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ORIGINAL ARTICLE

Autocrine/paracrine mechanism of interleukin-17B receptor promotes breast tumorigenesis through NF-κB-mediated antiapoptotic pathway

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Gain of function of membrane receptor was a good strategy exploited by cancer cells to benefit own growth and survival. Overexpression of HER2 has been found to serve as a target for developing trastuzumab to treat 20–25% of breast cancer. However, little or none of the other membrane receptor was found to be useful as a potential target for breast cancer treatment since then. Here, we showed that amplified signaling of interleukin-17 receptor B (IL-17RB) and its ligand IL-17B promoted tumorigenicity in breast cancer cells and impeded acinus formation in immortalized normal mammary epithelial cells. External signal transmitted through IL-17RB activated nuclear factor-κB to upregulate antiapoptotic factor Bcl-2 and induced etoposide resistance. Elevated expression of IL-17RB had a stronger correlation with poor prognosis than HER2 in breast cancer patients. Interestingly, breast cancer patients with high expression of IL-17RB and HER2 had the shortest survival rate. Depletion of IL-17RB in trastuzumab-resistant breast cancer cells significantly reduced their tumorigenic activity, suggesting that IL-17RB and HER2 have an independent role in breast carcinogenesis. Furthermore, treatment with antibodies specifically against IL-17RB or IL-17B effectively attenuated tumorigenicity of breast cancer cells. These results suggest that the amplified IL-17RB/IL-17B signaling pathways may serve as a therapeutic target for developing treatment to manage IL-17RB-associated breast cancer.

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Keywords: antiapoptosis; autocrine; IL-17RB; NF-κB; tumorigenesis

INTRODUCTION

Overexpression of membrane receptor is a common feature observed in a variety of cancers, such as epidermal growth factor receptor-1 in non-small-cell lung cancer[1] and epidermal growth factor receptor-2 (HER2/neu) in breast cancer.[2] Through interacting with specific ligands, constitutive activation of membrane receptors increases proliferation, survival and invasion ability of cancer cells. Importantly, the aberrant activation of signaling pathways also offers potential opportunities for pharmacological intervention. In breast cancer, trastuzumab (a.k.a. Herceptin)-targeted therapy has been used to treat HER2/neu+ tumor and it significantly improves clinical outcome.[3] However, critical issues including developing drug resistance, limited response rate and cancer recurrence remain to be resolved. Thus, identification of novel cell surface receptors involved in breast tumorigenesis is urgently needed to offer new potential therapeutic targets.

Accumulating evidence has suggested a strong association between chronic inflammation and cancer development among different types of cancer.[4] Cancer cells take advantage of cytokine or cytokine receptor overexpression to benefit their own growth or invasive ability via autocrine or paracrine loop. In breast cancer, several proinflammatory cytokines, such as interleukin (IL)-1,[5] IL-6[6] and tumor growth factor-β,[7] have been reported to promote proliferation or invasion. IL-17A (a.k.a. IL-17), which activates downstream signaling of signal transducer and activator of transcription 3 to upregulate several prosurvival genes, also promotes invasion of breast cancer.[8,9] Overexpression of IL-17RB in murine leukemia cells implicates an oncogenic role of this receptor.[10] However, the precise contribution of IL-17RB signaling in tumorigenesis remains to be substantiated.

The interactions among IL-17 ligands and receptors are intertwined. Previously, we found that IL-25 (a.k.a. IL-17E) secreted from non-malignant mammary epithelial cells induces breast cancer apoptosis.[11] The apoptotic activity of IL-25 is mediated by differential expression of its receptor, IL-25R, which is composed of IL-17RB and IL-17RA heterodimer.[12] On the other hand, IL-17RA and IL-17RB are also the receptors for IL-17A and IL-17B, respectively.[13,14] Thus, the ligand–receptor interactions may exert differential roles in a temporal and spatial manner. It is worth noting that high expression of IL-17RB was found to correlate with poor prognosis in breast cancer patients.[11] However, the precise contribution of IL-17RB/IL-17B signaling in breast carcinogenesis remains unclear.

In this study, we affirmed that the amplified IL-17RB/IL-17B signaling was critical for breast tumorigenesis by correlating its expression with poor prognosis based on two well-characterized independent cohorts of breast cancer patients. Gain or loss of function study of IL-17RB/IL-17B signaling in non-malignant mammary epithelial cells and cancer cells further supported this notion. Amplified IL-17RB/IL-17B signaling was found to activate Bcl-2 expression to exert antiapoptotic effect through nuclear factor-κB (NF-κB) pathway. Importantly, treatment with

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IL-17RB/IL-17B-specific antibodies significantly reduced tumorigenicity of breast cancer cells. These data indicate that the amplified IL-17RB/IL-17B signaling contributes to breast tumorigenesis and offers a potential therapeutic target for breast cancer.

RESULTS

High expression of IL-17RB promotes breast tumorigenesis

We first examined the expression of IL-17RB in a panel of non-malignant mammary epithelial cells (H184B5F5/M10 and MCF10A)

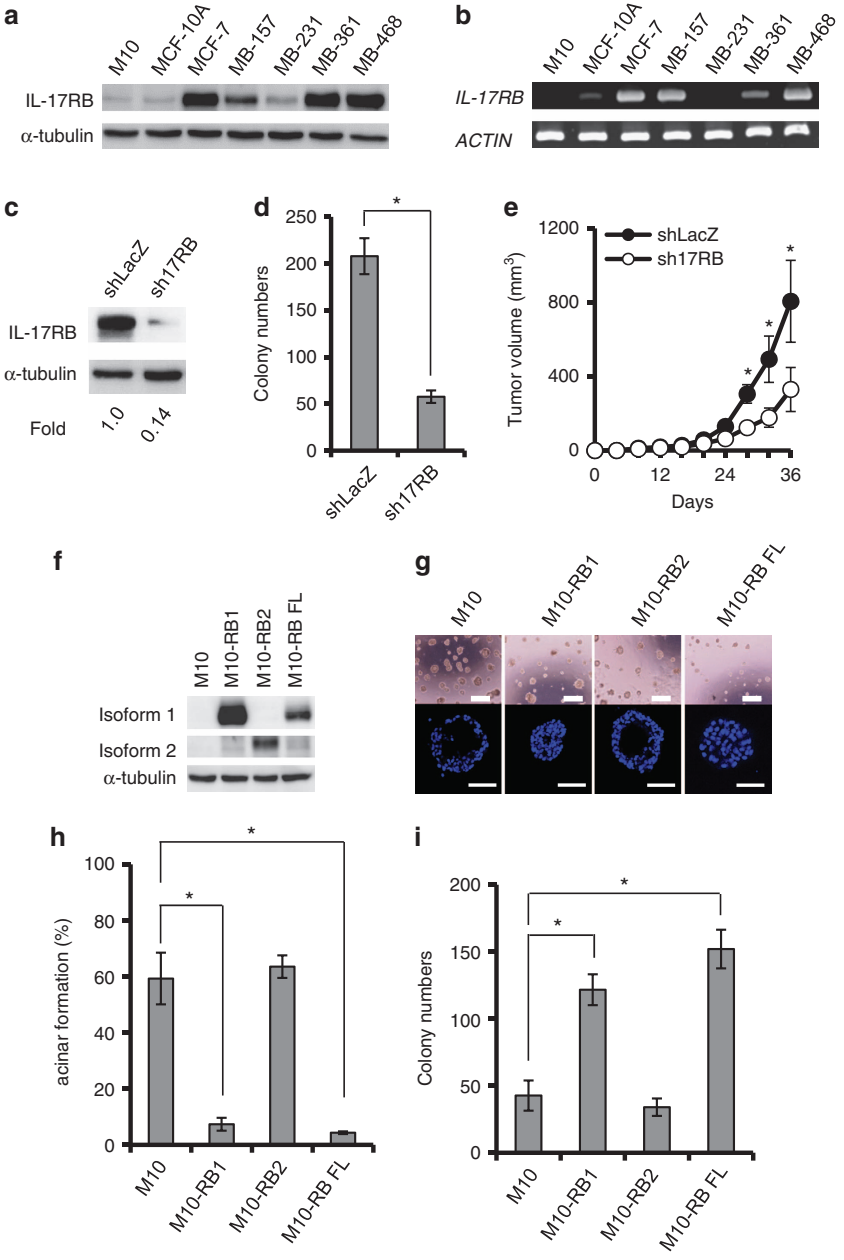


Figure 1. IL-17RB conferred anchorage-independent growth and impaired proper acinar structure formation. (**a** and **b**) Western blot (**a**) and reverse transcription–polymerase chain reaction analyses (**b**) showed that most breast cancer cell lines expressed higher levels of IL-17RB compared with non-malignant mammary epithelial cells (M10 and MCF10A). (**c**) Western blot analysis showed the IL-17RB protein amount in MDA-MB-361 cells transduced with IL-17RB shRNA. LacZ shRNA was used as a knockdown control. (**d**) Soft-agar colony formation assay showed that depletion of IL-17RB impaired the ability of anchorage-independent growth in MDA-MB-361 cells. All data points were at least triplicate and all experiments were performed at least three times with similar results. One representative result is shown. Data show means ± s.d. $*P<0.05$ (Student's *t*-test). (**e**) Xenograft tumorigenesis assay using NOD/SCID/γ^{null} mice injected with MDA-MB-361 shLacZ control or shIL-17RB cells. Six mice were used in each group. Data show means ± s.d. $*P<0.05$ (Student's *t*-test). (**f**) Western blot analysis showed the overexpression of membrane-bound IL-17RB isoform 1 (M10-RB1), secreted isoform 2 (M10-RB2) and full-length IL17RB (M10-RB FL) in M10 cells compared with the control cells (M10). (**g**) Abnormal acinar morphologies were observed in IL-17RB1- and IL-17RB-FL-overexpressing M10 cells, but not in control and IL-17RB2-overexpressing cells. The upper panels were the representative phase-contrast images of IL-17RB variants expressing M10 cells in 3D culture on day 16 (bar, 200 μm). The lower panels showed the 4′,6-diamidino-2-phenylindole -staining images after 16 days in 3D culture (bar, 100 μm). (**h**) The quantification results of acinar formation in **g**. Results were means ± s.d. of three independent experiments. (**i**) Overexpression of IL-17RB1 and IL-17RB-FL increased colony formation in M10 cells. Asterisk (*) indicates $P<0.05$, and Student's *t*-test was used to evaluate the statistical significance.

and breast cancer cell lines (MCF7, MDA-MB-157, MDA-MB-231, MDA-MB-361 and MDA-MB-468) by western blot and reverse transcription–polymerase chain reaction (PCR) analyses. Elevated expression of IL-17RB protein and mRNA were predominantly observed in many breast cancer cell lines (Figures 1a and b). Depletion of IL-17RB by its corresponding short hairpin (sh)RNA in two cell lines, MDA-MB-361 and MCF7, highly expressing IL-17RB (Figure 1c and Supplementary Figure S1A), resulted in a significant decrease in soft-agar colony formation (Figure 1d and Supplementary Figure S1B). IL-17RB depletion also significantly retarded tumor growth in a xenograft model using non-obese diabetic (NOD)/severe-combined immune deficiency (SCID)/γ^{null} mice (Figure 1e). Palpable tumors derived from the control (shLacZ) and IL-17RB-depleted cells (sh17RB) were both observed in the first week. However, from Days 20 to 36, tumors from control cells grew faster and larger than those from IL-17RB-depleted cells (Figure 1e). The wet weights of the tumors derived from IL-17RB-depleted cells were only 40% of those from the control cells (Supplementary Figure S1C), indicating that high expression of IL-17RB promotes tumor growth.

Membrane-bound IL-17RB is critical for promoting breast tumorigenesis

The human IL-17RB gene encodes two alternative spliced isoforms. Isoform 1 contains a transmembrane domain (hereafter referred as IL-17RB1), and isoform 2 (IL-17RB2) is a secreted form without the transmembrane domain.[10] The IL-17RB full-length (IL-17RB-FL) cDNA mainly transcribed IL-17RB1 and a very small amount of IL-17RB2 because it harbors an intron inside.[10] To pinpoint which isoform is critical for breast tumorigenesis, the non-malignant mammary epithelial cell line, M10, was transduced with retrovirus-carrying IL-17RB1, IL-17RB2 or IL-17RB-FL, respectively (Figure 1f). These cells were seeded in the three-dimensional (3D) Matrigel culture for testing their acinar-forming activity. The control and IL-17RB2-overexpressing M10 cells formed acinar structures with normal hollow lumens (Figures 1g and h), but M10 cells expressing IL-17RB1 or IL-17RB-FL failed to develop a proper lumen-like structure (Figures 1g and h). In addition, only cells expressing the membrane-bound IL-17RB promoted colony formation (Figure 1i). These results indicated that overexpression of the membrane-bound IL-17RB1 contributes to the transformation of normal cells to cancerous phenotypes.

IL-17RB/IL-17B signaling activates NF-κB pathway and exerts antiapoptosis via upregulation of Bcl-2

To elucidate how IL-17RB promotes tumorigenesis in breast cancer, we performed differential expression profiling using IL-17RB-overexpressing M10 cells and IL-17RB-depleted MDA-MB-361 cells. We found 72 upregulated and 70 downregulated genes with differential expression ratio >1.5-fold (Supplementary Tables S1 and S2). Using the bioinformatics database, DAVID (http://david.abcc.ncifcrf.gov/) and KEGG, we found that apoptosis and focal adhesion pathways were most likely regulated by IL-17RB (Supplementary Figure S2). The proapoptotic genes *TNFSF10*[15] and *TRADD*[16] were upregulated in IL-17RB-depleted cells. Conversely, antiapoptotic gene *BCL-2*[17] was upregulated in IL-17RB-overexpressing cells (Figure 2a). These results were further confirmed by real-time quantitative PCR analysis (Figures 2b and c). As IL-17RB signaling activates NF-κB in human renal cell lines[18] and NF-κB upregulates Bcl-2[17] in breast cancer cells, it is likely that overexpression of IL-17RB may block apoptosis via NF-κB-mediated Bcl-2 upregulation in breast cancer cells.

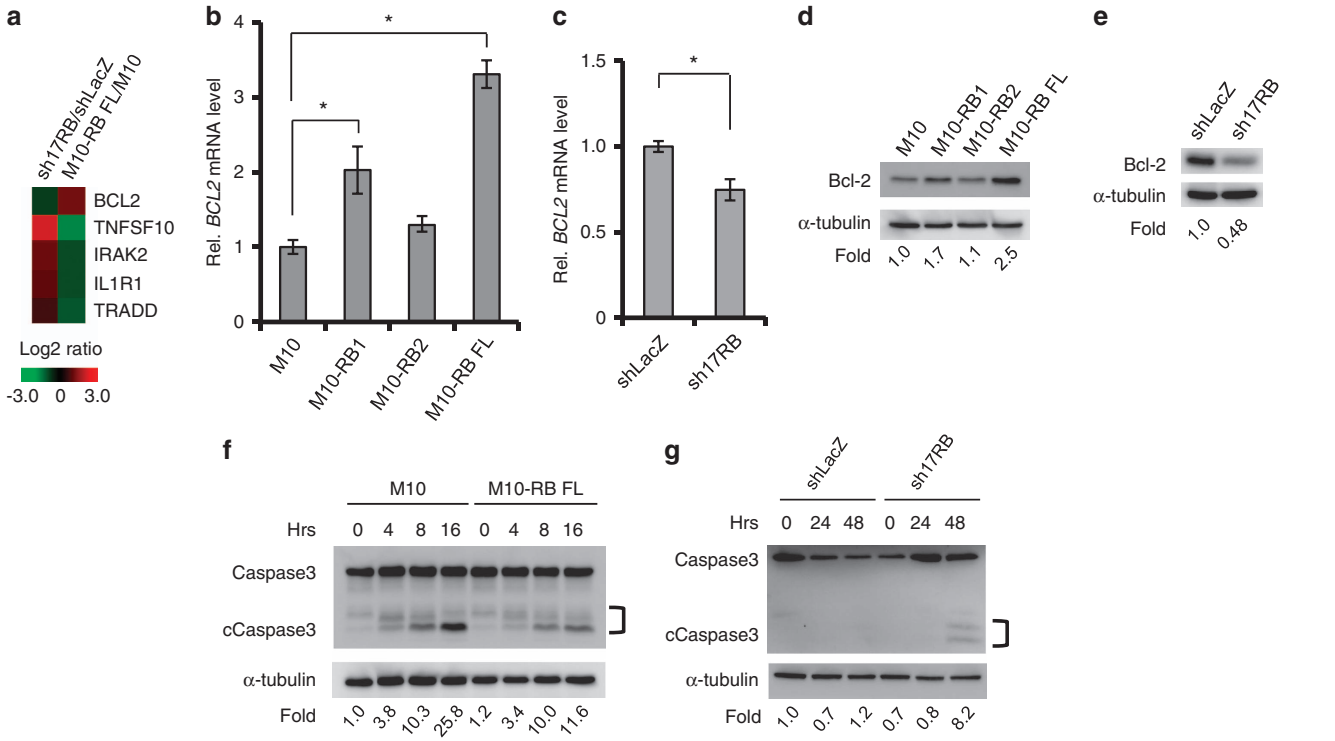


Figure 2. Overexpression of IL-17RB inhibited apoptosis through upregulation of Bcl-2. (**a**) Examples of apoptosis-related genes identified from expression profiling of cells with perturbed IL-17RB expression. (**b** and **c**) Real-time quantitative PCR analysis of *BCL-2* expression in IL-17RB-overexpressing M10 cells (**b**) and IL-17RB-depleted MDA-MB-361 cells (**c**). (**d** and **e**) Western blot analysis of Bcl-2 protein levels in IL-17RB-overexpressing M10 cells (**d**) and IL-17RB-depleted MDA-MB-361 cells (**e**). (**f**) Time-course experiment of etoposide (200 μM) treatment showed that overexpression of IL-17RB decreased the level of cleavage caspase-3 (cCapspase-3). The relative fold of cCaspase-3 induced by etoposide treatment was shown. (**g**) Knockdown of IL-17RB in MDA-MB-361 cells increased the amount of cCaspase-3 upon etoposide (400 μM) treatment. $^*P<0.05$. Student's *t*-test was used to evaluate the statistical significance. Fold: relative cCaspase-3 amount.

To test this possibility, we performed NF-κB reporter assay and found that the NF-κB promoter activity was upregulated in IL-17RB1- and IL-17RB-FL-overexpressing cells (Supplementary Figure S3A). Furthermore, Bcl-2 but not other NF-κB downstream prosurvival genes, including *XIAP*[19] and *SURVIVIN*,[20] were upregulated in IL-17RB1- and IL-17RB-FL-overexpressing cells (Figures 2b and d and Supplementary Figure S4). Conversely, in IL-17RB-depleted MDA-MB-361 cells, the NF-κB promoter activity (Supplementary Figure S3B) and the expression of Bcl-2 were reduced (Figures 2c and e). When treating with the cytotoxic agent, etoposide (VP-16, topoisomerase II inhibitor), activation of apoptotic marker cleavage caspase-3 was reduced in IL-17RB-overexpressing cells compared with the control (Figure 2f). In contrast, caspase-3 activation was enhanced in IL-17RB-depleted cells (Figure 2g). These results suggested that overexpression of IL-17RB inhibited apoptosis via NF-κB-mediated Bcl-2 upregulation.

IL-17B enhances tumorigenic activity through IL-17RB

IL-17B, the ligand of IL-17RB, was expressed in both normal and tumor cells by reverse transcription–polymerase chain reaction (Figure 3a); however, the level of the secreted ligand was barely detectable by enzyme-linked immunosorbent assay. To test whether ectopic addition of IL-17B enhances tumorigenic activity of breast cancer cells, we generated recombinant IL-17B (rIL-17B) protein from mammalian cell-expressing system (Figure 3b). A supplement with rIL-17B increased the colony-forming ability of MDA-MB-361 cells, which express high endogenous IL-17RB, in a dose-dependent manner. On the other hand, rIL-17B treatment failed to enhance colony formation of MDA-MB-231 cells, which express low levels of IL-17RB (Figure 3c and Supplementary Figure S5). Similar results were also observed in M10 cells expressing IL-17RB-FL, but not the control (Figure 3d). In contrast, depletion of the endogenous IL-17B in MDA-MB-361 cells (Supplementary Figure S6A) not only inhibited the colony formation (Figure 3e) but also decreased the NF-κB reporter activity (Figure 3f) and Bcl-2 expression (Supplementary Figures S6B and C). Consistently, the tumor size and weight were both reduced in IL-17B knockdown cells compared with shLacZ control in the xenograft model (Figures 3g and h). These findings suggested that IL-17B contributes to breast tumorigenesis specifically via IL-17RB.

IL-17B signaling activates NF-κB by enhancing TRAF6 recruitment to IL-17RB

Based on bioinformatics and amino-acid sequence analysis in IL-17RA and IL-17RB, we determined two types of putative functional domains, which may involve in IL-17RB signaling: (1) the extracellular ligand-binding domains (LBDs) (Thr28-Leu36, Thr89-Ser96 and Ser259-His264; Supplementary Figure S7A); and (2) the intracellular tumor necrosis factor receptor-associated factor 6 (TRAF6)-binding domain (from Pro339-Glu341), which is critical for IL-17RB signaling transduction.[21] To affirm that IL-17B signaling transduces through IL-17RB, two mutants, ΔLBD, deleted with LBD of Thr89-Ser96, and the other, ΔTRAF6, deleted with TRAF6-binding domain, were generated (Figure 4a). The expressions of these two mutants were comparable in M10 cells (Figure 4b). Compared with the wild-type receptor, expression of these two mutants abolished IL-17RB signaling, leading to the reduction of colony formation and NF-κB promoter activity (Figures 4c and d). Similarly, unlike the wild-type receptor, acinus formation of M10 cells expressing these mutants appeared to be unaffected (Supplementary Figures S7B and C). Consistently, addition of rIL-17B to those cells failed to enhance their colony formation (Figure 4e).

To trace the downstream effectors of this signaling, we tested whether IL-17B promotes the recruitment of TRAF6 to IL-17RB. TRAF6 is the factor directly binding to the TRAF6-binding domain in IL-17RB receptor upon ligand addition. This recruitment is also

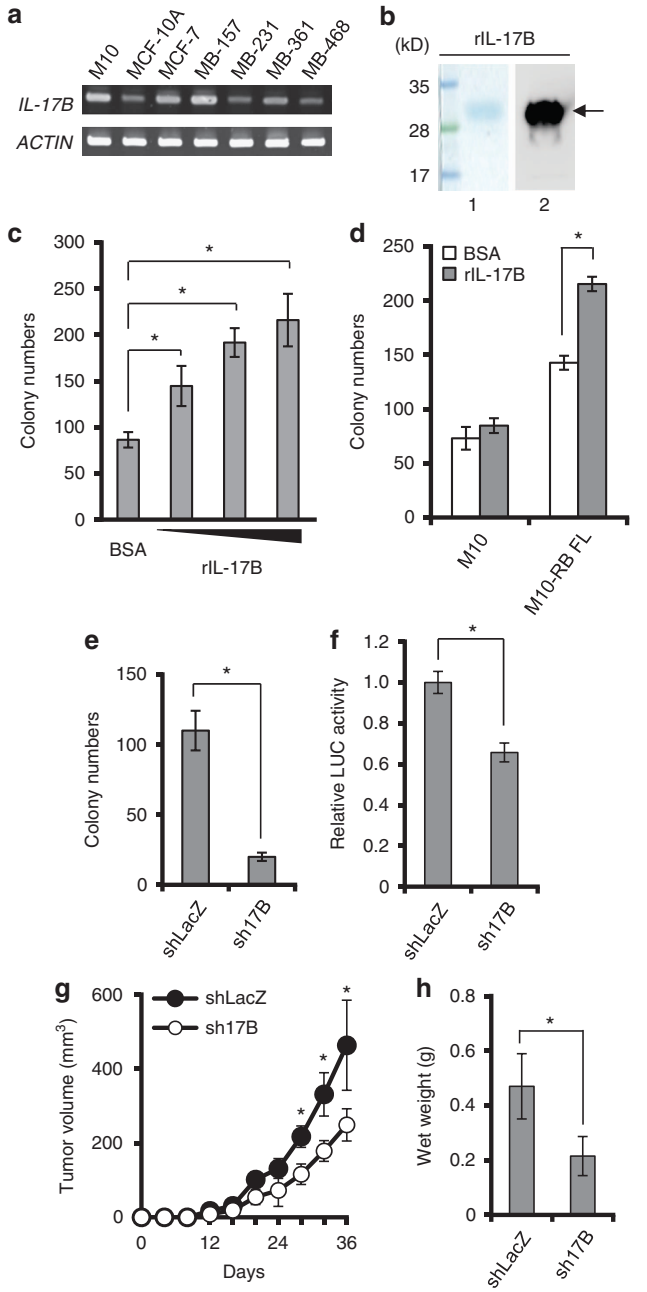


Figure 3. IL-17B enhanced anchorage-independent growth and tumorigenic activity. (**a**) Reverse transcription–polymerase chain reaction analysis of *IL-17B* gene expression in non-malignant mammary epithelial cells (M10 and MCF10A) and breast cancer cells. (**b**) PageBlue staining (left) and western blot analysis (right) of purified recombinant IL-17B protein. (**c**) A dose-dependent promotion of colony formation of MDA-MB-361 cells treated with rIL-17B (50, 100 and 200 ng/ml). Bovine serum albumin (BSA) was used as a control. (**d**) Addition of rIL-17B (200 ng/ml) increased the colony-forming ability of IL-17RB-overexpressing M10 cells. (**e**) Knockdown of IL-17B inhibited the colony-forming ability of MDA-MB-361 cells. (**f**) The NF-κB promoter activity was decreased in IL-17B-depleted MDA-MB-361 cells. (**g**) Tumorigenesis assay of NOD/SCID/γ^{null} mice injected with MDA-MB-361 shLacZ control or shIL-17B cells. Depletion of IL-17B reduced tumor growth. Tumor volume was determined every 4 days after injection. Six mice were used for each group. Data show means ± s.d. (**h**) IL-17B depletion reduced the weight of MDA-MB-361-derived tumor. All data points from **c** to **h** were performed in at least triplicate and all experiments were performed at least three times with similar results. One representative result is shown. Data show means ± s.d. *$P<0.05$ (Student's *t*-test). LUC, luciferase.

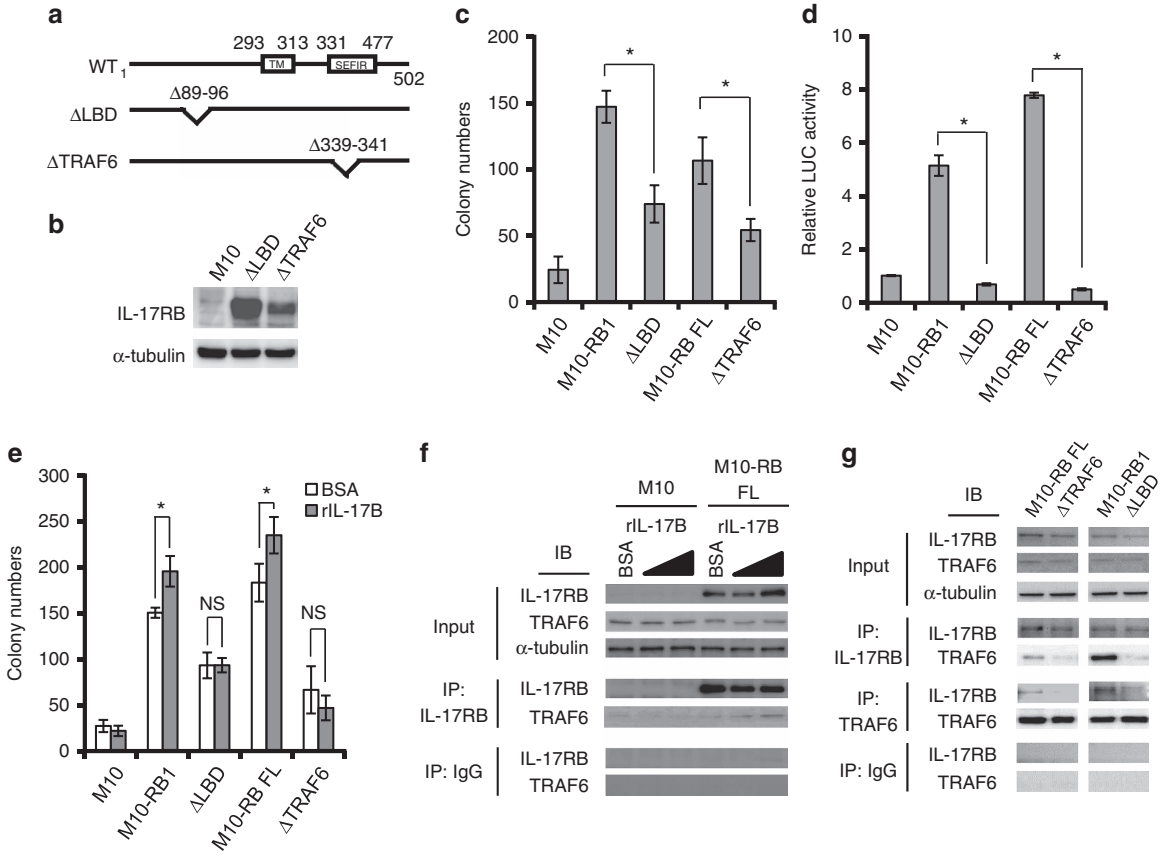


Figure 4. IL-17B transduced signals by enhancing TRAF6 recruitment to IL-17RB to activate NF-κB. (**a**) Schematic shows the wild-type (WT) of full-length IL-17RB, ligand-binding mutant (ΔLBD) with a deletion in putative LBD (amino acids 89–96) and ΔTRAF6 mutant with a deletion in TRAF6-binding domain (amino acids 339–341). (**b**) Western blot analysis showing the amount of IL-17RB mutants (ΔLBD or ΔTRAF6) ectopically expressed in M10 cells. (**c**) The increment of colony numbers was reduced in IL-17RB mutants (ΔLBD or ΔTRAF6) compared with IL-17RB isoform 1- (M10-RB1) or full length IL-17RB- (M10-RB FL) expressing cells that were ectopically expressed in M10 cells. All data points were performed in at least triplicate and all experiments were performed at least three times with similar results. One representative result is shown. Data show means ± s.d. $^*P<0.05$ (Student's *t*-test). (**d**) Deletion of IL-17RB ligand and TRAF6-binding domain reduced the NF-κB reporter luciferase (LUC) activity. (**e**) Addition of rIL-17B (200 ng/ml) failed to stimulate colony formation in ΔLBD and ΔTRAF6 mutants that were ectopically expressed in M10 cells. (**f**) Co-immunoprecipitation (IP) analysis revealed that IL-17B treatment (100 and 200 ng/ml) enhanced the association between IL-17RB and TRAF6. One-tenth of the input protein was shown. (**g**) Deletion of LBD and TRAF6-binding domain in IL-17RB abolished the recruitment and association of TRAF6. One-tenth of the input protein was shown. $^*P<0.05$ (Student's *t*-test). NS, no significant difference. BSA, bovine serum albumin; IgG, immunoglobulin.

critical for the NF-κB signaling transduction.[21,22] Upon rIL-17B treatment, the association between IL-17RB and TRAF6 was increased in a dose-dependent manner (Figure 4f). In contrast, both ΔTRAF6 and ΔLBD mutants failed to recruit TRAF6 (Figure 4g). These results suggested that IL-17B bound to the extracellular domain of IL-17RB and transduced the signal through its intracellular domain by recruiting TRAF6 to activate NF-κB activity.

Antibodies targeting IL-17RB/IL-17B inhibit tumorigenicity of breast cancer cells expressing IL-17RB

To further assess the importance of the IL-17RB/IL-17B signaling, we used antibodies specific to IL-17RB and IL-17B to examine their biological consequences. Addition of IL-17B antibodies to the M10 cells expressing IL-17RB or MDA-MB-361 cells inhibited their colony-forming activity (Figures 5a and b). Similarly, addition of IL-17RB antibody inhibited colony formation of MDA-MB-361 cells (Figure 5c). Importantly, the colony-forming ability of MDA-MB-231 cells, which expressed little or no IL-17RB, was not affected by treating with either IL-17B or IL-17RB antibodies (Supplementary Figure S8). Furthermore, treatment with IL-17RB antibodies retarded tumor growth of MDA-MB-361 cells in the xenograft model (Figure 5d). These results suggested that disruption of IL-17RB/IL-17B signaling inhibits breast tumorigenicity and use of the specific antibodies may provide a potential therapeutic strategy to treat IL-17RB-positive breast cancer (Figure 5e).

Elevated IL-17RB expression has a stronger correlation with poor prognosis than HER2-positive breast cancer

In a cohort with limited number of patients (69 patients), it was shown that the elevated expression of IL-17RB is correlated with poor prognosis.[11] To affirm this previous observation, an independent larger cohort of 179 breast cancer patients (Supplementary Table S3) was further examined by immunohistochemistry (IHC). Consistently, elevated IL-17RB expression was correlated with poor prognosis (Figures 6a and b, $P=0.02$). The correlation between IL-17RB expression and poor prognosis was statistically significant even when adjusted with several clinical parameters including age, tumor size, lymph node status and estrogen receptor expression (Figure 6c). In addition, we also performed real-time quantitative PCR to measure the amount of IL-17RB isoform 1 transcripts in an another independent cohort of

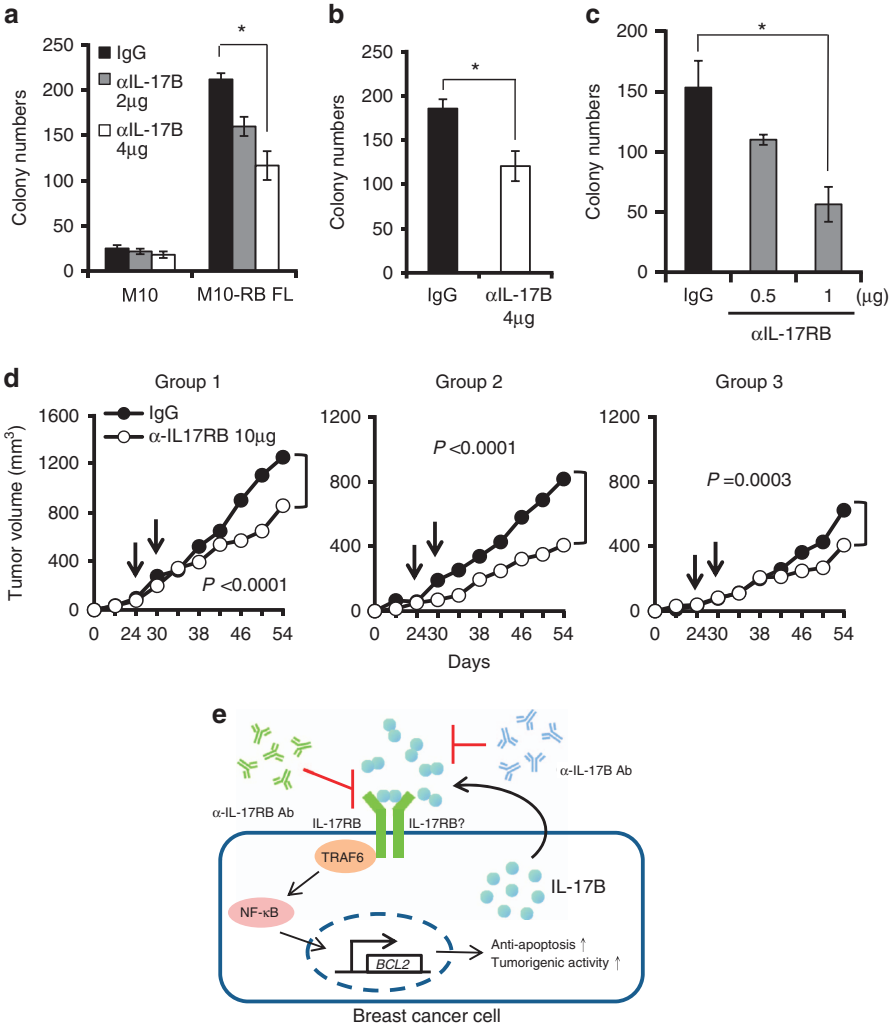
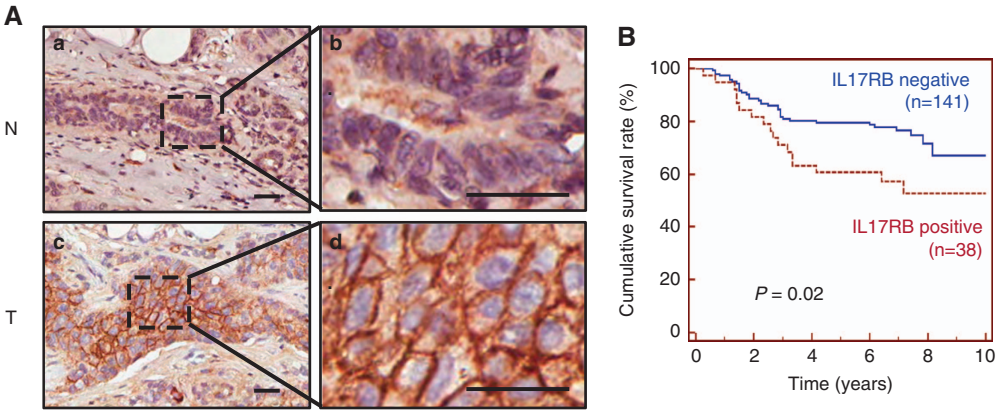


Figure 5. Neutralizing IL-17RB or IL-17B with specific antibodies reduced the tumorigenicity of breast cancer cells. (**a**) Neutralization of IL-17B by the addition of 2 and 4 μg/ml anti-IL17B antibody decreased the colony numbers of IL-17RB-overexpressing M10 cells. Immunoglobulin G (IgG) was used as a control. (**b**) IL-17B neutralization by the addition of 4 μg/ml anti-IL17B antibody significantly decreased the colony formation of MDA-MB-361 cells. (**c**) Purified mouse polyclonal antibody against IL-17RB (0.5 and 1 μg/ml) decreased colony numbers of MDA-MB-361 cells. (**d**) MDA-MB-361-derived tumors were treated with mouse normal IgG or purified mouse polyclonal IL-17RB antibody (10 μg per tumor) by intratumoral injection. Arrows indicate the days of antibody injection. (**e**) Schematic shows the IL-17RB/IL-17B signaling in breast cancer cells. When IL-17RB/IL-17B signaling was activated, TRAF6 was recruited to IL-17RB and activated NF-κB signaling pathway to upregulate the expression of antiapoptotic gene *BCL-2*. Targeting soluble IL-17B (blue antibodies) or the receptor IL-17RB (green antibodies) using specific antibodies is a potential therapeutic strategy for IL-17RB-associated breast cancer. All data points in **a**–**c** were performed in at least triplicate and all experiments were performed at least three times with similar results. One representative result is shown. Data show means ± s.d. $*P < 0.05$ (Student's *t*-test). Nonlinear regression (curve-fit) analysis was used to evaluate the statistical significance in xenograft models.

104 clinical breast cancer specimens (Supplementary Table S3), and used $-\Delta Ct = -7.55$ as a cutoff value based on a receiver operating characteristic curve analysis to define 'high or low' IL-17RB1 expression. Kaplan–Meier analysis showed that patients with high IL-17RB1 expression had a shorter survival compared to patients with low IL-17RB1 expression (Figure 6d, $P = 0.03$). The association of IL-17RB1 expression and poor prognosis was statistically significant after being adjusted with age, tumor size, lymph node status, grade and estrogen receptor expression (Supplementary Table S4). These results suggest that high expression of IL-17RB1 may serve as a poor prognosis marker for breast cancer patients.

Intriguingly, we found that IL-17RB expression was associated with HER2 amplification in breast cancer specimens (Supplementary Table S5). The coexistence of IL-17RB and HER2 overexpression was further affirmed by IHC in the serial paraffin-embedded breast cancer tissue sections (Figure 7a). Patients with both high IL-17RB expression and HER2 amplification had the shortest survival rate (Figure 7b). Interestingly, when we compared the IL-17RB- or HER2-positive group with the double-negative group of patients, elevated expression of IL-17RB showed a stronger correlation to poor prognosis than HER2 amplification (Figure 7c). Both of these correlations were strengthened (Figure 7d) when the triple-negative patients, who have the worst prognosis,[23] were excluded from the cohort. These findings suggest that IL-17RB may serve as an alternative target for patients who have both HER2 amplification as well as IL-17RB expression.

To address this issue, we used a trastuzumab (a.k.a. Herceptin)-resistant breast cancer cells.[24] Interestingly, these cells retained the expression of IL-17RB compared with parental cells (Figure 7e). To test whether the remaining IL-17RB offers an alternative target for further treatment, we depleted IL-17RB in parental SKBR3 and



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Variables	Univariate HR (95%CI)	*P*	Multivariate HR (95%CI)	*P*
IL17RB positive stain (vs.negativegroup)	1.97 (1.10-3.50)	**0.02**	2.08 (1.09-3.97)	**0.03**
Age (≥ 50 years old)*	0.67 (0.39-1.17)	0.16	0.73 (0.38-1.41)	0.35
Tumor size (percm)	1.13 (1.06-1.21)	**0.0002**	1.20 (1.06-1.36)	**0.004**
Lymph - node positive (vs. negative group)	2.58 (1.21-5.53)	**0.015**	4.36 (1.69-11.31)	**0.003**
Estrogen - receptor positive stain(vs.negative group)	0.47 (0.27-0.82)	**0.008**	0.28 (0.15-0.54)	**0.0002**

*Median age: 50 years old,HR:hazardratio,CI: confidence interval

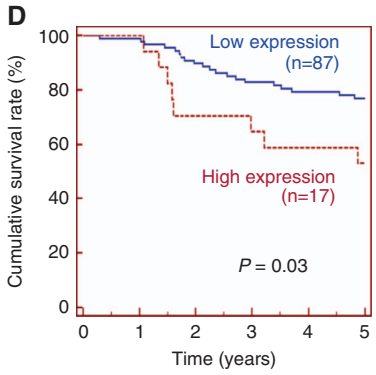

Figure 6. IL-17RB overexpression was correlated with poor prognosis in breast cancer patients. (**a**) IHC staining of IL-17RB. The pictures showed the negative (**a**, **b**) and positive (**c**, **d**) membrane staining of IL-17RB in normal (N) and breast cancer tissue (T), respectively (bar, 25 μm). (**b**) Kaplan–Meier survival analysis of patients with IL-17RB-positive and -negative IHC staining. (**c**) Univariate and multivariate proportional hazards analysis of mortality in breast cancer patients according to IL-17RB IHC staining. (**d**) Kaplan–Meier analysis showed correlation between cumulative survival and IL-17RB expression levels in breast cancer patients. Statistical significance of **b** and **d** were assessed with the log-rank test.

SKBR3-hr cells. As shown in Figure 7f, depletion of IL-17RB in parental cells reduced its colony-forming efficiency to about 50% of the control, whereas depletion of IL-17RB in SKBR3-hr cells drastically abolished their colony-forming ability (Figure 7f). These results suggested that IL-17RB had a role independent of HER2 in breast carcinogenesis, and targeting to IL-17RB may offer a viable approach to treat trastuzumab-resistant cells.

DISCUSSION

In this study, we affirmed that amplified IL-17RB/IL-17B autocrine signaling promoted tumorigenesis in breast cancer cells. IL-17RB/IL-17B transduced signals through TRAF6 to activate NF-κB, which in turn upregulated the expression of antiapoptotic gene *BCL-2*, resulting in etoposide resistance. Blocking this pathway with either IL-17RB or IL-17B antibodies reduced breast cancer tumorigenicity (Figure 5e). These results suggest that IL-17RB/IL-17B signaling has an important role in breast tumorigenesis and may serve as a potential therapeutic target for IL-17RB expression in breast cancer.

Interleukins are known to promote malignant cell transformation and metastasis through eliciting inflammatory microenvironments.[25] IL-17 (IL-17A) has been shown to promote tumor development through the induction of suitable microenvironments at tumor sites and myeloid-derived suppressor cells.[9,26] Consistently, loss of IL-17A in mice is associated with reduced expression of signal transducer and activator of transcription 3-regulated cytokines and reduced tumorigenesis.[27] Interestingly, in murine colon cancer cell-derived tumors, IL-17A has also been shown to reduce tumor growth and metastasis,[28] probably through promoting cytotoxic T-cell activation in tumor immunity,[29] suggesting that ligand–receptor interactions may exert differential roles in a temporal and spatial manner. On the other hand, IL-17B appears to promote breast tumorigenesis (Figure 3). Although the expression of IL-17B, the cognate ligand of IL-17RB,[18] is low in both normal and cancerous mammary epithelial cells (Figure 3a), cancer cells overexpressing IL-17B receptor could gain their growth advantages through IL-17RB/IL-17B autocrine signaling pathway.

The interaction among IL-17 ligands and receptors are intertwined. It has been suggested that the downstream signaling of IL-17RB depends greatly on the ligands and its interacting proteins. IL-17RB can transmit IL-17E signaling by heterodimerization with IL-17RA.[12] The binding of IL-17E to IL-17RB/IL-17RA induces apoptosis in breast cancer cells.[11] In contrast, IL-17RB/IL-17B transduces the prosurvival signaling through recruitment of TRAF6 to activate NF-κB (Figure 4) and induces antiapoptotic process through the upregulation of Bcl-2 (Figure 2). However, the detailed molecular mechanism of how IL-17B binds to IL-17RB in a homodimer manner to transmit the signal inside the cells remains to be elucidated.

IL-17RB expression is almost undetectable in normal mammary epithelial cells. Upregulation of this membrane receptor occurs in about 20% of breast cancer (Figure 6). Using both real-time quantitative PCR analysis specifically detecting IL-17RB1 and IHC staining of the membrane-bound IL-17RB, our data showed a significant correlation between IL-17RB1 expression and poor prognosis in breast cancer (Figure 6). These observations are

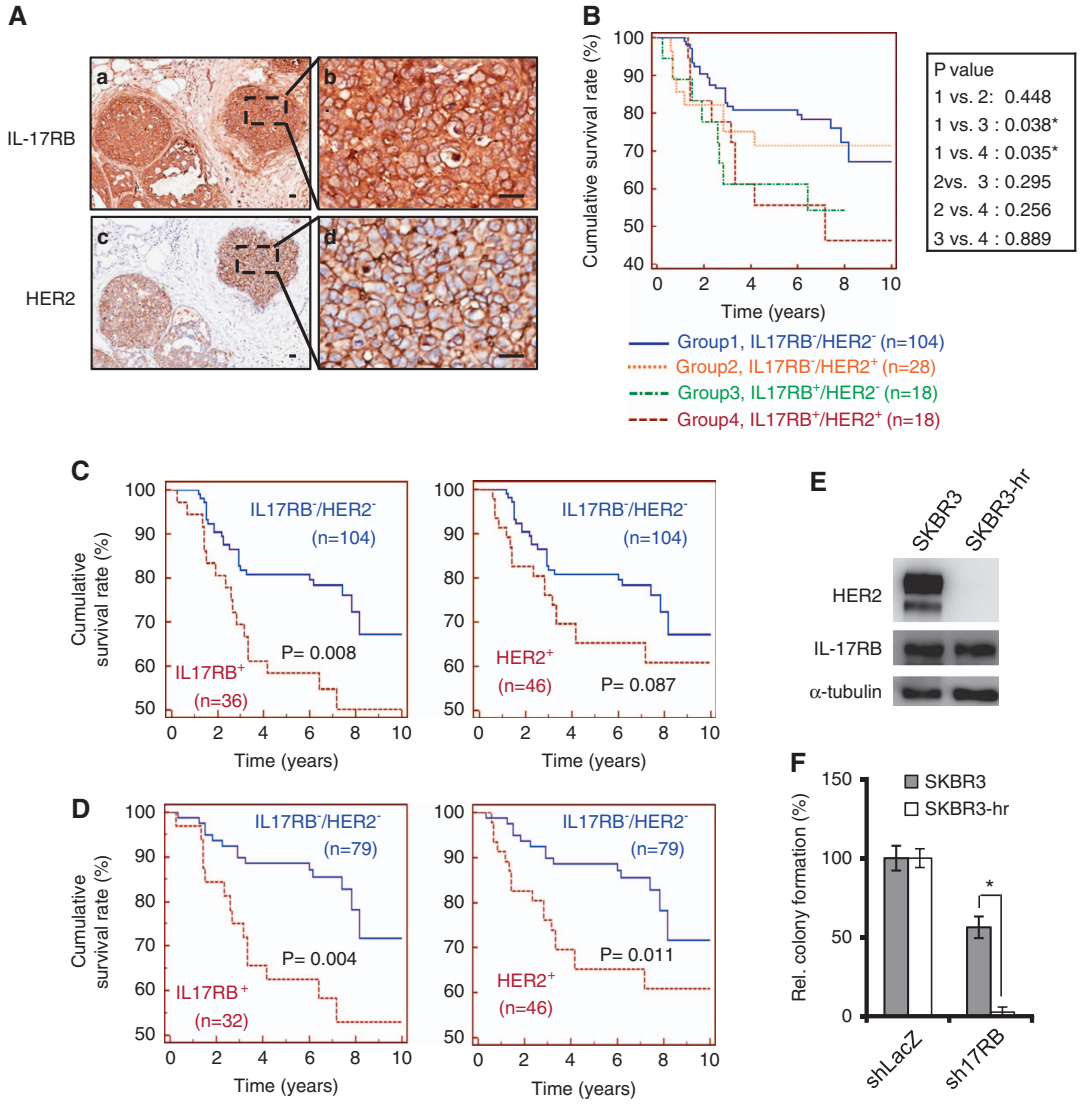

Figure 7. Comparison of the prognosis in breast cancer patients with elevated expression of IL-17RB or HER2. (**a**) IHC staining of IL-17RB (**a**, **b**) and HER2 (**c**, **d**) in serial paraffin-embedded sections showed the coexistence of IL-17RB and HER2 expression (bar, 25 μm). (**b**) Kaplan–Meier survival analysis showed the correlation of IL-17RB/HER2 amplification with cumulative survival in breast cancer patients. Patients with both IL-17RB expression and HER2 amplification (Group 4) have the shortest survival rate. The *P*-values between two groups are depicted. $^*P<0.05$. (**c**) Survival analysis of IL-17RB- and HER2-positive groups vs double-negative group of patients, respectively. Elevated expression of IL-17RB showed a stronger correlation to poor prognosis than HER2 amplification. (**d**) Survival analysis of IL-17RB- and HER2-positive groups vs double-negative group of patients excluded triple-negative patients, respectively. Elevated expression of either IL-17RB or HER2 shows a significant correlation with poor prognosis. Results of **b**–**d** were analyzed by log-rank test. (**e**) Western blot analysis showed the expression levels of IL-17RB and HER2 in parental and trastuzumab-resistant SKBR3-hr cells. (**f**) Depletion of IL-17RB dramatically reduced colony-forming ability in trastuzumab-resistant SKBR3-hr cells. Data points in **f** were performed in at least triplicate and all experiments were performed at least three times with similar results. One representative result is shown. $^*P<0.05$ (Student's *t*-test).

consistent with the previous finding that IL-17RB was over-expressed in murine leukemia cells and may be oncogenic.[10] However, Ma *et al.*[30,31] reported that the expression ratio of *HOXB13/IL-17RB* had a better clinical outcome in early-stage estrogen receptor-positive/lymph-node-positive breast cancer after receiving adjuvant tamoxifen monotherapy,[30,31] implicating that overexpression of IL-17RB may be a good prognostic marker in this subset of breast cancer. Although the precise reason for this discrepancy remains to be explored, it was noted that the IL-17RB isoforms detected by Ma *et al.*[30,31] were the total IL-17RB isoforms including membrane-bound and secreted, whereas our data indicated that only the membrane-bound IL-17RB isoform 1 promotes breast tumorigenesis.

Importantly, the expression of IL-17RB was highly associated with HER2 amplification and the patients with both IL-17RB and HER2 overexpression had the shortest survival rate (Figure 7). HER2 was overexpressed in 20–25% of breast cancers and associated with poor prognosis and drug resistance. As an epidermal growth factor receptor tyrosine kinase, HER2, in cooperation with other receptors, transmits extrinsic signals to turn on many genes involved in proliferation and survival.[32] Targeting HER2 with monoclonal antibody such as trastuzumab can successfully improve the prognosis; however, resistance to this treatment often occurs. Our findings that depletion of IL-17RB in trastuzumab-resistant cells dramatically reduced the tumorigenic activity (Figure 7) make IL-17RB a potential

therapeutic target in HER2-positive breast cancers, particularly in those resistant to trastuzumab.

MATERIALS AND METHODS

Cell lines

Human breast cancer cell lines MCF7, MDA-MB-157, MDA-MB-231, MDA-MB-361, MDA-MB-468, SKBR3 and SKBR3-hr were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics/antimycotics. Non-malignant mammary epithelial cell lines H184B5F5/M10 (M10) and MCF10A cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum and Dulbecco's modified Eagle's medium/F12 supplemented with 5% horse serum, 20 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin and antibiotics/antimycotics in a humidified 37 °C incubator supplemented with 5% CO_2. H184B5F5/M10 cell line was purchased from Bioresource Collection and Research Center (BCRC, Sinjhu City, Taiwan), and others were purchased from ATCC (Manassas, VA, USA).

Clinical specimens

All human samples were obtained from National Taiwan University Hospital (NTUH, Taipei, Taiwan). The samples were encoded to protect patient confidentiality and used under protocols approved by the Institutional Review Board of Human Subjects Research Ethics Committee of Academia Sinica (AS-IRB02-98042) and NTUH (no. 200902001R). Clinical information was obtained from pathology reports. Patients with at least 5-year follow-up were included in this study.

Soft-agar colony formation assay

In one well of a 12-well plate, 2500 cells were seeded in the culture medium containing 0.35% agar on top of a layer of the culture medium containing 0.5% agar (M10 cells also used MCF10A culture medium in soft colony formation assay). Cells were maintained in a humidified 37 °C incubator for 16 days and colonies were fixed with ethanol containing 0.05% crystal violet for quantification. For addition of rIL-17B protein or IL-17B/IL-17RB neutralization assays, anti-human IL-17B (R&D Systems, Minneapolis, MN, USA), anti-human IL-17RB antibodies or rIL-17B was added to the soft-agar culture every 2 days.

Xenograft assay in NOD/SCID/γ^{null} mice

Animal care and experiments were approved by the Institutional Animal Care and Utilization Committee of Academia Sinica (IACUC no. 080085). In total, 2×10^6 MDA-MB-361 breast cancer cells mixed with equal volume of Matrigel (BD Bioscience, San Jose, CA, USA) were injected into NOD/SCID/γ^{null} fat pads.[33] Tumor volumes were evaluated every 4 days after initial detection. Student's *t*-test was used to test the significant differences between shLacZ, shIL-17RB and shIL-17B cells derived from tumor growth. *In vivo* administration of IL-17RB antibody was initiated when tumors reached 50–100 mm^3, and the mice were divided into the same group with comparable tumor size. For each tumor, 10 μg of IL-17RB antibody in 20 μl sterile phosphate-buffered saline was administered by intratumoral injection. Nonlinear regression (curve fit) was used to evaluate the statistical significance of tumor growth between control and treated mice in each group.

IL-17RB antibody

Recombinant IL-17RB extracellular domain that carried only a single N-linked GlcNAc at each glycosylation sites was generated by ectopic overexpression in a suspension cell culture of *N*-acetylglucosaminyltransferase I-deficient (GnTI$^-$) strain HEK293 cells.[34] The resulting *N*-glycans, GlcNAc2Man5, was then treated with endoglycosidase Endo H to remove residual glycans. Polyclonal antibody generated through this immunogen was used throughout the entire work.

Immunoblotting

Immunoblot analysis was performed after 8 or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, with overnight incubation of 1:2000 dilution of mouse polyclonal anti-IL17RB, anti-Bcl-2 (OP60T; Merck, Whitehouse Station, NJ, USA), anti-caspase-3 (IMG144A; Imgenex, San Diego, CA, USA), or 1:1000 dilution of anti-IL17B (MAB1248; R&D Systems), anti-TRAF6 (Sc-8409; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-HER2 (GTX61656; GeneTex, Irvine, CA, USA) antibodies followed by a 1:10 000 dilution of horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (GeneTex). Signals were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA). Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA) before loading and verified by α-tubulin level using a 1:10 000 dilution of anti-α-tubulin antibody (GTX72360; GeneTex). The intensity of western blot bands was quantified using the Image J software (NIH, Bethesda, MD, USA).

Immunohistochemistry

Formalin-fixed, paraffin-embedded primary tumor tissue sections were used. Antigen retrieval was performed using ethylenediaminetetraacetic acid buffer (Trilogy, Cell Marque, Rocklin, CA, USA) heated for 10 min in a microwave. Endogenous peroxidase activity was eliminated by 3% H_2O_2. The slides were blocked in phosphate-buffered saline containing 10% fetal bovine serum and then incubated with purified mouse anti-IL17RB polyclonal antibody (1:100) or anti-HER2 rabbit antibody (1:100) overnight at 4 °C. Horse radish peroxidase-conjugated rabbit/mouse polymer (Dako REAL EnVision, Dako, Glostrup, Denmark) and liquid diaminobenzidine tetrahydrochloride plus substrate (DAB chromogen, Dako) were used for visualization. All slides were counterstained with hematoxylin, and the images were taken using an Aperio Digital Pathology System (Aperio, Vista, CA, USA). Samples were identified as IL-17RB positive if more than 5% of the tumor cells were positive for membrane staining.

3D Matrigel culture assay

In a well of an eight-well chamber slides (Labtek, Nunc., Waltham, MA, USA), approximately 5000 M10 or MCF10A cells were seeded in the growth medium supplemented with 2% Matrigel on top of a layer of Growth Factor Reduced Matrigel (BD Biosciences) as described.[35] The 3D morphogenesis was monitored by fluorescence microscopy confocal sectioning at day 16 after seeding.

Co-immunoprecipitation assay

The whole-cell protein extract was prepared using lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 2 mM $MgCl_2$ and 1% Triton X-100) at 4 °C and precleaned with protein A/G beads for 60 min at 4 °C. IL-17RB and TRAF6 were immunoprecipitated with 1 μg antibodies against IL-17RB and TRAF6 (Cell Signaling, Beverly, MA, USA), respectively, at 4 °C overnight. Normal mouse/rabbit immunoglobulin G was used as a control. The immunoprecipitated protein complex were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and followed by western blot analysis. In rIL-17B treatment experiment, the cells were serum starved for 6 h before being treated with rIL-17B for 5 min.

NF-κB reporter assay

Cells of 80% confluence were transfected using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA). For NF-κB reporter assay, 0.5 μg NF-κB luciferase reporter plasmid and 50 ng of the pGL4-74 Renilla luciferase plasmid (as a transfection efficiency control) were co-transfected into cells per well (24-well plate). Cell extracts were prepared at 24 h after transfection, and the luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA) following the manufacturer's instruction.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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