Splicing out Cancer: Implicating Novel Splicing Factors in Metastatic Breast Cancer

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ABSTRACT
Breast cancer is among the leading causes of cancer-related mortality among Canadian women; and despite significant research investment, a clear therapeutic target remains elusive. Splicing has generated some interest as a potential oncogenic system, with evidence suggesting that alternatively spliced protein products may aid oncogenesis, and recent work has implicated overexpression of the splicing machinery itself as oncoproteins and tumor suppressors in their own right. Employing mutagenesis assays, as well as bioinformatics we previously identified a novel splicing factor SFX as well as SF3X as potential drivers of metastatic breast cancer. Here, we demonstrate both SFX and SF3X are significantly upregulated in both human and murine models of breast cancer (p<0.001). Further, abatement of known oncogenic pathways, namely the BET domain system and the inflammatory signal cascade leads to the loss of the observed overexpression of SFX and SF3X. Finally, cells display significantly altered morphology and behaviour when expressions of these splicing factors are eliminated (by targeting the inflammatory cascade). We therefore demonstrate multiple, independent oncogenic systems converge to dramatically elevate expression of SFX and SF3X, implying a novel role for these factors in metastatic breast cancer, meriting further study.
INTRODUCTION

Breast cancer is the most frequently occurring malignancy among Canadian women representing over ¼ of new metastatic neoplasia diagnoses in 2016. Despite gradual advances in therapeutic strategies over past decades, it remains the 2nd leading cause of cancer-related mortality among women. However, in most cases the primary breast tumour itself is not the immediate cause of death, rather in an estimated 90% of cases, it is metastases of this tumor which prove fatal. Thus novel treatments are increasingly focusing on abating metastases, which offers both significant therapeutic potential in reducing mortality; and with growing appreciation of the heterogeneity of carcinogenesis, an ultimately more practical treatment. However, despite intense research and numerous candidate systems, an effective therapeutic target has, as yet, eluded detection.

Splicing has, on occasion, generated some interest as a potential metastatic system. This fundamental process, found ubiquitously in all eukaryotic life, removes introns from pre-mRNA and splices exons into a contiguous molecule facilitating translation into protein. In higher organisms, splicing can also be used to augment proteomic diversity, by generating multiple spicoforms or alternatively spliced transcripts from the same gene. Some 95% of the coding human genome is now believed to be involved in alternative splicing, which thus implies a largely unexplored regulatory role for splicing as well.

Unlike other potential carcinogenic systems, splicing itself is highly conserved across species. It requires five small ribonuceloproteins (U1,2,4,5,6) as well as over 200 other associated proteins, which collectively referred to as the spliceosome, facilitate: the looping of introns onto themselves, detaching it from the mRNA, and the splicing together of adjacent exons. Intriguingly, critical sequences on both the splicing machinery and the target mRNA drive assembly and function of the spliceosome; and it is well established that mutations on either components may significantly dysregulate splicing, leading to the generation of alternative protein products.

Unsurprisingly, such aberrant proteins have been implicated in an array of cancers. Jensen et al. demonstrated alternative splicing of the Tnc-C gene (a matrix protein) prompted generation of isoforms requisite for the development of skin hyperplasia. In gliomas, alternative spicing was identified among MYC associated factor X (MAX), which led to the direct promotion of glycolytic gene expression and subsequent proliferation. Of note, MYC is among the most frequently mutated genes in cancer, including in breast cancer, and is known to play regulatory roles in a variety of cell cycle regulation systems.

Increasingly, investigations have also identified splicing components as oncogenes and tumor suppressors in their own right, and not simply as mediators of alternative splicing. In correlative studies, overexpression of splicing factor 2 (SF2) demonstrated significant reduction of senescence among murine and human epithelial cells. Other investigations have also noted that downregulation of splicing factors promoted p-53 mediated cellular senescence - a fundamental and often mutated oncogenic pathway. However, despite promising early associations, few investigations have
demonstrated definitive mechanistic explanations of splicing directly promoting metastasis. Furthermore, despite a growing appreciation of the consequences of aberrant splicing, few studies have investigated the mechanistic regulation of splicing by cancer, or how known oncogenic systems may drive changes in splicing.

In a recent sleeping beauty transposon mutagenesis screen on Retinoblastoma-deficient mammary epithelium, we observed a novel splicing factor: SFX, as a significant gene-centric common integration site (gCISs), implying a potential role in driving metastatic breast cancer. Prior studies and bioinformatics data also revealed another splicing factor of interest: SF3X. The function of neither factor is as yet definitively established, though there is evidence that SFX is likely involved in locating the 3’ branch point of the mRNA, and SF3X may be involved in general assembly and function of the spliceosome itself. Herein, we describe the expression and mechanisms of expression of these factors in metastatic breast cancer, evidencing a novel role for SFX and 3X.

RESULTS

A. SFX & SF3X ARE UPREGULATED IN METASTATIC BREAST CANCERS

In ascertaining a potential role in cancer, we first sought to clarify the expression of SFX and SF3X in metastatic malignancies versus normal mammary tissue. To do so, we obtained samples of random murine mammary tumors (which demonstrated extensive metastasis) and normal mammary tissue from the same individual. Recognizing that mammary tumors are largely epithelial in origin, lineage depletion by magnetic separation was performed on the normal mammary tissue to isolate epithelial cells. Subsequently, the expression of SFX and SF3X were assessed by western blot.

Figure 1a: Expression of SFX and SF3X in random murine mammary cancer, in comparison to normal, lineage depleted mammary epithelial cells - controlled against GAPDH. Normal Mammary and Breast Tumor 1 were adjacent samples from the same individual, and Normal Mammary 2 and Breast Tumor 2 & 2B were likewise adjacent samples from a different random murine model. Figure 1B: Quantified difference of expression by average pixilation measure (see methods) of SFX and SF3X in cancerous and normal tissue. Statistical significance assayed by paired-t test, on sample of 3 independent exposures comparing normal and tumor.
Dramatic upregulation of SFX and SF3X was consistently observed; with pixilation estimations (see methods) observing significantly ($p < 0.001$) higher expression of both splicing factors in tumors compared to normal tissue, implying both factors are dynamically upregulated upon mammary cells becoming cancerous. However, recognizing the obvious limitations in translating murine model results to human cancer, we further examined expression of both factors in a variety of human model breast cancer cell lines, via western blotting. In all lines, we observed high expression of SFX and SF3X, with some isoform heterogeneity among slower growing lines, such as MDMBA-468, and H263T in the expression pattern of SFX. Intriguingly, SFX and 3X expression are consistent with each other, in that higher expression of one appears to occur concurrently with higher expression of the other.

Thus, in murine and modeled human breast cancers, consistently high expression of SFX and SF3X was observed; and in metastatic murine models, carcinogenesis appears to promote expression of both factors. Having demonstrated upregulation, we next sought to identify the systems by which SFX and SF3X were thus induced.

**B. LOSS OF THE BET-BROMO DOMAIN SYSTEM ELIMINATES SF3X EXPRESSION**

The BET proteins are a well-established oncogenic system associated in epigenetic and chromosomal regulation of tumor suppressors as well as exercising direct control on cell growth and proliferation via understood signal transduction pathways.\(^{16-18}\) This system has been targeted by a variety of drugs including JQ1 which can effectively prevent the interaction of the BET domains with chromatin, thus preventing their regulatory action.\(^{16-18}\)

Upon exposure to JQ1, human breast cancer cells (MDMBA-231) showed significantly less expression of SF3X, with complete loss of expression occurring at doses exceeding 50nM. Thus, the BET domain system may be involved in driving the upregulation of SF3X observed in cancerous tissue.
C. LOSS OF INFLAMMATORY SIGNALLING ELIMINATES SFX AND SF3X EXPRESSION

Inflammatory signalling is another well-established oncogenic system, which is known to perpetuate cell proliferation, tumor development as well as angiogenesis - which is heavily implicated in the most metastatic cancers.\textsuperscript{19-20} As with BET proteins, modes of signal transduction are understood, and numerous drugs exist which impede these pathways at various points. AMX, is one such drug which prevents the transduction of several inflammatory markers.

Upon exposure to AMX, expression of both SFX and SF3X are dramatically decreased in model breast cancer cells (MDMBA-231), with complete inhibition of both splicing factors evident at doses exceeding 500nM. Therefore, it is likely that inflammatory signalling may also be involved in upregulating SFX and SF3X in cancer.
CELL GROWTH AND MORPHOLOGY

Having observed stark loss of SFX and SF3X in the above model, we next asked whether these cancer cells (having lost SFX and SF3X expression) display any difference in morphology and growth rate in comparison to untreated models. Initially, no significant visual differences were observed among healthy cells treated with AMX. However, when cells were first starved, over 4 days without fresh media, and subsequently treated, visibly different cell behaviour is evident.

First, upon starvation, cells decrease in size, and appear to adopt a rounder morphology - perhaps indicating a propensity for metastasis. However, after exposure to varying doses of AMX, cells greatly increase in size, and form extensive appendages, analogous to neural networks. Here, a cell’s metastatic potency is limited as a larger size and increased cell/cell interaction impede detachment and ultimate invasion into the vasculature - both of which are requisite for metastasis. The cell density is also significantly reduced, going from perfect confluence to largely vacant, implying high mortality among cells with high AMX exposure. Here, we have not yet demonstrated loss of SFX and 3X as solely responsible for the change in cell character, as AMX mediates other factors - including its primary inflammatory targets, which may account for this change in behaviour. However, in this cursory correlation, we note that in cells incidentally lacking SFX and SF3X, we observe lower density and lower metastatic potency.

**Figure 4:** a - Live MDMBA-231 cells under shadow light microscopy at approximately 100X magnification, 12 hours after first exposure to AMX (at indicated concentration) after 4 day starvation. 4b - Average cell density per square millimetre, sampled 10 times per sample, per exposure, at varying time points after first exposure to AMX, with no statistical significant observed between exposures (p>0.81), however significant differences occurring between control (CTR) and all drug concentrations (p>0.05). Note: Error bars denote 1 standard deviation about mean density. 4c - Relative cell size by average pixilation measure upon exposure to increasing concentrations of AMX normalized to control (no exposure), sampled 10 times, per sample, per exposure. Statistically significant difference observed among CTR and 500nM and 1000nM exposure (p>0.01). Note: Error bars denote 1 standard deviation about mean cell size.
DISCUSSION

This preliminary investigation into the role of newly identified SFX, and its associated spliceosome partner SF3X, have noted that aberrant expression of both factors occur in breast cancer. Specifically, we noted than in random murine models, metastatic cancerous mammary tissue significantly overexpressed both SFX and SF3X. This was analogously demonstrated in a variety of human model breast cancer lines, which all demonstrated prominent expression of both factors, though some lines appeared to express alternative isoforms of SFX, evident by visibly different banding patterns. There is as yet no investigation into the basis of this difference of SFX among cells lines, thus meriting further study.

We further observed two independent, and well-established oncogenic systems, namely the inflammatory signalling cascade, and the BET-Bromo Domain contribute to SFX and SF3X expression, with the latter targeting SF3X only. This convergence of two unrelated oncogenic systems onto splicing provides strong evidence that splicing, and SFX and 3X in particular, may serve an important role in the development and progression of cancer, which has hitherto been unexplored. Further studies should attempt broader investigations examining alternate interactions to observe whether additional systems may be implicated in upregulating splicing and splicing machinery. This investigation also observed that in cells lacking SFX and SF3X, induced by exposure to AMX - cell density significantly decreases, while cell size increases, and simultaneously, morphology and cell/cell interactions are also visibly different. Here, we note again that this study cannot assert that the observed differences were due to SFX and SF3X loss alone, as AMX exposure may mediate alternative interactions which may have produced this difference. However, it is intriguing that in cells undergoing such dramatic transformation, both factors - which we have demonstrated to be involved in multiple oncogenic systems, are lost. Further studies, being conducted at present, will generate knockdowns of SFX, SF3X and both SFX & SF3X, employing lentiviral gene suppression strategies to identify what specific effect the loss of these factors alone will yield. Simultaneously, models over expressing these factors will also be assessed. Furthermore, prior studies have also observed that dramatic changes in splicing machinery may facilitate alternative splicing of RNA, yielding different protein products. Thus, future studies will also attempt to ascertain if the transcriptome of cells with mutated SFX and SF3X is significantly different by whole RNA sequencing.

It is noteworthy that the splicing machinery of a cell is known to be highly sensitized to mutation and alteration. Prior study has even demonstrated that mutations of the splicing machinery seem to occur almost mutually exclusively, in that cells cannot appear to tolerate more than a single major mutation in the core spliceosome at a given time. In fact, mechanistic studies in developmental biology have demonstrated that cells with mutated splicing components require a corresponding wild-type copy for survival, and the functional copy may inhibit expression and function of mutants. Our results corroborate this, as cells losing both SFX and SF3X expression (by AMX exposure) showed significantly reduced density and altered morphology. Furthermore, homozygous mutations which remain viable are found almost entirely at 3' identification sites, on 3 to 4 factors - with most others proving lethal. The fact that SFX’s role appears to be in the recognition of the 3’ branch site may thus
imply that it is among a select few splicing factors which cancerous systems may target. Overall, it is unclear why splicing systems remain so sensitive to mutation, yet this may prove a crucial target in combatting cancer as it appears, by the sheer scale of overexpression and the implication of multiple oncogenic pathways in this upregulation, that splicing and SFX and SF3X specifically, are implicated in metastatic breast cancer.

METHODS

MURINE EXPRESSION ANALYSES

All work involving mice were performed in accordance with practices outlined by the Canadian Animal Care Council guidelines, and under the oversight of the Toronto General Research Institute’s Animal Research Committee, and the Research Ethics board. Mice were obtained at random, to assay general expression of SFX and SF3X, and were obtained at time of sacrifice for other investigations. Tumor and normal mammary samples were obtained under typical aseptic surgery techniques, and were macerated and lyzed immediately.

LINEAGE DEPLETION

To extract epithelial cells from normal tissue magnetic cell sorting was performed where epithelial stem cells were labeled with magnetic beads and extracted, under standard depletion procedures outlined by Thermo Fisher Scientific® and prior study as noted by Lee et al. 26

ASSAYING EXPRESSION

The expression of all proteins was assessed by standard Tris-glycine gel based western blotting procedures, with antibodies obtained by Cedarlane®. Expression was quantified by comparing the pixelated area of the blot, where the boundaries were defined at points less than 25% different in colour from the centre of the blot, sampled visually. Relative expression was then established by normalizing the obtained area to that of the relevant control.

VISUALIZING CELLS

Cell lines were obtained from frozen stocks maintained by the Zacksenhaus laboratory and handled under standard aseptic techniques. Live cells were visualized under simple light microscopy, with shadow lighting, and no other staining procedure. Cells were imaged under constant magnification, and subsequently quantified for number by counting all cells within the prescribed field of view. To quantify size, the pixels occupied by cells were calculated by radially measuring the furthest arm of the cell from the centre (define visually) and the area of the circle defined as the cell size. As prior, cell size was normalized to the relevant control to ascertain relative change.
SLEEPING BEAUTY

Sleeping beauty transposon mutagenesis assays were conducted by the Zacksenhaus lab, in accordance with established procedures outlined by Moriarty et al.\textsuperscript{27}

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