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EXTRA VIEW

Relationship between RUNX1 and AXIN1 in ER-negative versus ER-positive Breast Cancer

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ABSTRACT

RUNX1 plays opposing roles in breast cancer: a tumor suppressor in estrogen receptor-positive (ER^+) disease and an oncogenic role in ER-negative (ER^-) tumors. Potentially mediating the former, we have recently reported that RUNX1 prevents estrogen-driven suppression of the mRNA encoding the tumor suppressor AXIN1. Accordingly, AXIN1 protein expression was diminished upon RUNX1 silencing in ER^+ breast cancer cells and was positively correlated with AXIN1 protein expression across tumors with high levels of ER. Here we report the surprising observation that RUNX1 and AXIN1 proteins are strongly correlated in ER^- tumors as well. However, this correlation is not attributable to regulation of *AXIN1* by RUNX1 or vice versa. The unexpected correlation between RUNX1, playing an oncogenic role in ER^- breast cancer, and AXIN1, a well-established tumor suppressor hub, may be related to a high ratio between the expression of variant 2 and variant 1 (v2/v1) of AXIN1 in ER^- compared with ER^+ breast cancer. Although both isoforms are similarly regulated by RUNX1 in estrogen-stimulated ER^+ breast cancer cells, the higher v2/v1 ratio in ER^- disease is expected to weaken the tumor suppressor activity of AXIN1 in these tumors.

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Introduction

In addition to their developmental roles, the 3 transcription factors in the mammalian RUNX family play context-specific roles in cancer as either tumor suppressors or oncogenes.[1-6] RUNX1 is a master regulator of haematopoietic cell fate determination and is frequently disrupted in leukemias.[7-11] Recently, its role in estrogen receptor-positive (ER^+) breast cancer suppression has been disclosed based on identification of recurrent somatic mutations and/or deletions in tumor biopsies.[12-14] Even though *Runx1* knockout is insufficient for breast cancer initiation,[15] its silencing in ER^+ breast cancer cells *in vitro* has been shown to increase cell proliferation and expression of stem cell markers, attributable to decreased expression of the tumor suppressor AXIN1.[1]

AXIN1 is a multidomain scaffold protein with a tumor suppressor activity mostly attributable to its role as a rate-limiting factor in the ß-catenin destruction complex.[16-18] Besides its well-known function as a negative regulator of Wnt/ß-catenin signaling,[17-19] AXIN1 has been implicated in coordinating several other pathways including TGFβ, SAPK/JNK, p53, YAP/TAZ and Myc.[20-24] Two major isoforms of AXIN1 have been described, with variant 1(v1) comprising 11 exons and v2 lacking exon 9, which encodes a PP2A binding domain that likely plays a role in destabilizing Myc by dephosphorylation of S62[22]. Although AXIN1 is a well-recognized tumor suppressor with multiple mutations identified in several different cancers,[25-28] no recurrent mutations in *AXIN1* have been identified in breast cancer.[14] In our recent study, however, we demonstrated that RUNX1 and estrogens combinatorially regulated the *AXIN1* gene in breast cancer cells, so that *AXIN1* expression was downregulated when RUNX1 was lost while ER was active.[1] Additionally, breast carcinogenesis is accompanied with an increase in the v2/v1 ratio between the 2 AXIN1 isoforms.[29]

Despite significant improvements to early detection and the development of effective hormonal and other therapies, breast cancer is predicted to claim more than 40,000 female lives in the United States in 2016[30]. Heterogeneity of breast cancer is critical in disease management. More than 2 thirds of all tumors are ER^+ and/or $PR^+/HER2^-$, 12% are triple negative breast cancer (TNBC), 10% are ER^+ and/or $PR^+/HER2^+$, and 5% are $ER^-/HER2^+$ [31]. $HER2^+$ and TNBC patients have poor survival compared with ER^+ breast cancer patients. Whereas ER^+ and $HER2^+$ patients can benefit from hormonal (Tamoxifen, Fulvestrant, Letrozole)[32-36] and anti-HER2 therapies (Trastuzumab, Lapatinib),[37,38] no targeted therapeutic approaches are available for aggressive TNBC.[39-42]

An increasing body of evidence points at context-dependent functional interaction between sex hormone steroid

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signaling and the roles that RUNX proteins play in cancer.[43-48] In breast cancer, interactions of RUNX proteins with estrogen signaling are critical for their roles as either tumor suppressors or oncogenes.[1,6,49,50] Recurrent inactivating mutations in *RUNX1* are specific to ER^+ tumors.[14,15] Rather than functioning as a tumor suppressor, RUNX1 expression in TNBC correlates with poor prognosis and its silencing in a cell culture model of TNBC ameliorates cancer-related phenotypes.[51-53] In this study we report the unexpected positive association between RUNX1 and AXIN1 in ER^- breast cancer and present evidence suggesting that the association of RUNX1 with tumor aggression in this breast cancer subtype can be explained in part by the preferential expression of AXIN1v2.

Materials and methods

Cells

The ER^+ MCF7 and the ER^- MDA-MB-231 breast cancer cell lines were obtained from the American Type Culture Collection and cultured in DMEM (Mediatech, Inc.) supplemented with 10% FBS (Gemini Bio-products) and in DMEM/F12 (Mediatech, Inc.) supplemented with 5% FBS, respectively. Construction of the MCF7/shRx1^{dox} cells and MDA-MB-231/shRx1^{dox} cells, conditionally expressing shRNAs for *RUNX1* upon doxycycline (dox) treatment, has been described previously.[1] For hormone depletion, cells were washed 3 times with PBS and maintained for 48 h in phenol-red free growth medium supplemented with 10% charcoal stripped serum (CSS) (Gemini Bio-products) before treatment.

RT-qPCR

Total RNA was isolated using Aurum^{TM} Total RNA mini-kit (BioRad) and cDNA was synthesized from 1 μg of total RNA with qScript^{TM} cDNA SuperMix (Quanta Biosciences). Quantitative Real Time PCR was performed in triplicate using Maxima SYBR Green/Fluorescein Master Mix (ThermoFisher Scientific) with CFX96 instrument (BioRad). Relative mRNA expression values were normalized to 18S RNA. Primers used for RT-qPCR were 5′ -CAAGCAGAGGTATGTGCAGGA- 3′ (Forward) and 5′ -CACAACGATGCTGTCACACG- 3′ (Reverse) for Axin1 v1; and 5′ -AAGCAGAGGACAAGATCGCA- 3′ (Forward) and 5′ -CGCAGAAGTAGTACGCCACA- 3′ (Reverse) for Axin1 v2.

Tissue microarray analysis

Breast cancer tissue microarray (TMA) slides used in this study were purchased from Protein Biotechnologies, Inc. (TM-1007). Represented in this TMA are 34 ER^- tumors, including 33 cases of invasive ductal carcinoma and 1 case of ductal carcinoma in situ. TMA slides were immunostained as described previously using antibodies against RUNX1 (#8529) or AXIN1 (#2087) from Cell Signaling Technology.[1] ER and Ki67 histoscores were provided by the manufacturer and presence or absence of RUNX1 and AXIN1 was determined by a certified surgical pathologist at USC. Association between the RUNX1 and AXIN1 status was tested using the Pearson chi-square test for the 2 × 2 table using MedCalc (http://medcalc.com).

Data mining

RNA-sequencing data for the breast cancer cohort of TCGA was downloaded from the TCGA Data Portal (http://cancergenome.nih.gov/). Isoform sequencing data and exon sequencing data for *AXIN1* was analyzed using Partek Genomics Suite 6.4 (Partek, Inc.).

Results

Correlation between RUNX1 and AXIN1 in ER^- breast cancer tumors

Based on clinical data mining of RUNX1-depleted ER^+ breast cancer cells and genome-wide analyses of ER^+ breast epithelial cells *in vivo* and *in vitro*, we have recently demonstrated that RUNX1 antagonized estrogen-mediated AXIN1 suppression.[1]

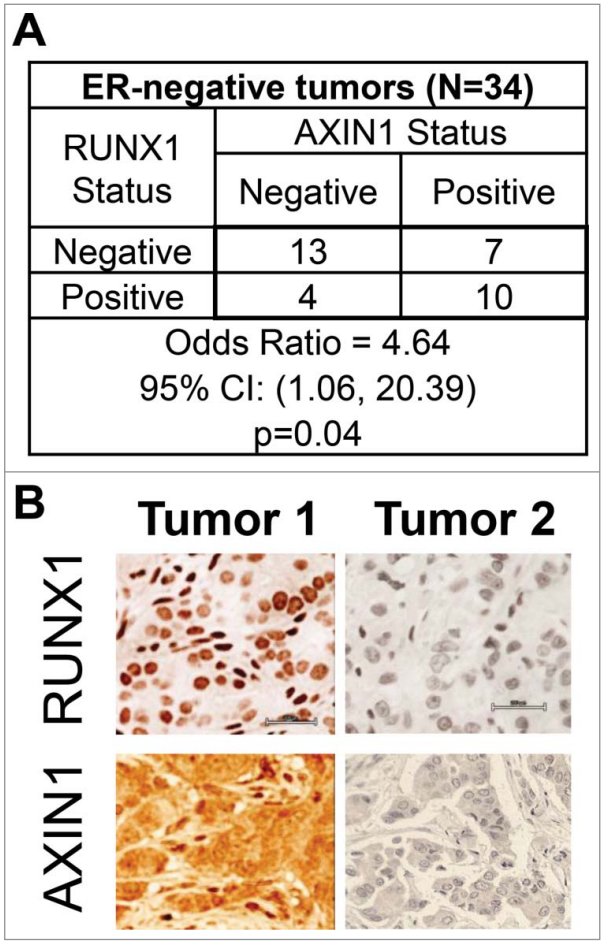

Figure 1. Association between RUNX1 and AXIN1 in ER^- breast cancer tumors. Breast cancer tumor microarray TMA-1007 from Protein Biotechnologies, Inc. was immunostained for RUNX1 and AXIN1. The ER^- invasive ductal carcinomas were designated as positive or negative for RUNX1 and AXIN1. (A) RUNX1 and AXIN1 status, and the odds ratio and 95% confidence intervals for the association between AXIN1 status and RUNX1 status in the ER^- tumors in the TMA. Association between the RUNX1 status and AXIN1 status was tested using the Pearson chi-square test for the 2 × 2 table. (B) RUNX1 and AXIN1 immunohistochemical staining of 2 representative ER^- tumors from the TMA illustrating the association between RUNX1 and AXIN1 expression.

Consistent with this model, immunohistochemical analysis of 31 ER$^+$ breast cancer tumors in a tissue microarray (TMA-1007 from Protein Biotechnologies) indicated positive correlation between RUNX1 and AXIN1 in a manner dependent on ERα expression.[1] Analysis of 34 ER$^-$ tumors that were represented in the same TMA revealed that RUNX1 and AXIN1 were strongly correlated in the ER$^-$ tumors as well (Fig. 1). This observation was unexpected because unlike ER$^+$ breast cancer cells, RUNX1 manipulation in ER$^-$ breast cancer cells did not affect AXIN1 expression.[1] Additionally, unlike in ER$^+$ tumors, RUNX1 does not appear to play a tumor suppressor role in ER$^-$ breast cancer. Not only is the RUNX1 locus devoid of recurrent mutation,[14] RUNX1 expression in ER$^-$ breast cancer actually correlates with disease aggression.[51-53]

Alternative splicing of AXIN1 in ER$^-$ vs. ER$^+$ breast cancer

RUNX1 appears to play opposite roles in ER$^+$ and ER$^-$ breast cancer, yet the tumor suppressor AXIN1 is positively correlated with RUNX1 in both ER$^+$ and ER$^-$ tumors (Fig. 1 and ref. 1). To resolve this conundrum, we hypothesized that ER$^-$ tumors express at relatively higher level of the cancer-associated variant 2 of *AXIN1* (*AXIN1v2*). We tested this hypothesis by interrogating the breast cancer RNA-seq database of TCGA. Indeed, although v2 is the transcript expressed at higher levels across all tumor subtypes, its levels are highest in the basal-like subtype and the v2/v1 ratio is higher in ER$^-$ (Basal-like and HER2-enriched) compared with ER$^+$ (Luminal A and Luminal B) tumors (Fig. 2A). Accordingly, a heat map describing expression of each AXIN1 exon across the breast cancer tumors in TCGA demonstrates reduced expression of AXIN1 exon 9 in ER- tumors, in particular the basal-like subtype (Fig. 2B).

Positive correlation of each of RUNX1 and AXIN1 with the Ki67 index in ER$^-$ breast cancer

We next calculated the correlation between each of RUNX1 and AXIN1 across the 34 ER$^-$ breast cancer tumors in the TMA-1007 tissue microarray with the Ki67 index provided by the manufacturer. Consistent with the proposed oncogenic role of RUNX1 in ER$^-$ breast carcinogenesis,[51-53] its expression was positively correlated with the Ki67 index ($p = 0.01$; Table 1). Less expectedly, AXIN1 expression was also positively correlated with the Ki67 index (Table 1). This finding suggests that AXIN1 plays a weak, if any, tumor suppressor role in ER$^-$ breast cancer, possibly related to the predominance of AXIN1v2 in these tumors.

Association between AXIN1 and RUNX1 in ER$^-$ breast cancer does not involve transcriptional control

The correlation between RUNX1 and AXIN1 across ER$^+$ breast cancer is attributable to RUNX1-mediated antagonism of *AXIN1* transcriptional repression by estrogens.[1] The positive correlation between RUNX1 and AXIN1 in the ER$^-$ tumors (Fig. 1) is misaligned with our observations that *RUNX1* silencing did not decrease AXIN1 expression in estrogen-deprived ER$^+$ MCF7 cells (CSS without E2 supplementation) or in ER$^-$ MBA-MB-231 cells.[1] We suspected that this misalignment was attributable to differential regulation of the 2 AXIN1 variants. However, RT-qPCR analysis revealed similar regulation of AXIN1v1 and AXIN1v2. They were both downregulated by RUNX1 silencing in MCF7 cultures in the presence of estrogens (Fig. 3B-C) and they were both unaffected by RUNX1 silencing in hormone depleted MCF7 cultures (Fig. 3A) and in the ER$^-$ MDA-

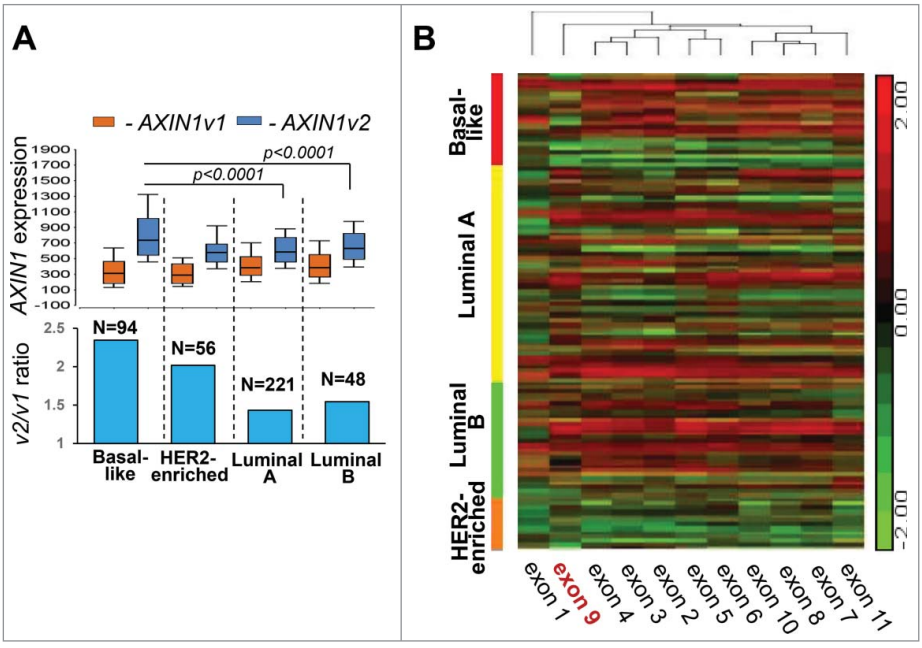

Figure 2. AXIN1 splice variant expression in breast cancer subtypes. (A) Box-whisker plot describing the differential expression of AXIN1 variants (upper panel) and their ratio (lower panel) in breast cancer subtypes. (B) Semi-supervised hierarchical clustering for expression of AXIN1 exons in breast cancer subtypes. Expression of AXIN1 variants was represented by RSEM normalized values of the individual isoforms and expression of AXIN1 exons was represented by RPKM values in the Level-3 RNA-seqV2 data downloaded from the TCGA data portal. P-values were calculated by ANOVA.

Table 1. Association between RUNX1 or AXIN1 and Ki67.

ER-negative tumors (N = 34)		
	Ki-67 status	
RUNX1 Status	Negative / Mild	Positive / Strong
Negative	13	6
Positive	3	11
	Odds Ratio = 7.94 95% CI: (1.69, 39.42) p = 0.01	
	(N = 34)	
	Ki-67 status	
AXIN1 Status	Negative / Mild	Positive / Strong
Negative	11	5
Positive	5	13
	Odds Ratio = 5.7 95% CI: (1.3, 25.05) p = 0.02	

MB-231 cell cultures (Fig. 3D). These results suggest that the positive correlation between RUNX1 and AXIN1 in ER$^-$ breast cancer (Fig. 1) is not attributable to a RUNX1-mediated control of *AXIN1* transcription.

Discussion

RUNX proteins are known to exert context-dependent opposing roles in breast cancer. Potentially reflecting subtype-specific features, RUNX1 somatic mutations occur recurrently in ER$^+$ but not in ER$^-$ breast cancer.[14] The tumor suppressor role of RUNX1 in ER$^+$ tumors[12-14] is in part is attributable to antagonism of estrogen-mediated *AXIN1* suppression.[1] In ER$^-$ tumors, on the other hand, RUNX1 predominantly plays an oncogenic role, reflected in positive correlation with disease aggression and mortality[52,53] as well as cancer-related phenotypes *in vitro*.[51,52] Apparently inconsistent with the oncogenic role of RUNX1 in ER$^-$ breast cancer, its expression in these tumors is positively correlated with AXIN1 (Fig. 1). Our findings suggest that RUNX1-positive ER$^-$ tumors remain aggressive despite AXIN1 expression because they express the AXIN1 variants differently than ER$^+$ tumors. Specifically, the ER$^-$ tumors express AXIN1 with a higher ratio between variant 2 and variant 1 (Fig. 2A).

The mechanism underlying the positive correlation between RUNX1 and AXIN1 in ER$^-$ breast cancer remains to be elucidated. Unlike in ER$^+$ breast cancer cells, RUNX1 does not regulate AXIN1 mRNA in ER$^-$ breast cancer cells (Fig. 3). Additionally preliminary studies demonstrated indifference of RUNX1 expression to IWR1-mediated upregulation of AXIN1 (data not shown) suggesting that AXIN1 does not regulate RUNX1 expression. We cannot rule out regulation of *AXIN1* by RUNX1 *in vivo* through a mechanism not supported in our culture models (Fig. 3). It is also possible that RUNX1 and AXIN1 are both regulated, independently, by a common upstream pathway.

Even though RUNX1 does not regulate *AXIN1* in ER$^-$ breast cancer cells (Fig. 3), and even though its correlation with AXIN1 expression (Fig. 1) is easier to interpret given the ratio between the AXIN1 isoforms (Fig. 2), the molecular mechanisms underlying the oncogenic role of RUNX1 (Table 1 and refs.[51-53]) are poorly understood. To fulfill its oncogenic role, RUNX1 might employ mechanisms similar to those employed by RUNX2 in promoting cancer aggression.[2,4,5,43,54-62] Indeed, about 2 thirds of the RUNX1 transcriptome is shared with the RUNX2 transcriptome in estrogen-deprived MCF7 cells.[1] Conceivably, these shared genes contribute to aggressive disease and high mortality of patients with ER$^-$ /RUNX1$^+$ tumors. The present study contends that high AXIN1 expression in these tumors does not provide sufficient tumor suppression because of the differential enrichment for variant 2 of AXIN1.

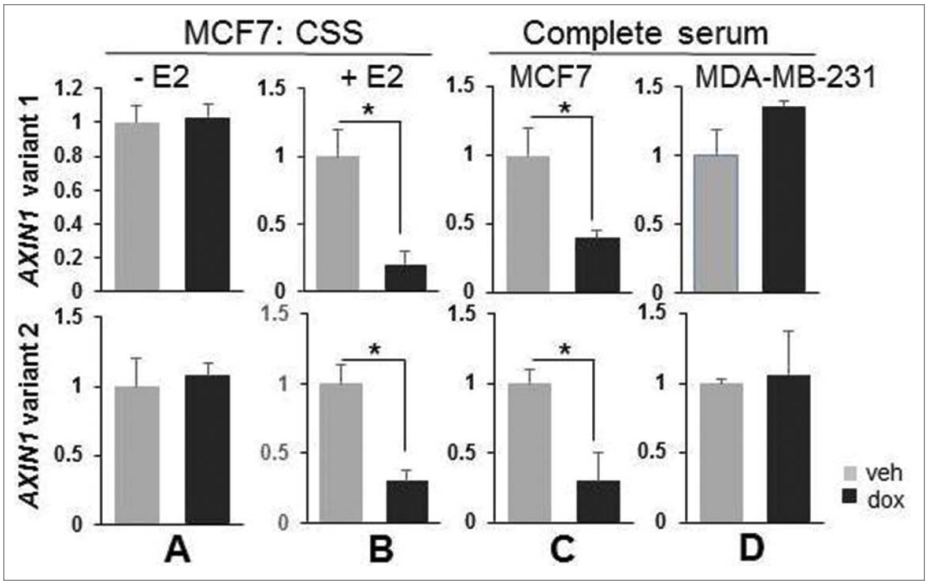

Figure 3. RUNX1 regulates both AXIN1v1 and AXIN1v2 in an estrogen-dependent manner. MCF7/shRx1^{dox} (A-C) and MDA-MB-231/shRx1^{dox} cells (D) were maintained in either 10% charcoal-stripped serum (A-B) or complete (estrogen-containing) 10% serum (C-D), and treated with dox to silence RUNX1 (A-D) and E2 (only B) for 48 h. Expression of *AXIN1* transcripts v1 and v2 was measured by RT-qPCR and corrected for 18S RNA levels (Mean ± SEM of 3 independent experiments). $^*p < 0.05$ by t-test.

Disclosure of potential conflict interest

No potential conflicts of interest were disclosed

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