β2-adrenoceptors on tumor cells play a critical role in stress-enhanced metastasis in a mouse model of breast cancer

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Abstract

Chronic stress accelerates metastasis - the main cause of death in cancer patients - through the activation of β-adrenoceptors (βARs). We have previously shown that β2AR signaling in MDA-MB-231^{HM} breast cancer cells, facilitates invadopodia formation and invasion *in vitro*. However, in the tumor microenvironment where many stromal cells also express βAR, the role of β2AR signaling in tumor cells in metastasis is unclear. Therefore, to investigate the contribution of β2AR signaling in tumor cells to metastasis *in vivo*, we used RNA interference to generate MDA-MB-231^{HM} breast cancer cells that are deficient in β2AR. β2AR knockdown in tumor cells reduced the proportion of cells with a mesenchymal-like morphology and, as expected, reduced tumor cell invasion *in vitro*. Conversely, overexpression of β2AR in low metastatic MCF-7 breast cancer cells induced an invasive phenotype. Importantly, we found that knockdown of β2AR in tumor cells significantly reduced the impact of stress on metastasis *in vivo*. These findings highlight a crucial role for β2AR tumor cell signaling in the adverse effects of stress on metastasis,

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

and indicate that it may be necessary to block β_2AR on tumor cells to fully control metastatic progression.

Keywords

triple negative breast cancer; β_2-adrenoceptor; chronic stress; metastasis; invasion

INTRODUCTION

Despite advances in cancer treatment, metastasis remains the greatest clinical challenge in the management of cancer and contributes to the majority of deaths in cancer patients (Mehlen and Puisieux, 2006). Studies have revealed that chronic stress drives cancer progression by accelerating metastasis in *in vivo* mouse models of various cancer types (Ben-Eliyahu et al., 2000; Campbell et al., 2012; Kim-Fuchs et al., 2014; Lamkin et al., 2015; Le et al., 2016; Liu et al., 2015; Sloan et al., 2010; Thaker et al., 2006; Zhao et al., 2015). This raises the possibility that targeting stress responsive signaling may help slow cancer progression and metastatic dissemination (Sloan et al., 2010; Thaker et al., 2006), and provide a much needed additional therapy for the treatment of cancer.

The sympathetic nervous system (SNS) plays an important role in the response to stress, which triggers the release of catecholaminergic neurotransmitters from SNS nerve fibers (Elenkov et al., 2000). These neurotransmitters activate β-adrenoceptors (βAR), which induces downstream signaling in responsive cells and leads to transcriptional changes (Elenkov et al., 2000). A number of different cell types present within the tumor microenvironment express βARs, and thus are able to respond to stress signaling. These include immune cells and endothelial cells (Abrass et al., 1985; Graf et al., 1993; Sanders et al., 1997), which have a critical role in driving cancer progression (Condeelis and Pollard, 2006; Folkman, 2002; Le et al., 2016). In response to stress, stromal cells contribute to metastasis by remodeling tumor architecture in ways that favor dissemination of tumor cells. This includes macrophage recruitment into the primary tumor (Sloan et al., 2010; Zhao et al., 2015) and vascular remodeling to increase blood vessel (Sloan et al., 2010; Thaker et al., 2006) and lymph vessel (Le et al., 2016) routes of tumor cell dissemination. Experimental strategies that prevent either macrophage recruitment or vascular remodeling block stress-enhanced metastasis (Le et al., 2016; Sloan et al., 2010; Thaker et al., 2006), demonstrating that regulation of the tumor stroma plays an important role in the effects of stress on cancer progression.

Tumor cells also express βARs (Pon et al., 2016; Reeder et al., 2015), and activation of βAR signaling increases invasion of tumor cells, as measured by *in vitro* assays (Creed et al., 2015; Kim-Fuchs et al., 2014; Pon et al., 2016; Yamazaki et al., 2014) and in explant cultures (Creed et al., 2015). Previously, we discovered that the β_2AR-selective agonist formoterol, but not the β_1AR-selective agonist xamoterol, induced the formation of invadopodia in breast cancer cells (Creed et al., 2015). Invadopodia are actin-rich cellular structures that localize matrix metalloproteases (MMPs) and degrade the extracellular matrix for tumor cell invasion (Murphy and Courtneidge, 2011). However, the role of β_2AR-

regulated invasion *in vivo* is less clear. Unlike in simple *in vitro* assays, in the tumor microenvironment contextual cues provided by stromal cells influence whether tumor cells are able to escape the primary tumor and disseminate to distant tissues (Bissell and Labarge, 2005; Devaud et al., 2014). Therefore, in the complex tumor microenvironment where stromal cells also respond to βAR stress signaling, it is unclear whether β_2AR signaling in tumor cells significantly contributes to metastasis. Previous studies that used systemic β-blockade to investigate βAR regulation of metastasis were unable to distinguish the contribution of βAR signaling in tumor cells, as systemic β-blockade indiscriminately targets both tumor cells and stromal cells (Campbell et al., 2012; Sloan et al., 2010; Thaker et al., 2006). While use of siRNA has shown that β_2AR signaling affects the growth of tumor cells injected directly into metastatic target organs (Thaker et al., 2006), it is not known if signaling from β_2ARs on tumor cells is required for the early stages of the metastatic cascade including tumor cell invasion and escape from the primary tumor.

To address this, we used an shRNA approach to generate breast cancer cells that were stably deficient in β_2AR. Using MDA-MB-231[HM] cells, a human breast cancer cell line that is highly responsive to βAR signaling, we investigated the effect of tumor cell β_2AR knockdown on metastasis from a primary orthotopic mammary tumor. Consistent with previous pharmacologic studies, genetic modulation of MDA-MB-231[HM] β_2AR reduced cell invasion, and prevented a shift to mesenchymal cell morphology. Conversely, upregulating β_2AR expression in MCF-7 tumor cells with low endogenous β_2AR expression increased invadopodia formation, demonstrating a central role for β_2AR in regulating tumor cell invasion. Furthermore, we show that β_2AR knockdown in MDA-MB-231[HM] tumor cells attenuated stress-enhanced metastasis from primary mammary tumors. These findings show that in this model of breast cancer, β_2AR-driven tumor cell invasion plays a significant role in the effects of stress on metastasis. These findings suggest that pharmacological strategies that block the effects of stress on metastasis may need to also target β_2AR on tumor cells to fully control metastatic progression.

METHODS

Genetic manipulation of tumor cell β_2AR expression

The human breast cancer cell line MDA-MB-231[HM] (a kind gift from Dr Zhou Ou, Fudan University Shanghai Cancer Center, China) (Chang et al., 2008) was transduced with a lentiviral vector containing codon optimized firefly luciferase under the control of the ubiquitin-C promoter as previously described (Le et al., 2016). The identity of the cell line was confirmed by karyotyping (CellBank Australia). Cells were maintained in DMEM (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies, USA) at 37°C with 5% CO_2. To silence *ADRB2*, cells were transduced with shRNA that specifically targets human *ADRB2* (shADRB2a: 5'-TGCTGTGACTTCTTCACGA-3'; shADRB2b: 5'-GCCATCAACTGCTATGCCA-3') or scramble control sequence (SCR): 5'-ATCTCGCTTGGGCGAGAGTAAG-3' (Dharmacon, USA). MCF-7 cells were acquired from ATCC (Manassas, Virginia) and grown in MEM supplemented 10% FBS. The β_2AR-GFP construct was made by sub-cloning the human β_2AR coding sequence 5' to a codon-humanized GFP gene derived from the vector pGFP[2]-N1 (Perkin Elmer), and sequence

verified. Cells were stably transduced and GFP-positive cells sorted by FACS. All experiments used bulk cell populations to avoid clonal effects. Cells were confirmed negative for mycoplasma using MycoAlert™ Mycoplasma Detection Kit (Lonza, Australia).

Functional analysis of β_2AR knockdown in tumor cells by cAMP accumulation assay

Tumor cells (4×10^4) were seeded into a 96-well plate and serum-starved overnight. Cells were then washed and incubated in pre-warmed stimulation buffer (140 mM NaCl, 5 mM KCl, 800 nM MgSO$_4$, 200 nM Na$_2$HPO$_4$, 440 nM KH$_2$PO$_4$, 5 mM HEPES, 1.3 mM CaCl$_2$, 5.6 mM glucose, 0.1% w/v BSA, 500 μM 3-isobutyl-1-methylxanthine, pH 7.4) at 37°C in 0% CO$_2$ for 30 minutes. Formoterol hemifumarate (β_2AR-selective agonist; Tocris, UK) at indicated concentrations was then added for 10 minutes before cells were lysed with ice-cold 100% ethanol, evaporated and rehydrated with 50 μL detection buffer (0.1% BSA, 5 mM HEPES, 0.3% Tween20, pH 7.4). cAMP in cell lysates (5 μL) was then incubated with 1 unit of AlphaScreen™ acceptor beads (Perkin Elmer, USA) diluted in detection buffer, followed by incubation with 1 unit of donor beads. Fluorescence signal was measured using a Fusion plate reader (Perkin Elmer, USA). cAMP accumulation was expressed as pmol/well.

Tumor cell proliferation

Tumor cells (5×10^4) were seeded into a 12 well plate and serum-starved overnight. Cells were then treated with 1 μM isoproterenol (non-selective β-agonist Sigma, USA) in vehicle (DMEM supplemented with 2% FBS). Isoproterenol was replenished every 24 hours. Cell numbers were quantified using the Tali™ Image-Based Cytometer (Invitrogen, USA) at indicated time points.

Cell morphology assay

Tumor cells (5×10^3) were seeded into a 96 well plate and serum-starved overnight. Cells were then treated with or without 1 μM isoproterenol in the absence or presence of ICI-118,551 (β_2AR-selective antagonist, 1 μM, Tocris, UK) in DMEM supplemented with 2% FBS. Isoproterenol and ICI-118,551 were replaced every 24 hr to minimize possible effects of auto-oxidation. Cells were imaged using a 20× long WD objective every 24 hr for 3 days using the Operetta High-Content Imaging System (Perkin Elmer, USA). At each time point, nine random fields of view from each well were captured and analyzed using Harmony® High Content Imaging and Analysis Software (version 3.5, Perkin Elmer). The roundness of individual cells was assessed using the proprietary analytical tools provided in the Harmony Analysis software. The cell roundness parameter ranged between 0 and 1 where a value of 1 is equivalent to perfect roundness. The parameter threshold to define a mesenchymal-like cell was set at 0.5 (Ren et al., 2015; Yang and Weinberg, 2008). The proportion of mesenchymal-like tumor cells in the population meeting this criterion was then quantified.

Invadopodia assay

Gelatin was labelled with Alexa Fluor-568 protein labeling kit according to the manufacturer instructions. Culture vessels were coated with an 8:1 ratio of unlabeled to labeled 0.2% gelatin and topped with a thin layer of unlabeled 10 μg/mL human fibronectin to promote

cell adherence. MCF7-WT or MCF7-β_2AR cells were serum-starved overnight. Cells were then seeded onto the prepared cultured vessels containing 10% serum, ± βAR agonists (0.5 μM isoproterenol or 0.5 μM formoterol hemifumarate) for 5 hours. Cells were then fixed in 4 % paraformaldehyde, actin stained with phalloidin and nuclei counterstained with 1 μg/ml Hoechst 33242. Invadopodia were imaged on a Leica SP8 Confocal using a 63× PL APO CS2 1.4NA objective. Images were captured through LAS AF software version 3.2 (Leica Microsystems, North Ryde Australia). Images were prepared as Tiff stacks for actin and gelatin using ImageJ and submitted to the Invadopodia Analysis Server (IAS) and analysed as previously described (Creed et al., 2015).

Gene expression analysis

Tumor cells (7×10^5) were seeded into a 6 well plate and serum-starved overnight before treatment with vehicle, isoproterenol (1 μM), and/or ICI-118,551 (β_2AR-selective antagonist, 1 μM) in DMEM supplemented with 2% FBS for the time indicated, prior to analysis. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Germany). qRT-PCR was used to quantify gene expression in 100 ng of total RNA using Taqman probes (Applied Biosystems, USA) targeting human βAR subtypes (*ADRB1* Hs02330048_s1, *ADRB2* Hs00240532_s1, *ADRB3* Hs00609046_m1) and MMP2 (*MMP2* Hs01548727_m1) and iScript One-Step RT-PCR kit (Biorad, USA) with 50 PCR amplification cycles of 15 seconds of strand separation at 95°C and 30 seconds of annealing and extension at 60°C. Transcript levels for genes of interest were normalized relative to scramble control cells.

Tumor cell invasion assay

Serum-starved tumor cells (1.5×10^5) were suspended in 300 μL DMEM supplemented with 2% FBS with or without 1 μM isoproterenol and seeded into Transwell® chambers (8 μm pores, PET membrane, Sigma, USA) that were pre-coated with 100 μL Matrigel (2mg/mL, BD, USA). For 24 hr, cells were allowed to invade toward the bottom chamber, which was filled with media supplemented with 10% FBS with or without 1 μM isoproterenol. Membranes were then fixed with 4% paraformaldehyde (Sigma, USA) and stained with Hoechst 33342 (1:1000, Sigma, USA) and imaged using a Nikon Ti-E microscope, fitted with 10× Plan Apo 0.3NA objective and a Photometrics CoolSNAP Myo camera. Fluorescent excitation and emission was provided by a Nikon UV-A filter cube (excitation 355/50 and emission 420LP). Images were captured using NIS Elements software (version 4.3, Nikon Instruments). Invaded cells were counted using the Fiji distribution of ImageJ (Schindelin et al., 2012).

Breast cancer model with restraint stress

To investigate spontaneous metastasis from an orthotopic primary tumor, 2×10^5 tumor cells in 20 μL PBS (Invitrogen, USA) were injected into the left 4th mammary fat pad of anesthetized (3% isoflurane) 8 week-old female BALB/c *nu/nu* mice (University of Adelaide, Australia). Mice were housed under PC2 barrier conditions (temperature and humidity controlled, 12 h dark/light cycle) and acclimatized in a home cage for a week prior to tumor cell inoculation. Primary tumors were monitored by digital caliper twice a week and volume calculated using the formula: (length × width^2)/2. Mice were randomly assigned to either stress or non-stress group (n = 5 per group) and acclimatized in a home cage for a

week prior to tumor cell inoculation. To induce chronic stress, mice were subjected to a well-characterized restraint stress paradigm (Sloan et al., 2010{Le, 2016 #3; Thaker et al., 2006). Mice were placed in a ventilated Perspex chamber that restricted movement for 2 hours per day for a total of 21 days, commencing 7 days before tumor cell injection. Mice in the non-stress group remained in their home cage throughout the 2 hour period. Distant metastasis to lymph nodes and lung was monitored longitudinally twice weekly using bioluminescence imaging, which allows repeated measure analysis of metastatic burden in the same mouse throughout the experiment. Mice were injected with *d*-luciferin (150 mg/kg, CHOICE Analytical) via tail vein and imaged using an IVIS Lumina II (Perkin Elmer) (Le et al., 2016; Sloan et al., 2010). Mice were euthanized 28 days after tumor cell injection and tissue-specific metastasis was confirmed in lung and axillary lymph node by *ex vivo* bioluminescence imaging. *In vivo* experiments were conducted in duplicate. All procedures involving mice were carried out under protocols approved by the Institutional Animal Ethics Committee and in accordance with National Health and Medical Research Council animal ethics guidelines.

Statistical analysis

Longitudinal mixed-effect linear models were used to determine the effect of stress on the trajectory of metastasis, and to evaluate if those effects were modified by tumor β_2AR knockdown (Verbeke and Molenberghs, 2009). We examined the stress×treatment (tumor cell β_2AR knockdown) interaction in a 2 (non-stress vs. stress) × 3 (scramble control vs. shADRB2a vs. shADRB2b) experimental design. Data were analyzed according to the model $Y_{ij} = \beta_1 d_{i1} t_{ij} + \beta_2 d_{i2} t_{ij} + \beta_3 d_{i3} t_{ij} + \beta_4 d_{i4} t_{ij} + \beta_5 d_{i5} t_{ij} + \beta_6 d_{i6} t_{ij} + \epsilon_{ij}$, where: Y_{ij} are mouse-specific and day-specific primary tumor volume or luciferase signals (Fig. 4a) for the ith mouse on the logarithmic scale; t_{ij} is the time (days of follow up), d_{ij} (j=1,…,6) are binary variables so that $d_{ij} = 1$ if the ith mouse belongs to the jth group and 0 otherwise; ϵ_{ij} is the random error term. Linear models were fitted using the routine lm while multiple comparisons used the routine glht in the package multicomp (Bretz et al., 2010). The effect of stress on the trajectory of metastasis or primary tumor volume was calculated using the difference in gradient of fitted lines between stress and non-stress conditions for each tumor cell type. Pearson's correlation analysis was performed to assess the relationship between the effect of stress on the trajectory of primary tumor volume and the effect of stress on the trajectory of metastasis. For *ADRB* expression in MCF7-WT and MCF7-β_2AR cells, unpaired t-tests were used to compared each βAR subtype between the different cell lines. For cell proliferation and cell morphology assays, the effect of isoproterenol on cell proliferation or mesenchymal-like phenotype of each cell lines were compared using one-way ANOVA repeated measures followed post hoc Tukey's planned comparison tests when applicable. For all other experiments, a two-way ANOVA test was first performed to identify the presence of interaction between treatments. Post hoc Tukey's planned comparison tests were performed when applicable, to test the simultaneous differences between pairs of treatment effects on experimental responses (*MMP2* gene expression, *in vitro* invasion, number of invadopodia, *ex vivo* bioluminescence signals and primary tumor mass). Experiments were conducted 2–4 times. Treatment effects showing *p*-values smaller than 0.05 were regarded as statistically significant. Statistical analysis was carried out in both R

computing environment (Team, 2014) and Prism 6 software (GraphPad Software, La Jolla, CA, USA).

RESULTS

Generation of MDA-MB-231^{HM} cells deficient in β$_2$AR

We have previously shown that β$_2$AR is the only functional βAR subtype in MDA-MB-231^{HM} cells (Pon et al., 2016). Therefore, we used two different hairpin sequences that target the gene encoding β$_2$AR (*ADRB2*) to knock down expression of β$_2$AR in MDA-MB-231^{HM} breast cancer cells, and generated shADRB2a and shADRB2b cell lines. To examine the efficiency of *ADRB2* silencing, we used qRT-PCR to quantify the expression of *ADRB2* in shADRB2a and shADRB2b cells relative to control cells transduced with a scrambled shRNA sequence (scramble control). *ADRB2* expression was reduced by > 50% in both shADRB2a and shADRB2b cells compared to scramble control cells ($p < 0.01$) (Fig. 1a). *ADRB1* expression was not affected by shRNA transduction, and *ADRB3* expression was not detected in these cell lines (Fig. 1a), consistent with our previous findings (Creed et al., 2015; Pon et al., 2016). Stable knockdown was confirmed with reduction of the *ADRB2* transcript maintained even after 6 weeks in culture (data not shown).

To investigate the effect of *ADRB2* knockdown on receptor function, we evaluated cAMP accumulation as an indicator of receptor signaling. Cells were stimulated with the β$_2$AR-selective agonist formoterol, which induced cAMP accumulation in scramble control cells ($E_{max} = 0.29 \pm 0.06$ pmol/well) (Fig. 1b). However, the cAMP response was blunted in β$_2$AR-deficient cell lines (shADRB2a: $E_{max} = 0.13 \pm 0.02$ pmol/well; shADRB2b: $E_{max} = 0.21 \pm 0.04$ pmol/well) (Fig. 1b). Reduction of the cAMP response in β$_2$AR-deficient cell lines was also demonstrated by a need for a greater concentration of formoterol to reach the EC_{50} (50% of the maximal response, control cells: 13.30 ± 9.25 nM vs. shADRB2a: 63.94 ± 38.67 nM; shADRB2b: 32.58 ± 13.21 nM) (Fig. 1b).

To determine if *ADRB2* knockdown affected tumor cell proliferation, we compared the *in vitro* proliferative activity of β$_2$AR-deficient cells to scramble control cells. β$_2$AR knockdown did not modulate the proliferation of tumor cells, either under baseline conditions (Fig. 1c), or after treatment with the non-selective βAR agonist isoproterenol (Supplementary Fig. 1). Collectively, these findings confirmed the generation of MDA-MB-231^{HM} tumor cell lines with reduced levels of *ADRB2* transcription and blunted β$_2$AR signaling, and without compromised tumor cell proliferative capability.

Characterization of tumor cell invasiveness in MDA-MB-231^{HM} cells deficient in β$_2$AR

Our previous studies using pharmacological modulation of β$_2$AR found a crucial role for tumor cell β$_2$AR signaling in promoting invadopodia formation and tumor cell invasion (Creed et al., 2015; Pon et al., 2016). To examine if genetic knockdown of β$_2$AR similarly affected tumor cell invasive capacity, we first assessed the effect on *MMP2* expression, as a transcriptional readout of tumor cell invasion. Treatment with the βAR agonist isoproterenol increased *MMP2* expression in scramble control tumor cells (black bars, Fig. 2a), consistent with previous findings that βAR signaling regulates *MMP* expression (Yang et al., 2006), but

the effect was attenuated in β_2AR knockdown cells (grey bars, Fig. 2a). Pharmacological blockade of β_2AR signaling using the β_2AR-selective antagonist ICI-118,551 abolished the effect of isoproterenol on *MMP2* expression in scramble control cells, providing pharmacological confirmation that *MMP2* expression is sensitive to β_2AR signaling (Fig. 2a). Of note, ICI-118,551 further reduced *MMP2* expression in isoproterenol-treated β_2AR-deficient cells to baseline levels (Fig. 2a), indicating that the incomplete knockdown of β_2AR (Fig. 1a) may be responsible for the residual *MMP2* expression observed in isoproterenol-treated knockdown cell lines (Fig. 2a).

To examine if β_2AR knockdown also affected tumor cell morphology, we used high-content screening to investigate the effect of β_2AR knockdown on MDA-MB-231^{HM} cell morphology. Treatment of scramble control cells with the βAR agonist isoproterenol (vs. vehicle) *in vitro*, steadily increased the proportion of mesenchymal-like cells over 3 days of treatment (black line, Fig. 2b). In contrast, β_2AR knockdown attenuated the effect of isoproterenol on mesenchymal morphology at day 3 ($p < 0.001$ for shADRB2a, $p < 0.05$ for shADRB2b; grey lines, Fig. 2b and 2c). Additionally, pharmacological blockade of β_2AR signaling using the β_2AR-selective antagonist ICI-118,551 abolished the effect of isoproterenol on mesenchymal morphology in scramble control cells (Supplementary Fig. 2a and 2b), pharmacologically confirming a role for β_2AR in tumor cell mesenchymal phenotype.

Morphological transition from epithelial-like to a mesenchymal-like phenotype has been linked with increased tumor cell invasiveness. To examine whether β_2AR knockdown also impaired the functional invasiveness of tumor cells, we treated MDA-MB-231^{HM} cells with the non-selective βAR agonist isoproterenol and examined tumor cell invasion through a basement membrane using a transwell invasion assay. Isoproterenol increased invasion in scramble control cells by 30-fold ($p < 0.05$) (Fig. 2d). However, this effect of isoproterenol was abrogated in tumor cells deficient for β_2ARs (Fig. 2d), confirming that β_2AR signaling in MDA-MB-231^{HM} tumor cells drives invasion. Collectively, these findings confirm that genetic knockdown of β_2AR modulates the tumor cell invasive phenotype in a way that replicates pharmacological β_2AR blockade (Creed et al., 2015; Pon et al., 2016).

MMPs are localized at subcellular structures called invadopodia that regulate invasion of tumor cells (Murphy and Courtneidge, 2011). We found previously that β_2AR regulation of invasion is mediated by formation of invadopodia (Creed et al., 2015). To independently validate the findings that genetic modulation of β_2AR affects tumor cell invasion, we overexpressed β_2AR in MCF-7 breast cancer cells which have low endogenous levels of β_2AR, compared to MDA-MB-231^{HM} cells (Fig. 3a), and investigated the effect on formation of invadopodia. MCF-7 cells were transduced with a construct encoding a β_2AR-GFP fusion protein and β_2AR overexpression was confirmed at both the transcript and protein level using qRT-PCR and fluorescence microscopy, respectively (Fig. 3b and 3c). Tumor cell invasive capacity was assessed by formation of invasive invadopodia structures that degrade underlying extracellular matrix (Fig. 3c). Treatment with the β_2AR-selective agonist formoterol or the non-selective agonist isoproterenol induced formation of invadopodia in cells that overexpressed β_2AR, but not in wildtype MCF-7 cells (Fig. 3d). Collectively, these *in vitro* findings confirm that genetically modulating β_2AR by either

over-expression or knockdown, affects isoproterenol-induced tumor cell invasiveness, as demonstrated by transcriptional, morphological and functional readouts (Fig. 2 and Fig. 3).

β₂AR-driven tumor cell invasion contributes to stress-enhanced metastasis *in vivo*

Having established that knockdown of β₂AR in MDA-MB-231[HM] cells limits βAR-regulated invasion *in vitro* (Fig. 2d) without compromising proliferation (Fig. 1c), we investigated whether knockdown of β₂AR in tumor cells also modulates the effect of stress on metastatic dissemination from a primary tumor *in vivo*. In the complex environment of the tumor, many stromal cell populations can also respond to β₂AR signaling, which plausibly may offset the effect of β₂AR-knockdown in tumor cells on metastasis. To model breast cancer, scramble control tumor cells or β₂AR knockdown tumor cells were injected into the mammary fat pad of mice and bioluminescence imaging was used to non-invasively quantify the onset and kinetics of metastasis to distant organs (Fig. 4a). Longitudinal analysis of spontaneous dissemination of metastatic tumor cells from the primary tumor found that under baseline (non-stress) conditions, knockdown of β₂AR had no effect on metastasis (Fig. 4a solid lines, and 4b). By day 28 of tumor progression, in mice with tumors derived from scramble control tumor cells, chronic restraint stress increased metastasis 10-fold, compared to non-stress conditions (Fig. 4a), consistent with previous findings (Lamkin et al., 2015; Le et al., 2016; Sloan et al., 2010). This overall increase in metastasis occurred as a consequence of a daily $9.9\% \pm 0.77\%$ increase in metastatic burden for the duration of the experiment (Fig. 4c). Significantly, knockdown of *ADRB2* in tumor cells reduced this cumulative effect of stress on metastasis, with mice bearing shADRB2a or shADRB2b tumors displaying a reduction in stress-enhanced metastasis by $61.41\% \pm 12.25\%$ ($p < 0.001$) and $85.86\% \pm 13.30\%$ ($p < 0.001$) respectively, compared to mice with scramble control tumors (Fig. 4a–c).

To evaluate the effect of β₂AR knockdown on tumor burden in metastatic target organs, we used *ex vivo* bioluminescence imaging to quantify metastatic tumor cells that had spread from the primary mammary tumor to lymph node and lung. *Ex vivo* analysis confirmed that β₂AR knockdown in MDA-MB-231[HM] tumor cells blocked stress from increasing metastasis to both lymph node and lung ($p = 0.001$, versus scramble control tumor cells) (Fig. 4d and 4e).

The rate of primary tumor growth is highly prognostic for the extent of tumor cell spread (Weigelt et al., 2005). Therefore, the effects of β₂AR signaling on metastasis may have occurred secondary to the effects on the primary tumor. In fact, assessment of primary tumor mass on day 28 of tumor growth revealed that stress produced a small, but significant, increase in primary tumor growth regardless of the β₂AR status of tumors ($p < 0.05$; Fig. 5a). We used regression analysis to evaluate the relationship between the effect of stress on primary tumor growth rate and the effect of stress on metastasis. Interestingly, the effect of stress on the primary tumor did not predict the effect of stress on metastasis in control tumor cells with intact β₂AR signaling (Fig. 5b). However, knockdown of β₂AR resulted in the expected positive correlation between primary tumor growth rate and trajectory of metastatic burden (Fig. 5c and 5d). Therefore, when β₂AR is present on tumor cells, stress drives metastasis independently of primary tumor growth. Collectively, these findings demonstrate

that even in the complex tumor microenvironment where multiple cell populations may respond to βAR signaling, $β_2$AR-driven tumor cell invasion significantly contributes to the effects of stress on accelerating metastasis. Furthermore, these findings demonstrate the feasibility of targeting $β_2$AR-driven tumor cell invasion to inhibit the adverse effects of stress on breast cancer metastasis.

DISCUSSION

Despite major advances in cancer treatment, metastasis remains a significant clinical challenge in the breast cancer clinic. We and others have shown that chronic stress acts through βAR signaling to drive metastasis (Kim-Fuchs et al., 2014; Le et al., 2016; Liu et al., 2015; Sloan et al., 2010; Thaker et al., 2006; Zhao et al., 2015), and our previous *in vitro* findings have demonstrated that $β_2$AR signaling in cancer cells enhances invadopodia formation and promotes tumor cell invasion *in vitro* (Creed et al., 2015; Pon et al., 2016). However, the contribution of direct activation of $β_2$AR signaling in tumor cells (rather than through effects on the tumor stroma) to metastatic dissemination from a primary tumor is unclear. Here, we extend our understanding by showing that $β_2$AR signaling in human MDA-MB-231[HM] breast cancer cells contributes to the deleterious effects of stress on metastatic dissemination from the complex microenvironment of a primary mammary tumor. We found that silencing $β_2$AR expression in MDA-MB-231[HM] breast cancer cells blocked the effect of stress on metastasis *in vivo*, which identifies tumor cells as an additional *in vivo* target of stress-induced βAR signaling. These results expand on earlier findings that demonstrated a role for stromal cells in the tumor microenvironment in the adverse effects of stress on metastasis, and suggests that βAR signaling in both tumor cells and stromal cells contributes to the effect of stress on metastasis. This raises the possibility that inhibiting stress signaling to either tumor cells (as shown here) or to the tumor stroma (Le et al., 2016; Sloan et al., 2010) may be sufficient to modulate metastasis.

Invasion is one of the critical steps along the metastatic cascade that determines successful metastatic outgrowth. Notably, incomplete knockdown of $β_2$AR in MDA-MB-231[HM] tumor cells (Fig. 1a) was sufficient to diminish stress-enhanced metastasis (Fig. 4a and 4c), suggesting that even partial modulation of tumor cell $β_2$AR may be sufficient to slow cancer progression. We also showed that the effects of stress-induced tumor $β_2$AR signaling on metastasis was independent of the effect on primary tumor size (Fig. 5b–d). These results extend our previous findings that pharmacological modulation of tumor cell $β_2$AR regulates tumor cell invasion *in vitro* (Creed et al., 2015; Pon et al., 2016), by showing that $β_2$AR signaling in tumor cells also plays a key role in driving metastasis *in vivo*.

It is possible that $β_2$AR signaling may regulate an epithelial-to-mesenchymal transition in tumor cells to increase metastatic invasion and dissemination. Here, we observed that isoproterenol treatment shifted MDA-MB-231[HM] cells towards a mesenchymal morphology (Fig 2a), an effect that was attenuated by $β_2$AR knockdown (Fig. 2a). It will be important to characterize if $β_2$AR agonism induces similar behavior in other tumor cell lines and *in vivo*. Notably, several recent *in vitro* studies suggest that βAR signaling induces an epithelial-mesenchymal transition in gastric, colon and lung cancer cell lines (Lu et al., 2015; Shan et al., 2014; Zhang et al., 2016). To better understand βAR regulation of epithelial-

mesenchymal transition it will be necessary to clarify the molecular mechanisms. While we found no evidence that βAR signaling regulates expression of the mesenchymal marker vimentin in MDA-MB-231[HM] breast cancer cells (data not shown), it will be important to evaluate other molecules that are known to influence epithelial-mesenchymal transition, including SNAIL and TWIST1. Such studies should control for catecholamine-induced auto-oxidation, which may also induce morphological changes.

While the *in vivo* findings described here are limited to the contribution of tumor cell β_2AR in metastatic dissemination in the MDA-MB-231[HM] model of breast cancer, findings that stress also increases metastasis in models of ovarian cancer (Thaker et al., 2006), pancreatic cancer (Kim-Fuchs et al., 2014), and colorectal cancer (Liu et al., 2015; Zhao et al., 2015), suggest that it will be important to examine the role of tumor cell β_2AR in metastatic dissemination of other tumor types. Additionally, while these results highlight βAR-regulated tumor cell invasion as a significant contributor to cancer progression, they do not exclude a role for βAR signaling to stromal cells in the adverse effects of stress on metastasis (Le et al., 2016; Sloan et al., 2010; Thaker et al., 2006). It is likely that the beneficial effects of βAR-targeted therapies (e.g., β-blockers) on slowing metastasis are due to effects on both tumor cells and stromal cells. Myeloid cells are sensitive to βAR signaling (Le et al., 2016; Powell et al., 2013), which leads to their accumulation in primary tumors where they remodel blood and lymphatic vasculature to provide additional routes for tumor cell dissemination (Le et al., 2016; Sloan et al., 2010; Thaker et al., 2006). To fully characterize the interactions between tumor cells and stromal cells that mediate the effects of stress on metastasis, it will be valuable to use additional strategies including *ADRB2* knockout mice and cell-specific *ADRB2* deletion mutants to probe the contributions of various cell types in the tumor microenvironment to stress-induced cancer progression.

There are three subtypes of βAR that are expressed on both stroma cells and tumor cells (Badino et al., 1996; Du et al., 2014; Reeder et al., 2015; Vandewalle et al., 1990). While β_2AR is the only functional βAR subtype in MDA-MB-231[HM] cells (Creed et al., 2015; Pon et al., 2016), other cancer cells have been shown to express β_1AR and β_3AR subtypes (Dal Monte et al., 2013; Kim-Fuchs et al., 2014; Magnon et al., 2013). However, the role of these receptor subtypes in cancer progression is only starting to be elucidated (Magnon et al., 2013). As the use of β-blockers to slow metastasis is clinically translated, it will be important to investigate the role of other βAR subtypes in cancer progression. Second generation β-blockers were developed for increased cardio-specificity by increasing their selectivity for β_1ARs, and are now more commonly used than older generation non-selective β-blockers (Salpeter, 2003). Our findings that β_2ARs on MDA-MB-231[HM] tumor cells also contribute to metastasis suggest that some breast cancers may be more effectively targeted with antagonists that also inhibit β_2ARs (e.g., propranolol). This is supported by a recent retrospective epidemiological study that found the use of non-selective β-blockers that target both β_1AR and β_2AR, but not β_1AR-selective blockers, was associated with improved cancer outcome (Barron et al., 2011). These findings provide insights into the contribution of tumor cell β_2AR signaling in stress-enhanced cancer progression and further support the use of β-blockers to modulate metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

β_2-adrenoceptors on MDA-MB-231^{HM} breast cancer cells contribute to stress-enhanced metastasis

β_2-adrenoceptor signaling regulates a shift to mesenchymal morphology in MDA-MB-231^{HM} breast cancer cells

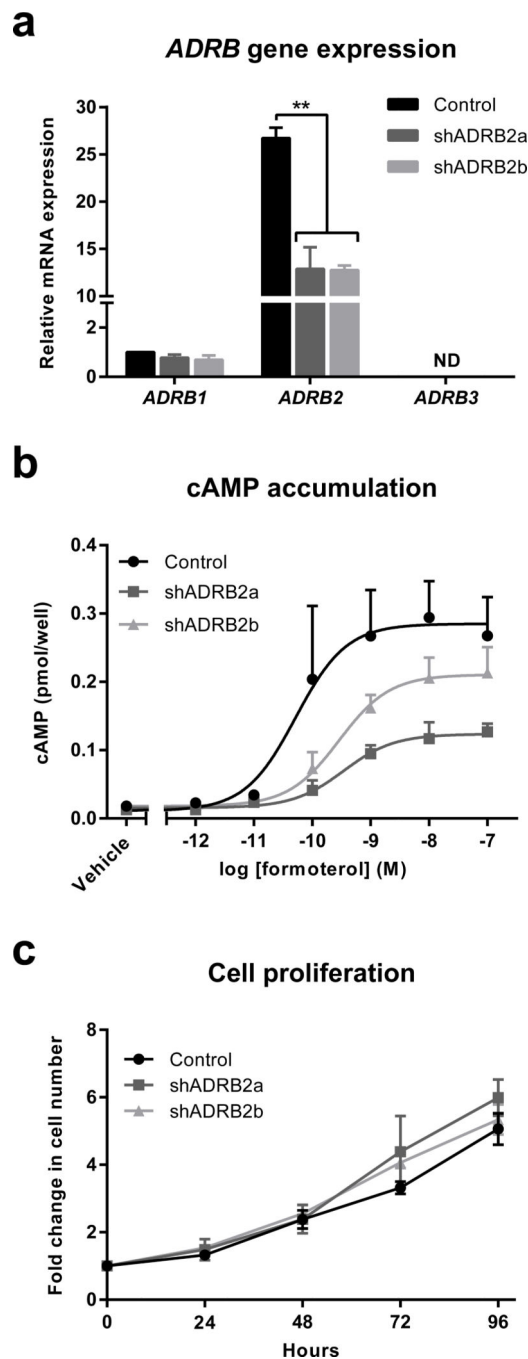Genetic modulation of β_2-adrenoceptor on breast cancer cells regulates invasion

Fig. 1. Generation of MDA-MB-231^{HM} breast cancer cells deficient in β_2AR
(a) Quantification of *ADRB* mRNA transcript levels using qRT-PCR in MDA-MB-231^{HM} cells transduced with shRNA scramble sequence (control) or shRNA targeting *ADRB2* (shADRB2a and shADRB2b) (n = 4). ND: not detected. (b) Quantification of cAMP accumulation in scramble control, shADRB2a and shADRB2b tumor cells in response to increasing concentrations of the β_2AR selective-agonist formoterol (n = 3). (c) Proliferation of control, shADRB2a and shADRB2b tumor cells (n = 3). Data represent mean ± standard error. ** $p < 0.01$ by one-way ANOVA with *post hoc* Tukey's planned comparison tests.
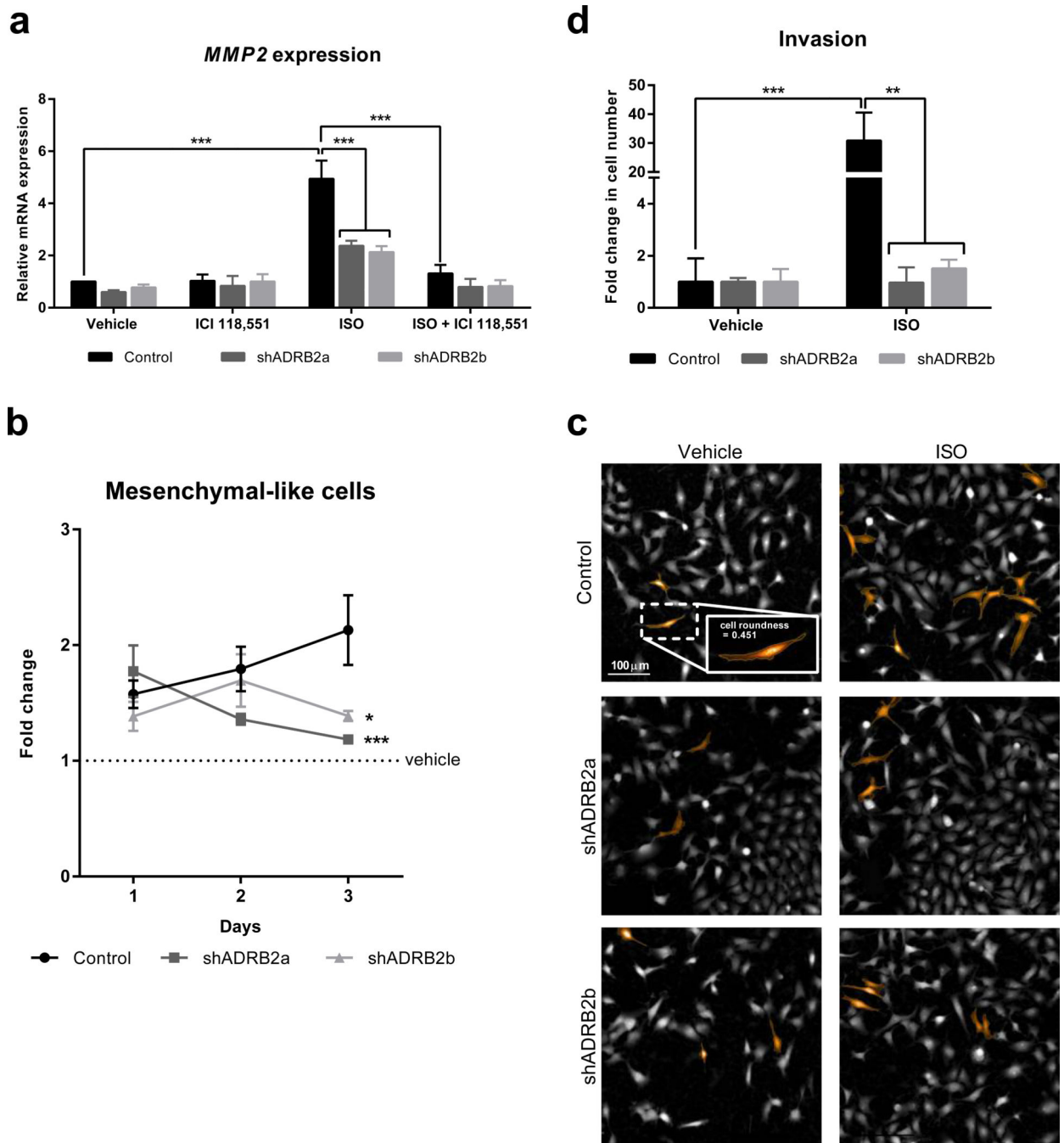
a

MMP2 expression

d

Invasion

b

Mesenchymal-like cells

c

Fig. 2. Characterization of tumor cell invasiveness in β₂AR-deficient MDA-MB-231^HM breast cancer cells

(a) Quantification of _MMP2_ mRNA transcript levels using qRT-PCR in scramble control, shADRB2a and shADRB2b tumor cells treated with vehicle, isoproterenol (non-selective βAR agonist, ISO, 1 μM) and/or ICI-118,551 (β₂AR-selective antagonist, 1 μM) ($n = 3 – 5$). (b) Fold change in proportion of tumor cells that exhibit mesenchymal-like morphology (defined by cell roundness < 0.5) in response to ISO (1 μM, normalized to vehicle) ($n = 5 – 6$). (c) Representative images of mesenchymal-like cells (roundness < 0.5) highlighted in orange, in cultures of scramble control, shADRB2a, shADRB2b cells after treatment with

vehicle or 1 μM ISO. Inset: a magnified view of a mesenchymal-like tumor cell. Scale bar: 100 μm). (d) Quantification of invasion through transwells by scramble control, shADRB2a and shADRB2b tumor cells in response to ISO (1 μM) treatment ($n = 4$). Data represent mean ± standard error. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ by (b) one-way ANOVA with repeated measures or (a and d) two-way ANOVA with *post hoc* Tukey's planned comparison tests. # $p < 0.05$, ### $p < 0.001$ for ISO vs. ISO + ICI-118551 within each cell line.
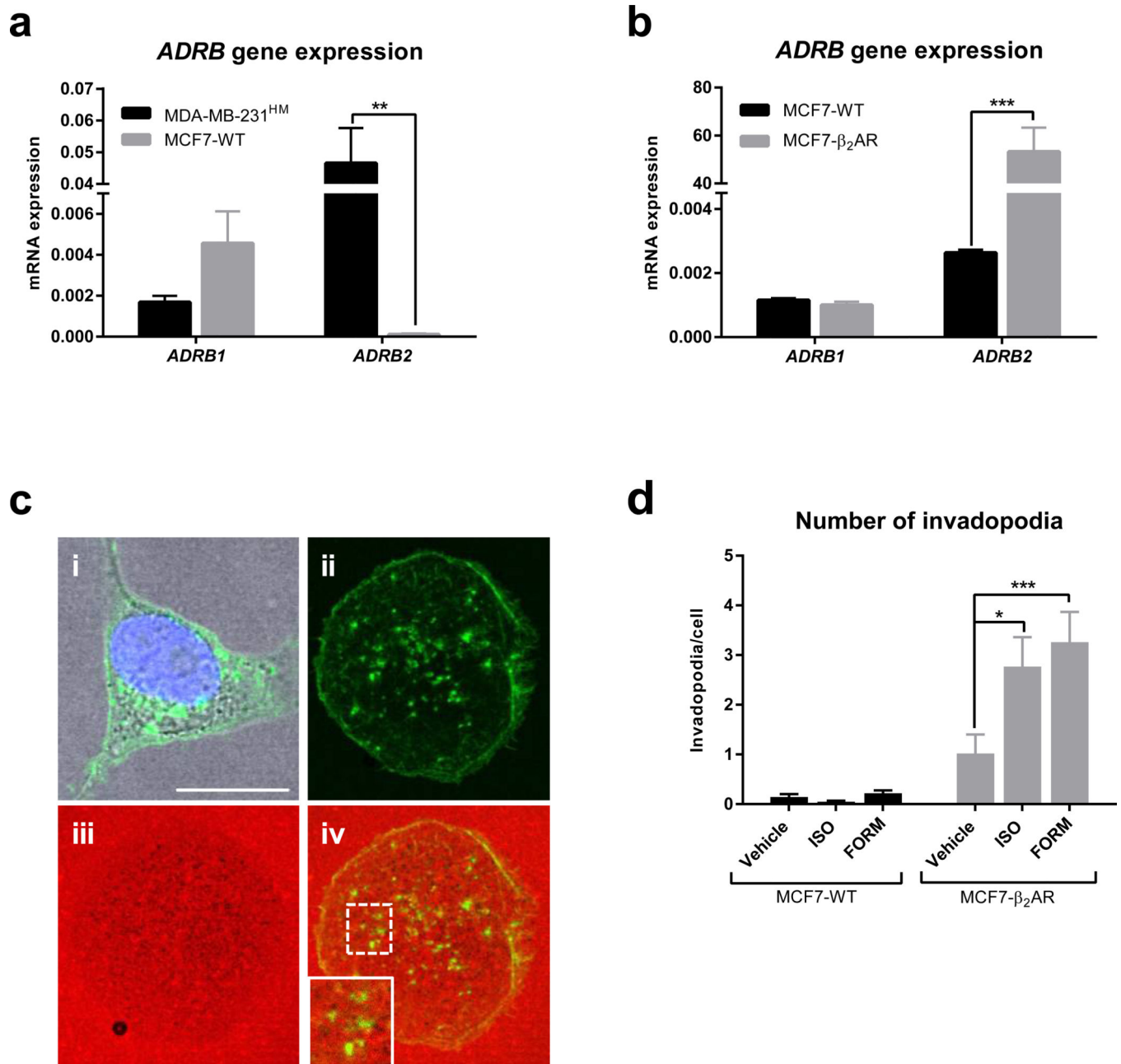
Fig. 3. Overexpression of β_2AR in MCF-7 cells increases tumor cell invasiveness
(a and b) Quantification of *ADRB* mRNA transcript levels using qRT-PCR in (a) MDA-MB-231[HM] and MCF-7 wildtype (MCF7-WT) cells ($n = 3$) and (b) MCF7-WT and MCF-7 overexpressing β_2AR (MCF7-β_2AR) cells ($n = 3$). Normalized to *ACTB* levels. (c) Representative images of invadopodia formation assay. Panel i. A cell showing β2AR-GFP (green). Panels ii–iv. A cell showing localization of actin foci (green, ii), matrix degradation (grey patches of degradation on red-fluorescent gelatin, iii), and merged image with inset panel showing active invadopodia that have degraded matrix (iv) Scale bar: 10 μm. (d) Quantification of invadopodia per cell in MCF7-WT cells and MC7-β_2AR cells treated with vehicle, isoproterenol (non-selective βAR agonist, ISO, 0.5 μM) or formoterol (β_2AR-

selective agonist, FORM, 0.5 μM) ($n > 80$ cells per condition). Data represent mean ± standard error. $*p < 0.05$ and $***p < 0.001$ by (a and b) unpaired t-tests or (d) one-way ANOVA with *post hoc* Tukey's planned comparison tests.
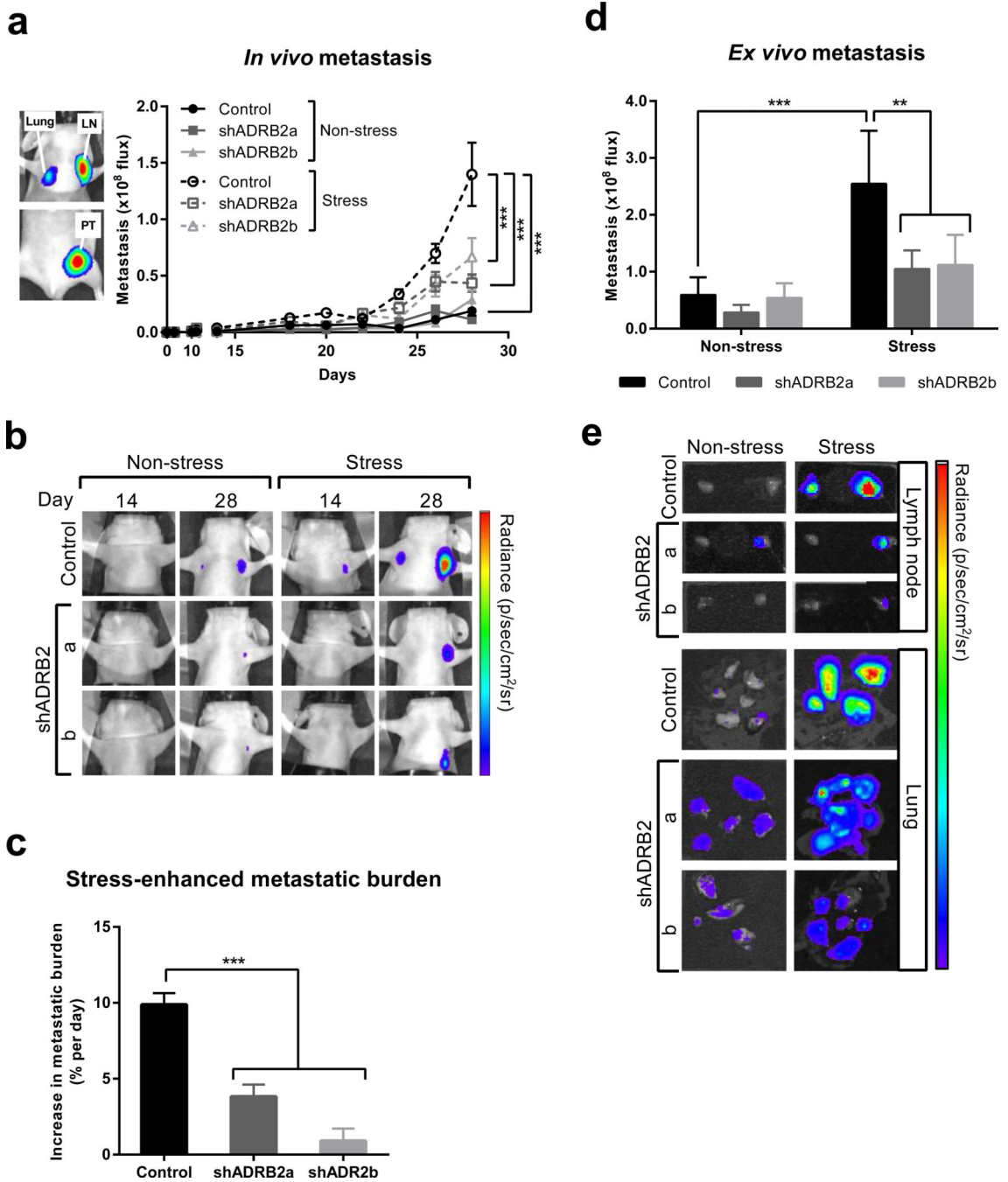
Fig. 4. β_2AR knockdown in MDA-MB-231^{HM} breast cancer cells impairs metastasis *in vivo*
(a) Left panel: Representative *in vivo* bioluminescence image of the orthotopic MDAMB-231 breast cancer model showing primary tumor (PT) and spontaneously disseminated metastasis in lymph node (LN) and lung 28 days after tumor cell injection. Right panel: Quantification of distant metastasis by bioluminescence imaging in mice with tumors derived from scramble control or β_2AR-deficient cells (shADRB2a and shADRB2b). Mice were exposed to non-stress or stress conditions. ($n = 5$ at each time point). (b) Representative images of metastasis *in vivo* at day 14 and 28 after tumor cell injection. Note:

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the primary tumor is not visible. Scale: min. 3×10^6 photons/sec; max. 1×10^8 photons/sec. (c) The magnitude of the effect of stress on metastatic burden was computed as the difference in the rate of metastatic progression under non-stress and stress conditions. (d) *Ex vivo* quantification of distant metastasis in target organs that were harvested from mice 28 days after tumor cell injection (n = 5). (e) Representative images of metastasis in lung and lymph node *ex vivo* at 28 days post tumor cell inoculation. Scale: lymph node: min. 5×10^6; max. 1×10^8 and lung: min. 2×10^5; max. 8×10^6 photons/sec. Data represent mean ± standard error. $*p < 0.05$ and $***p < 0.001$ by (c) one-way ANOVA or two-way ANOVA (a) with or (d) without repeated measures followed by *post hoc* Tukey's planned comparison tests.
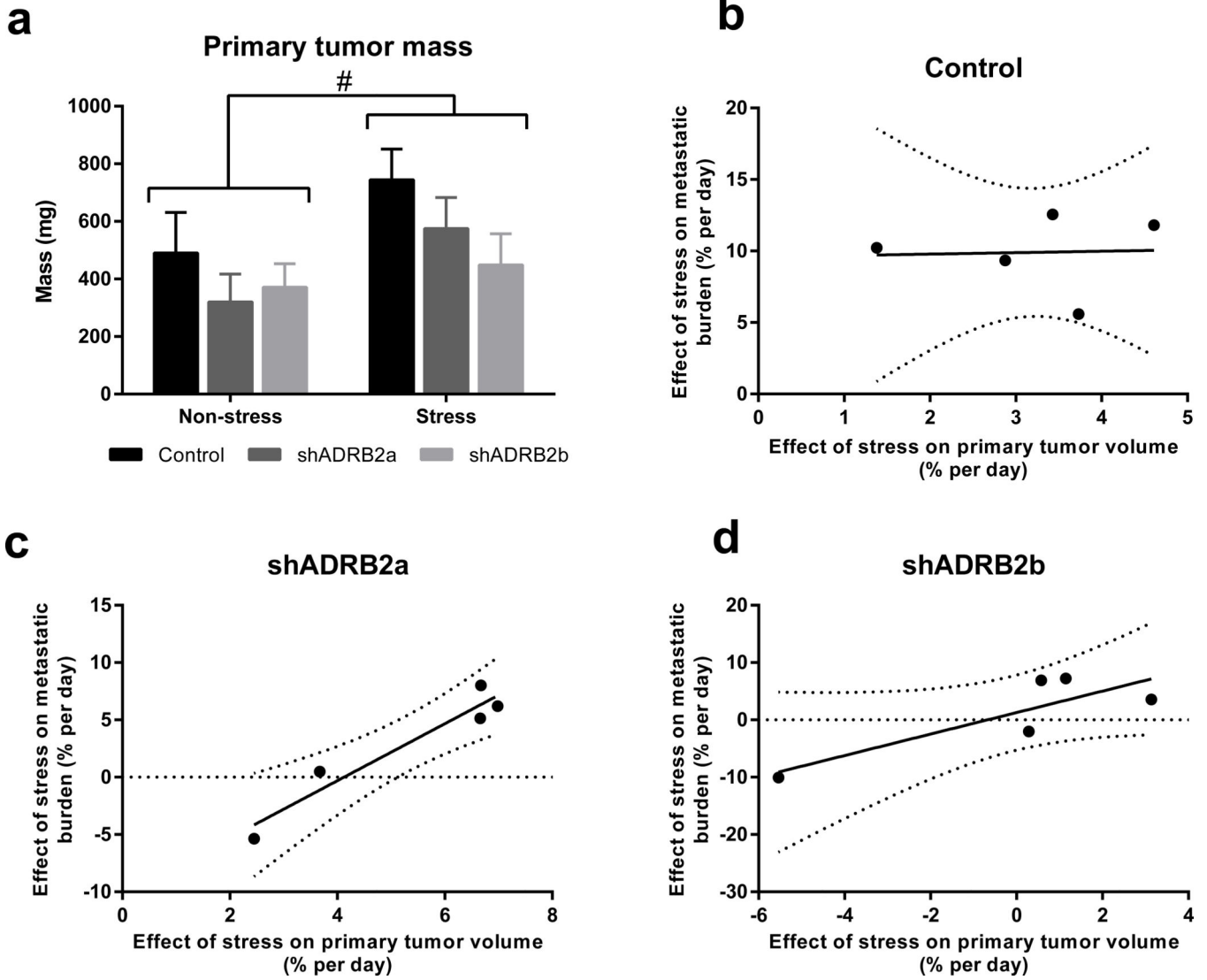
Brain Behav Immun. Author manuscript; available in PMC 2017 October 01.

Fig. 5. Stress-enhanced metastasis is independent of primary tumor size

(a) Mass of tumors derived from control or β_2AR-deficient cells, from mice exposed to non-stress or stress conditions ($n = 5$). (b – d) Pearson correlation analysis of the relationship between the effect of stress on the trajectory of metastasis and the effect of stress on the trajectory of primary tumor growth in mice bearing (b) control ($r^2 = 0.001938$, $p = 0.944$), (c) shADRB2a ($r^2 = 0.9224$, $p = 0.0094$), and (d) shADRB2b ($r^2 = 0.6995$, $p = 0.0775$) tumors. $\#p < 0.05$ for main stress effect by two-way ANOVA.