Research article

Dynamic regulation of CD24 and the invasive, CD44posCD24neg phenotype in breast cancer cell lines
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Abstract

Introduction The invasive, mesenchymal phenotype of CD44posCD24neg breast cancer cells has made them a promising target for eliminating the metastatic capacity of primary tumors. It has been previously demonstrated that CD44neg/lowCD24pos breast cancer cells lack the ability to give rise to their invasive CD44posCD24neg counterpart. Here we demonstrate that noninvasive, epithelial-like CD44posCD24pos cells readily give rise to invasive, mesenchymal CD44posCD24neg progeny in vivo and in vitro. This interconversion was found to be dependent upon Activin/Nodal signaling.

Methods Breast cancer cell lines were sorted into CD44posCD24pos and CD44posCD24neg populations to evaluate their progeny for the expression of CD44, CD24, and markers of a mesenchymal phenotype. The populations, separated by fluorescence activated cell sorting (FACS) were injected into immunocompromised mice to evaluate their tumorigenicity and invasiveness of the resulting xenografts.

Results CD24 expression was dynamically regulated in vitro in all evaluated breast cancer cell lines. Furthermore, a single noninvasive, epithelial-like CD44posCD24pos cell had the ability to give rise to invasive, mesenchymal CD44posCD24neg progeny. Importantly, this interconversion occurred in vivo as CD44posCD24pos cells gave rise to xenografts with locally invasive borders as seen in xenografts initiated with CD44posCD24neg cells. Lastly, the ability of CD44posCD24pos cells to give rise to mesenchymal progeny, and vice versa, was blocked upon ablation of Activin/Nodal signaling.

Conclusions Our data demonstrate that the invasive, mesenchymal CD44posCD24neg phenotype is under dynamic control in breast cancer cell lines both in vitro and in vivo. Furthermore, our observations suggest that therapies targeting CD44posCD24neg tumor cells may have limited success in preventing primary tumor metastasis unless Activin/Nodal signaling is arrested.

Introduction

The CD24 gene encodes a highly glycosylated, glycosylphosphatidylinositol anchored cell surface protein [1]. Thought to function as an adhesion molecule, it is known to bind Platelet Activation-Dependent Granule to External Membrane Protein (aka P-Selectin) [2] and facilitate intracellular signaling despite lacking a transmembrane domain [3]. In both normal and cancerous mammary tissue, CD24 positivity is frequently associated with a terminally differentiated, luminal phenotype [4-6]. In spite of this classification, the influence of CD24 expression on tumorigenicity and invasiveness is inconsistent, ranging from a positive [7-10] to a negative one [11-14].

Al-Hajj et al. [14] first described an impact of CD24 expression on breast cancer tumorigenicity by observing that CD44posCD24neg cells were highly tumorigenic in immunocompromised mice while CD44posCD24pos were nontumorigenic. Since then, the CD44/CD24 profile has been widely investigated in both primary tissues [4,15-22] and established breast cancer cell lines [13,23-31].
A relationship between CD24 and basal or luminal phenotype in breast cancer cell lines was reported by Fillmore and Kupperwasser [11]. Specifically, these authors demonstrated that cell lines with a high percentage of CD24pos cells expressed luminal keratins while cell lines with a high percentage of CD24neg cells expressed basal keratins. Consistent with these observations, CD44posCD24neg cells were found to possess a basal/mesenchymal phenotype relative to CD44negCD24pos cells [13]. Furthermore, using breast cancer cell lines, Sheridan et al. [27] demonstrated that CD44posCD24neg cells were more invasive than CD44negCD24pos cells. The invasive nature of CD44posCD24neg breast cancer cells has made this population a possible therapeutic target with the goal of eliminating the metastatic ability of primary tumors. Indeed, efforts to specifically target this population have been described [29-31].

Detailed comparisons between CD44neg/lowCD24pos and CD44posCD24neg breast cancer cells have been reported [4,13,32]. While CD44neg/lowCD24pos cells lack the ability to give rise to their invasive CD44posCD24neg counterpart [13], the regulation of CD24 and the invasive, CD44posCD24neg phenotype in CD44 positive breast cancer cells is less well understood. Our decision to work exclusively with CD44pos cells was a deliberate effort to focus specifically on CD44 and avoid the well-described influence of CD44 expression on cell behavior [33-36].

Herein, we report that CD24 is under dynamic regulation in vivo and in vitro in five breast cancer cell lines. Specifically, CD44posCD24neg cells readily give rise to CD44posCD24neg cells and vice versa. Furthermore, noninvasive, epithelial-like CD44posCD24pos cells give rise to invasive, mesenchymal CD44posCD24neg progeny in an Activin/Nodal dependent manner. In vivo, this interconversion resulted in CD44posCD24pos cells giving rise to xenografts which had a similar capacity for local invasion as those initiated with CD44posCD24neg cells. These observations have potential clinical implications as specific targeting of CD44posCD24neg cells will leave behind CD44posCD24pos cells capable of giving rise to invasive progeny unless Activin/Nodal signaling is arrested.

Materials and methods

Cell culture

MCF7, ZR75.1, and MDA MB 231 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). MDA MB 231 and MCF7 cells were maintained in Dulbecco’s Minimum Essential Medium (DMEM, Invitrogen, Gaithersburg, MD, USA) supplemented with 5% heat inactivated fetal bovine serum (FBS, Invitrogen), 10 μg/ml bovine insulin (Sigma, St. Louis, MO, USA), and 100 units/ml penicillin-streptomycin (Invitrogen). ZR75.1 cells were maintained in RPMI1640 (Invitrogen) supplemented with 10% heat inactivated FBS and 100 units/ml penicillin-streptomycin.

For generation of clonally derived cell lines, Ca1a cells were double-sorted and single cells plated directly into 96-well dishes containing conditioned DMEM/F12 media supplemented with 5% heat inactivated HS. Those wells containing a single cell were identified microscopically and expanded.

Flow cytometric analysis and sorting

Anti-human CD44-allophycocyanin (APC, clone G44-26, 0.2 μg/ml final concentration) and anti-human CD24-phycocerythrin (PE, clone ML5, 26.6 μg/ml final concentration) or anti-human CD24-fluorescein (FITC, clone ML5, 26.6 μg/ml final concentration) (unless otherwise noted, all antibodies were purchased from BD Biosciences, Franklin Lakes, NJ, USA) were used for both analysis and live sorting. 7-aminoactinomycin D (7AAD, 1 μg/ml final concentration, BD Biosciences) was used for live/dead cell distinction. For flow cytometric analysis, cells were stained with a PBS solution containing 0.1% BSA and 0.1% sodium azide (Sigma) for 25 min at 4°C followed by two washes with the same buffer. For dual staining of CD24 and vimentin (PE, clone VI-RE/1, 10 μg/ml final concentration, Becton Dickinson, Franklin Lakes, NJ, USA), cells were stained with a PBS solution containing 0.1% BSA and 0.1% sodium azide (Sigma). Cells not receiving 7-aminoactinomycin D were used for live sorting. 7-aminoactinomycin D (7AAD, 1 μg/ml final concentration, Becton Dickinson) was solubilized in dimethyl sulfoxide (DMSO, Sigma) and supplemented to media at a final concentration of 10 μM and a final DMSO concentration of 0.1%. Cells not receiving SB-431542 were treated with 0.1% DMSO.
siRNA experiments, cells were trypsinized 24 hr post-transfection. Cells were counted post-sorting using a Cellometer. Plates in triplicate in media containing 0.1% HS. Media conditioned and counted. For both experiments, 30,000 cells received a subcutaneous estrogen pellet (0.72 mg β-estradiol, Calbiochem, Gibbstown, NJ, USA); animals also side (VP-16) was administered intraperitoneally (ip, 30 mg/kg body weight, respectively) in 200 μl Hank’s Balanced Salt Solution (Invitrogen) prior to surgically exposing the gland for injection. Tumor size was measured weekly using a caliper. Experiments were terminated once a xenograft reached 1.0 cm in diameter or 75 d following injection of cells, whichever came first. Xenografts were removed, minced into < 1 mm pieces, and dissociated (F12 media containing 100 units/ml penicillin-streptomycin, 0.8 units/ml Dispase (Invitrogen), and 100 units/ml Collagenase type 3 (Worthington Biochemical Corp, Lakewood, NJ, USA)) at 37°C under rotating preparation. Following fixation, cells were blocked with 1% BSA. Primary antibodies (anti-Slug [clone D-19, 2 μg/ml final concentration] and anti-vimentin [clone H-84, 2 μg/ml final concentration], Santa Cruz Biotechnology, Santa Cruz, CA) were followed by the appropriate secondary antibody (anti-goat or anti-rabbit Alexa Fluor 594 or Alexa Fluor 488, 1:100 dilution, Invitrogen). Imaging was performed using the Carl Zeiss LSM510 confocal imaging system (Carl Zeiss MicroImaging, Thornwood, NY, USA) at 63× magnification or an Olympus IX51 microscope (Olympus, Center Valley, PA, USA) at 20× magnification.

siRNA mediated knockdown of CD24
Non-targeting and CD24 siRNA pools were purchased from Dharmacon (Lafayette, CO, USA). Ca1a cells were transfected with 50 nM siRNA using DharmaFECT 1. Cells were harvested 72 hr post-transfection.

Matrigel invasion assays
Cell invasion was assessed using Matrigel coated transwell chambers (8 μm, BD Biosciences). For analysis of sorted cells, cells were counted post-sorting using a Cellometer AutoT4 (Nexcelom Bioscience, Lawrence, MA, USA). For siRNA experiments, cells were trypsinized 24 hr post-transfection and counted. For both experiments, 30,000 cells were plated in triplicate in media containing 0.1% HS. Media containing 15% HS was used as the chemoattractant. Cells that had invaded 48 hr later were fixed with methanol, stained with 1% toluidine blue and counted under 20× magnification.

Realtime RT-PCR
Total RNA was isolated from cells using the QIAGEN RNeasy kit (Valencia, CA, USA). The QIAGEN AllPrep DNA/RNA kit was used to isolate genomic DNA. RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen) primed with oligo-dT and random hexamers. The cDNA was then subjected to realtime PCR amplification using gene specific primers and 2x Brilliant II Sybr Green QPCR Mastermix (Roche Applied Science, Indianapolis, IN, USA). Primer sequences and PCR conditions are provided (see Additional data file 1). GAPDH was employed as a housekeeping gene after confirming that it is expressed at similar levels between the CD44posCD24pos and CD44posCD24neg cells (see Additional data file 2). Data are presented as mean delta delta Ct relative to CD44posCD24pos cells.

Immunoflouresence and confocal microscopy
Cells were either grown on ibidi 8-well chamber slides (Research Products International, Mt. Prospect, IL, USA) and fixed/permeabilized with ice cold acetone or sorted live, fixed/permeabilized with ice cold acetone followed by cytosin preparation. Following fixation, cells were blocked with 1% BSA. Primary antibodies (anti-Slug [clone D-19, 2 μg/ml final concentration] and anti-vimentin [clone H-84, 2 μg/ml final concentration], Santa Cruz Biotechnology, Santa Cruz, CA) were followed by the appropriate secondary antibody (anti-goat or anti-rabbit Alexa Fluor 594 or Alexa Fluor 488, 1:1000 dilution, Invitrogen). Imaging was performed using the Carl Zeiss LSM510 confocal imaging system (Carl Zeiss MicroImaging, Thornwood, NY, USA) at 63× magnification or an Olympus IX51 microscope (Olympus, Center Valley, PA, USA) at 20× magnification.

Bisulfite sequencing
Bisulfite modification was performed on genomic DNA isolated from CD44posCD24pos or CD44posCD24neg sorted cells using the QIAGEN EpiTect Bisulfite Kit. Primers for PCR amplification were designed with MethPrimer [40] and a region spanning 366 bases and 28 CpG dinucleotides starting at -422 relative to the transcriptional start sight was queried (forward 5’ GGTATTTAAATTTTGTTATGG 3’, reverse 5’ ATCTTCCCAAAACTAAAAACC 3’). PCR products were cloned into DH5α cells by TOPO TA cloning (Invitrogen) and sequenced using M13 primers.

RNA stability assay
Following sorting into CD44posCD24pos and CD44posCD24neg populations, cells were seeded into six-well dishes. One day later, cells were treated with 10 μg/ml Actinomycin-D (Sigma) and collected at 0, 4, 8, or 16 hr. RNA was isolated using Trizol (Invitrogen). Changes in CD24 mRNA were monitored by realtime RT-PCR.
Statistics
Analysis of variance was performed using StatView 5.0.1 (SAS Institute, Cary, NC, USA). For analysis of realtime RT-PCR data, technical replicates for each gene from each of three independent experiments were averaged. Analysis of variance was performed on the resulting three independent values.

Results
CD24 expression is dynamically regulated in breast cancer cell lines
In an effort to understand the dynamics of CD24 expression in breast cancer cell lines, cells were sorted based on their CD44/CD24 expression and the CD44/CD24 expression of their progeny was evaluated. Nineteen breast cancer cell lines were initially screened for their expression of CD44 and CD24 (see Additional data file 3). Four cell lines (Ca1a, MCF7, SUM159 and MDA MB 231) were selected to evaluate the fluidity of CD24 expression in vitro. Cells were sorted into CD44pos/CD24neg and CD44pos/CD24pos populations (see Figure 1).

Figure 1

(a) CD24 expression is dynamically regulated in breast cancer cell lines. (a) CD44pos/CD24neg and CD44pos/CD24pos cells were sorted from Ca1a, SUM159, MCF7, or MDA MB 231 breast cancer cell lines and expanded in vitro. The CD44, CD24 expression profile of the sorted cells was assessed via flow cytometry after two passages. (b) CD24 promoter CpG islands as predicted by MethPrimer (top panel). A 366 bp region was queried by bisulfate sequencing analysis (BS1, -422 to -788 relative to transcriptional start site). Bisulfite sequencing analysis of CD24 promoter in CD44pos/CD24pos and CD44pos/CD24neg parental Ca1a cells. Methylated CG (filled circles) and unmethylated CG (open circles) are presented (middle panel). Percentages of methylation of each CpG in the CD24 promoter from region queried (bottom panel). (c) CD24 mRNA stability assessed in Actinomycin treated, sorted cells. CD44pos/CD24neg (blue line) and CD44pos/CD24pos (red line) differences are presented as delta delta Ct means +/- standard deviations around the mean.
Additional data file 4) and allowed to expand for two passages after which their CD44/CD24 expression was assessed by flow cytometry. For all four cell lines queried, CD44posCD24neg cells gave rise to CD44posCD24pos cells and vice versa (Figure 1a).

Data presented above suggests that CD24 expression is dynamically regulated in immortalized breast cancer cell lines. To evaluate if the CD24 gene was susceptible to dynamic transcriptional regulation, CpG methylation status of the CD24 promoter was queried in CD44posCD24neg and CD44posCD24pos populations sorted from the Ca1a cell line. A region spanning 366 bases (starting at -422 relative to the transcriptional start site) and 28 CpG dinucleotides was queried via bisulfite sequencing (Figure 1b). No differences in CpG methylation were observed between CD44posCD24neg and CD44posCD24pos cells. This suggests that rapid changes in CD24 transcription can occur without necessitating epigenetic modification of its promoter.

To further understand the regulation of CD24 expression, stability of the transcript was compared between CD44posCD24neg and CD44posCD24pos FACS sorted Ca1a cells. Following sorting, transcription was inhibited with Actinomycin-D and the rate of CD24 mRNA disappearance was evaluated. As indicated in Figure 1c, differences in CD24 abundance between CD44posCD24neg and CD44posCD24pos cells is not achieved by altered mRNA stability. CD24 expression as evaluated by flow cytometry could also be regulated at the translational level or by cell surface localization of the protein. However, given that cells devoid of the protein at the cell surface have markedly depressed levels of CD24 transcript (roughly one tenth that of CD24 positive cells) indicates that transcriptional regulation plays a considerable role in regulating CD24 protein expression.

Noninvasive CD44posCD24pos cells give rise to invasive CD44posCD24neg cells

We next set out to determine whether CD44posCD24pos cells could give rise to functional heterogeneity in addition to immunophenotypic heterogeneity as demonstrated above. It had been previously reported that CD44posCD24neg cells possess an invasive, mesenchymal phenotype relative to the epithelial-like phenotype of CD44dmposCD24pos cells [13,27]. After sorting Ca1a cells, we confirmed that relative to CD44posCD24pos cells, the CD44posCD24neg population expressed elevated levels of Slug and vimentin and reduced levels of E-cadherin (Figure 2a, b, c). To confirm vimentin expression, Ca1a cells were dual stained for CD24 and vimentin. Consistent with data in Figure 2b, 92% of CD44posCD24neg cells were vimentin positive and expressed the protein at elevated levels (median fluorescence intensity = 1,494). While 32% of CD44posCD24pos cells fell in the vimentin positive gate, these cells expressed the protein at markedly lower levels (median fluorescence intensity = 7) than CD24neg cells (see Additional data file 5). Furthermore, this population was nearly eight-fold more invasive through Matrigel than CD44posCD24pos cells (Figure 2d).

We took advantage of these differences between CD44posCD24pos and CD44posCD24neg cells to evaluate if either population possessed the ability to give rise to molecular and functional heterogeneity. Specifically, we set out to determine if the CD44posCD24neg progeny of noninvasive CD44posCD24pos cells possessed an invasive, mesenchymal phenotype. To address this question in the most stringent manner possible, clones were propagated from CD44posCD24neg or CD44posCD24pos Ca1a cells (Figure 3a). Following a double sort, single cells were deposited into 96-well dishes and expanded. Only wells confirmed to contain a single cell after sorting (determined microscopically) were evaluated. Less than 1.5% of CD44neg cells were able to generate clones, independent of CD24 status, indicating that these cells lack self-renewal properties (data not shown).

Seven clones were generated from sorted CD44posCD24pos cells and five clones were generated from CD44posCD24neg cells with roughly 30% of single cells giving rise to a successful colony, independent of CD24 expression (data not shown). For all clones, CD44posCD24neg cells gave rise to CD44posCD24neg cells, and vice versa (see Additional data file 6). FACS profiles of clones derived from a CD44posCD24pos cell or a CD44posCD24neg cell are presented in Figure 3b demonstrating the ability of a single CD44posCD24pos cell to give rise to isogenic CD44posCD24neg progeny, and vice versa. These observations confirmed data generated with bulk sorted Ca1a, SUM159, MCF7, and MDA MB 231 cells (Figure 1a).

As presented in Figure 2, the parental Ca1a cell line possesses two functionally unique populations (invasive CD44posCD24neg cells and noninvasive CD44posCD24pos cells). To determine if either CD44posCD24pos or CD44posCD24neg cells possessed the ability to give rise to this molecular and functional heterogeneity, the clones described above were sorted and queried for expression of mesenchyme-related genes as well as invasiveness through Matrigel. We observed that a single noninvasive, epithelial-like CD44posCD24pos cell had the ability to give rise to isogenic, CD44posCD24neg progeny possessing elevated levels of Snail and Slug and reduced levels of E-cadherin (Figure 3c). Furthermore, these CD44posCD24neg progeny were 5-fold more invasive than their CD44posCD24pos parental cell (Figure 3d). Likewise, a single CD44posCD24neg cell had the ability to give rise to noninvasive, epithelial-like, CD44posCD24pos progeny (Figures 3c, d). These data demonstrate that CD44posCD24pos cells are plastic and can readily give rise to progeny possessing molecular and functional characteristics unlike their own.
Xenografts derived from CD44^{pos}CD24^{pos} cells are locally invasive and contain CD44^{pos}CD24^{neg} progeny

Data presented above demonstrate that noninvasive CD44^{pos}CD24^{pos} cells readily give rise to invasive CD44^{pos}CD24^{neg} progeny. To determine if this is limited to in vitro conditions, three cell lines (Ca1a, MCF7 and ZR75.1) were sorted into CD44^{pos}CD24^{pos} and CD44^{pos}CD24^{neg} populations and injected into the abdominal fat pad of immunocompromised mice. Not surprisingly, we observed differences among cell lines in tumorigenicity (Figure 4a, b). In all cases, within cell lines, CD44^{pos}CD24^{pos} and CD44^{pos}CD24^{neg} populations were equally tumorigenic (Figure 4a, b). In the case of Ca1a, 10 cells from either CD44^{pos}CD24^{pos} or CD44^{pos}CD24^{neg} cells resulted in a similar frequency (Figure 4a) of equally sized tumors ($P = 0.89$, data not shown). Both populations gave rise to tumors greater than 1 cm in diameter within 50 days of injection. One thousand ZR75.1 cells, independent of CD24 status, resulted in 100% of mice developing tumors by 62 days post injection (Figure 4a) with CD44^{pos}CD24^{neg} cells yielding 1.9 fold larger tumors than CD44^{pos}CD24^{pos} cells ($P < 0.05$, data not shown). When 500 ZR75.1 cells were injected, 2/5 mice and 1/4 mice developed tumors by 75 days when injected with CD44^{pos}CD24^{pos} or CD44^{pos}CD24^{neg} cells, respectively (Figure 4b) without a difference in tumor volume ($P = 0.56$, data not shown). Likewise, the injection of 10,000 MCF7 cells resulted in 100% tumor incidence within 50 days (Figure 4a) with no difference in tumor volume ($P = 0.23$, data not shown).
Clones generated from a single CD44<sup>pos</sup>CD24<sup>pos</sup> or CD44<sup>pos</sup>CD24<sup>neg</sup> cell possess molecular and functional heterogeneity similar to that of parental line. (a) Clones were generated by sorting single Ca1a CD44<sup>pos</sup>CD24<sup>pos</sup> or CD44<sup>pos</sup>CD24<sup>neg</sup> cells into individual wells of 96-well plates. Only wells confirmed to contain a single cell were expanded. (b) CD44/CD24 expression of representative clones derived from a single CD44<sup>pos</sup>CD24<sup>neg</sup> or CD44<sup>pos</sup>CD24<sup>pos</sup> cell. From the experiment described in (a), seven clones were generated from sorted CD44<sup>pos</sup>CD24<sup>pos</sup> cells and five clones were generated from CD44<sup>pos</sup>CD24<sup>neg</sup> cells. The CD44/CD24 profiles of representative clones are presented. (c) Total RNA was isolated from isogenic CD44<sup>pos</sup>CD24<sup>neg</sup> and CD44<sup>pos</sup>CD24<sup>pos</sup> cells sorted from the clones described in (a, b) and transcript abundance was evaluated via real-time RT-PCR. Data generated with cells sorted from a clone derived from a single CD44<sup>pos</sup>CD24<sup>neg</sup> cell are presented in red. Data generated with cells sorted from clone derived from a single CD44<sup>pos</sup>CD24<sup>pos</sup> cell are presented in blue. * indicates \( P < 0.05 \). (d) Invasion through Matrigel by isogenic CD44<sup>pos</sup>CD24<sup>neg</sup> and CD44<sup>pos</sup>CD24<sup>pos</sup> cells. Data generated with cells sorted from a clone derived from a single CD44<sup>pos</sup>CD24<sup>neg</sup> cell are presented in red. Data generated with cells sorted from clone derived from a single CD44<sup>pos</sup>CD24<sup>pos</sup> cell are presented in blue. * indicates \( P < 0.05 \).
Once xenografts reached 1 cm in diameter they were removed, dissociated, and subjected to flow cytometric analysis. Contaminating host cells were excluded by gating out H-2Kd pos and mouse specific CD45 pos cells. While the CD44/CD24 profile of resulting xenografts is not identical to that of the parental cell line, CD44posCD24pos cells readily gave rise to CD44posCD24neg progeny in vivo, and vice versa (Figure 4c). This latter observation is consistent with our in vitro observations. More importantly, we observed that xenografts initiated with either CD44posCD24neg or CD44posCD24neg cells had a capacity for local invasion (Figure 4d). These observations confirmed that progeny of noninvasive CD44posCD24pos cells yield progeny capable of invading surrounding tissues.

Requirement for Activin/Nodal signaling in the generation of molecular heterogeneity

The role Activin/Nodal signaling plays in the generation of molecular and functional heterogeneity by CD44posCD24pos and CD44posCD24neg cells was explored with the use of SB-431542, a small molecule inhibitor of ALK4, -5, -7 [38,39]. Immediately post-sorting, vimentin expression was greatest in CD44posCD24neg cells and low/negative in CD44posCD24pos cells (Figures 2c, 5). As expected, 96 hours post-sorting, vehicle treated CD44posCD24pos cells and CD44posCD24neg cells gave rise to progeny with molecular heterogeneity (Figure 5). Specifically, epithelial-like, vimentin negative/low CD44posCD24pos cells gave rise to mixed prog-
eny; some expressed high levels of vimentin and others lacked the mesenchymal marker. Similarly, mesenchymal, vimentin positive CD44\textsuperscript{pos}CD24\textsuperscript{neg} cells expanded giving rise to a mixed population of vimentin negative and positive progeny. Following treatment with SB-431542, however, vimentin low/ negative CD44\textsuperscript{pos}CD24\textsuperscript{pos} cells gave rise to uniformly vimentin negative progeny. CD44\textsuperscript{pos}CD24\textsuperscript{neg} cells treated with SB-431542 gave rise to homogeneously vimentin positive progeny (Figure 5). These data demonstrate that active Activin/Nodal signaling is not required for expansion of either CD44\textsuperscript{pos}CD24\textsuperscript{pos} or CD44\textsuperscript{pos}CD24\textsuperscript{neg} cells. However, both populations require this pathway in order to give rise to molecular heterogeneity. Specifically, Activin/Nodal signaling is required for vimentin positive, CD44\textsuperscript{pos}CD24\textsuperscript{neg} cells to give rise to vimentin negative progeny and for vimentin negative, CD44\textsuperscript{pos}CD24\textsuperscript{pos} cells to give rise to vimentin positive progeny.

**Depletion of CD24 caused increased invasiveness without yielding a mesenchymal phenotype**

We next sought to evaluate whether the lack of CD24 expression is upstream or downstream of the mesenchymal phenotype associated with CD24 negativity. Seventy two hours following transient transfection using a pool of siRNA targeting CD24 yielded a seven-fold increase in the percentage of CD24\textsuperscript{neg} cells and a concomitant 26-fold decrease in median fluorescence intensity relative to cells transfected with non-targeting siRNA (Figure 6a). Depletion of CD24 expression did not yield a mesenchymal phenotype based on the expression of E-cadherin, Snail, Slug, and Twist (Figure 6b) but instead resulted in a reduction in Slug mRNA (P < 0.05). Consistent with an apparent lack of epithelial to mesenchymal transition, CD24 siRNA similarly failed to alter cell morphology (data not shown). Despite this lack of mesenchymal phenotype, CD24 siRNA transfected cells were 3.5-fold more invasive than non-targeting siRNA transfected cells (Figure 6c). In the invasion experiments, cells were counted and seeded to invasion chambers 24 h post transfection. The number of invading cells was counted 72 h post transfection. These data indicate that exogenous down regulation of CD24 is sufficient to yield increased invasiveness. However, it is unable to elicit a mesenchymal phenotype associated with endogenous down regulation of CD24.

**Discussion**

Herein, we demonstrate that noninvasive, epithelial-like CD44\textsuperscript{pos}CD24\textsuperscript{pos} cells readily give rise to invasive, mesenchymal CD44\textsuperscript{pos}CD24\textsuperscript{neg} progeny. This plasticity, which is dependent upon Activin/Nodal signaling, is the likely mechanism by which noninvasive, epithelial-like CD44\textsuperscript{pos}CD24\textsuperscript{pos} cells give rise to xenografts with locally invasive boundaries.

Cell motility is a fundamental aspect to early cancer metastasis. The ability of single cells to move from the primary tumor is...
frequently facilitated via the transition from an epithelial to a mesenchymal phenotype. Indeed, tumors that possess a mesenchymal gene signature correlate with tumor progression and poor prognosis \[41-43\]. As such, direct targeting of the invasive, mesenchymal component of primary breast cancer could be of substantial clinical benefit. The acquisition of a mesenchymal phenotype is associated with, among other things, the loss of E-cadherin \[44\] and increased vimentin expression \[45\]. Recently, CD44\textsuperscript{pos}\textsuperscript{CD24}\textsuperscript{neg} breast cancer cells were demonstrated to possess this mesenchymal phenotype \[13\] and we herein extended these observations. The specific targeting of CD44\textsuperscript{pos}\textsuperscript{CD24}\textsuperscript{neg} cells has proven effective at reducing the frequency of this population \[29-31\]. Our interest was in broadening the understanding of regulation of the CD24 gene and the invasive, mesenchymal CD44\textsuperscript{pos}\textsuperscript{CD24}\textsuperscript{neg} population in breast cancer cell lines.

Molecular and functional differences between CD44\textsuperscript{neg}\textsuperscript{dim}\textsuperscript{CD24}\textsuperscript{pos} and CD44\textsuperscript{pos}\textsuperscript{CD24}\textsuperscript{neg} cells have been eloquently described, including the observation that the former cannot give rise to the latter \[4,13,32\]. However, CD44 expression is known to profoundly impact cell behavior. Relative to CD44\textsuperscript{pos} cancer cells, those with low to no CD44 expression have reduced growth, invasiveness, and tumorigenicity, heightened susceptibility to chemotherapeutics, and reduced levels of pluripotent stem cell markers \[33,34,46-48\]. Indeed, we observed that fewer than 2% of CD44\textsuperscript{dim}/\textsuperscript{neg} cells (independent of CD24 status) gave rise to colonies \textit{in vitro}. Due to the well-characterized dominant effect of CD44 on cell behavior and the fact that previous work has compared CD44\textsuperscript{dim}/\textsuperscript{neg} to CD44\textsuperscript{pos} cells \[4,13,32\], the regulation of CD24 and its specific role in breast cancer cell behavior is largely unknown.

We demonstrated \textit{in vitro} and \textit{in vivo} that CD24 expression is dynamically regulated. Specifically, CD44\textsuperscript{pos}\textsuperscript{CD24}\textsuperscript{neg} cells readily gave rise to CD44\textsuperscript{pos}\textsuperscript{CD24}\textsuperscript{neg} progeny and \textit{vice versa}. This was stringently confirmed \textit{in vitro} by demonstrating that clones derived from a single CD44\textsuperscript{pos}\textsuperscript{CD24}\textsuperscript{pos} cell yielded CD44\textsuperscript{pos}\textsuperscript{CD24}\textsuperscript{neg} progeny. In non-transformed mammary epithelial cells, CD24 positivity is frequently associated with a terminally differentiated, luminal phenotype \[5,6,49\]. Such lineage commitment and long-term modification of gene expression is frequently achieved via alterations in promoter CpG dinucleotide methylation \[50,51\]. In our study, bisulfite sequencing analysis revealed that CD24 promoter methylation is similar between CD44\textsuperscript{pos}\textsuperscript{CD24}\textsuperscript{neg} and CD44\textsuperscript{pos}\textsuperscript{CD24}\textsuperscript{pos} cells suggesting that transcription can be rapidly altered without requiring changes in promoter methylation. Data presented herein do not rule out regulation of CD24 expression by modified translation or cell surface localization of the protein. However, these findings are consistent with our data demonstrating that the gene is indeed susceptible to dynamic transcriptional regulation. Furthermore, others have shown in MCF10A, a normal mammary cell line, that CD24 expression is under the regulatory control of Wnt signaling \[52\].

More importantly, the clones we generated confirmed that CD44\textsuperscript{pos}\textsuperscript{CD24}\textsuperscript{pos} cells give rise to functionally heterogeneous progeny. Specifically, we demonstrated that a single noninvasive, epithelial-like CD44\textsuperscript{pos}\textsuperscript{CD24}\textsuperscript{pos} cell could give rise to
CD44posCD24neg progeny with an invasive, mesenchymal phenotype. Similarly, xenografts initiated with CD44posCD24pos cells contained CD44posCD24neg progeny. Furthermore, these xenografts were as invasive as those initiated with CD44posCD24neg cells. These observations demonstrate that while CD44posCD24pos cells are noninvasive, they are fully capable of giving rise to invasive progeny.

Recently, Chang et al. [53] described a similar phenomenon in clones derived from Sca-1high and Sca-1low multipotent mouse hematopoietic cells. They reported that isogenic Sca-1high and Sca-1low cells, despite both being multipotent, had divergent global gene expression profiles and were functionally different. Furthermore, Sca-1high cells gave rise to Sca-1low cells and vice versa. Our findings, and those of Chang et al. [53], demonstrate the fundamental plasticity in functional heterogeneity present in isogenic mammalian cells.

Efforts are currently underway to specifically target CD44posCD24neg breast cancer cells due to their invasive, mesenchymal phenotype [29-31] and hypothesized role in seeding distant metastases. The data described herein have potential clinical implications as specific targeting of CD44posCD24neg cells will leave behind CD44posCD24pos cells that we demonstrate are capable of giving rise to invasive progeny. In an effort to address this, we sought to identify key pathways required by CD44posCD24pos cells to give rise to mesenchymal progeny. Relative to CD44posCD24neg breast cancer cells, Shipitsin et al. [4] found the TGFβ pathway was active in CD44posCD24pos cells. CD44 expression has been demonstrated to regulate TGFβ signaling [35,54], so we chose to evaluate the influence of CD44 expression on Activin/Nodal signaling and vice versa in CD44pos cells. To do so, we treated CD44posCD24neg and CD44posCD24pos cells with the Activin/Nodal inhibitor, SB-431542 [38,39]. These experiments demonstrated that Activin/Nodal signaling was not required for the expansion of either population, i.e. vimentin negative CD44posCD24pos cells expanded giving rise to vimentin positive progeny in the presence of the drug. Likewise, SB-431542 treated vimentin positive CD44posCD24neg cells gave rise to vimentin positive progeny. However, we demonstrated that both CD44posCD24pos and CD44posCD24neg cells require Activin/Nodal signaling in the generation of phenotypically diverse progeny. Most substantially, SB-431542 exposure to epithelial-like CD44posCD24pos cells blocked their ability to give rise to mesenchymal, vimentin positive progeny. These findings also demonstrate that despite the molecular and functional differences between CD44posCD24pos and CD44posCD24neg cells, both populations share a similar requirement for Activin/Nodal signaling in the generation of functionally heterogeneous progeny, thus making this pathway an exciting candidate to target clinically.

When CD24 expression was depleted exogenously, cell invasiveness increased. However, this invasiveness was not associated with changes in gene expression seen when CD24 expression is reduced endogenously. Increased invasiveness in the absence of elevated Snail or Slug expression has been previously reported in the literature. Specifically, β-catenin-lymphoid enhancer factor-1 expression yields increased invasiveness in colon carcinoma without increasing Snail or Slug expression [55]. Our observations suggest that the endogenous down regulation of CD24 is likely not an upstream event in the acquisition of the invasive, mesenchymal phenotype by CD44posCD24neg progeny of CD44posCD24pos cells. However, the current experiments were not able to determine if exogenous depletion of CD24 yielded a phenotype with similar levels of invasiveness as cells devoid of CD24 via endogenous means. A diagram outlining the proposed role of Activin/Nodal signaling in the regulation of CD24 and the invasive CD44posCD24neg phenotype is provided in Figure 7.

Conclusions

Herein we report that while CD44posCD24pos breast cancer cells represent a noninvasive, epithelial phenotype, they give rise to xenografts with a profound capacity for local invasion. This ability to form invasive tumors was ascribed to the fact that CD44posCD24pos cells readily give rise to CD44posCD24neg cells that possess an invasive, mesenchymal phenotype. The plasticity of CD44posCD24pos cells was blocked with SB-431542 indicating that ablation of Activin/Nodal signaling may be required in combination with therapies targeting CD44posCD24neg cells when breast cancer cell lines are used as models.
Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
MJM developed ideas, conceived the experiments and wrote and edited the manuscript. MJM, JMF, MAA, MP and EG conducted the experiments. JMF and EG edited the manuscript. BKV developed ideas and edited the manuscript. All authors contributed to the analysis of data.

Additional files
The following Additional files are available online:

**Additional file 1**
A table containing realtime PCR primer sequences and conditions. See http://www.biomedcentral.com/content/supplementary/bcr2449-S1.DOC

**Additional file 2**
A table containing GADPH Ct values for CD44posCD24neg and CD44posCD24pos cells. See http://www.biomedcentral.com/content/supplementary/bcr2449-S2.DOC

**Additional data file 3**
A table containing the estrogen receptor, progesterone receptor, HER2 amplification, and CD44/CD24 expression in 19 breast cancer cell lines. See http://www.biomedcentral.com/content/supplementary/bcr2449-S3.DOC

**Additional file 4**
A table containing the CD44/CD24 expression profile of clones derived from a single CD44posCD24pos or CD44posCD24neg cell. See http://www.biomedcentral.com/content/supplementary/bcr2449-S4.PPT

**Additional file 5**
A figure containing representative post sort analyses of sorted Ca1a, SUM 159 and MCF7 cells. See http://www.biomedcentral.com/content/supplementary/bcr2449-S5.PPT

**Additional file 6**
A figure containing representative flow cytometric quantitation of vimentin expression by CD44posCD24pos and CD44posCD24neg Ca1a cells. See http://www.biomedcentral.com/content/supplementary/bcr2449-S6.DOC

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