

Communication

Extracellular vesicle integrins distinguish unique cancers

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Abstract: The proteomic profile of extracellular vesicles (EVs) has been of increasing interest, particularly in understanding cancer growth, drug resistance, and metastatic behavior. Emerging data suggests that cancer-derived EVs carry an array of oncogenic cargo, including certain integrin proteins that may, in turn, promote cell detachment, migration, and selection of future metastatic sites. We previously reported a large comparison of secreted vesicle protein cargo across sixty diverse human cancer cell lines. Here, we analyze the distinct integrin profiles of these cancer EVs. We further demonstrate the enrichment of integrin receptors in breast cancer EVs compared to vesicles secreted from benign breast epithelial cells. Total EV integrin levels, including the quantity of integrins $\alpha 2$, αv , $\beta 4$, and $\beta 5$ correlate with breast tumor stage. In particular, integrin $\alpha 2$ also largely reflects progenitor cell expression, highlighting the utility of this integrin protein as a potential circulating biomarker of primary tumors. This study provides preliminary evidence of the value of vesicle-associated integrin proteins in cancer diagnosis and prediction of tumor stage. Differential expression of integrins across cancer cells, and selective packaging of integrins into EVs may contribute to further understanding the development and progression of tumor growth and metastasis across a variety of cancer types.

Keywords (3-10): Exosomes, mass spectrometry, proteomics, biomarkers, cancer, extracellular vesicles, microvesicles, oncosomes

1. Introduction

Integrins are well-conserved ubiquitous cell adhesion receptors that play important roles in extracellular matrix attachment and signal transduction, contributing to pathways involved in cell growth, survival, proliferation, and migration. Functional integrin molecules are heterodimers composed of two integral membrane glycoprotein subunits, one α and one β , held together by disulfide bonds. Serving as an extracellular matrix linker, the globular head of an integrin protein projects extracellularly, while the C-terminal tails of both subunits anchor actin cytoskeleton components inside the cell [1-9]. In vertebrates, the integrin family comprises 18 α subunits and 8 β subunits that can assemble into 24 heterodimers with various ligand-binding properties [3]. While a variety of ligands bind integrin receptors, non-collagenous matrix proteins containing the arginine-glycine-aspartate (RGD) sequence, including fibronectin, laminin, and vitronectin serve as major extracellular ligands for integrin molecules [4, 10]. The majority of integrin cytoplasmic tails bind anchor proteins such as talin, α -actinin, and filamin which connect bundles of actin filaments. The ability of integrins to bind both intra- and extra- cellular proteins facilitates transmission of force across the plasma membrane, and also contributes to their unique bidirectional signaling properties [11-15]. "Outside-in" integrin signaling describes the sequence of extracellular ligand binding and subsequent activation of intracellular cytoskeletal components as well as various protein enzymes, including focal adhesion kinase (FAK), Src-family kinases, small GTPases Ras and Rho, ABL-family kinases, and integrin-linked kinase (ILK) [16]. The mitogen activated protein kinase (MAPK)/

extracellular-signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) pathways are activated through these transduction mechanisms, providing integrin-dependent regulation of cell cycle and proliferation [17]. The FAK-Src complex also facilitates cell migration by disassembling focal adhesion complexes at the trailing edge of the cell [18]. Furthermore, “inside-out” signaling occurs following cytokine or chemokine activation of G-protein coupled receptors (GPCRs) which leads to integrin activation, assembly, and increased extracellular ligand affinity [3, 19].

The apparent multifaceted role of integrins in cell growth and migration has generated great interest in the contribution of this protein family to cancer progression. Accumulating evidence suggests integrin signaling plays multiple roles in the stepwise progression of cancer, including tumor growth, detachment, angiogenesis, intravasation, homing and extravasation, and finally the expansion of metastases [20-29]. It is believed that these mechanisms of cancer progression may be, in part, regulated by integrin-switching. In these scenarios, integrins that promote cell adherence and quiescence are downregulated, while those that foster the breakdown of cell adhesions and remodeling of the ECM may be simultaneously overexpressed [20, 30].

It has been well understood that cellular integrin trafficking occurs through constitutive caveolin- or clathrin- mediated endocytosis into endosomes for sorting, degradation, or recycling [31]. Integrin localization to endosomes may also be important for FAK signaling [32]. More recently, integrin secretion into small 40-200 nm extracellular vesicles (EVs), including exosomes and small microvesicles, secreted from cells has been described [33-41]. The roles of extracellular vesicles in multiple steps of cancer progression has also been of great interest [42]. In a seminal study published by Hoshino and colleagues in 2015, tumor-derived EVs were demonstrated to harbor integrins that were instrumental in preparing a pre-metastatic tumor niche, and indeed guided organ-specific metastasis based on EV-cell tropism [38]. Vesicles containing integrins $\alpha 6\beta 1$ and $\alpha 6\beta 4$ were found to be taken up specifically by lung fibroblasts and epithelial cells and were associated with lung metastasis of progenitor cancer cells. In contrast, vesicle $\alpha v\beta 5$ directed uptake of EVs by liver Kupffer cells and promoted liver metastasis. Small peptide inhibition of these respective integrin receptors significantly reduced cancer metastasis to distant sites [38]. In another study, prostate cancer EVs were shown to transfer integrin $\alpha v\beta 6$ to non-tumorigenic cells, resulting in increased migratory capacity of the recipient cells [37]. Similarly, EVs carrying $\alpha v\beta 3$ from prostate cancer cells have been observed to increase cell migration of surrounding recipient cells [35, 36]. In the context of viral tumorigenesis, increased levels of integrin subunits $\alpha 4$, αL , and $\beta 3$ were demonstrated to be secreted into EVs following infection with human tumor virus Epstein-Barr virus (EBV); while $\alpha 3$, $\alpha 6$, and $\beta 1$ integrins were decreased in EVs from EBV or Kaposi sarcoma herpesvirus infected cells [39]. Finally, persistent EV secretion of integrin subunits, such as $\beta 4$ in the setting of prostate carcinoma, may also be useful in predicting chemotherapy-resistant cells [34].

Altogether, a growing body of evidence suggests that integrins secreted into cancer EVs may confer invasive or migratory phenotypes to naïve surrounding cells, and can direct organotropic metastasis by site-specific uptake and microenvironment modification. Furthermore, EV integrins offer novel targets as biomarkers for cancer progression. In a previous study, we conducted a large scale proteomic analysis of extracellular vesicle cargo secreted from a panel of sixty human cancer cells [40]. Here we further analyze the EV integrin profiles from these cancer cells. We additionally compare the EV proteomes of breast cancer cells to that of a benign breast epithelial cell line, highlighting the overexpression of many integrin subunits secreted from tumorigenic cells. Finally, levels of several vesicle-associated integrins correlate with increasing tumor stage and reflect cellular levels. These findings support the utility of circulating integrins as potential cancer cell biomarkers, and emphasize the functional roles these proteins play in stepwise cancer progression.

2. Materials and Methods

2.1 Cell culture

Sixty cell lines from the National Cancer Institute (NCI-60) were acquired and cultured, as previously described [40]. MCF10a cells were grown using the Mammary Epithelial Cell Growth

Medium BulletKit (Lonza, CC-3150) comprised of the basal medium MEBM supplemented with the provided aliquots of bovine pituitary extract (BPE), hEGF, insulin, and hydrocortisone. Instead of the GA-1000 aliquot provided in the kit, 100 ng/mL of cholera toxin was added to the medium, as recommended by ATCC. At 90% confluence, complete medium was aspirated and cells were washed with warm sterile phosphate buffered saline (PBS). To minimize contaminating proteins, cells were grown in BPE-free medium for another 48 hours before EV enrichment.

2.2 Extracellular vesicle enrichment and protein quantification

NCI-60 and MCF10a EVs were processed as previously described in great detail [40, 43]. The efficacy and purity of samples has been demonstrated numerous times through nanoparticle tracking, immunoblot analysis, and electron microscopy by our laboratory [43-47]. Briefly, serum-free cell-conditioned medium was aspirated from cell culture plates in three biological replicates, and centrifuged at 500 g for 5 minutes, then at 2,000 g for 30 minutes before incubation overnight with a 1:1 volume of 2X PEG solution [16% (w/v) polyethylene glycol, 1 M NaCl]. Following the polyethylene glycol incubation, samples were centrifuged at 3,214 g for 60 minutes, and pellets were resuspended in PBS for an ultracentrifugation purification step (100,000 g for 70 minutes). Final pellets were lysed in strong lysis buffer [5% SDS, 10 mM EDTA, 120 mM Tris-HCl pH 6.8, 2.5% β -mercaptoethanol, 8 M urea] with HALT protease inhibitor (Thermo, 78438) added. Vesicle protein quantification was performed using the fluorescence-based EZQ™ Kit (Thermo, R33200) according to manufacturer's instructions.

2.3 SDS-PAGE and in-gel digestion

NCI-60 EV protein was further processed as previously reported [40]. Similarly in this study, 20 μ g of MCF10a EV protein was loaded into a 4-20% polyacrylamide gel (Lonza, 59511) for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), gel fixation, and Coomassie staining as described in detail [48]. Gel lanes were fractionated into five sections and then subdivided into 1 mm³ cubes for trypsin-digestion [43].

2.4 Mass spectrometry

Mass spectrometry and protein identification of NCI-60 EV cargo was reported in our previous study [40]. Additional analysis of NCI-60 integrin proteins was performed using Supplementary Table 1 published in the prior study. Trypsin-digested MCF10a EV protein samples were submitted to the Florida State University Translational Science Laboratory for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis, consistent with the protocol and parameters used previously for NCI-60 cell-derived EVs [40]. The same Thermo LTQ Orbitrap Velos nLC-ESI-LTQ-Orbitrap (high-resolution electrospray tandem mass spectrometer) was used in this study to analyze MCF10a EV protein. Raw peptide data each of the five fractions of MCF10a samples were pooled and analyzed in Scaffold software. Data was analyzed using three search engine databases (MS-Amanda Proteome Discoverer, Mascot, and Sequest) and a recent UniProt knowledgebase reviewed (Swiss-prot) human protein database. Fragment tolerance was set to 0.80 Da (monoisotopic). Fixed modifications included only carbamidomethyl (C), and variable modifications included oxidation (M), N-terminal acetylation, and phosphorylation (STY). Digestion mode was set specific for trypsin, with a maximum of two missed cleavages. False discovery rate was set to 0.01. MCF10a EV data was averaged amongst replicates, then normalized to previously analyzed NCI-60 EV protein by multiplying integrin spectral counts by a calculated normalization factor (average total spectral counts per sample across all benign and cancer breast EVs divided by the total spectral counts identified in each individual sample). Whole cell protein data published by Gholami et al. [49] was previously normalized across the NCI-60 panel [40] and integrin expression was re-analyzed in this study.

2.5 Protein enrichment analyses

Differential enrichment of biological processes between proteins identified in MCF10a EVs and proteins identified in all six breast cancer EV samples was compared using FunRich v3 [50, 51]. All

terms with an adjusted *p*-value less than 0.5 were determined to be significant. Displayed results reflect the processes containing the greatest percentage of proteins from each dataset. Figures were constructed using Microsoft Excel and CorelDraw X5 software.

3. Results

3.1. Extracellular vesicle integrin profiling across the NCI-60 panel

In a prior study, we reported a comprehensive comparison of extracellular vesicle proteins secreted from sixty human cancer cells, highlighting both common and differentially expressed cargo [40]. Here, we closely analyze the integrin profiles of EVs harvested across the panel of cancer cells. As we previously highlighted [40], integrin $\beta 1$ was present in varying levels within EVs secreted from all cells, while other integrins were more selectively expressed (Figure 1A and Figures S1 and S2). For instance, integrins $\alpha 2$, $\alpha 3$, $\alpha 6$, and αv were generally secreted into EVs from solid tumors. On the other hand, $\beta 2$ integrin subunits are present exclusively on the surface of leukocytes and were likewise found only to be secreted along with αL into EVs from leukemia cells in this study. Subunits $\alpha 2$, $\alpha 3$, $\alpha 6$, αv , $\beta 1$, and $\beta 4$ comprised the vast majority of integrins secreted into cancer EVs (Figure 1B). Integrin $\beta 1$ has been previously proposed to be more highly expressed in vesicles secreted from cancer cells than non-tumorigenic cells [38]. Because the $\beta 1$ and $\beta 4$ subunit encompassed the chief beta subunits identified in cancer EVs, commonly secreted alpha subunits were compared to beta subunit secretion. Spectral counts of integrins $\alpha 2$, $\alpha 3$, $\alpha 6$, and αv were plotted against integrin $\beta 1$ and $\beta 4$ spectral counts across the NCI-60 EVs (Figure S3). Integrin $\alpha 3$ was found to be most often secreted in a one-to-one ratio with the $\beta 1$ subunit (Figure 1C). This positive correlation and ratio highly suggests the abundance of integrin $\alpha 3\beta 1$ secreted into cancer EVs.

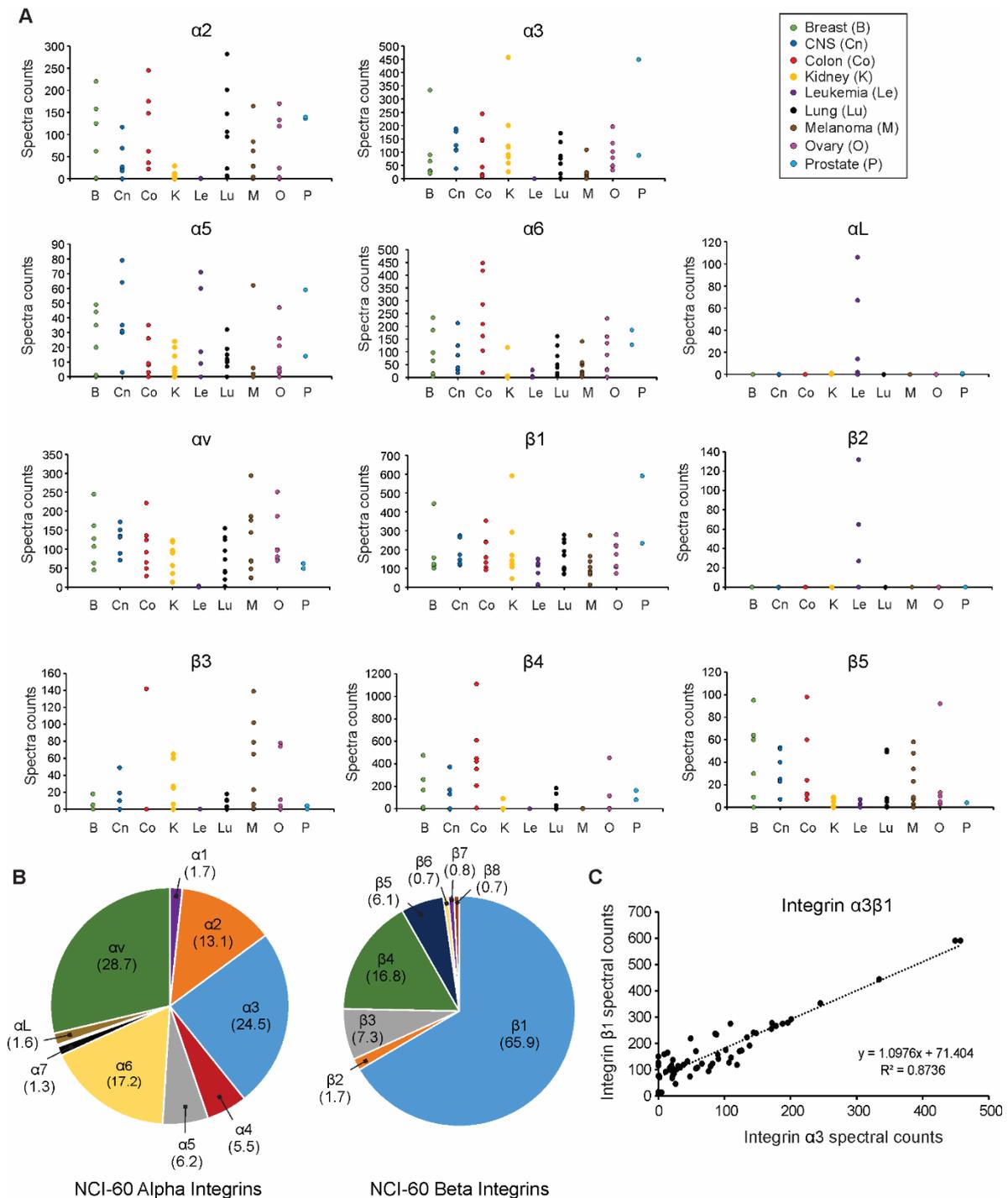


Figure 1. Tumor extracellular vesicles display distinct integrin profiles. (a) Spectral counts of alpha and beta integrin subunits secreted into tumor EVs were compared across the NCI-60 panel of human cancer cells. (b) Breakdown of EV alpha and beta integrin subunit composition (percentages) averaged across the NCI-60 panel. (c) Direct comparison of vesicular $\alpha 3$ and $\beta 1$ protein spectral counts secreted by each of the 60 cancer cell lines.

3.2 Integrin $\alpha 2$ reflects progenitor cell expression

In our previous study, we also reported that many proteins secreted into EVs reflect their progenitor cell levels, suggesting the utility of vesicle cargo as cellular biomarkers [40]. Here, normalized spectral counts of abundant integrin subunits secreted into breast cancer EVs were compared to whole cell levels previously published [49] (Figure 2). Integrin $\alpha 2$ levels in vesicles were noted to correlate highly with respective progenitor cell levels, in a nearly 1:1 ratio. Integrin $\beta 4$

also demonstrated a positive correlation between cell and vesicle expression, though notably with a lower coefficient of determination. Interestingly, integrin αv demonstrated a strongly negative correlation between cell and vesicle protein levels, perhaps suggesting a more selective packaging of the protein into EVs.

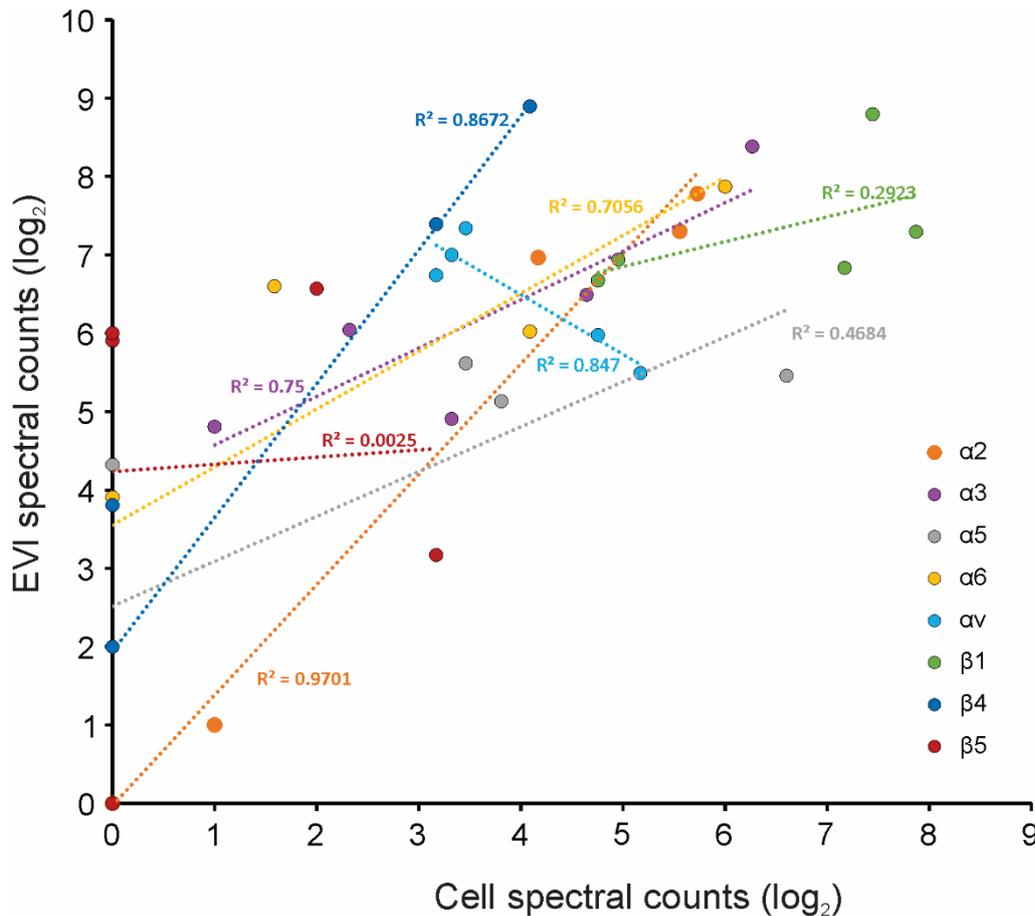


Figure 2. Vesicle-associated integrin $\alpha 2$ best represents progenitor cell expression across breast cancer lines. Spectral counts of most abundant alpha integrin subunits in breast cancer-derived EVs compared to respective whole cell spectral counts previously reported.

3.3 Integrin expression differs in cancer cell-derived EVs compared to benign EVs.

Extracellular vesicles from the benign breast epithelial cell line MCF10a were harvested and similarly purified for mass spectrometry analysis (Figure 3A). Proteins common to all six breast cancer-derived EVs were compared to those found in MCF10a EVs (Figure 3B). Significant overlap between common cancer and benign EV proteins was seen, although a number of proteins appeared to be present or absent only in breast cancer cell-derived EVs. In unbiased enrichment analyses, proteins enriched in breast cancer EVs were involved in biological processes including integrin surface interactions, $\beta 1$ integrin interactions, and syndecan-mediated signaling (Figure 3C). Interestingly, normalized spectral count comparison of integrin subunits across breast cell-derived EVs demonstrated significant overexpression of integrins present in cancer EVs (Figure 3D). Only integrins $\alpha 1$, $\alpha 6$, αv , and $\beta 1$ were identified in benign breast EVs. Notably, integrin $\alpha 1$ appeared to be present in higher levels in benign breast EVs than most cancer EVs. The breakdown of common alpha and beta integrin subunits found in breast cancer EVs closely resembled the general profile of cancer EV integrins (Figure 1B and 3E). While integrin $\beta 1$ remained the predominant beta subunit in benign breast EVs, integrin $\alpha 1$ comprised the majority of alpha subunits (Figure 3F). Integrins $\alpha 2$, $\alpha 3$, and $\alpha 5$ were not present in benign EVs compared to cancer EVs. Differences in these expression

profiles suggest that “integrin switching” may be reflected in vesicles secreted from progenitor cancer cells.

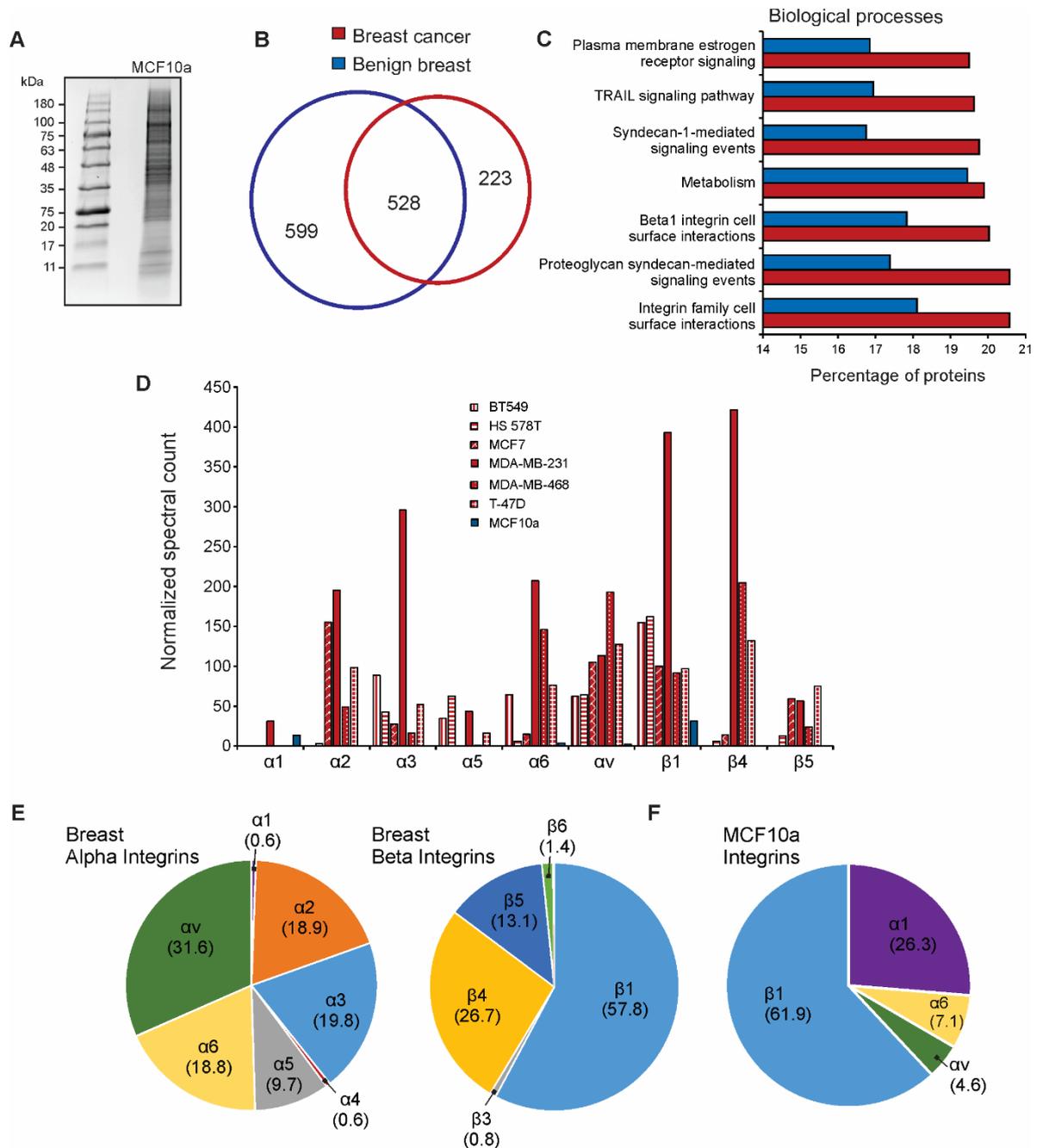


Figure 3. Breast cancer EV integrin profiles differ from benign breast cell-derived vesicles. (a) Coomassie-stained gel purification of MCF10a EV proteins. (b) Overlap of total vesicle proteins identified by mass spectrometry from MCF10a cells (benign breast) compared to those identified in all six breast cancer cell lines in the NCI-60 panel. (c) Enrichment analysis of proteins identified in benign breast EVs versus breast cancer cell-derived EVs. (d) Spectral count comparison of most abundant integrin subunits secreted by benign (blue) or tumor (red) breast cells. (e) Breakdown of EV alpha and beta integrin subunit composition (percentage of total alpha or beta proteins, respectively) secreted from breast cancer cells. (f) Vesicle composition of integrin subunits secreted by MCF10a breast epithelial cells (percentage of total integrins identified in samples).

3.4 EV integrin levels predict breast cancer stage.

Finally, breast cancer lines were categorized by relative tumor stage according to clinical information provided by the American Type Culture Collection (ATCC) database. In this study, benign MCF10a cells were classified as 0, while stage 1 denoted non-metastatic cells (HS578T), stage 2 denoted tumors that had spread to regional lymph nodes (BT549), and stage 3 included those with documented metastasis to distant organs (MCF7, MDA-MB-231, MDA-MB-468, and T47D). Total spectral counts of all integrin proteins identified in breast cell-derived EVs were seen to increase with tumor staging (Figure 4). In particular, higher levels of integrins $\alpha 2$, αv , $\beta 4$, and $\beta 5$ were secreted from more aggressive progenitor cancer cells. These preliminary findings demonstrate the potential utility of circulating integrin proteins to detect early tumors and predict more aggressive cancers.

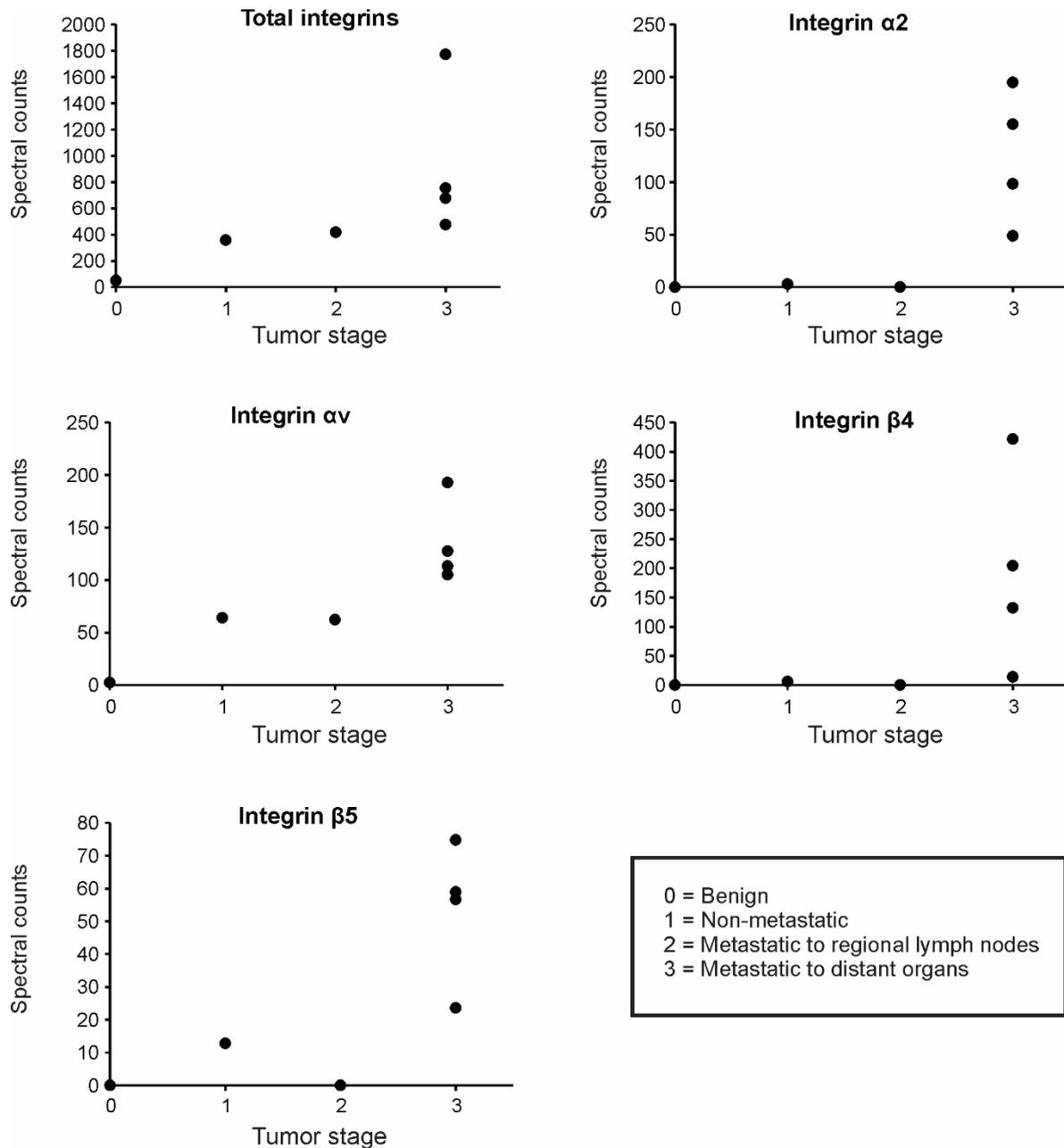


Figure 4. Breast cancer stage correlates with EV integrin levels. Spectral counts of representative vesicle integrin subunits compared across benign and tumorigenic breast lines. Tumor stage was categorized based on clinical information provided by the American Type Culture Collection (ATCC) database.

4. Discussion

Generating increasing excitement across many scientific fields, the trafficking patterns and content of extracellular vesicles have shed recent light onto mechanisms of cancer growth and metastasis. In this study, we focus on the differential expression and packaging of integrin proteins into EVs from a variety of cancer cell types. While EV integrin content may serve as a unique circulating fingerprint representing an underlying malignancy, several integrin proteins may be similarly packaged into many cancer-derived EVs. Here, we highlight integrin subunit $\beta 1$ as commonly secreted into the majority of EVs. We further show in cancer cell-derived EVs, that the $\beta 1$ subunit likely couples to a high degree with the $\alpha 3$ subunit to form a functional integrin protein heterodimer. Interestingly, the $\alpha 3\beta 1$ integrin has been previously demonstrated to preferentially bind laminins involved in extracellular matrix assembly [52, 53], and decreased expression has been noted in many epithelial cancer cells [23, 54-59]. Interaction of membrane-bound $\alpha 3\beta 1$ integrin with the adjacent extracellular matrix may serve to inhibit cellular invasion through outside-in signal transduction. Indeed, increased expression of several oncoproteins, including n-myc and c-myc have been found to be associated with decreased cellular $\alpha 3\beta 1$ integrin levels [60-62]. Given that $\alpha 3\beta 1$ integrin is known to interact with several tetraspanin proteins including CD63 and CD81 [63], it is possible that EV secretion of $\alpha 3\beta 1$ integrin may serve to decrease intracellular levels, thereby facilitating downstream oncogenic pathways. We have previously demonstrated CD63-dependent protein secretion into EVs as a major mechanism to regulate intracellular signaling activity in this manner [45, 47].

In this study, we also identified vesicle-associated integrin subunit $\alpha 2$ as highly correlated with cellular levels, suggesting the utility of this protein as a representative circulating biomarker. The $\alpha 2$ subunit has previously been proposed as a marker of advancing colorectal cancer [64], and has been additionally implicated in prostate cancer metastasis to bone [65]. Our findings here support the potential utility of EV-associated $\alpha 2$ protein as a marker of advancing breast cancer and reflective of cellular expression. Excitingly, several integrins appeared to be present in accumulating levels as breast cancer stage increased, including $\alpha 2$, αv , $\beta 4$, and $\beta 5$. Further investigation is clearly warranted to assess the clinical application of these vesicle integrins in the context of breast cancer diagnosis. Certainly future study of the unique EV integrin fingerprints from varying cancer types highlighted in this study may facilitate further application to diagnosing malignancies in clinical settings.

Finally, although beyond the scope of this study, our findings here present an opportunity to further explore the roles of vesicle integrins in cancer metastasis and tissue targeting. As mentioned above, significant work from the Lyden lab has provided evidence that EV integrins may guide tissue-specific metastases into lung or liver microenvironments [38, 66]. Here we expand on the proteomic analysis of cancer cell-derived EV integrins, creating a profile across sixty unique cancer cells with a propensity to seed to various metastatic sites. We also compare vesicle integrin expression between benign and tumorigenic breast cells, demonstrating increased integrin secretion and integrin-switching in cancer EVs that suggests a role of these vesicle proteins in cancer progression. Altogether, the analyses conducted in this study provide evidence of widespread differences in integrin secretion across various cancer types and further signify changes in breast cancer EV integrins compared to those secreted from benign breast cells. Given the growing body of evidence surrounding the roles of integrin proteins in the stepwise progression of cancer and the abundance of integrins present in cancer EVs, recognition and elucidation of differential integrin secretion from cancer cells may deliver novel means to understand global mechanisms of tumorigenesis and metastasis. Furthermore, future investigations into integrin-directed EV uptake will be essential to facilitating innovative vesicle engineering for targeted or therapeutic delivery of cargo.

Supplementary Materials: Table S1: Proteins identified in mass spectrometry analysis of MCF10a cell-derived EVs. Figure S1: Alpha integrin subunit expression secreted into EVs across the NCI-60 panel of cancer cells,

Figure S2: Beta integrin subunit expression secreted into EVs across the NCI-60 panel of cancer cells, Figure S3: Relation of most abundant alpha and beta integrin ratios secreted into cancer-cell derived vesicles.

Author Contributions: Conceptualization, S.N.H. and D.G.M.; validation, S.N.H.; formal analysis, S.N.H.; investigation, S.N.H.; resources, D.G.M.; data curation, S.N.H.; writing—original draft preparation, S.N.H.; writing—review and editing, S.N.H. and D.G.M.; supervision, D.G.M.; funding acquisition, D.G.M.

Funding: This research was funded by grants awarded to D.G.M. from the Florida Department of Health (4BB05) and the National Cancer Institute of the National Institutes of Health (R01CA204621).

Acknowledgments: We thank Xia Liu, Rakesh Singh, and the Florida State University Translational Science Laboratory for help in the mass spectrometry conducted in this study.

Conflicts of Interest: The authors declare no conflict of interest.

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