Intracellular estrogen receptor-binding fragment-associated antigen 9 exerts in vivo tumor-promoting effects via its coiled-coil region

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Abstract. Estrogen receptor-binding fragment-associated antigen 9 (EBAG9) is a tumor-promoting factor of largely unknown function. To assess a causative role of EBAG9 in advanced malignancies, we generated the EG7-OVA and MethA murine tumor cell lines that stably express full-length or truncated EBAG9 protein, using retroviral-mediated gene transduction. Upon subcutaneous inoculation into immunocompetent mice, both cell lines showed marked acceleration of in vivo tumor growth when full-length EBAG9 was overexpressed. Interestingly, deletion of the coiled-coil region, thereby producing truncated EBAG9 protein, abolished the tumor-acceleration effect, establishing the importance of this domain in EBAG9-mediated tumor promotion. However, there was no alteration in in vitro cell proliferation or expression levels of MHC class I and co-stimulatory molecules believed to play a role in immune evasion of tumor cells in these tumor cell lines expressing full-length or truncated EBAG9 protein. Furthermore, both full-length and truncated EBAG9 proteins showed a predominantly cytoplasmic localization in the tumor cells. Collectively, these results suggest that EBAG9 overexpression can be causative in enhancing the malignant properties of tumor cells, and that tumor promotion likely requires EBAG9 intracellular association with as yet unidentified binding partners via the coiled-coil region.

Introduction

Identification of the molecules responsible for the development and/or progression of certain malignant tumors has marked significant progress in clinical cancer research, as such molecules serve as prognostic markers and/or therapeutic targets. Recently, estrogen receptor-binding fragment-associated antigen 9 (EBAG9) has come under scrutiny (1). EBAG9 is a ubiquitously expressed protein encoded by an estrogen-responsive gene (1). Studies have suggested that EBAG9 is linked to advanced malignancies, although its function remains undetermined (2-5). Nakashima et al (6) demonstrated that EBAG9 was a type II membrane protein that had a C-terminal coiled-coil region at the cell surface that could bind to the putative receptor in T cells and NK cells, thereby inducing their apoptotic cell death (6), and named it receptor-binding cancer antigen expressed on SiSo cells (RCAS1). However, Engelsberg et al presented a new finding that EBAG9 was a Golgi-resident protein that modulates cell surface glycosylation by a series of biochemical and cellular imaging analyses (7-11). They proposed that EBAG9-overexpression might contribute to the antigenicity of tumor cells by generation of the tumor-associated O-linked glycan Tn antigen (for example, 22.1.1 antigen).

Recently, Ogushi et al demonstrated that the overexpression of EBAG9 in a murine renal carcinoma cell line enhanced in vivo tumorigenesis, providing the first evidence of a tumor-promoting function for EBAG9 (12). They also suggested the
involved in the process of developing structures and tissues.

A complete list of coefficients, along with their corresponding values, is provided in the supplementary material (10).

2. The biocompatibility of the scaffold is critical for the successful application of the scaffold in the tissue engineering field. This is because the scaffold must be able to support cell growth and function, as well as interact with the host tissue to facilitate tissue repair and regeneration. In this study, we evaluated the biocompatibility of the scaffold using in vitro and in vivo assays.

3. The in vitro cell culture experiments showed that the scaffold supported the attachment, proliferation, and differentiation of human bone marrow stromal cells (hBMSCs). The cells were shown to form a confluent monolayer within 7 days of culture, indicating good cell adhesion and viability on the scaffold.

4. The in vivo experiments demonstrated that the scaffold was able to promote the formation of new bone when implanted in a critical-sized defect in a mouse model. The new bone formation was assessed using histological and micro-CT imaging techniques, which showed significant bone regeneration within 8 weeks of implantation.

5. These results suggest that the scaffold has the potential for use in clinical applications for the repair of bone defects, such as fractures, spinal cord injuries, and craniofacial reconstructions. Further studies are needed to optimize the scaffold composition and design for specific applications.

6. In conclusion, the study presents a novel scaffold for bone tissue engineering with promising biocompatibility and bone-forming ability. Further research is recommended to investigate the long-term effects of the scaffold in animal models and its potential for use in human patients.
criteria were established to determine when an animal's health was so poor that it needed to be euthanized: in order, paleness of the paws and muzzle, weight loss, lethargy/cachexia, and finally hypothermia, they were necropsied. All remaining mice were euthanized at day 40.

Flow cytometry analysis. A flow cytometer (FACSCalibur) with CellQuest analysis software was used for analyses. Staining involved 1x10^4 cells incubated with primary monoclonal antibodies, washed with PBS/2% FBS, and secondary antibodies added. After the second incubation, cells were washed and resuspended in PBS/2% FBS for flow cytometry analysis. The antibodies used were anti-EBAG9 mAb clone 5E4 (Oncogene Research Products, San Diego, CA, USA) (isotype control; mouse IgG1, BD Pharmingen, San Diego, CA, USA), anti-RCAS1 mAb clone 22.1.1 (18) (kindly provided by T. Watanabe and M. Nakashima, and also purchased from MBL, Nagoya, Japan) (isotype control; mouse IgM, BD Pharmingen), phycocerythrin (PE)-conjugated anti-H-2K^d mAb clone AF6-88.5 (BD Pharmingen) (isotype control; PE-conjugated mouse IgG2a, BD Pharmingen), biotin-anti-B7-H1 mAb clone 1-111A (eBioscience, San Diego, CA, USA) (isotype control; mouse IgG2a, BD Pharmingen), biotin anti-B7-H4 mAb clone 9 (eBioscience) (isotype control; mouse IgG2b, BD Pharmingen), PE-conjugated rat anti-mouse IgM (BD Pharmingen), R-PE-conjugated rat anti-mouse immunoglobulin (Dako, Kyoto, Japan) and PE-streptavidin (BD Pharmingen). To stain the EBAG9 polypeptides residing intracellularly, cells were treated with the Cytotox/Cytoperm reagent (BD Pharmingen) according to the manufacturer's instructions and stained as described above. For the analysis of H-2K_d in EG7 cells by flow cytometry, cells were assessed 48 h after retroviral-mediated gene transduction.

Immunofluorescence and confocal microscopy. To stain intracellular EBAG9 polypeptide, MethA and Jurkat cells (1x10^6) were fixed, permeabilized (Cytofix/Cytoperm, BD Pharmingen) according to the manufacturer's instructions, then incubated with anti-EBAG9 mAb clone 5E4, washed with PBS/2% FBS, and finally stained with PE-conjugated rat anti-mouse IgG1 (BD Pharmingen) antibody. To stain the cell surface 22.1.1, 1x10^6 MethA and Jurkat cells were incubated with anti-RCAS1 mAb clone 22.1.1, washed with PBS/2% FBS, and then stained with PE-conjugated rat anti-mouse IgM (BD Pharmingen) antibody. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Dojindo Laboratories, Kumamoto, Japan). After staining, cells were put on glass slides, scanned and digitized using a confocal laser scanning microscope (Leica TCS SP2, Wetzlar, Germany) with a 63x/1.40 NA oil-immersion objective (Leica). Images were assembled with Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Western blot analysis. To assess the expression levels of actin and OVA, Western blot analysis was performed following previously described procedures (17). Briefly, cell lysates (50 µg) were first separated on a 4-20% gradient polyacrylamide gel (Daichi Pure Chemicals Co., Ltd., Tokyo, Japan). After electrotransfer onto a PVDF membrane, OVA protein and actin were respectively reacted with anti-chicken egg albumin mAb (clone OVA-14; Sigma) and anti-actin mAb (clone C-2; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). All the above mAbs are of mouse IgG1-isotype. Following the reaction with horseradish peroxidase-bovine anti-mouse IgG (Santa Cruz), each band was visualized using the ECL system (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA). Western blot analyses were quantified by densitometry using Image J 1.43 (National Institute of Health, Bethesda, MD, USA).

Inhibition of T cell proliferation. EG7 or MethA cells were seeded at 1x10^6 cells/well in 6-well plates. After 48 h, supernatant was collected and used after filtration through 0.45 µm filters. Splenocytes obtained from C57BL/6 mice were stimulated with anti-CD3 mAb (10 ng/ml, BD Pharmingen) in the presence of interleukin-2 (50 U/ml) at 1x10^5 cells/well in 96-well flat-bottom plates. Graded doses of the above supernatant samples were included in each well to assess their inhibitory effects on T cell proliferation. On day 3, the extent of T cell growth was determined using Cell Counting Kit-8 (Dojindo Laboratories), according to the manufacturer's instructions.

Statistical analysis. The results are presented as the mean ± standard deviation (SD) or standard error of the mean (SEM). Statistical significance of differences was calculated using one-way ANOVA followed by Tukey's test, and a P<0.01 was considered significant in all analyses. Tumor volumes were compared by Kruskal-Wallis ANOVA, followed by post hoc comparisons with Dunn's test (intergroup comparison).

Results

Generation of EBAG9-expressing murine tumor cell lines. To test whether overexpression of EBAG9 enhanced the malignant properties of tumor cells, we constructed the retroviral vectors GCDN/EBAG9-FL and GCDN/EBAG9-TR to express full-length- or truncated-EBAG9 protein, together with the EGFP in transduced cells (Fig. 1A). We have prepared EBAG9-FL and EBAG9-TR, as well as a control vector, GCDN/EGFP, harboring only EGFP cDNA (Fig. 1A).

We next transduced the well-characterized murine tumor cell lines, EG7 (13) and MethA (14), with the vectors expressing either EBAG9-FL or EBAG9-TR. After sorting the EGFP cell lines, EG7 (13) and MethA (14), with the vectors expressing either EBAG9-FL or EBAG9-TR. After sorting the EGFP positive population to enrich EBAG9 transgene-positive cells, we performed RT-PCR analysis using the primer pair specific for EBAG9 cDNA (P1 and P2, Fig. 1A). As shown in Fig. 1B, we found that minimum levels of endogenous EBAG9 mRNA was expressed in both untransduced EG7 and MethA cells (Fig. 1B, UT). In contrast, enhanced expression of EBAG9 mRNA was evident in both EG7 and MethA cells after transduction with the GCDN/EBAG9-FL or -TR vectors (Fig. 1B). Eventually, EG7 and MethA cell lines were successfully engineered to stably express the full-length or truncated EBAG9 transgene.

In vitro and in vivo growth of the EBAG9-expressing murine tumor cells. To explore whether overexpressed EBAG9 would promote cell proliferation, we performed an MTT-based colorimetric assay. Neither EG7 nor MethA cells exhibited significant acceleration of cell proliferation associated with overexpression of the EBAG9-FL or EBAG9-TR transgene (Fig. 2A), indicating that the forced expression of EBAG9 did not affect in vitro cell proliferation.
We then investigated the in vivo tumor formation and growth of EG7 and MethA cells overexpressing EBAG9 in immunocompetent strains of C57BL/6 and BALB/c mice, respectively. When inoculated with these EG7 cell lines, tumors did not grow in the UT and EBAG9-TR groups (Fig. 2B, upper panel). In the EBAG9-FL group, however, striking acceleration of tumor growth was observed in all inoculated mice (Fig. 2B, upper panel, P<0.01 versus other groups). Similarly, MethA cells overexpressing full-length EBAG9 (EBAG9-FL) exhibited a clear acceleration of in vivo tumor growth compared with the other two groups in BALB/c animals (Fig. 2B, lower panel, P<0.01 versus other groups). Collectively, these results demonstrated that these murine tumor cells were able to enhance their malignant properties in vivo in immunocompetent animals when engineered to overexpress full-length EBAG9 protein.

Cellular localization of full-length and truncated EBAG9. To investigate the cellular localization of overexpressed EBAG9 protein, we first examined the expression pattern of EBAG9 gene products with or without cell-permeabilization by flow cytometry. Analysis of non-permeabilized EG7 cells revealed cell surface expression of EBAG9 molecules, which was observed at comparable levels to the transduced EG7 cells (Fig. 3A, left panel). Remarkably, analysis of permeabilized
Figure 3. Cellular localization of full-length and truncated EBAG9. (A) Cell surface expression of EBAG9 in EG7 and MethA cells. Unfixed cells were stained with anti-EBAG9 mAb clone 5E4 and PE-conjugated rat anti-mouse IgG1 antibody before expression of EGFP and cell surface EBAG9 was analyzed by flow cytometry. (B) Intracellular expression of EBAG9 in EG7 and MethA cells. Cells were fixed, permeabilized, stained (see Materials and methods) and analyzed. Note the dim EGFP fluorescence intensities caused by the fixation/permeabilization, since GFP accumulates in the cytoplasm and then readily leaks out of permeabilized cells. (C) Confocal microscopic analysis of EBAG9 expression. MethA and Jurkat cells were fixed, permeabilized, and stained with anti-EBAG9 mAb clone 5E4 and PE-conjugated rat anti-mouse IgG1 antibody. Shown are EBAG9 in red (PE) and nuclei in blue (DAPI).
EG7 cells revealed that the overexpression of both full-length and truncated EBAG9 in EG7 cells enhanced EBAG9 expression in the cytoplasm (Fig. 3B, left panel). In contrast, in MethA cells we detected no EBAG9 staining on the cell surface, despite the presence of the transgene (Fig. 3A, right panel), but enhanced intracellular expression in transduced cells (Fig. 3B, right panel).

To explore the cellular localization of EBAG9-FL, and EBAG9-TR, we performed confocal immunofluorescence microscopy. No EBAG9 labeling was detected at any location in untransduced (MethA-UT and Jurkat) and EGFP-transduced control MethA cells (MethA-EGFP). In the EBAG9-transduced cells, both full-length and truncated EBAG9 proteins were shown to be localized predominantly in an intracellular location (Fig. 3C), which is consistent with the results that were obtained by flow cytometry analysis (Fig. 3A and B).

Taken together, these results suggest that overexpressed full-length and truncated EBAG9 proteins were mainly localized to intracellular compartments in the transduced EG7 and MethA cells, irrespective of their basal expression levels of endogenous EBAG9.

Effect of overexpressed full-length and truncated EBAG9 on cell surface 22.1.1 antigen expression. We next investigated whether EBAG9 overexpression could alter cell surface glycosylation as previously reported (9), using another mAb clone, 22.1.1, which detects the tumor-associated Tn antigen (22.1.1 antigen) induced on the cell surface (9). As shown in Fig. 4A (left panel), no visible 22.1.1-reactivity was observed in EG7 cells, even when transduced with EBAG9-FL and EBAG9-TR. In MethA cells, low-level basal staining was seen in untransduced cells (Fig. 4A, right panel, UT), but enhancement of 22.1.1 reactivity was not apparent in any of the transduced cells (Fig. 4A, EBAG9-FL and -TR).

Confocal immunofluorescence microscopy also revealed that the 22.1.1 antigen was detected only at the cell surface.
of untransduced Jurkat cells, but not in any of the MethA cell groups, even when transduced with EBAG9-FL and EBAG9-TR (Fig. 4B). These results demonstrated that the retroviral-mediated EBAG9 overexpression achieved in this study was not sufficiently high to modify cell surface glycosylation in transduced EG7 and MethA tumor cells, indicating that alteration of cell surface glycosylation may not be the cause for the observed EBAG9-mediated acceleration of in vivo tumor growth.

Expressions of OVA, MHC class I, and B7-H1/H4 in tumor cells overexpressing full-length and truncated EBAG9. EG7 cells express OVA protein as a model tumor-associated antigen (TAA) recognizable by immunocompetent hosts. The enhanced in vivo tumor growth observed in the EBAG9-FL group suggested the inhibition of immune responses against OVA. At first, we examined the expression levels of OVA in a series of EG7 cells. Western blot analysis showed comparable amounts of OVA protein in all the EG7 cell samples regardless of transgene expression (Fig. 5A).

We next investigated the expression of H-2Kb major histocompatibility complex (MHC) class I that may be involved in immune escape. Similar levels of H-2Kb expression were observed in EGFP-positive (EBAG9-expressing) and EGFP-negative (untransduced) populations in EBAG9-overexpressing EG7 cells (Fig. 5B, EBAG9-FL and -TR). Similarly, the reduction in MHC class I expression was not observed in EBAG9-overexpressing MethA cells (data not shown). These results suggest that the observed EBAG9-mediated acceleration of in vivo tumor growth is not attributable to the down-modulation of MHC class I expression and the subsequent reduction in TAA presentation.

We also examined whether the expression of cell surface molecules, which can lead to tumor cell immune evasion, might contribute to the EBAG9-mediated in vivo tumor promotion. Therefore, we examined the cell surface expression of B7-H1 and B7-H4, both of which have been recently implicated in cancer immune evasion (19-22). As shown in Fig. 5C, we found that both cell lines similarly expressed B7-H1 on the cell surface (left panel, UT), whereas they did not exhibit any detectable staining of B7-H4 (right panel, UT). In both cases, EBAG9 expression did not induce any alteration in the levels of these molecules (Fig. 5C, EBAG9-FL and -TR), thereby excluding the possibility of B7-H1- or B7-H4-mediated immune evasion as a mechanism of EBAG9’s in vivo tumor promotion.

Effect of supernatant from murine tumor cells overexpressing full-length and truncated EBAG9 on T cell proliferation. We finally assessed whether EBAG9-overexpression would induce the secretion of any T cell proliferation inhibitory factors from the transduced tumor cells. We prepared filtered supernatant samples from viable cultured tumor cells, and then added them to cultures of anti-CD3 mAb-stimulated splenic T cells at varying doses (Fig. 6). Interestingly, the supernatant obtained from untransduced EG7 cells showed dose-dependent inhibition of T cell proliferation (Fig. 6, upper panel, UT). Overexpression of the full-length- or truncated-version of EBAG9 did not significantly enhance this inhibitory activity (Fig. 6, upper panel, EBAG9-FL and -TR). The supernatant samples obtained from a series of MethA cells did not exhibit significant inhibitory effects on T cell activation, regardless of the presence or absence of overexpressed EBAG9 (Fig. 6, lower panel). These results indicate that EBAG9-overexpression did not lead to the secretion of factors that could lead to detectable levels of inhibition of T cell proliferation in either EG7 or MethA cells.

Discussion

We have demonstrated that overexpression of EBAG9 enhances the in vivo malignant properties of murine tumor cells. Although several researchers have suggested a potential link between advanced malignancy and high-level expression of EBAG9 in tumor tissue (3,23-28), evidence that this molecule itself exerts
Here we report the importance of the C-terminal coiled-coil region in EBAG9’s tumor-promoting function. The mutant we used, which lacks the 35 most-C-terminal amino acids, is predicted to be indistinguishable in biological characteristics from the one (RCAS1Δ179-213-GFP) that Engelsberg et al used in their analyses of EBAG9 membrane topology and subcellular localization (9). Considering their results, deletion of the 35 C-terminal amino acids is believed not to affect EBAG9’s Golgi-predominant localization, but to abolish the possibility that altered expression of certain immunomodulatory molecules plays a role in EBAG9-mediated tumor-promoting function. The recent identification of the EBAG9 binding partner, Snapin, suggests a possible role for EBAG9 in regulated secretory pathways (7). However, the supernatant obtained from the culture of tumor cells overexpressing full-length EBAG9 did not show any increased inhibitory effects on T cell proliferation (Fig. 6). These observations indicate that EBAG9-overexpression in our experimental system does not induce the secretion of T cell-inhibitory factors from EG7 and MethA cells to levels where the supernatant would show appreciable inhibition of T cell proliferation. However, the possibility that altered expression of certain immunomodulatory molecules plays a role in EBAG9-mediated enhanced tumor-genesis cannot be completely excluded.

Finally, altered secretion of soluble factors with immunoregulatory functions may be responsible for the enhanced malignant behavior of tumor cells due to EBAG9-overexpression, as the recent identification of the EBAG9 binding partner, Snapin, suggests a possible role for EBAG9 in regulated secretion pathways (7). However, the supernatant obtained from the culture of tumor cells overexpressing full-length EBAG9 did not show any increased inhibitory effects on T cell proliferation (Fig. 6). These observations indicate that EBAG9-overexpression in our experimental system does not induce the secretion of T cell-inhibitory factors from EG7 and MethA cells to levels where the supernatant would show appreciable inhibition of T cell proliferation. However, the possibility that altered expression of certain immunomodulatory molecules plays a role in EBAG9-mediated enhanced tumor-genesis cannot be completely excluded.

Here we report the importance of the C-terminal coiled-coil region in EBAG9’s tumor-promoting function. The mutant we used, which lacks the 35 most-C-terminal amino acids, is predicted to be indistinguishable in biological characteristics from the one (RCAS1Δ179-213-GFP) that Engelsberg et al used in their analyses of EBAG9 membrane topology and subcellular localization (9). Considering their results, deletion of the 35 C-terminal amino acids is believed not to affect EBAG9’s Golgi-predominant localization, but to abolish the ability to associate with cytoplasmic molecules through the coiled-coil region. As our data support the significance of the coiled-coil region in EBAG9-mediated tumor promotion,
identification of the binding partner will increase our understanding of the underlying mechanism(s). Since the recently identified EBAG9 partner, Snapin, reportedly binds to EBAG9 via the N-terminal region, not the C-terminal coiled-coil domain (7), other molecules that associate with EBAG9’s C-terminal region, and thus play a role in in vivo tumor-enhancement, may exist.

*In vivo* tumor promotion is most likely not merely the function of EBAG9, considering a ubiquitous expression pattern (1) and a suggested regulatory role in exocytosis (7) of this molecule. Since our retroviral system allows efficient gene transduction into various primary cells (16,29,30), its broader application in further studies will provide insights into EBAG9’s function in many areas, not only tumorigenesis, but also other non-cancerous situations.

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