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Runx1 is associated with breast cancer progression in MMTV-PyMT transgenic mice and its depletion *in vitro* inhibits migration and invasion

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Abstract

Runx1 is a transcription factor essential for definitive hematopoiesis, and genetic abnormalities in Runx1 cause leukemia. Runx1 is functionally promiscuous and acts as either an oncogene or tumor suppressor gene in certain epithelial cancers. Recent evidence suggests that Runx1 is an important factor in breast cancer, however its role remains ambiguous. Here, we addressed whether Runx1 has a specific pathological role during breast cancer progression and show that Runx1 has an oncogenic function. We observed elevated Runx1 expression in a subset of human breast cancers. Furthermore, throughout the course of disease progression in a classical mouse model of breast cancer (i.e., the MMTV-PyMT transgenic model), Runx1 expression increases in the primary site (mammary gland) and is further upregulated in tumors and distal lung metastatic lesions. *Ex vivo* studies using tumor epithelial cells derived from these mice express significantly higher levels of Runx1 than normal mammary epithelial cells. The tumor cells exhibit increased rates of migration and invasion, indicative of an aggressive cancer phenotype. Inhibition of Runx1 expression using RNA interference significantly abrogates these cancer-relevant phenotypic characteristics. Importantly, our data establish that Runx1 contributes to murine mammary tumor development and malignancy and potentially represents a key disease-promoting and prognostic factor in human breast cancer progression and metastasis.

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Competing interests

The authors declare that they have no competing interests.

Keywords

Runx1; breast cancer progression/metastasis; MMTV-PyMT

Introduction

Breast cancer is the most frequently diagnosed cancer in women and the leading cause of cancer-related mortality among women worldwide, accounting for almost a quarter of all cancers and 14% of cancer deaths (Jemal et al., 2011). Metastasis is often a fatal step in breast cancer progression, characterized by tumor cell intravasation, survival in circulation, and extravasation to distal sites such as the lung (Langley and Fidler, 2007). Perou and colleagues established over a decade ago that breast cancer progression and metastasis are intimately coupled with specific complex molecular transcriptional programs and downstream signaling pathways (Perou et al., 2000). In order to realize effective targeting of such molecular components for therapeutic benefit, it is vital that a more extensive understanding of the dysregulated molecular pathology of breast cancer is achieved.

Transcription factors are master regulators of signaling pathways in both normal and abnormal physiological processes, and have been shown to play key roles in cancer (Vaquerizas et al., 2009). Runt-related transcription factor 1 (Runx1, also known as AML1, CBF α 2 or PEBP2 α B) is one of a family of three transcription factors – Runx1, Runx2 and Runx3 – each associated with specific human cancers (Ito, 2004, Cameron and Neil, 2004, Chuang et al., 2013, Blyth et al., 2005). Runx1 functions as an activator or repressor of transcription by binding DNA through the Runt domain in complex with its binding partner core binding factor β (CBF β) (Speck and Terry, 1995). Runx1 regulates a variety of physiological processes, including cell survival, proliferation, differentiation and cell cycle progression and is most recognized for its role in hematopoiesis and leukemia (Blyth et al., 2005, Friedman, 2009, Mikhail et al., 2006, Ichikawa et al., 2013, Lambert et al., 2012). Recent reports have associated Runx1 with breast cancer (Janes, 2011, Chimgé and Frenkel, 2013, Kadota et al., 2010, van Bragt et al., 2014, Taniuchi et al., 2012, Ferrari et al., 2014), and genome-wide sequencing of cohorts of breast cancer patients revealed it is one of the most prominently mutated and/or deleted genes in breast cancer (2012, Ellis et al., 2012, Banerji et al., 2012).

This study addresses if Runx1 expression correlates with breast cancer progression and metastasis and if Runx1 is important for maintenance of a cancer-related cell phenotype. To this end, a clinically informative *in vivo* mouse model for mammary tumor development that permits molecular and histological analysis of tumor progression and metastasis as well as complementary cell models were investigated (Chimgé and Frenkel, 2013, Taniuchi et al., 2012, Janes, 2011, Wotton et al., 2002, Cheon and Orsulic, 2011, Lin et al., 2003). In the transgenic mouse model used, mammary gland specific expression of a polyoma middle T-antigen (PyMT) transgene is achieved using the mouse mammary tumor virus (MMTV) promoter (Guy et al., 1992). The potent PyMT oncoprotein, which acts as a membrane scaffold protein, impacts on signal transduction pathways that are also altered in human breast cancer including the Ras/Raf/MEK and PI3K/Akt pathways (Rodriguez-Viciana et

al., 2006). This results in a disease progression similar to human breast cancer, with the development of multiple mammary adenocarcinomas as well as metastatic lesions in the lung with almost 100% penetrance (Lin et al., 2003). MMTV-PyMT mice develop well-differentiated, luminal-type adenomas that progress to metastatic, poorly differentiated adenocarcinoma within 15 weeks (Lin et al., 2003, Herschkowitz et al., 2007). One of the major advantages of this model is that it can be used to study both primary mammary tumor development and metastasis.

Here, we confirmed the clinical relevance of Runx1 in breast cancer. Significantly, our *in vivo* interrogation of the MMTV-PyMT mouse model demonstrates that Runx1 expression increases concomitant with disease progression. Moreover, complementary *in vitro* studies establish that Runx1 is associated with higher migration and invasion ability; the knockdown of Runx1 supports its functional role in contributing to maintenance of a more aggressive tumor cell phenotype. Thus, these studies reveal the oncogenic potential of Runx1 in the progression and metastasis of breast cancer.

Materials and Methods

Mice

Animal studies were conducted in accordance with approved Institutional Animal Care and Use Committee (IACUC) protocols and the NIH Guide for Care and Use of Laboratory Animals. Female FVB/NJ mice (Jackson Laboratory, Bar Harbor, ME, USA) were crossed with male FVB mice that were transgenic (+/-) for PyMT antigen under the control of the MMTV promoter. Genotyping was performed by PCR as described previously for the PyMT transgene (Guy et al., 1992). Female mice from this cross that were PyMT^{+/-} were saved for further analysis. Mice were sacrificed at 4, 8, 10, 12, 13 and 15 weeks of age and whole mammary glands, tumor (if present) and/or lungs excised. The 15 week time point was considered to be the time point shortly before tumor burdens in mice reached a humane end point. To avoid non-biological variation, mice were sacrificed (and processed) at random ages from different litters at different times. Portions of tissues were either snap frozen for RNA extraction or fixed in 10% Zinc-Formalin solution and paraffin embedded for histological analysis.

Immunohistochemistry and semi-quantitative analysis

Formalin fixed paraffin embedded mammary gland, tumor and lung tissues from MMTV-PyMT mice were sectioned at 4µm on a Leica 2030 paraffin microtome (Leica Microsystems, Buffalo Grove, IL, USA). Before immunohistochemical procedures were carried out, routine hematoxylin and eosin staining was performed on each sample (Fischer et al., 2008).

The same immunohistochemical procedure was carried out for both the human tissue microarray and mouse tissue sections, except that only the mouse tissues were baked for one hour at 60°C. Following deparaffinization and rehydration, antigen retrieval was performed using DAKO Target Retrieval Solution (DAKO, Carpinteria, CA, USA), pH6.0 in 50% glycerol at 95°C for 20 minutes. Sections were blocked for endogenous peroxidase using

hydrogen peroxide in methanol followed by treatment with 1% bovine serum albumin, 10% normal goat serum and 0.1% Triton X-100. The tissue was incubated overnight at room temperature with anti-AML1 antibody (rabbit polyclonal, 1:100) (Cell Signaling, Danvers, MA, USA). The anti-AML1 antibody was validated to confirm its specificity (Supplementary Material Fig. S1A). The reaction was visualized using VectaStain ABC Elite Rabbit IgG and DAB (Vector Laboratories, Burlingame, CA, USA) according to manufacturer's instructions. Images were captured on an Olympus BX50 microscope (Olympus, Center Valley, PA, USA) using a QImaging Retiga 2000R camera with attached CRI color filter (QImaging, Surrey, BC, Canada). One positive and two negative controls were included with each staining procedure (Supplementary Material Fig. S1B).

Semi-quantitative analysis of Runx1 in the human breast cancer tissue microarray (Catalog # BR1503b; US Biomax, Rockville, MD, USA) was achieved in three different ways: 1) by assessment of the presence or absence of Runx1 staining; 2) by assessment of the Runx1 staining intensity, graded on a scale of None, Weak, Moderate and Strong; and 3) by estimation of the % of Runx1 positive cells present (See Table 1 and Supplementary Material Fig. S2). Staining was graded in a blind manner by two independent investigators (GB & NMB), with inter-observer agreement found to be substantial with a kappa value of 0.75 (Cohen's kappa coefficient test).

Runx1-stained mouse sections were evaluated based on immunoreactivity and scored on a staining scale system of absent (0) to strong (+4), with each score associated with an arbitrary value for graphical representation of the data (Buesa, 2005). Following staining procedures (as described), sections were scored as follows: absence of staining received a negative (-/0) designation and positive staining received a +1/25, +2/50, +3/75, or +4/100 score. A panel with images representing the above staining classification scores was created as a reference for each tissue (Supplementary Material Fig. S1C); subsequently, all images were scored based on the aforementioned reference/classification system. Sections were randomly selected from $n = 3$ animals per time point per group per tissue and were evaluated blindly by two independent investigators (GB & NMB) with <5% deviation in results from the two independent investigators.

Cell culture

The mouse MMTV cell line, a tumor epithelial cell line derived from the tumor of a 13 week old PyV-MT^{+/+} female mouse, was obtained from Dr. Leslie M. Shaw (University of Massachusetts Medical School) who developed the cell line (Nagle et al., 2004). These cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin. The NMuMG normal mouse mammary epithelial cell line were purchased from and validated by the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were cultured following instructions from ATCC and used within six months of purchase. Media and supplements were obtained from Life Technologies, Carlsbad, CA, unless otherwise stated. All cells were cultured at 37°C in a humidified 5% CO₂ atmosphere.

siRNA transfection

MMTV cells (~60–70% confluent) were transfected with control non-silencing siRNA (siNS – 500nM) or the On-Target Plus Mouse Runx1 siRNA smartpool (Thermo Scientific, Lafayette, CO, USA) (siRunx1 – 500nM) using Lipofectamine2000 (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. Controls in which cells were exposed to Lipofectamine2000 only (Mock) were also included where indicated. Gene and protein knockdown of Runx1 was assessed 48 hours after transfection, and growth, migration and invasion assays were initiated 24 hours after transfection ('0 hours' time point).

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from mouse tissues and cell lines using Trizol according to manufacturer's instructions (Life Technologies, Carlsbad, CA, USA) and purified with a Zymo RNA purification kit (Zymo Research, Irvine, CA, USA). cDNA was synthesized using the Superscript First-Strand Synthesis System according to manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). qRT-PCR was performed using SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA) and gene specific primers (Runx1 3': GGCCATGAAGAACCAGGTAG; Runx1 5': CAACTTGTGGCGGATTTGTA; RPL19 3': TTGCTCTAGTGTCTCCGC; RPL19 5': GGAATGGACAGTCACAGGC) in an Applied Biosystems Viia 7 system (Life Technologies, Carlsbad, CA, USA). After normalization to the reference gene (as indicated), relative expression levels of each target gene were calculated using the comparative C_T ($-C_T$) method.

Western blot analysis

Whole cell protein lysates were generated using RIPA buffer; nuclear and cytoplasmic protein lysates were generated using the NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific, Lafayette, CO, USA). Both protein isolation reagents were supplemented with 25 μ M MG132 and complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Lysates were separated on a 10% acrylamide gel and immobilized on PVDF membranes (Millipore, Billerica, MA, USA). Blots were blocked using 5% non-fat dry milk (Bio-Rad Laboratories, Hercules, CA, USA) before being incubated overnight at 4°C with the following primary antibodies: anti-AML1 (rabbit polyclonal, 1:1000) and anti-GAPDH (rabbit monoclonal, 1:5000) (Cell Signaling, Danvers, MA, USA) and anti-Lamin A/C (goat polyclonal, 1:2000) (Santa Cruz Biotechnology, Dallas, TX, USA). GAPDH and Lamin A/C were used as loading controls. Secondary antibodies conjugated to HRP (Santa Cruz Biotechnology, Dallas, TX, USA) were used to detect proteins in conjunction with an enhanced chemiluminescence kit and Chemidoc XRS + imaging system (both Bio-Rad Laboratories, Hercules, CA, USA).

Migration assays

For the scratch assay, 3.5×10^5 MMTV and NMuMG cells were seeded in duplicate in 6-well plates and allowed to adhere overnight. Cells were serum-starved for 8hrs prior to beginning the assay as follows: At 95–100% cell confluency, a scratch was made using a pipette tip and cell migration monitored by taking pictures at indicated time points post-

scratch. The area of the scratch was quantified using the MiToBo plug-in for ImageJ software and plotted as a percentage of total area for each of three independent experiments. For studies using siRNAs, the same procedure was performed with the following exceptions: 2×10^5 MMTV cells were seeded and transfected (as described) the following day; the scratch was made 24–48 hours later when cells reached 95–100% confluence.

For the trans-well migration assay, 5×10^4 MMTV and NMuMG cells were seeded in triplicate in migration chambers (BD Biosciences, Bedford, MA, USA) in serum-free medium. Cells were allowed to migrate through 8µm pores toward medium containing 10% FBS for 48 hours. Non-migrating cells were removed and cells that migrated through the membrane were fixed with ice-cold 100% methanol. Fixed cells were stained with 0.5% crystal violet solution and photographed before solubilizing the stain with 10% glacial acetic acid and measuring absorbance at 595nm in three independent experiments. For studies using siRNAs, the same procedure was followed with the following exceptions: 2×10^5 MMTV cells were seeded and transfected (as described) the following day. Twenty-four hours post-transfection, cells were trypsinized and seeded in migration chambers as described above.

Analysis of cell invasion

For the invasion assay, 5×10^4 MMTV and NMuMG cells were seeded in triplicate in growth factor reduced Matrigel invasion chambers (BD Biosciences, San Jose, CA, USA) in serum-free medium. Cells were allowed to invade through the Matrigel and 8µm pores toward medium containing 10% FBS for 48 hours. Non-invading cells were removed using a cotton swab and cells that invaded through the membrane were fixed with ice-cold 100% methanol. The procedure for trans-well migration described above was then followed to complete the experiments.

Statistics

Data were expressed as mean $\pm$ standard error of the mean. Statistically significant differences were determined using two-tailed unpaired Student's *t*-tests where indicated. In all cases, $P < 0.05$ was considered significant.

Results

Runx1 expression is increased in a subset of human breast cancers and increases with disease progression in mammary glands of MMTV-PyMT mice

To address the clinical relevance and pathological role of Runx1 in breast cancer progression and metastasis, we initially conducted a pilot study to determine Runx1 protein expression in a human breast cancer tissue microarray (TMA) as well as Runx1 RNA expression in a relevant publicly available dataset. As shown in Fig. 1A, Runx1 is present in the epithelial cells of the normal breast. Notably, Runx1 expression was detected and elevated in a subset of breast tissue disease states including ductal carcinoma *in situ* and invasive ductal carcinoma. Moreover, in cancerous tissues where Runx1 was present, the percentage of Runx1 positive cells was substantially more than in normal breast tissue suggesting a role for Runx1 in breast cancer (Fig. 1A and Table 1). To complement data

obtained from analysis of Runx1 expression in the TMA, an Agilent gene expression microarray dataset generated by Gluck *et al* (Gluck et al., 2012) that contained 154 invasive breast carcinoma cases and 4 normal breast tissues was analyzed (available at www.oncomine.org). Probing of this dataset revealed that 75% (3/4) of normal breast and 96% (148/154) of invasive breast cancer samples expressed *Runx1*. Importantly, of the *Runx1*-expressing invasive breast carcinoma cases, 72% (107/148) expressed more *Runx1* than the average reported for normal breast samples (Supplementary Material Fig. S3). Taken together, these findings prompted us to further investigate Runx1 in breast cancer using the MMTV-PyMT transgenic mouse model in order to examine Runx1 expression at multiple time points during disease progression and metastasis.

To determine the changes in Runx1 expression at the primary site of cancer initiation, mammary glands from mice positive for the transgene (MMTV⁺) and age-matched negative control mice (MMTV⁻) were sacrificed at 4, 8, 10, 12, and 13 weeks of age. These time points represent early through late stages of disease progression. Mammary glands from MMTV⁺ mice at 15 weeks were not used in this analysis because the mammary gland could not be cleanly excised separate from the tumor due to the extent of tumor involvement.

H&E staining of mammary glands (Fig. 1B, upper panels) shows the morphological changes during disease progression and is in line with previously published work (Lin et al., 2003). The morphology of mammary glands from control mice (MMTV⁻) did not change significantly over time (other than moderately increasing in size with age) and therefore one image from a representative mouse mid-way through the time course (10 weeks) is shown (Fig. 1B). Runx1 staining was present at consistently low levels in MMTV⁻ mammary luminal epithelial cells only. In contrast, Runx1 expression in MMTV⁺ mammary glands increased concomitant with disease progression, with strong nuclear expression evident at 13 weeks (Fig. 1B, lower panels). This observation was validated when gene and protein expression were evaluated: *Runx1* RNA levels remained at steady-state low levels in MMTV⁻ mice and increased significantly over controls at almost all time points in MMTV⁺ mice ($P < 0.05$, Fig. 1C). Further, semi-quantitative analysis was performed on Runx1 immunostained tissues and protein levels remained consistently low in MMTV⁻ mice over 13 weeks (Fig. 1D). Conversely, elevated Runx1 persisted in MMTV⁺ mice and was significantly increased over MMTV⁻ mice at almost all time points ($P < 0.05$, Fig. 1D).

Tumors and metastatic lung lesions from MMTV-PyMT mice express high levels of Runx1

Given the finding of increased Runx1 in both a subset of human breast tumors (Fig. 1A and Table 1) and throughout disease progression in the mammary gland of MMTV⁺ mice (Fig. 1B–D), the expression of Runx1 in the associated tumors was further evaluated. Tumors were excised at three time points representing early (10 weeks), mid (12 weeks) and late (15 weeks) stage tumor progression. Analysis of H&E staining of tumor sections revealed sheets of transformed epithelial cells, which became more densely packed as the tumor developed over the course of 5 weeks (Fig. 2A, upper panels). Positive nuclear Runx1 staining was detected in tumor epithelium at all time points examined and increased with tumor development (Fig. 2A, lower panels; Fig. 2B). Semi-quantitative analysis of Runx1 immunostaining demonstrated a trend for increasing Runx1 protein levels over time;

however, this change was only significant ($P < 0.05$) at the latest time point when compared with the earliest (15 vs. 10 weeks) (Fig. 2B). Similar to protein levels, *Runx1* RNA expression did not change significantly over time yet remained elevated (at a level comparable with that of MMTV⁺ mammary glands), particularly at later time points (12 and 15 weeks), suggesting Runx1 involvement throughout tumor development (Fig. 2C).

Lung and lymph nodes are the favored sites of metastasis in the MMTV-PyMT model. Previous work showed that lung metastases are readily observed both macroscopically and microscopically in MMTV-PyMT mice from about 12 weeks of age, with more lesions present at later time points (Guy et al., 1992, Franci et al., 2013). Therefore, to detect changes in Runx1 expression during the onset and progression of metastatic disease, MMTV⁺ and age-matched negative control lungs were examined histologically at four time points – 10, 12, 13 and 15 weeks – during which tumors were developing and growing. Similar to the mammary gland, although there was Runx1 expression in the MMTV⁺ lung epithelium, levels of Runx1 did not change over time and so only one representative image from a 10 week old MMTV⁺ mouse is shown (Fig. 2D). Metastasis was readily detectable by morphological assessment of H&E stained MMTV⁺ lung sections at 13 and 15 weeks, however no metastasis was detected at earlier time points (10 or 12 weeks) or in MMTV⁻ mice (Fig. 2D, upper panels). Importantly, Runx1 expression increased significantly over controls at 13 and 15 weeks ($P < 0.05$ and $P < 0.01$, respectively), with elevated Runx1 expression present in the multiple metastatic lesions in the lung, particularly at the latest stage assessed (15 weeks) (Fig. 2D–F). Higher magnification images of two different mice are shown to highlight the increased nuclear Runx1 expression in mice at 15 weeks, relative to 13 weeks in the metastatic cell deposits in the lung (Fig. 2F). The increased staining intensity at 15 weeks is consistent among all mice ($n = 3$) and reflects an increased number of stained cells per field of view (see Materials and Methods and Supplementary Material Fig. S1). Collectively, these findings indicate a robust expression of Runx1 in the tumor cells that is retained in those cells which colonize in lung metastases.

MMTV-PyMT tumor cells express markedly higher Runx1 than normal mammary gland cells

To determine if Runx1 contributed to any cancer-relevant phenotypes in breast cancer, an *in vitro* cell model complementary to the mouse model was used. The MMTV cells (Fig. 3A) are an epithelial tumor cell line derived from the tumor of a 13 week old MMTV-PyMT mouse (Nagle et al., 2004). The control cell line used was NMuMG (Fig. 3A), a normal mouse mammary gland epithelial cell line (Owens et al., 1974). *Runx1* RNA expression was significantly higher in the MMTV cells than the NMuMG cells ($P < 0.001$), with MMTV cells expressing 4-fold more Runx1 RNA than control cells (Fig. 3B, left). Similarly, Runx1 protein levels were 4-fold higher ($P < 0.001$) in MMTV cells compared with NMuMG cells (Fig. 3B, right). The differences in Runx1 expression between these two cell lines were analogous with the differences seen in early versus late stage disease in the MMTV-PyMT⁺ mice (Fig. 1B–D and Fig. 2A–F), and thus were considered suitable for further studies.

To reveal the significance of high Runx1 expression in the MMTV cells, siRNAs were used to diminish Runx1 expression. Morphological changes as a result of expressing siNS or

siRunx1 were not observed (Fig. 3C). RNA and protein levels were measured from MMTV cells transfected with either a non-silencing control siRNA (siNS) or a Runx1 siRNA smartpool (siRunx1) to determine knockdown efficacy (Fig. 3D). The level of *Runx1* RNA expression in the siRunx1-transfected cells was reduced (Fig. 3D, left panel) and the siRunx1-transfected cells expressed almost 70% less Runx1 protein than the siNS control ($P<0.05$) (Fig. 3D, right panel). Moreover, Runx1 was present almost exclusively in the nuclear protein fraction (Fig. 3D, right panel).

MMTV cells exhibit significantly greater ability to migrate and invade than normal mammary gland cells

As Runx1 expression was strongly associated with tumor growth and metastasis in the MMTV-PyMT model of breast cancer, we proposed that the Runx1-expressing MMTV cells may have a more aggressive cancer-related phenotype. To test this hypothesis, the ability of MMTV cells to migrate and invade was evaluated in comparison with the NMuMG control cells. In a scratch assay, MMTV cells demonstrated a significantly higher ability to migrate than controls at both 12 hours ($P<0.05$) and 24 hours ($P<0.001$) post-scratch, as determined by measuring the area of the scratch at the time points indicated (Fig. 4A). Consistent with these findings, the ability of MMTV cells to migrate in a trans-well assay was significantly greater (2.5-fold, $P<0.001$) than that of NMuMG cells (Fig. 4B). Moreover, using an invasion assay, the ability of MMTV cells to invade through a matrigel-coated membrane was significantly higher ($P<0.05$) than that of NMuMG cells (Fig. 4C). To complement the representative field of view images (Fig. 4B, C, right panel), quantitation of migration/invasion by measurement of the absorbance of solubilized crystal violet stain retained by migrated/invaded cells is shown (Fig. 4B, C, left panels) for the global representation of the wells. Together, these studies demonstrate that robust Runx1 expression is associated with a more aggressive cancer-related cell phenotype.

Runx1 depletion in MMTV cells significantly diminishes cancer-promoting migration and invasion phenotypes

The strongest cancer-associated phenotypes demonstrated for MMTV cells were their superior migration and invasion abilities (Fig. 4A–C). Thus, we postulated that these characteristics would be modulated upon Runx1 depletion. Consistent with this hypothesis, the ability of siRunx1-transfected MMTV cells to migrate as well as to invade was significantly reduced compared with controls (Fig. 5A–C). Fig. 5A shows representative images of the scratch assay, both at the time of the scratch and 24 hours later; clearly, siRunx1-transfected cells have a decreased ability to migrate (Fig. 5A, left). The images are corroborated by associated quantitative analysis that confirmed migration of Runx1-depleted cells was significantly reduced compared with the siNS control ($P<0.05$) (Fig. 5A, right). Likewise, migration of siRunx1-transfected cells in a trans-well migration assay was significantly less ($P<0.05$) than the control cells, as evidenced by representative images and quantitative data shown in Fig. 5B. Moreover, invasion was significantly inhibited relative to controls ($P<0.05$) when Runx1 expression was decreased in MMTV cells (Fig. 5C). In summary, these findings confirm the oncogenic potential of Runx1 in breast cancer cells.

Discussion

Runx protein function in cancer is dichotomous, because these proteins act as either tumor suppressors or oncogenes, depending on cellular context (Chimge et al., 2012, Pratap et al., 2009, Chimge and Frenkel, 2013, Wotton et al., 2002). The key findings of this study support an oncogenic function for Runx1 in breast cancer: Runx1 expression increases concomitant with disease progression in the mammary gland of MMTV-PyMT mice and is highly expressed in metastatic lesions in lung. Complementary *in vitro* studies demonstrate that Runx1 expression is significantly higher in tumor epithelial cells compared to normal epithelial control cells. Runx1 positive tumor cells exhibit a more aggressive phenotype, with significantly higher migration and invasion ability than control cells. Moreover, Runx1 knockdown in the MMTV cells results in a marked abrogation of the aggressive cancer-related phenotype. Thus, these data provide strong evidence indicating the oncogenic potential of Runx1 in breast cancer.

Recent genetic studies have highlighted a potential role for Runx1 in breast cancer (Scheitz et al., 2012, Banerji et al., 2012, Ellis et al., 2012), with one study reporting that *Runx1* is in the top 1% of over-expressed genes in breast cancer based on a meta-analysis of microarray studies comparing *Runx1* gene levels in tumor versus normal tissue (Scheitz et al., 2012). Our study examined Runx1 protein and RNA levels in normal human breast and breast cancer patient samples by immunohistochemical staining of tissue microarrays and analysis of publicly available microarray datasets, respectively. The results show that Runx1 is expressed not only in normal breast epithelium, but also highly expressed in some breast cancers. Our results are in line with a very recent study in which a comprehensive analysis of Runx1 protein in breast cancer patients was performed (Ferrari et al., 2014). It was found that high Runx1 expression significantly associated with poorer survival in a cohort of breast cancer patients.

Corroborating the immunohistochemical findings in human tissues, examination of Runx1 in the clinically informative MMTV-PyMT transgenic mouse model of breast cancer revealed that Runx1 expression in the mammary gland epithelium of diseased mice is increased over controls as early as 8 weeks, and further increases throughout breast cancer progression. This pattern of expression is consistent with studies in other cancers of epithelial origin. For example, Planaguma and colleagues demonstrated that Runx1 is overexpressed in invasive endometrial carcinoma in relation to cancer progression from normal atrophic endometrium through hyperplasia and subsequent carcinoma (Planaguma et al., 2004). We also show that Runx1 expression is consistently elevated in MMTV-PyMT breast tumors. This result agrees with data from other epithelial cancers such as epithelial ovarian cancer (EOC), where Runx1 protein is overexpressed in high grade tumors in a study carried out on a cohort of 117 EOC patient samples (Keita et al., 2013). In contrast, one study reported down-regulation of the Runx1 gene in high grade vs. low grade breast tumors from a cohort of 29 breast tumor samples; however, this group did not investigate protein expression in these patients and the sample size was limited (Kadota et al., 2010). We further demonstrate that Runx1 is highly expressed in the multiple metastatic lesions found in the lungs of MMTV-PyMT mice. Consistent with this result, Runx1 has been shown to be over-expressed in EOC metastasis as well as endometrial cancer metastasis (Doll et al., 2009,

Keita et al., 2013). Collectively, our *in vivo* data strongly suggests that Runx1 expression is intimately involved with breast cancer progression and metastasis.

Using a complementary *in vitro* tumor epithelial cell model derived from the MMTV-PyMT mouse (Nagle et al., 2004) (as well as a normal mouse mammary gland epithelial cell control), we evaluated cancer-related phenotypic properties of the cells including invasion and migration. The MMTV cells, which express 4-fold more Runx1 than the control cells, exhibit a significantly greater ability to migrate and invade compared with the control cells, and when Runx1 is knocked down in the MMTV cells, the phenotype is significantly diminished. This result is comparable with related studies in human ovarian cancer cells, where knockdown of Runx1 in SKOV3 cells inhibited migration and invasion (Keita et al., 2013).

Our finding that MMTV-derived tumor cells exhibit strong Runx1 expression is in line with our *in vivo* observations of robust Runx1 expression in tumors from MMTV-PyMT mice. We considered whether this increase in Runx1 expression was solely a consequence of differences in epithelial cell content between MMTV⁺ and MMTV⁻ mice, particularly as the proportion of epithelial cells in the MMTV⁻ mice was substantially lower. However, this is unlikely based on the similarity of Runx1 expression in our models and that of another study: Zhu *et al* performed expression profiling of mouse mammary tumor models including the MMTV-PyMT model, using pregnant mouse mammary glands as their control because they are highly enriched for epithelial cells (Zhu et al., 2011). Upon probing their dataset for Runx1 expression, we found the differences between tumor and control to be very much in line with our data. Indeed, we also see the same difference in Runx1 expression in our tumor vs normal epithelial cells in the *in vitro* model. This finding supports the idea that Runx1 is dysregulated in tumor epithelial cells and is an important player in breast cancer progression and metastasis.

Another of the Runx family members – Runx2 – has been shown in numerous studies to be involved in breast cancer metastasis, particularly to bone (Pratap et al., 2006, Pratap et al., 2005, Pratap et al., 2011). A recent study by Blyth's group using an MMTV mouse model engineered to over-express Runx2 demonstrated that although development and differentiation in the mammary gland were perturbed, there was a lack of overt carcinoma and no effect on metastasis was reported (McDonald et al., 2014). It is interesting to postulate that this may be due to context-specific differential expression and function of Runx factors, where Runx2 may be more important in cancers that metastasize to bone and Runx1 may be more important in cancers that metastasize to lung. We propose that Runx1 exhibits pleotropic effects throughout breast cancer progression, being important in the malignant transformation of epithelial cells in the mammary gland and contributing to disease progression in breast cancer. Runx1 may play a significant role in a more aggressive disease phenotype by promoting migration and invasion of cancer cells during tumor development and metastasis.

Intriguingly, two very recent studies have associated Runx1 with hormonal status in breast cancer (Ferrari et al., 2014, van Bragt et al., 2014). This is particularly interesting in light of several genetic studies that drew attention to the clinical relevance of Runx1 and breast

cancer, yet focused almost exclusively on Runx1 in human estrogen receptor positive (ER+) breast cancers (Banerji et al., 2012, Cancer Genome Atlas Network, 2012, Ellis et al., 2012). van Bragt *et al* reported that Runx1 is a key regulator of ER+ mammary luminal epithelial cells and their findings of Runx1 expression patterns *in vivo* are very much in line with our findings in normal breast and at the earliest stages of breast cancer development in the MMTV-PyMT model (van Bragt et al., 2014). Moreover, Ferrari *et al* reported data to suggest that Runx1 may contribute to tumor progression in ER- (and progesterone- (PR-) and HER2-) breast cancers (Ferrari et al., 2014). Of note, the MMTV-PyMT model is a luminal model of breast cancer and ER (as well as PR) status is reported to change throughout cancer progression: early stage disease is ER+ but this switches to ER- in the latest stages of disease (Kouros-Mehr et al., 2008, Lin et al., 2003). Also, the MMTV cells used in this study (derived from the tumor of a late-stage disease mouse) were found to be negative for ESR1 (encoding ER α) (G. Browne, unpublished observation). Thus, our findings that 1) Runx1 expression increases during disease progression and particularly at late stages in a mouse model that switches from ER+ to ER- and 2) the Runx1-high ER- MMTV breast cancer cells exhibit a more aggressive phenotype (abrogated upon Runx1 depletion) are clinically relevant. This conclusion is further supported by the strong correlation between Runx1 expression and poor prognosis in triple negative human breast cancer tissues (Ferrari et al., 2014).

The most striking results presented in this study establish the oncogenic potential of Runx1 in breast cancer: Runx1 is markedly associated with disease progression, tumor development and metastasis in the MMTV-PyMT model of breast cancer. Moreover, Runx1 is associated with maintenance of more aggressive cancer-associated characteristics and its depletion results in significant abrogation of this phenotype. In light of our findings and other recent reports, more in-depth investigations are warranted to fully appreciate the molecular mechanisms of Runx1 action in breast cancer. Indeed, some studies have begun to explore this potential: ablation of Runx1 in other epithelial cancers dramatically reduces tumor burden (Scheitz et al., 2012). Further, a recently discovered small-molecule inhibitor of the Runx1-CBF β interaction is capable of reducing tumor burden in a murine leukemia model (Cunningham et al., 2012). These data, together with the results of this study, highlight the therapeutic potential of targeting the oncogenic activity of Runx1 in breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Runx1 expression during breast cancer progression in a sub-set of human breast cancer patient samples and the mammary gland of MMTV-PyMT mice

(A) Representative images from Runx1 immunohistochemical staining of a human breast cancer tissue microarray containing normal breast as well as Runx1 positive and negative breast cancer patient samples (scale bar 100 μ m; mag. 40x). Insets (bottom left, boxed off region) are lower magnification (20x) images of respective cores to show a more global view of individual breast cancer samples. (B) H&E (upper panels; scale bar 2mm; mag. 2x) and Runx1 (lower panels; scale bar 100 μ m; mag. 40x) staining of representative mammary glands from mice positive for the transgene (MMTV⁺) at multiple time points (4, 8, 10, 12 and 13 weeks) representing early (~4–8 weeks), mid (~8–12 weeks), and late (~12–15 weeks) stage breast cancer disease progression. A representative negative control (MMTV⁻) at 10 weeks is also shown. (C) Scatter plot showing qRT-PCR of Runx1 RNA expression at time points indicated in mammary glands from diseased (MMTV⁺) and age-matched negative control (MMTV⁻) mice with $N = 3-5$ and $N = 5-6$ mice per time-point per MMTV⁻ and MMTV⁺ group, respectively. Expression levels are relative to the control gene RPL19. (D) Semi-quantitative analysis of Runx1 protein expression as assessed by scoring immunohistochemically processed mammary glands from $n = 3$ representative mice per time point per group. All quantitative/semi-quantitative data are depicted as mean $\pm$ S.E. per group. * $P < 0.05$, ** $P < 0.01$ (student's t -test). White T = transformed/tumor tissue; white * = lymph node.

Fig. 2. Runx1 expression in MMTV-PyMT breast tumors and metastatic lung lesions

(A) H&E (upper panels; scale bar 1mm; mag. 4x) and Runx1 (lower panels; scale bar 100µm; mag. 40x) staining of representative tumors at early (10 weeks), mid (12 weeks), and late (15 weeks) stage tumor progression. (B) Semi-quantitative analysis of Runx1 protein expression as assessed by scoring immunohistochemically processed tumors from $n = 3-5$ representative mice per time point per group. (C) Scatter plot showing qRT-PCR of Runx1 RNA expression at time points indicated in tumors from $N = 4-5$ MMTV⁺ mice per time point per group. Expression levels are relative to the control gene RPL19. NS = no significant difference, $P > 0.05$. (D) H&E (upper panels; scale bar 1mm; mag. 4x) and Runx1 (lower panels; scale bar 500µm; mag. 10x) staining of representative lungs at time points indicated from MMTV⁺ mice and a representative 10 week negative control (MMTV⁻). (E) Semi-quantitative analysis of Runx1 protein expression as assessed by scoring immunohistochemically processed lungs from $n = 3$ representative mice per time point per group. (F) High magnification images of two representative MMTV⁺ mice (denoted #1 and #2) at 13 and 15 weeks (scale bar 100 µm; mag. 40x) highlighting significantly increased Runx1 expression in metastatic lesions. All quantitative data are depicted as mean $\pm$ S.E. per group. * $P < 0.05$, ** $P < 0.01$ (student's t -test).

Fig. 3. Characterization of Runx1 expression in normal breast epithelial cells (NMuMG) and tumor epithelial cells (MMTV), with and without Runx1 depletion

(A) Phase contrast microscopy images (mag. 10x) of model cell lines to evaluate morphological differences in cells used in this study: NMuMG, normal mouse mammary gland epithelial cells and MMTV, tumor epithelial cells derived from the tumor of a 13 week old MMTV-PyMT⁺ mouse. (B) Left: qRT-PCR of *Runx1* RNA expression in NMuMG and MMTV cells from $N = 3$ independent samples performed in triplicate. Values are shown relative to RPL19. Right, upper: representative western blot demonstrating endogenous Runx1 expression in two independent samples from NMuMG and MMTV cell lines. Lamin A/C was used as the loading control. Right, lower: Runx1 protein quantitation

(plotted relative to Lamin A/C control) from $N = 3$ independent experiments performed in duplicate. (C) MMTV cells transfected with non-targeting siRNA (siNS) or Runx1-targeting siRNA smartpool (siRunx1) were subject to phase contrast microscopy. Images (mag. 10x) of transfected cells were used to evaluate any morphological changes present. (D) Left: qRT-PCR of Runx1 RNA expression in siNS- or siRunx1-transfected MMTV cells from $N = 3$ independent samples performed in triplicate. Values are shown relative to RPL19. Right, upper: representative western blot demonstrating nuclear (*Nuc*) and cytoplasmic (*Cyt*) Runx1 protein expression in MMTV cells transfected as above. Lamin A/C and GAPDH were used as the loading control for *Nuc* and *Cyt* Runx1 protein expression, respectively. Right, lower: Runx1 protein quantitation (plotted relative to Lamin A/C control) from $N = 3$ independent experiments. All quantitative data are depicted as mean $\pm$ S.E. per group. * $P < 0.05$, *** $P < 0.001$ (student's *t*-test).

Fig. 4. Evaluation of migration and invasion ability in NMuMG and MMTV cells

(A) Representative phase contrast images (magnification 100x) of NMuMG and MMTV cells subjected to a scratch assay for times indicated. The area of the scratch was plotted as a percentage of total area for $N = 3$ independent experiments carried out in duplicate. (B) Light microscopy images (mag. 12x) of stained cells from a representative (1 of $N = 3$) trans-well migration assay experiment with NMuMG and MMTV cells (*left*); quantitation of migrated cells assessed by measurement of the absorbance of solubilized crystal violet stain retained by migrated cells (*right*). (C) Light microscopy images (mag. 12x) of stained cells from a representative (1 of $N = 3$) trans-well matrigel invasion assay experiment with NMuMG and MMTV cells to evaluate invasion (*left*); quantitation of invaded cells assessed by measurement of the absorbance of solubilized crystal violet stain retained by invaded cells (*right*). For all assays, three independent experiments were carried out in triplicate. All quantitative data are depicted as mean $\pm$ S.E. per group. * $P < 0.05$, *** $P < 0.001$ (student's t -test).

Fig. 5. Effect of Runx1 depletion on migration and invasion ability of MMTV cells
 MMTV cells treated with transfection reagent only (Mock) or transfected with non-targeting siRNA (siNS) or Runx1-targeting siRNA smartpool (siRunx1) were assayed as follows: (A) Representative phase contrast images (mag. 10x) of MMTV cells treated as above were subjected to a scratch assay for times indicated. The area of the scratch was plotted as a percentage of total area for $N = 3$ independent experiments carried out in duplicate. (B) Light microscopy images (mag. 12x) of stained cells from a representative (1 of $N = 3$) trans-well migration assay experiment with MMTV cells treated as above (*left*); quantitation of migrated cells assessed by measurement of the absorbance of solubilized crystal violet stain retained by migrated cells (*right*). (C) Light microscopy images (mag. 12x) of stained cells from a representative (1 of $N = 3$) trans-well matrigel invasion assay experiment with MMTV cells treated as above to evaluate invasion (*left*); quantitation of invaded cells assessed by measurement of the absorbance of solubilized crystal violet stain retained by invaded cells (*right*). For all assays, three independent experiments were carried out in duplicate. All quantitative data are depicted as mean $\pm$ S.E. per group. * $P < 0.05$ (student's t -test).

Table 1

Runx1 and other clinic-pathologic characteristic of normal and breast cancer tissues from a tissue microarray (n = 140 total)

	No. of normal cases (% of total)	No. of DCIS cases (% of total)	No. of IDC cases (% of total)
<i>User determined</i>			
Runx1 status			
Negative	1 (17%)	7 (50%)	34 (28%)
Positive	5 (83%)	7 (50%)	86 (72%)
Runx1 staining			
None	1 (17%)	7 (50%)	34 (28%)
Weak	1 (17%)	3 (21%)	45 (37%)
Moderate	2 (33%)	4 (29%)	27 (23%)
Strong	2 (33%)	0	14 (12%)
% of Runx1 positive cells			
10%	5 (100%)	3 (42%)	23 (27%)
11–40%	0	2 (29%)	32 (37%)
41–79%	0	2 (29%)	16 (19%)
80%	0	0	15 (17%)
<i>BioMax determined</i>			
Age			
50	4 (67%)	8 (57%)	52 (43%)
50	2 (33%)	6 (43%)	68 (57%)
Histologic grade			
1 (well differentiated)	-	-	8 (7%)
2 (moderately differentiated)	-	-	89 (74%)
3 (undifferentiated)	-	-	15 (13%)
ER			
Negative	1 (17%)	10 (71%)	81 (68%)
Positive	5 (83%)	4 (29%)	39 (32%)
PR			
Negative	2 (33%)	8 (57%)	90 (75%)
Positive	4 (67%)	6 (43%)	30 (25%)
HER2			
Negative	6 (100%)	14 (100%)	95 (79%)
Positive	0	0	25 (21%)
AR			
Negative	0	12 (86%)	82 (68%)
Positive	6 (100%)	2 (14%)	38 (32%)
Ki67			

	No. of normal cases (% of total)	No. of DCIS cases (% of total)	No. of IDC cases (% of total)
Negative	6 (100%)	14 (100%)	65 (54%)
Positive	0	0	55 (46%)
p53			
Negative	3 (50%)	9 (64%)	40 (33%)
Positive	3 (50%)	5 (36%)	80 (67%)

IDC, invasive ductal carcinoma; DCIS, ductal carcinoma in situ; cyst., cystsarcoma; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; AR, androgen receptor