using a QuickGene DNA whole blood kit L (Kurabo Industries, Osaka, Japan). All exons of the *CARD14* gene were amplified by PCR, and the products were sequenced on an ABI 3130xl Genetic Analyser (Applied Biosystems ABI, Carlsbad, CA). (For specific details about materials and methods see Supplementary Data online).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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An Actin-Binding Protein Espin Is a Growth Regulator for Melanoma

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TO THE EDITOR

Effective therapies for melanoma are limited despite the fact that the incidence is increasing at a greater rate than that of any other cancers (Chen *et al.*, 1996). Identification of key molecules regulating growth, progression, and metastasis in melanoma is essential to provide novel therapeutic strategies. We previously developed *RET*-transgenic mice of line 304/B6 carrying oncogenic *RET* (*RFP/RET*) under regulation of the metallothionein-I promoter (RET-mice), in which skin melanoma develops spontaneously (Kato *et al.*, 1998). As melanoma in RET-mice histopathologically resembles human melanoma, RET-mice have been used worldwide as a standard model for melanoma (Kato *et al.*, 1998; Kumasaka *et al.*, 2010).

The *Espin* gene encodes an actin filament–binding protein (Bartles *et al.*, 1996; Sekerková *et al.*, 2006). Espin affects the actin cytoskeleton, resulting in a special association with microvillar specializations of sensory cells (Sekerková *et al.*, 2004). Our recent study showed that Espin expressed in melanoma cells in mice and humans affects metastasis through the regulation of invasion via lamellipodia formation (Yanagishita *et al.*, 2014). That was the first report showing a correlation between Espin and cancer cells. However, there has been no study showing whether Espin regulates the proliferation of cancer cells. In this study, we examined the effect of Espin on anchorage-dependent and -independent growth of melanoma cells.

Anti-Espin rabbit polyclonal antibody (Yanagishita *et al.*, 2014), murine

Abbreviations: GFP, green fluorescent protein; RET-mice, RET-transgenic mice

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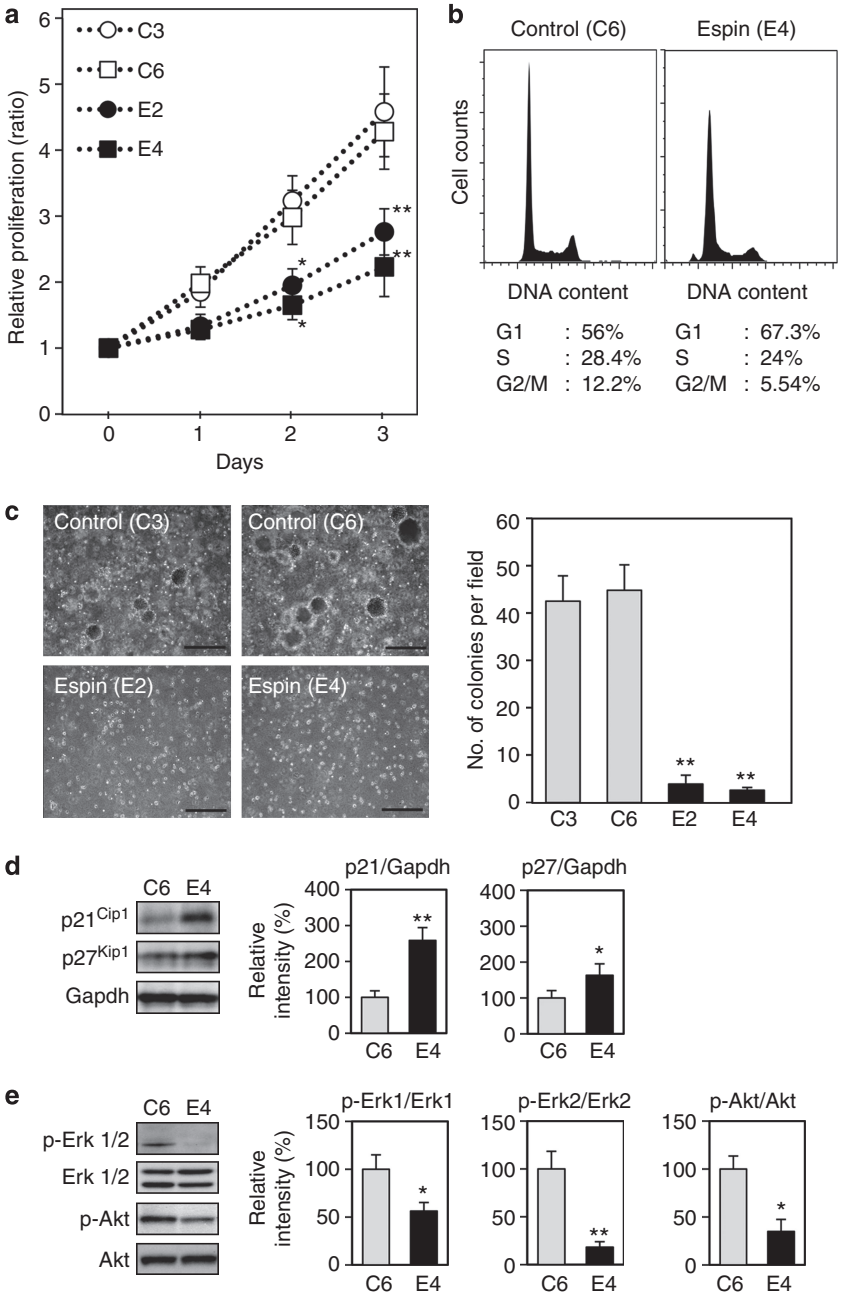

Figure 1. Espin-mediated regulation of growth *in vitro*. (**a**) Levels (mean ± SD; $n = 3$) of anchorage-dependent growth at the indicated days in control (clones C3 and C6) and Espin-depleted Mel-ret cells (clones E2 and E4) are presented. (**b**) Ratios of G_1, S, and G_2/M phases analyzed by laser flow cytometry using propidium iodide in control and Espin-depleted cells are presented. The data shown are representative of three experiments. (**c**) Representative images of colony assays in control and Espin-depleted cells are presented. Numbers of GFP-positive colonies per field (mean ± SD; $n = 3$) in both control and Espin-depleted cells are presented in the graphs. Bar = 1 mm. (**d**, **e**) Protein expression levels (mean ± SD; $n = 3$) of $p21^{Cip1}$, $p27^{Kip1}$, Gapdh, p-Erk1/Erk1, p-Erk2/Erk2, and p-Akt/Akt in control and Espin-depleted cells determined by immunoblot analysis are presented. Levels of p-Erk1/Erk1, p-Erk2/Erk2, and p-Akt/Akt in Espin-depleted cells are presented as ratios to those in control cells in the graphs.

Mel-ret melanoma cells derived from a tumor in an RET-mouse (Kato *et al.*, 1999), and stable clones of control Mel-ret cells with a green fluorescent protein (GFP) tag (Clones C3 and C6) and Espin-depleted Mel-ret cells with a GFP tag (Clones E2 and E4) (Yanagishita *et al.*, 2014) by shRNA expression that were developed in our previous study were used in this study. Analyses of anchorage-dependent and -independent growth, laser flow cytometry, immunoblot analysis, immunohistochemistry, TUNEL, and xenografting were performed according to the

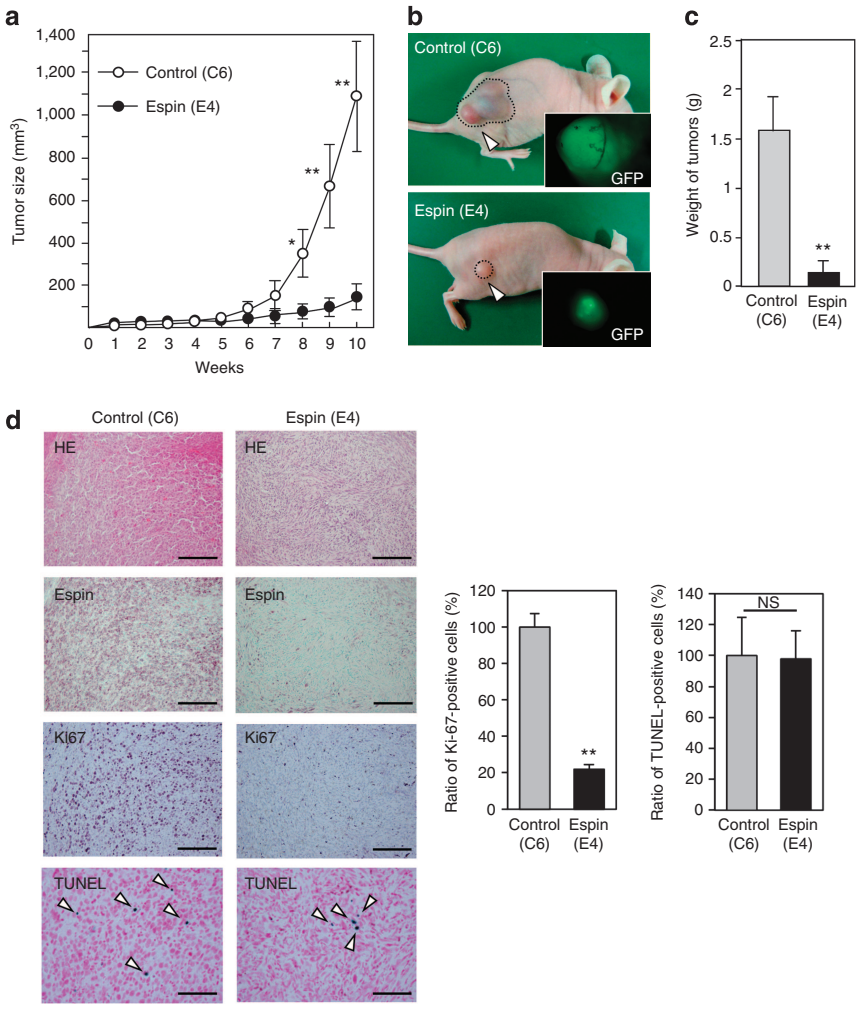

Figure 2. Espin-mediated regulation of growth *in vivo*. (**a**) Tumor sizes (mean ± SD; $n = 7$) at the indicated weeks after inoculation of control (clone C6) and Espin-depleted (clone E4) cells with a green fluorescent protein tag into nude mice. (**b**) Representative macroscopic appearances of tumors from control and Espin-depleted cells at 10 weeks after inoculation. (**c**) Weights (mean ± SD; $n = 7$) of tumors from control and Espin-depleted cells at 10 weeks after inoculation. (**d**) Results of HE staining, immunohistochemical analysis of Espin and Ki67 (purple), and TUNEL staining in xenografted tumors derived from Espin-depleted cells (E4) and control cells (C6). Levels of Ki-67-positive cells and TUNEL-positive cells in Espin-depleted cells (mean ± SD; $n = 5$) are presented as ratios to those in control cells, respectively, in the graphs. Arrowheads show TUNEL-positive cells (blue). Bar = 200 μm.

methods described previously (Hasegawa *et al.*, 2008; Ohgami *et al.*, 2010; Yajima *et al.*, 2012). This study was performed in Chubu University and Nagoya University and was approved by the Animal Care and Use Committee (approval no. 2410062 in Chubu University and 25444 in Nagoya University) and Recombination DNA Advisory Committee (approval no. 12-03 in Chubu University and 13-35 in Nagoya University). Statistical analysis was performed according to the method described previously described (Kato *et al.*, 2011). Significant differences (**$P<0.01$, *$P<0.05$) from the control based on Student's *t*-test are presented.

The results for anchorage-dependent growth on days 1–3 (Figure 1a) are shown as ratios to that on the day of starting the culture (day 0). The growth of Espin-depleted Mel-ret melanoma cells for 3 days was 40–51% reduced compared with that of control Mel-ret melanoma cells (Figure 1a). A comparative study of the cell cycle profiles of control and Espin-depleted cells by laser flow cytometry showed a >10% increase of G_1 phase in Espin-depleted cells compared with that in control cells (Figure 1b). Moreover, anchorage-independent growth of Espin-depleted cells was 90–95% reduced compared with that of control cells (Figure 1c). To clarify the molecular mechanism of Espin-mediated regulation of growth, regulators for G_1 phase were examined (Sherr and Roberts, 1999). Levels of $p21^{Cip1}$/Gapdh and $p27^{Kip1}$/Gapdh protein expression in Espin-depleted cells are presented as ratios to those in control cells (Figure 1d). Protein expression levels of cyclin-dependent kinase inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ in Espin-depleted cells were higher than those in control cells (Figure 1d), whereas expression of $p16^{Ink4a}$ protein

was undetectably low in both Espin-depleted and control cells (data not shown). As the cell proliferation rate of Mel-ret cells with both Espin and $p21^{Cip1}$ depletion was significantly higher than that of cells with only depletion of Espin (Supplementary Figure S1 online), Espin might work upstream of $p21^{Cip1}$ in the same pathway and regulate cell proliferation. Activities of Erk and Akt, both of which are cell proliferation–associated proteins (Ohshima *et al.*, 2010), in Espin-depleted cells were lower than those in control cells (Figure 1e). Previous studies showed that $p21^{Cip1}$ is associated with cell proliferation via the Erk/Akt pathway (Cmielová and Rezáčová, 2011). Taken together, $p21^{Cip1}$ might be correlated with Espin-mediated regulation of cell proliferation and Erk/Akt activity.

Our *in vivo* experiment on anchorage-independent growth by subcutaneous inoculation of Mel-ret melanoma cells into nude mice showed that tumor size (Figure 2a and b) and weight (Figure 2c) in Espin-depleted cells were 92% and 94% smaller than those in control cells, respectively. Immunohistochemical analysis of a proliferation marker (Ki67) in xenografted tumors showed that the ratio of proliferating cells in Espin-depleted cells was decreased compared with that in control cells (Figure 2d), whereas there was no difference in the ratio of TUNEL-positive apoptotic cells between Espin-depleted cells and control cells (Figure 2d). These results suggest that Espin-mediated growth inhibition *in vivo* is dependent on decreased cell proliferation rather than increased apoptotic cell death.

We demonstrated for the first time that Espin regulates both anchorage-dependent growth and anchorage-independent growth in melanoma cells through G_1 arrest and modulates the expression and/or activity of $p21^{Cip1}$, $p27^{Kip1}$, Erk, and Akt. There has been no study other than our previous study (Yanagishita *et al.*, 2014) and the present study with melanoma cells showing the roles of Espin in cancer cells. Our previous study using human melanoma cells further showed higher expression levels of Espin protein in five melanoma cell lines with BRAF mutation and one melanoma cell line with NRAS mutation compared with the expression level in primarily cultured melanocytes (Yanagishita *et al.*, 2014). Our previous study (Yanagishita *et al.*, 2014) and the present study showed that Espin controls both cell proliferation and invasion. As it was previously reported that regulation of both cell proliferation and invasion is important for the control of metastasis of melanoma (Chin, 2003), we speculate that Espin-mediated reduction in proliferation can secondarily suppress metastasis of melanoma. Thus, our recent findings for Espin may provide a potential benefit for Espin-targeting therapy for melanoma. Further studies are needed to evaluate the usefulness of Espin as a biomarker and/or a molecular target for therapy in melanoma.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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