2015-01-01

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RESEARCH COMMUNICATION

A laminin 511 matrix is regulated by TAZ and functions as the ligand for the α6Bβ1 integrin to sustain breast cancer stem cells

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Understanding how the extracellular matrix impacts the function of cancer stem cells (CSCs) is a significant but poorly understood problem. We report that breast CSCs produce a laminin (LM) 511 matrix that promotes self-renewal and tumor initiation by engaging the α6Bβ1 integrin and activating the Hippo transducer TAZ. Although TAZ is important for the function of breast CSCs, the mechanism is unknown. We observed that TAZ regulates the transcription of the α5 subunit of LM511 and the formation of a LM511 matrix. These data establish a positive feedback loop involving TAZ and LM511 that contributes to stemness in breast cancer.

Supplemental material is available for this article.

Received October 10, 2014; revised version accepted November 21, 2014.

Most tumors contain a distinct population of cells with stem cell characteristics, including the ability to self-renew and populate new tumors. This population is often referred to as tumor-initiating or cancer stem cells (CSCs) (Al-Hajj et al. 2003; Baccelli and Trumpp 2012; Visvader and Lindeman 2012). CSCs are likely responsible for tumor recurrence in response to therapy and may contribute to metastasis (Dean et al. 2005; Calcagno et al. 2010; Pinto et al. 2013). For this reason, deciphering the mechanisms that generate and sustain CSCs is a problem of paramount importance. This issue has been the focus of intense investigation in recent years, resulting in considerable advancements in the understanding of the nature of CSCs (Visvader and Lindeman 2012; Beck and Blanpain 2013; Kreso and Dick 2014) and the realization that their genesis and function can be determined by their microenvironment (Scheel and Weinberg 2012). Given this surge in CSC biology, it is surprising that the contribution of the extracellular matrix (ECM) has not been investigated more rigorously. Although the ECM is presumed to impact the genesis and function of CSCs (Lane et al. 2014; Wong and Kumar 2014), much remains to be learned about the nature of this involvement and the mechanisms involved.

The α6β1 integrin is an established marker of breast and other CSCs (Lathia et al. 2010; Goel and Mercurio 2013; Goel et al. 2013). Recently, however, we discovered that the α6Bβ1 integrin, a specific splice variant of the α6 cytoplasmic domain, is a determinant of breast CSC function, a function that cannot be executed by α6Aβ1, the other α6 splice variant (Goel et al. 2014; Seguin et al. 2014). Given that the α6 integrins function primarily as laminin (LM) receptors (Mercurio 1990), this finding implies that α6Aβ1 and α6Bβ1 differ in their response to LM matrices and that a specific LM functions as the preferred ligand for α6Bβ1 to sustain CSC function. This hypothesis is consistent with a large body of literature implicating the LMs in mammary gland biology and breast cancer (e.g., Streuli et al. 1995; Pouliot and Kusuma 2013). The challenge here is to identify LMs that regulate α6Bβ1 specifically and elucidate the mechanisms by which the LM/α6Bβ1 interaction contributes to the function of breast CSCs. In pursuit of this problem, we discovered that LM511 is associated with α6Bβ1 and functions as the preferred ligand for this splice variant. Importantly, we observed that high LM511 expression and the formation of a LM511 matrix niche characterize breast CSCs. Our data also reveal that LM511/α6Bβ1 activate the Hippo transducer TAZ, which has been implicated in the function of breast CSCs (Cordenonsi et al. 2011), providing a novel mechanism for LM regulation of CSC function. Unexpectedly, we discovered that the α5 subunit of LM511 is a TAZ target gene and that TAZ regulates the formation of a LM511 matrix, establishing one mechanism for how TAZ contributes to CSC function.

Results and Discussion

Breast CSCs produce a LM511 matrix that functions as the ligand for the α6Bβ1 integrin to promote self-renewal and tumor initiation

The goal of this study was to identify the α6Bβ1 ligand that enables this integrin splice variant to promote self-renewal and initiate new tumors and determine the mechanisms involved. We used two model systems

[*Keywords*: TAZ; cancer stem cell; extracellular matrix; integrin; laminin]
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Article is online at http://www.genesdev.org/cgi/doi/10.1101/gad.253682.114.

initially to achieve this goal. The CD44$^+$/CD24$^-$ population isolated from Src-transformed MCF10A cells (Iliopoulos et al. 2011) consists of distinct epithelial (EPTH) and mesenchymal (MES) populations that differ in the relative expression of the α6A and α6B splice variants and stem cell properties (Goel et al. 2014). Specifically, the MES population is enriched in α6Bβ1 expression and exhibits self-renewal and tumor-initiating ability compared with the EPTH population, which is enriched for α6A integrin expression. We also engineered SUM1315 cells to express either the α6Aβ1 or α6Bβ1 splice variants at equivalent levels of surface expression and demonstrated that α6Bβ1-expressing cells exhibit CSC properties in comparison with α6Aβ1-expressing cells (Goel et al. 2014). The gene expression profiles of the EPTH and MES populations were compared by RNA sequencing (RNA-seq) analysis (Supplemental Table S1). The data obtained revealed distinct differences in the expression of specific LM subunits between these populations, which we confirmed by real-time quantitative PCR (qPCR). Specifically, the MES population and the α6Bβ1-expressing SUM1315 cells exhibited a significant increase in the mRNA expression of the LMα5 and LMβ1 subunits and a concomitant decrease in the LMα3, LMβ2, LMβ3, and LMγ2 subunits (Fig. 1A). These results were corroborated by immunoblotting (Fig. 1B).

The LMα5 subunit is a component of LM511 and LM521 (Aumailley et al. 2005; Miner 2008). The fact that LMβ2 expression is repressed in the MES and α6Bβ1/SUM1315 cells infers that LM511 correlates with α6Bβ1 expression and stem cell properties and that it appears to be the preferred ligand for α6Bβ1. To assess this hypothesis, we assayed adhesion to LM511, LM111, fibronectin (FN), and collagen I (COL 1). Indeed, the MES population and the α6Bβ1/SUM1315 cells adhered better to LM511 than to the other matrix proteins (Fig. 1C). Also, the MES and α6Bβ1/SUM1315 cells adhered significantly better to LM511 than the EPTH and α6Aβ1/SUM1315 cells (Fig. 1C). Titration of matrix protein concentration revealed that α6Bβ1-expressing cells adhered much more avidly to LM511 than to either LM111 or FN (Fig. 1D, left; Supplemental Fig. S1A). More definitive evidence to implicate α6Bβ1 as the receptor for LM511 was obtained using transcription activator-like effector nucleases (TALENs) to disrupt the alternative splicing site in the α6 mRNA, which results in loss of α6B expression (Goel et al. 2014). TALEN-mediated depletion of α6Bβ1 inhibited adhesion to LM511 without affecting adhesion to LM111 (Fig. 1D, right). Adhesion to LM111 was not affected because depletion of α6Bβ1 increases α6Aβ1 (Goel et al. 2014), which likely functions as a LM111 receptor. The residual adhesion of MDA-231–α6B–TALEN cells to LM511 at a high concentration (5 μg/mL) appears to be mediated by α6Aβ1 because it was inhibited significantly by GoH3, an α6 inhibitory antibody (Supplemental Fig. S1B).

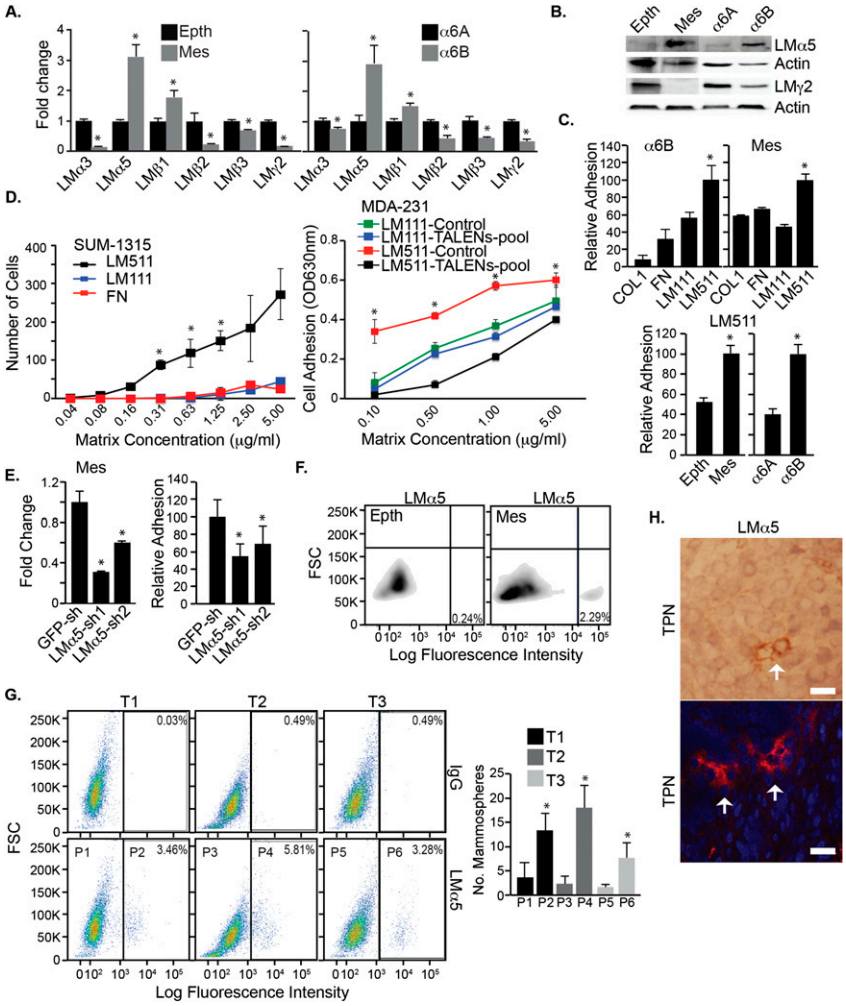

Figure 1. LM511 is the preferred ligand for integrin α6Bβ1. (*A*) Relative mRNA expression of LMα5, LMβ2, LMα3, LMβ3, LMβ1, and LMγ2 in the MES and EPTH populations of CD44$^+$/CD24$^-$ Src-transformed MCF10A cells and α6Aβ1- and α6Bβ1-expressing SUM1315 cells was quantified by qPCR. (*B*) The expression of LMα5, LMγ2, and actin was assessed by immunoblotting in these cells. (*C*) The cells described in *A* were assayed for their ability to adhere to COL 1, FN, LM111, and LM511 (1 μg/mL). (*D*, *left*) The ability of α6Bβ1-expressing SUM1315 cells to adhere within 30 min to increasing concentrations of FN, LM111, and LM511 was determined. (*Right*) Control and α6B-depleted MDA-MB-231 cells were compared for their ability to adhere to increasing concentrations of LM111 and LM511. α6B expression was depleted using TALENs as described (Goel et al. 2014). (*E*) LMα5 expression was diminished in the MES population of CD44$^+$/CD24$^-$ Src-transformed MCF10A cells using shRNAs, and the ability of these cells to adhere to glass was assayed. (*F*) Flow cytometric analysis of surface-bound LMα5 expression in EPTH and MES cells. (*G*) Three primary human breast tumors (T1, T2, and T3) were dissociated and sorted by FACS using a LMα5 Ab. Cells with low surface-bound LMα5 (P1, P3, and P4) were compared with cells with high surface-bound LMα5 (P2, P4, and P6) for their ability to form mammospheres (bar graph). (*H*) Frozen sections of human triple-negative breast cancers were stained with a LMα5 Ab, 4C7, using either immunohistochemistry (*top*) or immunofluorescence (*bottom*). Arrows depict individual cells with intense staining. Bar, 100 μm.

Our data suggest that LM511 is produced by breast CSCs and that it functions as the ligand for α6Bβ1 to promote self-renewal and tumor initiation. To test this hypothesis, we depleted LMα5 expression in MES cells and observed a significant decrease in their adhesion to glass (Fig. 1E). The possibility that breast CSCs produce a LM511 matrix was substantiated by sorting the MES cells using a LMα5 Ab. This process revealed a relatively small population of tumor cells (~2%) that exhibited high surface-bound LMα5 (Fig. 1F). A similar approach was used to analyze three primary breast tumors, and we found that each tumor contained a relatively small population of cells with high surface-bound LMα5 (Fig. 1G). Importantly, this population has an increased ability to form mammospheres (Fig. 1G) and expresses more of a MES marker (vimentin) and less of an EPTH marker (E-Cadherin) than the bulk population (Supplemental Fig. S1C,D). Also, immunohistochemistry staining of breast tumors identified a small number of cells that exhibited high LMα5 expression compared with other tumor cells (Fig. 1H).

Subsequently, we investigated the contribution of LMα5 to self-renewal and tumor initiation more rigorously. The LMα5-blocking Abs (4C7 and 8G9) reduced the ability of MES cells to form primary mammospheres, an effect that was synergistic in the presence of both Abs (Fig. 2A). Depletion of LMα5 in these cells using shRNAs resulted in a significant decrease in self-renewal, as assessed by serial passaging of mammospheres (Fig. 2B). Orthotopic injection of the shLMα5 cells into the mammary fat pad resulted in a significant increase in tumor-free survival compared with control cells (Fig. 2C). We also made use of a transgenic model of breast cancer in which the Rb pathways were inactivated in the mammary epithelium by the SV40 large T-antigen (T121) along with conditional alleles of *p53* and *Brca1* (Kumar et al. 2012; Goel et al. 2013). These *TgMFT121; Brca1f/f p53f/f; TgWAP-Cre* mice (referred to as TBP) develop poorly differentiated carcinomas with a triple-negative phenotype (Kumar et al. 2012). We isolated a population of cells from TBP tumors ($\alpha6^{high}/\beta1^{high}$) enriched for cells with stem cell properties (Shackleton et al. 2006; Stingl et al. 2006), which constitute a relatively small fraction of tumor cells (Fig. 2D). This population, which expresses α6B, exhibited substantially more LMα5 expression and ability to form mammospheres compared with the non-CSC populations (Fig. 2D). The ability of this CSC population to form mammospheres and initiate new tumors is dependent on its expression of LMα5 (Fig. 2E,F).

LM511/α6Bβ1 promote TAZ activation

The data provided thus far indicate that LM511 is the preferred ligand for α6Bβ1 and that it functions in this capacity to promote self-renewal and tumor initiation. Insight into the mechanism by which LM511 promotes these functions was obtained by analyzing our RNA-seq data (Supplemental Table S1). This analysis revealed that the MES population is enriched for the expression of genes regulated by the Hippo transducers TAZ and YAP (Varelas 2014) compared with the EPTH population. The significance of this observation is supported by the report that TAZ is necessary for the function of breast CSCs (Cordenonsi et al. 2011). We confirmed our RNA-seq data by comparing the expression of TAZ target genes in these populations by qPCR (Fig. 3A) and determining that TAZ nuclear localization is significantly higher in the α6Bβ1-expressing (Fig. 3B) and MES cells (Fig. 3C) than in the EPTH and α6Aβ1-expressing cells. Interestingly, YAP nuclear localization did not differ as much between these populations (Fig. 3C). Also, the activity of TEADs, the dominant transcription factors that anchor TAZ on DNA (Varelas 2014), was significantly higher in α6Bβ1-expressing cells (SUM1315) and MES compared with α6Aβ1-expressing (SUM1315) and EPTH cells (Fig. 3D; Supplemental Fig. S1E). The importance of TAZ in mammosphere formation and self-renewal was also confirmed (Fig. 3E).

More definitive evidence to implicate LM511 in regulating TAZ activity was obtained by comparing TAZ nuclear localization and target gene expression in cells plated on LM111 and LM511. Clearly, LM511 attachment promotes TAZ activation (Fig. 3F) and TAZ target gene expression (Fig. 3G) more robustly than LM111.

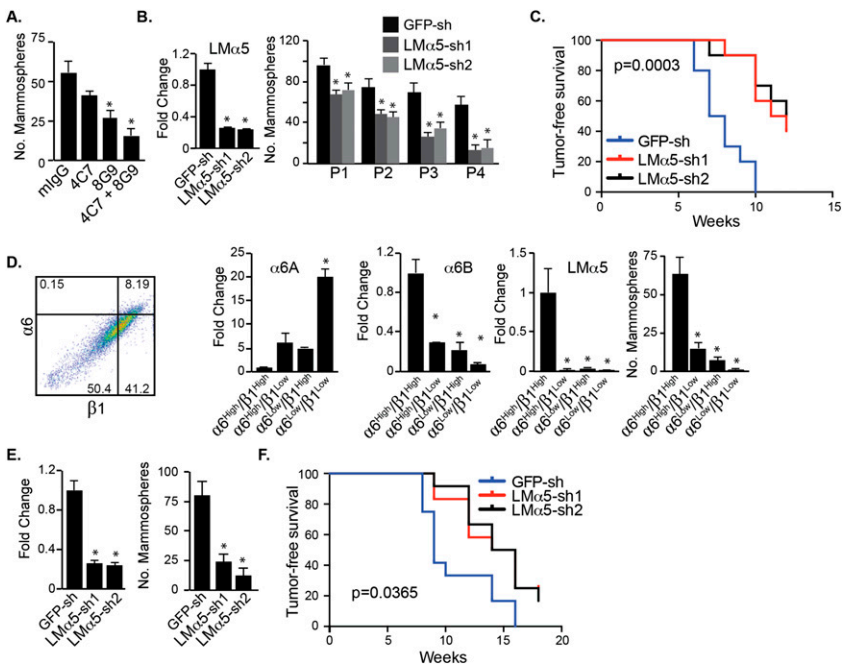

Figure 2. Autocrine LM511 is necessary for self-renewal and tumor initiation. (*A*) Mammosphere cultures of MES cells were treated with LMα5-blocking antibodies (4C7 and 8G9) daily for 1 wk and quantified. (*B*) LMα5 expression was diminished in α6Bβ1-expressing SUM1315 cells using shRNAs, and these cells were used for serial passaging of mammospheres. (*C*) Control (shGFP) and LMα5-diminished MES cells were injected into the mammary fat pads of NSG mice, and tumor formation was assessed by palpation. The curve comparison was done using a log rank test ($P < 0.05$). (*D*) TBP mammary tumor cells were sorted by FACS using α6 and β1 integrin Abs. The four populations generated were analyzed for α6B and LMα5 expression and mammosphere formation. Subsequently, LMα5 expression was diminished in the $\alpha6^{high}/\beta1^{high}$ population using shRNAs, and the impact on mammosphere formation and tumor-free survival ($P < 0.05$) was determined. (*E*,*F*) LMα5 expression was diminished in the $\alpha6^{high}/\beta1^{high}$ population using shRNAs, and the impact on mammosphere formation (*E*) and tumor-free survival (*F*) ($P < 0.05$) was determined.

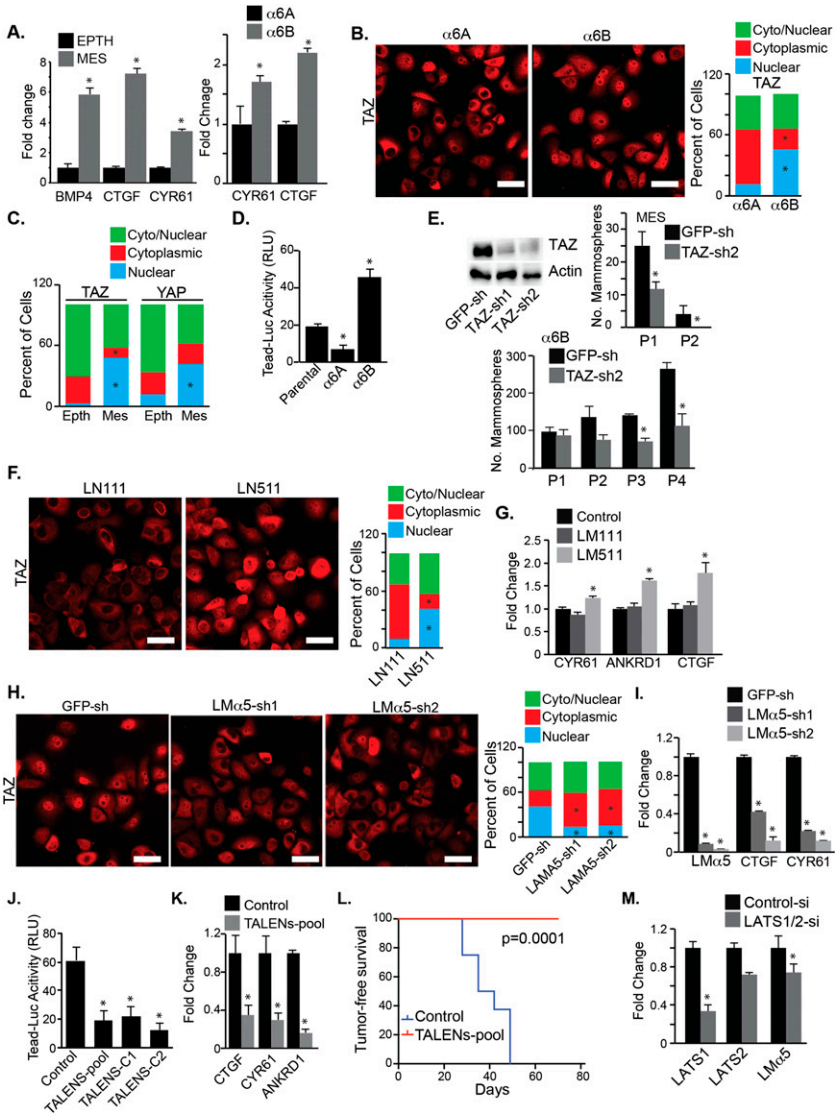

Figure 3. LM511/α6Bβ1 promotes TAZ activation. (*A*) Relative mRNA expression of TAZ target genes in the MES and EPTH populations of $CD44^+/CD24^-$ Src-transformed MCF10A cells and α6Aβ1- and α6Bβ1-expressing SUM1315 cells was quantified by qPCR. (*B*) α6Aβ1- and α6Bβ1-expressing SUM1315 cells were plated on a LM511 matrix, and the localization of TAZ was assessed by immunofluorescence. (*C*) The same assay as in *B* was performed using the MES and EPTH populations of $CD44^+/CD24^-$ Src-transformed MCF10A cells, and both YAP localization and TAZ localization were assessed. (*D*) TEAD transcriptional activity was assayed in α6Aβ1- and α6Bβ1-expressing SUM1315 cells and parental cells by transient expression of the 8XGTIIC-luciferase reporter construct. (*E*) TAZ expression was diminished using shRNA in the MES and α6Bβ1-expressing SUM1315 cells, and the impact on serial mammosphere passage was evaluated. (*F*) TAZ localization was assessed by immunofluorescence in confluent cultures of SUM1315 cells on either a LM111 or LM511 matrix. (*G*) Expression of TAZ target genes was quantified by qPCR in SUM1315 cells plated on either LM111 or LM511. (*H*,*I*) LMα5 expression was diminished in SUM1315 cells, and the impact on the expression of TAZ localization (*H*) and TAZ target genes (*I*) was determined. (*J*,*K*) Expression of integrin α6B was depleted in MDA-MB-231 cells using α6B-specific TALENs (see Fig. 1E). These cells (Puro alone, TALENs-pool, TALENs-C1, and TALENs-C2) were used to assay TEAD transcriptional activity (*J*) and expression of TAZ target genes (*K*). (*L*) Control (Puro alone) and TALENs-pool cells were injected into the mammary fat pads of NSG mice, and tumor formation was assessed by palpation. The curve comparison was done using a log rank test ($P < 0.05$). (*M*) Expression of Lats1/2 was diminished in EPTH cells using siRNAs, and mRNA expression of Lats1/2 and LMα5 was quantified by qPCR. Bar graphs in this figure represent the average of three independent experiments, and the *P*-value was determined using Student's *t*-test. Bars, 100 μm.

Depletion of LMα5 expression resulted in a significant decrease in TAZ nuclear localization and target gene expression (Fig. 3H,I). We also validated the contribution of α6Bβ1 to TAZ activation directly by comparing the activity of a TEAD reporter construct and expression of TAZ target genes in cells in which α6B had been deleted using TALENs (Goel et al. 2014) to control cells (Fig. 3J,K). Importantly, TALEN-mediated deletion of α6B also prevented tumor formation upon orthotopic injection (Fig. 3L).

The regulation of TAZ by LM511 appears to be independent of Hippo signaling based on our observations that the ability of LM511 to activate TAZ is independent of cell confluence (data not shown) and that knockdown of Lats1 did not increase LMα5 expression (Fig. 3M). Although we do not exclude the involvement of Hippo signaling, our observations are consistent with other reports of Hippo-independent YAP/TAZ activation (e.g., Dupont et al. 2011).

TAZ regulates LMα5 expression

Although TAZ has been implicated in the function of breast CSCs (Cordenonsi et al. 2011), the mechanisms involved have not been established. Given our observation that both TAZ target genes and LMα5 are enriched in cells with stem-like properties, we investigated the possibility that TAZ regulates LMα5 expression. Indeed, we discovered that knockdown of TAZ, but not YAP, diminished LMα5 mRNA expression significantly (Fig. 4A,B). This effect was also observed on LMα5 protein expression (Fig. 4A,B). These results prompted us to pursue the possibility that LMα5 is a TAZ target gene. We cloned the LMα5 promoter and detected a twofold increase in its activity in MES cells compared with EPTH cells (Fig. 4C, left). To establish that this activity is dependent on TAZ, we cotransfected the promoter construct with or without exogenous TAZ expression in HEK293 cells and observed that TAZ expression increased promoter activity significantly compared with vector control (Fig. 4C, right).

TAZ does not have a DNA-binding site and functions as a transcriptional coactivator (Kanai et al. 2000). In silico motif analysis of the LMα5 promoter identified multiple TEAD-binding motifs (Fig. 4D). The TEAD transcription factor is the predominant mediator of TAZ function in the Hippo pathway (Varelas 2014). Chromatin immunoprecipitation (ChIP) was used to establish binding of TAZ to these TEAD-binding sites (Fig. 4D). To control for specificity, no TAZ binding was detected in exons of the LMα5 gene (Fig. 4D). Exogenous expression of TAZ in the LMα5-low population of cells sorted from three PDX tumors was sufficient to increase their expression of

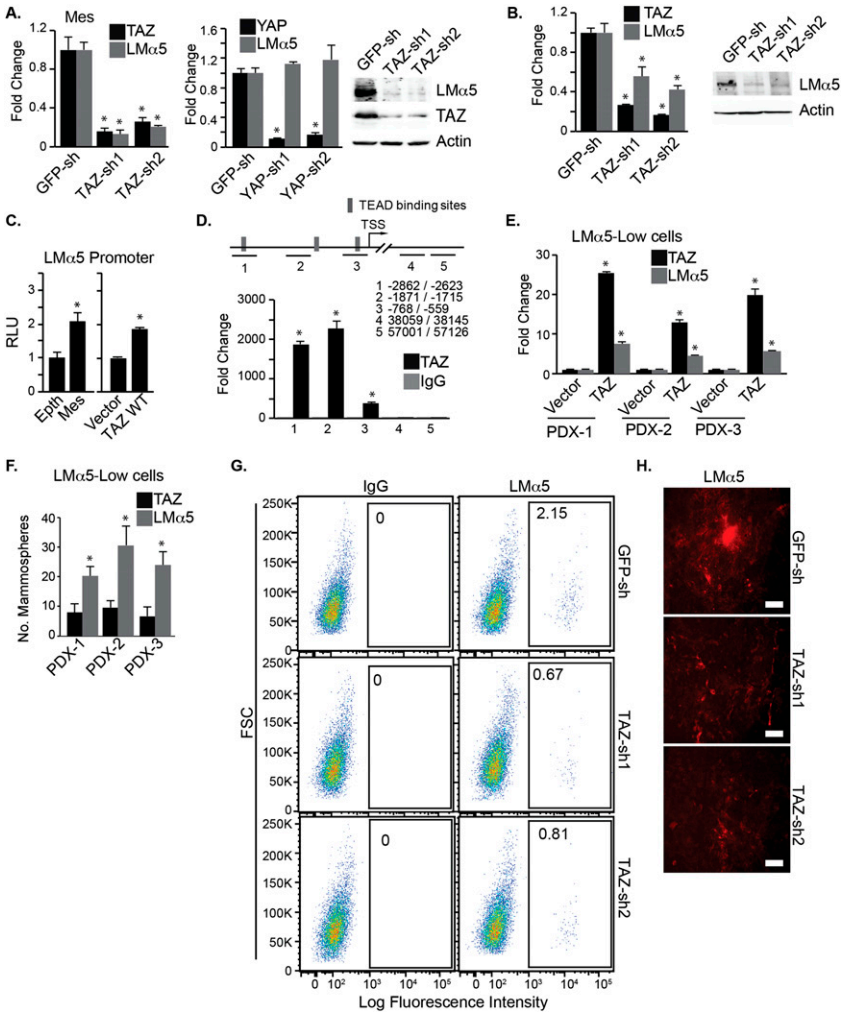

Figure 4. TAZ regulates LMα5 expression. (*A*) Expression of either TAZ or YAP was diminished in MES cells, and the impact on LMα5 mRNA expression was quantified by qPCR. LMα5 protein expression in TAZ-depleted cells was also evaluated by immunoblotting. (*B*) TAZ expression was diminished in SUM1315 cells, and the impact on LMα5 mRNA and protein expression was determined. (*C*, *left*) A luciferase construct containing the LMα5 promoter was generated and used to assay LMα5 transcriptional activity in the MES and EPTH cells. (*Right*) LMα5 promoter activity was assayed using the same reporter construct in control and TAZ-expressing HEK293 cells. (*D*, *top*) Schematic of the LMα5 promoter indicating the location of the putative TEAD-binding sites. (*Bottom*) Binding of TAZ to the LMα5 promoter was assayed by ChIP. (*E*,*F*) Cells with low surface-bound LMα5 (from PDX breast tumors) were isolated and infected with lentiviral particles expressing TAZ. (*E*) Expression of TAZ and LMα5 mRNA expression was quantified by qPCR. (*F*) These transfected cells were also assayed for their ability to form mammospheres. (*G*) Surface-bound LMα5 was quantified by flow cytometry in the EPTH cells transfected with empty vector or TAZ shRNAs. (*H*) MES cells were cultured for 4 d and detached using EDTA (25 mM), and the exposed matrix was analyzed for LMα5 expression by immunofluorescence.

LMα5 mRNA and ability to form mammospheres significantly (Fig. 4E,F).

The above findings infer that TAZ regulates the expression of a LM511 matrix. To examine this hypothesis, we assessed the impact of TAZ knockdown on surface-bound LMα5 by flow cytometry. As shown in Figure 4G, diminishing TAZ significantly decreased the frequency of the small population of cells with high surface-bound LMα5 (see Fig. 1F,G). We also found that TAZ knockdown reduced the ability of cells to deposit a LM511 matrix in culture (Fig. 4H).

In this study, we identified LM511 as the ligand for α6Bβ1 and demonstrated that LM511/α6Bβ1 signaling promotes stem cell properties by activating the Hippo transducer TAZ. The regulation and function of YAP and TAZ in cancer have been the focus of intense investigation (Piccolo et al. 2013; Yu and Guan 2013). However, aside from the report that an MT1–MMP/β1 integrin cascade promotes YAP/TAZ nuclear localization in skeletal stem cells (Tang et al. 2013), nothing is known about ECM/integrin regulation of YAP/TAZ in cancer. For this reason, our discovery that breast CSCs produce a LM511 matrix that functions to sustain TAZ activation and stem cell properties is a significant advancement that highlights the importance of the ECM in regulating Hippo effectors (Yu and Guan 2013). Moreover, our data reveal that high LM511 expression is a useful marker for identifying tumor cells with stem cell properties and that such cells can be isolated by flow cytometry using LMα5 Abs.

The second major conclusion of this study is that LMα5 is a TAZ target gene and that TAZ regulates the formation of a LM511 matrix. This conclusion is significant because it provides insight into the mechanism by which TAZ contributes to the function of CSCs. Indeed, our finding that TAZ contributes to the regulation of LMα5 transcription implicates the ECM as a critical effector of TAZ-mediated functions. Our data also indicate that breast CSCs generate a LM511 matrix using a positive feedback loop that involves LM511/α6Bβ1-mediated activation of TAZ and TAZ-mediated regulation of LM511. These findings imply that breast CSCs generate their own matrix niche that functions to maintain stemness by sustaining TAZ activation.

Materials and methods

Cells

ER-SRC-transformed MCF-10A cells were provided by Dr. Kevin Struhl (Harvard Medical School, Boston, MA). Isolation of the $CD44^+/CD24^-$ population from these Src-transformed MCF10A cells and characterization of distinct EPTH and MES populations have been described (Goel et al. 2014). The generation of SUM1315 cells that express either α6Aβ1 or α6Bβ1, the use of TALENs to target the splicing site in the α6 subunit, and the generation of α6B-depleted cells have also been described (Goel et al. 2014). HEK293 cells were purchased from American Type Culture Collection. Flow cytometry was used to analyze surface expression of the α6 integrin, CD44, and CD24.

RNA-seq analysis

RNA was extracted from the indicated cells and sent to Beijing Genomics Institute (BGI) for quantification, sequencing, and analysis. Sequencing was performed on the single end of the mRNA fragment with a reading length of 50 base pairs (bp). Each sample had a sequencing depth of 5 million.

Additional details about the materials and methods are provided in the Supplemental Material.

Accession numbers

The accession number for the REN-seq data is SRX767003. The project accession number for the REN-seq data is PRJNA268321.

Acknowledgments

National Institutes of Health grant CA168464 was the major support for this work. Support was also provided by a Swedish Cancer Society grant (to M.P.).

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Genes Dev. 2015 29: 1-6
Access the most recent version at doi:10.1101/gad.253682.114