Different molecular profiles are associated with breast cancer cell homing compared with colonisation of bone: evidence using a novel bone-seeking cell line

Faith Nutter¹, Ingunn Holen¹, Hannah K Brown¹, Simon S Cross², C Alyson Evans¹, Matthew Walker¹, Robert E Coleman¹, Jules A Westbrook³, Peter J Selby³, Janet E Brown^{1,3} and Penelope D Ottewell¹

¹Academic Unit of Clinical Oncology, Cancer Research UK (CR-UK), and Yorkshire Cancer Research (YCR) Sheffield Cancer Research Centre ²Academic Unit of Pathology, University of Sheffield, Beech Hill Road, Sheffield S10 2RX, UK ³Leeds Institute of Molecular Medicine, CR-UK Cancer Research Centre, University of Leeds, Leeds, UK

Correspondence should be addressed to P D Ottewell

Email
P.D.Ottewell@sheffield.ac.uk

Abstract

Advanced breast cancer is associated with the development of incurable bone metastasis. The two key processes involved, tumour cell homing to and subsequent colonisation of bone, remain to be clearly defined. Genetic studies have indicated that different genes facilitate homing and colonisation of secondary sites. To identify specific changes in gene and protein expression associated with bone-homing or colonisation, we have developed a novel bone-seeking clone of MDA-MB-231 breast cancer cells that exclusively forms tumours in long bones following i.v. injection in nude mice. Bone-homing cells were indistinguishable from parental cells in terms of growth rate *in vitro* and when grown subcutaneously *in vivo*. Only bone-homing ability differed between the lines; once established in bone, tumours from both lines displayed similar rates of progression and caused the same extent of lytic bone disease. By comparing the molecular profile of a panel of metastasis-associated genes, we have identified differential expression profiles associated with bone-homing or colonisation. Bone-homing cells had decreased expression of the cell adhesion molecule fibronectin and the migration and calcium signal binding protein S100A4, in addition to increased expression of interleukin 1B. Bone colonisation was associated with increased fibronectin and upregulation of molecules influencing signal transduction pathways and breakdown of extracellular matrix, including hRAS and matrix metalloproteinase 9. Our data support the hypothesis that during early stages of breast cancer bone metastasis, a specific set of genes are altered to facilitate bone-homing, and that disruption of these may be required for effective therapeutic targeting of this process.

Key Words

- ▶ bone
- ▶ breast
- ▶ metastasis

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Introduction

As many as 75% of patients with advanced breast cancer will develop bone metastasis, and these have a poor prognosis with median survival rates of ~2 years following initial diagnosis of bone involvement (Major Cook *et al.* 2009). New therapeutic approaches are therefore needed to improve outcome for patients with tumour spread to the skeleton. Identification of specific molecular determinants involved in tumour cell homing and colonisation of bone are fundamental for the development of effective therapeutic regimes. During metastatic conversion, breast cancer cells acquire molecular changes enabling invasion of surrounding tissue, vascular spread to distant sites, and proliferation and survival in secondary sites (Lawson Blatch & Eddins 2009, Ding Ellis *et al.* 2010). It is likely that metastasis is a result of a stepwise accumulation of genetic mutations, with different molecular alterations being required for different stages in the metastatic process. Therefore molecules involved in tissue homing are likely to be distinct from those involved in subsequent colonisation, and evidence indicates that only a small subset of tumour cells is able to undergo these alterations (Klein 2004, Karakosta *et al.* 2005).

It is widely accepted that initiation of metastasis is driven by genetic and phenotypic adaptations in tumour cells, changing them from an epithelial to a mesenchymal cell type. This process is driven by the overexpression of mesenchymal proteins such as fibronectin and metalloproteinases (Kang *et al.* 2003, Yang & Weinberg 2008) in addition to loss of cell–cell adhesion molecules including E-cadherin and B-catenin. E-cadherin is a key protein in this process; loss of this molecule results in reduced adhesion of epithelial cells to desmosomes, causing cellular mobility and metastatic dissemination of tumour cells into the circulation (Adams & Nelson 1998). Once in the circulation tumour cells must home to a secondary environment and will only establish metastases at this distant site if the microenvironment is appropriate (Chambers *et al.* 2002). Two primary cell types contribute to the metastatic bone niche in bone: stromal cells and transient cells. Stromal cells originate from mesenchymal cells in the marrow and include adipocytes, fibroblasts and osteoblasts. They support the differentiation and proliferation of cancer cells via molecules such as vascular cell adhesion molecule 1, syndecan-1 and matrix metalloproteinase 2 (MMP2; Michigami *et al.* 2000, Maeda *et al.* 2004, Lipton 2010). Transient cells include erythrocytes, T cells and platelets, all of which have been shown to aid tumour growth and metastasis (Bussard *et al.* 2008).

In addition, the continuous process of bone remodelling involves osteoclast-mediated release of an abundance of growth factors, cell adhesion molecules and cytokines that make it an attractive site for metastatic tumour cells.

Bone-homing of breast cancer cells is most prominent in the highly vascular trabecular areas of the long bone metaphysis, where they lodge in a niche identical to, or largely overlapping with, the haematopoietic stem cell niche (Mishra *et al.* 2011, Shiozawa *et al.* 2011). It has been proposed that the mechanics of the sluggish sinusoidal vascular supply within the metaphysis give the haematopoietic precursors, as well as invading tumour cells, ample opportunity to move in and out of the marrow. Following extravasation into the bone marrow, tumour cells reactivate their epithelial cell properties. Cell adhesion molecules, such as E-cadherin, are re-expressed enabling tumour cells to interact with the recently colonised tissue (Wells *et al.* 2008). Within bone, tumour-specific growth factors are believed to upregulate fibronectin in resident fibroblasts providing a permissive niche for incoming tumour cells. Tumour cells then lodge in the haematopoietic stem cell niche through fibronectin binding, resulting in enhanced MMP9 expression, which in turn serves to break down basement membranes, releasing growth factors into the local environment to support the growth of newly introduced cells (Kaplan *et al.* 2005). Although the process of epithelial to mesenchymal transition and its role in tumour cell shedding from the primary site has been extensively studied (reviewed in Leopold *et al.* (2012)), there is still much to be discovered about the molecular mechanisms that regulate tumour cell colonisation of bone. Furthermore, molecules involved in tissue specific homing of breast cancer cells to the bone environment remain to be clearly distinguished from those involved in the subsequent colonisation of bone.

The human breast cancer cell line MDA-MB-231 forms tumours in mouse long bones following injection via the left cardiac ventricle and is therefore one of the most commonly used models of bone metastasis (Bellahcène *et al.* 2007). Once in the bone microenvironment, a subpopulation of these cells localises to particular niches where they are triggered to proliferate and form metastatic colonies (Henriquez *et al.* 2007). Interestingly, the majority of the injected tumour cells do not colonise bone: studies have shown that most cells entering the bone die 5–7 days post injection, implying that only a small population of these cells express the relevant

molecular phenotype for bone colonisation (Phadke *et al.* 2006). It is well established that MDA-MB-231 cells form bone tumours following intracardiac injection, but not following injection into the peripheral circulation (Bellahcène *et al.* 2007). Therefore, it has been proposed that these cells do not specifically home to bone; instead, arterial pressure is required to drive them into the bone environment where they get trapped in the capillaries (Bellahcène *et al.* 2007). In support of this hypothesis, i.v. injection of MDA-MB-231 cells is commonly used as a model for lung metastasis in which tumours grow following entrapment in the capillaries of the lung (Zhou *et al.* 2012). In addition, MDA-MB-231 cells lack crucial molecules that are required for true homing, such as CXCR4 (reviewed Parker & Sukumar (2003)). In light of this, we have used repeated *in vivo* passaging of MDA-MB-231 (MDA-P) cells to establish a novel bone-seeking clone that reproducibly forms long bone tumours following i.v. injection in mice (MDA-IV). Using this unique tool, we have identified genetic changes that are specifically associated with breast cancer bone homing, or subsequent colonisation of the bone microenvironment (Fig. 1).

Materials and methods

Animals

All experiments were carried out in 6- to 8-week-old female Balb/c nude mice (Charles River, Margate, Kent, UK). Mice were maintained on a 12 h light:12 h darkness cycle with free access to food and water. Experiments were carried out in accordance with local guidelines and with home office approval under project licence 40/3531, University of Sheffield, UK.

Generation of MDA-IV cells

Human breast cancer MDA-MB-231 cells (European Collective of Cell Cultures, Salisbury, Wiltshire, UK) transfected with eGFP (MDA-P cells) as described previously (Ottewell *et al.* 2008) were cultured in DMEM supplemented with 4500 mg/l glucose, L-glucosamine, pyruvate (Invitrogen–Gibco) and 10% FCS. 1×10^5 MDA-P cells were injected into the left cardiac ventricle of five mice and into the tail vein of five mice. The cells that formed colonies in mouse tibiae were extracted by scraping out of the marrow cavity and pooled before culture *in vitro* in 5% CO₂ at 37 °C. Following 1 week in culture, 1×10^5 cells were re-injected into the left cardiac ventricle or tail vein ($n=5$ per group). This procedure was

repeated seven times until tumour growth in mouse long bones was detected following i.v. injection (MDA-IV cells; see Fig. 1). To establish whether MDA-IV cells consistently colonised long bones following i.v. injection, these cells were isolated *in vitro* as described above and re-injected into the tail vein of five mice.

Characterisation of MDA-P and MDA-IV cells *in vitro*

All experiments were carried out in triplicate and repeated three times: cell proliferation was monitored every 24 h for 96 h by cell counting with a 1/400 mm² haemocytometer (Hawksley, Lancing, Sussex, UK). Cell–cell adhesion was assessed as percentage of cells adhering to a monolayer of the same cell type; cells were cultured to form a confluent monolayer and 20 000 cells of the same type (MDA-P or MDA-IV) were labelled with 1 nM dialkyl carbocyanine membrane dye (DiD) (Invitrogen, Molecular Probes, Paisley, Renfrew, UK) for 30 min in serum-free media before being seeded onto the equivalent confluent cells. Two hours following co-culture, media containing floating cells were discarded and confluent cells were trypsinised. The percentage of DiD-labelled cells that had adhered to non-labelled cells of the same type were quantified by flow cytometry. Migration of tumour cells was investigated using a wound closure assay: the cells were seeded onto 0.2% gelatine, once confluent 10 µg/ml mitomycin C was added to inhibit cell proliferation, and a 50 µm scratch was made across the monolayer. The percentage wound closure was measured at 6 and 24 h. Tumour cell invasion was assessed using 6 mm Transwell plates with an 8.0 µm pore size (Costar, Corning Incorporated, St Louis, MO, USA) either uncoated or coated with matrigel (Invitrogen). The cells were seeded into the inner chamber at a density of 5×10^5 per assay in RPMI without FCS and RPMI supplemented with FCS was added to the outer chamber. After 4, 6 or 24 h following seeding cells were removed from the top surface of the membrane and cells that had invaded through the pores were stained with haematoxylin and eosin (H&E). Invasion was calculated as the percentage of cells that invaded through the basement membrane compared with cells that had moved to the underside of uncoated plates. The numbers of cells were counted using a DMRB microscope (Leitz, Biebrach, Baden-Württemberg, Germany) and OsteoMeasure XP v1.2.0.1 program (Osteometrics, Inc., Decatur, GA, USA).

Analysis of tumour growth *in vivo*

For tumour growth in bone, 1×10^5 MDA-P cells were injected into the left cardiac ventricle and 1×10^5 MDA-IV

Figure 1

Diagrammatic representation showing (a) differences between tumour cell homing to bone and bone colonisation and (b) generation of MDA-IV cells. Parental MDA-MB-231 (MDA-P) cells were inoculated into the left cardiac ventricle of 12-week-old female Balb/c nude mice. Following tumour growth in the long bones, tumour cells were extracted and cultured *ex vivo*.

These cells were then re-inoculated into the left cardiac ventricle or lateral tail vein of new Balb/c nude mice. This procedure was repeated seven times before tumour cells gained the ability to colonise mouse long bone following i.v. inoculation (MDA-IV cells).

cells were injected into the tail vein of the female mice ($n=25$ per group). Tumour take was monitored using a GFP imaging system (LighTools, Encinitas, CA, USA). The animals were killed at 7, 14, 21, 28 and 35 days following tumour cell inoculation ($n=5$ per group) by cardiac exsanguination and cervical dislocation. Serum was stored at -80°C for future analysis. Right tibiae were fixed in 4% paraformaldehyde, analysed by microcomputed tomography imaging (μCT) for osteolytic lesions, decalcified in a solution of 1% paraformaldehyde/0.5% EDTA in PBS for 4 weeks and then embedded in paraffin wax. Intratibial tumours from

the left tibiae were placed in RNAlater (Ambion, Paisley, Renfrew, UK) and stored at -80°C before RNA extraction. Intratibial tumour volume was measured by drawing round tumours on four H&E-stained $5\text{ }\mu\text{m}$ (non-serial) histological sections per sample using Osteomeasure Software (Osteometrics, Inc.) and a computerised image analysis system. For subcutaneous tumour growth, 0.5×10^6 MDA-P or MDA-IV cells were injected into both flanks of 12-week-old female mice ($n=10$ per group). Tumour growth was monitored by calliper measurements and animals were killed once the first tumour reached 1 cm^3 .

Microcomputed tomography imaging

Analysis of bone volume was carried out using a Skyscan 1172 X-ray-computed microtomograph (Skyscan, Aartselaar, Belgium). Pixel size was set to 4.37 μm and scanning was initiated from the top of the proximal tibia or the distal femur. For each sample, 275 cross-sectional images were reconstructed with NRecon Software (version 1.4.3, Skyscan). For trabecular bone, the volume of interest included cancellous bone and excluded the cortices. Trabecular bone volume fraction (BV/TV) – the ratio of the volume of bone present (BV) to the volume of the cancellous space (TV) – was calculated for 1 mm of the bone, starting 0.2 mm from the growth plate. For cortical bone, the volume of interest included only the cortices. Cortical volumes of the tibia and femur were calculated for 1.5 and 0.9 mm of bone respectively. Modelling and analysis were performed with the use of CTAn (version 1.5.0.2) and CTvol (version 1.9.4.1) Software (Skyscan).

Real-time PCR

Total RNA was extracted with TRIzol (Invitrogen AB) before RT using SuperScript III (Invitrogen). cDNA from MDA-P tumours or MDA-IV tumours extracted from the tibiae, grown subcutaneously or from cells grown *in vitro* were analysed on low-density metastasis array microfluidic plates, each comprising 96 genes associated with human breast cancer metastasis (plate number: 4414229, Applied Biosystems). Expression profiles of genes assessed as being associated with either bone homing or colonisation of bone were confirmed independently in three samples per group (intratibial, subcutaneous or cells grown *in vitro*) for MDA-P and MDA-IV cells using TaqMan gene expression assays. Relative mRNA expression of *FN1* (Hs00365058_m1), *HRAS* (Hs00610483_m1), *IL1B* (Hs00174097_m1), *MMP9* (Hs00234579_m1), *NME1* (Hs02621161_m1), *S100A4* (Hs00243202_m1 and Hs01569256_m1) and *SERBP1* (Hs00167155_m1) was compared with that of the housekeeping gene *GAPDH* (Hs99999905_m1) (Applied Biosystems) using an ABI 7900 PCR System (PerkinElmer, Applied Biosystems, Foster City, CA, USA) and TaqMan universal master mix (Applied Biosystems). Relative mRNAs were determined using the formula: $\Delta\text{CT} = \text{CT (target gene)} - \text{CT (GAPDH)}$.

Immunohistochemistry

Immunohistochemistry of tumours was carried out on 5 μm paraffin sections using rabbit polyclonal antibodies that

recognised human γ CATENIN (2309s, 1:500 dilution, New England Biolabs, Ipswich, MA, USA), HRAS (ab97488, 1:200 dilution, Abcam plc, Cambridge, UK), interleukin 1B (IL1B; ab2105, 1:200 dilution, Abcam plc), fibronectin (ab32419, 1:1000 dilution, Abcam plc) and S100A4 (ab40722, 1:500 dilution, Abcam plc) or rat MAB to E-CADHERIN (DECMA-1, 1:50 dilution, Abcam plc) followed by a biotin-conjugated anti-rabbit or anti-rat secondary antibody (1:200 dilution, Vector Laboratories, Peterborough, UK). The secondary antibody was detected by incubation with 3,3-diaminobenzidine (Vector Laboratories).

MDA-P and MDA-IV cells were plated onto chamber slides at a density of 1×10^4 cells/chamber, 24 h before immunofluorescent staining with the antibodies described followed by fluorescein-conjugated anti-rabbit secondary antibody (1:200, Vector Laboratories). Slides were mounted with Vectashield HardMount containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and imaged using a LEICA DMI4000B microscope.

IL1 β expression was assessed on a tissue microarray (TMA) containing primary breast cancer tumour cores taken from 150 patients included in the large clinical trial known as AZURE (Coleman *et al.* 2011). The samples were taken for pre-treatment and patients were subsequently randomised to standard adjuvant therapy with or without the addition of zoledronic acid for 5 years (Coleman *et al.* 2011). The TMA was stained for IL1B (ab2105, 1:200 dilution, Abcam plc) and scored blindly by a histopathologist, Prof. S S Cross. Tumours were assessed as either IL1B-negative or -positive and then linked to disease recurrence (any site) or disease recurrence specifically in bone (+/– other sites).

ELISA

IL1B present in mouse serum, from cell lysates or from conditioned media following 24 h exposure to cells, was detected using a Human IL1B ELISA Kit (EH2IL1B, Pierce Biotechnology, Thermo Scientific, Rockford, IL, USA) as per the manufacturer's instructions. Results were expressed in $\mu\text{g}/\text{cell}$.

Statistical analysis

Statistical analysis of experiments using non-clinical material was carried out by unpaired *t*-test using GraphPad Prism Software, version 5.0 (GraphPad prism software Inc. La Jolla, CA, USA). Statistical significance was defined as $P > 0.05$. The log-rank test was used for the analysis of disease outcomes, according to IL1B status of the primary breast tumours.

Figure 2

Differences between MDA-P and MDA-IV cells *in vitro*. Graphs showing mean $\pm$ S.E.M. for (a) the growth dynamics of MDA-P compared with MDA-IV cells over a 96 h time period, (b) percentage of cells that have adhered to cells of the same type 2 h following seeding onto a confluent monolayer;

(c) photomicrographs of and (d) percentage of wound closure by MDA-P or MDA-IV cells at 6 and 24 h as assessed by scratch assay, and (e) percentage of tumour cells that have invaded through a basement membrane.

* $P < 0.05$ by unpaired Student's *t*-test.

Results

Comparison of MDA-P and MDA-IV cells *in vitro*

The primary aims of this study were to identify molecular alterations acquired by breast cancer cells resulting in specific homing to bone and to separate these from alterations associated with bone colonisation (Fig. 1a). We generated a clone of MDA-MB-231 cells that reproducibly home to and form tumours in the mouse long bones following injection into the lateral tail vein (MDA-IV). MDA-IV cells were established following repeated *in vivo* passaging of MDA-MB-231 cells through the long bones of mice and re-injection via the left cardiac ventricle. Seven rounds of *in vivo* passaging were performed before a line of cells capable of homing to the bone following i.v. injection was identified (Fig. 1b). MDA-IV cells specifically home to and grow in the bone microenvironment and no tumour growth was detected in any extra-osseous organs of nude mice following i.v. injection of MDA-MB-231 *in vivo* passages 1–6.

To assess whether the increased bone homing by MDA-IV cells was associated with altered tumour cell growth, we compared the growth dynamics of MDA-P and MDA-IV cells in culture over a 96 h time period. No significant differences in the rate of proliferation of

tumour cells were detected between MDA-P and MDA-IV cells any time point (Fig. 2a).

The initial stages of the metastatic process are associated with a loss of cell–cell adhesion, as well as increased migration and invasion of cancer cells. We investigated whether these processes were altered in cells with an increased capacity to home to the bone, comparing MDA-P and MDA-IV cells (Fig. 2). Analysis of the percentage of cells that were able to adhere to a monolayer of cells of the same type demonstrated that the bone homing MDA-IV cells exhibited significantly decreased levels of cell–cell adhesion compared with MDA-P cells (22.53 ± 0.37 vs $30.29 \pm 0.39\%$, $P < 0.01$; Fig. 2b). In contrast, MDA-IV cells exhibited significantly decreased migration of cells in a wound-healing assay compared with MDA-P cells (Fig. 2c and d). At 6 h, MDA-IV cells repopulated $13.7 \pm 2.6\%$ of the wound area compared with $23.3 \pm 3.5\%$ for MDA-P cells ($P < 0.05$) and at 24 h wound closure increased to $74.4 \pm 3.8\%$ for MDA-IV cells and $99.4 \pm 0.4\%$ for MDA-P cells ($P < 0.01$). Despite differences in cell–cell adhesion and migration being observed between MDA-IV and MDA-P cells, both cell types were highly invasive and no differences in invasion were detected between the two cell lines after 4, 6 or 24 h following seeding (Fig. 2e).

Characterisation of tumour growth *in vivo*

To characterise the *in vivo* growth dynamics of MDA-IV compared with the original MDA-P cells, mice received injections of MDA-IV cells via the lateral tail vein or of parental MDA-P cells via the left cardiac ventricle ($n=25$ per group); five to seven mice per group were culled at weekly intervals to enable the measurement of tumour growth and the extent of osteolytic bone disease. No significant differences in time until first detection of tumour growth or take-rate in bone were observed between MDA-P or MDA-IV cells: GFP imaging identified tumours growing in the long bones of 25% of mice that had received injections of MDA-P cells and in 40% of mice that received injections of MDA-IV cells at day 14, with no tumour growth being observed in either group before this time point. By day 28, large tumours were clearly visible in 80% of mice that received MDA-P cells and 83% that received MDA-IV cells. No significant differences in tumour growth in bone were observed between the two cell lines (Fig. 3a). Histological analysis showed no significant differences in tumour area in mouse long bones bearing MDA-P or MDA-IV cells (Fig. 3b) and both MDA-P and MDA-IV tumours were associated with a lytic bone lesions. μ CT analysis of tibiae bearing both MDA-P and MDA-IV tumours shows an increase in bone loss correlating with increased tumour area, with the extent of lytic disease being similar for both cell lines (Fig. 4). Interestingly, detectable tumour growth outside of bone

was exclusive to lungs and lung tumours were only observed following intracardiac injection of MDA-P cells (Fig. 3a). Taken together, these data confirm that the only overt difference between MDA-P and MDA-IV cells is in their capacity to home to bone following i.v. injection.

To differentiate between molecular alterations associated with tumours growing in an *in vivo* environment as opposed to cells growing in an *in vitro* culture, 0.5×10^6 of either MDA-P or MDA-IV cells were injected into both flanks of female Balb/c nude mice and tumour growth was assessed before analysis of gene and protein expression. Similarly to cells grown *in vitro* and in bone, no differences in growth rates were detected between subcutaneous MDA-P and MDA-IV tumours at any point over a 30 day time period, mean tumour area of $4.4 \pm 1.1 \text{ mm}^2$ being recorded for MDA-P cells and $3.4 \pm 1.7 \text{ mm}^2$ for MDA-IV cells at 35 days (Fig. 3c).

Genetic alterations associated with bone-homing

Intravenous injection of MDA-IV cells, but not MDA-P cells, results in tumour growth in bone. Therefore, MDA-IV cells must have undergone molecular alterations required for homing to bone during the repeated *in vivo* passaging. To identify some of the genetic alterations associated with this bone homing, we compared expression of genes associated with metastasis between MDA-IV cells and MDA-P cells grown *in vitro* (Fig. 5). Analysis of low-density array plates

Figure 3

Growth of MDA-P and MDA-IV cells *in vivo*. (a) LighTools images of GFP-expressing tumours in mouse long bones, 7, 21 and 35 days following tumour cell inoculation (white arrows show presence of small GFP-expressing tumours) and a table showing the percentage of mice with

detectable tumour growth in and outside the long bones. (b) Mean tumour area $\pm$ s.e.m. in bone and (c) mean subcutaneous tumour area $\pm$ s.e.m. No significant differences in tumour growth were detected between MDA-P and MDA-IV cells.

Figure 4

The effects of bone homing (MDA-IV) and parental (MDA-P) cells on bone volume. (a) Representative cross sectional μ CT images from femours of mice 21 and 35 days post inoculation. (b) Mean trabecular bone volume (percentage BV:TV) $\pm$ s.e.m. in tumour-bearing bone. Tumour-bearing mice showed reduced trabecular bone volume and increased formation of osteolytic lesions from day 28 onwards compared with naïve controls (indicated by dashed line).

containing 96 genes associated with human breast cancer metastasis identified 19 genes, whose expression was increased greater than twofold in MDA-IV cells compared with MDA-P cell grown *in vitro*, and four genes whose expression was decreased. *EPHB2*, *ETV4*, *FGFR4*, *GNRH1*, *HRAS*, *HTATIP*, *IL1B*, *MCAM*, *MMP1*, *MMP2*, *MMP3*, *MMP4*, *NME1*, *RB1*, *RBL1*, *RBL2*, *SERBP1*, *SMAD2* and *TACSTD1* were increased; *CEACAM1*, *DCC*, *FN1* and *MMP10* were decreased (Supplementary Table 1, see section on supplementary data given at the end of this article).

Genes found to display differences in expression between MDA-IV and MDA-P cells *in vitro* were further investigated by RT-PCR (Table 1). This verified that expression of the cell proliferation and differentiation-associated gene *Il1b* was significantly increased in MDA-IV compared with MDA-P cells (28.83 ± 3.05 -fold increase; $P < 0.01$). In addition, expression of the cell adhesion, migration and growth-associated gene *Fn1* was reduced by 5.38 ± 0.19 -fold and the migration and calcium signal-

binding protein *S100A4* by 3.5 ± 0.12 -fold in MDA-IV cells compared with MDA-P cells ($P < 0.05$ and $P < 0.01$ respectively).

Genetic alterations associated with bone colonisation

As both MDA-P and MDA-IV cells generated tumour growth in bone, both cell lines must contain a population of cells with bone-colonising properties. However, the majority of cells reaching the bone die within a few days and tumour colonies are formed from a subset of the initial cell population (Phadke *et al.* 2006), implying that most cells entering the bone microenvironment are not equipped with the necessary adaptations to form metastatic tumours. We have therefore investigated the genetic differences between MDA-P and MDA-IV cells grown in bone *in vivo* with the same cells growth *in vitro*. To ensure that alterations in gene expression were specifically associated with growth in bone, rather than in an unspecific site *in vivo*, the expression profiles of tumour cells grown subcutaneously were first subtracted from those of the same tumour cells grown in bone, before comparing the profiles of cells grown *in vitro* (see Fig. 5). Of our panel of 96 genes, expression of 16 was altered greater than twofold between tumours extracted from bone and the same cell line grown *in vitro*, indicating that these are associated with tumour cell colonisation of and/or progression in bone. There was an increase in the expression of four genes (*FN1*, *GNRH1*, *MMP9* and *S100A4*) in both MDA-P and MDA-IV tumours in bone compared with cells grown *in vitro*. Expression of *CTDP1*, *ETV4*, *EGFR4*, *IL1B*, *MCAM*, *MMP1*, *RB1* and *SMAD2* was reduced in MDA-P and MDA-IV tumour cells grown in bone, when compared with the same cells grown *in vitro*. In addition, genes altered in both cell lines, *EPHB2*, *MMP3*, *PTGS2* and *RBL1*, were downregulated in MDA-IV tumours in bone compared with expression *in vitro* (Supplementary Table 2, see section on supplementary data given at the end of this article).

Validation of gene expression profiles associated with bone colonisation by quantitative RT-PCR identified a 54.28-fold increase in *FN1* expression in MDA-P cells and a 147.68-fold increase in MDA-IV cells grown *in vivo* compared with *in vitro* ($P < 0.005$); *HRAS* was increased by 13.34-fold in MDA-P cells ($P < 0.05$) and 9.85-fold in MDA-IV cells ($P < 0.05$), and *MMP9* was increased by 22.35-fold in MDA-P ($P < 0.005$) and 16.23-fold in MDA-IV cells ($P < 0.005$). *IL1B* was downregulated when the cell lines were grown *in vivo*, rather than being specific to bone homing. All tumours expressed significantly lower levels of *IL1B* compared with cells grown *in vitro* with

Figure 5

Flow diagram of gene analysis for establishing (a) genes associated with tumour cell homing and (b) genes associated with tumour cell colonisation.

subcutaneous tumours expressing less *IL1B* than tumours growing in bone. In contrast to the array data, *S100A4* was differentially expressed in MDA-P and MDA-IV cells showing a 5.59-fold decrease in MDA-P cells and an 142.88-fold increase in MDA-IV cells grown in bone compared with *in vitro* ($P < 0.005$ and 0.0005 respectively). *NME1* and *SERBP1* were not significantly altered in expression between tumour cells growing in bone or *in vitro* (Table 2).

Expression profile of proteins associated with tumour cell homing and colonisation of bone

Gene profiling data shown above suggest that the ability of cells to home to the bone microenvironment is associated with reduced levels of *FN1* and *S100A4* and an increase in *IL1B* in MDA-IV cells, compared with MDA-P cells grown *in vitro*. In contrast to bone-homing, the genetic profile associated tumour cell colonisation and progression in bone involved increased expression of *FN1*, *MMP9* and *HRAS*. We therefore investigated the protein expression of these molecules in MDA-P and MDA-IV cells *in vitro* and *in vivo*. Immunohistochemical staining indicated decreased levels of fibronectin and S100A4 and increased levels of IL1 β protein in MDA-IV cells compared with MDA-P cells *in vitro* (Fig. 6). ELISA analysis of IL1 β from cell lysates revealed a 16-fold increase in IL1 β expression in MDA-IV cells compared with MDA-P cells (38.224 ± 1.251 vs 1.962 ± 1.387 pg from 1×10^6 cells respectively), with MDA-IV cells secreting significantly more IL1 β into the media (14.11 pg/ml per 1×10^6 cells) compared with MDA-IV cells (IL1 β expression was below the level of detection). These data confirm that bone-homing MDA-IV cells have a

distinct molecular phenotype to parental cells. Staining of tumours in mouse tibiae reveals increased levels of S100A4 protein in MDA-IV tumours compared with MDA-P tumours. Interestingly, no differences in fibronectin protein or IL1 β were detected between MDA-IV and MDA-P tumours in bone (Fig. 6) and in mouse serum, IL1 β protein was below the level of detection by ELISA, indicating that

Table 1 Expression profile of genes associated with homing of breast cancer cells to the bone microenvironment

Assay	<i>In vitro</i> MDA-IV/MDA-P
<i>FN1</i>	
Fold change	$-5.38 \pm 0.19^*$
Δ CT	$8.27 \pm 0.99/5.76 \pm 1.02$
<i>HRAS</i>	
Fold change	1.98 ± 0.13
Δ CT	$7.54 \pm 1.05/8.44 \pm 1.12$
<i>IL1B</i>	
Fold change	$28.83 \pm 3.05^+$
Δ CT	$8.12 \pm 0.97/12.87 \pm 1.06$
<i>MMP9</i>	
Fold change	-1.17 ± 0.12
Δ CT	$16.25 \pm 1.34/16.12 \pm 1.05$
<i>NME1</i>	
Fold change	2.11 ± 0.53
Δ CT	$2.99 \pm 1.19/3.69 \pm 0.86$
<i>S100A4</i>	
Fold change	$-3.5 \pm 0.12^+$
Δ CT	$11.21 \pm 0.89/9.32 \pm 1.00$
<i>SERPINE1</i>	
Fold change	1.61 ± 0.14
Δ CT	$5.79 \pm 1.23/6.29 \pm 1.11$

Data show the fold increase or decrease (–) of genes from bone-seeking, MDA-IV, compared with parental, MDA-P, cells grown *in vitro* and bone tumours. Subcutaneous tumours were used as controls. RT-PCR was performed in triplicate with data representing mean $\pm$ s.e.m. Data highlighted in bold are statistically significant according to the unpaired *t*-test. * $P < 0.05$, + $P < 0.01$.

Table 2 Expression profile of genes associated with colonisation of breast cancer cells in the bone microenvironment

Assay	MDA-P bone/cell (A)	MDA-P bone/ subcutaneous (B)	MDA-IV bone/cell (C)	MDA-IV bone/ subcutaneous (D)	Change associated with colonisation of MDA-P cells (A-B)	Change associated with colonisation of MDA-IV cells (C-D)
<i>FN1</i>						
Fold change	57.29 ± 1.77 [†]	3.01 ± 0.88	145.55 ± 23.42	-2.13 ± 0.53	54.28	147.68
ΔCT	-0.08 ± 0.93/5.76 ± 1.03	-0.08 ± 0.93/1.88 ± 1.69	2.88 ± 2.42/8.27 ± 0.99	2.88 ± 2.42/1.88 ± 1.69		
<i>HRAS</i>						
Fold change	1.33 ± 0.17	-12.01 ± 6.76	7.58 ± 1.26*	-2.27 ± 0.68	13.34	9.85
ΔCT	8.04 ± 0.99/8.44 ± 1.12	8.04 ± 0.99/5.02 ± 2.31	6.40 ± 2.33/7.54 ± 1.05	6.40 ± 2.33/5.34 ± 1.79		
<i>IL1B</i>						
Fold change	-6.37 ± 0.66*	-52.1 ± 24.48	-8.5 ± 3.68 [†]	-15.20 ± 4.79	45.73	6.7
ΔCT	15.51 ± 0.99/12.87 ± 1.06	15.51 ± 0.99/10.23 ± 2.59	12.71 ± 2.86/8.12 ± 0.97	12.71 ± 2.86/8.93 ± 2.51		
<i>MMP9</i>						
Fold change	6.0 ± 0.5 [†]	-16.35 ± 4.65	14.76 ± 1.91 [†]	-1.47 ± 0.95	22.35	16.23
ΔCT	13.56 ± 1.39/16.12 ± 1.05	13.56 ± 1.39/9.79 ± 2.27	10.64 ± 2.48/16.25 ± 1.34	10.64 ± 2.48/10.87 ± 1.92		
<i>NME1</i>						
Fold change	-1.28 ± 0.12	-5.18 ± 0.82	1.37 ± 0.28	2.31 ± 0.72	3.9	-0.94
ΔCT	2.99 ± 1.19/3.69 ± 0.86	1.14 ± 2.33/4.39 ± 1.03	1.14 ± 2.33/2.99 ± 1.19	4.39 ± 1.03/3.69 ± 0.86		
<i>ST00A4</i>						
Fold change	-3.5 ± 0.12 [†]	2.09 ± 0.55	164.8 ± 51.36	21.92 ± 0.69 [†]	-5.59	142.88
ΔCT	11.21 ± 0.89/9.32 ± 1.00	5.76 ± 2.33/4.86 ± 1.03	5.76 ± 2.33/11.21 ± 0.89	4.86 ± 1.03/9.32 ± 1.01		
<i>SERBP1</i>						
Fold change	1.61 ± 0.14	3.38 ± 0.66	2.33 ± 0.36	1.13 ± 0.08	-1.77	1.2
ΔCT	5.79 ± 1.23/6.29 ± 1.11	4.84 ± 2.97/6.33 ± 0.93	4.85 ± 2.9/5.79 ± 1.23	6.33 ± 0.93/6.29 ± 1.11		

Data show the fold increase or decrease (–) of genes present in bone tumours compared with those grown *in vitro* (bone/cell) and as subcutaneous tumours (bone/subcutaneous). These comparisons were performed for both parental, MDA-P and bone-seeking MDA-IV cells. RT-PCR was performed in triplicate with data representing mean ± s.e.m. Data highlighted in bold are statistically significant by unpaired t-test, * $P < 0.05$, [†] $P < 0.01$ and [‡] $P < 0.001$.

Figure 6

Photomicrographs of immunohistochemical staining for fibronectin, HRAS, IL1B and S100A4 protein expression in (a) MDA-P and MDA-IV cells grown *in vitro* (green staining indicates protein expression and blue shows DAPI stained cell nuclei) and (b) MDA-P and MDA-IV tumours grown in bone and subcutaneously *in vivo* (protein expression is shown as brown staining). Immunohistochemical staining showed a decrease in fibronectin and HRAS

and an increase in expression of IL1B and S100A4 in bone-homing MDA-IV cells compared with MDA-P cells grown *in vitro*. Although fibronectin was not expressed in bone tumours, IL1B and S100A4 were increased in MDA-IV compared with MDA-P bone tumours and no differences in HRAS were observed between bone-homing and parental cells growing in bone. Scale bars show 0–50 μ m.

IL1B may be reduced in MDA-IV cells and fibronectin may be downregulated in parental cells once they are established *in vivo*. As the cell-division-associated gene *HRAS* was found to be upregulated in MDA-IV cells grown *in vivo* compared with parental cells and with cells grown *in vitro*, we also investigated the expression of this protein. In accordance with gene expression, similar levels of HRAS protein were detected in MDA-P and MDA-IV cells when grown in culture; however, immunohistochemical staining of

metastatic bone tumours shows an increase in HRAS in tumours that developed from MDA-IV cells, compared with those that developed from MDA-P cells.

Expression profile of IL1B in primary breast tissue

Immunohistochemistry for the presence of IL1B was carried out on a TMA, containing 150 primary breast tumour cores taken from patients presenting with

stage II/III breast cancer. With a median follow-up of 84 months, significant correlations between expression of IL1B and both disease recurrence at any site ($P < 0.0001$) and metastasis to bone ($P < 0.0001$) were seen. 49% of patients with detectable IL1B in their primary tumours developed a recurrence compared with 7% of patients with IL1B-negative primary tumours (Fig. 7c). Similarly, 37% of patients with detectable IL1B in their primary tumours developed bone metastasis compared with 5% of patients with IL1B negative primary tumours (Fig. 7d).

Discussion

The primary aims of this study were to identify and distinguish between molecular expression profiles that are specific to breast cancer homing to bone and bone colonisation. In order to investigate these separate processes, following i.v. injection (MDA-IV), we generated a clone of MDA-MB-231 cells that specifically homed to the long bones of Balb/c nude mice and compared these cells with the parental cell line (MDA-P). We confirmed that bone-homing properties were specific to MDA-IV cells by injecting both cell types IV into mice. In this experiment, only MDA-IV cells were able to form tumours in the long bones and over an 8-week time period tumour growth was never observed following i.v. injection of

MDA-P cells. Despite differences in bone-homing properties, MDA-IV and MDA-P cells colonised bone with the same frequency following i.v. and intracardiac injection, respectively, and grew at the same rate in the bone microenvironment, forming lytic lesions of similar size, implying that differences between these two cell lines are specific to bone-homing rather than colonisation/growth in bone. Other research groups have adopted similar techniques to investigate genes associated with breast cancer metastasis to bone. Kang *et al.* (2003) and Bellahcène *et al.* (2007) both carried out microarray analysis on separately derived bone-seeking clones of MDA-MB-231 cells. In these studies, bone-seeking MDA-MB-231 cells were compared with parental cells grown under *in vitro* culture conditions. Importantly, subsequent analysis has shown a close correlation in proteins changed between parental and bone-homing MDA-MB-231 cells grown *in vitro* and proteins changed between primary and bone-metastatic deposits of clinical samples acquired from the same patient (Dumont *et al.* 2012). Therefore, alterations between parental and bone-homing variants of MDA-MB-231 cells are likely to be clinically relevant to breast cancer patients. None of these studies investigated genetic alterations in cells grown *in vivo* or in the bone microenvironment, despite describing genetic differences found between the cell lines as being related to breast

Figure 7

IL1B expression in human primary breast cancer biopsies from untreated breast cancer patients. Photomicrographs showing positive (a) and negative (b) staining for IL1B. Survival curves showing association between

IL1B expression and (c) disease-free interval (metastasis to any site or death) or (d) time taken until skeletal metastasis. The log-rank test was employed to assess significance.

cancer bone metastasis. Furthermore, previous studies have made no attempt to differentiate between molecules that are specific to bone-homing and those that are related to subsequent colonisation.

It is widely accepted that metastasis is initiated by tumour cell shedding into the circulation following genotypic changes in the cancer cells from an epithelial to a mesenchymal cell type; commonly referred to as epithelial to mesenchymal transition (EMT). This process is thought to be driven by the initial loss of cell adhesion molecules including E-cadherin, and α , β and γ catenins (Yang & Weinberg 2008). The *in vivo* model system used in the current study involved inoculating tumour cells directly into the circulation and therefore did not mimic tumour cell shedding. Neither MDA-P or MDA-IV cells were found to express E-cadherin, β or γ -catenin (data not shown) and both of these cell lines were highly invasive *in vitro*, indicating that alterations between the two cell lines are not linked to changes associated with EMT. We can therefore be confident that differences between the cell lines are specific to the later stage of metastasis, bone-homing.

We found that bone homing was associated with decreased cell–cell adhesion and migration, coupled with significantly reduced levels of the cell adhesion and migration molecule fibronectin. In agreement with this, comparative analysis of a separate bone-seeking clone of MDA-MB-231 (B02) cells with their parental line carried out by Bellahcène *et al.* (2007) also revealed significant reductions in fibronectin. In the B02 study, loss of fibronectin as well as S100A4 was reported to be associated with breast cancer metastases in bone; however, our findings do not support this hypothesis; following colonisation of MDA-IV and MDA-P cells in the bone microenvironment, we found a substantial increase in fibronectin and differential changes in the levels of S100A4 with significantly decreased levels in MDA-P cells and increased levels in MDA-IV cells. S100A4 expression was reduced in both cell lines grown *in vitro*, indicating that loss of these molecules is specifically associated with breast cancer cell homing to bone and not colonisation. Interestingly, bone-homing was associated with increased IL1B. No previous reports have shown a specific link between IL1B expression and bone metastasis, autocrine production of IL1B has been associated with poor prognosis of a variety of human carcinomas, including breast, ovarian and pancreatic ductal carcinoma (Kiefel *et al.* 2012). It has been suggested that IL1B expression in breast cancer cells promotes a motile and invasive cell phenotype, accounting for the poor prognosis observed

in patients (Kiefel *et al.* 2012). We have now clarified the role of IL1B in breast cancer metastasis, showing that increased expression of this protein in primary tumours appears to be strongly linked with adverse clinical outcome and a significantly increased likelihood of developing bone metastasis. We plan to validate this potentially important clinical observation in a larger dataset of tumours to investigate the possibility that this molecule could be used as a companion diagnostic to help select patients most likely to benefit from bone-targeted treatments.

For tumour cells to colonise the bone microenvironment, they must be able to leave the circulation and lodge within the stem cell niche before proliferation. Our data demonstrate that this process is associated with increased levels of MMP9, HRAS and fibronectin. MMP9 has strong associations with increased tumour cell invasion in a number of cancer types including lung, non-Hodgkin's lymphoma and giant cell tumours (Ueda *et al.* 1996, Chu *et al.* 1997, Friedrich *et al.* 2010). Furthermore, increased MMP9 expression has been shown to increase the capacity of tumour cells to extravasate as well as to cause activation of bone-resorbing osteoclasts (Friedrich *et al.* 2010), possibly accounting for the lytic lesions observed in long bones of mice following colonisation of MDA-P and MDA-IV cells. In addition to the pro-invasive properties of MMP9, HRAS overexpression has been shown to increase the invasion of MCF10A breast cells and to transform these cells from an endothelial to an epithelial cell type, processes that are both strongly linked with tumour cell colonisation of a metastatic site (Kim *et al.* 2003). Therefore, it is likely that increased MMP9 and HRAS expression may drive bone colonisation through increasing the tumour cell extravasation once they reach their target metastatic organ (bone). Fibronectin, on the other hand, has well characterised properties as a cell adhesion molecule and has been shown to be upregulated in bone metastatic deposits compared with primary breast tumours (Dumont *et al.* 2012). It is likely that increased expression of this molecule enables tumour cells to adhere to the stem cell niche once they have successfully entered the bone microenvironment (van der Pluijm *et al.* 1997, Saad *et al.* 2002).

Altered expression profiles of fibronectin, S100A4, HRAS and MMP9 in primary breast tissue have previously been shown to be associated with poor clinical outcome: high fibronectin expression has been linked with increased tumour aggressiveness and significantly reduced life expectancy (Bae *et al.* 2013). Tissue array analysis of

samples taken from 110 breast cancer patients has shown strong correlation between high fibronectin expression and higher probability of distant metastasis. In addition, the same study revealed a strong association between MMP9 expression and low overall survival (Fernandez-Garcia *et al.* 2014). Using a similar array, Watson *et al.* (1991) showed a strong correlation between HRAS and a trend towards lymph node involvement, early re-occurrence and death from cancer irrespective of ER, grade and clinical stage. Similarly, S100A4 is well recognised as an inducer of metastasis and indicator of poor prognosis in breast cancer (Andersen *et al.* 2011). In a study of 92 patients, increased expression of S100A4 was shown to be an early step in the metastatic process and it has been speculated that this protein can be used as an early predictor of metastasis in early-stage breast cancer (Lee *et al.* 2004). All available clinical data on these molecules are currently taken from their expression profiles in primary tumour samples. The purpose of our study was to investigate molecular alterations associated with tumour cell homing and colonisation of the bone environment, these data cannot be surmised from looking at the primary tumour alone. In order to study this in patients' material, circulating tumour cells from blood and bone metastatic deposits from the same patient are required. This material is difficult to obtain in sufficient quantities for statistically viable molecular analysis; therefore, we have used *in vivo* modelling to investigate these differences.

In conclusion, our data demonstrate that different molecules drive breast cancer cell homing to bone and subsequent colonisation of the bone microenvironment. Bone-homing is associated with loss of cell–cell adhesion accompanied by reduced levels of fibronectin as well as increased IL1B. Bone colonisation is associated with increased tumour cell migration and adhesion, as well as increased expression of fibronectin, HRAS and MMP9. These findings warrant further investigation into the *in vivo* metastatic functions of these molecules with respect to bone metastasis with the aim of developing future diagnostic and therapeutic modalities.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-13-0158>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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