FXYD5 (dysadherin) may mediate metastatic progression through regulation of the β-Na\(^+\)-K\(^+\)-ATPase subunit in the 4T1 mouse breast cancer model

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FXYD5 belongs to a group of single-span transmembrane proteins named after the invariant extracellular motif Phe-Xxx-(14, 19, 22, 23, 27, 39, 42). The seven mammalian family members of this proteins are called Na\(^+\)-K\(^+\)-ATPase regulators, expressed in a variety of normal epithelial tissue, such as lung, kidney, intestine (25). As other family members, FXYD5 specifically interacts with the Na\(^+\)-K\(^+\)-ATPase and modulates its kinetic properties (8). FXYD5 is structurally different from other FXYD family members. Its extracellular domain is much longer than that of other FXYD proteins (e.g., 145 vs. <30 amino acids) and it is rich in S, P, and T residues, suggesting extensive O-glycosylation.

Despite its role in carcinogenesis, FXYD5 is expressed in a variety of normal epithelial tissue, such as lung, kidney, and intestine (25). As other family members, FXYD5 specifically interacts with the Na\(^+\)-K\(^+\)-ATPase and increases the pump’s V\(_{\text{max}}\) when expressed in Xenopus oocytes (25) or mammalian cells (27). No other partners for interaction have been described for FXYD5; however, many of the reported observations cannot be readily explained by modulation of Na\(^+\)-K\(^+\)-ATPase kinetics.

The Na\(^+\)-K\(^+\)-ATPase pump is composed of catalytic α- and regulatory β-subunits. There are four different α-subunits (α1, α2, α3, and α4) and three β-subunits (β1, β2, and β3). The dominant and most investigated combination in most mammalian cells is α1β1. Several studies have suggested that, in addition to ion pumping, the Na\(^+\)-K\(^+\)-ATPase has both structural and signaling functions. These include cardiac glycoside-induced signaling (20; involvement in cell adhesion and intercellular interaction (47); and cytoskeletal organization and motility (36). Some of the above are mediated by its β-subunit and its sugar branched structure (47, 48). A cancer-promoting function has been attributed to the β1 and β2 isoforms. It has been shown that loss of β1-subunit expression is associated with a poorly differentiated phenotype that is found in carcinoma cells (6, 35). In normal epithelial cells, downregulation in β1 expression is associated with epithelial-mesenchymal transition (EMT) (29, 34). The β2-subunit has been initially described as an adhesion molecule on glia that mediates neuron-astrocyte adhesion and neural cell migration (10). It has also been suggested that the loss of β2 plays a role in the invasion pattern in the malignant progression of gliomas (41).

The relationship of FXYD5 and β1 has been reported previously. We demonstrated that FXYD5 can induce structural changes in the β1 isoform by modifying its glycosylation state in Xenopus oocytes and mammalian cell lines (22, 25). In addition, it has been demonstrated that FXYD5 impairs epithelial barrier function by disrupting the intercellular interactions between β1-subunits (4).

In this study we have adopted the 4T1 breast cancer model system and employed it to investigate causal relationships between the expression of FXYD5 and lung metastases. This in vivo system, as well as in vitro cell culture work in 4T1 cells, enabled us to examine a number of key steps in the mechanism...
underlying FXYD5 mediated phenotypes. We have found that FXYD5 induces metastasis spread, without having a substantial effect on primary tumor growth. This phenotype is not mediated by FXYD5 alone, but rather by a FXYD5/Na⁺-/K⁺-ATPase complex. FXYD5-dependent downregulation of β1 mimics the E-cadherin decrease reported for other cell systems which promotes metastasis by effecting transcellular and intracellular events.

MATERIALS AND METHODS

4T1-luc mouse breast cancer model. The mouse breast cancer cell line 4T1 was generously provided by Prof. Zvi Granot (The Hebrew University-Hadassah Medical School, Jerusalem, Israel). This cell line was isolated from a single spontaneously arising mammary tumor from a BALB/cfC3H mouse. 4T1-luc cells were transduced with a lentiviral vector (pLVX-Luc) to stably express firefly luciferase for quantitative assessment of tumor burden in visceral organs. The cells were cultured in DMEM supplemented with 10% FCS (Biological Industries, Beit Haemek, Israel) and penicillin-streptomycin (Biological Industries, Beit Haemek, Israel) in a humidified atmosphere of 5% CO₂ in air. FXYD5-silenced clones were generated by two shRNA sequences: one within the coding sequence (Sigma TRCN0000079352) and the other to its 3' untranslated region (Sigma TRCN0000079348), stably transected into parental cells using JetPei transfection system, according to manufacturer’s instructions. pLKO.1-puro nontarget shRNA control plasmid DNA was used as negative control. Positive colonies were selected by puromycin resistance at a concentration of 2 μg/ml. Positive colonies were selected by puromycin resistance. pLKO.1-puro nontarget shRNA control plasmid DNA was used as negative control.

Animals and procedures. Six- to eight-week-old BALB/c female mice were purchased from the Jackson Laboratory and Harlan (Israel). All experiments involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Weizmann Institute of Science. Subcutaneous low passage 4T1-luc cells (5 × 10⁶) were suspended in 0.1 ml PBS and either injected into the mammary fat pad in anesthetized mice (ketamine 0.05 mg/g body wt) or intravenously injected into the tail vein. The number of mice studied and treatment schedule of the experiment are described in each figure legend.

In vivo imaging. Prior to animal injection, luciferase activity was validated and found similar in 4T1-luc WT and FXYD5-silenced clones. To visualize the cells in vivo, mice were treated with 50 μl of luciferin (30 mg/ml, Regis Technologies) via intraperitoneal injection. Animals were shaved and anesthetized in an isofluorane chamber, and the tumor burden in lung and mammary fat pad was evaluated by measuring luciferase activity using in vivo bio-photic imaging (IVIS Spectrum System).

Lung metastasis negative India ink staining. To identify and quantify surface pulmonary nodules, mice were euthanized in a CO₂ chamber and the chest cavity was exposed through a midline chest incision. The trachea was penetrated with a 20-gauge needle, and the lungs were slowly perfused with 15% India ink solution. The lungs were rinsed in water and destained in Fekete’s solution (100 ml of 70% ethanol mixed with 10 ml of 4% formaldehyde and 5 ml of 100% glacial acetic acid). Surface pulmonary nodules appeared white against a black background of normal lung parenchyma.

RNA isolation and quantitative real-time PCR. RNA was isolated from cultured cells using an RNeasy kit (QIAGEN) and reverse transcribed from the poly A⁺ tail using a Super-Script II Reverse Transcriptase kit (Invitrogen). PCR reactions were carried out using the Power SYBR Green PCR kit (Applied Biosystems by Life Technologies) according to the manufacturer’s instructions. GAPDH mRNA was used for quantitative normalization.

RNA sequencing. The procedure was performed by the Israel National Center of Personal Medicine (INCPM). Triplicates of WT 4T1 and two FXYD5-silenced clone (sh48, sh52) samples of 500 ng total RNA each were processed using the TruSeq RNA Sample Preparation Kit v2 protocol (Illumina). Libraries were evaluated by Qubit and TapeStation. The nine samples were pooled together with different barcodes to allow sequencing in one lane. Sequencing reads were mapped to mouse genome assembly (mm10) using TopHat (version 2.0.10) and counted with HTSeq-count (version 0.6.1p1), using intersection-strict mode. Differential expression was analyzed with DESeq2.

Surface biotinylation and Western blotting. Surface biotinylation procedure was done as described previously (23). In brief: Confluent monolayers were surface biotinylated by a 10-min incubation at 4°C with 1.5 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce) in PBS without calcium or magnesium. The unbound biotin was quenched with 100 mM glycine in PBS, and cells were lysed by rocking for 1 h at 4°C in RIPA buffer supplemented with protease inhibitors (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 0.1% SDS, 1 mM PMSF, 20 mg/ml leupeptin, and 20 mg/ml pepstatin A). Cell debris was removed by centrifugation at 5,000 g for 5 min, 2–10% of the volume was taken as “total protein” sample, and the rest (~700 μl) was incubated overnight at 4°C with 100 μl streptavidin agarose resin slurry (Pierce catalog no. 20353). The beads were then washed and streptavidin-bound proteins were eluted by incubation with SDS sample buffer (cell surface fraction). When indicated, eluted cell surface proteins were subjected to PNGaseF treatment (New England BioLabs) according to manufacturer’s instructions. Total and cell surface proteins were resolved electrophoretically on 7.5% acrylamide Tris glycine gels (Bio-Rad) and blotted onto PVDF membranes (Bio-Rad).

Image acquisition and quantification. Chemiluminescent signal from horseradish peroxidase-conjugated secondary antibodies was generated by Advansta WesternBright ECL reagent and detected by digital imaging charge-coupled device camera (LASS 4000, GE Healthcare). Multiple exposures were taken at linear response range, and saturated images were avoided or disqualified. Quantitative data of the relative expression of the target proteins were generated with ImageJ software (National Institutes of Health, Bethesda, MD). Each band was probed with a corresponding loading control within the same lane. Statistical information was gathered from at least three independent experiments.

Gelatin zymogram. Eighty to ninety percent confluent cells were cultured in serum-free medium for 24 h to eliminate the influence of bovine serum proteases. Conditioned medium was concentrated by Amicon ultra centrifugal filters (Millipore). Samples were mixed with 0.1% SDS loading buffer lacking reducing agents and loaded without denaturing on 7.5% (wt/vol) polyacrylamide SDS gels, containing 0.1% gelatin. After electrophoresis, the gels were soaked in reaction buffer (100 mM Tris-HCl, 200 mM NaCl, 10 mM CaCl₂, pH 8.0) for 12 h at 37°C and then stained with GelCode (Thermo Fisher Scientific). Metalloproteinase proteolytic activity was visualized by clear zones against the dark Coomassie background.

Coimmunoprecipitation. Coimmunoprecipitation (co-IP) assay was performed as previously described (23), with minor modifications. In brief: confluent 100-mm plates were rinsed twice with ice-cold Ca²⁺/Mg²⁺ PBS and solubilized in 2 ml of lysis buffer [1 mg/ml C12E10, 5 mM Tris, pH 7.6, 100 mM NaCl, 5 mM ouabain, 2 mM CaCl₂, 1 mM PMSF, and protease inhibitor cocktail (Sigma)]. Cells were centrifuged at 5,000 g for 5 min at 4°C, and the supernatant, containing solubilized proteins, was precleared for 1 h with agarose G beads (Sigma). These supernatants were then rotated for 4 h at 4°C with the respective antibody, and then with agarose G beads overnight. The bound proteins were eluted in nonreducing SDS sample buffer at 37°C for 10 min, followed by low-speed centrifugation for 1 min to discard
the beads. Proteins were resolved on polyacrylamide Tris-tricine gels together with a sample of total cell lysate and blotted onto PVDF membrane, and Western blot analysis was done with indicated antibodies.

**Adhesion assay and image acquisition.** Cell adhesion assay was done as previously described (23). In brief, 4T1 cells were plated at low density in eight-well chamber plates (μ-slide ibidi, Germany). Time-lapse images of predefined areas were recorded using an Eclipse Ti microscope in an O.KOlab incubator at 37°C with 5% CO₂ and 80% humidity.

**Fluorescent microscopy.** For visualization of cell junction markers and plasma membrane proteins, 4T1-luc cells were cultured in cell chambers (ibidi) and fixed with 3% paraformaldehyde plus 0.5% Triton X-100. These were incubated for 1 h at room temperature with polyclonal antibodies to either β-catenin (1:500) or monoclonal antibodies to β1-Na⁺-K⁺-ATPase (1:100) followed by 3 × 5 min washing in PBS and a 30-min incubation at room temperature with Cy3-coupled goat anti-rabbit/mouse antibody (1:500, Jackson Laboratories). Samples were washed three times in PBS, covered with mounting medium (Immuno-mount, Thermo Scientific), and visualized by confocal microscopy (Olympus).

**RESULTS**

**FXYD5 silencing prevents lung metastasis spread in 4T1 mammary breast cancer model.** To investigate the possible involvement of FXYD5 in carcinogenesis, we have used an in vivo model system based on injection of 4T1 cells into syngeneic mice. 4T1 cells derived from mouse mammary tumor is one of only a few breast cancer models with the capacity to metastasize efficiently to sites affected in human breast cancer. Upon injection into mammary fat pad of syngeneic BALB/c mice, these cells cause a primary tumor that metastasizes first to lung and later to liver, bone, spleen, and brain (17, 44). Similar and faster metastases develop following tail vein injection of these cells. Lung lesions caused by tail vein injection have the same genetic profile as those obtained by orthotopic injection of these cells. Lung lesions caused by tail vein injection of FXYD5-silenced cells than in the wild-type (WT) 4T1-luc cells (Fig. 1, B1 and B2, and Supplemental Videos S1 and S2; Supplemental Material for this article is available at the Journal website). Thus, 4T1 cells behaved in culture as was found in a number of cancer-derived and normal cells studied by us before (23).

In the first set of in vivo experiments, WT and FXYD5-silenced (shFXYD5) 4T1-luc cells were injected into the fat pad of BALB/c mice, and tumor development was followed over time (Fig. 2A1). Four weeks later, tumors of >0.5 cm³ were observed at the point of injection in both groups. Although FXYD5-silenced xenografts exhibited a prolonged initiation period, both groups eventually achieved similar tumor sizes (see growth measurement quantification at Fig. 2A2). However, only FXYD5-expressing tumors exhibited development in lung metastases, which were not seen at all in the
FXYD5 silencing reduces E-cadherin expression and induces the expression of β1-, Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. The results above provide considerable evidence for FXYD5 involvement in metastatic development; however the molecular mechanism underlying its effect is not clear yet. Previously, two mechanisms were suggested. First, FXYD5 promotes metastasis by downregulating E-cadherin, thereby decreasing cell-cell adhesion and permitting expression of a motile phenotype (14). Second, FXYD5 induces metastasis by an E-cadherin-independent CCL2-mediated mechanism. Increased secretion of CCL2 has been correlated with FXYD5 upregulation in several tumor cell lines, and could be a key element in the FXYD5-mediated effect (30). The underlying mechanisms are likely to include the chemoattraction of tumor-promoting leukocytes, and promotion of tumor cell migration and angiogenesis (40, 51). However, the above suggested pathways do not reconcile with the 4T1 model, since these cells do not secrete significant amounts of CCL2, and the relationship between FXYD5 and E-cadherin is opposite to that previously described. E-cadherin is significantly upregulated at the transcriptional level and is highly abundant in the plasma membrane of FXYD5-expressing cells (Fig. 3, A and B). Despite E-cadherin downregulation, FXYD5-silenced cells exhibit the morphology of epithelial cells as was earlier demonstrated in Fig. 1B. The implication of the above results will be addressed in the DISCUSSION section.

To reveal and quantify genes affected by FXYD5 expression, we performed high-throughput cDNA sequencing (RNA-seq) on 4T1-luc WT cells and both FXYD5-silenced clones.
FXYD MEDIATED METASTASIS IN BREAST CANCER MODEL

Fig. 3. Opposite effect of FXYD5 on E-cadherin expression in 4T1 cells. A: Western blot analysis of surface biotinylated fraction with anti E-cadherin. α1-Subunit was used as a loading control (bottom). B: E-cadherin RNAseq analysis. All data summarized from WT 4T1 cells (WT) and two shFXYD5 clones (sh48, sh52). *** P < 0.001. FPKM, fragments per kilobase of transcript per million mapped reads.

(sh48, sh52). The cells were processed as described in MATERIALS AND METHODS. The parameters were set to include only hits that were modified by both FXYD5 shRNAs. The results revealed 35,000 unannotated transcripts; ~700 of them were identified as differentially expressed between WT and FXYD5-silenced cells. β1-Na+K+-ATPase was prominent among the high fold upregulated genes. The Na+K+-ATPase profile, summarized in Table 1, demonstrates the dominance of the α1/β3 isoform, complemented with FXYD5. A small amount of FXYD3 transcripts was also apparent in WT cells, but it was not detectable at the protein level (data not shown). The silencing of FXYD5 did not evoke expression of any other of the FXYD proteins. Nevertheless, it was correlated with significant upregulation of the β1-subunit of Na+K+-ATPase.

We also examined the Na+K+-ATPase expression profile in 4T1 cells using isoform-specific antibodies. The results in Fig. 4A demonstrate that there is no change in α-subunit abundance due to FXYD5 silencing. The dominating isoform in 4T1 cells is α1, as recognized with an α1-specific antibody. However, the β isoform-specific antibodies validated significant β1 upregulation in FXYD5-silenced cells, in place of β3-subunit expressed in WT cells. The fact that there is no correlation between FXYD5 expression and changes in the β3 transcriptional profile as opposed to significant alteration in β3 surface protein expression is not surprising. The 1:1 stoichiometry

Table 1. FXYD5 affects Na-K-ATPase isoform profile in 4T1 cells

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>WT</th>
<th>sh48</th>
<th>sh52</th>
<th>Fold Change, sh48/WT</th>
<th>Fold Change, sh52/WT</th>
<th>P Value</th>
</tr>
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<tr>
<td>Aplα1α1</td>
<td>21,960.3</td>
<td>15,401</td>
<td>18,685</td>
<td>0.70</td>
<td>0.85</td>
<td>&lt;0.0005</td>
</tr>
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<td>0</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aplα1α3</td>
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<td>1</td>
<td>1.33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aplβ1α1</td>
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<td>2,677.66</td>
<td>1,179</td>
<td>31.38</td>
<td>13.82</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Aplβ1β2</td>
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<td>4.33</td>
<td>4.33</td>
<td></td>
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</tr>
<tr>
<td>Aplβ1β3</td>
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<td>3,051.33</td>
<td>3,066.67</td>
<td>2.28</td>
<td>2.29</td>
<td>0.02</td>
</tr>
<tr>
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<td>0</td>
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<tr>
<td>Fxyα1α1</td>
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<td>0</td>
<td>0.67</td>
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<td>Fxyα1α2</td>
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<tr>
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<tr>
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<td>0</td>
<td>0</td>
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<tr>
<td>Fxyβ1β2</td>
<td>1,897.33</td>
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<td>228.67</td>
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<td>3.33</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
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</tbody>
</table>

The numbers represent mRNA mean values and statistical analysis obtained from high-throughput RNA sequencing in 4T1 wild-type (WT) and FXYD5-silenced clones (sh48, sh52). Bolded values, key proteins described in the present study.

Fig. 4. Na+K+-ATPase expression profile in 4T1 cells. A: surface expression of WT 4T1-luc cells (WT) and two shFXYD5 clones (sh48, sh52). All cell types were surface biotinylated. Streptavidin-bound proteins were treated with PNGase F for N-glycan removal, resolved electrophoretically and blotted with isoform-specific antibodies to Na+K+-ATPase (anti-α1 1:2,000, anti-β1 1:4,000, anti-β3 1:1,000). β1 and β3 isoforms are marked by arrows. The figure is a representative experiment repeated at least three times. B: cellular distribution of β1. Left: WT 4T1-luc cells (WT) and shFXYD5 cells (sh48) were grown in glass chambers. The cells were fixed, stained for β1 (1:100), and visualized by confocal microscopy, as described in MATERIALS AND METHODS. Right: transmission images of corresponding cells (marked Tm). Scale bar, 20 μm.
metric ratio between Na\textsuperscript{+}-K\textsuperscript{+}-ATPase subunits does not correlate with free β3 in plasma membrane, which is rapidly internalized. Abundance of β1 was also confirmed by immunocytochemistry (Fig. 4B). The protein staining was observed only in the plasma membranes of FXYD5-silenced 4T1 cells and was mainly localized at the cell-cell contacts areas.

**FXYD5 silencing promotes β1- Na\textsuperscript{+}-K\textsuperscript{+}-ATPase/Annexin A2 interaction and reduces MMP-9 secretion.** The FXYD5-facilitated β1-to-β3 exchange in 4T1 cells led us to search for β-mediated pathways. One mechanism that may link β1 to intracellular processes was suggested by Barwe et al. (2). These authors reported that the adaptor protein Annexin A2 binds to the cytoplasmic NH\textsubscript{2}-terminal of the pump’s β1-subunit. Annexin A2 is a calcium-dependent, phospholipid-binding protein found in various cell types. It is upregulated in various tumor types and plays multiple roles in regulating cellular functions, including angiogenesis, proliferation, apoptosis, cell migration, invasion, and adhesion (for review see ref. 21). Annexin A2 binding to various tumor types and plays multiple roles in regulating cellular functions, including angiogenesis, proliferation, apoptosis, cell migration, invasion, and adhesion (for review see ref. 21). Annexin A2 binding to FXYD5-silenced 4T1 cells was mainly localized at the cell-cell contacts areas. The protein staining was observed only in the plasma membranes of FXYD5-silenced 4T1 cells and was mainly localized at the cell-cell contacts areas.

To verify the involvement of Annexin A2 in FXYD5-mediated signaling events leading to metastatic spread, we silenced it in WT cells with a mix of specific siRNA. The silencing reduced the total Annexin A2 level by ~80% which in turn significantly reduced MMP-9 secretion, confirming a direct relationship between Annexin A2 and MMP-9 in 4T1 cells (Fig. 6A). To establish a chain of events leading from MMP-9 secretion to alteration of cell phenotype, we first analyzed for variations in cell morphology and adhesion. Both 4T1 WT and siAnxA2 cells were seeded at similar concentrations, and the variations in cell morphology and adhesion. Both 4T1 WT and siAnxA2 cells were seeded at similar concentrations, and the rate of adhesion and lamellipodia formation was monitored by Ti microscopy in an OKOlab incubator. The results in Fig. 6B indicate that such polarization occurred at a much higher rate.

![Fig. 5. FXYD5 β isoform regulation effects Annexin A2/MMP-9 axis.](http://ajpcell.physiology.org/)
in the Annexin A2-silenced cells than in WT 4T1-luc cells and was comparable to that observed in both FXYD5 silenced clones. We also injected silenced cells (siAnxA2) intravenously into mouse tail vein and monitored tumor progress for 7 days. Metastasis became apparent in the thoracic regions 7 days after the injection in WT 4T1-injected mice, but to a much lower extent in siAnxA2-injected mice (Fig. 6, C1 and C2).

**DISCUSSION**

Despite advances in the treatment of early stage breast cancer, metastasis remains the most lethal outcome of cancer progression. Metastasis depends on the cancer cells’ ability to undergo several adaptations, and much remains to be discovered about the molecular mechanisms that control this process. FXYD5 has been previously shown to be upregulated in a number of cancerous tumors (for review see ref. 30), and its expression has been correlated with poor prognosis. Several studies have suggested that the underlying mechanism of FXYD5 interaction is either due to downregulation of E-cadherin (14) or due to upregulation of CCL2 chemokine receptor (31). Likewise, both pathways describe transcriptional changes induced by FXYD5 presence and do not propose any direct associations connecting it to metastasis. In fact, the only direct interacting partner reported for FXYD5 until now is with the Na\(^+\)-K\(^+\)-ATPase pump. The current study establishes an in vivo causal relationship between FXYD5 and breast cancer metastasis in mouse 4T1 BALB/c model. The evidence presented in this paper proposes the possibility of FXYD5 involvement in more than one step of metastatic progression. We also suggest the possibility of a novel role of β-Na\(^+\)-K\(^+\)-ATPase as mediator of the FXYD5-dependent mechanism (for summary see Fig. 7).

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Fig. 6. Annexin A2 silencing rescues cells from FXYD5 phenotype. A1: WT 4T1-luc cells were silenced with mixture of Annexin A2-specific siRNA for 48 h. Protein lysates were resolved by electrophoresis and blotted with anti-Annexin A2 (top). Tubulin was used as a loading control (middle). Bottom: gelatin zymogram of secreted fractions from 4T1 WT and Annexin A2-silenced cells. A2: quantification of the Western blots. B: WT 4T1-luc transfected with control mix and siAnxA2 cells were plated. Fields of 100–150 cells were imaged at 30-min intervals over 20 h, and the rate of cell elongation was quantified by determining the percentage of cells in the field that maintain a radial shape as a function of time. Data points are means ± SE of 4 fields for each cell type. C1: WT 4T1-luc transfected with control mix and siAnxA2 (10\(^6\)) cells were injected into the tail vein of a normal female BALB/c mouse. Images were taken after 7 days. The relationship between color and light intensity in arbitrary units (counts) for the whole animal images is given by the color bar at the right side of the figure. C2: quantification of results from 3 independent experiments ± SE (4–5 animals per treatment in each experiment), ***P < 0.001.

Fig. 7. Proposed mechanism for FXYD5 involvement in metastatic development in 4T1 cells. 1. FXYD5-mediated substitution of β1 to β3 completely abolishes epithelial behavior and promotes a more motile phenotype, which promotes metastasis. 2. FXYD5-mediated replacement of β1 to β3 allows Annexin A2 secretion, which in turn promotes MMP-9 activation, ECM degradation, and metastatic spread. 3. FXYD5 presence in 4T1 is coupled to E-cadherin expression which promotes MET and enables colony establishment in distant epithelial tissues.
FXYD5 expression promotes metastasis in 4T1 breast cancer model. We have reported previously that FXYD5 affects cell adhesion and motility by modifying the anterior-posterior polarity of newly adhered cells (23). Consistent with these findings, our present work in 4T1 cells shows that the above phenotype is in good correlation with metastasis progression in FXYD5-expressing cells. Metastatic burden in mice lungs, injected with FXYD5 silenced 4T1 cells, is dramatically lower compared with those injected with WT 4T1-luc cells, either injected directly into bloodstream or developed spontaneously from primary tumor site. Thus, extravasation, attachment to the lung ECM, and/or survival in this tissue appear to be key factors in the FXYD5-dependent metastasis. However, we cannot rule out FXYD5 involvement in initial stages of metastatic development. The primary tumor growth was not as radically changed by FXYD5 knockdown, which is in agreement with the fact that FXYD5 has no effect on cell proliferation (22, 23). Interestingly, the kinetics of tumor growth is different between 4T1-luc WT and FXYD5-silenced cells. The initial period of lump development in FXYD5-silenced xenograft is slower than with WT 4T1-luc cells. Recently, it was recognized that tumor microenvironment is an essential ingredient in cancer malignancy (11). Therefore one possible explanation for this behavior is that FXYD5 might have an inductive effect on the host compartment that encourages growth and proliferation. Further investigation is required to establish whether it is a real effect that occurs during spontaneous cancer development in mammary tissue, or an artifact of experimental implantation of 4T1 cells.

FXYD5 upregulates E-cadherin and induces metastasis by promoting mesenchymal-epithelial transition. Several studies proposed FXYD5 as an inducer of epithelial-mesenchymal transition (EMT), since its expression is associated with a loss of normal epithelial architecture, namely loose cell-cell contacts, reduced single cell polarization, and increased motility (19, 22, 23). The above morphological changes have also been confirmed at the molecular level. In M1 cells, FXYD5 overexpression is associated with downregulation of ZO-1, which is often linked to EMT (9, 22). In murine airway epithelial cells, FXYD5 expression is accompanied with an increase of the mesenchymal marker vimentin (12, 16, 28). Finally, it has been shown that FXYD5 is inversely correlated to E-cadherin, which is considered to be central to EMT (14, 16).

Unexpectedly, in the current study we observe the opposite. Silencing of FXYD5 results in significant downregulation of E-cadherin mRNA and surface-expressed protein. This is not an isolated event and is also accompanied with downregulation of E-cadherin-inducing transcription marker. Despite E-cadherin loss, the FXYD5-silenced cells do not manifest any metastatic ability.

Interestingly, a similar phenomenon was reported for the human MDA-MB-231 breast carcinoma cell line. The lung metastases, developed from highly aggressive and E-cadherin-negative orthotopic mammary fat pad tumors, were shown to express E-cadherin (50). In agreement, a number of additional reports related E-cadherin membrane staining in the metastases but not in the primary carcinoma sites (13, 18, 33, 38). These data provide significant evidence that carcinoma cells may reconstitute E-cadherin expression in later stages to establish colonization within distant epithelial tissue. Just as the loss of E-cadherin is considered to be a central event in EMT, the reexpression of E-cadherin has been proposed to be the important hallmark of MET (50). The results in this paper support the notion that some level of E-cadherin is necessary for metastatic progression and provide experimental evidence that this mechanism might be mediated by FXYD5.

Although we cannot rule out FXYD5 effect on E-cadherin during EMT, it is not manifested in the 4T1 breast cancer model. EMT is critical to this initial escape by enabling individual cell migration and invasion through tissue barriers (49). Therefore, in principle, the metastatic potential of FXYD5-silenced 4T1 cells should have been restored by bypassing the primary EMT events and introducing the cells directly into the bloodstream. However, this is not the case here, emphasizing that FXYD5 has more extensive effects on additional stages of the metastatic process.

FXYD5 metastatic effect may be mediated through β-Na⁺-K⁺-ATPase. Despite the lack of E-cadherin, FXYD5-silenced 4T1 cells exhibit restoration of epithelial phenotype. To our knowledge, no other cadherin family protein is upregulated in FXYD5-silenced cells. Therefore, an alternative mechanism, which might compensate for E-cadherin absence, is required.

Recently, it has been demonstrated that intercellular β1-β1 interaction is important for initiation and maintenance of adherent junctions in epithelial cells (47). Subsequently, a unique β1 trans-dimerization domain was identified, which led to the conclusion that these transcellular β1-β1 bridges can be sustained only between two identical β1 isoforms (45, 46). Interestingly, the Na⁺-K⁺-ATPase profile in WT 4T1 cells displays the dominance of the β3 isofrom, as demonstrated by both mRNA and protein. However marked upregulation of β1 is noticed following FXYD5 silencing. The data on the β3-subunit are rather limited, probably owing to its low abundance and difficulty in detection. It has been found primarily in rat lung and testes, and its function has been mainly characterized in the context of pump kinetics (1, 15). In contrast, a loss of β1 has been previously proposed as a cancer-promoting mechanism in kidney carcinoma culture cells and in patients’ tumor samples (34).

In this study, we have suggested the possibility that downregulation of the Na⁺-K⁺-ATPase β1-subunit is a part of the FXYD5-mediated mechanism to promote tumor metastasis. Previously, FXYD5 has been shown to modify the glycosylation status of the Na-K-ATPase β1-subunit (22, 24, 25). Since sugar moieties are important for β1-β1 bridge formation, it has been proposed as a mechanism to weaken cell-cell contacts and impair epithelial barrier function (22). The current study suggests an additional, FXYD5-dependent regulation level of β1. FXYD5-mediated substitution of β1 to β3 could abolish cell-cell junctions and promote a more motile phenotype. Therefore we suggest that FXYD5-dependent loss of β1 in 4T1 cells may be equivalent to FXYD5-mediated downregulation of E-cadherin in other cell systems.

FXYD5-mediated β-isoform selectivity promotes metastasis through activation of Annexin A2/MMP-9 pathway. Cellular adhesion and motility have also been shown to be affected by intracellular β1-Na-K-ATPase interaction. β1 association with the adaptor protein Annexin A2, through β1 intracellular domain, was proposed to reduce cell motility through activation of Rac1/PI3K pathway (2).

Annexin A2 is a well-established player in cancer progression and metastatic development. Interestingly, its prevalence
in association with cancer is very similar to the one reported for FXYD5. Many studies have reported increased expression of Annexin A2 in cancer compared with normal tissues. The upregulation of Annexin A2 expression in pancreatic, colorectal, and brain tumors has been directly correlated with advanced clinical stage. In addition, higher Annexin A2 expression has been observed in metastatic breast cancer and colon cancer cells as compared with nonmetastatic cells (for review see ref. 21). Secretion of Annexin A2 has been also reported for various tumor types (5, 52). Annexin A2 promotes metastasis by colocalization with its binding proteins, which in turn facilitates the proteolytic cascade leading to the activation of MMPs and selective degradation of ECM components (for review see ref. 26).

In the current study we have shown that expression of FXYD5 in 4T1 cells induces secretion of Annexin A2, without affecting its total protein level or mRNA. Modification in Annexin A2 subcellular localization has been correlated with increased MMP-9 secretion (for review see ref. 21). The metalloproteinase MMP-9 is associated with the malignant phenotype of tumor cells because of their unique ability to degrade components of the basement membrane (32). We established that silencing of Annexin A2 significantly inhibited metastatic spread in lung tissue, when injected into mouse tail vein. These data place Annexin A2 downstream to the mechanism of FXYD5. The balance between FXYD5 and annexin II by a radioimmunoassay. J Immunol Methods 188: 81–89, 1995.

REFERENCES


