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SHORT COMMUNICATION

Heterogeneity in ERK activity as visualized by *in vivo* FRET imaging of mammary tumor cells developed in MMTV-Neu mice

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Human epidermal growth factor receptor2/Neu, which is overexpressed in about 30% of human breast cancers, transduces growth signals in large part via the Ras–Raf–MEK–ERK pathway. Nevertheless, it is a matter of controversy whether high ERK activity in breast cancer tissues correlates with better or worse prognosis, leaving the role of ERK activity in the progression of breast cancers unresolved. To address this issue, we live-imaged ERK activity in mammary tumors developed in mouse mammary tumor virus-Neu transgenic mice, which had been crossed with transgenic mice expressing a Förster resonance energy transfer biosensor for ERK. Observation of the tumor by two-photon microscopy revealed significant heterogeneity in ERK activity among the mammary tumor cells. The level of ERK activity in each cell was stable up to several hours, implying a robust mechanism that maintained the ERK activity within a limited range. By sorting the mammary tumor cells on the basis of their ERK activity, we found that ERK^{high} cells less efficiently generated tumorspheres *in vitro* and tumors *in vivo* than did ERK^{low} cells. In agreement with this finding, the expressions of the cancer stem cell markers CD49f, CD24 and CD61 were decreased in ERK^{high} cells. These observations suggest that high ERK activity may suppress the self-renewal of mammary cancer stem cells.

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INTRODUCTION

Cancer cells display phenotypic and functional heterogeneity. The concept of cancer stem cells (CSCs)/tumor-initiating cells (TICs) is a representative example of such heterogeneity within a single tumor.[1] CSCs/TICs have the ability to self-renew and generate a diverse range of tumor cells, and thus serve as promising anti-cancer drug targets.[2] Nevertheless, methods to study CSCs/TICs *in vivo* are limited because the identification of CSCs/TICs depends on the expression of cell surface markers.

Human epidermal growth factor receptor2 (HER2)/Neu is overexpressed in some 30% of breast cancers and associated with poor prognosis.[3] It has been suggested that HER2/Neu may regulate the CSC/TIC property. In HER2-overexpressing carcinoma cell lines, cells with a CSC/TIC property presented increased HER2 levels compared with the bulk cell population.[4] HER2 over-expression in normal mammary epithelial cells increases the proportion of stem and progenitor cells.[5] In line with these observations, it has been shown that the effects of HER2 overexpression on breast CSCs are blocked by the HER2 inhibitor trastuzumab.[4,5] Moreover, overexpression of the polycomb protein EZH2, which has been linked to breast cancer progression, causes RAF1 amplification and, thereby, ERK activation to promote CSC/TIC expansion.[6]

The receptor-type tyrosine kinases, including HER2/Neu, transduce growth signals in large part via the Ras–Raf–MEK–ERK pathway.[7] In mouse mammary tumor virus (MMTV)-Neu transgenic mice, which are widely used as a mouse model of HER2/Neu-positive luminal-type breast cancer,[8,9] ERK regulates the self-renewal of mammary stem and progenitor cells by upregulation of cyclin D1 kinase activity[10] and contributes to malignancy by increasing the activity of the Notch pathway.[11] ERK also regulates many other cancer cell phenotypes, such as invasion and metastasis.[7] In agreement with these observations, high ERK activity in breast cancer tissues has been shown to correlate with poor prognosis of patients.[12] It has also been reported that activation of the ERK pathway drives resistance to paclitaxel.[13]

Surprisingly, however, in some clinical studies high ERK activity in breast cancer tissues is associated with improved survival.[14–16] This seemingly contradictory observation might involve the inhibitory effect of ERK on the self-renewal of stem cells.[17,18] Even though the inhibitory role of ERK on maintenance of the undifferentiated state is cell type-specific,[19] it should be of great concern whether ERK plays a positive or negative role in the self-renewal of CSCs/TICs of mammary tumors, considering the fact that many anti-cancer drugs target the HER2/Neu–Ras–ERK pathway. To answer this question, we visualized the ERK activity in mammary tumors developed in MMTV-Neu transgenic mice that had been crossed with transgenic mice expressing a Förster resonance energy transfer (FRET) biosensor for ERK.[20] Observation of the transgenic mice by two-photon microscopy revealed significant heterogeneity in ERK activity among the primary breast cancer cells. By sorting the mammary tumor cells on the basis of their ERK activity, we found that ERK^{high} cells generated

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tumorspheres in tissue culture and tumors after orthotopic implantation less efficiently than did ERK^{low} cells.

RESULTS AND DISCUSSION

Heterogeneity in ERK activity as visualized by *in vivo* FRET imaging

To study the role of ERK activity in mammary tumors, we crossed MMTV-Neu$^{+/+}$ mice with EKAREV$^{+/-}$ mice, which express a FRET biosensor for ERK, EKAREV.[21] With EKAREV$^{+/-}$ mice, ERK activity in mouse tissues can be visualized by the yellow fluorescent protein/cyan fluorescent protein (CFP) ratio image (Supplementary Figure S1A). The obtained EKAREV$^{+/-}$/MMTV-Neu$^{+/-}$ mice were indistinguishable from the parent MMTV-Neu$^{+/-}$ mice in size, the susceptibility to mammary tumor development, and the histological appearance of the developed tumors. We first observed normal mammary glands of EKAREV$^{+/-}$ mice by two-photon microscopy (Figure 1a). The EKAREV biosensor was expressed both in luminal epithelial cells and myoepithelial cells. The level of ERK activity was higher in myoepithelial cells than luminal epithelial cells. Tissue autofluorescence of mammary tissues was negligible in our experimental setup (Supplementary Figure S1B and C).

The EKAREV$^{+/-}$/MMTV-Neu$^{+/-}$ mice developed mammary tumors from 24 weeks after birth. We found that tumor cells exhibited significant heterogeneity in ERK activity without forming any clusters with similar activity levels (Figure 1b). The standard deviations of the FRET/CFP ratio of luminal epithelial cells and mammary tumors were 0.069 and 0.13, respectively (Figures 1a and b). There was no correlation between the FRET signal and the expression level of the biosensor, suggesting that the divergence in the FRET signal of mammary tumor cells indeed reflected the heterogeneity in the ERK activity (Figure 1c). We confirmed the heterogeneity in the ERK activity by immunostaining with an anti-phospho-ERK antibody (Figure 1d).

To examine whether such diversity in ERK activity was caused by temporal noise or population noise, the mammary tumor cells were live-imaged for six hours. The ERK activities relative to the average for the cell population did not change remarkably in most cells during the course of observation, suggesting that each tumor cell maintained ERK activity homeostasis within a limited range by means of a robust mechanism (Figure 1e, Supplementary Figure S1D).

Real-time pharmacodynamics of anti-cancer drugs against ERK activity in the primary and secondary tumors

The heterogeneity in the ERK activity among tumor cells inspired us to examine its biological meaning. To investigate this issue, mice bearing the mammary tumors were administered with several anti-cancer drugs. To gain insight into the role of microenvironments, in addition to the primary mammary tumors (P0) we also used passaged mammary tumors (P1), which appeared in one month after the injection of the primary tumor into the mammary fat pad of syngeneic FVB mice. The MEK inhibitor PD0325901 immediately suppressed ERK activity in both the P0 mammary tumor cells and the P1 mammary tumor cells with a half-time of ~10 min (Figures 2a and b). We observed no remarkable differences in the response to PD0325901 between

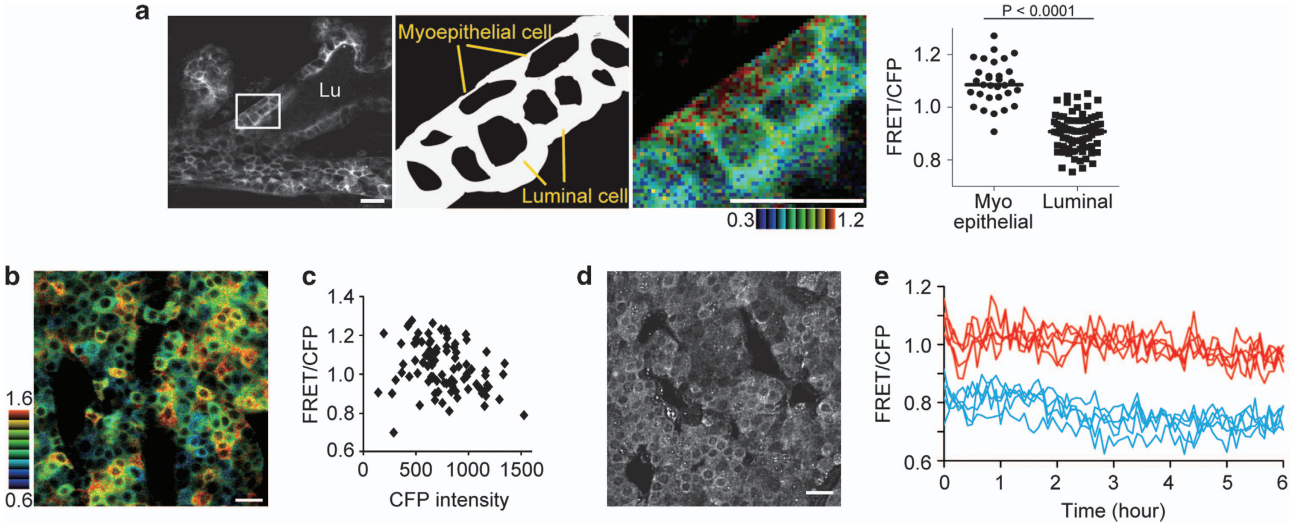

Figure 1. Heterogeneity in ERK activity as visualized by *in vivo* FRET imaging. (**a**) The transgenic mice expressing an ERK FRET biosensor EKAREV were reported previously.[21] Founder animals were backcrossed with FVB/N for at least two generations before crossing with MMTV-Neu$^{+/+}$ mice (FVB/N; The Jackson Laboratory, Bar Harbor, ME, USA). Mice were anesthetized with 1.5% inhalation of isoflurane (Abbott Laboratories, Abbott Park, IL, USA) at 0.5 l/min. Surgical exposure of a tissue flap containing the mammary gland was performed as described previously.[34] The mammary gland in a twelve-week-old female EKAREV$^{+/-}$ mouse was observed under an IX81/FV1000 inverted microscope equipped with a ×30 silicon oil-immersion objective lens (UPLSAPO 30xS; Olympus, Tokyo, Japan), which was connected to a Mai Tai DeepSee HP Ti: Sapphire Laser (Spectra Physics, Santa Clara, CA, USA). The excitation wavelength was 840 nm. Acquired images were analyzed with MetaMorph software (Molecular Devices, Sunnyvale, CA, USA) and Imaris Software (Bitplane AG, Zurich, Switzerland) as described previously.[21,35] An XY slice, a scheme and an enlarged FRET/CFP ratio image are shown. The FRET/CFP ratio image is shown in intensity-modulated display (IMD) mode. In the IMD mode, eight colors from red to blue are used to represent the FRET/CFP ratio, with the intensity of each color indicating the mean intensity of FRET and CFP. The upper and lower limits of the ratio range are shown at the bottom of the figure. The FRET/CFP ratios of myoepithelial cells (Myo, $N = 32$) and luminal cells (Lumi, $N = 90$) are shown in the histogram. (**b**) A FRET image of mammary tumors developed in EKAREV$^{+/-}$/MMTV-Neu$^{+/-}$ mice and observed under the 2P microscope. (**c**) The CFP intensity and FRET/CFP value in each primary tumor cell were plotted ($N = 50$). (**d**) Immunostaining of a mammary tumor. Paraffin-embedded sections were fixed with 4% paraformaldehyde, blocked with 3% BSA, incubated with an anti-phosphor-ERK antibody (1:200; catalog number 4370S; Cell Signaling Technology, Danvers, MA, USA), and further incubated with Alexa 546-conjugated anti-rabbit IgG (1:1000; number A-11035; Life Technologies, Grand Island, NY, USA). The section was imaged under a fluorescent microscope. (**e**) Mammary tumors in EKAREV$^{+/-}$/MMTV-Neu$^{+/-}$ mice were timelapse-imaged for 6 h under a 2P microscope. Red or blue lines represent the time courses of the top or bottom 12.5% of cells (five cells each). *P*-value was calculated using a two-tailed Student's *t*-test. 2P, two-photon.

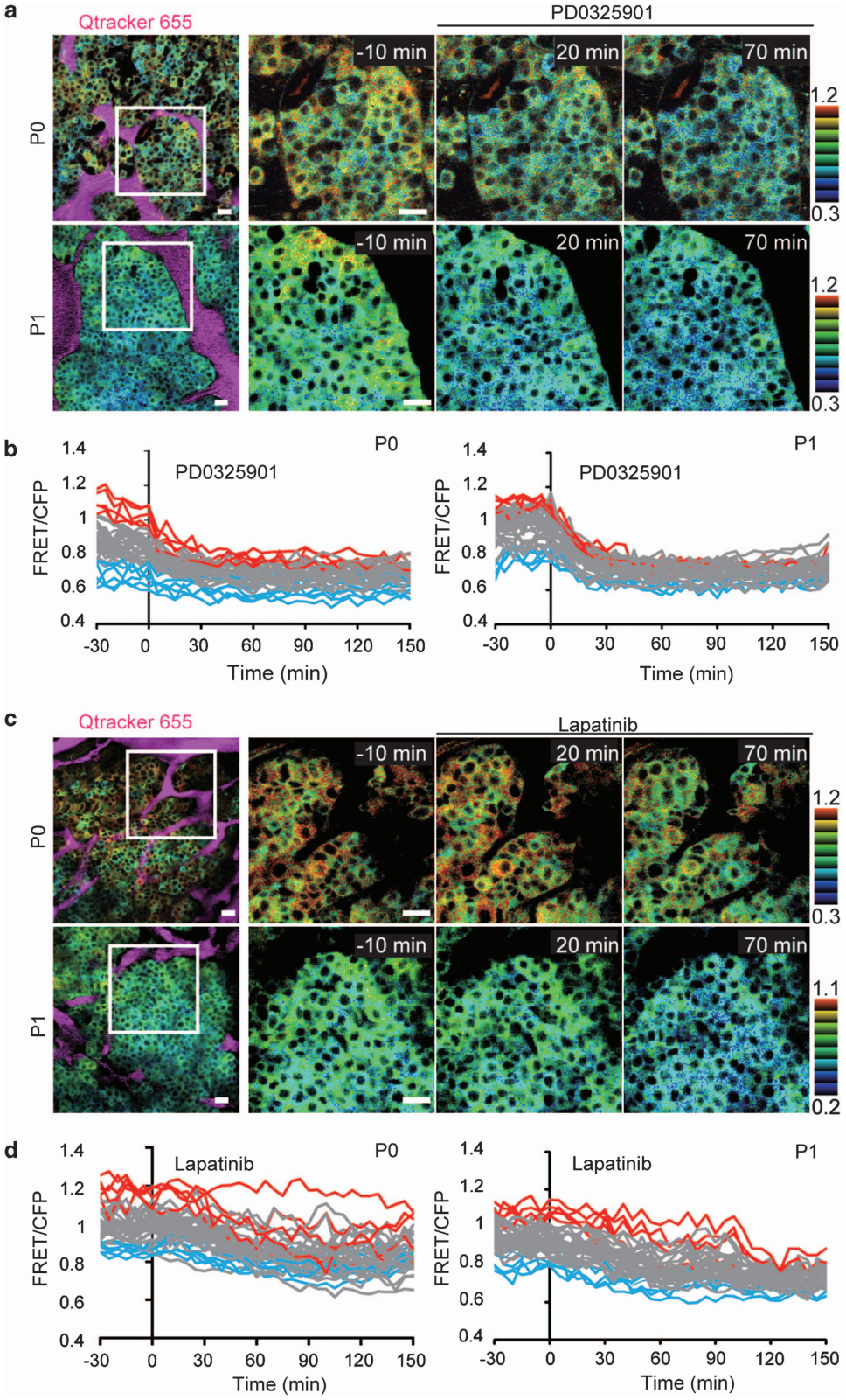

Figure 2. Timelapse *in vivo* imaging of ERK activity in EKAREV$^{+/-}$/MMTV-Neu$^{+/-}$ mice treated with an MEK or a tyrosine kinase inhibitor. The primary tumors in EKAREV$^{+/-}$/MMTV-Neu$^{+/-}$ mice and the secondary tumors in syngeneic mice were observed by 2P microscopy. (**a**) An MEK inhibitor, PD0325901, was injected intravenously (5 mg/kg) at time zero. The white-boxed regions are magnified in the right panels. (**b**) FRET/CFP ratios of individual cells were plotted against time. The red and blue lines indicate cells belonging to the top and bottom 12.5%, respectively, at the beginning of imaging ($N = 40$). (**c** and **d**) An EGFR/HER2 dual tyrosine kinase inhibitor, Lapatinib, was injected intravenously (50 mg/kg) at time zero. Data were obtained as in (**a** and **b**) ($N = 40$). Error bars indicate s.d. Scale bar, 20 μm. 2P, two-photon, EGFR, epidermal growth factor receptor.

cells close to and those remote from the blood vessels, which were visualized by Qtracker 655 co-injected with the reagents. Notably, the decrease in ERK activity was larger in cells with higher ERK activity than in cells with lower ERK activity (Supplementary Figure S2A). The low ERK-activity cells in the P0 tumors appeared to be the least-responsive cell population. Lapatinib, a dual tyrosine kinase inhibitor against epidermal growth factor receptor and epidermal growth factor receptor2 (HER2), gradually decreased the ERK activity from 10 min after injection in both the P0 mammary tumor cells and the P1 mammary tumor cells (Figures 2c and d). Again, the low ERK-activity cells in the P0 tumors responded to Lapatinib least efficiently (Supplementary Figure S2A). To examine whether the delay might have been caused by the difference in the pharmacokinetics, we examined the effect of PD0325901 and Lapatinib *in vitro* with primary mammary tumor cells derived from the transgenic mice (Supplementary Figure S2B). As observed *in vivo*, ERK activity in the Lapatinib-treated cells started decreasing with ~5 min delay, suggesting that the delay was caused by the sensitivity of the tumor cells to Lapatinib. With the *in vitro* tumor cells, we also examined the response of mammary tumor cells to EGF. In contrast to the response to the inhibitors, no significant correlation was observed between the basal ERK activity and the EGF-induced increment (Supplementary Figure S2C). Taken together, these results imply that the heterogeneity in ERK activity in mammary tumor cells may contribute to the difference in the sensitivity to anti-cancer drugs.

In vivo imaging of protein kinase activity by two-photon microscopy has opened a new window for the assessment of pharmacodynamics. With transgenic mice expressing FRET biosensors, the effect of an MEK inhibitor on the ERK activity of neutrophils or the effect of a cAMP analog on the PKA activity of muscles and neurons has been video-imaged in living mice.[21] More recently, the effect of dasatinib on Src activity has also been visualized with subcutaneously implanted pancreatic cancer cells that stably express a FRET biosensor for Src.[22] Here, we showed the drug sensitivity of the mammary tumors developed in MMTV-Neu transgenic mice. We chose PD0325901 and Lapatinib, which are a non-ATP-competitive MEK inhibitor under phase I trial[23] and a dual tyrosine kinase inhibitor of epidermal growth factor receptor and HER2 in clinical use,[24] respectively. Upon intravenous injection of inhibitors of MEK or HER2, the ERK activity decreased more significantly in high ERK-activity cells than in low ERK-activity cells (Figure 2). On the basis of this observation, we may be able to speculate that the inhibitors of the HER2/Neu-ERK pathway are less effective for treatment of low ERK-activity cells.

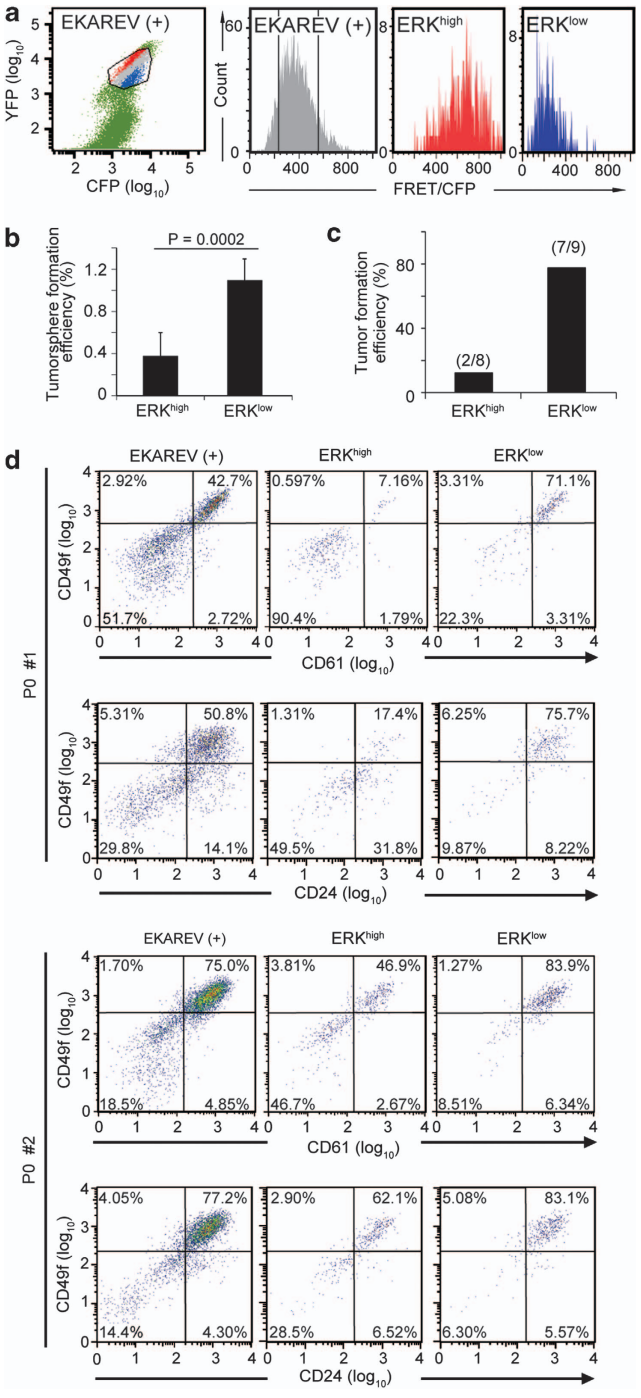

Figure 3. Characteristics of the ERK^{high} and ERK^{low} populations. Mammary tumors were dissected from EKAREV$^{+/-}$/MMTV-Neu$^{+/-}$ mice to obtain a single-cell suspension essentially as described previously.[36] Briefly, a portion of the tumor was minced into small pieces with sterile scissors, washed in PBS, digested in collagenase/trypsin for 30 min at 37 °C with occasional mixing, and washed once with DMEM/F12 (Life Technologies). Mammary epithelial cells were enriched by Mouse Epithelial Cell Enrichment kit (StemCell Technologies, Vancouver, BC, Canada). Single-epithelial cell suspensions were resuspended in L-15 Medium (Life Technologies) and analyzed and/or sorted with a FACSAria (Becton Dickinson, Franklin Lakes, NJ, USA). (**a**) Representative FACS dots plots showing the biosensor-expressing mammary tumor cells developed in EKAREV$^{+/-}$MMTV-Neu$^{+/-}$ mice. Mammary tumor cells in the highest and lowest decile with respect to the FRET/CFP ratio were named the ERK^{high} and ERK^{low} populations, respectively. The sorted ERK^{high} and ERK^{low} cells were re-analyzed for the validation of the sorting (shown in red and blue histograms, respectively). (**b**) The ERK^{high} and ERK^{low} cells were plated in poly-HEMA-treated six-well plates (Becton Dickinson) at a density of 10 000 cells/ml in phenol red-free Mammary Epithelial Cell Basal Medium (TAKARA BIO INC., Otsu, Japan) supplemented with 20 ng/ml EGF (Sigma-Aldrich, St Louis, MO, USA), 20 ng/ml basic fibroblast growth factor (Sigma), 5 μg/ml insulin (Sigma) and 2% B27 supplement (Life Technologies). The tumorsphere formation was evaluated two weeks after plating. The result represents averages of three biological replicates. (**c**) The ERK^{high} and ERK^{low} cells (50 cells each) were injected into fat pads of syngeneic mice. Development of tumors was assessed three months after inoculation. (**d**) Representative flow cytometric profiles for CD24/CD49f or CD61/CD49f were obtained from ERK^{high} and ERK^{low} populations in two representative primary tumors ($N=4$). We used anti-CD49f antibody conjugated with Alexa-647 or PE/Cy7 (BioLegend, San Diego, CA, USA), anti-CD24 antibody conjugated with PE/Cy7 (BioLegend) and anti-CD61 antibody conjugated with Alexa-647 (BioLegend). Error bars indicate s.d. *P*-value was calculated using a two-tailed Student's *t*-test.

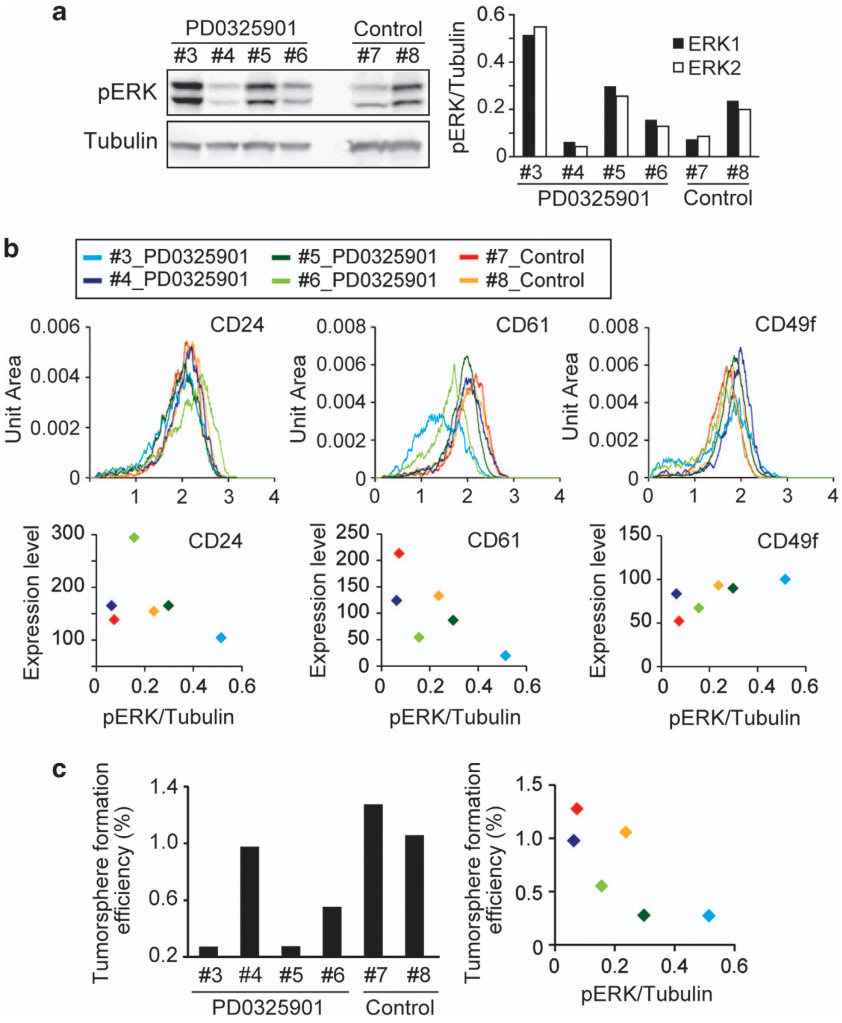

Figure 4. Inverse correlation of ERK activity with the expression of the cancer stem cell markers. EKAREV$^{+/-}$MMTV-Neu$^{+/-}$ mice bearing mammary tumors were orally administered with 50 mg/kg PD0325901 for one week. Single-cell suspension of tumor cells were prepared as described in the legend to Figure 3. (**a**) Part of cells was subjected to immunoblotting analysis with anti-phospho-ERK antibody or anti-tubulin antibody (Merck Millipore, Billerica, MA, USA). (**b**) Expression of cancer stem cell markers were examined as described in the legend to Figure 3. Mode values of cells treated with PD0325901 was divided by those without PD0325901 to show the expression level. (**c**) The suspended cells were plated in poly-HEMA-treated six-well plates at a density of 10 000 cells/ml as described in the legend to Figure 3. The tumorsphere formation was evaluated two weeks after plating.

Decreased tumorsphere formation efficiency and expression of CSC markers in tumor cells with high ERK activity

To elucidate the role of the heterogeneity of ERK activity among mammary tumor cells, we sorted the cells on the basis of their ERK activity (Figure 3a). A fluorescent dye that accumulates in dead cells, 7AAD, was included to select for live cells. After selecting single living cells with the forward-scatter and side-scatter signals, biosensor-expressing cells were identified by the CFP and yellow fluorescent protein signals. For cell sorting, the FRET/CFP ratio was used as the index of FRET efficiency as in 3-dimensional imaging. Mammary tumor cells in the highest and lowest decile with respect to the FRET/CFP ratio were named the ERK^{high} and ERK^{low} populations, respectively (Figure 3a). We next compared the biological activity by a tumorsphere formation assay (Figure 3b). The ERK^{high} population exhibited lower tumorsphere formation efficiency ($0.3 \pm 0.2\%$) compared with the ERK^{low} population ($1.3 \pm 0.2\%$). To understand the role of ERK in tumorsphere formation, we followed the ERK activity of ERK^{high} and ERK^{low} cell populations *in vitro*. The fraction of high ERK-activity cells in the ERK^{high} population decreased within nine days to the level of those of the ERK^{low} population (Supplementary Figure S3A). Timelapse imaging revealed that the high ERK-activity cells died within three days (Supplementary Figure S3B), implying that the low tumorsphere formation efficiency of ERK^{high} cells is at least partially attributable to the vulnerability of ERK^{high} cell. The ERK^{high} and ERK^{low} populations were further analyzed by an *in vivo* transplantation assay (Figure 3c). Again, the ERK^{high} population yielded tumors less efficiently than did the ERK^{low} population. Because the tumorsphere formation assay and the transplantation assay were used to examine the stem cell property of cancer cells, these results strongly suggested that high ERK activity may suppress the stemness of mammary tumor cells.

To explore this possibility, we examined the expression of mammary stem cell markers on the ERK^{high} and ERK^{low} cell populations. As reported previously,[25,26] a large portion of the MMTV-Neu tumor cells expressed three mammary stem cell markers, CD49f, CD24 and CD61 (Figure 3d). We found that CD61^{-}/CD49f^{-} cells or CD24^{-}/CD49f^{-} cells were significantly increased in the ERK^{high} population, suggesting that high ERK activity may suppress the stem cell property of mammary tumor

cells. The percentage of $CD61^-/CD49f^-$ cells or $CD24^-/CD49f^-$ cells in the ERK^{high} population varied depending on the mouse age, but the pattern was similar in multiple mice ($N = 3$), showing a reproducible decrease in $CD61^-/CD49f^-$ cells or $CD24^-/CD49f^-$ cells in the ERK^{high} population of MMTV-Neu mammary tumor cells.

Finally, we attempted to increase mammary tumor cells bearing the stem cell property by the pretreatment of mice bearing the tumors with the MEK inhibitor. Against our expectation, we failed to observe the decrease in the ERK activity of P0 mammary tumor cells by the daily administration of the MEK inhibitor PD0325901 (Figure 4a). The discrepancy between the data by FRET imaging (Figure 2) and the anti-phospho-ERK immunoblotting could be explained by several reasons. First, the variation in the ERK activity of each tumor could be greater than the effect of PD0325901 on each tumor. Second, the daily administration of PD0325901 to the tumor-bearing mice could render the tumor cells resistant to this compound. Third, the FRET biosensor detects the phosphorylation level of the ERK substrates; whereas anti-phospho-ERK immunoblotting detects the phosphorylation of ERK.

Notably, however, the ERK activity in each tumor correlated inversely with the expression levels of CD24 and CD61, but not of CD49f (Figure 4b), and also with the tumorsphere formation efficiency (Figure 4c), suggesting that high ERK activity suppresses the CSC property. The failure to suppress ERK activity *in vivo* urged us to use J3B1 cells, which are derived from the non-tumorigenic murine EpH4 mammary epithelial cell line.[27] We found that PD0325901 suppressed ERK activity and increased the expression of CD24 and CD61 (Supplementary Figure S4), indicating that ERK negatively regulated the expression of CD24 and CD61. Again, CD49f expression was not affected by the decrease in the ERK activity. Altogether, these observations are compatible with the proposal that high ERK activity suppresses the CSC property in the MMTV-Neu mammary tumor model.

Previous studies have identified CD24, CD49f and CD61 as markers of the mammary stem cells and/or luminal progenitor cells.[28–31] The identified markers have also been used to purify CSCs/TICs in MMTV-Neu mice. Liu *et al.*[25] demonstrated a dramatic increase in the $CD49f^+CD24^+$ population in mammary tumors, which efficiently form tumorspheres. In the initial attempt to use CD61 as a CSC/TIC marker, Vaillant *et al.*[32] did not find significant enrichment of CSCs/TICs in the $CD61^{high}$ population; however, a later study identified the $CD49f^{high}$ $CD61^{high}$-sorted fraction as a TIC-enriched population of MMTV-Neu mice, which displayed increased tumorsphere formation ability, enhanced tumorigenicity *in vivo* and drug resistance to paclitaxel and doxorubicin.[26] We found that ERK^{high} cells exhibited not only the decrease of cells that were positive for CD49f, CD24 and CD61 but also a decrease of the tumorsphere efficiency *in vitro* and tumor formation efficiency in the fat-pad injection assay (Figure 3). Furthermore, ERK activity in mammary tumors inversely correlated with the expression level of CD24 and CD61 and the tumorsphere formation efficiency (Figure 4). In mouse ES cells, it has been established that ERK activity must be suppressed for the elimination of differentiation-inducing signaling.[33] In agreement with this report, we found that mammary tumor cells with higher ERK activity were selectively eliminated *in vitro* (Supplementary Figure S4). Thus, low ERK activity may be beneficial for self-renewal of the CSCs/TICs of mammary tumor cells as in EC cells.

It has been established that ERK has critical roles in the expansion of CSCs/TICs of breast cancer.[6,10,11] Intuitively, this observation would seem to undermine the finding that ERK^{high} cells isolated from mammary tumor tissues were less tumorigenic than ERK^{low} cells (Figure 3). Previous studies have demonstrated the roles of ERK by comparing cells with and without oncogene expression, indicating that the cancer cell population, on average, shows high ERK activity, rapid cell growth, and enrichment of CSCs/TICs in comparison with control cells. Therefore, it is plausible that, within breast cancer tissues, the cell population with lower ERK activity is enriched in CSCs/TICs and that the cell population with higher ERK activity is enriched in rapidly expanding and, at the same time, vulnerable cells.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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