein changes were also reflected by changes at the mRNA level, we measured p19ARF mRNA and detected significant decreases in all three SAFB1−/− MEFs compared with SAFB1+/+ (Fig. 2C). In SAFB1−/− MEF pair 3, which did not express p19ARF protein, we detected very low mRNA, suggesting that the genomic locus was intact in all SAFB1−/− MEFs. We did not detect any effect of SAFB1 on a p19ARF promoter (−1,680bp) activity using transient reporter assays in MEFs (Fig. 2D) and in MCF-7 cells (data not shown), suggesting that it was unlikely that SAFB1 directly regulates expression of p19ARF transcription. We therefore hypothesized that SAFB1 may indirectly...
that has also been shown to regulate p19ARF and senescence (17), using additional MEF pairs (Fig. 3). Cells were plated at confluency, and growth was measured. Points, mean of experiments done in triplicate; bars, SE. Similar results were obtained in two independent experiments. B, SAFB1+/+ and SAFB1−/− MEFs (passage 7) were kept at confluency for 3 to 4 weeks. Pictures are representative of three independent experiments. C, quantitative analysis of anchorage-dependent, oncogene-induced (SV40T, myc/SV40T, and ras/SV40T) foci formation assay in primary MEFs. Similar results were obtained in at least three independent experiments. D, growth properties of MEFs stably transformed with ras/SV40T. Representative growth curves for three independent transformed MEF clones for each genotype. Points, mean of experiments done in triplicate; bars, SE. E, anchorage-independent growth of SAFB1+/+ and SAFB1−/− MEFs stably transformed with ras/SV40T. Pictures at low (left) and high (right) magnifications. F, columns, mean number of foci; columns corrected for number of fluorescent cells (to correct for transfection efficiency); bars, SE. * P < 0.05. D, growth properties of MEFs stably transformed with ras/SV40T. Representative growth curves for three independent transformed MEF clones for each genotype. Points, mean of experiments done in triplicate; bars, SE. E, anchorage-independent growth of SAFB1+/+ and SAFB1−/− MEFs stably transformed with ras/SV40T. Pictures at low (left) and high (right) magnifications. F, columns, mean number of foci; columns corrected for number of fluorescent cells (to correct for transfection efficiency); bars, SE.

Figure 4. Lack of SAFB1 leads to a partially transformed phenotype. A, postconfluent growth of MEFs. Left, SAFB1+/+ and SAFB1−/− MEFs (passage 7) were plated at confluency, and growth was measured. Points, mean of experiments done in triplicate; bars, SE. Similar results were obtained in two independent experiments. Right, SAFB1+/+ and SAFB1−/− MEFs (passage 10) were kept at confluency for 3 to 4 weeks. Pictures are representative of three independent experiments. B, anchorage-dependent, oncogene-induced (SV40T, myc/SV40T, and ras/SV40T) foci formation assay in primary MEFs. Similar results were obtained in at least three independent experiments. C, quantitative analysis of anchorage-dependent, oncogene-induced (SV40T, myc/SV40T, and ras/SV40T) foci formation assay in primary MEFs (n = 3 per genotype). Columns, mean number of foci; columns corrected for number of fluorescent cells (to correct for transfection efficiency); bars, SE. * P < 0.05. D, growth properties of MEFs stably transformed with ras/SV40T. Representative growth curves for three independent transformed MEF clones for each genotype. Points, mean of experiments done in triplicate; bars, SE. E, anchorage-independent growth of SAFB1+/+ and SAFB1−/− MEFs stably transformed with ras/SV40T. Pictures at low (left) and high (right) magnifications. F, columns, mean number of foci; columns corrected for number of fluorescent cells (to correct for transfection efficiency); bars, SE.

Discussion

Here, we provide critical data about the role of SAFB1 in cellular immortalization and transformation. MEFs lacking SAFB1 show altered expression of two proteins involved in the senescence and immortalization processes: low levels of p19ARF and high levels of TBX2, a known repressor of p19ARF. Although SAFB1−/− MEFs are not fully transformed, they are able to proliferate in growth-restricting conditions and show increased anchorage-independent growth in the presence of oncogenes.

The failure of SAFB1−/− MEFs to senesce and, as a consequence, to undergo spontaneous immortalization places SAFB1 into a unique set of genes regulating this process (20). We show that loss of SAFB1 leads to a reduction in p19ARF levels but not to a unique set of genes regulating this process (20).

was not altered (Fig. 3B), suggesting that SAFB1 specifically regulates expression of TBX2. This regulation was not at the level of RNA stability because we did not detect a difference in TBX2 RNA half-life between SAFB1+/+ and SAFB1−/− MEFs (data not shown).

Overexpression of a dominant-negative TBX2, previously described by Vance et al. (12), into SAFB1−/− MEFs resulted in up-regulation of p19ARF (Fig. 3C). Together, these findings provide support for a model, in which SAFB1 loss results in increased TBX2 expression associated with lack of induction of p19ARF and thus lack of senescence and spontaneous immortalization.

Loss of SAFB1 leads to features of transformed phenotype. Attainment of cell immortality is a prerequisite for transformation (18), and several MEF models, which undergo spontaneous immortalization, also display increased transformation capabilities. To determine whether SAFB1−/− MEFs showed increased transformation characteristics, we tested their ability to proliferate in growth-restricting conditions. When cells were grown at confluency, SAFB1+/+ MEFs showed contact inhibition and were growth arrested (days 5-7), whereas SAFB1−/− MEFs continued to proliferate and achieved a higher density cellular monolayer (Fig. 4A, left). This loss of contact inhibition was even more pronounced when confluent cells were cultured long term (4 weeks), resulting in formation of multiple foci in SAFB1−/− MEFs (Fig. 4A, right).

MEFs lacking p19ARF fail to undergo senescence provoked by constitutive signaling of oncogenic ras (19) but are instead transformed. Infection of SAFB1−/− MEFs with rasV12 did not result in full transformation (data not shown); thus, loss of SAFB1 alone does not cooperate with ras in cellular transformation. In contrast, overexpression of SV40T, myc/SV40T, or ras/SV40T resulted in increased number and size of foci (Fig. 4B), suggesting that SAFB1 loss results in increased efficiency of transformation by cooperating oncogenes. To determine whether number of foci was also increased in SAFB1−/− MEFs, we cotransfected the oncogenes with enhanced green fluorescent protein (EGFP) and corrected the numbers of foci for the number of EGFP-positive cells. Overexpression of SV40T and ras/SV40T resulted in increased number of foci as shown in Fig. 4C.

We also generated stable clones of ras/SV40-transformed SAFB1−/− and SAFB1+/+ cells. There was no difference in anchorage-dependent growth between ras/SV40T-transformed SAFB1−/− and SAFB1+/+ cells (Fig. 4D; n = 3 per genotype). However, anchorage-independent growth in soft agar was dramatically increased as a consequence of SAFB1 loss in the stable clones (Fig. 4E and F). These data provide further evidence that loss of SAFB1 results in increased transformed phenotypes.
complete abrogation of expression. This effect was not direct, but indirect, possibly caused by increased expression of TBX2. TBX2 is a T-box transcription factor, which has been shown to repress p16\textsuperscript{INK4a} promoter activity and whose overexpression leads to cell immortalization (16, 17). Indeed, inactivation of TBX2 using a dominant-negative TBX2 construct resulted in up-regulation of p10\textsuperscript{ARF} providing strong support for a model, in which loss of SAFB1 causes an increase in TBX2 levels that in turn down-regulate p10\textsuperscript{ARF} promoter activity. The question remains as how SAFB1 regulates TBX2 expression. Preliminary experiments in our laboratory failed to establish a direct link in transient assays, suggesting a more complicated mechanism, which will be the focus of our future studies. An interesting possibility is that SAFB1 regulates TBX2 expression through effects on chromatin organization because the TBX2 5' - regulatory region contains a S/MAR, a consensus element that can be recognized by SAFB1.

Immortalization requires either biallelic loss of p19\textsuperscript{ARF} or inactivation of p53. For instance, p19\textsuperscript{ARF} heterozygous MEFs do not immortalize unless they lose the second allele (19). Interestingly, SAFB1\textsuperscript{−/−} MEFs are able to immortalize despite incomplete loss of p19\textsuperscript{ARF} expression. This suggests that loss of SAFB1 causes additional changes in as-yet-unknown target genes that cooperate with low p19\textsuperscript{ARF} levels in immortalization. Such genes are unlikely to be downstream effectors of p19\textsuperscript{ARF} (including p53) because reintroduction of p19\textsuperscript{ARF} into SAFB1\textsuperscript{−/−} MEFs readily inhibits cell proliferation, as one would expect from a functional p19\textsuperscript{ARF}−p53 pathway.

Previous work from the DePinho group has established that p19\textsuperscript{ARF} and p16\textsuperscript{INK4a} loss do not cooperate with T antigen in MEF transformation (21). Therefore, our findings that loss of SAFB1 cooperates with T antigen support the notion from above that loss of SAFB1 may cause additional p19\textsuperscript{ARF}-independent alterations.

Collectively, our data suggest that loss of SAFB1 facilitates immortalization of primary MEFs and increased cell transformation. Our study is particularly important because it connects a gene involved in breast tumorigenesis to the senescence process. Ongoing and future experiments will determine whether the mechanism(s) by which SAFB1 interferes with the senescence program in MEFs is also conserved in human cells.

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References


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