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Quantitative analysis of the secretome of TGF- β signaling-deficient mammary fibroblasts

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Abstract

Transforming growth factor β (TGF- β) is a master regulator of autocrine and paracrine signaling pathways between a tumor and its microenvironment. Decreased expression of TGF- β type II receptor (T β RII) in stromal cells is associated with increased tumor metastasis and shorter patient survival. In this study, SILAC quantitative proteomics was used to identify differentially externalized proteins in the conditioned media from the mammary fibroblasts with or without intact T β RII. Over 1000 proteins were identified and their relative differential levels were quantified. Immunoassays were used to further validate identification and quantification of the proteomic results. Differential expression was detected for various extracellular proteins, including proteases and their inhibitors, growth factors, cytokines, and extracellular matrix proteins. CXCL10, a cytokine found to be up-regulated in the T β RII knockout mammary fibroblasts, is shown to directly stimulate breast tumor cell proliferation and migration. Overall, this study revealed hundreds of specific extracellular protein changes modulated by deletion of T β RII in mammary fibroblasts, which may play important roles in the tumor microenvironment. These results warrant further investigation into the effects of inhibiting the TGF- β signaling pathway in fibroblasts because systemic inhibition of TGF- β signaling pathways is being considered as a potential cancer therapy.

Keywords

Cell biology; Fibroblasts; Secretome; Stable isotope labeling with amino acids in cell culture; TGF- β type II receptor

1 Introduction

The reciprocal effects of the tumor–host interaction are critical for tumor growth, invasion, and metastasis [1, 2]. During tumorigenesis, stromal cells are thought to be stimulated by adjacent carcinoma cells. In response, these stromal cells show accelerated proliferation, increased angiogenesis, altered extracellular matrix (ECM) deposition, and amplified inflammatory cell recruitment [3]. Further evidence shows that stromal cells have important roles in cancer cell proliferation and invasion. Stromal fibroblasts can trigger both proteolytic and structural modification of the ECM to create tracks that permit the invasion of following carcinoma cells [4]. When non-transformed mammary epithelial cells are transplanted into mouse fat pads containing fibroblasts that have been exposed to a radiation dose causing sub-lethal DNA damage, an increase in mammary cancer incidence was observed, when compared with transplantation of similar epithelial cells into mouse fat pads with non-radiated fibroblasts [5]. Studies also suggest that genetic alterations in stromal cells can contribute significantly to epithelial cell tumorigenesis and invasion by altering the interactions between tumor epithelial cells and the microenvironment [6, 7]. Furthermore, gene expression signatures of the stromal response to tumor invasion may predict prostate and breast cancer patient survival [8].

Transforming growth factor- β (TGF- β) is a master regulator of paracrine signaling pathways used by tumor cells and surrounding fibroblasts to influence the biological behaviors of tumor initiation, progression, and metastasis [9, 10]. The TGF- β type II receptor (T β RII) is required for all TGF- β intracellular signaling and cells lacking the receptor cannot respond to TGF- β [11]. Mice with the T β RII knockout specifically in stromal fibroblasts (Tgfr2^{fspKO} mice) developed prostatic intraepithelial neoplasia and invasive squamous cell carcinoma of the forestomach [12, 13]. Tgfr2^{fspKO} mammary fibroblasts co-grafted with polyomavirus middle T antigen (PyVmt) mammary carcinoma cells or 4T1 mammary carcinoma cells demonstrated increased tumor growth and metastasis, compared with carcinoma cells co-grafted with floxed control fibroblast cells (Tgfr2^{flox/flox}) [14, 15]. Reductions in T β RII in human colon tumor-associated stroma are also associated with increased lymph node metastasis and shorter patient survival [16]. These results, in combination with other studies, suggest that stromal fibroblasts significantly contribute to the regulation of the adjacent epithelium. Further investigation of the molecular mechanisms related to TGF- β mediated stromal-epithelial interactions in mammary tumor development is needed.

Secretory proteins exported into extracellular fluids represent a major class of proteins, collectively referred to as the secretome [17]. The secretome is associated with broad cellular processes, including homeostasis, immune mechanisms, developmental regulation, proteolysis, development of the ECM and cell adhesion. These proteins are also involved in the interaction of a tumor with its surrounding stroma. A number of extracellular growth

factors, proteases, cytokines, and chemokines are expressed at elevated levels in carcinoma-associated fibroblasts, compared with normal fibroblasts [18, 19]. Systematic identification of secretome changes associated with stromal fibroblasts will improve our understanding of these important stromal–epithelial interactions.

High-throughput proteomic techniques can be utilized to investigate the complex signaling networks altered by interruption of the TGF- β pathway. The quantitative proteomic technique stable isotope labeling with amino acids in cell culture (SILAC) is an effective method for identification of proteins differentially expressed between cell lines [20, 21]. In this technique, two different cell lines are grown in culture media containing normal essential amino acids, or stable-isotope-labeled essential amino acids (*e.g.* ^{12}C - and ^{13}C -labeled L-Lysine). In the isotope-containing culture medium, naturally occurring amino acids are replaced by their isotope-labeled analogues after six cell divisions. The labeled and normal peptides preserve their exact protein ratios and can be quantitatively analyzed simultaneously using MS.

In this study, SILAC was used to investigate protein changes in the conditioned media (CM) from murine mammary fibroblasts with or without intact T β RII. Immunoassays were then performed to verify the proteomic results. Bioinformatic analysis shows that the differentially externalized proteins are involved in several important biological processes. The tumor-promoting effect for one of the identified proteins was further studied.

2 Materials and methods

2.1 SILAC Cell culture

A custom DMEM lacking arginine and lysine was purchased from Invitrogen (Carlsbad, CA, USA). [$^{13}\text{C}_6$, $^{15}\text{N}_4$]-arginine and [$^{13}\text{C}_6$]-lysine (heavy) were purchased from Cambridge Isotopes (Andover, MA). [$^{12}\text{C}_6$, $^{14}\text{N}_4$]-arginine and [$^{12}\text{C}_6$]-lysine (light) were purchased from Sigma (St. Louis, MO, USA). Tgfr2^{flox/flox} and Tgfr2^{fspKO} fibroblasts were characterized previously [14, 15]. Tgfr2^{flox/flox} fibroblasts were grown in this custom DMEM with a combination of heavy arginine and heavy lysine, along with 10% dialyzed FBS and antibiotics. Similarly, Tgfr2^{fspKO} fibroblasts were grown in DMEM with a combination of light arginine and light lysine. The final concentrations of arginine and lysine in the media were 0.398 mM and 0.798mM, respectively. The cells that grew more than seven passages in these custom media were used for the experiments. After Tgfr2^{flox/flox} and Tgfr2^{fspKO} cells grew to 80% confluence, the cells were washed and incubated for 24 h in the same isotopically labeled serum-free medium. The CM containing the secretory proteins was collected and filtered using a 0.45 μm filter (Millipore, Bedford, MA, USA) and subsequently concentrated using a 5000 Da molecular mass cutoff Amicon Ultra Centrifugal Filter Device (Millipore). Cell viability was analyzed with trypan blue staining.

2.2 Sample preparation and MS analysis

The concentrations of the CM were determined using the Bradford protein assay (Biorad, Hercules, CA, USA). A validation study was first performed to check the SILAC quantifications of the proteins with different mixing ratios. A total of 15 μg protein from the

two cell lines were mixed with ratios of 1:3, 1:1, 3:1 (light:heavy) and precipitated using trichloroacetic acid separately. The protein pellets were run 1 cm into a 10% SDS-PAGE gel, after which the protein bands were cut out as a single fragment from each gel. These protein gel fragments were further cut into small pieces prior to reduction, alkylation, and tryptic digestion as described previously [22]. The tryptic peptide fragments were desalted using a C-18 RP salt trap (Michrom, Auburn, CA, USA). After desalting, the tryptic peptides were analyzed with RP microcapillary LC-ESI-MS/MS as described previously [23]. Briefly, a fritless microcapillary column (100 μ m id) was packed with 10cm of 5 μ m C18 RP material (Synergi 4u Hydro RP80a, Phenomenex). The peptides were loaded onto the RP column equilibrated in buffer A (0.1% formic acid and 5% ACN). The column was placed in-line with an LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA, USA). Peptides were eluted using a 60 min linear gradient from 0 to 60% buffer B (0.1% formic acid, 80% ACN) at a flow rate of 0.3 μ L/min. During the gradient, eluting ions were analyzed by one full precursor MS scan (m/z 400–2000) followed by five MS/MS scans on the five most abundant ions detected in the precursor MS scan while operating under dynamic exclusion.

After the validation study, a total of 200 μ g of protein from the two cell lines were mixed in a 1:1 ratio and digested using trypsin. A more in-depth protein identification method named multidimensional protein identification technology was utilized to maximize the number of proteins being identified [24, 25]. Fritless microcapillary columns (100 μ m id) were packed with 9 cm of 5 μ m C18 RP material (Phenomenex) followed by 3 cm of 5 μ m strong cation exchange material (Partisphere SCX, Whatman), and finally 2 cm of C18 material. The trypsin-digested peptides were loaded directly onto triphasic columns equilibrated in 0.1% formic acid, 2% ACN. The triphasic column was placed inline with an LTQ linear ion trap mass spectrometer. An automated 12 cycle multidimensional chromatographic separation was performed using buffer A (0.1% formic acid, 5% ACN), buffer B (0.1% formic acid, 80% ACN), and buffer C (0.1% formic acid, 5% ACN, 500mM ammonium acetate) at a flow rate of 0.3 μ L/min. The first cycle was a 20 min isocratic flow of buffer B. Cycles 2–12 consisted of 3 min of buffer A, 2 min of X% buffer C, 5 min of buffer A, and a 60 min linear gradient to 60% buffer B. Cycles 2–12 used X=5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% of buffer C, respectively. During the linear gradient, eluting peptides were analyzed by one full precursor MS scan (m/z 400–2000) followed by five MS/MS scans on the five most abundant ions detected the precursor MS scan while operating under dynamic exclusion.

The program extractms2 was used to generate the ASCII peak list and identify +1 or multiply charged precursor ions from the native MS data files. Tandem spectra were searched using SEQUEST-PVM [26] against the Refseq mouse protein database (Released May 2005) containing 26 304 entries with no protease specificity. Only fully tryptic peptides were considered as candidates. Protein database searches were performed with fixed modification for cysteine (+57 Da) and variable modifications for arginine (+10 Da) and lysine (+6 Da). The mass tolerance for precursor ions was 3.0 Da. Considering the analysis algorithm in SEQUEST already has an internal nonzero minimum fragment ion tolerance based on binning peaks to approximate unit Da bins, 0.0 Da mass tolerance for fragment ion

was selected. This 0.0 Da mass tolerance corresponded to a tolerance of ~0.5 Da for fragment ion due to the built-in unit mass binning. The SEQUEST output was consolidated into hypertext files and further analyzed using the PeptideProphet (version TPP v4.2) [27] and ProteinProphet programs (version TPP v4.2) [28]. These programs assign probability values to each peptide and protein identification, indicating the likelihood that the respective peptide or protein has been correctly identified. The definition and deviation of “protein probability” has been described in detail previously [28]. Briefly, multiple distinct peptides assigned to MS/MS spectra were used for protein identification. The protein identification probability is based on the correction and combination of the corresponding peptide identification probabilities.

A 95% identification probability cutoff corresponding to an estimated false discovery rate of 0.5% was selected based on the ProteinProphet algorithm. Detection of a minimum of two unique peptides was further required for each protein identification. The relative quantification for each protein was calculated from the relative areas of the extracted ion chromatograms of the precursor ions and their isotopically distinct equivalents using the XPRESS algorithm (version TPP v4.2) [29]. The overall distributions of the protein quantifications from the two SILAC experiments were normalized to the 1:1 protein mix ratio result obtained from the validation study.

2.3 Western blot, zymography, and ELISA analysis

Equal amounts of the protein from *Tgfb β 2^{fspKO}* and *Tgfb β 2^{flox/flox}* CM were run into 4–20% SDS gels. After protein transfer and blocking, the membranes were exposed to different primary antibodies (Igfbp3, 1:200; Igfbp4, 1:200; Timp3, 1:1000; App, 1:20000; Postn, 1:100; Ube2n, 1:800; Tkt, 1:200; Vcl, 1:500; Calr, 1:2000) in an overnight incubation at 4°C. After being incubated for 1 h at room temperature in the presence of the secondary antibody (1:10000), peroxidase activity was visualized using the ECL Western blotting detection system according to the manufacturer's instructions (GE Healthcare). The antibodies for Igfbp3 (Catalog No. sc-6004), Igfbp4 (Catalog No. sc-6005), and Vcl (Catalog No. sc-7648) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the Timp3 antibody (Catalog No. ab39184) was purchased from Abcam (Cambridge, UK); the App antibody (Catalog No. 1565-1) was purchased from Epitomics (Burlingame, CA); the Postn antibody (Catalog No. AF2955) was purchased from R&D Systems (Minneapolis, MN, USA); the Ube2n antibody (Catalog No. ARP34089) was purchased from Aviva Systems Biology (San Diego, CA, USA); the Tkt antibody (Catalog No. 11039-1-AP) was purchased from Proteintech Group (Chicago, IL, USA); and the Calr antibody (Catalog No. 56259) was purchased from QED Bioscience (San Diego, CA, USA). The respective secondary antibodies were anti-rabbit antibody (Catalog No. 31426) and anti-goat antibody (Catalog No. 31402) from Thermo Scientific (Rockford, IL, USA). Gelatin zymography of matrix metalloproteinase (MMP)-2 from *Tgfb β 2^{fspKO}* and *Tgfb β 2^{flox/flox}* CM was performed using a similar procedure as described previously [30].

CXCL10 levels in the *Tgfb β 2^{fspKO}* and *Tgfb β 2^{flox/flox}* CM were analyzed using ELISA immunoassays (Quantikine ELISA kit, R&D Systems) according to the manufacturer's protocol. Briefly, CM containing equal amounts of total proteins from the two cell lines

were added to a 96-well plate and incubated for 2 h at room temperature. After the wells were aspirated and washed, a secondary conjugate was added and incubated for 2 h at room temperature, and then with substrate solution for 30 min. The reaction was terminated with 2M H₂SO₄, and the plate was read at 450nm on a Bio-Rad microplate reader (Benchmark Plus™ 550). The CXCL10 concentration in each sample was determined by interpolation to the plotted standard protein concentration curve.

2.4 Cell proliferation and migration assays for CXCL10

For cell proliferation assay, PyVmT cells were plated in 96-well plates and grown to approximately 70% confluency. CXCL10 (Shenandoah Biotechnology, Warwick, PA, USA) of differing concentrations (0, 1, 10, or 100 ng/mL) in DMEM with 0.5% FBS was added to each well. After 48 h, 10 µL of WST-1 (Roche, Indianapolis, IN, USA) was added to each well and cell proliferation was analyzed according to the manufacturer's protocol. The WST-1 colorimetric assay is designed for spectrophotometric quantification of cell proliferation based on cellular enzymatic activity for cleavage of tetrazolium salts [31, 32]. The Benchmark Plus™ Bio-Rad microplate reader was used for the measurement of absorbance of samples at 450nm (reference wavelength 690 nm).

Wound closure assay was performed to evaluate PyVmT mammary carcinoma cell migration. Briefly, PyVmT cells were plated and permitted to reach 100% confluency in 6-well chamber plates. The cell layer was then scratched through, down to the culture plate, with a sterile pipette tip, and CXCL10 at 0, 1, 10, or 100 ng/mL in DMEM with 0.5% FBS was added. The open gap, or wound, was then inspected using a phase contrast microscope over time as the cells moved in and filled the gap. The percentages of wound closure were measured at 0, 24, 36, 48, and 60 h time points, or until the wounds were closed.

2.5 Bioinformatic and statistical analysis

Ingenuity Pathway Analysis Software (Ingenuity Systems, Redwood City, CA, USA) was used to analyze the predicted biological functions of the differentially externalized proteins. Fisher's exact test was used to calculate a *p*-value indicating the probability that each biological function assigned to the data set is assigned by chance. Two-tailed Student's *T*-test was performed for the analyses of cell proliferation and migration with escalating doses of CXCL10 treatment. Pearson's correlation coefficient was calculated to evaluate the reproducibility of the list of cognate proteins that were identified in each of the two SILAC experiments.

3 Results

3.1 Protein identification and quantification using SILAC

Differential protein externalization from the Tgfbr2^{fspKO} and Tgfbr2^{flox/flox} mammary fibroblasts CM were compared using SILAC analysis. The overall schematic outline of the SILAC experiment is shown in Fig. 1A. After 24-h serum-free media incubation, the CM from the cells with viability greater than 97% was used for proteomic analysis. The protein quantification using the SILAC technique was first assessed by mixing a total of 15 µg of protein from the two cell lines at ratios of 1:3, 1:1, and 3:1 (Tgfbr2^{fspKO}:Tgfbr2^{flox/flox}),

respectively. After trypsin digestion, the peptide mixtures were analyzed using RP LC-MS/MS. Opposite protein quantification distributions were observed for the two samples with 1:3 and 3:1 mixing ratio (Figs. 1B and C). The distribution of the sample with the 1:1 mixing ratio centered on the midline (Fig. 1D). Thus, the different distributions of the protein quantification values among the three samples correlated well with the corresponding mix ratios.

To increase the number of identified proteins, a total of 200 µg of protein was mixed at a ratio of 1:1. After trypsin digestion, the peptide mixtures were first separated using a 12-step gradient strong-cation exchange chromatography. Each of the strong-cation exchange fractions was further analyzed using the RP LC-MS/MS. Samples prepared from two different cell passages were analyzed separately. After protein database searches, a total of 1786 unique protein groups with over 95% confidence score were identified using the ProteinProphet algorithm [28] from the two experiments. A total of 966 protein groups were identified from both of the SILAC experiments, whereas 388 and 146 protein groups were found in only one of the replicates, respectively (Fig. 2A). The overall distribution of the fold changes ($Tgfr2^{FspKO}/Tgfr2^{lox/lox}$) for all of the identified proteins in the two experiments is shown in Fig. 2B. A majority of the identified proteins showed similar levels of externalization between the two cell lines. The numbers of proteins with differential externalization were distributed relatively evenly between the two cell lines. $T\beta RII$ is a potent regulator of a wide range of biological functions and signaling pathways, and loss of $T\beta RII$ function both upregulates and downregulates a range of proteins [9, 33].

To further evaluate the correlation between the abundance ratio estimates for cognate proteins from both SILAC experiments, Pearson's correlation analysis was performed. The correlation coefficient was 0.72, which reflects the overall reproducibility of multiple experiment steps, including cell line growth and isotope labeling, sample concentration and trypsin digestion, and multidimensional protein identification technology analyses. (Supporting Information Fig. 1). The proteins that were identified in both experiments with more than twofold changes in $Tgfr2^{FspKO}$ cells or in $Tgfr2^{lox/lox}$ cells are listed in Table 1. The protein accession numbers, fold changes (mean $\pm$ SD), the number of peptides used for quantification, protein sequence coverage, and protein names are included. Complete information for each of the identified proteins from the two experiments, including protein accession number, identification probability, sequence coverage, fold change, number of peptides for quantification, number of unique peptides for identification, and protein names are listed in Supporting Information Table 1.

3.2 Validation of proteomic results with Western blots and zymography

To further confirm the SILAC protein identification and quantification, Western blot and zymography were used to analyze a list of proteins. The proteins were selected because they had antibodies available and were differentially externalized between the two cell lines. Figure 3A shows the list of proteins identified by SILAC presented at higher levels in $Tgfr2^{FspKO}$ CM, when compared with results from $Tgfr2^{lox/lox}$ cells. The Western blots also demonstrate that these proteins are relatively more highly externalized in $Tgfr2^{FspKO}$ cells. Similarly, Fig. 3B shows the list of proteins identified by SILAC as externalized at

higher levels in Tgfb β 2^{flox/flox} cells, when compared with Tgfb β 2^{fspKO} cells. The Western blots and zymography (for MMP-2 only) show similar patterns of externalization or enzyme activity between the two cell lines. In addition, Fig. 3C shows the proteins determined by SILAC to have approximately equal levels between the two cell lines CM. Overall, these analyses based on SILAC and Western blots show the same trend of protein differential externalization between the two cell lines.

3.3 Classification of differentially externalized proteins based on the biological function

To discern potential biological functions of the differentially externalized proteins in the Tgfb β 2^{fspKO} and Tgfb β 2^{flox/flox} cell lines, the proteins with over twofold changes were analyzed using Ingenuity Pathways Analysis. This functional analysis identified the predicted biological functions that were most significant to the Ingenuity Pathways Knowledge Base, with the input proteins which showed greater than twofold change in either cell line. Proteins associated with different biological groups related to tumor progression were identified ($p < 0.0001$). Figure 4 shows the number of proteins in each functional group, and the specific proteins included in each category and their p -values are shown in Supporting Information Table 2.

3.4 Effect of CXCL10 on the PyVmT mammary carcinoma cell proliferation and migration

Based on the previous bioinformatic analysis, CXCL10 was found to be associated with various biological functions related to tumor progression, such as cell-mediated immune response, cell-to-cell signaling and interactions, inflammatory response, and cell morphology (Supporting Information Table 2). CXCL10 was identified as one of the proteins present at higher levels in Tgfb β 2^{fspKO} CM compared with Tgfb β 2^{flox/flox} CM (Supporting Information Table 1). Figure 5A shows the corresponding peptide quantification based on the MS ion intensities for CXCL10. The paired SILAC signals in the same spectrum are the “light” and “heavy” peptide ions, and the ratios of the peptides were derived from these “light” and “heavy” ion intensity ratios. The 10Da difference between the peaks is the mass difference between the light and the heavy R in the peptide. Figure 5B shows the MS/MS spectra of the selected peptide ions for the identification of amino acid sequences of [CNCIHIDDGPVR]. The b and y fragment ions demonstrate correct fragment ion coverage of this amino acid sequences correspond to CXCL10. Figure 5C shows the quantitative results from the CXCL10 ELISA assays of the CM from the two cell lines. The differential externalization of CXCL10 in the Tgfb β 2^{fspKO} and Tgfb β 2^{flox/flox} cell lines was found to be consistent with the MS results.

The effects of CXCL10 in PyVmT breast cancer cell line have not been previously characterized. Therefore, CXCL10 externalization and potential effects on PyVmT breast cancer cell were further analyzed. PyVmT carcinoma cells were treated with CXCL10 and assessed changes in cell proliferation and migration. Increased PyVmT cell proliferation was observed with increasing dosages of CXCL10 ($p < 0.001$) (Fig. 5D). In addition, PyVmT cells that were stimulated with higher dosages of CXCL10 exhibited higher rates of wound closure ($p < 0.001$) (Fig. 5E). These results indicated that CXCL10 can promote PyVmT cell proliferation and migration, which is consistent with the previous findings that Tgfb β 2^{fspKO} fibroblasts stimulate PyVmT tumor growth and invasion [14].

4 Discussion

TGF- β is a master regulator of autocrine and paracrine signaling pathways between a tumor and its microenvironment. Our study for the first time systematically characterized the proteomic changes in the extracellular space for mammary fibroblasts with impaired TGF- β signaling pathways. Various proteins of interest, including cytokines, proteases and their inhibitors, growth factors, and ECM proteins, demonstrated differential externalization. Further examination of these proteins' functions will be important for the strategy of targeting TGF- β signaling pathways as a cancer treatment strategy.

Some limitations related to quantitative secretome analysis need to be addressed. First, although incubation of cell lines in serum-free medium for 24–48 h is a necessary and commonly adapted procedure for secretome analysis [34–37], the potential serum starvation effects on the biological functions of cell lines need to be acknowledged. The cellular physiological state during the first few hours could be different from the latter time period, despite most cells in our study remaining viable at the end of incubation period. To reduce this effect, the CMs from Tgfr2^{fspKO} and Tgfr2^{flox/flox} cell lines were analyzed using identical procedures, and the proteins with different expression levels are likely to be associated with loss of T β RII expression in the mammary fibroblasts. Second, selection of an arbitrary cutoff in fold changes has been performed in different SILAC studies [38, 39]; however, an arbitrary cutoff such as twofold may represent different selection probabilities for different proteins. Third, the differences between MS-based and antibody-based protein quantification should be addressed. For MS-based quantification methods, the relative quantitative results are influenced by the MS peak intensity. Lower peaks have inherently greater errors, and their exclusion or inclusion by the algorithm would influence the analysis. In addition, antibody-based quantification methods may have different detection specificities, sensitivities, and dynamic ranges when compared with an MS-based approach. Therefore, some protein quantification using these two different methods may not show perfect correlation.

Various chemokines attract bone marrow-derived cells into a tumor microenvironment and facilitate tumor progression. One cytokine highly externalized in Tgfr2^{FspKO} cells is CXCL10, which is a C-X-C motif chemokine that attracts T-lymphocytes and NK cells through activation of CXCR3 [40, 41]. Our study also shows that CXCL10 can directly stimulate PyVmT mammary carcinoma cell proliferation and migration, which is consistent with the previous finding that PyVmT carcinoma cells co-grafted with Tgfr2^{FspKO} fibroblasts exhibit higher rates of tumor growth and metastasis [14]. Various human breast cancer cell lines express CXCR3, and its expression is upregulated *via* stimulation by CXCL10 [42]. Studies have also shown that CXCL10 promotes colorectal and papillary thyroid carcinoma growth and invasion [43, 44]. Expression of CXCL10 could be modulated by activation of Ras proteins in breast cancer cells [45]. Administration of a small-molecule antagonist of CXCR3 inhibited lung metastasis in a murine metastatic breast cancer model [46]. Furthermore, endothelial cells and epithelial cells that do not have detectable CXCR3 also express a functional receptor that can bind to CXCL10, which suggests other potential roles for CXCL10 in breast cancer development [47].

Colony stimulating factor (CSF-1) was another cytokine found to be presented at higher levels in $Tgfb2^{FspKO}$ cell CM, and its expression has been detected in human breast tumor-associated stroma [48–50]. CSF-1 is one of the major chemo-attractants that recruit macrophages to breast cancer [51]. Tumor-associated macrophages (TAMs) are a major component of leukocytic infiltration into tumors. TAMs are known to secrete angiogenic factors, proteases, growth factors, and cytokines to stimulate tumor progression [52, 53]. When CSF-1-null mutant mice were crossed with MMTV-PyVmT mice, TAMs were severely depleted in the mice without CSF-1 [54]. The rates of tumor progression to a more malignant state and metastasis to the lung were also significantly reduced in these mice. Local reintroduction of CSF-1 to the mammary epithelium recruited TAMs [54].

Multiple extracellular proteases and protease inhibitors were found to be differentially externalized in our study. Urokinase-type plasminogen activator (uPA) was found to be more highly externalized in $Tgfb2^{FspKO}$ cells than in $Tgfb2^{flox/flox}$ cells. uPA is a serine proteinase involved in ECM- and tissue-remodeling during tumor cell invasion [55]. uPA converts plasminogen into plasmin, which can directly degrade most proteins in the ECM. In addition, uPA can activate other MMPs to further dissolve ECM components [56]. In human breast cancer, *in situ* hybridization has shown that uPA is expressed specifically in myofibroblasts adjacent to tumor cells [57], while uPA production in cultured human breast fibroblasts has been shown to be downregulated by TGF- β [58]. It has been suggested that breast cancer cells recruit uPA-producing myofibroblast cells to facilitate the process of pericellular proteolysis for invasion [59, 60]. On the other hand, some protease inhibitors were also found to be presented at higher levels in $Tgfb2^{FspKO}$ cell CM, such as tissue inhibitors of metalloprotease-2 and -3 (TIMP-2 and TIMP-3). TIMPs are natural MMP inhibitors and are involved in other biological functions such as tumor angiogenesis, cell growth, and apoptosis [61, 62]. In invasive human mammary ductal tumors, the expression of TIMP-3 by fibroblasts, not tumor cells, correlates positively with the occurrence of distant metastases [63]. MMP-2 and MMP-3 levels were decreased in $Tgfb2^{FspKO}$ cells when compared with controls, which may be associated with increased levels of TIMP-2 and TIMP-3. Decrease of these MMPs in $Tgfb2^{FspKO}$ cells may also be associated with the loss of TGF- β signaling as TGF- β has been suggested to positively regulate MMP-2 expression in a variety of cell lines [64–66].

Several members of the insulin-like growth factor (IGF) family were found to be presented at higher levels in $Tgfb2^{FspKO}$ cell CM, including IGF-1, IGF-binding protein-2 (IGFBP-2), IGFBP-3, IGFBP-4, and IGFBP-6. IGF-1 plays an important role in the regulation of cell differentiation, metabolism, survival, and proliferation [67, 68]. IGF-1 is a mitogen and is known to stimulate breast cancer cell proliferation [69]. IGF-1 was readily found in mammary fibroblasts, but not in breast cancer cell lines, which indicates that IGF-1 functions *via* paracrine signaling pathways during breast cancer development [69–71]. The biological effects of IGF-1 are mediated by the IGF-1 receptor, which belongs to the family of receptor tyrosine kinases [72]. Upon ligand binding, IGF-1 receptor, which is overexpressed in malignant breast epithelial cells, activates the mitogen-activated protein kinases pathway and the phosphatidylinositol-triphosphate kinase pathway [73, 74]. The IGFBPs function as carrier proteins, prolonging the activity of IGF-1 and regulating its

bioavailability [75, 76]. In addition, IGFBPs exert bioactivity independently of the IGFs as growth modulators [77]. For example, IGFBP-3 has been shown to potentiate proliferation of mammary cancer cells stimulated by epidermal growth factor [78]. IGFBP-2 expression levels were found to be associated with breast cancer progression [79], and increased expression of IGFBP-2 has been found in various human cancers [80]. IGFBP-2 was shown to regulate PTEN and to contribute to human breast cancer progression [81]. The IGF protein family has also been studied in connection with breast cancer risk. In a prospective case-control study which included over 3000 study participants, increasing IGF-I and IGFBP-3 concentrations were associated with a significant increase in breast cancer risk in women who developed breast cancer after 50 years of age [82].

In addition to the aforementioned potential mechanisms, our previous studies showed that T β RII was only knocked out in a subset of fibroblasts *in vivo*. Cancer-associated fibroblasts are heterogeneous and possess complex mechanisms to facilitate tumor development. Human carcinoma-associated-fibroblasts are a heterogeneous population and overexpress different sets of protein markers [83]. It is likely that the cross-talk among different types of fibroblasts may also be important for tumor progression.

Overall, our study has revealed the complex proteomic changes in the CM from T β RII knock-out mammary fibroblasts. The differentially externalized proteins may play important roles in tumor microenvironment and influence mammary tumor development. As various strategies of inhibiting TGF- β pathways are being developed for the treatment of human neoplasia [84], this study will improve our understanding of the potential effects of loss of TGF- β signaling in fibroblasts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CM	conditioned media
CSF	colony stimulating factor
ECM	extracellular matrix

IGF	insulin-like growth factor
IGFBP	IGF-binding protein
MMP	matrix metalloproteinase
PyVmT	polyomavirus middle T antigen
SILAC	stable isotope labeling with amino acids in cell culture
TAM	tumor-associated macrophage
TGF	transforming growth factor
Tgfr2^{flox/flox} fibroblasts	floxed control fibroblasts
Tgfr2^{fspKO} fibroblasts	TβRII knockout fibroblasts
TIMP	tissue inhibitors of metalloprotease
TβRII	TGF beta type II receptor
uPA	urokinase-type plasminogen activator

Figure 1.

Overall scheme of the SILAC experimental design and distributions of the differentially externalized proteins identified from Tgfr2^{FSPKO} and Tgfr2^{lox/lox} cell line CM. (A) Murine mammary fibroblasts with (Tgfr2^{lox/lox}) or without (Tgfr2^{FSPKO}) intact T β RII were grown in “heavy” and “light” culture media, respectively. Equal amounts of CM from the two cell lines were mixed and analyzed simultaneously, minimizing variation associated with the downstream proteomic analysis. (B–D) Three different distributions of protein quantification correlate well with the samples' different protein mixing ratios of 1:3, 3:1, and 1:1 (Tgfr2^{FSPKO}:Tgfr2^{lox/lox}).

Figure 2. Protein identification and quantification using SILAC combined with Multidimensional Protein Identification Technology. (A) Two SILAC experiments were performed and 966 proteins were identified in both experiments, whereas 388 and 146 proteins were found only in the first and second replicates, respectively. (B) The overall distribution of the fold changes for all of the identified proteins in the two SILAC experiments.

Figure 3.

Validation of the SILAC protein externalization results by Western blotting and zymography. Equal amounts of CM from the Tgfr2^{FSPKO} and Tgfr2^{flox/flox} cell lines were used for the analyses. (A) The proteins that were presented at higher levels in the Tgfr2^{FSPKO} cell CM. (B) The proteins that were presented at higher levels in the Tgfr2^{flox/flox} cell CM. (C) The proteins that were presented at equal levels in the Tgfr2^{FSPKO} and Tgfr2^{flox/flox} cell CM.

Figure 4. Distribution of the differentially externalized proteins based on the predicted biological functions. Fisher's exact test was used to calculate a p -value indicating the probability that each biological function assigned to the data set is assigned by chance. A total of 14 different predicted biological functions related to tumor progression were identified as significant ($p < 0.005$).

Figure 5.

Validation of CXCL10 differential externalization and its effects on PyVmT mammary carcinoma cell proliferation and migration. (A) The "light" and "heavy" peptide ion intensities were used to calculate their corresponding differential protein level ratio. (B) The MS/MS spectra correlate with the sequences of [CNCIHIDDGPVR] confirming the identification of CXCL10. (C) The result from the CXCL10 ELISA assays of the CM from the two cell lines. (D) Escalating dosage of CXCL10 is shown to directly stimulate PyVmT cell proliferation using WST-1 cell proliferation assay (* $p < 0.001$, ** $p < 0.0001$). (E) Escalating dosage of CXCL10 is shown to directly stimulate PyVmT cell migration using wound closure assay (* $p < 0.01$, ** $p < 0.001$). The experiments were performed three times, with consistent results. Error bar: SD.

Table 1
List of proteins differentially externalized in the CM from Tgfr2^{FSPKO} and Tgfr2^{flox/flox} cells

Protein	SILAC 1 fold-changes (no. of peptides, sequence coverage)	SILAC 2 fold-changes (no. of peptides, sequence coverage)	Protein name
NP_031768	2.20±0.56 (970, 62.1%)	2.00±0.39 (435, 33.6%)	Collagen, type I, α1
NP_033170	2.01±0.42 (166, 61%)	2.19±0.36 (49, 45.8%)	Secreted frizzled-related protein 2
NP_659046	2.20±0.44 (25, 18.6%)	2.01±0.15 (20, 20.6%)	Meteorin, glial cell differentiation regulator like
NP_032934	2.16±0.38 (61, 62.3%)	2.07±0.27 (41, 43.9%)	Peptidylprolyl isomerase C
NP_034642, NP_908941	2.07±0.21 (18, 27.1%)	2.28±0.20 (11, 27.1%)	IGF-1 isoform 1 preproprotein; IGF-1 isoform 2 preproprotein
NP_038495	2.07±0.56 (53, 24.8%)	2.42±0.42 (17, 25.3%)	Aldehyde dehydrogenase 1A1
NP_033268	2.46±0.64 (747, 63.6%)	2.19±0.44 (268, 52.3%)	Secreted acidic cysteine rich glycoprotein
NP_033996	2.35±0.76 (44, 20.9%)	2.34±0.48 (7, 6.3%)	Cadherin 11
NP_766604	2.10±0.33 (5, 6.5%)	2.61±0.90 (4, 17.7%)	Vesicle amine transport protein 1 homolog-like (<i>Torrea californica</i>)
NP_032786	2.82±0.79 (95, 38.3%)	2.22±0.45 (42, 28.9%)	Osteoglycin
NP_031425	2.48±0.73 (13, 8.5%)	2.58±0.56 (3, 4.5%)	ADAM metalloproteinase domain 10
NP_035837	2.32±0.60 (7, 9.2%)	2.75±0.89 (6, 8.4%)	Vitronectin
NP_032721	2.82±0.86 (211, 45.9%)	2.27±0.44 (92, 22%)	Nidogen 2
NP_032535	2.70±0.67 (49, 26.4%)	2.42±0.65 (23, 22.6%)	Lipoprotein lipase
NP_031497	2.30±0.35 (33, 20.3%)	2.87±0.00 (3, 1.4%)	Amyloid β (A4) precursor protein
NP_031763	2.89±0.72 (84, 13.3%)	2.33±0.35 (22, 10%)	Procollagen, type V, α2
NP_034647	2.95±0.74 (47, 43.3%)	2.43±0.18 (21, 25.6%)	IGFBP-4
NP_031765	2.23±0.34 (14, 9.2%)	3.30±0.29 (6, 7.4%)	Procollagen, type VIII, α1
NP_032369	3.05±0.53 (29, 19.6%)	2.61±0.44 (11, 12%)	IGFBP-3
NP_033157	3.00±0.53 (26, 14%)	2.73±0.29 (17, 11.9%)	C-type lectin domain family 11, member a precursor
NP_033784	2.43±0.92 (114, 10.5%)	3.59±1.22 (69, 6.9%)	Albumin
NP_034018	2.97±1.00 (41, 17.1%)	3.23±0.62 (12, 7.5%)	Complement component factor H
NP_080715	3.23±0.71 (53, 19.7%)	3.03±0.54 (23, 11.6%)	Steroid-sensitive protein 1
NP_038554	2.76±0.42 (22, 18.3%)	3.53±0.83 (12, 17%)	Growth differentiation factor 6
NP_032019	3.47±1.29 (52, 6.3%)	2.93±0.96 (17, 5.4%)	Fibrillin 1
NP_031512	3.23±0.66 (30, 54.5%)	3.24±0.29 (4, 36%)	Rho, GDP dissociation inhibitor β
NP_032899	3.42±1.18 (140, 58.2%)	3.35±0.78 (69, 40.4%)	Plasminogen activator, urokinase
NP_034310	3.46±0.73 (22, 13%)	3.77±0.75 (22, 8.4%)	Fibulin 1
NP_032499	3.17±0.00 (1, 3.7%)	4.13±0.18 (3, 8.9%)	Keratin complex 2, basic, gene 1
NP_032246, NP_058652	3.40±0.01 (5, 6.1%)	4.05±0.27 (6, 6.8%)	Hemoglobin, β adult major chain; Hemoglobin, β adult minor chain;
NP_034060	3.62±1.47 (1100, 62.9%)	3.95±1.37 (485, 43.7%)	Collagen, type III, α1
NP_543120	3.41±1.11 (88, 23%)	4.98±0.96 (72, 20.6%)	Serpin peptidase inhibitor, clade C, member 1
NP_032607	4.97±2.71 (132, 23.9%)	5.12±1.76 (55, 15.4%)	Melanoma antigen
NP_059067	3.57±1.54 (19, 3.5%)	7.31±2.57 (19, 3.5%)	Hemopexin
NP_796277	5.25±2.29 (62, 20.9%)	6.00±3.09 (12, 14.9%)	Leucine-rich repeat neuronal 4

Protein	SILAC 1 fold-changes (no. of peptides, sequence coverage)	SILAC 2 fold-changes (no. of peptides, sequence coverage)	Protein name
NP_444473	6.02±2.60 (52, 11.8%)	5.57±2.24 (49, 13.4%)	Protease, serine, 1
NP_061345	6.01±2.12 (16, 10.4%)	5.73±1.71 (9, 10.7%)	Mesothelin
NP_032318	4.15±1.32 (4, 4.6%)	9.21±5.19 (5, 4.6%)	Hydroxysteroid (17-β) dehydrogenase 4
NP_031778	0.53±0.11 (218, 31.1%)	0.53±0.14 (144, 27.1%)	Ceruloplasmin isoform b
NP_038617	0.49±0.06 (31, 6.6%)	0.53±0.14 (25, 5.3%)	Latent TGF-β-binding protein 2
NP_036151	0.41±0.08 (26, 25.7%)	0.57±0.12 (48, 46.7%)	Peroxiredoxin 5 precursor
NP_035303	0.48±0.05 (21, 7.0%)	0.48±0.14 (16, 9.2%)	Protein S, α
NP_031817	0.47±0.04 (13, 34.2%)	0.48±0.14 (15, 16.6%)	Cysteine and glycine-rich protein 1
XP_484897	0.47±0.13 (5, 2.9%)	0.47±0.21 (6, 2.5%)	PREDICTED: procollagen, type VI, α3 isoform 1
NP_032515	0.38±0.11 (71, 26.6%)	0.53±0.14 (43, 26.0%)	Lipopolysaccharide-binding protein
NP_034492	0.44±0.10 (62, 48.3%)	0.45±0.11 (45, 45.8%)	Glutathione S-transferase omega 1
NP_034644	0.34±0.06 (5, 13.3%)	0.54±0.12 (13, 30.6%)	IGF-2 precursor
NP_032636	0.28±0.11 (136, 50.6%)	0.60±0.20 (90, 39.9%)	MMP-2
NP_033281	0.41±0.08 (32, 17.6%)	0.42±0.09 (23, 14.6%)	Serine (or cysteine) proteinase inhibitor, clade E, member 2
NP_056599	0.35±0.10 (174, 39.1%)	0.45±0.18 (170, 46.1%)	Periostin, osteoblast specific factor
NP_034939	0.38±0.09 (23, 25.3%)	0.33±0.08 (27, 17.1%)	MMP3
NP_031454	0.19±0.10 (38, 21.2%)	0.47±0.23 (37, 28.9%)	Angiotensinogen
NP_031462	0.26±0.06 (21, 19.6%)	0.36±0.15 (18, 15.2%)	Aldehyde dehydrogenase 3A1
NP_001012495	0.14±0.06 (26, 18.0%)	0.38±0.15 (51, 43.8%)	Stromal cell-derived factor 1 isoform γ precursor;
NP_038683,			Stromal cell-derived factor 1 isoform β precursor
NP_068350			Stromal cell-derived factor 1 isoform α precursor

The externalization ratio for each protein is derived from Tgfr2FspKO/Tgfr2flox/flox. Proteins with over twofold changes are included.