Chapter 15

Function of KLF4 in Stem Cell Biology

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Additional information is available at the end of the chapter

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1. Introduction

The Kruppel-like factor family is a group of zinc finger containing transcription factors, which are highly homologous with the Drosophila Kruppel protein. The feature that distinguishes the KLF family from other zinc finger containing transcriptional factors is the presence of three highly conserved C2H2 containing zinc finger motifs at the C-terminus [1-3]. These fingers enable KLFs to bind to the GC-box or CACCC-boxes on DNA with different affinities [4]. KLF4, as a member of KLF family, expresses in a wide range of tissues in mammals, and plays a critical role in regulating a diverse array of cellular processes including proliferation, differentiation, development, maintenance of normal tissue homeostasis and apoptosis. KLF4 can also acts either as a tumor suppressor or an oncogene depending on differing cellular context and cancer types.

The role that KLF4 plays in stem cell biology has attracted much more attention in recent years. For instance, in 2006, Takahashi K et al [5] reprogrammed somatic cells into pluripotent stem cells using KLF4 in combination with three other transcription factors: Oct4, Sox2 and c-Myc. Numerous recent literatures have further proved that KLF4 is essential for both embryonic stem (ES) cells self-renewal and maintenance, additionally our recent work revealed a critical role of KLF4 in maintenance of breast cancer stem cells [6]. Furthermore, we found that KLF4 is expressed in mouse skin hair follicle stem cells and such expression contributed to mouse cutaneous wound healing [7]. In this review, functions of KLF4 in stem cells, especially breast cancer stem cells and mouse hair follicle stem cells will be discussed, and the signaling pathways possibly involved will be addressed as well.

2. Identification and characterization of KLF4

Mouse KLF4 was first identified in 1996 independently by two groups and separately given two different names - GKLF (gut enriched Kruppel like factor):due to its high expression in the

gastrointestinal tract [8], and EZF (epithelial zinc finger) since it was highly expressed in differentiated epithelial cells of the skin [9]. Human KLF4 cDNA was cloned from human umbilical vein endothelial cell cDNA library [10] and later renamed as KLF4 to avoid confusion.

The human KLF4 gene locus is mapped on chromosome 9q31 whereas mouse KLF4 is on chromosome 4B3. Mouse KLF4 has a single ORF of 1449 bp that encodes a polypeptide of 483 amino acids with a predicted molecular weight of 53 Kd; while human KLF4 has an ORF of 1444 bp coding for a 470 amino acid protein with an estimated molecular mass 50 Kd. At the amino acid level the human and mouse KLF4 are shown to have 91% sequence similarity. The three tandem zinc finger motifs are conserved completely in the human and mouse sequences. Except skin and colon [8, 9], KLF4 is also found in lung, testis, small intestine [8, 9], thymus [11], cornea [12], cardiac myocytes [13] and lymphocytes [14]. In testis, four KLF4 transcripts with alternative polyadenylation were found and they generated different RNA species in various testicular cells, strongly suggesting translational regulation of KLF4 in spermatogenesis [15, 16].

3. General functions of KLF4

3.1. Inhibition of cell proliferation

KLF4 is known to induce growth arrest, inhibiting cell proliferation by regulating the expression of key cell cycle genes. Elevated expression of KLF4 in NIH3T3 subjected to serum starvation [8] has been shown to inhibit DNA synthesis. Microarray analysis confirms that a number of genes were up- or down-regulated upon KLF4 induction, most of which are involved in cell cycle control [17]. For example, the expression of cell cycle inhibitor p21/Cip1 was elevated [18], while cell cycle promoter Cyclin D1 was depressed [19]. KLF4 has been shown to inhibit cell proliferation by blocking G1/S progression of the cell cycle and to mediate p53 dependent G1/S cell cycle arrest in response to DNA damage [20, 21]. Furthermore, KLF4 plays an important role in maintaining the integrity of the G2/M checkpoint following DNA damage. While wild type HCT 116 colon cancer cells were arrested at the G2/M phase checkpoint upon γ-irradiation, p53 -/- cells were able to enter M phase even after irradiation. It was observed that upon introduction of KLF4 into p53 -/- cells, the mitotic indices were considerably reduced and the Cyclin B1 levels were also risen [22]. These studies suggest that KLF4 is a critical factor in regulating entry of the cells into the mitotic phase. Finally, KLF4 was found both necessary and sufficient in preventing centrosome amplification following γ–irradiation-induced DNA damage by transcriptionally suppressing cyclin E expression [23].

3.2. Promotion of cell differentiation

Microarray analysis has shown that many keratin genes were upregulated on KLF4 induction, indicating its role in epithelial differentiation. Additionally, KLF4 has been reported to transactivate promoters of epithelial genes including CYP1A1 [24], laminin α 3A [25], laminin 1 [26], keratin 4 [27], keratin 19 [28]. Recent studies demonstrated that KLF4 plays a vital role in goblet cell differentiation in the intestine [29, 30], conjunctiva [31], and also in the formation of the epi-

thelial barrier of the skin [32]. KLF4 null mice died one day after birth due to loss of barrier function of the skin. It appears that KLF4 influences the formation of the cornified envelope in the late-stage differentiation process that was supported by upregulation of Sprr2a, a cornified envelope gene, in KLF4 knockout mice. Two additional cornified envelope proteins: repetin (encoded by Rptn) and plasminogen activating inhibitor 2 (encoded by Planh2) were found later. KLF4 may regulate these genes resulting in an imbalance in cornified envelope assembly or composition, thereby altering the structural scaffold on which the lipid lamellae are organized. A differential role of KLF4 has also been reported in smooth muscle cells [33], monocytes [34], testes [15], T cells [11, 35] and murine tooth development [36].

3.3. Other functions

KLF4 is thought to be involved in chronic inflammatory disease since it has been shown to mediate proinflammatory signaling in human macrophages in vitro [37, 38] and regulate the expression of interleukin-10 in RAW264.7 macrophages [39]. KLF4 is also essential for differentiation of mouse inflammatory monocytes and involved in the differentiation of resident monocytes [34, 40]. The inflammation-selective effects of loss-of-KLF4 and gain-of-KLF4-induced monocytic differentiation in HL60 cells identify KLF4 as a key regulator of monocytic differentiation and a potential target for translational immune modulation [40]. KLF4 positively regulates human ghrelin expression [41], which is expressed in the gastrointestinal tract. In addition, it was found that KLF4 is an immediate early gene for Nerve Growth Factor [42]. A recent study showed that glutamatergic stimulation can trigger rapid elevation of KLF4 mRNA and protein levels, and that the over expression of KLF4 can regulate neuronal cell cycle proteins and sensitize neurons to NMDA-induced caspase-3 activity [43]. Another study demonstrated that KLF4 is involved in regulating the proliferation of CD8+ cells [44]. The transcription factor ELF4 directly activated the tumor suppressor KLF4 'downstream' of T cell antigen receptor signaling to induce cell cycle arrest in naive CD8+ T cells [44].

KLF4 has been implicated in the regulation of apoptosis [45, 46]. During DNA damage, cells can take two routes - either pass into the next phase overcoming the checkpoint or get arrested at the checkpoint and activates the repair machinery. As discussed previously, over expression of KLF4 in RKO colon cancer cells, when subjected to UV radiation, reduced the percentage of apoptotic cells [47]. In esophageal cancer cell lines, KLF4 has been shown to bind to the promoter and repress the activity of the surviving gene *in vivo* [48], which is necessary for caspase inactivation and therefore acts as a negative regulator of apoptosis.

4. KLF4 in stem cell biology

4.1. KLF4 function in embryonic stem cells

Embryonic stem (ES) cells are characterized by a self-renewal ability and pluripotency. Self-renewal is the capability of ES cells to be maintained in a proliferative state for prolonged periods of time, whereas pluripotency is the ability of ES cells to differentiate into a diverse array of specialized cell types. It has been shown that self renewal and maintenance of pluri-

potency in mouse ES cells requires leukemia inhibitory factor (LIF). LIF is a member of the IL6 cytokine family and is used to maintain ES cell cultures in an undifferentiated state through activation of the *Stat3* gene. Oct4, Sox2, and Nanog are all thought to be the master regulators of ES cell pluripotency. Although Oct4 and Sox2 are not direct targets of Stat3 [49], they have been identified as two essential transcription factors that form a heterodimer which binds to the Nanog promoter and regulates the expression of downstream genes that contribute to the maintenance of self-renewal [50]. KLF4 acts as a fast responding mediator to LIF-Stat3 signal changes, and directly binds to the promoter of Nanog to help Oct4 and Sox2 in regulating the expression of Nanog [51]. This observation confirms the critical role of KLF4 in ES cell self renewal as well as pluripotency.

4.2. KLF4 function in generation of induced pluripotent stem cells

ES cells are believed to hold great promise for regenerative medicine due to their unique ability to differentiate into any cell type. However, the application of human eggs or embryos encounters big ethical problems. This dilemma was broken in 2006 by Dr. Shinya Yamanaka's group. They picked four transcription factors, including Oct4, Sox2, c-Myc, and KLF4, to introduce into mouse embryonic fibroblasts via retroviral transfection [5]. The modified embryonic fibroblasts were found to be reprogrammed to a pluripotent state similar to that observed in ES cells. Later the finding was further confirmed by using either mouse or human adult fibroblasts [52-57]. The discovery of these "induced pluripotent stem cells" (iPS cells) was regarded as a great achievement in stem cell research and gave new insights into the feasibility of clinical application of stem cells.

A panel of assays has been performed to compare iPS cells with ES cells in morphology, surface marker expression, epigenetic status, formation of embryoid bodies *in vitro*, directed differentiation into neural cells and beating cardiomyocytes, teratoma formation *in vivo* and chimera contribution. The results indicated that iPS cells resemble ES cells by all measured criteria. Not only fibroblasts, but also other terminally differentiated cells can be reprogrammed to pluripotent cells [58]. After the introduction of pluripotency from terminally differentiated cells, the applications of the iPS cells have also been explored. By using a humanized sickle cell anemia mouse model, mice can be rescued after transplantation with hematopoietic progenitors obtained from autologous iPS cells *in vitro*. Mechanistically, the rescue was due to the correction of the human sickle hemoglobin allele by gene specific targeting. This report provides the first proof of principle for using iPS cells for disease treatment in mice [59] and demonstrates the therapeutic potential of iPS cells for human diseases.

Although iPS cells based on somatic cells avoid ethical issues, the use of oncogenes and retrovirus still raised safety concerns. For example, reactivation of the c-Myc retrovirus, increased tumorigenicity in the chimeras and progeny mice, hindering clinical applications [60]. Another problem is that iPS cells are refractory to differentiation and thereby increase the risk of immature teratoma formation after directed differentiation and transplantation into patients. Even if only a small portion of cells within each iPS cell clone shows impaired differentiation, then those cells might be sufficient to produce immature teratomas [61].

Nevertheless, the iPS cell technology potentially can overcome two important obstacles associated with human ES cells: immune rejection after transplantation and ethical concerns regarding the use of human embryos [61]. The advantage of iPS cell technology is that iPS cells can be generated using a few programming factors in any laboratory using standard techniques and equipment. Establishment of a stable and self-sustainable ES-specific transcriptional regulatory network is essential for reprogramming [62]. iPS cells still have the scope for clinical applications provided that proper ways are established to precisely evaluate each iPS cell clone and to select appropriate sub clones prior to clinical application.

4.3. KLF4 function in breast Cancer Stem Cells (CSC)

Cancer stem cells (CSCs) are a subpopulation of tumor cells that possess the stem cell properties of self renewal and differentiation, which allows them to generate the heterogeneous lineages of cancer cells that comprise the tumor. In 1997, a hierarchy in human acute myeloid leukemia cells was first reported, which improved the understanding of tumorigenesis and cast new light on cancer therapy [63]. CSCs in other types of hematological malignancies were identified later, and then CSC research was expanded to solid tumors shortly after. The identification of CSCs in solid tumors depends on specific biomarker. Recently, CSCs have been identified in numerous solid tumors, including pancreas [64], colon [65], prostate [66], bladder [67], lung [68] and breast cancer [69].

In breast cancer the first evidence of CSC was based on a combination of specific cell-surface antigen profile CD44+/CD24-/Lin- in 2003 [69]. More recently, aldehyde dehydrogenase (ALDH) was used as stem cell marker in a series of 577 breast carcinoma and 33 human breast cell lines [70]. ALDH is a detoxifying enzyme that oxidizes intracellular aldehydes and is thought to play a role in the differentiation of stem cells via the metabolism of retinal to retinoic acid [71]. Side population (SP) was also defined as a characteristic of breast CSC, which indicated an inherently high resistance to chemotherapeutic agents [72]. Since the CSCs have the capacity for self-renewal, differentiation into multiple cancer cell lineages, extensive proliferation as normal stem cells, and are responsible for tumor recurrence and chemotherapeutic resistance, it is necessary to figure out the key regulators and related signaling pathways that regulate the CSC in the process of carcinogenesis and tumor metastasis.

As discussed previously, KLF4 plays a critical role in ES self renewal and pluripotency, and is one of the four transcription factors creating iPS cells. Therefore, it's very worthy to explore the relationship between KLF4 and breast CSCs along with underlying mechanisms. Our recent work provides evidence for the first time that KLF4 is essential for the maintenance of breast CSCs and cell migration and invasion [7]. This evidence may offer important clues to understand how KLF4 promotes breast cancer development.

Earlier reports have shown that elevated KLF4 expression is detected in nearly 70% of breast carcinomas and that nuclear localization of KLF4 is associated with a more aggressive phenotype in early-stage breast cancer [73, 74]. However, the ability of KLF4 to initiate aggressive tumors in vivo has not been examined yet. Our study showed that KLF4 was highly expressed in CSC-enriched populations in mouse primary mammary tumor and human

breast cancer cell lines (Figure 1). Knockdown of KLF4 in breast cancer cell MCF-7 and MDA-MB-231 inhibits cell migration, invasion and adhesion *in vitro*, and the self-renewal of breast CSCs (Figure 2). Tumor growth in mouse xenograft mode was suppressed as well (Figure 3), suggesting that KLF4 could act as an oncogenic protein in breast cancers.

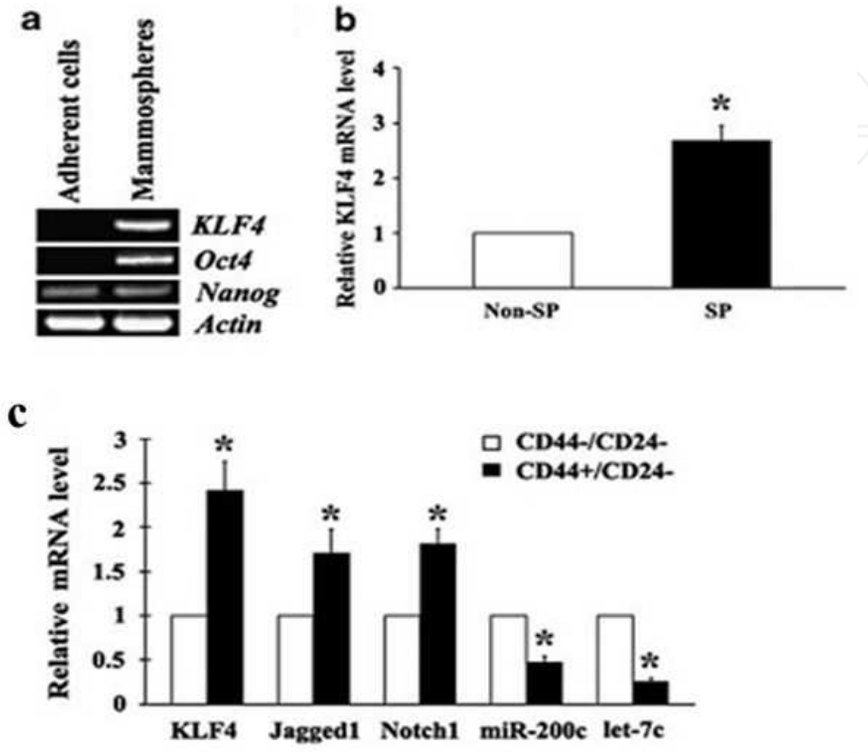

Figure 1. KLF4 was highly expressed in CSC-enriched population. (a) KLF4 expression was examined in adherent cells and mammospheres of primary tumors originated from MMTV-Neu transgenic mice. Oct4 and Nanog were used as positive and negative controls, respectively. (b) KLF4 expression was examined in SP and non-SP cells of MCF-7. The symbol * indicates $P<0.05$ vs non-SP cells group. (c) KLF4 expression was determined in CD44+/CD24- and CD44-/CD24- populations isolated by flow cytometry. The symbol * indicates $P<0.05$ vs CD44-/CD24- group.

The anti-proliferative function of KLF4 is associated with inhibition of cell cycle promoter cyclin-D1 [19] and activation of the cell-cycle inhibitor p21/Cip1 [18]. Since inactivation of either protein not only neutralizes the cytostatic effect of KLF4 but also collaborates with KLF4 in oncogenic transformation [75], thus further highlighting the importance of p21/Cip1. Although p21/Cip1 status might be a switch that determines the tumor suppressor or oncoprotein function of KLF4, the exact mechanism has not been elucidated yet. Moreover, a cellular mechanism by which KLF4 contributes to the aggressive characteristics of breast cancers remains unknown. Our current studies indicate that KLF4 is required for the maintenance of breast CSCs and the knockdown of KLF4 significantly decrease the self-renewal of breast CSCs by examining several different CSC markers. Notably KLF4 exerted an anti-apoptotic function in many cancer cell lines, so it is possible that the decreased CSC population upon KLF4 knockdown may be a result of the increased apoptosis mediated by KLF4 reduction. However, the fact that cell viability

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of KLF4 knockdown cells was comparable to that of the control cells would argue against this possibility. We have not performed limiting-dilution assays to determine the tumor-initiating capacities of CSC cells in non-obese diabetic/severe combined immunodeficiency mice yet, which is a traditional method in CSC studies. Nevertheless, our results not only provide additional experimental support for the important function of KLF4 in stem cell biology, but also are important for breast cancer studies. CSCs have been shown to foster blood vessel formation and promote cell motility. They are also resistant to chemotherapy and radiotherapy [76] and have been implicated in breast cancer metastasis that remains the number one cause of cancer-related mortality in women [77]. Our study suggested that overexpression of KLF4 was sufficient to drive cell migration and invasion. 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.

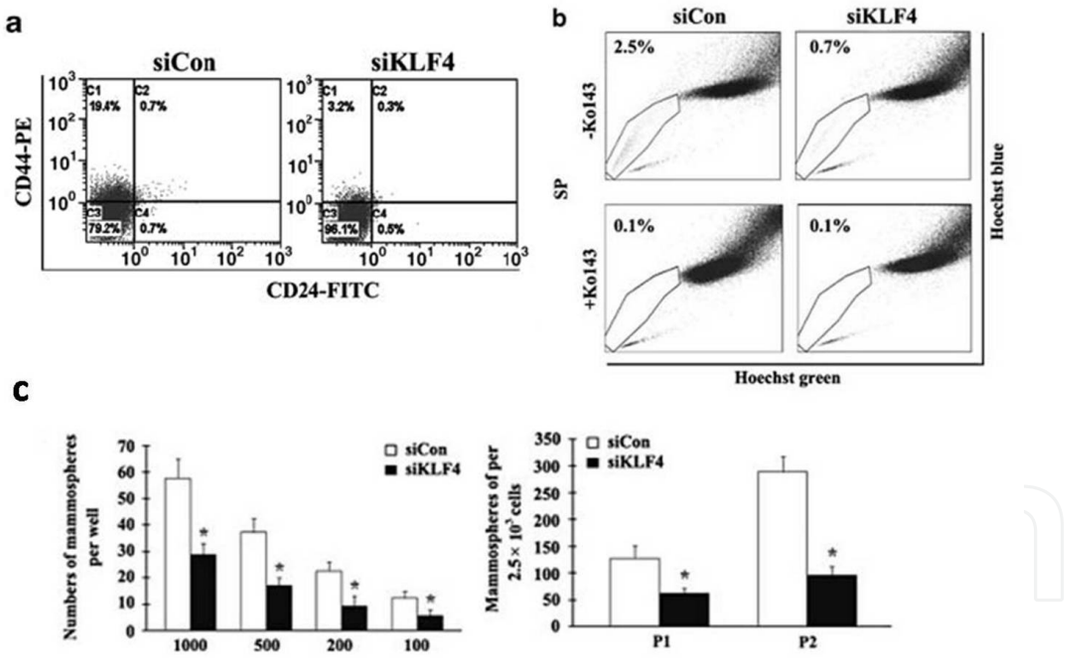

Figure 2. Knockdown of KLF4 resulted in a reduced stem cell population and decreased self-renewal of breast cancer stem cells. (a) Freshly isolated siCon and siKLF4 MCF-7 cells were labeled with CD24 (fluorescein isothiocyanate (FITC)) and CD44 (phycoerythrin (PE)) antibodies to identify CD44+/CD24-population using a FACSCalibur flow cytometer. (b) SP population in MCF-7 stable cells was determined by Hoechst 33342 efflux assays. (c) Left, MCF-7 cells (siCon and siKLF4) were grown in ultra-low attachment surface plates at a density of 1000, 500, 200, and 100 per well. Assays were conducted after 10 days (left). The symbol * indicates $P<0.05$ vs siCon group. Right, primary (P1) and secondary (P2) mammosphere formation under suspension culture conditions were evaluated in MCF-7 mammary tumor cell lines.

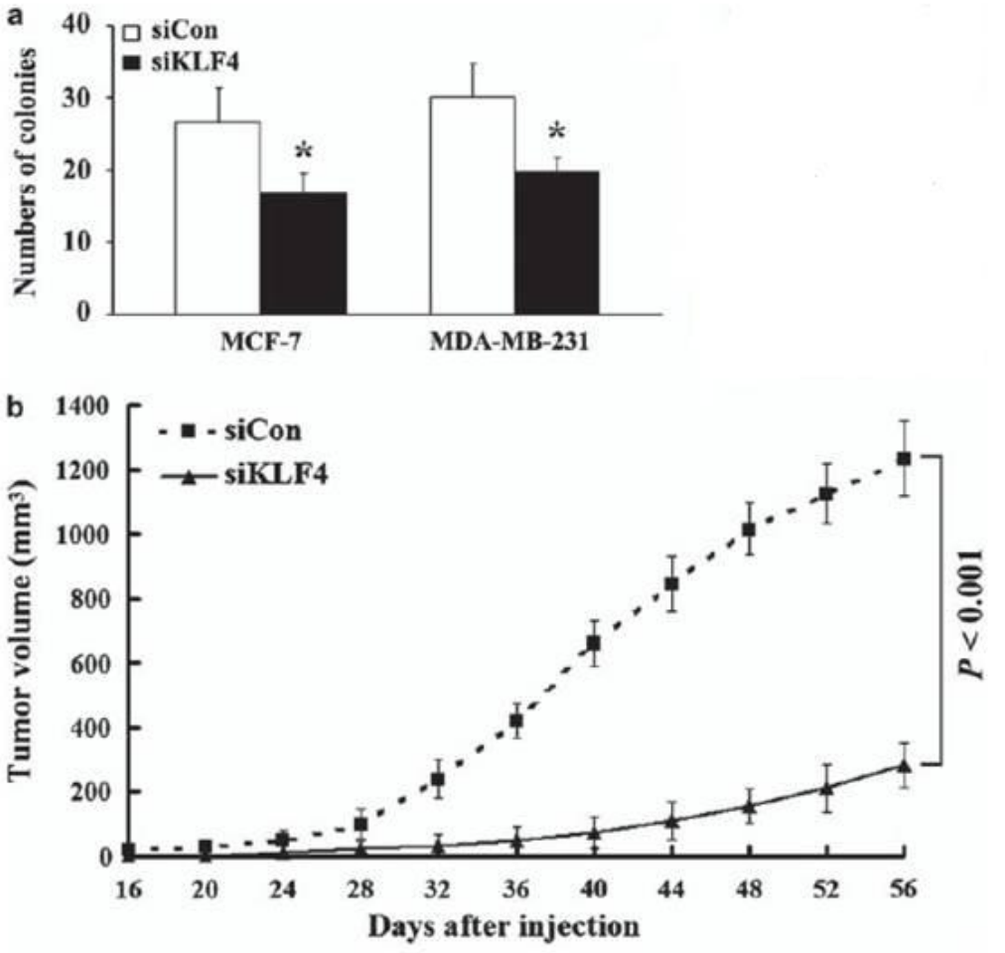

Figure 3. Knockdown of KLF4 reduced tumorigenesis in vitro and in vivo. (a) Colony-forming abilities of siCon and siKLF4 cells were assessed. The symbol * indicates $P<0.05$ vs siCon group. (b) Tumor growth curves were plotted for immunocompromised non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice injected with KLF-knockdown (siKLF4, solid line) and control cells (siCon, dashed line). Data are shown as mean size ± s.e.m. of tumors in five mice per cell line.

The function of KLF4 in maintenance of CSCs has been further 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 that Kenpaullone-induced iPS cells display characteristics of pluripotent ES cells [78]. We tested KLF4 expression in Kenpaullone-treated breast cancer cell lines and found that it decreased at both of the mRNA and protein levels. Additional reporter assays showed that KLF4 promoter activity was significantly inhibited by Kenpaullone treatment, suggesting that Kenpaullone-mediated downregulation of KLF4 occurred at a transcriptional level. KLF4 downregulation was also accompanied by decreased expression of two previously reported down-stream targets [79, 80]: p53 and intestinal alkaline phosphatase. This further validates the regulation of KLF4 by Kenpaullone. Since a maximal downregulation of KLF4 was observed at a 4 h time point after Kenpaullone treatment, we postulate that KLF4 may be an early responsive gene after Kenpaullone treatment, and after this point, the

expression of KLF4 gradually recovered. 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 CSCs and extended a function of Kenpaullone from the induction of iPS cells to the maintenance of mammary CSCs.

Our research also indicates that KLF4 might promote epithelial-mesenchymal transition (EMT) in breast cancers. EMT is a unique process by which epithelial cells undergo remarkable morphological changes (leading to increased motility and invasion) and believed to be reminiscent of 'cancer stem-like cells', showing characteristics similar to many cancer systems [81, 82]. It has been reported that KLF4 interacts with transforming growth factor-β, a well established regulator of EMT [83], and β-catenin, one of the most important mesenchymal markers. Based on the pivotal role of KLF4 in CSCs, in combination with its links to the transforming growth factor-β signaling pathway, we highly suspected that KLF4 improved 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 mesenchymal to epithelial transition [84] and a reversal of EMT. Fibronectin and vimentin, two critical mesenchymal-associated markers, were both decreased in KLF4 downregulated cells, which were consistent with reduced ability of migration and invasion of these cells. However, E-cadherin expression and localization, a hallmark of the EMT phenotype, showed no significant difference after KLF4 was knocked down. Contrary to our results though, KLF4 was reported to inhibit EMT in non-transformed MCF-10A cells by another group [85]. 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.

4.4. KLF4 function in mouse hair follicle stem cells

Skin is renewed throughout life by proliferation of a multipotential stem cell population and terminal differentiation of stem cell progeny. Epidermal renewal is thought to be controlled by stem cells located either in the basal layer of the interfollicular epidermis (IFE) or in the deepest portion of permanent hair follicle called bulge [86]. Mouse hair follicle stem cells which reside in the hair follicle bulge are characterized by expression of CD34 and CD49 [87-89], retention of either DNA or histone labels over long periods [90, 91], and expression of Leucine-rich repeats and immunoglobin-like domain protein 1 (Lrig1) [92, 93]. Wound healing is an important response of skin in order that it might repair itself after an injury. Regeneration of epidermis after wounding involves activation, migration and proliferation of keratinocytes from both the surrounding epidermis and the adnexal structures such as hair follicles [94-96]. The discovery of properties of epidermal stem cells led to the hypothe-

sis that these stem cells play a critical role in epidermal repair after wounding. Previous work has reported that bulge stem cells rapidly respond to wounding and migrate towards the IFE to help with the rapid hair-follicle regeneration, and that bulge-derived cells are transient amplifying cells committed to differentiation [93, 95, 97]. However, the role and contribution of keratinocytes derived from hair follicle bulge stem cells to cutaneous wound healing needs further elucidation.

It has been proven that KLF4 is essential for establishing the barrier function of skin. However, KLF4 expression and potential function in epidermal stem cells has not been studied before. In our current study, we have shown that KLF4 is likely expressed in mouse epidermal stem cells. A decreased number of hair bulge stem cells was observed in KLF4 knockout mice, which was accompanied by a decreased ability of colony formation from these cells when compared to those from control mice, suggesting that KLF4 may be required for the maintenance of skin hair follicle stem cells. Notably, KLF4 deficiency delayed the process of mouse cutaneous wound healing, during which KLF4-expressing multipotent cells migrated towards the wound area [6].

Using the wild type mice and KLF4/EGFP mouse model, we found that KLF4 was expressed in CD34+/CD49f+ bulge stem cell-enriched populations. However, KLF4 gene expression in CD34+/CD49f+/Lrig1+ cells was about 2.2 fold higher than in CD34+/CD49f-/Lrig1- cells sorted from wild-type mice. High levels of KLF4 expression in most differentiated, post mitotic skin epithelial cells [98] and low percentage of skin epidermal stem cells may be reasons why a difference has not been observed. Nevertheless, our studies collectively provide the first evidence that KLF4 was likely expressed in mouse hair follicle stem cells, especially in bulge stem cells.

The label retention cell (LRC) assay was used to confirm the quiescent nature of KLF4-expressing cells (Figure 4). Three-day-old KLF4/EGFP mice were injected with BrdU and left for an extended period. Twelve weeks later, the proportion of KLF4-positive cells in LRCs was 4.1%, suggesting that only a subset of these LRCs expressed KLF4. These results reveal a heterogeneous nature of LRCs. However, the difference between KLF4-expressing and KLF4-non-expressing LRCs and the related functional influence in wound healing still remain unknown. By lineage tracing to the KLF4/CreERTM/ Rosa26RLacZ mouse model, a multipotent and clonal nature of KLF4 expressing cells was identified as well (Figure 5). Our studies have also shown that KLF4 knockout decreased the population of CD34+/CD49f+ cells accompanied by reduced self-renewal ability of these cells. Together with the label retaining ability of KLF4 expressing cells, our results indicated KLF4 plays an important role in the homeostasis of skin bulge stem cells. In addition, expression of KLF4 in rare skin stem cells and in the bulk of differentiated keratinocytes may suggest that the functions of KLF4 in these populations are different. It has been reported that different KLF4 isoforms may exist and exhibit different functions in pancreatic cancer cell [99]. Characterization of different KLF4 isoforms and/or separation of distinct KLF4 expressing cells will be necessary for dissecting specific functions of KLF4 in skin homeostasis as well as pathogenesis including wound healing.

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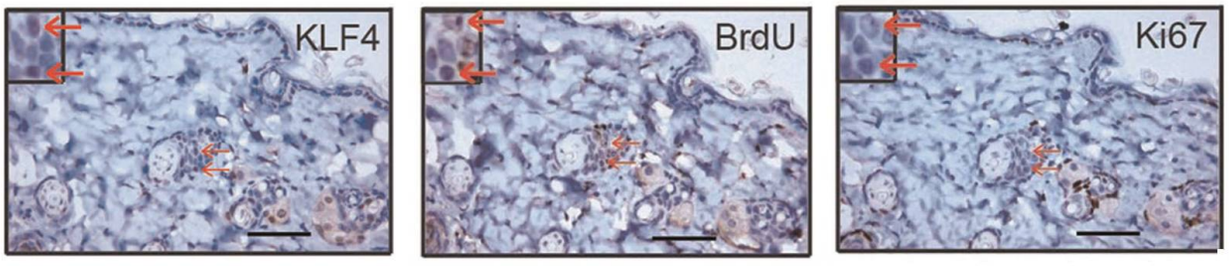

Figure 4. KLF4-expressing cells possessed label retaining property. 3-day-old KLF4/EGFP mice were injected with BrdU (75mg/kg) for 5 consecutive days. BrdU-positive cells were examined 3 months later by immunohistochemical staining. Anti-KLF4, anti-BrdU, and anti-Ki67 antibodies were used to stain consecutive slides. Insets show enlarged portion of the staining indicating co-localization of KLF4 and BrdU positive cells with no Ki67 signals (red arrows). Scale bars, 50 mm.

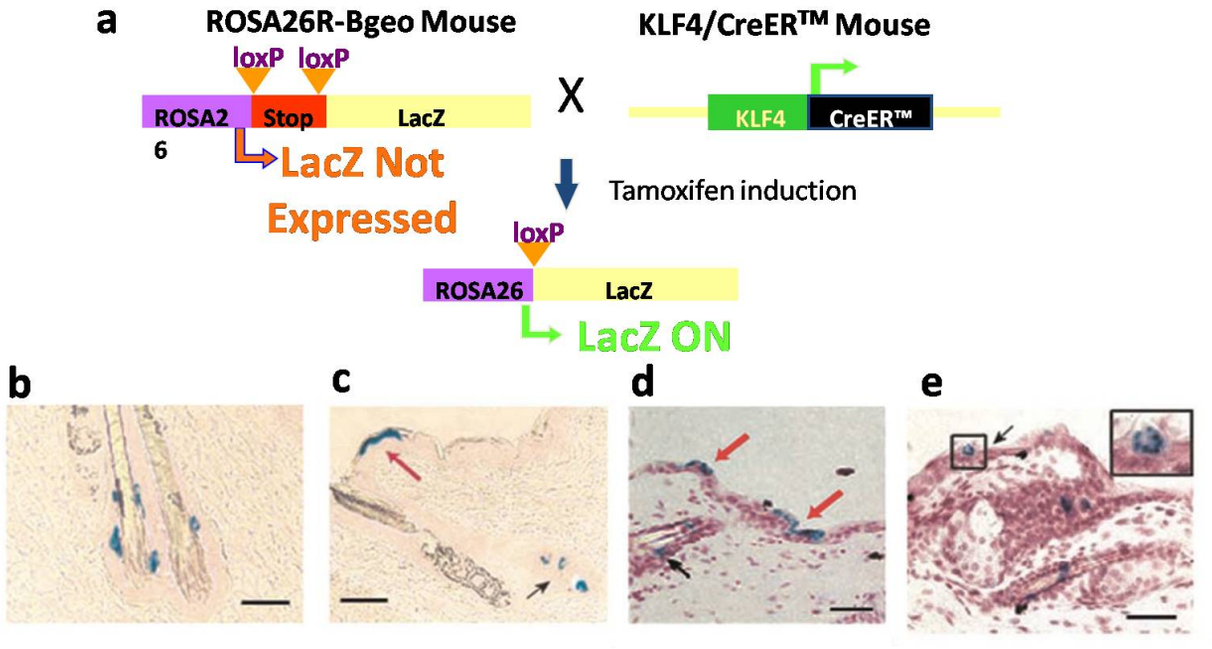

Figure 5. KLF4-expressing hair follicle stem cells were examined by lineage tracing. KLF4/CreERTM/Rosa26RLacZ mice were induced by tamoxifen (100mg/kg) for 5 consecutive days at 6-week-old (a).4 weeks later X-gal staining was performed. Potential KLF4 expression in interfollicular epidermis (shown by red arrows in c, d) and bulge area (b, and black arrows in c, d) was shown. A typical epithelial proliferation unit was shown in e (inset). Note that fixation was performed without xylene in a and b. Scale bars, 80 mm.

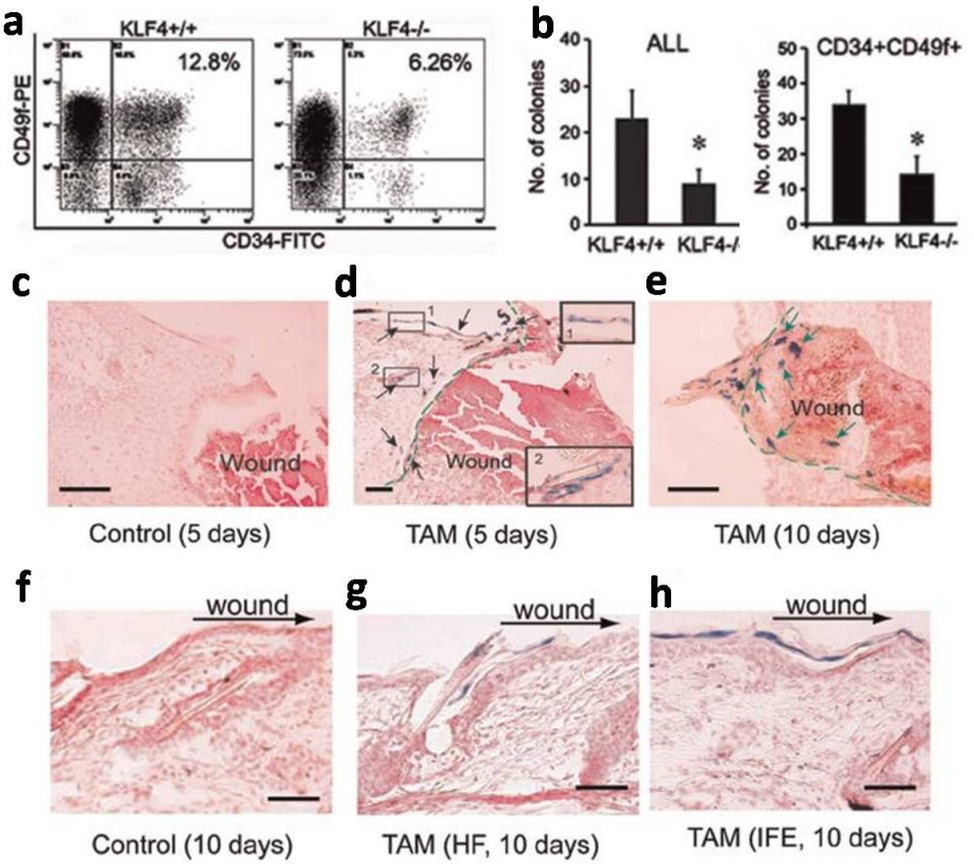

Figure 6. Knockout of KLF4 decreased hair follicle stem cell population and self-renewal potential *in vitro* and retarded would healing *in vivo*. (a) Dorsal skin keratinocytes isolatedfrom control (KLF4+/+) and KLF4 knockout (KLF4-/-) mice were analyzed by flow cytometry using mouse epidermal stem cell markers CD34 and CD49f. (b) Quantitation of the colony numbers from 2000 seeded keratinocytes. Data shown were the mean ± SM of three separate experiments. **P* <0.05 vs. control. 5mm wounds were introduced into the backs of KLF4/CreER^{TM}/Rosa26RLacZ mice 5 (c, d) or 10 days (e–h) after using control (c, f) or tamoxifen (d, e, g, h) induction and X-gal staining was performed. Blue strips on epidermis were shown in d (inset 1) and h. Blue cells was indicated by black arrows outside (d) and by green arrows inside (e) the conjunction of the wound (separated by dashed green lines). Inset 2 in d showed blue cells around hair follicles. Migration of KLF4 expressing multipotent cells from hair follicles (g) and interfollicular epidermis towards the wound area was detected similarly.. Scale bars, 80 mm.

Previous work has demonstrated that stem cells located in the bulge area [95] and isthmus [100] contribute to wound healing. Our work has shown that KLF4-expressing multipotent cells participate in re-epithelialization during cutaneous wound healing. It known that cutaneous wounds heal with an acute delay in re-epithelialization in the absence of hair follicles [101]. From our study we learned that KLF4 expression in possible hair follicle stem cells may contribute to the wound healing (Figure 6). We also observed that KLF4-expressing stem cells remained quiescent as evidenced by rarely detectable blue cells eight months after the cells were labeled. However, they were readily activated and detectable when the cutaneous wound occurred. This observation is consistent with a recent proposal for olfactory neural stem cells. In this pattern, stem cells within the LRC population serve as a reservoir of long-lived progenitors that remain largely quiescent during normal neuronal turnover or

even after acute, selective loss of mature neurons; meanwhile previously identified progenitors are largely responsible for tissue maintenance. Surprisingly after extensive injuries that deplete resident neuronal precursors, these quiescent stem cells transiently proliferate and reconstitute the neuroepithelium to maintain homeostasis [102]. Moreover, KLF4 deficiency delayed the process of wound healing and cell migration. It has been proven that KLF4 is essential for establishing skin barrier function because KLF4 deficiency selectively perturbed the late-stage differentiation structures including the cornified envelope [32]. It is not clear though, whether the role of KLF4 in barrier function is also involved in wound healing in our setting. Finally, our wound healing model did not limit for contraction. Although this simple method allowed us to observe an obvious phenotype, more rigorous models should be used in the future in order to define the role of KLF4 in the complex wound healing process. Nonetheless, our results suggest a critical function of KLF4-expressing epidermal multipotent stem cells in cutaneous wound healing.

4.5. Signaling pathways regulating KLF4 and stem cell biology

Stem cells often reside in locations called stem cell niches. Specifically, stem cell niches are defined as particular locations or microenvironments that maintain the combined properties of stem cell self-renewal and multipotency [103]. A combination of genetic and molecular analyses has identified many factors that support stem cell niches that also control stem cell identity. These factors include components of Notch, Wnt, and Hedgehog signaling pathways, all of which KLF4 is thought to be involved in [104-106].

5. Notch signaling and KLF4

Notch signaling is involved in cell proliferation and apoptosis, which affects the development and function of many organs. The signal is initiated by interaction of a Notch receptor with a Notch ligand on an adjacent cell. Upon activation, Notch is cleaved, releasing intracellular domain of the Notch (ICN) through a cascade of proteolytic cleavages by the metalloprotease tumor necrosis factor-α-converting enzyme (TACE) and γ-secretase. ICN then translocates to the nucleus where it displaces corepressor complexes that are prebound with CSL. The following recruitment of coactivators, including Mastermind-like proteins and CBP/p300, then activates gene expression of downstream target genes [107].

It has been reported that altered Notch signaling affects the function of a variety of mammalian stem cells such as hematopoietic, intestinal, and skin stem cells, and intestinal stem cells in Drosophila and germ stem cells in C. elegans [103, 105, 108]. KLF4 is proposed as the downstream target of Notch signaling pathway and KLF4 promoter activity is inhibited by Notch, but 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 [107, 109, 110]. Recently, downregulation of Notch1 gene expression in keratinocytes by KLF4 has also been reported [111].In our current study on breast CSCs, we found that the expression of Notch1, Notch2 and Jagged1 were

significantly decreased in KLF4 knockdown cells, and upregulated by overexpression of KLF4. Unexpectedly, inhibition of the Notch pathway by CompE, a γ-secretase inhibitor, had no effect on stem cell numbers and self-renewal potential of breast cancer cells. This result suggested that the Notch signaling pathway is not required for KLF4-mediated maintenance of stem cells in breast cancer cells (Figure 7). On the other hand, inhibition of Notch signaling by CompE in KLF4-overexpressing cells led to decreased migration and invasion ability, 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 [112]. However, in breast cancer cells, inhibitors of canonical Notch1 signaling suppressed the transformation induced by Notch1 whereas it had no effect on the transformation by KLF4, indicating KLF4-induced transformation requires Notch1, canonical Notch1 signaling is not required, and Notch1 may signal through a distinct pathway in cells with increased KLF4 activity. These results suggest that KLF4 could contribute to breast tumor progression by activating synthesis of Notch1 and by promoting signaling through a non-canonical Notch1 pathway [113].

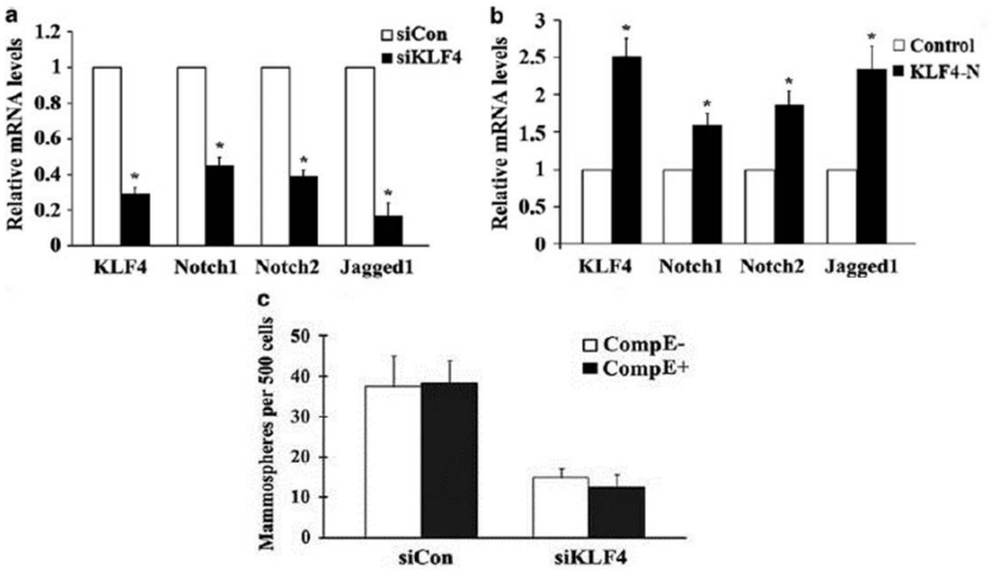

Figure 7. Notch signaling pathway is activated but not required for KLF4-mediated maintenance of stem cells in breast cancer cells (a) Levels of Notch1, Notch2 and Jagged1 expression in siCon and siKLF4 MCF-7 cells were detected by real-time PCR. The symbol * indicates $P<0.05$ vs siCon group. (b) Similar 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 CompE at a concentration of 1 mM.

6. Wnt signaling and KLF4

Wnt signaling is an ancient and highly conserved system that is involved in embryogenesis, development, cell polarization, differentiation and proliferation [114-116]. Wnt sig-

naling cascades have traditionally fallen into two categories: canonical and non-canonical, differentiated by their dependence on β-catenin. Canonical Wnt signaling is initiated when a Wnt ligand engages co-receptors of the Frizzled (Fzd) and low-density lipoprotein (LDL)-related protein (either Lrp5 or Lrp6), ultimately leading to β-catenin stabilization, nuclear translocation and activation of target genes. The canonical Wnt/β-catenin pathway plays a crucial role in stem and cancer stem cells' self-renewal and/or differentiation of skin, intestine and mammary gland [117].

In the absence of Wnt stimulus, β-catenin is held in an inactive state by a multimeric "destruction" complex comprised of adenomatous polyposis coli (APC), Axin, glycogen synthase kinase 3β (GSK3β) and casein kinase1α (CK1α) [118]. Nearly 90% of colon cancer harbors Wnt/β-catenin signaling mutations that result in β-catenin mutation. The most common type of mutation in colon cancer results in the inactivation of APC, thus driving constitutive activation of β-catenin [119-121]. KLF4 binds the transcriptional activation domain of β-catenin and inhibits β-catenin-mediated transcription in colorectal cancer cells, suggesting that the cross talk between KLF4 and β-catenin plays an important role in intestinal homeostasis and colorectal carcinogenesis [122]. A growing body of evidence illustrates a critical role of β-catenin in CSCs. For example, stem-like colon cells with a high level of β-catenin signaling have a much greater tumorigenic potential than counterpart cells with low β-catenin signaling [123]. The latest report shows that in stem cells and cancer cells, TERT, the enzymatic subunit of telomerase complex controlling telomere length, is directly regulated by β-catenin, and klf4 is required for β-catenin to localize to the *Tert* promoter [124].

In over 50% of clinical breast cancer cases a stabilization of β-catenin has been demonstrated. Inhibition of Wnt/β-catenin signaling in the mouse mammary gland blocks organ development and pregnancy-induced proliferation and heavily reduces the numbers of alveolar progenitor cells [125]. Wnt/β-catenin has also been implicated in mediating the radiation resistance of mouse mammary gland progenitor cells. Our recent study shows that KLF4 is required for maintenance of breast CSCs and for cell migration and invasion along with Notch signaling pathway [7]. However, the reaction of KLF4 and Wnt/β-catenin signaling in this setting still remains unknown and needs further investigation. Our other work showed that KLF4 contributes to cutaneous wound healing [6]. Additionally, the canonical Wnt signals are required in the normal skin to instruct bulge stem cells toward the hair cell fate [126], while in epidermal tumors, they control the maintenance of skin CSCs [84]. Therefore it is speculated that both of KLF4 and Wnt/β-catenin signaling are implicated in this process, and the relationship between them needs further investigation as well.

7. Hedgehog signaling and KLF4

Under normal conditions, HH signaling plays important roles in embryonic development and is also involved in tissue regeneration in adults [127, 128]. Activating events in the HH pathway are involved in numerous human cancers, including melanoma [129], glioma [130], and basal cell carcinoma (BCC) [131]. Mammalian HH signaling is initiated when one of

three HH ligands (Sonic, Indian, and Desert HH) binds the dodecatransmembrane receptor Patched (Ptch1). Ligand/receptor interactions occur through an autocrine or paracrine manner, depending on the context. Receptor engagement results in activation of the heptatransmembrane Smoothened (Smo), which is held in an inactive state in the absence of a ligand. Smo activation in turn regulates the activity of transcription factors Gli1, Gli2 and Gli3. Gli1/2/3 function to regulate transcription of genes involved in HH signaling such as Gli1 and Ptch1, and importantly genes involved in epithelial-mesenchymaltransition (EMT), such as SNAIL1[127, 128].

HH-GLI signaling was found to modulate normal dorsal brain growth by controlling precursor proliferation [132]; it was also found to have an essential role in controlling the behavior of CD133+ glioma cancer stem cells [130]. However, HH pathway-driven tumorigenesis depends on canonical Wnt/β-catenin signaling in BCC [131]. Recently, CSC/tumor initiating cells (TIC) in human melanomas were found in a collection of human melanomas obtained from a broad spectrum of sites and stages by using non-adherent spheres and ALDH enzymatic activity. Both pharmacological inhibition of HH signaling by the SMO antagonist cyclopamine and GLI antagonist GANT61, and stable expression of shRNA targeting either SMO or GLI1 result in a significant decrease in melanoma stem cell self-renewal *in vitro* and a reduction in the number of ALDH high melanoma stem cells, indicating an essential role of the HH-GLI1 signaling in of melanoma CSC/TIC. Notably, melanomaspheres express not only high levels of Hedgehog pathway components, but also high levels of embryonic pluripotent stem cell factors Sox2, Nanog, Oct4 and KLF4 [129]. This is the first report that reveals a possible correlation of HH signaling and KLF4 in CSC, though the underlying mechanism appears entirely unknown.

8. Concluding remarks

Since the identification and characterization of KLF4 over 10 years ago, significant progression has been made to understand its biological function, including its role in cell proliferation, differentiation, apoptosis and maintenance of normal tissue homeostasis. However, a novel role of KLF4 in stem cell biology further opens a window to study KLF4 in a different area. KLF4 is believed to play a significant role in ES cell self-renewal and pluripotency. Notably, KLF4 collaborating with other transcription factors including Oct4, Sox2 and c-Myc, drives somatic cells into iPS cells. CSCs have been identified in various tumors, and KLF4 can be speculated to have similar functions in CSCs based on its function in ES cell [133]. Our work provides evidence for the first time that KLF4 is essential for the maintenance of breast CSC and cell migration and invasion, which may be helpful to develop new therapeutic strategies for breast cancer. Apart from just breast CSCs, our work also demonstrates that KLF4 is highly expressed in skin hair follicle stem cells and facilitates the process of cutaneous wound healing. Many papers have confirmed the underlying molecular mechanism that KLF4 exerts its action in stem cell biology by integration of different signaling pathways, including Notch, Wnt and HH. Notch signaling pathway is responsible for KLF4-mediated mobility characteristics of breast cancer cells, while Wnt/β-catenin signaling recruits KLF4 to

regulate TERT expression in stem cells and cancer cells. As to HH signaling and KLF4, the research is still just beginning, but considering the crosstalk between Wnt/β-catenin and HH, it is very important to discern the communication between them. Nevertheless, understanding the signaling circuitries regulating stem cell fate decisions might provide important insights into novel therapeutic strategies for cancer and regeneration medicine.

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