Research paper

A role for kinesin-1 subunits KIF5B/KLC1 in regulating epithelial mesenchymal plasticity in breast tumorigenesis

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Background: Epithelial mesenchymal plasticity (EMP) is deemed vital in breast cancer progression, metastasis, stemness and resistance to therapy. Therefore, characterizing molecular mechanisms contributing to EMP are in need enabling the development of more advanced therapeutics against breast cancer. While kinesin superfamily proteins (KIFs) are well known for their role in intracellular cargo movement, our knowledge of their function in breast tumorigenesis is still limited.

Methods: Various breast cancer cell lines representing different molecular subtypes were used to determine the role of kinesin-1 subunits KIF5B/KLC1 in regulation of EMP.

Findings: In breast cancer, we show that kinesin family member 5B (KIF5B) and its partner protein kinesin light chain 1 (KLC1), subunits of kinesin-1, to play differential roles in regulating EMP and tumorigenesis. Indeed, we found KIF5B to be expressed in triple negative (TN)-basal-like/c Claudin low breast cancer subtype and to be an inducer of epithelial-mesenchymal transition (EMT), stemness, invasiveness, tumor formation and metastatic colonization. Whereas, we found KLC1 to be expressed in epithelial/luminal breast cancer subtypes and to be a suppressor of EMT, invasion, metastasis and stem cell markers expression as well as to be an inducer of epithelial/luminal phenotype. Interestingly, in TN-basal-like/c Claudin low cells we found a novel nuclear accumulation of KIF5B and its interaction with the EMT transcriptional regulator Snail1 independent of KLC1. In addition, TGF-β mediated pro-invasive activity was found to be dependent on KIF5B expression. In contrast, the epithelial differentiation factor and EMT suppressor prolactin (PRL) was found to repress KIF5B gene expression and KIF5B-Snail1 nuclear accumulation, but enhanced KLC1 gene expression and KIF5B-KLC1 interaction.

Interpretation: Together, these results highlight a new paradigm for kinesin-1 function in breast tumorigenesis by regulating EMP programing and aggressiveness.

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1. Introduction

Despite improvements in early detection and advances in treatment options, breast cancer progression to a metastatic disease remains a major clinical challenge. Epithelial mesenchymal plasticity (EMP) is now well recognized cellular process contributing to cancer cell diversity and intra-tumor heterogeneity associated with disease progression and impaired response to therapy [1,2]. The role of EMP in promoting aggressive breast cancer phenotype is further emphasized in recent molecular subclassification of breast cancer. Indeed the mesenchymal-basal-like (claudin low) TNBC subtype, frequently characterized by high histological grade and poor differentiation, to be associated with unfavorable pathological features and poor patient outcome in comparison to the epithelial/luminal subtypes [3,4]. Furthermore, oncogenic and pro-metastatic growth factors such as TGF-β are known to potently induce EMP and promote the transition of breast cancer cells from non-invasive epithelial to invasive mesenchymal with stem-like phenotype [5,6]. Conversely, EMP suppressors such as prolactin hormone (PRL), is shown to supress the mesenchymal properties and induce an epithelial phenotype in breast cancer cells and subsequently suppress their invasive and tumorigenic behaviour [7,8]. Therefore, regulators of EMP represent important targets for the development of novel therapeutics in breast cancer. Clinically, whereas current targeted breast cancer treatments are directed toward the epithelial/luminal subtypes, there are no targeted treatments for the
Research in context

Evidence before this study

Kinesin superfamily proteins (KIFs) are well known to be involved in intracellular movement and cytoplasmic transport of membranous organelles and macromolecules including complex proteins as well as RNA along the microtubules network. Kinesin-driven transport along microtubules is mediated by the concerted function of two kinesin subunits, the kinesin heavy chain (KHC) and the kinesin light chain (KLC). To date the role of kinesin-1 in cancer including breast cancer has not been extensively studied. Furthermore, it is not known whether the role of kinesin in breast tumorigenesis is limited to its function as a motor protein.

Added value of this study

In this study, we found the subunits of kinesin-1, KIF5B and KLC1, to be critical regulators of EMP in breast cancer showing opposite roles. We found that KIF5B to be highly expressed in the most aggressive basal/claudin low TNBC breast cancer subtype and is essential for cell viability, migration, invasion, stemness, tumorigenesis and metastasis. In contrast, we found its classical partner protein KLC1 to be expressed in luminal/epithelial breast cancer cells and to possess anti-invasive activity. Importantly, we found that loss of KLC1 expression in luminal breast cancer subtypes resulted in nuclear accumulation of KIF5B and acquisition of mesenchymal, invasive and stem-like phenotype with loss of epithelial properties.

Implications of all the available evidence

This study revealed a new understanding of the role of kinesin-1 in breast cancer mediating EMP programing. Our results highlight for the first time a KIF5B/KLC1 switch regulating EMP in breast cancer that may prove useful for EMP-targeted therapies.
like/claudin low cells (MDA-MB-231, SCP2 and SUM159) in comparison to luminal A (T47D), luminal B (BT474), Her-2E (SKBR3) and TN-LAR (MDA-MB-453) cells both at the m-RNA and protein levels.

To further evaluate the role of Kinesin-1 components, KIF5B and KLC1 in breast cancer, we next examined their clinical significance using Curtis dataset, (ONCOMINE database) containing gene profiling data of 1700 breast cancer cases [29]. Interestingly, we found KIF5B mRNA levels to be significantly higher in invasive breast carcinoma (1556 cases) compared to normal breast tissue (144 cases) (P = 5.47E-5) (Fig. 1C, left panel). In contrast, KLC1 mRNA levels were found to be significantly lower in invasive breast carcinoma (1556 cases) compared to normal breast tissue (144 cases) (P = 1.83E-24) (Fig. 1C, right panel). We next examined the mRNA levels of KIF5B using a cohort of 1411 breast cancer cases in GOBO database. We found higher KIF5B mRNA levels in the poorly differentiated grade III tumors compared to grades II and I tumors while we found KLC1 mRNA levels to be higher in well differentiated grade I tumors compared to grade II and III with a P value of (P = .03047) and (P = .08243) respectively (Fig. 1D). Next, we examined KIF5B gene expression in relation to clinically relevant breast cancer molecular subtypes [30] in GOBO database (containing 1881 human breast cancer cases) using HU and PAM50 sub-classifications [31], we found KIF5B mRNA levels to be highest in the basallike subtype and lowest in the luminal A subtype (P ≤ .00001) (Fig. 1E, left panel). In contrast, we found that KLC1 mRNA levels to be highest in luminal subtypes and lowest in basal-like subtype, (Hu P ≤ .00001; PAM50 P = .0084) (Fig. 1E, right panel). Lastly, we analyzed the association between KIF5B mRNA levels and patient outcomes represented as distant metastasis free survival (DMFS) and relapse free survival (RFS). For these analyses we used Kaplan Meier plotter database which allow monitoring of survival of a large number of breast cancer patients for >10 years [32]. Interestingly, patients with higher KIF5B mRNA levels showed worse outcome presented as reduced DMFS and RFS (Fig. 1F). On the other hand, when analyzing the association between KIF5B mRNA levels and patient outcomes, we found that patients with higher KLC1 mRNA levels show significantly better RFS outcome (Fig. 1G) while there was no prognostic value for DMFS (Supplementary Fig. S3A). Taken together, these findings demonstrate that KIF5B and KLC1 are differentially expressed in the different clinically relevant breast cancer molecular subtypes and suggest that these two components of the kinesin-1 complex have independent and potentially opposite functions in breast cancer.

2.2. TN-basal-like/claudin low breast cancer cells show dependency on KIF5B for viability/metabolic activity and invasion activity

We next investigated the functional role of KIF5B in breast tumorigenesis by means of RNA interference, using two independent KIF5B-specific shRNAs in the TN-basal-like/claudin low MDA-MB-231 cells. Effectiveness of KIF5B knockdown was verified at both m-RNA and protein levels for each shRNA (Fig. 2A). As can be seen in Fig. 2B, blocking KIF5B expression resulted in a significant loss of cell viability, most evident at 96 h time point. Our data also showed that KIF5B knockdown significantly decreased the migratory property of the MDA-MB-231 cells (Supplementary Fig. S2A). Moreover, KIF5B knockdown also resulted in a significant loss in the invasive capacity of the MDA-MB-231 cells (Fig. 2C). To avoid the limitation of a single cell line, these findings were reproduced in another aggressive TN-basal-like/claudin low cell model system SUM159, in which KIF5B was also found to be highly expressed and showed the same effects as that seen in MDA-MB-231 cells (Fig. 2D). Moreover, upon knockdown of KIF5B, we observed a change in cell morphology of the TN-basal-like/claudin low cells MDA-MB-231 and SUM159 from mesenchymal to cuboidal shape (Supplementary Fig. S2B). To further elaborate on the role of KIF5B in breast cancer, we suppressed KIF5B expression in the luminal A T47D cells normally expressing low levels of KIF5B. Interestingly, no change in cell viability or morphology was observed following KIF5B knockdown (Fig. 2E & Supplementary Fig. S2C). Altogether these results highlight a pro-angiogenic role for KIF5B in mediating cell viability, migration and invasion capacities in TN-basal-like/claudin low cells.

2.3. KIF5B plays a central role in inducing the EMT program, stemness and tumorigenic potential in TN-basal-like/claudin low cells

It is well known that the invasive capacity and distant metastasis properties of TN-basal-like/claudin low cells are mediated through acquisition of molecular signals that activate the function and/or expression of various EMT transcription factors and markers while suppressing the expression of epithelial markers [33,34]. To further decipher the role of KIF5B in breast cancer we then examined the role of KIF5B in regulating the EMT process. For this we examined the expression of EMT markers in MDA-MB-231 cells following knock-down of KIF5B. As shown in Fig. 3A, our data revealed that blocking KIF5B expression significantly decreased the m-RNA levels for several EMT transcription factors (ZEB1, ZEB2, Slug and Snail1) and mesenchymal markers (vimentin and FN1). Loss of protein expression levels of Snail–1, ZEB1 and vimentin were also confirmed by western blot and immunofluorescence analyses (Fig. 3B). On the other hand, loss of KIF5B resulted in a significant up-regulation in gene expression levels of the epithelial markers E-cadherin, CK18 as well as of the PRLR (Fig. 3C). The gain in E-cadherin and CK18 protein expression was further confirmed using western blot and immunofluorescence analyses (Fig. 3D). Importantly, our immunofluorescence data show that the re-expressed E-cadherin was not restricted only to the cell membrane but also associated with intracellular structures. Together, these results underscore the critical role of KIF5B in mediating induction of EMT reprogramming and suppression of epithelial differentiation pathways in TN-basal-like/claudin low tumors.

Breast cancer stem-like cells (BCSCs) are largely responsible for the aggressive phenotype, high invasive capacity and high rate of recurrence in TN-basal-like/claudin low tumors. In particular, the CD44low/CD24low stem-like cell sub-population has been identified as a mesenchymal-tumorigenic subpopulation with high metastatic activity [35–37]. We thus examined the role of KIF5B in regulating the stem cell phenotype of TN-basal-like/claudin low cells. Interestingly, loss of KIF5B expression in MDA-MB-231 cells resulted in a significant reduction in the stem-like cell sub-population CD44+/CD24+ with a significant increase in the number of the non-tumorigenic CD44−/CD24− cell subpopulation (Fig. 3E). Furthermore, examining individual stem cell

Fig. 1. KIF5B and KLC1 expression in relation to breast cancer molecular subtypes and patient outcome. A. Left panel, expression of KIF5B was examined using q-RT-PCR in T47D (control), MCF7, BT474, SKBR3, MDA-MB-453, MDA-MB-231, SCP2 and SUM159 cells. Results are expressed as relative expression of triplicates of three independent experiments ***p ≤ .0001 (one-way ANOVA). Right panel, T47D, MCF7, BT474, SKBR3, MDA-MB-453, MDA-MB-231, SCP2 and SUM159 cells were lysed and western blotting was carried out using monoclonal antibodies against KIF5B and β-tubulin. B. Left panel, m-RNA levels of KLC1 using q-RT-PCR in T47D (control), MCF7, BT474, SKBR3, MDA-MB-453, MDA-MB-231, SCP2 and SUM159 cells were determined by q-RT-PCR. Results are expressed as relative expression of triplicates of three independent experiments **p ≤ .001 (one-way ANOVA). C. Left panel and KLC1 (right panel) mRNA expression levels for each cell line were normalized to the housekeeping gene (18S). D. Left panel, expression of KIF5B and KLC1 in breast cancer cell lines. Expression level of KIF5B and KLC1 was determined by q-RT-PCR. E. Left panel, expression of KIF5B and KLC1 in breast cancer cell lines. Expression level of KIF5B and KLC1 was determined by q-RT-PCR. F. Kaplan-Meier survival curves of KIF5B gene expression in association with patient outcome (1535 patients, KM-plotter database) using DMFS as an end point (left panel), Kaplan-Meier survival curves of KIF5B gene expression in association with patient outcome (3554 patients, KM-plotter database) using RFS as an end point (middle panel). G. Kaplan-Meier survival curves of KIF5B gene expression in association with patient outcome (3951 patients, KM-plotter database) using RFS as an end point.
marker expression level, we found that loss of KIF5B led to a significant decrease in CD44 but not CD24 mRNA levels (Fig. 3F). This was followed by a significant decrease in gene expression levels of the self-renewal transcriptional factors OCT4, NANOG and SOX2 (Fig. 3G).

Next, we assessed the overall effect of loss of KIF5B expression on the tumorigenic capacity of MDA-MB-231 cells using colony formation assay. As shown in Fig. 3H, loss of KIF5B expression resulted in a significant decrease of the clonogenic capacity of MDA-MB-231 cells. On the other hand, there was no effect of loss of KIF5B on the clonogenic capacity of the luminal A T47D cells (Supplementary Fig. S2B). We then tested the effect of loss of KIF5B in modulating BCSC self-renewal, using tumorsphere formation assays. MDA-Scr and MDA-KIF5BShRNA cells were seeded at 1000, 500, 100, 50, 10 and 1 cells/well and tumorsphere formation was monitored. While we observed no differences in tumorsphere formation capacity between MDA-Scr and MDA-KIF5BShRNA cells at 1000 and 500 cells/well, we found a significant
loss of tumorsphere number in MDA-KIF5BShRNA cells in comparison to MDA-Scr cells starting at 100 cells/well (Fig. 2I). Next To address the role of KIF5B in driving breast tumorigenesis, we next examined the effects of loss of KIF5B expression on the tumorigenic potential of MDA-MB-231 cells using a mammary fat-pad orthotopic mouse model. While all mice injected with the control MDA-MB-231 cells expressing the scrambled ShRNA developed large tumors within the mammary fat-pad, only (3/7) mice injected with MDA-MB-231 cells expressing the KIF5B specific ShRNA developed tumors that were also significantly smaller than MDA-MB-231/Scr tumors (Fig. 3J). Together, these results clearly demonstrate a critical role for KIF5B in breast tumorigenesis generating a mesenchymal and stem-like phenotype characteristics of EMP and the aggressive TN-basal-like/claudin low phenotype.
2.4. KLC1 is required to maintain an epithelial and non-invasive breast Cancer phenotype

To address the role of KIF5B partner protein, KLC1, in breast cancer, we next suppressed KLC1 expression in the luminal A (T47D) and Her-2E (SKBR3) cells, using specific KLC1 siRNA (Fig. 4A). While knocking down KLC1 expression did not affect T47D and SKBR3 cell viability (Fig. 4B), importantly, we found that loss of KLC1 in T47D and SKBR3 cells reprogramed the normally non-invasive cells to an invasive phenotype (Fig. 4C). Furthermore, suppression of KLC1 resulted in a pronounced increase in expression of the EMT markers (ZEB1, ZEB2, Snail1, Slug, Vimentin and FN1) in the two epithelial/luminal breast cancer subtypes (Fig. 4D). The gain in expression of the EMT markers, Snail-1 and ZEB1, was also confirmed by immunofluorescence analyses (Fig. 4E). On the other hand, loss of KLC1 resulted in a significant down-regulation of mRNA levels of the epithelial markers E-cadherin and the PRLR (Fig. 4F). Loss of E-cadherin protein expression level was also confirmed by immunofluorescence analysis (Fig. 4G). Next, examining individual stem cell marker expression levels, we found that loss of KLC1 led to a significant increase in CD44 mRNA level while CD24 m-
RNA level was significantly decreased (Fig. 4H). The gain in protein expression of CD44 marker was further confirmed using western blot analyses in T47D and SKBR3 cells (Fig. 4I). To rule out off-target effects of the siRNA, the above findings were reproduced using another siRNA targeting KLC1 in luminal A (T47D) cells (Supplementary Fig. S3).

The above data implicated KLC1 in maintaining a non-invasive epithelial phenotype in breast cancer cells. Therefore, next we examined the overall effect of loss of KLC1 in driving organ metastasis and lung colonization in vivo. For this, we assessed the effects of loss of KLC1 gene expression on lung metastasis using tail vein injection preclinical xenograft mouse model. Interestingly, while none of the 6 mice injected with the control T47D cells expressing the control siRNA developed lung micrometastases, 3/6 mice injected with T47D-Si-KLC1 cells developed lung metastasis (Fig. 4J) supporting a role for KLC1 in suppressing metastasis in vivo. Together, these results demonstrate a role for KLC1 in maintaining non-invasive epithelial phenotype in breast cancer cells. Altogether, these findings highlight a clear association between KLC1 expression and maintenance of an epithelial, non-invasive and non-metastatic cellular phenotype emphasizing an EMP suppressor role for KLC1 in breast cancer contrary to KIF5B.

2.5. Nuclear accumulation of KIF5B and KIF5B/SNAIL interaction in TN-basal-like/claudin low cells independent of KLC1

Having found a new role for KIF5B in promoting EMP, independent of KLC1, this prompted us to investigate whether KIF5B plays a nuclear role in breast cancer. Therefore, next we examined the subcellular localization of KIF5B in the different breast cancer molecular subtypes, using immunoblotting of nuclear fractions. Interestingly, as shown in Fig. 5A, we found high nuclear accumulation of KIF5B in the TN-basal-like/claudin low cells (MDA-MB-231, SC2P and SUM159) while no nuclear signal for KIF5B could be detected in the epithelial/luminal breast cancer cells. In contrast, we could not detect any KLC1 nuclear accumulation in all breast cancer cell types. Moreover, immunofluorescence analysis of KIF5B showed strong nuclear localization of KIF5B in TNBC MDA-MB-231 cells, while remaining unfocalized in luminal A T47D cells (Fig. 5B). In contrast, KLC1 showed cytoplasmic localization in both T47D and MDA-MB-231 cells (Fig. 5C). To investigate whether the nuclear accumulation of KIF5B is determined by KLC1 expression levels, we examined KIF5B nuclear levels following suppression of KLC1 expression in the luminal breast cancer cells T47D and Her-2 SKBR3 cells. We examined KIF5B nuclear levels following suppression of KLC1 expression in the luminal breast cancer cells T47D and Her-2 SKBR3 cells. Interestingly, loss of KLC1 expression in the two luminal molecular subtypes led to KIF5B nuclear accumulation while no change in total KIF5B expression level was observed (Fig. 5D), suggesting that KLC1 expression levels influence and control KIF5B nuclear localization. These results highlight a potential nuclear function for KIF5B in TN-basal-like/claudin low cells deriving EMP independent of KLC1.

Next, we examined whether this nuclear accumulation of KIF5B in TN-basal-like/claudin low cells permits a new function for KIF5B in the nucleus. Therefore, we examined whether there is a physical interaction between KIF5B and the transcriptional regulators of EMT in TN-basal-like/claudin low cells. Indeed, as can be seen in Fig. 5E, all TN-basal-like/claudin low cells, MDA-MB-231, SC2P and SUM159, showed KIF5B interaction with Snail1. Screening the possible interaction between KIF5B and transcription factors ZEB1, ZEB2 as well as Slug, our data did not detect any interaction between KIF5B and these proteins (data not shown). To address whether KIF5B regulates Snail1 function, we examined Snail1 expression in MDA-MB-231 cells following loss of KIF5B expression. Interestingly, as can be seen in Fig. 5F, MDA-MB-231-Sh1-KIF5B cells showed loss of Snail1 nuclear accumulation and its re-localization to the cytoplasm, highlighting an important function for KIF5B in regulating Snail1 nuclear accumulation in TN-basal-like/claudin low cells. Next, to investigate whether Snail1 exerts any regulatory role in KIF5B function, we suppressed Snail1 expression in MDA-MB-231 cells using a specific Snail1 siRNA (Supplementary Fig. S4A, left panel). As can be seen in Supplementary Fig. S4A right panel, no change in the nuclear accumulation of KIF5B was observed. Altogether, these findings indicate that KIF5B nuclear accumulation in breast cancer is context dependent and is regulated by the expression of KLC1 promoting EMT transcriptional programming.

2.6. KIF5B is essential for metastatic colonization propensity and is a clinically relevant marker of high-grade invasive breast cancer

The above data suggested that KIF5B is a critical inducer of EMT, stemness and invasion in breast cancer cells. Therefore, next we examined the role of KIF5B in driving organ metastasis in vivo. For this, we monitored the effects of loss of KIF5B gene expression on lung metastasis using tail vein injection preclinical xenograft mouse model. Remarkably, while 6 out of 6 mice injected with the control MDA-MB-231 cells expressing the scrambled shRNA developed lung micrometastases, none of the 6 mice injected with MDA-MB-231-Sh1-KIF5B cells developed lung metastasis (Fig. 6A). These results demonstrate that KIF5B is a driver of metastatic colonization, the functional endpoint of EMP. As cancer cells adopt the EMP programing and invasive/metastatic behavior, they acquire cellular features clinically associated with high-grade invasive malignancy [38,39]. Next, we investigated the clinical features of breast cancer cases expressing KIF5B protein. For this, we used a TMA of 102 cases including 97 breast cancer cases and 5 normal/benign breast tissues. Importantly, we found KIF5B protein expression to be significantly higher in invasive ductal carcinoma in comparison to in situ carcinoma (p = .04). We then investigated whether KIF5B expression correlated with the different molecular and histological subtypes of breast cancer. We found a trend of high KIF5B protein expression in triple negative molecular subtype of breast cancer. Moreover, KIF5B protein expression was associated with poorly differentiated tumors (grade III) (81.25%) in comparison to the moderately
Fig. 5. KIF5B shows high nuclear accumulation and interaction with Snail1 in TN-basal-like/claudin low cells. A. Immunoblot analysis of nuclear extracts of breast cancer cells using monoclonal antibodies against KIF5B and Lamin-B1. B. Left panel, confocal immunofluorescence images of KIF5B (green), phalloidin (red) and nucleus (Dapi) (blue) of T47D (upper panel) & MDA-MB-231 (lower panel) cells. Scale bar, 10 μm. Right panel, confocal immunofluorescence images of KIF5B (green) and nucleus (Dapi) (blue) of MDA-Sh1-KIF5B cells (negative control). Scale bar, 10 μm. C. Left panel, confocal immunofluorescence images of KLC1 (green), phalloidin (red) and nucleus (Dapi) (blue) of T47D (upper panel) & MDA-MB-231 (lower panel) cells. Scale bar, 10 μm. Right panel, confocal immunofluorescence images of KLC1 (green) and nucleus (Dapi) (blue) of T47D-Si-KLC1 cells (negative control). Scale bar, 10 μm. D. Left panel, immunoblot analysis of nuclear extracts of T47D-Si-control (control) and T47D-Si-KLC1 cells. Right panel, immunoblot analysis of total cell lysates of T47D-Si-control and T47D-Si-KLC1 cells using monoclonal antibodies against KIF5B, Lamin-B1 and β-tubulin. E. MDA-MB-231, SCP2 and SUM159 cells were lysed and immunoprecipitations were performed using a goat polyclonal antibody against Snail1 or control normal goat IgG. Western blotting was carried out using a rabbit monoclonal antibody against KIF5B. F. Confocal immunofluorescence images of Snail1 (red) and nucleus (Dapi) (blue) of MDA-MB-231-Scr (control) & MDA-MB-231-Sh1-KIF5B cells. Scale bar, 10 μm.

Fig. 6. Breast cancer shows dependency on KIF5B for invasive capacity and metastasis in preclinical mouse model. A. Representative gross photo of lungs as well as H&E histological images of NOD-SCID tail vein mouse xenografts of MDA-MB-231-Scr MDA-MB-231-Sh-KIF5B. Black arrow heads indicate macro and micrometastasis. B. Left panel, positive immunohistochemical staining of KIF5B in normal adjacent tissue, in situ and invasive breast cancer lesions (10X and 40X). Right panel, associations between KIF5B protein expression and different clinicopathological parameters.

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2.7. KIF5B/KLC1 function downstream of TGF-β and PRL in TN-basal-like/claudin low cells

Our results so far emphasized a key role for kinesin-1 components KIF5B/KLC1 in regulating EMP in breast cancer. Extensive research has identified the growth factor TGFβ as a critical inducer of EMT, stemness, invasion and tumorigenesis in TN-basal-like/claudin low cells. Whereas, we have previously shown PRL to block TGFβ function and to suppress EMT, invasion and tumorigenesis of TN-basal-like/claudin low cells [40-42]. Therefore, next we examined whether KIF5B regulates TGFβ-mediated pro-invasive function. Interestingly, we found that loss of KIF5B expression in MDA-MB-231 cells significantly blocked TGFβ-mediated cell invasion (Fig. 7A, left panel). To further study the role of KIF5B in mediating TGFβ regulation of EMT, we examined TGFβ-induced expression of several EMT markers including ZEB1, ZEB2, Snail1 and Slug in MDA-Scr and MDA-Sh1-KIF5B cells. Interestingly, we found that loss of KIF5B abrogated TGFβ-induced EMT markers expression implicating KIF5B in TGFβ-induced EMT and cellular invasion capacity (Fig. 7A, right panel). To further study the role of TGFβ in regulating KIF5B expression, we next assessed the expression of KIF5B following TGFβ treatment of MDA-MB-231 cells. Our data did not show a significant change in KIF5B expression levels concomitant with a significant increase in KLC1 m-RNA expression levels. These results were further confirmed using the TN-basal-like/claudin low cell line MDA-MB-231 cells engineered to overexpress the PRLR upon doxycycline treatment, designated as MDA-MB-231/PRLR cells [8]. The ectopic expression of PRLR in MDA-MB-231/PRLR cells was confirmed using IF analyses (Fig. 7C, left panel). As can be seen in Fig. 7C, right panel, upon doxycycline treatment, whereas PRL treatment suppressed KIF5B expression, it resulted in increased expression of KLC1. We then evaluated the role of PRL in regulating KIF5B and Snail-1 nuclear accumulation, using the MDA-MB-231/PRLR cell model system. As shown in Fig. 7D, our data show that PRL treatment of MDA-MB-231/PRLR cells induced the expression of KLC1 and Snail-1 in MDA-MB-231 cells, whereas PRL treatment suppressed KIF5B expression, it resulted in increased expression of KLC1. We then evaluated the role of PRL in regulating KIF5B and Snail-1 nuclear accumulation, using the MDA-MB-231/PRLR cell model system. As shown in Fig. 7D, our data show that PRL treatment of MDA-MB-231/PRLR cells induced the expression of KLC1 and Snail-1 in MDA-MB-231 cells, whereas PRL treatment suppressed KIF5B expression.
PRLR, resulted in the re-localization of both KIF5B and Snail-1 to the cytoplasm, while no change in re-localization of the two proteins was observed in control cells. Finally, we also found that PRL treatment of MDA-MB-231/PRLR cells promotes the physical interaction between KIF5B and KLC1 (Fig. 7E). Together, these results emphasize a role for KIF5B/KLC1 in TGFβ and PRL regulation of EMP, highlighting a central role for KIF5B in mediating TGFβ-pro-invasive activities and establishing the ability of PRL to regulate and suppress KIF5B function in TN-basal-like/ Claudin low cells.

3. Discussion

Kinesins encompass a large family of proteins that are well-known to mediate intracellular movement and cytoplasmic transport of membranous organelles and macromolecules along the microtubules network. Kinesin-driven transport is mediated by the concert function of two subunits forming a complex of the motor protein (KHC) and the adaptor protein (KLC) [10,11]. Here we present evidence highlighting for the first time that kinesin-1 subunits, KIF5B and KLC1, as distinct regulators of EMP thereby contributing to breast cancer heterogeneity and aggressiveness (Fig. 8).

3.1. The differential expression of KIF5B vs KLC1 in breast Cancer

The roles of Kinesin-1 subunits KIF5B and KLC1 in breast tumorigenesis are still to be fully determined. Here using IHC analysis of breast cancer clinical cases we found KIF5B to be highly expressed in invasive ductal carcinoma and to be associated with poorly differentiated tumors. Moreover, and in agreement with a previous report [20], our IHC data also showed that TNBC clinical cases to exhibit high expression levels of KIF5B in comparison to other breast cancer subtypes. This finding was further confirmed using large bioinformatics dataset showing KIF5B to be enriched in the basal subtype based on PAM50 and Hu et al., subclassifications. These results together implicate KIF5B as a novel biomarker of high-grade invasive breast cancer. To further examine the expression of KIF5B in relation to breast cancer subtypes, we made use of breast cancer cell lines representative of the various breast cancer molecular subtypes [24–26]. Importantly, our data showed that KIF5B to be overexpressed in breast cancer cell lines characterized as TN-basal-like/Claudin low subtype and least expressed in cell lines representative of the luminal/epithelial subtype. Importantly, cell fractionation experiments showed enrichment of KIF5B within the nuclear compartment of only TN-basal-like/Claudin low cells. On the other hand, expression of KLC1, was found to correlate with favorable patient outcome and was found to exhibit different expression pattern than KIF5B. Interestingly, breast cancer cell lines as well as bioinformatics data of clinical breast cancer cases, showed KLC1 to be most expressed in luminal breast cancer subtypes including luminal A, luminal B and Her2-2E and least expressed in basal-like subtype. Off note, no nuclear accumulation of KLC1 was observed in all breast cancer tumors examined. Together our data emphasizes the differential expression and highlight possible independent functions of these two proteins in breast cancer.

3.2. Role of Kinesin-1 subunits (KIF5B/KLC1) in determining EMP

EMP is believed to be a critical regulator of cancer heterogeneity, disease progression and metastasis. When fully implemented cancer cells will acquire stem-like mesenchymal features exhibiting invasive/metas-tatic behavior resulting in high grade malignancy and resistance to available therapies. EMP may also contribute to molecular subtype conversion. Indeed, it has been shown that metastatic breast tumors of luminal but not basal-like subtype may undergo interconversion to more aggressive subtype [43]. These considerations underscore the interest in identifying further markers and molecular players driving the transition and switch from epithelial to mesenchymal states providing closer insights into understanding breast cancer progression and opening new avenues to more advanced therapies. TN-basal-like/Claudin low breast cancer cells are known to be enriched for genes associated with EMT and to exhibit full EMT [24,44]. Our data showed that loss of KIF5B expression in these basal-like/ Claudin low breast cancer cells resulted in suppression of cell viability, EMT, migration, invasion, stemness and metastatic colonization of the lung. This result highlight KIF5B as a critical regulator of the EMP programming associated with the TN-basal-like/Claudin low breast cancer subtype. On the other hand, KLC1 was found to be required to maintain an epithelial phenotype and to suppress EMT as well as stem cell markers endowing the cells with less invasive and less aggressive features. How kinesin1 regulates EMP is still to be fully discovered and it may involve various mechanisms. A previous report did show KIF5B to contribute to cell migration as part of the formation of invadopodia within the cytoplasm in the context of NT-basal-like/Claudin low breast cancer cells [20]. Importantly, our data point to a new mechanism through which KIF5B may contribute to EMP. Indeed, we found KIF5B to localize in the nucleus in NT-basal-like/Claudin low breast cancer cells. Moreover, we found KIF5B to interact with the EMT inducer Snail1 transcription factor in these cells. Still, further IHC analyses showed heterogenous nuclear co-localization of KIF5B and Snail1, suggesting that KIF5B may have additional nuclear functions independent of Snail1. Additionally, we found that loss of KIF5B in TN-basal-like/Claudin low breast cancer cells led to the re-localization of Snail1 to the cytoplasm suggesting that KIF5B may play a role in Snail1 nuclear localization in these cells. Interestingly, PRL was found to regulate both KIF5B and Snail1 nuclear localization. Indeed, following PRL treatment of TN-basal-like/Claudin low breast cancer cells KIF5B and Snail1 were excluded from the nucleus and partially co-localized in the cytoplasm. Moreover, we found that loss of KLC1 to be a determinant in the nuclear accumulation of KIF5B. These data suggest that KIF5B/KLC1 determine the transition between epithelial and mesenchymal phenotypes thereby defining the EMP status and aggressiveness of breast cancer.

3.3. Regulation of the EMP inducer TGFβ and the EMP suppressor PRL of kinesin-1 subunits KIF5B/KLC1 in breast cancer

Extensive studies have placed TGFβ-ligands “center-stage” in regulating EMP leading to breast cancer cell invasion, metastasis and stemness [45]. On the other hand, PRL is known to play an essential role in regulating mammary alveologenesis during pregnancy lactation cycle [45–47]. Recently, PRL was shown to have direct effects in inducing apical/basal polarity and mammary luminal/epithelial stem cell terminal differentiation [23]. Interestingly, PRL was also found to have negative cross-talk with TGFβ-Smad pathway and to suppress EMT, invasion and the tumorigenic phenotype of TNBC cells highlighting PRL as a critical suppressor of EMP [78,42]. Interestingly, our data implicates a central role for KIF5B/KLC1 loop in TGFβ- and PRL regulation of EMP in breast cancer. Whereas TGFβ-pro-invasive activity requires KIF5B, PRL blocks KIF5B function through stable KIF5B/KLC1 complex formation thereby suppressing EMP.

This manuscript highlights the role of kinesin-1 subunits KIF5B/KLC1 in breast cancer. While kinesin-1 is a large superfamily compromising various protein members, further studies are needed to investigate the role of other KIFs and KLCs in relation to their role in breast cancer. Collectively, our study revealed a new understanding of the role of kinesin-1 in breast cancer mediating EMP programing. We propose here that the expression pattern of the two components of kinesin-1, KIF5B and KLC1, play an important role in determining breast cancer phenotype and aggressiveness.

4. Material and methods

4.1. Antibodies, plasmids and reagents

Antibodies: anti-KIF5B rabbit monoclonal antibody (abcam #ab167429), anti-UKHC rabbit polyclonal antibody (Santa-Cruz #ab167429), anti-UKHC rabbit polyclonal antibody (Santa-Cruz #ab167429)
4.2. Cell culture

Normal mammary epithelial cells: mouse HC11 cells were obtained from N. Hynes (Friedrich Miescher Institute, Basel, Switzerland) and were maintained in RPMI-1640 containing 10% fetal bovine serum (FBS) (Multicell Invitrogen). Human breast cancer cells: MDA-MB-231 obtained from Dr. Shafaat Rabbani (McGill University), SCP2 and SUM159 were obtained from N. Hynes (Friedrich Miescher Institute, Basel, Switzerland), MDA-MB-453, SKBR3 and BT474 were obtained from Dr. Morag Park (McGill University), SCP2 and SUM159 were obtained from N. Hynes (Friedrich Miescher Institute, Basel, Switzerland), MDA-MB-453, SKBR3 MCF7 and SCP2 cells were maintained in DMEM media (Multicell Invitrogen) containing 10% fetal bovine serum (FBS) (Multicell Invitrogen). Human breast cancer cells T47D, MCF7, BT474, SKBR3, MDA-MB-453, and SKBR3 cells were maintained in DMEM media (Multicell Invitrogen) containing 10% fetal bovine serum (FBS) (Multicell Invitrogen). SUM159 were maintained in DMEM media (Multicell Invitrogen) containing 10% fetal bovine serum (FBS) (Multicell Invitrogen). Human breast cancer cells T47D, MCF7, BT474, SKBR3, MDA-MB-453, and SKBR3 cells were maintained in DMEM media (Multicell Invitrogen) containing 10% fetal bovine serum (FBS) (Multicell Invitrogen).

4.3. KIF5B stable knock-down in human breast cancer cells

Lentiviral particles expressing human shRNA against KIF5B was obtained from Sigma and scramble shRNA were obtained from Addgene. The scramble shRNA is in pLKO.1 (Addgene plasmid 338651) and human KIF5B MISSION shRNA in bacterial Glycercolyst (TRCN#0000338651). (CCCCGGGCGCACTTATCGGAATCTGGTATACCGGATATCCTGCTAAAGTTCGGGATTTCCTTGGGATTTC) and (TRCN#0000338580) (CCCCGGGCGCACTTATCGGAATCTGGTATACCGGATATCCTGCTAAAGTTCGGGATTTCCTTGGGATTTC). MDA-MB-231, SUM159 and T47D cells were infected with lentiviral particles. Stable cell lines were then generated using puromycin selection (InvivoGen) 1 μg/ml puromycin for MDA-MB-231 and 2 μg/ml for SUM159 and T47D cells.

4.4. KLC1 transient knock-down in human breast cancer cells

Silencer pre designed SiRNA against human KLC1 and Negative control SiRNA were obtained from Thermo Fisher Scientific AM51331 and AM16708. T47D and SKBR3 cells were infected with 28 nM SiRNA using lipofectamine 2000 protocol obtained from Thermo Fisher Scientific.

4.5. Snail1 transient knock-down in human breast cancer cells

Silencer pre designed SiRNA against human Snail1 and Negative control SiRNA were obtained from Thermo Fisher Scientific AM16708. MDA-MB-231 cells were infected with 28 nM SiRNA using lipofectamine 2000 protocol obtained from Thermo Fisher Scientific.

4.6. Western blotting analysis

Nuclear extraction lysates were collected by hypotonic buffer (10 mM HEPES-KOH pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 1 mM Na3VO4, 20 mM NaF and Protease inhibitors cocktail). Then, the pellet was washed with 1× PBS 3 times. After wash, High salt buffer (20 mM HEPES-KOH pH 7.9-25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA pH 8.0, 1 mM Na3VO4, 20 mM NaF and Protease inhibitors cocktail) were used to get the nuclear extract from the pellet. 20 μg protein was loaded in the SDS-PAGE gel.

Total protein lysates were obtained using RIPA lysis buffer (50 mM Tris pH 8, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na3VO4 and Protease inhibitors cocktail). Then, the pellet was washed with 1× PBS 3 times. After washing the beads, western blotting with SDS-PAGE gel was performed with specific antibodies.

4.7. Immunoprecipitation

RIPA lysis buffer (50 mM Tris pH 8, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na3VO4 and Protease inhibitors cocktail) was used to obtain the total protein lysates. Mixter of 1 μg of anti-KIF5B antibody, protein A/G beads (20 μl) and 500 μg of cell lysates were incubated for 3 h at 4 °C. After washing the beads, western blotting with SDS-PAGE gel was performed with specific antibodies.

4.8. Immunofluorescence

Cells were grown on coverslips with 80% confluency. Fixation process were performed of coverslips coated with cells in 4% Paraformaldehyde for 15 min at room temperature, followed by permeabilization process with 0.1% Triton X-100 (Fisher). Cells were subsequently immunostained with primary antibody for an overnight period at 4 °C and followed by secondary antibody and Dapi for 1 h at room temperature. Mounting media (Lerner # 13800) was used to mount the coverslips and stored at 4 °C. Confocal microscopy was performed using Zeiss LSM 780 confocal microscope equipped with a Plan-Apochromat x63/1.4 oil immersion objective.

4.9. RNA isolation and RT-qPCR

HCl1 cells were grown to confluence and were then allowed to undergo differentiation for 24 h in media containing 10% FBS, insulin and hydrocortisone. Then, cells were starved or treated with ovine PRL (Sigma) for 24 h. Breast cancer cells T47D, MCF7, BT474, SKBR3, MDA-MB-453, MDA-MB-231, SCP2 and SUM159 were also grown to confluence before RNA extraction was performed. MDA-MB-453 cells, MDA-MB-231/Vecto and MDA-MB-231/PRLR were grown to confluence then were starved or treated with recombinant human PRL for 48 h. All cells...
were lysed in 1 ml of trizol. Total RNA was isolated following RNA extraction protocol (Abcam, United States).

NanoDrop was used to quantify RNA concentrations at 260 nm. Total RNA 1 μg was used for reverse transcription by using (iScript Reverse Transcription supermix kit # 170-8841). RT-qPCR of KIF5B, Slug, Snail, Twist, FN1, Vimentin, ZEB1, ZEB2, PRLR E-cadherin, CK18, CD44, CD24, OCT4, NANOG and SOX2) was performed.

4.10. MTT assay

5.103 cells were seeded into 96-well plate and grown for a period 2 to 8 days. Then, cells were incubated with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) at 37 °C for 2 h.

4.11. Scratch assay

5 × 10^3 cells were seeded on 6 well plate and grown until reached confluency. A straight scratch was obtained by yellow pipette tip and scratch or wound was monitoring by taking picture at 0, 24 and 48 h.

4.12. Invasion assay

80 × 10^3 cells were seeded in 24-well plates HTS multi-well insert system coated with Matrigel. Invasion assays were performed for 24h migrated cells were counted using five fields of triplicates for each experimental point.

4.13. Soft agar transformation assay

30 × 10^3 cells were seeded into 24-well plate coated with 1% agar gel and grown in growth media with 0.6% agar for 3 weeks. Colonies were stained by 0.05% crystal blue. Number of colonies was counted using low power lens microscopy.

4.14. Tumosphere assay

MDA-Scr and MDA-Sh-KIF5B cells were seeded in ultra low-attachment 24-well plate (Corning), and cultured in serum-free DMEM medium supplemented with 10 ng/ml EGF, 10 ng/ml bFGF and 1 × B27 (Invitrogen). The plate was incubated at 37 °C with 5% CO2 for 7 days, without moving the plate. Tumospheres were counted using ImageJ software.

4.15. Gene expression analysis

Publically available (ONCOMINE) and (GOBO) databases were used to determine associations between KIF5B and KLC1 m-RNA expression levels and different clinicopathological parameters in large human breast cancer cohorts. KM plotter was used to determine associations of gene expression in relation to patient outcome.

4.16. Tissue microarray

TMA of 102 cases was commercially purchased from Pantomics (Richmond, CA; BRC1021). TMA include information containing; age, grade, stage and TNM. The virtual H & E slides for those cases were available and were reviewed by a pathologist to confirm the diagnosis and that they are representative of the tumor.

4.17. Immunohistochemistry

Immunohistochemical staining was performed to paraffin embedded slides. After deparaffinization and rehydration slides were immersed in retrieval solution (sodium citrate 10 mM, pH 6.0 buffer). The slides were incubated in hydrogen peroxide block s, followed by Ultra V Block. Slides were incubated with a rabbit anti-KIF5B Antibody, UltraVision LP Detection System HRP Polymer & DAP Plus Chromogen (Thermo Fisher Scientific, Fremont CA) was used for detection. The TMA slides were scanned using Aperio XT slide scanner (Leica Biosystems).

4.18. Immunohistochemistry scoring

Quantitative IHC scoring systems were used to evaluate KIF5B immunostaining. In brief, a representative pathologist-annotated malignant region was selected for each core using images of 40× magnification from digital IHC-stained TMA slides. The mean positive pixel count (PPC) for each representative region was obtained using positive pixel count (PPC) algorithm (Aperio). The lower PPC count indicates higher IHC staining intensity. The staining intensity was divided equally into 4 categories. For KIF5B cases with PPC ≥ 95 considered +3, 96–110 PPC considered +2, 111–127 considered +1 and cases with PPC ≤ 128 considered as score 0. ER, PR, HER-2 and Ki67 classification into molecular subtypes was done as previously described [48].

4.19. Animal models

All experimental animal work was performed in a specific-pathogen-free animal facility according to the guidelines and ethical regulations of the Research Institute McGill University Health Centre approved animal used protocol (#2014-7492) in accordance with Canadian Council of animal care guidelines.

4.20. KIF5B mammary fat pad NSG mouse xenografts

14 Female NSG mice were purchased from Charles River Laboratories (Sain-Constant, QC, Canada), housed and maintained under specific pathogen-free conditions (RI-MUHC animal facility). The mice were randomly divided into two groups (n = 7 mice per group). At 7 to 9 weeks of age, the first group was injected in the fourth-left mammary fat pad with 1 × 10^6 MDA-MB-231-Scr and the second group was injected with MDA-MB-231-Sh-KIF5B. Tumor growth were monitored up to 7 weeks after injection. When tumors were detectable, tumor size was measured with a vernier caliper (Mitutoyo, Kawasaki, Japan) and calculated using the formula [length + width2]/2. Mice were sacrificed by CO2 asphyxiation.

4.21. KIF5B tail vein NOD-SCID mouse xenografts

12 Female NOD-SCID mice were purchased from Charles River Laboratories (Sain-Constant, QC, Canada), housed and maintained under specific pathogen-free conditions (RI-MUHC animal facility). The mice were randomly divided into two groups (n = 6 mice per group). At 7 to 9 weeks of age, the first group was injected in the tail vein with 5 × 10^9 MDA-MB-231-Scr and the second group was injected with MDA-MB-231-Sh-KIF5B. Mice were monitored up to 5 weeks after injection. Mice were sacrificed by CO2 asphyxiation and lungs were collected.

4.22. KLC1 tail vein NSG mouse xenografts

12 Female NSG mice were purchased from Charles River Laboratories (Sain-Constant, QC, Canada), housed and maintained under specific pathogen-free conditions (RI-MUHC animal facility). The mice were randomly divided into two groups (n = 6 mice per group). At 7 to 9 weeks of age, the first group was injected in the tail vein with 5 × 10^9 T47D-Si-KLC1. Mice were monitored up to 5 weeks after injection. Mice were sacrificed by CO2 asphyxiation and lungs were collected.
4.23. Statistical analysis

Statistical analysis were performed using GraphPad prism 6 software using Student’s t-test or one-way ANOVA analysis accordingly. Results were shown as mean ± SEM and P < 0.05 was considered as cut-off for significant association.

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Authors' contributions

AM: Designed, performed experiments and drafting the article. IH: Performed and analyzed IHC experiments, some bioinformatics data NB: Performed the nuclear extraction assay, immunoprecipitation assay and contributed to drafting the article NW: Performed in vivo experiments JJJL: Contributed design and revising the article, SA: Principal research design and supervision of the project and drafting of the article.

Conflicts of interest

The authors declare no conflicts of interest.

Data deposition and materials sharing

https://www.panomics.com/tissue-arrays/reproductive-system/breast/brc1021

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