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ORIGINAL PAPER

Tumour but not stromal expression of β3 integrin is essential, and is required early, for spontaneous dissemination of bone-metastatic breast cancer

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Abstract

Although many preclinical studies have implicated β3 integrin receptors (αvβ3 and αIIbβ3) in cancer progression, β3 inhibitors have shown only modest efficacy in patients with advanced solid tumours. The limited efficacy of β3 inhibitors in patients could arise from our incomplete understanding of the precise function of β3 integrin and, consequently, inappropriate clinical application. Data from animal studies are conflicting and indicate heterogeneity with respect to the relative contributions of β3-expressing tumour and stromal cell populations in different cancers. Here we aimed to clarify the function and relative contributions to metastasis of tumour versus stromal β3 integrin in clinically relevant models of spontaneous breast cancer metastasis, with particular emphasis on bone metastasis. We show that stable down-regulation of tumour β3 integrin dramatically impairs spontaneous (but not experimental) metastasis to bone and lung without affecting primary tumour growth in the mammary gland. Unexpectedly, and in contrast to subcutaneous tumours, orthotopic tumour vascularity, growth and spontaneous metastasis were not altered in mice null for β3 integrin. Tumour β3 integrin promoted migration, protease expression and trans-endothelial migration *in vitro* and increased vascular dissemination *in vivo*, but was not necessary for bone colonization in experimental metastasis assays. We conclude that tumour, rather than stromal, β3 expression is essential and is required early for efficient spontaneous breast cancer metastasis to bone and soft tissues. Accordingly, differential gene expression analysis in cohorts of breast cancer patients showed a strong association between high β3 expression, early metastasis and shorter disease-free survival in patients with oestrogen receptor-negative tumours. We propose that β3 inhibitors may be more efficacious if used in a neoadjuvant setting, rather than after metastases are established.

Keywords: breast cancer; β3 integrin; bone metastasis; syngeneic mouse model; vitronectin; DisBa-01

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No conflicts of interest were declared.

Introduction

β3 integrins (αvβ3 and αIIbβ3) mediate cellular adhesion to extracellular matrix (ECM) substrates, including vitronectin, bone sialoprotein, osteopontin and fibrinogen, and are attractive therapeutic targets for metastatic cancers [1]. Studies employing αv or β3 inhibitors demonstrate that αvβ3 integrin regulates multiple cellular responses required for metastasis, including cell survival, migration, invasion through the ECM and angiogenesis [2]. However, while high β3 integrin expression is reported in several cancer types [3–8], its prognostic significance is still unclear. Tumour expression of αvβ3 integrin correlates inversely with invasive and metastatic behaviours in some melanoma and ovarian cancer lines [9,10] and is associated with better survival in ovarian cancer patients [10]. Enhanced αvβ3 levels in bone metastases compared to matched

primary breast tumours have been reported in some [4,11], but not all [5], studies.

Preclinical studies evaluating the function of αvβ3 in breast cancer bone metastasis have been limited by lack of robust and clinically relevant animal models. High levels of αvβ3 in a bone metastatic subline of human MDA-MB-231 breast tumour cells (BO2) [12], or exogenous expression of β3 integrin in parental cells [5], correlate with increased adhesion to cortical bone and an increased number of osteolytic lesions in mice compared to parental cells, following tail vein injection. We showed previously that exogenous expression of β3 integrin in weakly metastatic 66 cl4 mammary carcinoma cells, that otherwise do not express αvβ3 or spread to bone from the mammary gland, is sufficient to promote their spontaneous metastasis to bone without altering orthotopic tumour growth in immunocompetent mice [13]. While the above studies employing β3 over-expression showed that tumour αvβ3 integrin can 'contribute' to bone metastasis, whether expression of αvβ3 is 'essential' for spontaneous bone metastasis has yet to be demonstrated. Clinically, this distinction is important, since the effectiveness of therapies targeting tumour αvβ3 and their impact on bone metastasis and patient survival will be dictated by the dependency of tumours on αvβ3 integrin for successful metastasis. To our knowledge, the efficacy of αvβ3 integrin antagonists specifically against bone metastases has not been evaluated in patients.

Discrepancies also exist with regard to the precise contribution of β3 integrin expressed on stromal lineages to tumour growth and metastasis [14–20]. MMTV-c-neu transgenic mice null for either β3 or β3 and β5 integrins show no apparent changes in mammary tumour growth and vascularization compared to normal MMTV-c-neu mice [20]. This contrasts with the anti-tumour/anti-angiogenic effects of αv or β3 inhibitors against subcutaneous melanoma and breast tumours [21–23] and with the enhanced growth and angiogenesis of transplanted melanoma, lung and colon tumours in β3-null mice [16,19]. Moreover, while experimental melanoma metastasis to bone is decreased in β3-null compared to wild-type mice [17], loss of β3 integrin has no impact on the spontaneous metastasis of mammary tumours to lung in MMTV-c-neu mice null for β3 [20]. Importantly, since none of these models metastasize spontaneously to bone, the role of stromal β3 integrin in spontaneous metastasis to bone remains unknown. Collectively, these observations, derived from various animal models of metastasis, indicate that regulation of tumour growth, vascularization and metastasis by tumour and stromal β3 integrin is likely to vary between tumour types and sites of tumour growth. These differences may account for the limited efficacy of integrin inhibitors in advanced cancer patients with solid tumours [2,24–34]. Accordingly, improvement in the efficacy of β3 integrin inhibitors in patients with metastatic cancer may require a reappraisal of the precise contribution of β3-type receptors to the growth and metastasis of each tumour type.

Here, using clinically relevant mouse models of breast cancer metastasis to bone [35–37], a combination of *in vitro* assays, gene knockdown and β3-null mice, we demonstrate that β3 integrin in tumour cells, but not in stromal cells, is essential for spontaneous breast cancer metastasis and is required early for vascular dissemination to bone and other tissues. These findings have important implications for the design of therapies targeting β3 integrin in breast cancer patients.

Materials and methods

Cell culture

66 cl4 mammary carcinoma cells stably expressing β3 integrin (66cl4pBabeβ3^{high}) and control empty pBabe-puro retroviral vector (66cl4pBabe) were described previously [13]. Bone-metastatic 4 T1.2 with hygromycin resistance and 4 T1BM2 with mCherry expression were derived from 4 T1 cells [35–38]. All lines were maintained for up to 4 weeks in α-minimal essential medium (αMEM)/5% fetal calf serum (FCS)/1% penicillin–streptomycin at 37 °C, 5% CO_2. bEnd.3 murine microvascular endothelial cells, provided by Dr R Hallman (Jubileum Institute, Sweden), were cultured as described [13].

Knockdown of β3 integrin

Two oligonucleotides targeting β_3 integrin (NM-016780: sh1, AAGGATGATCTGTCCACGATC; and sh2, AGCAAACAACCCGCTGTATAA, start position 2387) were inserted into pRetroSuper (sh1) or pLMP (sh2) retroviral vectors, using standard methodology [35]. A non-targeting sequence (AGTACTGCTTACGATACGG) was used as control. Viral supernatants from transfection of PT67 packaging cells were used to infect 4 T1.2 (sh1 hairpin) and 4 T1BM2 (sh2 hairpin) cells. Cells expressing low levels of β3 integrin were isolated by flow cytometry, expanded in culture and frozen. Changes in integrin receptor expression were analysed by standard flow cytometry [13,39]. The primary antibodies used are described in Supplementary materials and methods (see supplementary material).

In vitro assays

Proliferation, adhesion and migration assays [13,35,36,39], zymography [40] and immunoblotting [41] are described in Supplementary materials and methods (see supplementary material). For trans-endothelial migration, bEnd.3 cells (1×10^5) were seeded in triplicate Transwell inserts and incubated at 37 °C for 24 h to form a monolayer. Adherent cells were washed with phosphate-buffered saline (PBS) and calcein-labelled tumour cells (2×10^5) were added to the insert in 200 µl serum-free αMEM/glutamine (2 mM)/bovine serum albumin (BSA; 0.05%)/sodium pyruvate (1 mM) and antibiotics. Medium containing 10% FCS was added to

the bottom chambers as a chemoattractant. After 48 h of incubation at 37 °C, migrated tumour cells on the underside [green (calcein) cytoplasm and red (propidium iodide) nucleus] (three random × 20 fields) were photographed on an Olympus BX-61 microscope and counted using Metamorph (Molecular Devices).

Tumour growth and metastasis

Procedures involving mice were completed in accordance with National Health and Medical Research Council ethics guidelines and approved by the Peter MacCallum Animal Ethics Committee. Wild-type female Balb/c mice were purchased from the Walter and Eliza Hall Institute (Australia). β3-null mice [42] were backcrossed to a Balb/c background (10 generations).

For spontaneous metastasis assays, 8–10 week-old mice were inoculated with 10^5 cells into the fourth mammary fat pad. Tumour growth was measured using electronic calipers [13,35] and the mice were harvested as a group on day 30, or earlier if showing signs of distress due to metastatic disease. Analysis of tumour microvascular density [43] is described in Supplementary materials and methods (see supplementary material). Lungs, femurs and spines were snap-frozen in liquid nitrogen before processing for relative tumour burden (RTB) quantitation by real-time qPCR of the tumour-expressed reporter gene, as described previously [13,35,40]. For experimental bone metastasis assays, 5×10^4 (4 T1.2 and 4 T1BM2 cells) or 1×10^5 (66 cl4) cells/100 µl PBS were injected into the left cardiac ventricle [44]. These mice were harvested as a group on day 15, or earlier if showing signs of distress. Specific primers and probes are detailed in Supplementary materials and methods (see supplementary material). When comparing metastatic burden from two tumour lines, RTB values were adjusted for reporter gene copy number, calculated from the level in genomic DNA in cultured cells.

Statistical analysis

Data were analysed using Prism 5.01 for Windows. For *in vitro* assays comparing multiple groups, a one-way ANOVA Tukey's multiple comparisons test was completed. Proliferation assays were evaluated by two-way ANOVA with Bonferroni post-test. For *in vivo* assays, Fisher's exact test was used for metastatic incidence and a Mann–Whitney test was completed for metastatic burden analysis. $p \leq 0.05$ was considered significant.

Results

Knockdown of β3 integrin in bone metastatic tumour cells

Exogenous expression of β3 in weakly lung-metastatic 66 cl4 cells contributes to their spontaneous metastasis from the mammary gland to bone [13]. However, this approach does not reveal an essential requirement for bone metastasis, a critical consideration for anti-metastatic therapy. To address this, β3 integrin expression was reduced by stable expression of RNA interference vectors encoding separate hairpins in 4 T1.2 (sh1) and 4 T1BM2 (sh2) cells. These models were chosen for their clinical relevance, with both expressing αvβ3 integrin and, unlike xenograft models, metastasizing spontaneously to bone, with high incidence from mammary tumours in immunocompetent mice [36,37].

β3 expression was substantially reduced in 4 T1BM2-β3lo compared to 4 T1BM2-ctrl and parental 4 T1BM2 cells and accompanied by down-regulation of αv integrin at the cell surface (Figure 1A, B; see also supplementary material, Figure S1A). Suppression of β3 transcript levels in 4 T1BM2-β3lo cells was confirmed by qRT–PCR (Figure 1C). Despite reduced surface levels of αv integrin, *αv* mRNA expression was not decreased (Figure 1D). Moreover, western blot analysis of whole-cell lysates showed that total αv protein levels in 4 T1BM2-ctrl and 4 T1BM2-β3lo cells were similar to parental cells (87% and 85% of parental cells, respectively) and not significantly different from each other when normalized to tubulin (Figure 1E). Thus, surface localization, rather than expression of αv integrin, is impaired by suppression of β3 integrin. Sustained, coordinated down-regulation of surface β3 and αv integrins was also observed using a different β3-targeting shRNA in 4 T1.2 bone-metastatic cells (see supplementary material, Figure S1B). The overall expression pattern of other integrins was comparable between 4 T1BM2 and 4 T1.2 parental lines (see supplementary material, Figure S2A, B), with the exception that α2 was expressed at low levels in 4 T1.2 but not in 4 T1BM2 cells. The expression of α2, α3, α5, α6, β1, β4, β5 and β6 integrin subunits was not significantly altered in 4 T1BM2-β3lo cells compared to 4 T1BM2-ctrl cells, whereas 4 T1.2 cells showed a small increase in α6 and β4 subunits following β3 down-regulation.

Suppression of β3 integrin inhibits spontaneous metastasis to bone and soft tissues without altering primary tumour growth

Adhesion of all lines to uncoated plastic (30 min) was negligible, irrespective of β3 integrin levels (Figure 2A; see also supplementary material, Figure S3A). As expected, adhesion of 4 T1BM2-β3lo and 4 T1.2-β3lo cells to vitronectin, the classical αvβ3 integrin ligand, was decreased (45–60% inhibition) compared to cells expressing a non-targeting shRNA. Adhesion to laminin (LM)-511 was unaffected by β3 suppression. Similarly, haptotactic migration towards vitronectin, but not LM-511, was significantly inhibited by β3 suppression (Figure 2B; see also supplementary material, Figure S3B), indicating that reduced adhesion and migration of cells with low β3 expression are substrate-specific. Proliferation of 4 T1BM2 and 4 T1.2 cells was unaffected by β3 integrin down-regulation (Figure 2C; see also supplementary material, Figure S3C).

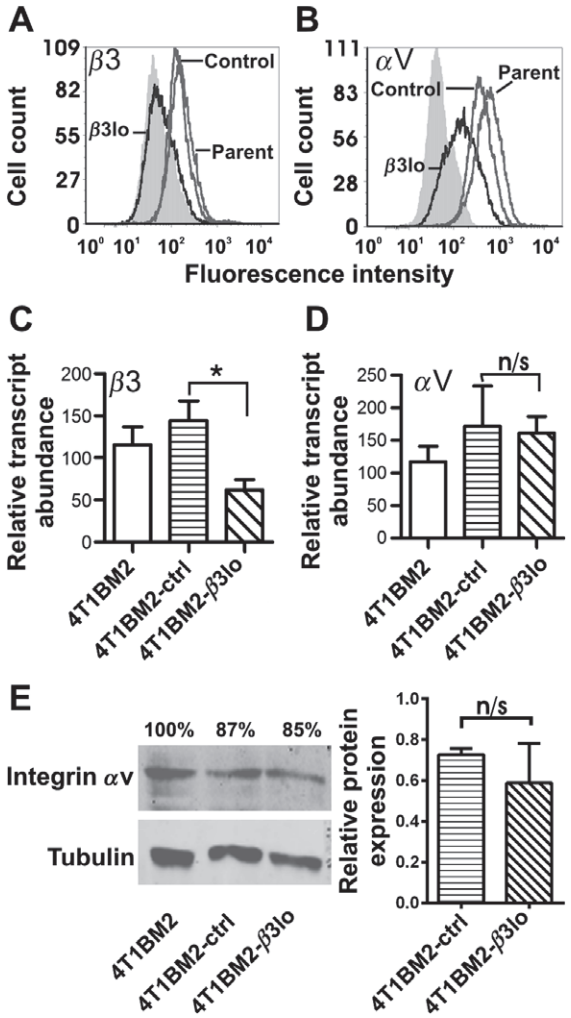

Figure 1. Stable suppression of β3 integrin expression induces coordinated down-regulation of αv integrin subunits at the cell surface. Parental 4T1BM2 cells (Parent) and cells expressing a non-targeting (Control) or a β3-targeting shRNA (β3lo) were analysed for the expression of β3 (A) and αv (B) integrins by standard flow cytometry; solid grey, isotype control antibody. β3 (C) and αv (D) mRNA transcripts were analysed by qRT–PCR; graph shows mean transcript abundance relative to *GAPDH* ± SEM of nine independent replicates; $*p < 0.05$; one-way ANOVA, Tukey's multiple comparisons test. (E) Representative western blot analysis of total integrin αv and tubulin (loading control) detected in whole-cell lysates (left) and quantitation of triplicate samples of 4T1BM2-ctrl versus 4T1BM2-β3lo (right) by densitometry; n/s, not significant; $p = 0.700$

In vivo, 4T1BM2-ctrl and 4T1BM2-β3lo tumours grew at the same rate (Figure 2D, E). However, visual examination of mice at harvest and quantitation of metastatic burden, using a sensitive quantitative PCR (qPCR)-based assay [35], revealed a dramatic effect of β3 down-regulation on spontaneous metastasis. Semi-quantitative measurement (presence or absence) of metastasis indicated a significantly lower incidence of mice developing bone but not lung metastases in the 4T1BM2-β3lo group (Figure 2F). However, visual inspection of lungs at harvest showed fewer and smaller metastatic nodules in mice bearing 4T1BM2-β3lo tumours, indicating a lower overall lung metastatic burden (Figure 2F, right panels). These observations were confirmed and quantitated by qPCR, with 4T1BM2-β3lo metastatic burden in lung, femur, spine and bone (combined femur and spine) (Figure 2G–J) decreased significantly compared to 4T1BM2-ctrl bearing mice ($p < 0.01$ in all organs). Suppression of β3 integrin similarly reduced metastatic burden in lung, femur, spine and bone but not tumour growth in the 4T1.2 model (see supplementary material, Figure S3D–G). These results demonstrate conclusively that expression of β3 in mammary tumour cells is required for efficient spontaneous metastasis to multiple organs.

Loss of stromal β3 integrin expression does not alter orthotopic primary tumour growth or metastasis

Conflicting results exist regarding the contribution of stromal cell populations expressing β3 integrin to primary tumour growth [16,19–21] and metastasis [17,20]. Importantly, no study has investigated the role of stromal β3 integrin in spontaneous breast cancer metastasis to bone. Therefore, we compared the orthotopic growth and metastatic dissemination of 4T1BM2 (Figure 3) or 4T1.2 tumours (see supplementary material, Figure S4) in β3-null versus wild-type littermates. We found no difference in primary tumour growth (Figure 3A; see also supplementary material, Figure S4A), final tumour weight (Figure 3B; see also supplementary material, Figure S4B) or metastatic burden in lung, femur, spine or bone (combined femur and spine; Figure 3C–F; see also supplementary material, Figure S4C–F, respectively) between integrin β3-null and wild-type mice.

The lack of effect of stromal β3 deletion on primary tumour growth contrasts with enhanced subcutaneous growth and vascularization of melanoma, colon and lung carcinomas in β3-null mice [16,19] and could be due to differences in tumour type or to the site of tumour growth. To address this, we compared the growth of 4T1BM2 cells inoculated in the mammary fat pad or subcutaneously in wild-type and β3-null mice. Orthotopic 4T1BM2 tumours grew at the same rate in wild-type and β3-null mice (Figure 3G–I). Importantly, while subcutaneous growth of 4T1BM2 tumours in wild-type or β3-null mice was visibly slower than in the orthotopic site (cf Figure 3G, J), it was significantly enhanced in β3-null mice compared to wild-type littermates (Figure 3J–L). Moreover, quantitation of microvascular density revealed no difference in orthotopic tumours growing in wild-type and β3-null mice (Figure 3M) but a significant increase in microvascular density in subcutaneous tumours growing in β3-null compared to wild-type mice (Figure 3N). Collectively, these results indicate that, unlike subcutaneous tumours, orthotopic growth and vascularization of mammary tumours are not affected by stromal β3 integrin deletion.

Tumour β3 integrin is required early to promote metastasis to bone

To assess the stage at which tumour β3 is required for metastasis to bone, we bypassed the formation of

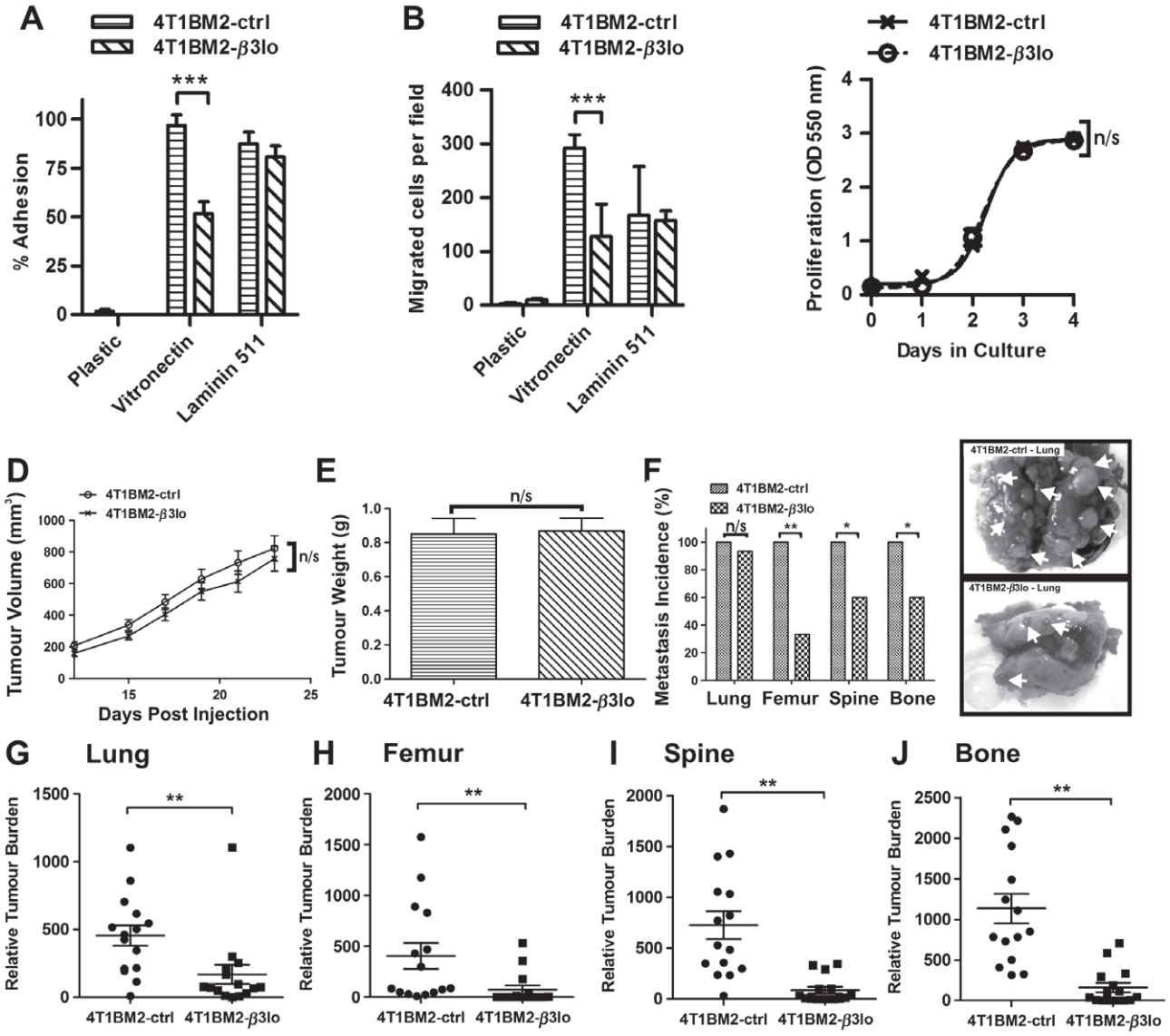

Figure 2. Down-regulation of αvβ3 integrin impairs vitronectin-mediated adhesion and migration and inhibits 4T1BM2 spontaneous metastasis to bone and lung. (A) Short-term adhesion (30 min) was measured in uncoated (plastic) or vitronectin- or LM-511-coated 96-well plates, as indicated. Data show % total cell input ± SD of representative experiments ($n = 3$), each completed in triplicate; $^{***}p < 0.001$; one-way ANOVA, Tukey's multiple comparisons test. (B) Haptotactic migration towards vitronectin or LM-511 was measured in Transwell chambers after 4 h at 37°C in the absence of serum; data show mean number of migrated cells ± SEM of nine replicate images (three random fields of view/Transwell membrane × three membranes; $^{***}p < 0.001$, one-way ANOVA, Tukey's multiple comparisons test). (C) Proliferation was measured in 96-well plates in the presence of 5% serum and quantitated every 24 h, using a sulphorhodamine B colorimetric assay; data are presented as mean ± SEM of six replicate wells/condition and are representative of three independent experiments; n/s, not significant; $p = 0.634$). (D) 4T1BM2-ctrl and 4T1BM2-β3lo orthotopic tumour growth was monitored thrice weekly by caliper measurements ($p = 0.254$; two-way ANOVA). (E) Tumour weight at harvest (day 26, $p = 0.782$, Mann–Whitney test): data in (D, E) show mean ± SD of 15 mice/group. (F) The incidence of mice developing metastases in lung, femur, spine or bone (combined femur + spine) was assessed by visual inspection and confirmed by qPCR detection of a marker gene (*mCherry*) relative to *vimentin*. Organs with a qPCR amplification signal above background compared to a naïve mouse were considered positive (n/s in lung, $p = 1.00$, $^{*}p < 0.05$, $^{**}p < 0.01$; Fisher's exact test): (right panels) representative images of lungs from 4T1BM2-ctrl and 4T1BM2-β3lo tumour-bearing mice; arrows, metastases. Metastatic burden in lung (G), femur (H), spine (I) and combined bone score (J) was determined by genomic qPCR detection of *mCherry* DNA relative to *vimentin* DNA; data show one point for each mouse ($n = 15$/group) and mean burdens (horizontal bar) ± SEM ($^{**}p < 0.01$; Mann–Whitney test)

primary tumours by inoculating 4T1BM2 cells directly into the left cardiac ventricle of wild-type mice. In contrast to the strong reduction in spontaneous bone metastasis, suppression of tumour β3 integrin did not reduce experimental metastasis to femur, spine or bone (femur and spine combined) (Figure 4A–C). We also compared the bone metastatic ability of 66cl4β3^{high} cells that over-express β3 integrin and metastasize spontaneously to bone to that of non-expressing 66cl4pBabe cells that do not spread spontaneously to bone from the mammary gland [13]. Surprisingly, extensive metastasis to femur, spine or both (Figure 4D–F) was observed, regardless of β3 expression. Thus, tumour β3 integrin is not essential for homing, survival and colonization of bone.

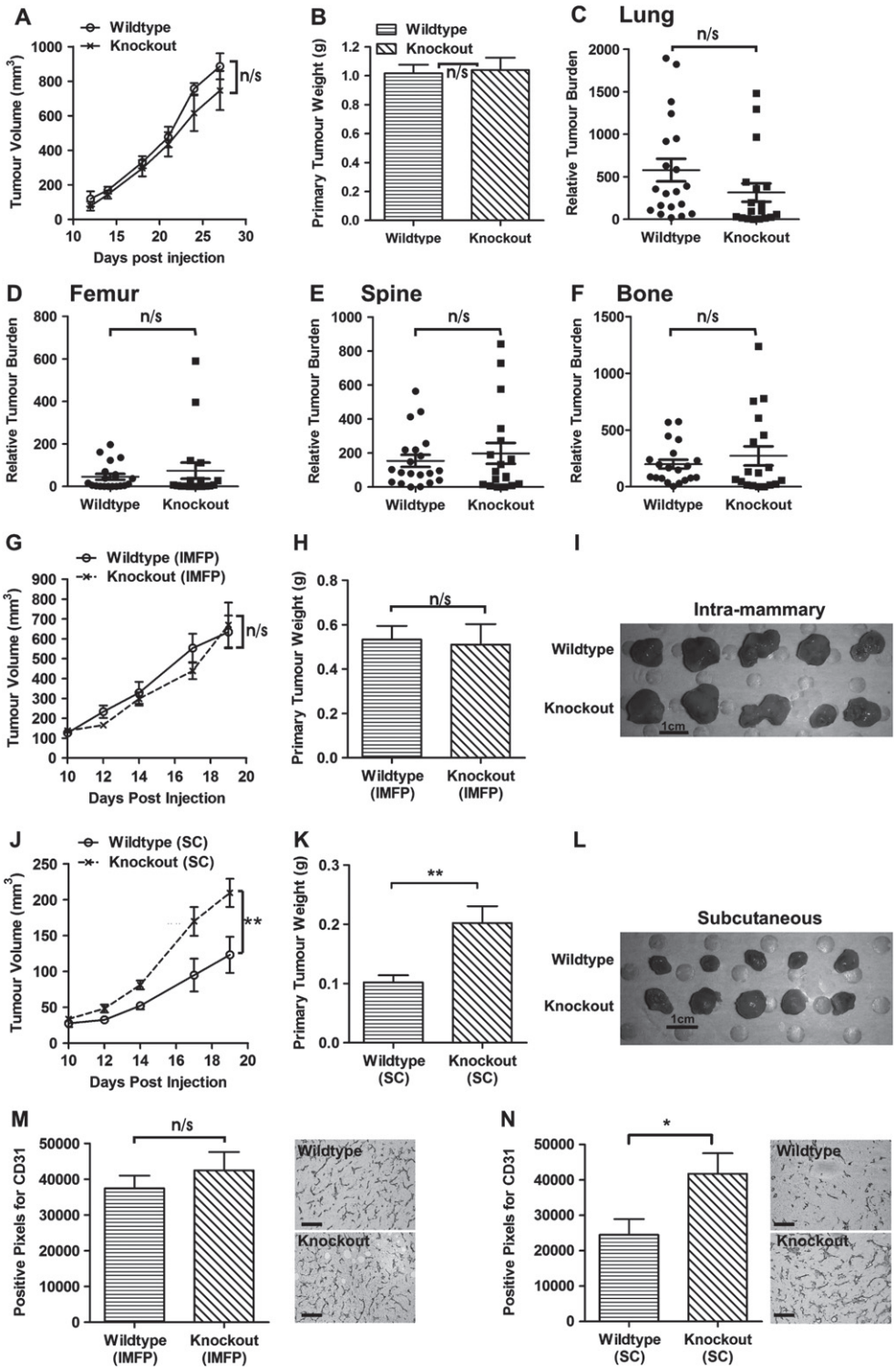

Figure 3. Stromal deletion of β3 integrin does not alter 4T1BM2 orthotopic tumour growth and spontaneous metastasis, but enhances subcutaneous tumour growth and vascularization; parental 4T1BM2 cells (1×10^5) were inoculated orthotopically into wild-type ($n = 20$) or littermate β3 knockout ($n = 18$) syngeneic Balb/c mice. Tumour growth rate (A), tumour weight at harvest (day 27) (B) and metastatic burden in lung (C), femur (D), spine (E) and bone (F) were measured as described in the legend to Figure 2. Data show one point for each mouse and mean burdens (horizontal bar) ± SEM; no statistical differences (n/s) in tumour growth rate ($p = 0.848$; two-way ANOVA), tumour end weight ($p = 0.895$; Mann–Whitney test) or metastatic burden between WT and KO mice were observed in lung ($p = 0.056$), femur ($p = 0.578$), spine ($p = 0.793$) or bone ($p = 0.530$); Mann–Whitney test. Parental 4T1BM2 cells (1×10^5) were inoculated orthotopically (IMFP; G–I) or subcutaneously (SC; J–L) into wild-type or β3 knockout mice (five mice/group) as indicated. (G, J) Tumour growth was monitored over 19 days and differences in growth rate analysed by two-way ANOVA, Bonferroni post-test (n/s, not significant; $p = 0.590$; $^{**}p < 0.01$). (H, K) Tumour weight at harvest (Mann–Whitney test; n/s, $p = 0.854$, $^{**}p < 0.01$). Data in (G, H, J, K) show mean ± SD of five replicates/group. (I, L) Images showing size comparison between orthotopic (I) or subcutaneous (L) tumours growing in wild-type and β3 knockout mice; scale bar = 1 cm. Microvascular density in orthotopic (M) and subcutaneous (N) tumours was analysed by IHC detection of CD31; data show mean pixels ± SEM from 15 replicates (three fields/section ± five sections/tumour; $n = 5$ mice/group; n/s, $p = 0.444$, $^{*}p < 0.05$, Mann–Whitney test). Representative CD31 stainings in wild-type and β3 knockout mice are shown in the right panels. Scale bar = 100 μm

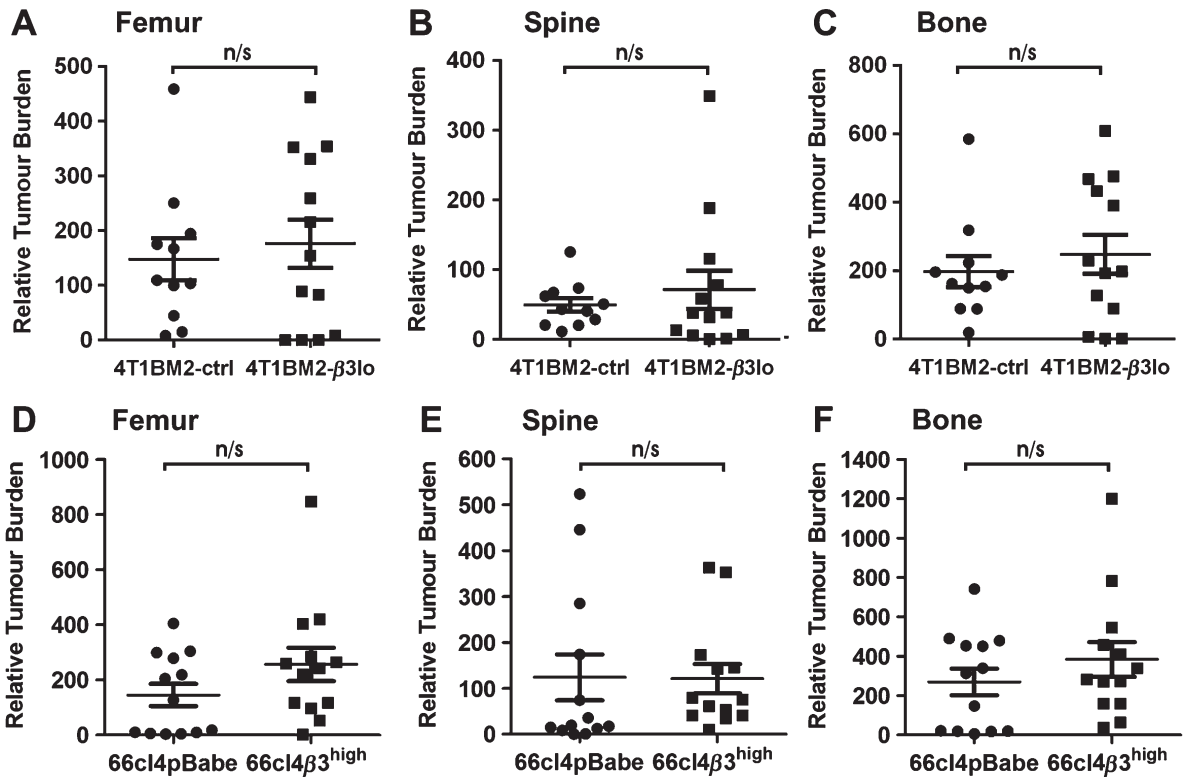

Figure 4. Experimental bone metastasis is not affected by changes in tumour β3 integrin expression. Balb/c mice were inoculated into the left ventricle of the heart with 4T1BM2-ctrl versus 4T1BM2-β3lo cells (5×10^4/mouse, $n = 11$ and 13 mice/group, respectively) (A–C) or 66cl4pBabe versus 66cl4β3^{high} cells (10^5/mouse, $n = 13$ mice/group) (D–F). The mice were sacrificed after 14 days and metastatic burden in femurs (A, D), spine (B, E) or bone (C, F) analysed by genomic qPCR detection of *mCherry* or the puromycin resistance gene relative to *vimentin*; data show one point for each mouse and mean burdens (horizontal bar) ± SEM. No statistical differences (n/s) in metastatic burden were found between 4T1BM2-ctrl and 4T1BM2-β3lo femur ($p = 0.368$), spine ($p = 0.495$) or bone ($p = 0.511$) and between 66cl4pBabe and 66cl4β3high femur ($p = 0.140$), spine ($p = 0.964$) or bone ($p = 0.315$) (Mann–Whitney test)

Next, we compared the ability of 4T1BM2-ctrl and 4T1BM2-β3lo or 4T1.2-ctrl and 4T1.2-β3lo cells to migrate towards a gradient of serum, a rich source of soluble vitronectin [45]. Integrin β3 suppression impaired migration towards serum by approximately 50% ($p < 0.001$; see supplementary material, Figure S5A, B). To further confirm that serum chemotaxis was specifically dependent on β3 integrin, we used DisBa-01, a potent snake venom-derived disintegrin that targets αvβ3 integrin [46]. DisBa-01 dose-dependently inhibited haptotatic migration of 4T1BM2 towards vitronectin, but not towards collagen-IV, demonstrating its specificity towards αvβ3 integrin substrates (see supplementary material, Figure S5C). Importantly, chemotactic migration towards serum was also inhibited by DisBa-01 in a dose-dependent manner (see supplementary material, Figure S5D).

4T1-derived tumour lines secrete abundant MMP9, which contributes to their migration and invasion [13,36]. We found that 4T1BM2-β3lo and 4T1.2-β3lo cells secrete significantly less MMP9 than control cells (see supplementary material, Figure 6A, B). Moreover, migration across a monolayer of endothelial cells was enhanced significantly by elevated β3 expression in 66cl4 cells (Figure 5A) and was inhibited by suppression of β3 integrin in 4T1BM2 cells (Figure 5B). Reduced chemotaxis, protease secretion and trans-endothelial migration following β3 down-regulation would be expected to prevent or delay tumour cell migration and intravasation. Indeed, qPCR signal for the *mCherry* tumour marker in blood on day 26 was near or below the limit of detection in the majority of 4T1BM2-β3lo tumour-bearing mice (mean value = 1.132) compared to control mice (mean value = 16.22; $p = 0.01$) (Figure 5C).

In a second series of experiments, blood was collected by cardiac puncture when tumours were small (~0.5 cm^3) and viable tumour cells scored by colony formation *in vitro*. Tumour weights were similar in both groups ($p = 0.944$; Figure 5D) and, while macro-metastases were not visible in either group at this early stage, qPCR quantitation revealed a higher lung ($p = 0.05$) and spine ($p = 0.012$) tumour burden in control mice (Figure 5E). Importantly, only blood from 4T1BM2-ctrl-bearing mice gave rise to colonies ($p = 0.0006$; Figure 5F), confirming that down-regulation of tumour β3 significantly impairs the ability 4T1BM2-β3lo cells to enter the vasculature.

Since αvβ3 integrin controls an early step required for metastatic dissemination to multiple sites, high β3 expression in breast cancer patients would be expected to be associated with poor clinical outcome. To assess clinical relevance, we first ran a differential expression analysis for integrin β3 in Oncomine 4.4.4.3 [47],

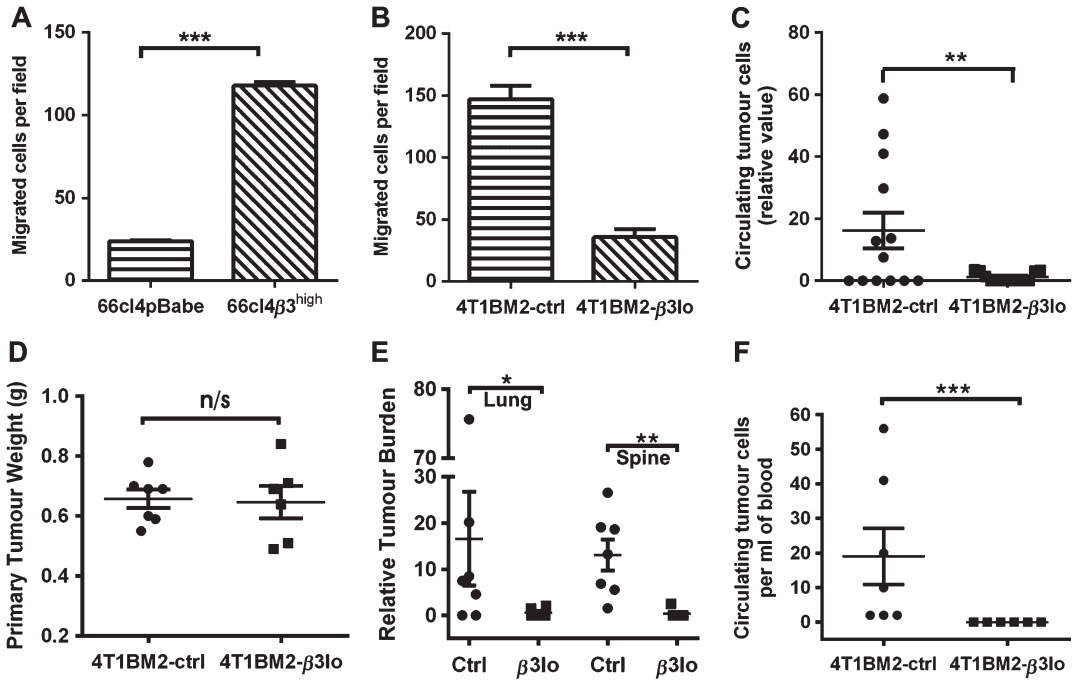

Figure 5. Integrin αvβ3 promotes trans-endothelial migration and intravasation of tumour cells. (A) β3 integrin over-expression enhances trans-endothelial migration *in vitro*. (B) β3 integrin suppression inhibits trans-endothelial migration *in vitro*. Migration of tumour cells (1×10^5 cells/well) through a monolayer of bEnd.3 endothelial cells was measured after 48 h. Data in (A, B) show mean number of migrated cells ± SD of nine replicate images (three random fields of view/Transwell membrane × three membranes) from a representative experiment ($n = 3$; $^{***}p < 0.001$, Mann–Whitney test). (C) β3 Integrin suppression inhibits intravasation *in vivo*: 4T1BM2-ctrl and 4T1BM2-β3lo tumour cells (1×10^5) were inoculated orthotopically into syngeneic Balb/c mice and blood collected by cardiac puncture after 26 days; relative number of circulating tumour cells was quantitated by genomic qPCR detection of *mCherry* gene relative to *vimentin*; data show one point for each mouse and mean (horizontal bar) ± SEM ($n = 13$ mice/group; $^{**}p = 0.01$; Mann–Whitney test). (D–F) β3 Integrin suppression delays vascular dissemination: mice were inoculated with 4T1BM2-ctrl ($n = 7$) and 4T1BM2-β3lo cells ($n = 6$), as above; blood was collected by cardiac puncture when mammary tumours reached ~0.5cm^3 and viable circulating tumour cells scored by colony formation in culture (0.5 ml/dish). (D) Tumour weight at harvest. (E) Metastatic burden in lung and spine was determined by genomic qPCR. (F) Colony formation assay: the number of colonies after 10 days (> 50 cells) was counted and the data expressed as the number of circulating tumour cells/ml blood. Data in (D–F) show one point/mouse and mean values (horizontal bar) ± SEM (n/s, not significant; $p = 0.944$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; Mann–Whitney test)

focusing on clinical outcome in breast cancer patients (fold-change ≥ 1.5; $p \leq 0.05$). In all, 10/13 analyses indicated that high β3 was associated with metastasis and recurrence (see supplementary material, Figure S7A, B). The prognostic value of β3 integrin expression was further investigated in molecular subtypes of breast cancer, using the BreastMark prognostic biomarker analysis tool [48]. High β3 integrin expression was correlated with shorter disease-free survival in patients with oestrogen receptor-negative (ER^-) tumours (Figure 6A, B) or with lymph node metastasis (LN^+; Figure 6E, F). No significant association was found between β3 expression and progesterone receptor (PR) status (Figure 6C, D), high tumour grade (Gr3; Figure 6G) or HER2 status (data not shown; $HER2^+$, $p = 0.121$; $HER2^-$, $p = 0.282$). Multivariate analyses showed that high β3 expression is significantly associated with reduced survival in ER^-/PR^- (Figure 6H), ER^-/LN^+ (Figure 6I) and $ER^-/LN^+/Gr3^+$ tumours (Figure 6J). These observations in human breast tissues are consistent with the lack of ER and PR in 4T1BM2 primary tumours and the aggressive nature of this mouse model of metastasis (see supplementary material, Figure S7C).

Discussion

The diversity of experimental approaches, animal models and tumour types used to investigate the contribution of β3 integrin to tumour growth and metastasis has made it difficult to reconcile some of the discrepancies between earlier studies. Unlike xenograft models, the 4T1BM2 and 4T1.2 models used herein have the unique ability to metastasize spontaneously and aggressively in immunocompetent mice [35–37]. Our data demonstrate conclusively that tumour-associated αvβ3 integrin is essential for efficient spontaneous metastasis to bone and lung, but not for growth in the mammary gland. Integrin αvβ3 mediates tumour cell attachment to several bone-derived ECM proteins, and is thought to be a critical for homing and colonization of bone [13,49,50]. Unexpectedly, we found that β3 down-regulation in 4T1BM2 and 4T1.2 decreased spontaneous, but not experimental, bone metastasis. Moreover, exogenous expression of β3 in 66cl4β3^{high} cells, which promotes spontaneous metastasis to bone [13], did not enhance experimental bone metastatic burden compared to control cells. Clearly, 66cl4 cells do not require αvβ3 to

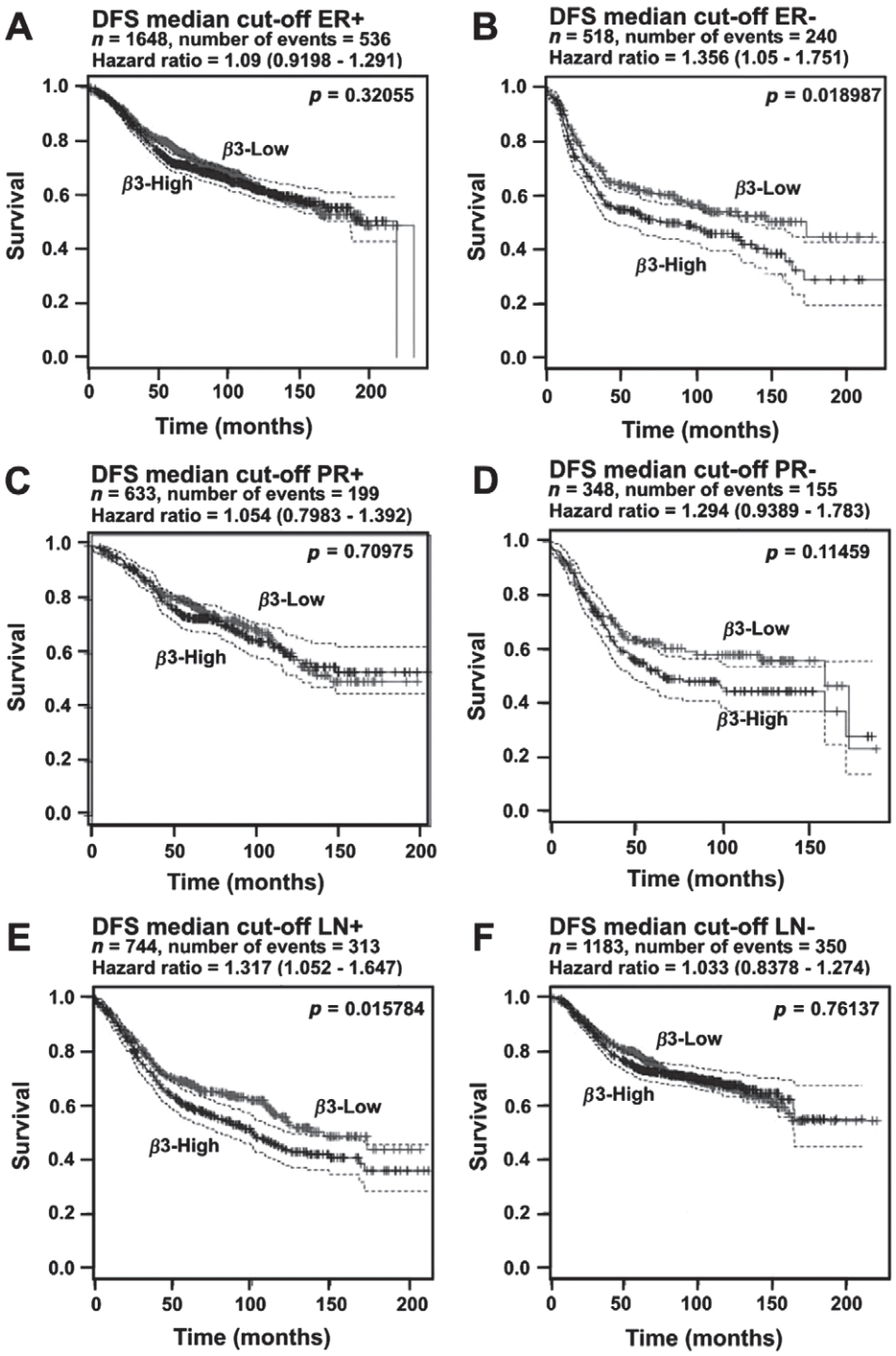

Figure 6. Association between β3 integrin expression and disease-free survival (DFS). The association between β3 expression and DFS in human breast tumour samples was interrogated in public databases, using the BreastMark prognostic biomarker analysis tool [48]. $\beta3^{high}$ and $\beta3^{low}$ (median cut-off) are shown in black and grey, respectively; dotted lines show confidence intervals. (A) Oestrogen receptor-positive (ER^+) tumours. (B) Oestrogen receptor-negative (ER^-) tumours. (C) Progesterone receptor-positive (PR^+) tumours. (D) Progesterone receptor-negative (PR^-) tumours. (E) Lymph node-positive (LN^+) tumours. (F) Lymph node-negative (LN^-) tumours. (G) Grade 3 (Gr3) tumours. (H) Oestrogen receptor-negative/progesterone receptor-negative (ER^-/PR^-) tumours. (I) Oestrogen receptor-negative/lymph node-positive (ER^-/LN^+) tumours. (J) Oestrogen receptor-negative/lymph node-positive/grade 3 (ER^-/LN^+/Gr3) tumours

home and colonize bone, since they formed experimental bone metastases in 100% of animals (see Figure 4), even though they do not express this receptor [13]. These observations indicate that tumour αvβ3 is essential primarily during the early, rather than late, steps of breast cancer metastasis.

Suppression of tumour β3 reduced MMP9 secretion, serum chemotaxis and trans-endothelial migration. These responses are expected to contribute to the early dissemination of αvβ3-expressing breast tumours *in vivo* through interactions with its ligands, a conclusion further supported by decreased circulating tumour cells

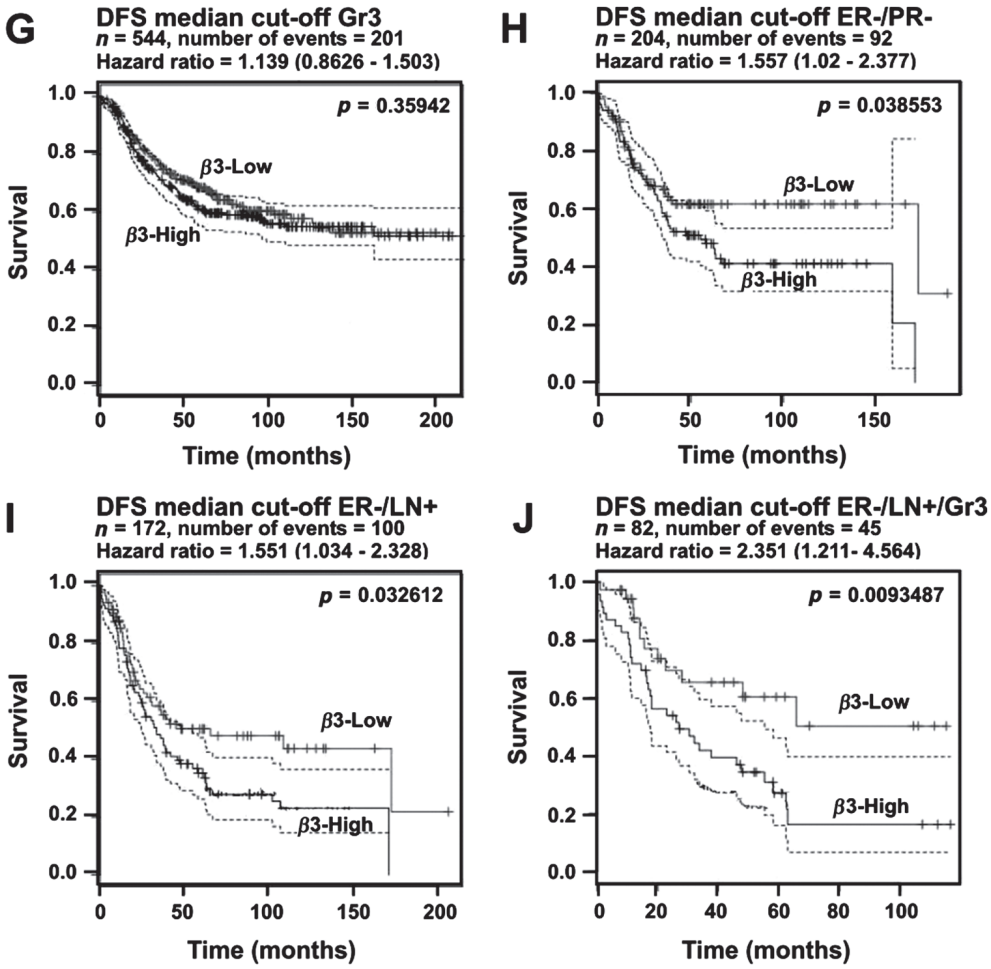

Figure 6. Continued

observed in 4T1BM2-β3lo tumour-bearing mice. Consistent with this, the expression of the αvβ3 ligand, vitronectin, is elevated in small vessel walls surrounding cancer cells in patients with early stage breast cancer, and the concentration of vitronectin in serum is elevated in advanced breast cancer patients [51]. We therefore conclude that the most critical role of tumour αvβ3 is to facilitate the early escape and intravasation of breast tumours, resulting in enhanced vascular dissemination and subsequent metastasis to multiple organs. The clinical relevance of our findings in mouse models is strongly supported by the correlation observed between high β3 expression, metastatic disease and poor clinical outcome in ER-negative breast cancer patients.

Our data appear to be at odds with earlier reports showing increased experimental bone metastasis in β3-over-expressing MDA-MB-231 variants [5,12]. However, it should be noted that, while mice inoculated with these cells showed increased number of osteolytic lesions, the overall incidence of mice developing experimental bone metastases was, in agreement with our study, high in both control and β3-over-expressing groups. Tumour αvβ3 integrin does not directly stimulate metastatic growth in bone, but promotes the recruitment of active osteoclasts in proximity to metastatic lesions [5,13]. Degradation of the bone matrix and the subsequent release of growth factors could provide a growth stimulus for breast tumour cells in bone. Consistent with this, β3 inhibitors reduce the formation of osteolytic lesions more potently when used as pretreatment [18], or as long-term daily treatments [5], at concentrations that also inhibit osteoclast activity. The extent to which αvβ3-expressing osteoclasts contribute to bone colonization is likely to vary between tumours. MDA-MB-231 cells are aggressively osteolytic compared to 4T1 or 66cl4 cells, and presumably their growth in bone may be more dependent on the release of bone-derived growth factors. Collectively, data from the above studies and ours indicate that, while there is heterogeneity amongst tumour lines in their dependency on osteoclasts to colonize bone, tumour αvβ3 is not essential for bone metastases to develop.

Most surprising was the lack of effect of stromal β3 ablation on tumour growth, vascularization and spontaneous metastasis, given the numerous studies that have implicated endothelial cells or platelets in these processes [16,19,52–54]. Studies employing subcutaneous tumour transplantation models reported enhanced tumour growth and vascularization following stromal β3 deletion [16,19]. While we could replicate these observations in mammary tumour cells implanted into

the subcutis, stromal deletion of β3 integrin did not alter tumour growth in the mammary gland. These observations indicate that stromal β3 regulates tumour growth and angiogenesis in a tissue-specific manner, and argue against a critical role for endothelial β3 in promoting the orthotopic growth and angiogenesis of breast tumours. Our data support those reported in the MMTV-c-neu/$\beta3^{-/-}$ transgenic model of breast cancer metastasis [20] and resolve the apparent conflicts with earlier tumour transplantation studies employing β3-null mice [55].

Most studies supporting the role of platelet αIIbβ3 in metastasis have made use of *in vitro* surrogate assays or *in vivo* experimental metastasis models, in which tumour cells are injected directly into the vasculature [5,12,52,53,56]. In one study, pharmacological inhibition of platelet αIIbβ3 significantly reduced experimental melanoma metastasis to bone, which was attributed to disruption of tumour cell-induced platelet aggregation [17]. Conceivably, injection of a large bolus of cells could enhance experimental metastasis by promoting excessive tumour cell clumping and/or exaggerating tumour-induced platelet aggregation, processes known to promote embolic arrest of tumour cells [53,57,58]. Interestingly, the well-documented correlation between the ability of tumour cells to induce platelet aggregation and metastatic potential has not been observed consistently in breast cancer metastasis models [59]. To minimize tumour cell clumping and non-specific tumour–platelet interactions in our experimental metastasis assays, the number of cells injected (5×10^4–1×10^5) was significantly lower than that employed in most xenograft studies [5,12]. We do not interpret our results as evidence that tumour–platelet interactions are not required for spontaneous metastasis of breast tumours. Rather, we propose that platelet αIIbβ3 function is not essential for efficient breast cancer metastasis to bone and lung. Indeed, liposome-encapsulated Cilostazol, a platelet aggregation inhibitor, reduces spontaneous metastasis of 4T1 tumours to lung by 50% [60]. However, the effects of Cilostazol on bone metastasis and of αIIbβ3 integrin inhibition on spontaneous metastasis were not investigated in this study.

Current experimental and clinical evidence indicates that not all tumour types (or anatomical sites) could benefit from therapies employing αvβ3 integrin antagonists such as cilengitide [25,61]. Our study demonstrates for the first time that tumour rather than stromal β3 integrin is a critical determinant of metastatic potential in breast cancer and is essential for efficient spontaneous metastasis to multiple sites, including bone. Regulation of early steps of metastasis to multiple organs by tumour β3 integrin is consistent with the association between high β3 expression and poor clinical outcome in ER-negative breast cancer patients. Our findings have important implications for the design of anti-metastatic therapies targeting β3 integrin in breast cancer, and could explain in part the limited therapeutic response observed in clinical trials testing β3 inhibitors in patients with advanced metastatic disease [26–30]. We propose that to achieve optimal efficacy in breast cancer patients, β3 inhibitors should be used in a neo-adjuvant setting to target early steps of metastatic progression, rather than after metastases are established.

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Author contributions

NP and RLA conceived and managed the study and wrote the manuscript; experimental work was carried out by NP, RZC, KCM, AN, SP-F, ACBMM, DD, XL, S-HK and RT; HAS provided purified DisBa-01 and expert guidance; and RPR completed the prognostic analyses. All authors revised and approved the final manuscript.

Abbreviations

ECM, extracellular matrix; ER, oestrogen receptor; LM, laminin; MMTV, mouse mammary tumour virus; PR, progesterone receptor.

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SUPPLEMENTARY MATERIAL ON THE INTERNET

The following supplementary material may be found in the online version of this article:

Supplementary materials and methods

Figure S1. Stable suppression of β3 integrin expression induces coordinated down-regulation of αv integrin subunits at the cell surface

Figure S2. Flow-cytometric analysis of the repertoire of integrin subunits following β3 integrin down-regulation

Figure S3. Down-regulation of αvβ3 integrin impairs vitronectin-mediated adhesion and migration and inhibits 4T1.2 spontaneous metastasis to bone and lung

Figure S4. Stromal deletion of β3 integrin does not alter 4T1.2 orthotopic tumour growth and spontaneous metastasis

Figure S5. Suppression of β3 integrin expression or function impairs αvβ3-dependent migration

Figure S6. β3 integrin down-regulation inhibits MMP-9 expression

Figure S7. Differential expression analysis of integrin β3 in ER-positive and -negative human cancer cases