Kruppel-like factor 4 (KLF4) is required for maintenance of breast cancer stem cells and for cell migration and invasion

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Abstract

Kruppel-like factor 4 (KLF4) is highly expressed in more than 70% of breast cancers and functions as an oncogene. However, an exact mechanism by which KLF4 enhances tumorigenesis of breast cancer remains unknown. In this study, we show that KLF4 was highly expressed in cancer stem cell (CSC)-enriched populations in mouse primary mammary tumor and breast cancer cell lines. Knockdown of KLF4 in breast cancer cells (MCF-7 and MDA-MB-231) decreased the proportion of stem/progenitor cells as demonstrated by expression of stem cell surface markers such as aldehyde dehydrogenase 1 (ALDH1), side-population (SP), and by in vitro mammosphere assay. Consistently KLF4 overexpression led to an increase of the cancer stem cell population. KLF4 knockdown also suppressed cell migration and invasion in MCF-7 and MDA-MB-231 cells. Furthermore, knockdown of KLF4 reduced colony formation in vitro and inhibited tumorigenesis in immunocompromised NOD/SCID mice, supporting an oncogenic role for KLF4 in breast cancer development. Further mechanistic studies revealed that the Notch signaling pathway was required for KLF4-mediated cell migration and invasion, but not for CSC maintenance. Taken together, our study provides evidence that KLF4 plays a potent oncogenic role in mammary tumorigenesis likely by maintaining stem cell-like features and by promoting cell migration and invasion. Thus, targeting KLF4 may provide an effective therapeutic approach to suppress tumorigenicity in breast cancer.

Keywords

KLF4; cancer stem cell; breast cancer; migration; invasion

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Introduction

Although the cancer stem cell (CSC) hypothesis was originally generated from studies of hematological malignancies, it is now finding acceptance for solid tumors, as well, e.g., colon, pancreas, prostate, brain, and breast cancers (Bonnet and Dick, 1997; O’Brien et al., 2007; Singh et al., 2004). There is substantial evidence from human breast tumors, mouse mammary tumor models, and established human and mouse breast cancer cell lines for the existence of a heterogeneous cancer stem cell population. Cancer initiating cells and/or cancer stem cells with specific cell surface markers have been reported to exhibit CSC characteristics on the basis of their enhanced tumorigenesis and self-renewal potential in immunodeficient mice (Gudjonsson et al., 2002; Kelly et al., 2007; Shackleton et al., 2006).

Initial identification of breast CSCs was based on a combination of CD44+, CD24−, and Lin− markers (Al-Hajj et al., 2003). Later, a number of markers such as ALDH1 and side population (SP) were defined (Ginestier et al., 2007; Hirschmann-Jax et al., 2004). The cancer stem cell hypothesis postulates that carcinogenesis originates from tumor stem/progenitor cells possibly due to dysregulation of normal stem cell self-renewal pathways. Therefore, it is necessary to characterize specific CSC markers and the related signaling pathways that regulate the mammary stem/progenitor cell population and drive carcinogenesis and tumor metastasis.

The Kruppel-like factor (KLFs) family of gene regulatory proteins are transcription factors implicated in the regulation of a wide range of cellular processes, including proliferation, apoptosis, differentiation, inflammation, migration, and tumor formation (Huang et al., 2005; Segre et al., 1999; Shields et al., 1996). KLF4 is a Kruppel-like factor that is highly expressed in the post-mitotic cells of both the gut and skin (Shields et al., 1996; Zheng et al., 2009). Downregulation of KLF4 in colon adenomas, gastric cancer, intestinal adenomas, esophageal cancer, prostate cancer, and lung cancer may contribute to cellular hyperproliferation and malignant transformation, which is consistent with its role in cell cycle arrest and growth inhibition (Choi et al., 2006; Wei et al., 2005). However, high levels of KLF4 expression are also reported in primary breast ductal carcinoma and oral squamous carcinoma (Foster et al., 2005; Pandya et al., 2004). This paradox is partially resolved by a recent study showing that p21Cip1 status might be a switch that determines the tumor suppressor or oncoprotein function of KLF4 (Rowland et al., 2005). An exact mechanism by which KLF4 switches between these two opposing modes remains largely unknown.

Recently, it has been reported that KLF4 is expressed in embryonic stem (ES) cells and forced expression of a combination of four transcription factors, including Oct4, c-myc, Sox2, and KLF4, is capable of reprogramming fibroblasts into induced pluripotent stem cells (iPS) that are similar to ES cells, hinting that KLF4 is indispensable for the maintenance of stem cells (Takahashi and Yamanaka, 2006; Wernig et al., 2007). Forced expression of KLF4 in ES cells inhibited differentiation into erythroid progenitors, and increased their capacity to generate secondary embryoid bodies, suggesting a role for KLF4 in self-renewal (Bruce et al., 2007; Zhang et al.). In addition, recent evidence demonstrates that KLF4 plays a role in cellular migration and invasion during embryogenesis (Garvey et al.). In colon cancer cells, KLF4 overexpression potently inhibits colony formation, migration, and invasion, which facilitates the suppression of tumorigenesis by KLF4 (Dang
et al., 2003). Urokinase-type plasminogen activator receptor (u-PAR) and laminin-5 that play critical roles in cell migration, growth and invasion have been demonstrated to be regulated or associated with KLF4 (Miller et al., 2001; Wang et al., 2004; Zhang et al., 2006). Furthermore, KLF8 (another KLF family member), whose structure and function are closely related to KLF4 (Eaton et al., 2008), promotes both a mesenchymal and stem cell population in kidney (Wang et al., 2007). KLF8 can induce motility and invasiveness in normal epithelial cell lines through regulation of E-cadherin gene expression (Wang and Zhao, 2007). Taken together, these findings suggest that KLF4 may play important roles in the regulation of stem cell self-renewal and cell motility, the two processes that are now believed to contribute to tumorigenesis. However, whether and how KLF4 plays a role in cancer stem cells and carcinoma cell invasion and metastasis is currently uninvestigated.

In this study, we provide data showing that KLF4 is essential for maintaining cancer stem cells and for promoting migration and invasion, resulting in tumor formation in vivo. Our research on the function of KLF4 in cancer stem cells and cell motility may facilitate us to better understand its role in tumorigenesis.

Results
KLF4 is highly expressed in cancer stem cell-enriched populations

Previous reports showed that overexpression of KLF4 promoted self-renewal of embryonic stem cells (Li et al., 2005) and it was required for iPS induction (Takahashi and Yamanaka, 2006), suggesting an important function of KLF4 in stem cell biology. It has been shown that breast cancer stem cells can be enriched in suspension cultures as mammospheres (Al-Hajj et al., 2003). To determine if KLF4 is highly expressed in cancer stem cells, we isolated single cell suspension from primary breast cancers originated from MMTV-Neu transgenic mice and grew them as adherent cells or mammospheres. As expected, KLF4 mRNA levels were much higher in mammospheres than that in adherent cells (Figure 1a).

Cancer stem cells can be isolated by their ability to efflux Hoechst 33342 dye and are referred as the “side population” (SP) (Yu et al., 2007). We purified SP and non-SP cells from MCF-7 and found that KLF4 expression in SP cells was 2.6-fold higher than in non-SP cells (Figure 1b). In addition, both mRNA and protein levels of KLF4 in mammospheres of MCF-7 cells were significantly higher than that in adherent cells (Figure 1c).

Previous studies identified a subpopulation (CD44+/CD24−/ESA+) of breast cancer cells that displayed a stem/progenitor cell phenotype in both human tumors and mouse models and were able to form tumors in NOD/SCID mice (Al-Hajj et al., 2003; Fillmore and Kuperwasser, 2008; Wright et al., 2008). In luminal breast cancer cell lines, including MCF-7 and SUM225, 100% cells are ESA positive. Thus, sorting cells for the CD44+/CD24− phenotype is sufficient to enrich cancer stem cell in MCF-7 cells. We, therefore, sorted and detected KLF4 expression in CD44+/CD24− and CD44−/CD24− populations. We found that KLF4 expression in the CD44+/CD24− population was 2.5-fold higher than that in the CD44−/CD24− population (Figure 1d). Expression of let-7c and miR-200c, which had been previously demonstrated to inhibit self-renewal of CSCs (Wellner et al., 2009; Yu et al., 2007), was remarkably reduced in the CD44+/CD24− population. In addition,
increased expression of Jagged1 and Notch1, which play positive roles in the maintenance of CSCs, were observed in the CD44+/CD24− population. These results suggest that high level of KLF4 expression is associated with CSC-enriched populations in breast cancer cells.

**KLF4 is required for the maintenance of breast CSCs**

To test the importance of KLF4 for the maintenance of CSCs, we generated KLF4-knockdown stable cells (designated as siKLF4) by transduction with shRNA vector and KLF4-overexpressing stable cells (designated as KLF4-N) by transduction with an N-terminal version of KLF4. Both mRNA and protein levels of KLF4 in KLF4-knockdown and KLF4-overexpressing stable cells were confirmed by real-time PCR and Western blot (Supplementary Figure 1). Supporting the importance of KLF4 in maintaining CSCs, we found that KLF4 knockdown was associated with a significant decrease of the CD44+/CD24− population (from 19.8% ± 3.2% for siCon cells to 3.5% ± 0.84% for siKLF4 cells) (Figure 2a). As expected, overexpression of KLF4 increased the CD44+/CD24− population from 0.57% to 3.32% (Supplementary Figure 3a). Recently, SP (as characterized by the efflux of Hoechst 33342 vital dye) and ALDH activity (as determined by the Aldefluor assay) have gained importance as potential markers of the breast cancer stem cell phenotype. As shown in Figure 2b, KLF4 knockdown strongly reduced SP abundance from 2.5% to 0.7%. Cells overexpressing KLF4 retained significantly more dye when compared with parental MCF-7 cells (0.1% up to 0.4%) (Supplementary Figure 3b). Furthermore, Knockdown of KLF4 decreased the number of ALDH-positive cells from 3.9% to 1.9% in MCF-7 cells and from 2.4% to 1.4% in MDA-MB-231 cells (Figure 2c, 2e). Overexpression of KLF4 increased the ALDH-positive cells from 1.3% to 7.2% in MCF-7 cells and from 3.1% to 7.5% in MDA-MB-231 cells (Supplementary Figure 3c, 3e). A recent study demonstrated that ESA+/PROCR+ populations in MDA-MB-231 cells were highly enriched with cancer stem/progenitor cell populations which exhibited the ability to self renew and divide asymmetrically (Hwang-Verslues et al., 2009). Based on these data, we analyzed ESA+/PROCR+ populations in MDA-MB-231 cell lines. siCon cells contained an ESA+/PROCR+ population of 8.1%, whereas KLF4 knockdown reduced this population to less than 1.8% (Figure 2d). Conversely, KLF4 overexpression increased the ESA+/PROCR+ population from 1.9% to 6.0% (Supplementary Figure 3d). Taken together, these results indicate that KLF4 is required for the maintenance of the cancer stem cell population.

To test whether KLF4 plays a role in self-renewal of CSCs, we cultured siCon or siKLF4 cells under non-adherent conditions. Although siKLF4 cells formed multipotent mammospheres, they did so at a significantly lower frequency ($P < 0.05$) than did siCon cells (Figure 2f, left). In addition, siKLF4 cells formed 3.3-fold fewer secondary mammospheres than siCon cells ($P < 0.05$), indicating a defect in self-renewal of siKLF4 cells (Figure 2f, middle). Moreover, the mammospheres formed in siKLF4 cells were significantly smaller, as compared with those in the siCon group ($P < 0.05$) (Figure 2f, right), suggesting that there are significantly fewer stem cells in siKLF4 cells.
Knockdown of KLF4 inhibits migration and invasion of breast cancer cells

One of the important properties of tumor cells is their increased mobility. To evaluate whether KLF4 regulates cell migration and invasion, a Matrigel invasion assay and scratch assay were performed in MCF-7 and MDA-MB-231 cells. While non-metastatic MCF-7 cells developed aggregated spheres and showed restrained cell motility (data not shown), metastatic MDA-MB-231 cells (siCon cells) formed branched structures, invaded through an 8-mm Matrigel and adhered to the bottom of the plates (Figure 3a, left). However, MDA-MB-231 cells with KLF4 knockdown (siKLF4 cells) formed a spherical shape on Matrigel (Figure 3a, right), indicating that their ability to invade a Matrigel-coated membrane was completely inhibited. Furthermore, at 12 h after scratch, the percentage of wound closure for MDA-MB-231 siCon cells was 67.2%, whereas siKLF4 cells showed 20.4% closure, indicating that KLF4 was essential for migration in MDA-MB-231 cells (Figure 3b, bottom). Similar results were observed in MCF-7 cells (Figure 3b, top). It is well established that cell attachment or detachment with the matrix where the tumor grows in the microenvironment is the “hallmark” of cell migration and invasion during metastatic processes (Yang and Weinberg, 2008). Consistent with decreased potential of migration and invasion, we found that siKLF4 cells had decreased capacity of attachment with fibronectin compared to siCon cells (Figure 3c).

Inhibition of KLF4 by a chemical inhibitor suppresses cancer stem cell self-renewal and cell migration

In addition to developing KLF4-knockdown stable cells, we proceeded to confirm KLF4 function by using a chemical inhibitor of KLF4. A previous study demonstrated that a small molecule, Kenpaullone, was able to replace KLF4 in the reprogramming of primary and secondary fibroblasts and NPCs (Lyssiotis et al., 2009), suggesting a possible regulation of KLF4 by Kenpaullone. To test this, we performed quantitative real-time PCR and Western blot to detect KLF4 expression in Kenpaullone-treated breast cancer cell lines. As shown in Figure 4a, Kenpaullone, at a non-cytotoxic concentration of 5 μM, suppressed KLF4 expression in a time-dependent manner, and reached a maximum of 60% at the 4 h time point. At the 8 h time point, KLF4 expression was 40% of the control (data not shown). Similar effects were observed in MDA-MB-231 cells (Figure 4a, right). Western blot confirmed the inhibitory effect of Kenpaullone on KLF4 protein levels (Figure 4b). Additional reporter assays showed that KLF4 promoter activity was significantly inhibited by Kenpaullone treatment (Supplementary Figure 4a), suggesting that Kenpaullone-mediated downregulation of KLF4 occurred at a transcriptional level. KLF4 downregulation was also accompanied by decreased expression of two previously reported downstream targets (Hinnebusch et al., 2004; Wassmann et al., 2007): p53 as detected by Western blot (Figure 4b) and intestinal alkaline phosphatase (IAP) as detected by the reporter assay (Supplementary Figure 4b), thus further validating the regulation of KLF4 by Kenpaullone.

A number of signaling pathways have been implicated in stem cell self-renewal (Faherty et al., 2007; O’Brien et al.) To determine through which signaling pathways Kenpaullone affected KLF4 expression, specific inhibitors were used in the assay. Treatment with a PI3K/Akt inhibitor (Wortmannin) or ERK inhibitor (PD98059) had no effect on KLF4 expression (Supplementary Figure 5). However, treatment with H89, a specific PKA
pathway inhibitor, significantly enhanced KLF4 expression by 2.77-fold (Figure 4c, left). Furthermore, a combination of Kenpaullone and H89 resulted in a 3.7-fold increase of KLF4 expression as compared with Kenpaullone treatment alone and a 62% decrease of KLF4 expression as compared with H89 treatment alone (Figure 4c, left), suggesting that the decrease in KLF4 expression by Kenpaullone was partially mediated by the PKA signaling pathway. Similar results were also observed at protein levels (Figure 4c, right).

To determine whether Kenpaullone-treated cells exhibited similar phenotypes as KLF4-knockdown cells, stem cell self-renewal and cell motility were determined. As we expected, Kenpaullone-treated cells displayed a significantly decreased mammosphere formation at a frequency of approximately 1/62.5 (16 ± 1 colonies per 1000 cells), when compared to 1/19.6 (51 ± 3.6 colonies per 1000 cells) in mock-treated cells (Figure 4d, left). In addition, Kenpaullone-treated mammospheres were roughly 14-fold smaller in volume (Figure 4d, right), indicating a defect in self-renewal. Moreover, flow cytometric analysis showed that Kenpaullone treatment in MCF-7 cells reduced the proportion of CD44+/CD24− cells from 24.8% to 9.5% (Figure 4e). To examine if KLF4 mediated the effect of kenpaullone, we first performed flow cytometric analysis. In siCon cells, Kenpaullone treatment decreased the CD44+/CD24− population from 20.2% ± 2.8% to 11.0% ± 1.2%, whereas little difference was observed in siKLF4 cells (from 3.9% ± 0.5% to 3.5% ± 0.7%) (Supplementary Figure 6). Next, we evaluated whether Kenpaullone influenced the migration and invasion ability of MDA-MB-231. As shown in Figure 4f, the percentage of wound closure in MDA-MB-231 cells with or without Kenpaullone treatment was 9.2% and 34.5% (P < 0.05), respectively. Moreover, Kenpaullone treatment suppressed the invasion of MDA-MB-231 cells into Matrigel in a dose-dependent manner (Figure 4g), suggesting that Kenpaullone treatment may cause similar effects as KLF4 knockdown (Figure 3a, 3b). Finally, Kenpaullone treatment apparently inhibited the wound closure in siCon cells from 62.7% to 14.3%, whereas little effect was observed in siKLF4 cells (Supplementary Figure 7). Collectively, these data suggest that Kenpaullone-mediated reduction of CD44+/CD24− cells and inhibition of cell migration are likely dependent on KLF4, which further confirms the function of KLF4 in the cancer stem cell maintenance and cell motility.

Knockdown of KLF4 suppresses colony formation in vitro and inhibits tumorigenesis in vivo

One reliable measurement of the tumorigenic nature of cells in vitro is the ability to form colonies in soft agar. KLF4 knockdown reduced the number of colonies by almost 42.8% when compared to siCon cells in MCF-7, and by almost 38.6% in MDA-MB-231 cells (Figure 5a). In addition, siCon colonies contained significantly more cells than the siKLF4 colonies (data not shown).

To investigate whether the stem cell phenotype and invasive capacity by KLF4 could extend to in vivo conditions, we used a mouse xenograft model to examine tumorigenesis in vivo. 5 × 10^5 KLF4 knockdown or control MDA-MB-231 cells were injected into the mammary fat pad of immunocompromised NOD/SCID mice. Control cells formed palpable tumors in 16 days after injection. However, KLF4 knockdown cells formed palpable tumors in 28 days after injection (Figure 5b). In addition, while tumors produced by the control cells displayed
fast growth and reached a diameter of 20 mm 9 weeks after injection, tumors produced by KLF4 knockdown cells grew much slower and never reached the 20-mm size (Figure 5b, 5c).

**Notch signaling pathway is required for KLF4-mediated cell migration and invasion**

The Notch signaling pathway is not only responsible for maintaining self-renewal of cancer stem cell, but is also involved in the migration and invasion of cancer cells (Dontu et al., 2004; Farnie and Clarke, 2007). To determine whether KLF4 could maintain the stem cell phenotype and increases cell motility in our model via activation of the Notch pathway, we first examined the relationship between KLF4 and the expression levels of Notch signaling molecules. Real-time PCR showed a significant reduction in the expression of Notch1, Notch2 and Jagged1 in siKLF4 cells when compared with siCon cells (Figure 6a). Conversely, overexpression of KLF4 increased the expression of those molecules (Figure 6b). To investigate whether the Notch signaling pathway was involved in KLF4-induced phenotypes as posited above, we repressed Notch signaling by using Compound E (CompE), an inhibitor of the Notch processing enzyme γ-secretase. CompE treatment did not block the formation of mammospheres in siCon or siKLF4 cells (Figure 6c, P > 0.05), which suggested that Notch signaling was not required for cancer stem cell self-renewal that was maintained by KLF4. To detect whether the Notch signaling pathway was involved in cell migration and invasion, CompE was used to treat MDA-MB-231 cells. As shown in Figure 6d, MDA-MB-231 control cells formed branched structures and some of them exhibited invasion capacity. However, the majority of KLF4-overexpressing cells (KLF4-N cells, Figure 6d) invaded through Matrigel and formed a monolayer at the bottom of the plates within 5 days. CompE treatment completely abrogated invasion of both control and KLF4-N cells. In addition, at 6 h after scratch, the percentage of wound closure for MDA-MB-231 KLF4-N cells was 48.4%, whereas KLF4 control cells exhibited much less wound closure (22.6% closure) (Figure 6e), which was consistent with the positive role of KLF4 in cell migration. Moreover, CompE treatment almost entirely abrogated the migration ability of KLF4-N cells, as shown by the reduced percentage of wound closure from 48.4% (untreated group) to 3.7% (CompE treated group, P < 0.05, Figure 6e).

**Discussion**

Although earlier reports have shown that 70% of breast carcinomas have elevated KLF4 expression and that increased nuclear staining for KLF4 is associated with a more aggressive phenotype (Foster et al., 2000; Pandya et al., 2004), the ability of KLF4 to initiate aggressive tumors in animal models has not been examined *in vivo*. By performing a colony formation assay and using a xenograft tumor model, we confirmed that KLF4 knockdown inhibited mammary tumor development *in vitro* and *in vivo* (Figure 5), suggesting that KLF4 could act as an oncogenic protein in breast cancers.

Earlier studies showed that the anti-proliferative function of KLF4 is associated with inhibition of cyclin-D1 (Shie et al., 2000) and activation of the cell-cycle inhibitor p21⁠CIP1 (Zhang et al., 2000). However, inactivation of either protein not only neutralizes the cytostatic effect of KLF4, but also collaborates with KLF4 in oncogenic function by an *in
vitro study (Rowland et al., 2005), thus further highlighting the importance of p21CIP1. Although this study provided a possible mechanistic explanation for the context-dependent oncogenic or tumor-suppressor functions of KLF4, it has not been validated in vivo. In addition, a cellular mechanism by which KLF4 contributes to the aggressive characteristics of breast cancers remains elusive. Our current studies indicate that KLF4 is required for the maintenance of breast cancer stem cells (Figure 2), and KLF4 knockdown significantly delayed tumor development of breast cancer cells in a xenograft mouse model (Figure 5). In our studies, we provided data demonstrating that knockdown of KLF4 significantly decreased CSC-enriched populations by using several different CSC markers. It should be noted that we cannot exclude other explanations of our results. As we mentioned before, KLF4 exerted an anti-apoptotic function in many cancer cell lines. It is possible that the reduced CSC population in KLF4 knockdown cells may be a result of the increased apoptosis mediated by KLF4 reduction. However, the fact that cell viability of KLF4 knockdown cells was comparable to that of the control cells would argue against this possibility (data not shown).

In addition, we have not performed limiting-dilution assays (LDAs) to determine the tumor-initiating capacities of CSC cells in NOD/SCID mice, which is a traditional method in CSC studies. However, the fact that stem cell properties in KLF4 knockdown cells were characterized by specific cell surface markers, side population, and aldehyde dehydrogenase, importance of which has widely been confirmed in tumorigenicity, and that KLF4 knockdown cells showed decreased tumorigenesis in NOD/SCID mice would still support an important role of KLF4 in maintenance of breast cancer stem cells. Nevertheless, our results not only provide additional experimental support for the important function of KLF4 in stem cell biology, as shown before in embryonic stem cells (Li et al., 2005) and in iPS cells (Takahashi and Yamanaka, 2006; Wernig et al., 2007), but also are important for breast cancer studies. Cancer stem cells have been shown to foster blood vessel formation and promote cell motility. They have marked therapeutic resistance (Charafe-Jauffret et al., 2008) and have been implicated in breast cancer metastasis that remains the number one cause of cancer-related mortality in women (Lawson et al., 2009). Our study suggested that overexpression of KLF4 was sufficient to drive cell migration and invasion (Figure 6d, e). Additional studies on the mechanisms by which KLF4 maintains cancer stem cell phenotype will be very helpful to develop novel therapeutic strategies targeting KLF4 or the related signaling pathway to treat malignant breast cancer and metastasis.

The function of KLF4 in maintenance of cancer stem cells has been confirmed in our study by using Kenpaullone, a small molecule inhibitor of KLF4. Previous work has demonstrated that Kenpaullone is able to replace KLF4 in the reprogramming of primary and secondary fibroblasts and Kenpaullone-induced iPS cells display characteristics of pluripotent ES cells (Lyssiotis et al., 2009). However, no difference of KLF4 expression has been observed after Kenpaullone treatment. Our study indicated that Kenpaullone treatment led to decreased expression of KLF4 both at the mRNA and protein levels (Figure 4a, b). This discrepancy may have arisen from two different systems, the previous one dealing with iPS cells and ours with breast cancer cells. More importantly, we postulate that KLF4 may be an early responsive gene after Kenpaullone treatment. In the iPS studies, KLF4 expression was detected after 5-day Kenpaullone incubation. The early responsiveness of KLF4 to
Kenpaullone may have been missed. In our system, a maximal down-regulation of KLF4 was observed at a 4 h time point after Kenpaullone treatment (Figure 4a), after which its expression gradually recovered. Nevertheless, Kenpaullone-treated cells possessed phenotypes similar to KLF4 knockdown cells in our studies, which, from another point of view, confirmed the indispensable role of KLF4 in cancer stem cells and extended a function of Kenpaullone from the induction of iPS cells to the maintenance of mammary cancer stem cells.

The epithelial-mesenchymal transition (EMT), as a unique process by which epithelial cells undergo remarkable morphological changes (leading to increased motility and invasion), is believed to be reminiscent of “cancer stem-like cells”, showing characteristics similar to many cancer systems (Mani et al., 2008; Yang and Weinberg, 2008). TGF-β is a well established regulator of EMT during development and tumor progression (Miettinen et al., 1994). King et al. observed that TGF-β induced KLF4 synthesis in vascular smooth muscle cells (King et al., 2003). In addition, β-catenin, one of the most important mesenchymal markers, has been verified to interact with KLF4 in the intestinal crypts, with the crosstalk of KLF4 and β-catenin playing a critical role in the homeostasis of the normal intestine as well as in the tumorigenesis of colorectal cancers (Zhang et al., 2006). Based on the pivotal role of KLF4 in cancer stem cells, in combination with its links with TGF-β signaling pathway, we highly suspected that KLF4 promoted EMT in breast cancers. In our studies, KLF4 knockdown MCF-7 cells exhibited a well-spread morphology, with the majority of cells forming a rounded, epithelial-like form and aggregating together in groups, a typical characteristic of MET and a reversal of EMT (data not shown). In addition, two critical mesenchymal-associated markers, fibronectin and vimentin, were decreased in siKLF4 (MCF-7 and MDA-MB-231) cells (data not shown), which were consistent with reduced ability of migration and invasion of siKLF4 cells. However, E-cadherin expression and localization, a hallmark of the EMT phenotype, showed no significant difference between siCon and siKLF4 cells. Recently, KLF4 was reported to inhibit EMT in non-transformed MCF-10A cells (Yori et al.), which was entirely the opposite of our results. Our major argument is that MCF-10A cells are spontaneously transformed cells with no potential of tumorigenesis. Therefore, the results from MCF-10A cells may not be readily applicable to other mammary tumor cells. In their study, MDA-MB-231 tumor cells with KLF4 overexpression had also been used. However, results from our studies, using KLF4 knockdown and overexpression stable cells, supported a positive connection between KLF4 and EMT. Clearly, more studies are necessary to examine whether the difference of the two systems or the genetic background of specific MDA-MB-231 clones contributes to the discrepancies between the previously reported results and our current results.

It has been reported that Notch signaling plays a critical role in normal human mammary development by acting on both stem cells and progenitor cells (Dontu et al., 2004), suggesting that abnormal Notch signaling may contribute to mammary carcinogenesis by deregulating the self-renewal of normal mammary stem cells. In this current study, we found that the expression of Notch1, Notch2 and Jagged1 was significantly decreased in KLF4 knockdown cells (Figure 6a). Unexpectedly, inhibition of the Notch pathway by a GSI had no effect on stem cell numbers and self-renewal potential of breast cancer cells (Figure 6c),

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suggesting that the Notch signaling pathway is not required for KLF4-mediated maintenance of stem cells in breast cancer cells. On the other hand, inhibition of Notch signaling by CompE in KLF4-overexpressing cells led to decreased migration and invasion ability (Figure 6d, e), which indicated that the Notch signaling pathway was responsible for KLF4-mediated mobility characteristics of breast cancer cells. These results are consistent with the role of Notch signaling as potent drivers during tumor progression and in converting polarized epithelial cells into motile, invasive cells (Sahlgren et al., 2008). The relationship between the Notch signaling pathway and KLF4 appears dependent on different cellular contexts. Our early work and that of others suggest that KLF4 is inhibited by Notch in the gastrointestinal tract (Ghaleb et al., 2008; Real et al., 2009; Zheng et al., 2009). Recently, downregulation of Notch1 gene expression in keratinocytes by KLF4 has also been reported (Lambertini et al.). However, in our current studies, it appears that KLF4 upregulates gene expression of Notch1, Notch2, and Jagged1 (Figure 6a, b). This regulation likely occurs at the transcriptional level, as recently reported (Liu et al., 2009), though an indirect effect cannot be ruled out.

In conclusion, our study provides evidence for the first time showing that KLF4 is essential for the maintenance of breast cancer stem cells and cell migration and invasion, which may offer important clues to understand how KLF4 promotes breast cancer development. Additional studies on the underlying mechanism will be very helpful to develop KLF4-based therapeutic strategies to treat breast cancer. In addition, whether KLF4 has the similar functions to maintain other cancer stem cells and to facilitate cell motility remains a topic for further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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KLF4 was highly expressed in CSC-enriched population. (a) KLF4 expression was examined in adherent cells and mammospheres of primary tumors originated from MMTV-Neu transgenic mice. Primary tumor cells of MMTV-Neu transgenic mice were isolated, as described in Materials and Methods. $1 \times 10^4$ primary tumor cells were cultured as adherent cells or mammospheres. Relative mRNA levels of KLF4 were determined by semi-quantitative RT-PCR. Oct4 and Nanog were used as positive and negative controls, respectively. (b) KLF4 expression was examined in side population (SP) and non-SP cells of MCF-7. MCF-7 cells were stained with 5 μM Hoechst 33342 in the absence or presence of 1 μM BCRP inhibitor Ko143. The cell population that disappeared in the presence of Ko143 was identified as SP cells. $5 \times 10^4$ SP and non-SP cells in MCF-7 were purified by a FACS sorter and KLF4 expression was analyzed by quantitative real-time PCR. * $P < 0.05$ vs non-SP cells group. (c) KLF4 expression was determined in adherent cells and mammospheres of MCF-7 cells by quantitative real-time PCR (left) and Western blot (right). For mammosphere culture, MCF-7 cells were grown in Ultra-Low Attachment Surface plates at a density of $5 \times 10^3$/plate. Ten days after seeding, RNAs from both adherent cells and mammosphere were extracted and real-time PCR and Western blot were performed to detect KLF4 expression. * $P < 0.05$ vs adherent cells group. Tubulin was used as an internal control. (d) KLF4 expression was determined in CD44+/CD24− and CD44−/CD24− populations isolated by flow cytometry. $1 \times 10^5$ cells in each population were sorted and quantitative real-time PCR was executed to detect KLF4 expression. * $P < 0.05$ vs CD44−/CD24− group.
Figure 2.
Knockdown of KLF4 resulted in a reduced stem cell population and decreased self-renewal of breast cancer stem cells. (a) FACS analysis was performed to examine CD44+/CD24− population in KLF4 knockdown (designated as siKLF4) cells. Freshly isolated siCon and siKLF4 MCF-7 cells were labeled with CD24 (FITC) and CD44 (PE) antibodies. For identification of CD44+/CD24− population, samples were analyzed using a FACSCalibur flow cytometer and CellQuest software. (b) SP population in MCF-7 stable cells was determined by Hoechst 33342 Efflux Assays. 1 × 10^6 MCF-7 stable cells were incubated with 5 μM Hoechst 33342 in the absence (top) or presence (bottom) of 1 μM BCRP inhibitor Ko143. SP cells were analyzed by flow cytometry, as described in Materials and Methods. ALDH activities in MCF-7 (c) and MDA-MB-231 (e) stable cells were assessed by utilizing the Aldefluor assay. MCF-7 and MDA-MB-231 stable cells were incubated with 1.5 μl of activated ALDEFLUOR substrate at 1 × 10^6 cells/ml. DEAB was used to establish the baseline fluorescence of these cells and to define the ALDEFLUOR-positive region. Incubation of cells with ALDEFLUOR substrate in the absence of DEAB induces a shift in BAAA fluorescence, defining the ALDEFLUOR-positive population. (d) FACS analysis was perform to examine ESA+/PROCR+ population in MDA-MB-231 stable cells. Freshly isolated siCon and siKLF4 cells were labeled with ESA (FITC) and PROCR (PE) antibodies. (f) Left, MCF-7 cells (siCon and siKLF4) were grown in Ultra-Low Attachment Surface plates at a density of 1000, 500, 200, and 100/well. Assays were conducted after 10 days (left). * P < 0.05 vs siCon group. Middle, Primary (P1) and secondary (P2)
mammosphere formation under suspension culture conditions were evaluated in MCF-7 mammary tumor cell lines. Right, Images showed typical mammospheres that formed after 10 d in non-adherent cultures of control (siCon) and KLF4 knockdown cells (siKLF4).
Figure 3.

KLF4 knockdown reduced cell migration, invasion and adhesion in vitro. (a) Knocking down KLF4 inhibited MDA-MB-231 cells invasion quantified by a Matrigel assay. MDA-MB-231 cells (siCon and siKLF4) were plated in Matrigel-coated plates at a density of $2 \times 10^3$ cells/well. Insets showed representative fields of invasive cells. (b) Knocking down KLF4 reduced migration of MCF-7 (top) and MDA-MB-231 (bottom) cells measured by scratch assay. MCF-7 or MDA-MB-231 cells were seeded into 6-well plates at a density of $1 \times 10^6$ cells/well. The 6-well plates were scratched (time 0) and photographed at 12 h. (c) KLF4 knockdown cells had reduced adhesion capacity measured by cell attachment assay. MCF-7 (left) and MDA-MB-231 (right) cells were plated onto 96-well plates treated with fibronectin at a density of $2 \times 10^3$ cells/well in triplicate. Cells were allowed to adhere for 2 h and adherent cells were stained with Crystal Violet and quantified using the absorbance as a measurement at 550 nm. * $P < 0.05$ vs siCon group.
Figure 4. Kenpaullone, a small molecule inhibitor of KLF4, reduced self-renewal of breast cancer stem cells and cell motility in vitro. MCF-7 and MDA-MB-231 cells were seeded in 6-well plates and incubated with Kenpaullone (5 μM, designated as Ken +). At the indicated time points (0, 1, 2, and 4 h), total RNA and proteins were extracted. Real-time PCR (a) and Western blot (b) were performed to detect the expression of KLF4 and p53. *P < 0.05 vs Kenpaullone-untreated group (Ken−). In b, Tubulin was used as internal controls. (c). KLF4 expression was detected in MCF-7 cells treated with PKA pathway-specific inhibitors H89, Kenpaullone and combination of H89 and Kenpaullone. 24 hours after treatment, total RNA and proteins were extracted. Real-time PCR (left) and Western blot (right) were performed to detect KLF4 expression. *P < 0.05 vs Ken+ group; #P < 0.05 vs H89 group. Tubulin was used as an internal control. (d) Mammosphere formation under suspension culture conditions was evaluated in MCF-7 mammary tumor cell lines. MCF-7 cells were seeded in Ultra-Low Attachment Surface plates and incubated with Kenpaullone (5 μM) for 10 days. *P < 0.05 vs Ken− group. Images showed typical mammospheres that formed after 10 d in non-adherent cultures (right). (e) Mammary stem cell markers (CD24 and CD44) were detected by flow cytometry in Kenpaullone-treated MCF-7 cells. MCF-7 cells were treated with Kenpaullone (5 μM) for 7 days. Cells were labeled with CD24 (FITC) and CD44 (PE) antibodies. Migration (f) and invasion (g) of MDA-MB-231 cells were evaluated after Kenpaullone treatment.
Knockdown of KLF4 reduced tumorigenesis in vitro and in vivo. (a) Colony-forming abilities of siCon and siKLF4 cells were assessed. MCF-7 and MDA-MB-231 stable cells were grown in soft agar (0.3%) at a density of 2.5 × 10^3 cells/10-cm plate in triplicate for 3 weeks. Eight random fields were selected and colonies were counted using a 10 × objective. Mean number of colonies per plate was calculated and data were presented as the means ± S.M. * P < 0.05 vs siCon group. (b) Tumor growth curves were plotted for immunocompromised NOD/SCID mice injected with KLF-knockdown (siKLF4, solid line) and control cells (siCon, dashed line). Five immunocompromised NOD/SCID female mice in the age of 4–6 weeks were injected with the siKLF4 or siCon MDA-MB-231 cells subcutaneously into the mammary fat pad number four according to standard injection procedures. Once tumors were palpable, tumor growth was monitored until the tumor size reached to 20 mm in diameter in either direction. Data are shown as mean size ± S.M. of tumors in five mice per cell line. (c) Representative tumors were shown that were grown in immunocompromised NOD/SCID mice injected with siKLF4 and siCon cells.
Figure 6.
Inhibition of the Notch signaling abrogated KLF4-induced cell migration and invasion. (a) Levels of Notch1, Notch2, and Jagged1 expression in siCon and siKLF4 MCF-7 cells were detected by real-time PCR. * $P < 0.05$ vs siCon group. (b) Similar to (a) except that Control and KLF4-N (KLF4 overexpression) MCF-7 cells were used. (c) MCF-7 cells (siCon and siKLF4) were seeded into Ultra-Low Attachment Surface plates and incubated with Compound E (CompE) at a concentration of 1 μM. Assays to detect mammospheres were conducted after 10 days. Invasion (d) and migration (e) of MDA-MB-231 cells (Control and KLF4-overexpresing cells) were evaluated after CompE incubation (1 μM).