Separation and Characterization of Epithelial and Mesenchymal-like Murine Mammary Tumor Cells Reveals Epithelial Cell Differentiation Plasticity and Enhanced Tumorigenicity of Epithelial-enriched Tumor Cells

Katie A. Palen · Weiqing Jing · James J. Weber · Sara B. Tilkens · Andrew M. Chan · Bryon D. Johnson · Jill A. Gershan

Received: 7 September 2011 / Accepted: 21 December 2011 / Published online: 12 January 2012
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Abstract Tumors are composed of heterogeneous populations of cells including tumor-initiating cells (TICs) and metastatic precursors. While the origin of these cells is unknown, there is evidence that tumor cells can transdifferentiate from an epithelial to a mesenchymal phenotype, a property referred to as epithelial-to-mesenchymal transition (EMT). This cellular plasticity may explain the heterogeneous nature of tumors and differences in the tumorigenic and invasive properties of cells. Understanding the origin of these cells and the contribution of external factors that influence the acquisition of cellular properties is critical for the development of

therapeutics to eradicate cancer. In this study, we show that primary murine tumor cells harvested from FVB/N Tg (MMTV/Neu) spontaneous mammary tumors possess differentiation plasticity and can be enriched to be epithelial or mesenchymal-like using selected culture media conditions, and we show evidence of EMT in a clonal population of primary epithelial tumor cells when cultured in fibroblast growth factor-1 (FGF-1) or transforming growth factor- β (TGF- β). We also determined that in contrast to the identification of mesenchymal-like tumor cells as TICs in orthotopic xenograph models of tumorigenicity, epithelial-enriched

K. A. Palen · W. Jing · J. J. Weber · S. B. Tilkens · A. M. Chan · B. D. Johnson · J. A. Gershan (✉)
Department of Pediatrics, Section of Hematology-Oncology,
Medical College of Wisconsin,
8701 Watertown Plank Road,
Milwaukee, WI 53226, USA
e-mail: jgershan@mcw.edu

K. A. Palen
e-mail: kpalen@mcw.edu

W. Jing
e-mail: wjing@mcw.edu

J. J. Weber
e-mail: jweber@mcw.edu

S. B. Tilkens
e-mail: stilkens@mcw.edu

A. M. Chan
e-mail: axchan@mcw.edu

B. D. Johnson
e-mail: bjohnson@mcw.edu

K. A. Palen · W. Jing · J. J. Weber · S. B. Tilkens · A. M. Chan · B. D. Johnson · J. A. Gershan
The Children's Research Institute,
Children's Hospital of Wisconsin,
Milwaukee, WI 53226, USA

K. A. Palen · J. J. Weber · J. A. Gershan
Department of Pediatrics, Section of Hematology-Oncology,
MFRC 6008, Medical College of Wisconsin,
8701 Watertown Plank Road,
Milwaukee, WI 53226, USA

W. Jing · B. D. Johnson
Department of Pediatrics, Section of Hematology-Oncology,
MFRC 6072, Medical College of Wisconsin,
8701 Watertown Plank Road,
Milwaukee, WI 53226, USA

S. B. Tilkens · A. M. Chan
Department of Pediatrics, Section of Hematology-Oncology,
MFRC 6033, Medical College of Wisconsin,
8701 Watertown Plank Road,
Milwaukee, WI 53226, USA

murine mammary tumor cells were more tumorigenic as compared to mesenchymal-enriched cells when transplanted back subcutaneously into syngeneic immune competent mice. Together, these data suggest that EMT plasticity can be induced in primary murine mammary tumor cells, and that tumorigenicity of epithelial or mesenchymal-like cells may be influenced by factors such as the site of tumor inoculation or the immune state of the host (xenogenic immune compromised versus syngeneic immune competent).

Keywords EMT · Plasticity · Tumorigenicity · Mesenchymal · Epithelial · Fibroblast growth factor-1 · Tumor microenvironment

Abbreviations

E	Epithelial
M	Mesenchymal-like
EMT	Epithelial to mesenchymal transition
FGF-1	Fibroblast growth factor-1
MEGM	Mammary epithelial growth media
Tg/Neu	FVB/N Tg MMTV/Neu
TIC	Tumor-initiating cell
TGF- β	Transforming growth factor beta
s.c.	Subcutaneous

Introduction

Breast cancer is the second leading cause of cancer deaths in women and recurrence is the most common cause of mortality. Greater than 95% of breast cancers are epithelial, however within the tumor, there are subpopulations of cells that express epithelial and mesenchymal markers or a combination of both. This cellular heterogeneity has been attributed to differentiation plasticity of tumor cells and the ability to acquire mesenchymal features through epithelial to mesenchymal transition or EMT. During EMT, adherent epithelial cells lose basal-apical polarity and acquire a mesenchymal phenotype that includes enhanced migratory capacity, invasiveness, resistance to apoptosis and an increased production of extracellular matrix components [1]. Some of the hallmarks of EMT include loss of CD24 cell surface expression [2–4], downregulation of epithelial cytokeratins 8, 18 and 19 and E-cadherin, upregulation of vimentin, and mesenchymal transcriptional regulators such as Snai1 (Snail), Snai2 (Slug), Zeb1, Zeb2, Twist, FOXC1, and FOXC2 as reviewed in [5]. Mesenchymal cancer cells are more resistant to conventional chemotherapy and radiotherapy and correlate with tumor recurrence and a poor prognosis [6], [7]. Recently, a loss of HER2/neu (ErbB2) expression has been associated with a gain in mesenchymal phenotype in the human JIMT-1 breast cancer cell line [8].

This loss of ErbB2 expression during EMT may account for the acquired resistance to trastuzumab treatment in patients diagnosed with HER2/Neu⁺ breast cancer.

Reduced CD24 cell surface expression is associated with EMT, and has also been identified as a marker for tumorigenic self-renewing cancer stem cells. Human breast cancer cells with tumor-initiating potential have been identified as CD24^{−/lo} by their ability to initiate and form tumor in immune-compromised mice as well as the ability to grow as mammospheres in non-adhesive in vitro conditions [3, 9–11]. There is evidence that CD24 expression is dynamically regulated in tumor cell lines such that non-invasive CD24⁺ cells can give rise to invasive CD24^{−/lo} cells however, defining tumor-initiating cells (TICs) based on CD24 expression has been inconsistent between human xenograph and murine syngeneic animal models [2, 10]. In murine tumor models, there are conflicting reports regarding the expression of CD24 on TICs derived from mammary tumors. For example, murine TICs have been characterized as CD24^{high} in MMTV-wnt-1 [12], CD24⁺ in MMTV-Neu [13], CD24^{high} in p53-null [14] and CD24^{low} in Neu relapsed variant [15] models of murine mammary carcinogenesis. Given the variability of CD24 expression on cells with tumor-initiating potential in different experimental systems, we sought to further analyze the expression of CD24 as well as other TIC and EMT molecular markers on epithelial-enriched and mesenchymal-enriched tumor cells derived from spontaneous murine mammary tumors and determine whether there is a difference in the tumorigenicity of these tumor cell types. We chose the FVB/N Tg (MMTV/Neu) (hereafter referred to as Tg/Neu) mouse model for study because these mice develop spontaneous Neu expressing luminal mammary tumors, and tumor cells can be purified in vitro based on Neu expression. Furthermore, based on reports of EMT-associated loss of Neu expression [8], Neu expression can be used as a marker to separate epithelial and mesenchymal-like tumor cells.

In this report, we show that both epithelial-enriched and mesenchymal-enriched cells can be cultured from primary mammary tumors using different culture media conditions. A downregulation of Neu and an upregulation of *CD44s* and mesenchymal-associated transcription factor transcripts were more consistent markers of mesenchymal-enriched cells than CD24. We also show that primary mammary tumor cells possess the property of differentiation plasticity as EMT occurred when a clonal population epithelial tumor cells (derived from a spontaneous mammary tumor) upregulated mesenchymal-associated transcription factor transcripts when exposed to FGF-1 and TGF- β . Contrary to reports that describe enhanced tumorigenicity of mesenchymal-like human tumor cells in orthotopic xenograph models, we found that epithelial-enriched cells were more tumorigenic than mesenchymal-like cells when inoculated

subcutaneously into syngeneic Tg/Neu mice. Explanations for the difference in tumorigenicity of epithelial and mesenchymal cells may include the influence of the host microenvironment at the site of inoculation, or the immune constitution of the host as xenograph experiments were performed in immune-compromised hosts and the experiments in this study were performed in syngeneic immune competent hosts.

Methods

Mice Female FVB/N Tg (MMTV/Neu) 202MUL/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in the Biomedical Resource Center at the Medical College of Wisconsin. For some experiments, mice were inoculated subcutaneous (s.c.) in the hind flank with tumor cells and followed for tumor growth. Tumors were measured using routine caliper measurements, and when tumors exceeded 250 mm² or 35 mm² with ulceration, the mice were considered as moribund and euthanized. All experiments were approved by the Medical College of Wisconsin Animal Care and Use Committee.

Cell Lines and Primary Tumor Cultures The cell line (NT) derived from a spontaneous Tg/Neu mammary tumor was a gift from Dr. Elizabeth Jaffe, Johns Hopkins University. Human mammary tumor cell lines, MDA MB 231 and MCF7, were purchased from ATCC (Manassas, VA). To obtain primary tumor cells, spontaneous focal mammary tumors harvested from Tg/Neu mice were processed into single-cell suspensions by passage through 1.0 mm pore mesh screens. Cells were cultured in either: (1) MEGM® (Lonza, Switzerland) supplemented with 2% fetal bovine serum (FBS) and proprietary concentrations of bovine pituitary extract, epidermal growth factor, insulin, hydrocortisone, gentamycin and amphotericin (referred to as low serum/GF media), or (2) RPMI supplemented with 20% FBS, 12 mM HEPES buffer, 2 mM L-glutamine, 10 µM non-essential amino acids, 1 mM sodium pyruvate, 100 µg/ml streptomycin, and 100 U/ml penicillin (referred to as high serum media). For some experiments, cells were cultured in either low serum/GF media or high serum media with or without 10 ng TGF-β or 20 or 50 ng/ml fibroblast growth factor-1 (FGF-1) with 50 µg/ml heparin sulfate. The association of FGF-1 with heparin sulfate is required for interaction of FGF-1 with FGF receptors.

To obtain purified cultures of Neu⁺ mammary tumor cells, cells were stained with anti-c-ErbB2/c-neu mouse monoclonal antibody clone 7.16.4 (Calbiochem-EMD Chemicals, Gibbstown, NJ) and phycoerythrin (PE)-conjugated goat anti-mouse (Jackson ImmunoResearch, West Grove, PA)

secondary antibody, and incubated with anti-PE microbeads (Miltenei Biotech, Auburn, CA). Cells were then separated by positive selection using automated immunomagnetic sorting (AutoMACS, Miltenei Biotech). To obtain epithelial or mesenchymal-like enriched cells, the cells were stained with the anti-c-ErbB2/c-Neu monoclonal antibody and goat anti-mouse PE followed by staining with either a fluorescein isothiocyanate (FITC) or peridinin-chlorophyll-cyanine PerCP-Cy5.5 labeled anti-CD24 clone M1/69 monoclonal antibody (eBioscience, San Diego, CA). Labeled cells were then sorted by flow cytometry based on Neu and CD24 expression using a FACSARIA III flow cytometer and FACSDiva software (Becton-Dickinson, Franklin Lakes, New Jersey).

Flow Cytometry To determine cell surface expression of Neu and CD24, cells were stained with anti-c-ErbB2/c-Neu antibody and goat anti-mouse PE, followed by staining with either FITC or PerCP-Cy5.5-conjugated anti-CD24. Stained cells were analyzed on a Becton Dickinson FACS Calibur flow cytometer and FlowJo analysis software (Treestar, Inc., Ashland, OR).

Microscopy For bright field microscopy, cells were imaged using a 10x objective (Zeiss, Thornwood, NY) on a Zeiss Axiovert 200 M microscope. For immunofluorescent microscopy, cells were permeabilized with 0.05% Triton X (1:20 dilution of SurfactAmp, Pierce, Rockford, IL) and were incubated with a purified anti-mouse Zeb2 (SIP1) polyclonal rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with the Alexa Fluor 568-conjugated goat-anti-rabbit (Invitrogen, Carlsbad, CA) secondary antibody. As negative controls, cells were stained with the Alexa Fluor 568 secondary antibody only. Cells were imaged using a 20x objective (Zeiss) on a Zeiss Axio Z1 upright microscope and images were analyzed using AxioVision rel 4.8 (Zeiss) software. Images were merged using the AxioCam MR (Zeiss) software.

qPCR Tumor cells were suspended in Trizol® and immediately frozen at -80° C. RNA was processed according to the Trizol® reagent manufacturer's protocol (Invitrogen). The RNA optical density and ratio of absorbance at 260 and 280 nm (A_{260/280}) was determined using a Nanodrop-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). cDNA was synthesized using 2 µg of RNA, the Superscript® VILO™ (Invitrogen), iScript™ cDNA synthesis kit (BIO-RAD, Hercules, CA) or the QuantiTect reverse transcription cDNA kit (Qiagen, Valencia, CA) per manufacturer's protocol. cDNA (2 µl at a 1:10 or 1:100 dilution) was amplified in a 20 µl reaction volume in the CFX C1000 Real-time Thermal Cycler (Bio-Rad) with the primer sets listed on Table 1, using reagents in the Quantifast SYBR green PCR Kit (Qiagen) or the SsoFast™ EvaGreen®

Table 1 qPCR Primers. Including Accession number, primer sequence, amplicon length, amplification of unique splice variants and qPCR efficiency

Gene	Accession	5' Primer 5'–3'	3' Primer 5'–3'	Amplicon length bp	Targeted splice variants	PCR Efficiency/ r^2
<i>Rattus rattus</i>						
ErbB2 (Neu)	NM_017003	TACCGGCACAGACATGAAGT	TGTGAGCGATGAGCATGTAA	188		107.6%/0.990
<i>Mus musculus</i>						
CD24	NM_009846	GGAGCGGACATGGGCAGAGC	GAGCTGCCACCCCTCTGGT	179		100.6%/0.996
CD44	NM_009851	CCCCGGAACACACAGCCTCCT	CCACGTCTCTGTGGGTAGC	122	Isoform a	104.8%/0.998
CD44	NM_001039150	AGCACCTTGGCCACCACTCCT	GGGCCGAAGCAGTTGTCCCT	145	Isoform b	98.1%/0.997
CD44	NM_001039151	GCACCTTGGCCACCAAGATCG	AGCCAGTGCCAGGAGAGATGC	194	Isoform s	108.2%/0.970
Zeb1	NM_011546	GCAGGAGCGCCAGTGAAGG	TGGGTGGCGTGGAGTCAGAGT	118		111.6%/0.998
Zeb2	NM_015753	CCCCACACTTCGGGGCTTC	GGGGCCATCCGCCATGATCG	184		101.9%/0.998
Snai1 (Snail)	NM_011427	AGCTGCAGGACGCGTGTGTG	GAAGCGGGGGGTTGAGGAC	103		103.5%/0.996
Snai2 (Slug)	NM_011415	ACAGCGAACTGGACACACACACA	TGGAGTGGAGCTGCCGACGA	145		108.7%/0.961
Fgf-1	NM_010197	GGCACCTCTGTACAGCGCAA	GGCAGCAAGCAGCGGTGGTA	125		109.6%/0.965
Tgfb1	NM_011577	CCGGAGCCCGAAGCGGACTA	GGGGTTCCGGCACTGCTTCC	152		108.8%/0.998
Vimentin	NM_011701	CGCCAGGCCAAGCAGGAGTC	CTCATCTGCAGGGCGGCCAA	250		99.0%/0.995
<i>Homo sapiens</i>						
CD24	NM_013230	AGCGGTTCTCCAAGCACCCAGCA	GGCCACCAATTGCTCTGCCCATGT	79		128.3%/0.993
CD44	NM_001001391	CCCTGTCTACCAGAGACCAAGACACA	TGCTCCACCTTCTTGACTCCCA	106	Isoform s	107.5%/0.997
Zeb1	NM_001128128	ACCTGCCAACAAGACCAAGACAGTGT	CCTGAGCTTCAGGCCCCCAAGGA	123		92.2%/0.980
Zeb2	NM_014795	ACACCCCTTGGCACAACAACGA	TGCGGTCTGGATCGTGGCTTC	106		111.8%/0.972
Snai2 (Slug)	NM_003068	AAGGCGTTTTCAGACCCCTGGT	TGCTCTGTTCAGTGAAGGCAAGA	90		96.9%/0.989

Supermix (Bio-Rad) using the following amplification protocols: 95⁰ C for 5 min or 30 s followed by 39 cycles of 95⁰ C for 10 or 3 s and 60⁰ C for 30 or 3 s, respectively. Samples were then heated to 95⁰ C for 10 s followed by a melt curve from 65⁰ C to 95⁰ C with 0.5⁰ C increments for 5 s. The 18 s reference gene was amplified using QuantiTect 18 s primers (Qiagen). All reactions were done in triplicate wells with duplicate well no template controls (NTC). To calculate fold-change in gene expression, the average $\Delta\Delta C_q$ values from triplicate wells were combined from at least 3 separate experiments.

Scratch Assay Approximately 10⁶ cells were seeded per well in a 6 well plate for 6 days with and without 50 ng/ml

FGF-1 and 50 μ g/ml heparin sulfate. When cells were confluent, three scratch wounds were introduced with P200 tips per well and cells were allowed to migrate into the wound site. Bright field photomicrographs were captured from duplicate wells with five images taken per well at 0 and 18 h. The wound areas were measured with the ImageJ software and cell motility is expressed as the relative area migrated.

Statistical Analysis Data from the qPCR assays were compared using the two-tailed one sample Student's *t* test with Prism 5.0a software (GraphPad, San Diego, CA). Survival curves were compared using the log-rank (Mantel-Cox) test with Prism 5.0a software. *P* values ≤ 0.05 were considered as significant.

Fig. 1 Epithelial and mesenchymal-like tumor cells were enriched when separated based on Neu and CD24 expression. (a) A representation of Neu expression on tumor cells after immunomagnetic cell sorting using a mouse anti-c-ErbB2/c-neu antibody, PE-conjugated anti-mouse secondary antibody and anti-PE microbeads. (b) Expression of Neu and CD24 on cells cultured in low serum/GF media. Two populations of cells are shown in the following gates: Neu^{high}CD24^{high} (Gate 1) and Neu^{low}CD24^{low} (Gate 2). (c) Bright field microscopy of the cells analyzed in Fig. 1b showing epithelial cell clusters [E] and fibroblastoid mesenchymal-like cells [M]. (d) Bright field microscopy of epithelial-like cells that were flow sorted from Gate 1 in Fig. 1b, and cultured for

9 days in low serum/GF media. (e) Bright field microscopy of fibroblastoid mesenchymal-like cells that were flow sorted from Gate 2 in Fig. 1b, and cultured for 9 days in low serum/GF media. (f) Bright field microscopy of tumor cells harvested from a spontaneous tumor that have an epithelial morphology when cultured only in high serum media (g) Bright field microscopy of cells sorted from Gate 1 in Fig. 1b, and cultured over time in low serum/GF media showing the presence of fibroblastoid mesenchymal-like [M] and epithelial [E] cell clusters. (h) Neu expression on the cells in Fig. 1d (E cells) and Fig. 1e (M cells). The data is representative of 1 of 2 spontaneous tumors analyzed

Results

Epithelial and Mesenchymal-like Murine Primary Mammary Tumor Cells were Separated based on Neu and CD24 Expression

To obtain purified tumor cell cultures, Tg/Neu spontaneous mammary tumors were processed into single cell suspensions, cultured for approximately 48 h in low serum/growth factor (GF) media (see [Methods](#)), then purified based on Neu expression using immunomagnetic sorting. Figure 1a is a representative example of Neu expression on tumor cells after immunomagnetic sorting. Neu-positive tumor cells were then cultured in either high serum media (see [Methods](#)) or low serum/GF media. Figure 1b shows that when cells were cultured in low serum/GF media, there were populations of cells with high and low(er) expression of Neu and CD24. Morphologically this culture contained both tight epithelial (*E*) cell clusters and elongated fibroblastoid mesenchymal-like (*M*) cells (Fig. 1c). To separate *E* and *M* cells, cells were stained with fluorescently labeled Neu and CD24 antibodies and cells in the gates shown in Fig. 1b were sorted by flow cytometry. After short-term (~9 days) culture in low serum/GF media, the cells that were derived from Gate 1 in Fig. 1b had an epithelial morphology (Fig. 1d). The cells that were derived from Gate 2 in Fig. 1b were mesenchymal-like (Fig. 1e). As shown in Fig. 1h, the epithelial cells in Fig. 1d had higher Neu expression as compared to the fibroblastoid cells in Fig. 1e. When cells harvested from a spontaneous mammary tumor were cultured only in high serum media they became epithelial and maintained a stable epithelial morphology (Fig. 1f), while the epithelial cells derived from Gate 1 (Fig. 1b) that were cultured in low serum/GF media reverted to a mixed population of epithelial and mesenchymal-like cells (Fig. 1g).

M-enriched Tumor Cells have Decreased Neu Expression and Increased Gene Expression of Mesenchymal-associated Transcription Factors, and *CD44s* as Compared to *E*-enriched Cells

To enrich for subcultures of *E* and *M* tumor cells, cells were cultured in either high serum (enriched for *E* cells) or low serum/GF (enriched for *M* cells) media. Figure 2a shows Neu and CD24 expression on cells from two different mammary tumors when cultured long-term (~100 days) in either media. To further phenotype and show that cells from the *M*-enriched cultures were more mesenchymal than *E*-enriched cells, gene expression associated with TIC and EMT markers was compared. CD44 is a marker of TICs in many tissues including human breast, pancreas, head and neck, gastric, ovarian, prostate and colon, as reviewed in [16],

Fig. 2 *M*-enriched tumor cells harvested from spontaneous tumors show increased transcription of mesenchymal-associated transcription factors, *CD44s*, and a decrease in Neu expression relative to *E*-enriched cells (a) Expression of Neu and CD24 on cells harvested from 2 different spontaneous mammary tumors when cultured for approximately 100 days. Cells that were cultured in high serum media were enriched for Neu^{high} epithelial cells [*E*], and cells that were cultured in low serum/GF media were enriched for Neu^{low} mesenchymal-like cells [*M*]. (b and c) qPCR of *E* and *M* cells derived from Spontaneous Tumor 1 (b) and Spontaneous Tumor 2 (c) where the transcription of the TIC markers and mesenchymal markers was compared. The results are graphed as expression in *M* cells relative to *E* cells; #*P*≤0.05, **P*≤0.01, ^*P*≤0.001, and +*P*≤0.0001. Gray bars represent TIC markers, and black bars represent mesenchymal markers. (d) qPCR of cDNA made from the invasive MDA MB 231 and non-invasive MCF7 human mammary tumor cell lines. The data are graphed as expression in MDA MB 231 cells relative to MCF7 cells; **P*≤0.01, and +*P*≤0.0001. Gray bars represent TIC markers, and black bars represent EMT markers. (e) Fluorescent microscopy of *E* and *M*-enriched cells cultured from Spontaneous Tumor 1 and stained with a rabbit polyclonal antibody specific for Zeb2 and a goat anti-rabbit Alexa-Fluor 568-conjugated secondary antibody. Panels a, d and g show dapi staining, panels b and e show fluorescence of Zeb2 staining in epithelial (*E*, panel b) and mesenchymal-like cells (*M*, panel e). As a control, panel h was stained with the secondary goat anti-rabbit Alexa-Fluor 568-conjugated antibody only. Panels c, f and i are merged images of dapi and Alexa-Fluor staining. The data is from 1 of 3 replicate experiments

however, there are multiple isoforms of CD44 that contain up to 10 variant exons. To determine if there was differential gene expression of *CD44* isoforms in *E* and *M*-enriched mammary tumor cells, primers specific for isoforms a, b and the standard isoform, s, were used. Isoform a contains variant exons 2–10, isoform b contains variant exons 6–10 and isoform s does not contain variant exons. Figure 2b shows the qPCR results from Spontaneous Tumor 1 *E* and *M* cell cultures. The *M*-enriched cells had a significant increase in expression of the *CD44s* isoform and decreased expression of isoforms a and b as compared to *E*-enriched cells. When EMT-associated gene expression was examined, *M*-enriched cells had significantly increased expression of EMT transcriptional regulators including *Zeb1*, *Zeb2*, *Snail*, *Slug*, and the mesenchymal protein marker, *vimentin*, as compared to *E*-enriched cells. Since the epithelial and mesenchymal-like-enriched cells were initially sorted based on Neu and CD24 expression, there was an expected decrease in the expression of *Neu* in the *M*-enriched cell population, but there was an unexpected increase in the expression of *CD24* in the *M*-enriched cells of Spontaneous Tumor 1. This data suggests that CD24 expression may not be a consistent mesenchymal marker in this model system. Figure 2c shows the gene expression profile of subcultures derived from Spontaneous Tumor 2. While the gene expression changes between the epithelial and mesenchymal-enriched cells was not as dramatic as for Spontaneous Tumor 1, there was an upregulation of *CD44s* and mesenchymal-associated transcription factors and a down regulation of *Neu* in the *M*-enriched cell culture as

compared to the *E*-enriched culture. To show that *M* and *E*-enriched cells harvested from murine mammary tumors had an expression profile similar to invasive and non-invasive human mammary cells, qPCR of *CD24*, *CD44s* and mesenchymal-associated transcription factors was compared in the invasive MDA MB 231 and non-invasive MCF7

human breast cancer cell lines. Similar to the murine mammary tumor *M*-enriched cells *CD44s*, and mesenchymal-associated transcription factors were increased in the invasive MDA MB 231 cells relative to the non-invasive MCF7 cells (Fig. 2d). This result suggests that *M*-enriched cells have a gene signature similar to invasive human mammary

tumor cells. To show a correlation of protein expression with the qPCR results, there was greater protein expression of *Zeb2* in *M* cells as compared to *E* cells (Fig. 2e panels e and b, respectively).

Culture with FGF-1 and TGF- β Induces EMT in a Clonal Population of Primary Tumor Epithelial Cells

The ability to enrich for epithelial and mesenchymal-like cells using different media conditions suggest that primary tumor cells cultured in vitro have an inherent plasticity and differentiate according to environmental cues. To show that primary epithelial tumor cells can be induced to undergo EMT, *E*-enriched cells were cultured in low serum/GF media with FGF-1 to induce transdifferentiation to a more mesenchymal phenotype [17]. After 45 days in culture, the cells treated with FGF-1 consisted of both epithelial and fibroblastoid cells with fibroblastoid cells elongating from the edge of the epithelial cell cluster, while the cells cultured without FGF-1 remained as small tight epithelial clusters (Fig. 3a).

To further support the findings that primary mammary tumor cells can be induced to undergo EMT, a clonal population of spontaneous tumor-derived *E* cells were cultured for 7 days in high serum media with or without 50 ng/ml of FGF-1 with 50 μ g of heparin sulfate or 10 ng/ml TGF- β and gene expression was analyzed by qPCR. When cells were exposed to TGF- β , there was a significant increase in *CD44s*, *Zeb1*, *Zeb2* and *Slug* expression, and when cells were exposed to FGF-1 there was a significant increase in *CD44s* and *Slug* expression indicating that exposure to both TGF- β and FGF-1 induced expression of mesenchymal-associated genes in clonal primary epithelial murine mammary tumor cells (Fig. 3b). To determine if there was a difference in *FGF-1* and *TGF- β* expression in epithelial or mesenchymal-like cells, qPCR analysis on clonal *E* and *M* cells was performed. Figure 3c shows a relative increase in transcript expression of *FGF-1* in epithelial tumor cells and a relative increase expression of *TGF- β* in mesenchymal-like cells. Since exposure to FGF-1 resulted in an increase in expression of mesenchymal-associated transcription factors, it was of interest to test if treatment with FGF-1 would alter the motility these cells. To test this, 3 clonal epithelial cell lines (A1, C2 and E5) that were derived from the epithelial-enriched primary tumor cells were subjected to scratch wound assays in the presence or absence of FGF-1. While individual clonal lines displayed variable migratory capacity, the addition of FGF-1 significantly enhanced cell motility by 20–60% (Figs. 3d and e).

Epithelial-enriched Tumor Cells are more Tumorigenic than Mesenchymal-enriched Tumor Cells

Based on reports in the literature [3], we hypothesized the *E* and *M*-enriched cells obtained from our cultures would

exhibit different tumorigenic properties. To determine if there was a difference in the tumorigenicity of these cells, Tg/Neu mice were challenged subcutaneously into the hind flank with 500, 5,000 or 50,000 *E* or *M*-enriched cells. Survival experiments of mice injected with the cells cultured from Spontaneous Tumors 1 and 2 (shown in Fig. 2a) were conducted. When tumors reached greater than 250 mm², or at least 35 mm² with ulceration, the mice were considered moribund and were euthanized. Analysis of the survival data show that the *E*-enriched cells were more tumorigenic than the *M*-enriched cells with approximately a one log difference in the dose required to induce 100% mortality (Figs. 4a and b). A similar difference was also observed in the tumor growth kinetics (Figs. 4c and d).

Discussion

The results of this study show that primary murine mammary tumors contain both epithelial and mesenchymal-like cells. The *M*-enriched cells had a decrease in *Neu* expression and a gene expression profile similar to the invasive MDA MB 231 human mammary tumor cell line with increased expression of *CD44s* and the mesenchymal-associated transcription factors, *Zeb1*, *Zeb2*, and *Slug*. Since CD24 is a luminal epithelial cell marker and reduced expression is associated with EMT, and the *E* and *M*-enriched tumor cells were initially separated based on Neu and CD24 expression we expected the *E*-enriched cells to have higher expression of Neu and CD24 than the *M*-enriched cells. However, *CD24* transcript was increased in the Spontaneous Tumor 1 Neu^{low} *M*-enriched cells and was not changed in the Spontaneous Tumor 2 *M*-enriched cells as compared to the *E*-enriched cells. These data indicate that CD24 may not be a reliable marker to define epithelial versus mesenchymal-like cell populations in this murine model of mammary carcinoma.

The increased expression of the *CD44s* isoform in *M*-enriched cells as well as in the MDA MB 231 invasive human breast cancer cell line is an interesting finding since the expression of CD44 isoforms have been implicated as markers that correlate with the invasive potential of tumor cells. Currently, there is conflicting evidence for an association between CD44 isoform expression and invasiveness. Expression of variant exons 3, 6, 8 and 10 have been associated with the invasive potential of tumors in numerous studies, as reviewed in [19]. However, studies have also indicated that expression of CD44s is associated with breast cancer metastasis [20, 21], and there is recent evidence that expression of CD44s accelerates EMT and breast cancer progression [22]. Our data supports the association of CD44s with a mesenchymal phenotype.

Fig. 3 Culture with FGF-1 and TGF- β induces expression of mesenchymal transcription factors in a clonal population of primary tumor epithelial cells. **(a)** $10\times$ bright field microscopy of an *E*-enriched cell culture derived from a spontaneous tumor and cultured for 45 days in low serum/GF media with or without 20 ng/ml of FGF-1 and 50 μ g/ml of heparin sulfate. **(b)** qPCR comparing gene expression in a clonal population of primary tumor *E* cells after 7 days of culture in high serum media with or without 10 ng/ml TGF- β or 50 ng/ml of FGF-1 with 50 μ g/ml of heparin sulfate. The data are graphed as expression in TGF- β or FGF-1 treated tumor cells relative to expression in tumor cells cultured in media only; # $P<0.05$ and * $P<0.01$. Gray bars represent TIC markers and black

bars represent EMT markers. **(c)** qPCR comparing FGF-1 and TGF- β transcript expression in clonal epithelial and clonal mesenchymal-like cells. The data are graphed as expression in *E* cells relative to *M* cells; # $P<0.05$. **(d)** Scratch assays were performed on three representative *E* cell clones, A1, C2 and E5. The area migrated after 18 h was quantified from at least 5 scratch wounds. Results represent 5–9 data points from duplicated wells performed in the presence (+) or absence (–) of 50 ng/ml FGF-1 and 50 μ g/ml of heparin sulfate. *** $p<0.0005$. Representative images of scratch wounds from A1 clones in the presence (panels **b**, **d**) or absence (panels **a**, **c**) of FGF-1 at time 0 (panels **a**, **b**) and at 18 h (panels **c**, **d**)

The transdifferentiation of cells between epithelial and mesenchymal states occurs during development and

provides cellular plasticity during embryogenesis and other normal tissue reorganizing processes. There is also evidence

Fig. 4 Epithelial-enriched tumor cells are more tumorigenic than enriched mesenchymal-like tumor cells. **(a)** Kaplan-Meier survival curves for mice ($n=4$) given s.c. injections into the hind flank of different numbers of the *E* or *M* cells from Spontaneous Tumor 1 (as shown in Fig. 2a); # $P \leq 0.05$. **(c)** Tumor growth in mice that received limiting dilutions of *E* and *M* cells from Spontaneous Tumor 1. Tumor

size was determined by routine caliper measurements. **(b)** Kaplan-Meier survival curves for mice ($n=4$) given s.c. injections into the hind flank of different numbers of the *E* or *M* cells from Spontaneous Tumor 2 (as shown in Fig. 2a); * $P \leq 0.01$. **(d)** Tumor growth in mice that received limiting dilutions of *E* and *M* cells from Spontaneous Tumor 2

that EMT occurs in breast cancer cells [23]. Our data suggests that mesenchymal-like cells expressing genes associated with EMT and an invasive phenotype can be enriched under selective in vitro culture conditions. Furthermore, molecular evidence for EMT was shown with upregulation of *CD44s*, *Zeb1*, *Zeb2*, and *Slug* expression in a clonal population of epithelial tumor cells cultured short term in FGF-1 or TGF- β . The appearance of fibroblastoid cells at the border of epithelial clusters (Fig. 3a) and an increase in mobility when cells were cultured in FGF-1 (Figs. 3d and e) also support evidence by others that EMT can be induced in vitro in primary tumor cells [25, 26]. The expression of *Zeb1*, *Zeb2*, *Snail* and *Slug* have all been implicated in EMT induction and the acquisition of an invasive cell phenotype, as reviewed in [24]. Altered expression of FGF-1 and FGF-2 have also been associated with a dedifferentiated and invasive cell phenotype [27], and there is evidence that factors such as TGF- β [28, 29] and epidermal growth factor (EGF) [28, 30] induce EMT. Our findings support the paradigm that epithelial and mesenchymal tumor cells are present in primary murine mammary tumors and that phenotypic

plasticity can be induced in vitro by external factors including FGF-1 and TGF- β .

Lastly, subcutaneously tumors grew more rapidly when syngeneic mice were challenged with epithelial-enriched cells as compared to mesenchymal-enriched cells. These data show that in the subcutaneous microenvironment of a syngeneic host, epithelial-enriched tumor cells are more tumorigenic than mesenchymal-enriched cells. This finding does not support previous findings reported in human xenograph models whereby mammary tumor-initiating cells are found in the mesenchymal-like population of cells [3, 18]. The differences in the tumor microenvironment and involvement of immune regulatory factors between human immune-compromised xenograph models and this immune-competent syngeneic model may account for the differences in the ability of *E*-enriched *M*-enriched subpopulations of tumor cells to seed and form tumor. It is possible that in the presence of the appropriate microenvironment (i.e. a mammary fat pad versus subcutaneous), the tumorigenic potential of *E* and *M* cells may differ.

In summary, in this Tg/Neu model of murine mammary carcinoma, (1) Primary cell tumor cultures were enriched in vitro for cells with epithelial or mesenchymal-like properties, (2) A decrease in Neu expression as well as an increase in transcript expression of *CD44s* and mesenchymal-associated transcription factors were consistent markers of the mesenchymal phenotype, (3) In vitro exposure to FGF-1 and TGF- β induced EMT in a clonal population of primary epithelial tumor cells and, (4) Epithelial-enriched tumor cells were more tumorigenic than mesenchymal-enriched tumor cells when introduced subcutaneously into syngeneic immune-competent hosts.

Acknowledgements Support for this work was provided by the Kathy Duffey Fogarty Greater Milwaukee Foundation, the Midwest Athletes Against Childhood Cancer (MAACC) Fund, and the Cedarburg Junior Woman's Club. We thank Elizabeth Jaffe for supplying the NT tumor cell line and Michael Dwinell for supplying the MDA MB 231 and MCF7 cell lines. We also thank Alexander Liska, Katie McShane and Rachel Wilkinson for technical support.

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