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14. ABSTRACT: Signal transduction networks such as the PI3K-AKT and EGFR pathways are important regulators of cell fate decisions, including cell proliferation, differentiation, apoptosis, and homeostasis. Furthermore, these pathways integrate and influence one another when cells are within an appropriate microenvironment. Using a proteomic approach, we identify stratifin, a protein which regulates AKT and EGFR signaling as well as the cell cycle, to be upregulated in T4-2 cells cultured in 3D lrECM. Expression of stratifin decreases to S1 levels upon phenotypic reversion; these effects were not seen when cells were cultured in 2D, supporting a possible role of stratifin in crosstalk. In the mouse mammary gland, stratifin expression was restricted to myoepithelial cells, and was expressed predominantly during periods of branching morphogenesis and ductal infiltration. Taken together, these data suggest a novel role of stratifin in epithelial cell proliferation and migration. The signaling networks regulated by stratifin will be assessed by shRNA knockdown in T4-2 cells.

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INTRODUCTION:

The fundamental goal of this project is to understand how signalling networks become integrated when cells are cultured in a physiologically relevant context, to elucidate potential nodes of pathway crosstalk, and to investigate how the interplay of signalling networks becomes deregulated in the case of malignancy. The proposed approach was to measure activation of the signalling networks through the use of fluorescent probes which assess the kinetics of pathway activation either at the canonical transcriptional endpoints of the cascades, or at potential nodes of crosstalk. As stated in the 2006 annual report, this approach has been somewhat difficult to implement in the HMT-3522 progression series, as the cell lines are notoriously difficult to transfect. In addition, potential caveats to the approach, such as cytotoxicity resulting from the fluorophores, differential kinetics of fluorescent protein maturation, degradation, and photobleaching, and the lack of sufficient resources to carry out kinetic assays in real time (such as through the use of FRET-based probes), indicate the initially proposed approach may not be ideal for understanding the biological role of signalling network integration in the normal breast and in malignancy, at least with the available resources.

Given these insurmountable technical obstacles and the proposed overall goal of uncovering novel nodes of signalling pathway integration when cells are cultured in 3D lrECM, and how these pathways become deregulated in tumorigenesis, it seems more appropriate at this time to take a more targeted approach to address the research goal of this proposal. Previous experiments performed in the Bissell lab have evaluated the proteomic profiles of HMT3522 S1 and T4-2 cells grown in Matrigel (3D lrECM). I began preliminary analysis of this data as a complement to the proposed goals of this project, in order to identify potential signalling pathways and nodes of crosstalk that become altered during tumorigenic progression.

To generate this data set, cells were cultured in 3D lrECM over a time course of 10 days, with cell lysates and conditioned media collected in two-day intervals. In collaboration with Daojing Wang (Life Sciences Division, LBNL) the samples were subjected to proteomic analysis in order to identify proteins upregulated in T4-2 cells relative to S1, as well as to identify potentially paracrine-acting factors released by T4-2 into their extracellular environment. Cell lysates and concentrated conditioned media were resolved by 2D gel electrophoresis with isoelectric focusing (pH 3-10 non-linear gradient) in the first dimension and 10% polyacrylamide gel in the second dimension (Invitrogen^{TM}). Spots in the T4-2 gels which increase in intensity over time, but are not present or did not increase in the S1 conditioned media to a proportional extent, were removed and proteolyzed by trypsin for mass spectrometry (MS) analysis as previously described[1].

Using this approach, stratifin (SFN; 14-3-3σ) was found to be upregulated in both the intracellular and extracellular proteomes of T4-2 cells (Table 1 and 2). This protein belongs to the 14-3-3 class of adaptor proteins, which are known to interact with regulators of cell signaling, apoptosis, adhesion, proliferation, differentiation, and survival (for recent reviews, see [2-4]). The diversity of cellular processes regulated by this protein class is related to their promiscuity in interacting partners. There are at least 7

different 14-3-3 isoforms recognizing a collective of 200 targets, and which bind to phosphoserine residues on target proteins as a dimer. SFN contains unique structural motifs to preferentially homodimerize[5], and is known to be an important regulator of the G2/M checkpoint[6]. The protein is a direct transcriptional target of p53 which is upregulated in response to chemical or irradiation-induced DNA damage, causing G2/M arrest[7]. In the skin, keratinocytes begin to overexpress SFN as the cells differentiate into stratified keratin squames (hence the protein name "stratifin"), at which point the cells begin to release soluble SFN into their microenvironment[8, 9]. This extracellular SFN has been shown to potently stimulate collagenase (MMP1) activity in dermal fibroblasts[9, 10].

The correlation of SFN expression with cancer progression is an area of active debate. There are reports of SFN expression being silenced by promoter hypermethylation in high frequency within several cancers, including breast[11, 12], prostate[13], liver[14], and ovary[15]. Interestingly, SFN overexpression has been characterized by several groups in pancreatic cancer[16-18], the most lethal of all cancers. A recent study investigated the levels of SFN in malignant and non-malignant primary breast tissues through a combined immunohistochemical and proteomic approach[19]. The authors concluded that silencing of SFN expression is a sporadic event occurring in only 3 of the 68 tumors, and that immunoreactivity in both the normal and malignant breast tissues was of greater intensity in basal layers corresponding to myoepithelial cells[19]. This was in agreement with other previous work suggesting that SFN is a marker of basal/myoepithelial cells in the breast in vivo[20].

As SFN is known to regulate signalling through the Ras-Raf-MEK-ERK and AKT pathways, to regulate MMP activity, and to regulate the cell cycle, with SFN being overexpressed or silenced in a number of cancers, this protein was selected as a potential signalling protein involved in pathway integration and crossmodulation which becomes disregulated in HMT3522 malignant progression. To validate the 2D gel/mass spectrometry data, western blot analysis of whole cell lysates and conditioned media was performed (Fig. 1). As expected, SFN was found to be abundant in T4-2, both intracellularly and extracellularly, with expression returning to S1 levels upon reversion with 100 nM AG 1478 (tyrphostin). Interestingly, no difference in abundance was observed in S1 and T4-2 cells grown on conventional (plastic) substratum, suggesting the upregulation of SFN in T4-2 cells, analogous to the pathway crosstalk between the EGFR and AKT pathway, depends on cells being cultured in a physiologically relevant context.

I would like to propose shifting the focus of the specific aims of this award to further investigate the role of SFN as a potential signal integrator in the HMT3522 progression series. In doing so, the overarching goals of the proposal would still be fulfilled. Uncovering the signalling pathways affected by the upregulation of SFN in T4-2 cells when cultured in 3D lrECM, as well as the role of SFN within the context of normal mammary gland development, are proposed to be evaluated in further detail in the attached revised Specific Aims.. Understanding the biology of this important signalling intermediate, both in normal mammary gland development and in the case of malignancy, may shed light on the stages involved in the step-wise progression of breast cancer, or a subset of breast cancers.

BODY:

Revised Specific Aim 1: Elucidate the biochemistry of stratifin within the context of normal mammary gland development and homeostasis.

Background: In order to have a comprehensive understanding of the role of SFN in malignancy, it is important to understand the role of the protein in normal mammary gland development and homeostasis. One model commonly used to understand the biological role of a given protein is to look at the mammary gland developmental stages in mice, and at what stage of development the protein is overexpressed. Within the mouse, rudimentary epithelial cells begin to form terminal end buds at around 3 weeks of age, with ductal elongation, infiltration of the fat pad, and branching morphogenesis continuing until the fat pad of the mammary gland is filled with epithelial ducts in adult mice at around 12 weeks of age[21]. In the adult, the epithelial alveolar structures at the end buds undergo cyclic rounds of proliferation and regression that coincide with the mouse oestrus cycle in preparation for pregnancy. During pregnancy, the alveolar structures undergo extensive proliferation and start to fill with milk around day 18 of pregnancy, with milk secretion into the ducts occurring at parturition. After the pups are weaned, the gland is extensively remodelled to a state similar to that of an adult virgin mouse in what is termed involution. This period is marked by pronounced apoptosis[21]. In summary, by evaluating when a protein that is involved in mammary gland development is expressed in the developmental stages of the breast, one can hypothesize whether the protein may be responsible for proliferation, differentiation, migration, morphogenesis, apoptosis, and invasion of mammary epithelial cells, and in an oestrogen-dependent or independent manner.

Methods: SFN expression has been previously described to occur predominantly within myoepithelial cells surrounding the luminal epithelial cells of the ducts and alveoli, in normal and neoplastic human breast [20]. As myoepithelial cells represent a minor constituent of the entire mammary gland biomass[21], immunohistochemical analysis was employed to qualitatively assess if and when SFN is expressed during development. Mammary gland tissue was harvested from CD1 mice at different developmental and pregnancy stages, fixed overnight in formalin, embedded in paraffin, and cut into 5 μm serial sections (provided by Jamie Bascom, LBNL Bissell lab). Sections were stained for SFN essentially as described [20]. Briefly, sections were deparaffinized in xylenes, hydrated in graded ethanols, and pressure-cooked (autoclaved) for 3 minutes in 10 mM citrate, pH 6.0, for antigen retrieval. Goat anti-SFN (C-18; Santa Cruz) was used at 1:40 dilution, with the antibody pretreated with blocking peptide at a 5:1 ratio of peptide:IgG to validate antigen specificity in competition studies. Slides were counterstained with hematoxylin, dehydrated in graded ethanols, and mounted in Permount mounding media. Mouse skin sections, T4-2 xenograft sections (provided by Aylin Rizki, LBNL Bissell lab), and formalin-fixed paraffin embredded human breast tissue sections (ProSci, Inc) were used as positive controls. Samples were treated and processed identically and simultaneously.

Results: T4-2 xenografts were highly abundant in SFN expression, as predicted from the preliminary data (Fig. 2). Staining was very specific for the T4-2 tumor, with no staining occurring in the surrounding mouse stroma. However, staining is also prominent in regions on the slide containing mouse skin (Fig. 3). This is expected, as SFN is known to be expressed within basal keratinocytes of the epidermis, with expression lost as keratinocytes differentiate into keratin squames[9]. Pre-treatment of the primary antibody with blocking peptide greatly reduced staining. Similar to previous reports[20], myoepithelial cells within the normal human breast were highest in SFN expression, with faint staining of luminal epithelial cells (Fig. 3).

In the mouse mammary gland, SFN expression was found to be restricted to myoepithelial cells. Interestingly, SFN is expressed most predominantly when the mouse is at 3 weeks and 8 weeks of age, with expression being greatly reduced at 16 weeks of age (Fig. 4). This window of development coincides to when ductal elongation and branching morphogenesis occurs, as terminal end buds form at 3 weeks of age and infiltrate the gland until the fat pad is filled at 12 weeks[21]. The termination of SFN expression at 16 weeks of age suggests that SFN may be directly involved in branching morphogenesis, and may be necessary for proper ductal elongation. It may be that, analogously, SFN regulates the invasion and/or migration of malignant epithelial cells during tumorigenic progression. This will be explored in Revised Specific Aim 2.

There is a mouse model that for SFN. The repeated-epilation (*Er*) mouse allele has been demonstrated by two independent groups to result from a single nucleoside insertion within SFN, resulting in a premature C-terminal truncation of the protein[22, 23]. *Er* mice have a dramatic thickening of the epidermis due to excessive keratinocyte proliferation and lack of differentiation into keratin squames. The excessive skin thickening closes the mouths of pups, resulting in suffocation of homozygous $SFN^{Er/Er}$ pups shortly after birth. Furthermore, heterozygous Er mice are more prone to spontaneous and chemical-induced skin carcinomas[24]. To assess whether SFN is necessary for branching morphogenesis in the mammary gland, rudimental mammary gland epithelia removed from *Er* pups could be transplanted into the cleared fat pad of a wild-type recipient to assess whether branching is abrogated. In addition, mice can be monitored histologically to assess whether there is a delayed branching in heterozygous *Er* mice compared to wild-type littermates between 3 and 14 weeks of age. We will pursue acquiring the mouse strain from Inder Verma (The Salk Institute for Biological Studies, La Jolla, CA) and will obtain all necessary animal use approvals from the local IACUC as well as DOD, and generate the human equivalent of the *Er* allele to understand biochemically what the consequences of the truncation are (dominant-negative, constitutive-active, or non-functional protein), if this revised specific aim is approved for research.

Revised Specific Aim 2: Evaluate the role of SFN in HMT3522 tumorigenic progression

Background: As SFN is known to regulate several signalling pathways and is a direct regulator of the G2/M transition through sequestration of cdc2 and cyclin B from the nucleus[7], the upregulation of this protein in T4-2 cells when grown in 3D, but not in 2D, is intriguing. While SFN has been described as a tumor suppressor, the overexpression of

the protein in high grade tumors, especially in pancreatic cancers, has been well-documented and is not accounted for[16-18]. As the Bissell lab employs a cell culture model which is physiologically relevant and is an in vitro correlate of malignant progression, the model is ideal for uncovering novel mechanisms by which proteins such as SFN regulate, and are regulated by, signalling cascades.

Methods: Stable T4-2 cell lines expressing shRNAs targeting SFN for knockdown will be established. Lentiviral-encoded shRNA hairpins were synthesized according to previously validated shRNA sequences[25]. Sense and antisense oligonucleotides were synthesized commercially (Bio-synthesis), ligated, and annealed into pENTR/pTER^{+} (provided by Eric Campeau, Life Sciences Division, LBNL) at BglII and HindIII sites. The shRNA sequences, encoded by Tet-inducible H1 promoter, were recombined into pLenti RNAi Puro DEST #2 (provided by Eric Campeau) and co-transfected with pLP1, pLP2 and pLP1-VSVG into 293FT cells for viral production. Viral supernatants were collected in 24 hour intervals over 2 days, concentrated by ultracentrifugation, and aliquoted. T4-2 cells were infected twice in the presence of 4 ug/ml polybrene, and were selected with puromycin at 1ug/ml.

Cells will be harvested to assess knockdown efficiency by western blot analysis. Viral particles giving >70% knockdown efficiency will be employed in future studies. Cells will be plated in 3D lrECM to assess whether SFN knockdown is sufficient to revert T4-2 cells. In addition, cells will be assessed for whether SFN knockdown will reduce the invasive, migratory, and anchorage-independent growth properties of T4-2 cells in modified Boyden chamber[26], wound healing, and soft-agar colony assays. These may be followed with xenograft injections into mice to assess whether SFN knockdown is sufficient to abrogate the T4-2 malignant properties. Again, all necessary animal use approvals will be obtained before beginning any xenograft experiments. If SFN knockdown influences an aspect of T4-2 malignancy, western blot analysis using phospho-specific antibodies to AKT, MAPK, and other pathways will be performed to evaluate pathways influenced by SFN knockdown. In addition, as extracellular SFN released from keratinocytes is known to stimulate MMP activity in dermal fibroblasts [9, 10], conditioned media collected from cells in which SFN is knocked down, along with cells infected with a scrambled shRNA, will be tested for its ability to stimulate MMP activity and invasion of cells through a modified Boyden chamber assay[26]. However, these experiments depend on whether SFN knockdown influences cell invasion and will not be proposed in greater detail. In summary, this revised specific aim, if approved for funding, would evaluate whether the preliminary finding of SFN upregulation in T4-2 cells serves a functional consequence towards malignant progression.

Revised Specific Aim 3: Assess whether SFN upregulation may be a property of a subset of breast tumors

Background: The HMT-3522 cells show basal-like properties when their microarray gene expression profile is clustered according to a set of classifier genes[27, 28] used to molecularly describe tumor subsets (Kenny et al., 2007; manuscript in submission). In vivo, T4-2 cells form tumors in nude mice which share many marker genes in common

with basal carcinomas, such as p63 and β1 integrin (Rizki et al., 2007; manuscript in submission). β1 integrin has been shown to be downregulated in luminal cancers but upregulated in basal carcinomas, and similarly to SFN, expressed predominantly in the normal basal/myoepithelial layer[29-31]. Breast carcinomas of the basal subtype are aggressive in their pathology, express myoepithelial markers, and are associated with a particularly poor prognosis in patients[28]. Given that basal carcinomas are very aggressive, that HMT-3522 can be considered a progression model of basal tumorigenesis, and that SFN is a marker of basal/myoepithelial cells in the normal breast, it may be that SFN is upregulated in a subset of breast cancers in vivo, reinforcing the clinical importance of understanding the biology of this protein in the HMT3522 progression series.

Methods: To assess whether SFN upregulation is a property of a subset of tumorigenic breast cells, a panel of human breast cancer cell lines (courtesy of Joe Gray, life sciences division, LBNL) will be cultured in 3D lrECM, and protein and mRNA isolated from the cells will be surveyed for expression of SFN by western blot and RT-PCR, respectively. Cell lines will be selected based on their microarray gene expression profile [32] when clustered according to the classifiers of Perou[27]. These experiments would reveal whether all breast tumor cell lines show elevated SFN expression when cultured in lrECM, or whether this property is limited to a subset of malignant breast epithelial cell lines, such as those which are categorized as basal-like. If a subset of breast cancer cell lines (in particular those with basal-like properties) are found to overexpress SFN, it will be investigated whether this is true in primary breast cancer tissue obtained from the UCSF Breast Cancer SPORE and of which the molecular classification by microarray analysis has been performed [32, 33]. If this work goes forward, human subjects approval will be acquired for receipt of tissue from the UCSF facility. Immunohistochemistry of sections from formalin-fixed, paraffin-embedded tissues will be performed using the procedure and SFN antibody previously employed. If approved, these experiments will reveal whether the overexpression of SFN is a property of the aggressive, basal-like subclass of breast tumors, and could uncover SFN as a novel therapeutic target specific for the cancer subtype, leading to rational drug design.

KEY RESEARCH ACCOMPLISHMENTS:

- Validated preliminary 2D gel/MS analysis by showing that T4-2 cells have a much higher expression of SFN than S1 cells when cultured in 3D lrECM, both intracellularly and extracellularly, and demonstrated that SFN expression in T4-2 cells is reduced upon reversion with AG 1478.

- Became familiar with immunohistochemistry and optimization steps necessary for adequate staining
    - Demonstrated that T4-2 cells form tumours in nude mice which have high SFN expression
    - Verified that SFN is a myoepithelial marker in the normal breast
    - Demonstrated that SFN expression is highest in myoepithelial cells of virgin mouse mammary glands during periods of proliferation and ductal branching morphogenesis, and is greatly reduced in adult mice

- Became familiar with RNAi technology, including production of lentivirus encoding shRNA hairpins.
    - Produced siRNA expression constructs giving ~50% knockdown in transient transfection of T4-2 cells (data not shown)
    - Produced high-titre viral stocks encoding shRNA hairpins targeting SFN or scrambled sequences
    - Very recently completed generation of stable pools of T4-2 cells infected with lentivirus encoding shRNAs, and am currently assessing whether SFN knockdown influences T4-2 malignancy

REPORTABLE OUTCOMES:

- Mentored an undergraduate student (Jason Jung, Department of Molecular and Cell Biology, UC Berkeley) two semesters for his honours thesis research. His honours poster presentation, entitled “The potential role of stratifin in normal mammary gland development and in tumorigenesis”, was among the top five of 78 posters recognized as “most outstanding poster” at the Department of Molecular and Cell Biology Honours Research Symposium

- Gave a poster presentation at the Department of Comparative Biochemistry annual meeting and reception

- generated stable pools of T4-2 cells infected with shRNA-encoding lentivirus targeting SFN for knockdown

CONCLUSIONS:

Proteomic analysis has identified SFN, a signalling intermediate in AKT and MAPK signalling cascades which directly regulates the cell cycle and which has deregulated expression in a number of cancers, to be upregulated in T4-2 cells when cultured in 3D lrECM. This finding was validated by western blot analysis, which also demonstrated that SFN levels are "reverted" to S1 expression levels when T4-2 cells are treated with AG1478. These results are not observed when cells are cultured on 2D substrata, indicating that the upregulation of SFN, similar to signalling pathway crosstalk, depends on cells being cultured in a physiologically relevant context. In the mouse mammary gland, as in the human breast, SFN expression was found to be restricted to myoepithelial cells, and was highest in expression during periods of epithelial cell proliferation, ductal elongation, and branching morphogenesis. Taken together, these results indicate that SFN, in response to the correct cellular microenvironment, may play a novel role in regulation of epithelial cell proliferation and migration. This possibility will be explored in greater detail using stable pools of T4-2 cells infected with lentivirus encoding shRNA hairpins targeting SFN, with an emphasis on elucidating the pathways under regulation by SFN in T4-2 cells.

Understanding the biology of a central regulator of cell proliferation and signalling within the context of normal mammary gland development is essential for a clear understanding of what goes wrong in the case of breast cancer. In addition, identification of novel mechanisms by which such proteins influence the malignant progression of a subset of carcinomas, in particular the basal-like breast carcinomas which have poor clinical outcome, will facilitate development of targeted breast cancer therapeutics for use in a subset of aggressive carcinomas that otherwise respond poorly to conventional therapies.

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SUPPORTING DATA:

Table 1: Intracellular proteins abundant in HMT-3522 cells grown in 3D lrECM, as identified by Q-TOF mass spectrometry

Spot Number	*T4 vs. S1 (fold)*	*Protein Name*	*SwissProt Accession Number*	*Molecular Weight kDa (theoretical)*	*pI (theoretical-unmodified)*
1046	2.3	14-3-3 protein sigma (phosphorylated at Ser63, Ser69 and T228, with 82% sequence coverage) *DJW101304_003*	P31947	27.7	4.7
1038	2.2	Superoxide dismutase [Mn] *DJW101304_002*	P04179	24.7	8.35
1442	9.4	Superoxide dismutase [Mn] (-Mn2+) *DJW121003_006*	P04179	24.7	8.35
1038 +1442	2.5	Superoxide dismutase [Mn]			
1106	9.0	Triosephosphate isomerase (phosphorylated at Ser20) *DJW121003_004*	P00938	26.5	6.51
1138	3.1	Annexin A1 *DJW121003_003*	P04083	38.6	6.64
1146	2.0	T-plastin *DJW121003_002*	P13797	70.4	5.50
1312	T4 only	Adenine phosphoribosyltransferase *DJW121003_005*	P07741	19.5	5.79
1596	0.25	Aldose reductase	P15121	35.7	6.55
		Annexin A1	P04083	38.6	6.64
		Glyceraldehyde 3-phosphate dehydrogenase	P04406	35.9	8.58
		Annexin A2	P07355	38.5	7.56
		Mitochondrial 39s ribosomal protein L39 *DJW121003_008*	Q9NYK5	34.2	6.47
1858	0.22	Ubiquinol-cytochrome C reductase iron-sulfur subunit	P47985	29.7	8.55
		3-hydroxyacyl-CoA dehydrogenase type II *DJW121003_010*	Q99714	26.9	7.65
1598	0.12	Electron transfer flavoprotein beta-subunit	P38117	27.8	8.25
		Sepiapterin reductase *DJW121003_009*	P35270	28.0	8.25
1630	0.09	Isocitrate dehydrogenase [NAD] subunit alpha *DJW121003_007*	P50213	39.6	6.46

* Note: For spots #1596, #1858 and #1598, more than one protein was identified from the digested peptides, suggesting that these proteins co-migrated in the gels. However the first in the list is the most significant hit.

Table 2: Extracellular proteins released from T4-2 cells grown in 3D lrECM, as identified by Q-TOF mass spectrometry

Spot Number	SwissProt Entry Name	Protein Name	Accession Number	Molecular Weight (kDa)	pI (theoretical)
V1028	CO3_human	Complement component 3	P01024	187.2	6
V1150	PDA3_human	Protein disulfide isomerase A3 (ER60 precursor)	P30101	56.7	6.3
V1030	MASP_human	Maspin (protease inhibitor 5)	P36952	42.1	6
V1038	A1AT_human	Alpha-1 protease inhibitor	P01009	46.7	5.5
V1040	NDR1_human	N-Myc downstream regulated gene 1 protein	Q92597	42.8	5.7
V1010	PAI1_human	Human plasminogen activator inhibitor type-1	P05121	45	7.1
V1044	PMG1_human	Phosphoglycerate mutase 1	P18669	28.8	7.2
V1050	LDHB_human	L-lactate dehydrogenase B chain	P07195	36.6	6
V1232	G3P2_human	Glyceraldehyde-3-phosphate dehydrogenase	P04406	36	8.8
V1090	LDHA_human	L-Lactate dehydrogenase A	P00338	36.7	8.6
V1060	143S_human	14-3-3-protein sigma (79% sequence coverage, with no phosphorylated site found)	P31947	27.7	4.7
V1052	GTP_human	Glutathione S-transferase P	P09211	23.3	5.5
V1138	LAMA_human	Lamin A	P02545	74.1	7
V1098	PDX6_human	Peroxiredoxin 6	P30041	25	6.3
V1026	TPIS_human	Triosephosphate isomerase	P60174	26.6	6.9
V1144	GDIB_human	Rab GDP dissociation inhibitor beta	P50395	50.6	6.4

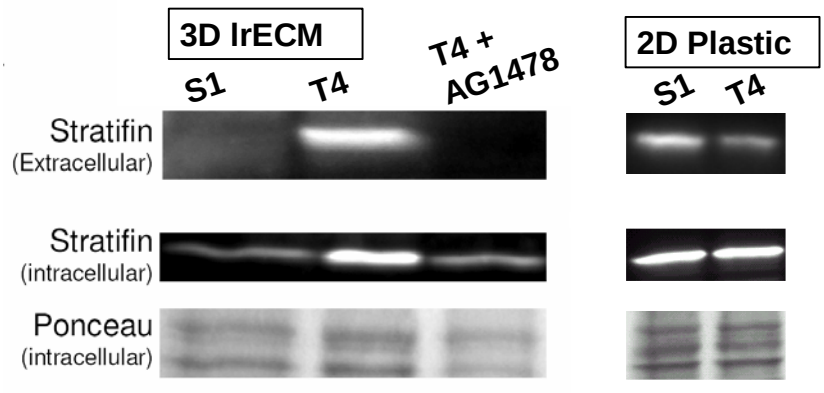

Figure 1: Stratifin expression and release is increased in T4-2 cells grown in 3D lrECM, but not when grown in 2D. Conditioned media was collected from cells over 48 hours (between days 2-4; or between days 6 and 8 for S1 in 2D; in triplicate) and was concentrated in a 5kDa molecular weight cutoff centrifugal filter (Millipore™). Both extracellular and intercellular stratifin were evaluated after the 48 hour collection period. Intercellular protein was collected by extracting cell colonies from the lrECM using PBS-EDTA extraction, followed by lysis in RIPA buffer, or by lysis in RIPA buffer for cells grown on 2D plastic. SDS-PAGE gels were normalized to protein concentration. Protein loading of concentrated conditioned media was normalized to the total number of cells present in the culture as aassessed from isolated genomic DNA (data not shown).

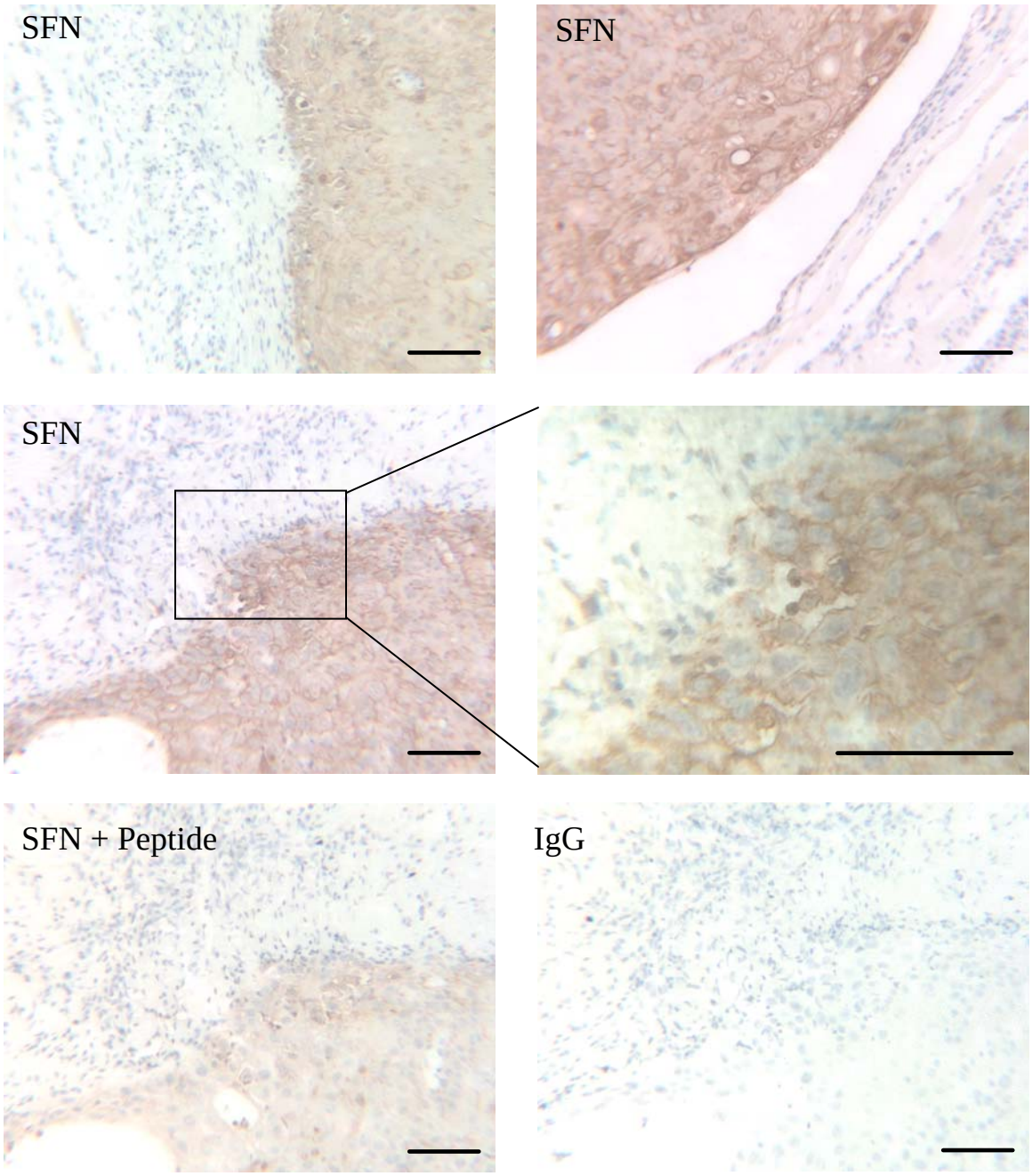

Figure 2: T4-2 cells form tumors expressing high levels of SFN when injected into nude mice. T4-2 cells were injected subcutaneously into nude mice in the presence of 3D lrECM by Aylin Rizki. Serial sections were stained with a SFN-specific antibody (SFN), antibody pretreated with a blocking peptide (SFN + Peptide), or a non-specific IgG (IgG) as indicated. Scale bar = 100 μM

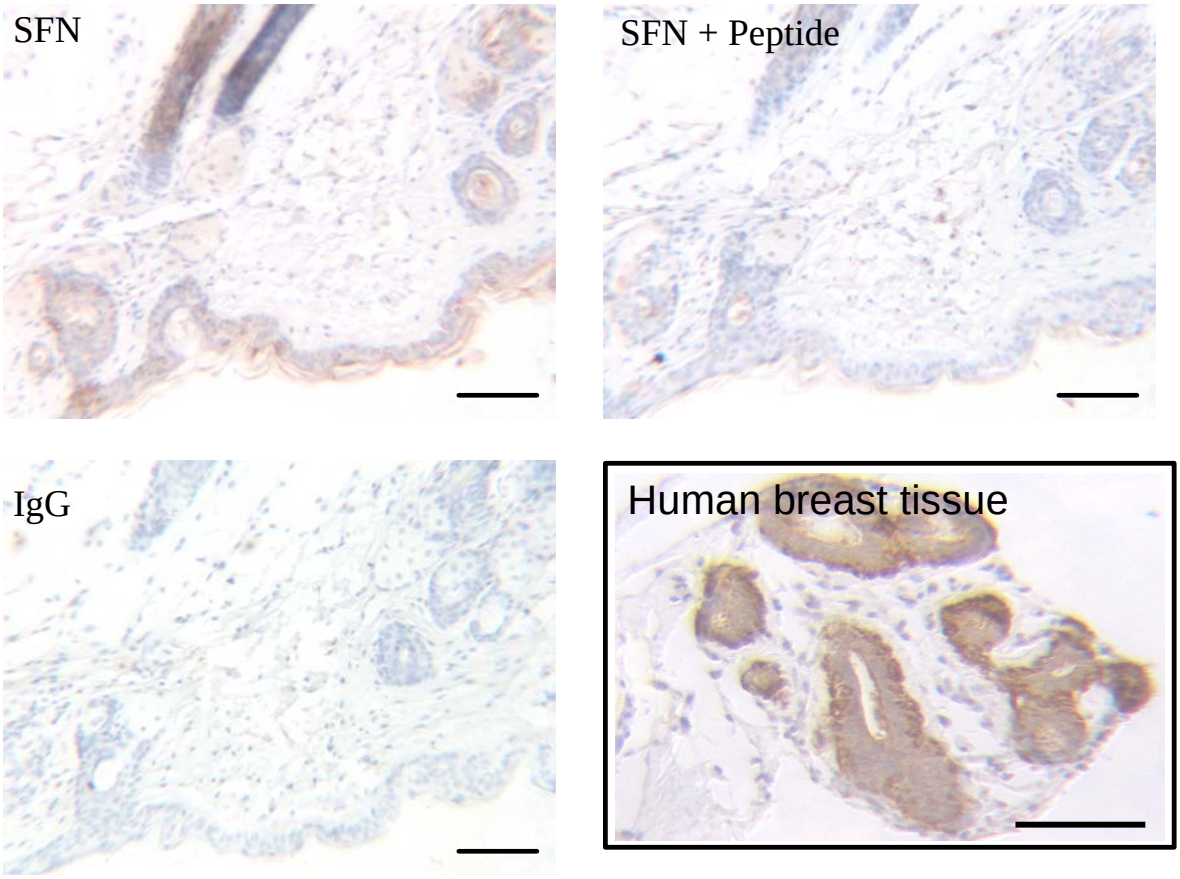

Figure 3: Basal keratinocytes in the mouse skin and myoepithelial cells within human breast tissue are positive for SFN expression. Regions of the T4-2 xenograft slides containing mouse skin were positive for SFN expression as expected. Serial sections were stained with a SFN-specific antibody (SFN), antibody pretreated with a blocking peptide (SFN + Peptide), or a non-specific IgG (IgG) as indicated. Staining of human breast tissue (obtained commercially) showed expression of SFN within myoepithelial cells, with some staining of luminal epithelial cells. Scale bar = 100 μM

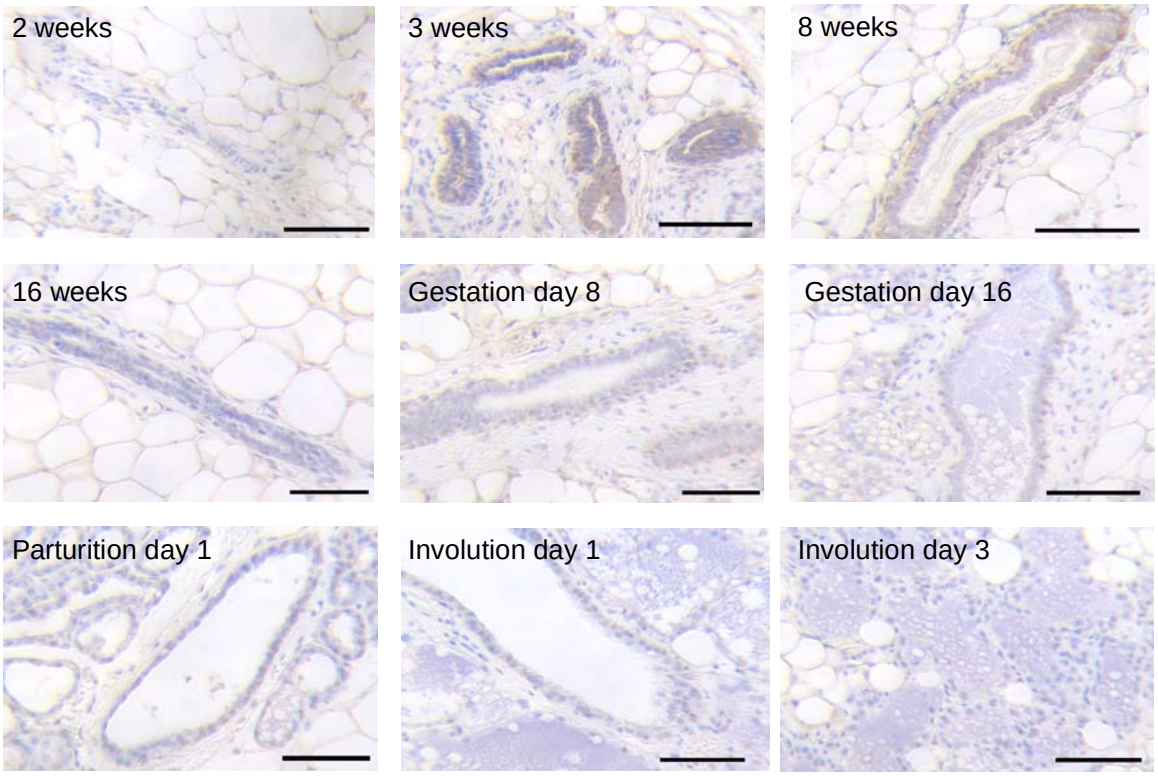

Figure 4: SFN is expressed during stages of branching morphogenesis within the mouse mammary gland. Sections of formalin-fixed, paraffin-embedded tissues (provided by Jamie Bascom) at the stages of mammary gland development indicated were labeled for SFN expression by immunohistochemistry. Staining was strongest at 3 and 8 weeks of age, with marginal staining during day 8 of gestation. Antigen retrieval was carried out by pressure cooking for 3 minutes in 10 mM citrate buffer, pH 6.0. Samples were treated identically and simultaneously. Scale bar = 100 μM.