FEBS Letters 587 (2013) 631–638

FEBS Letters

journal homepage: www.FEBSLetters.org

A feedback regulation between Kindlin-2 and GLI1 in prostate cancer cells

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ARTICLE INFO

Article history:
Received 22 October 2012
Revised 5 December 2012
Accepted 21 December 2012
Available online 18 January 2013

Edited by Zhijie Chang

Keywords:
Kindlin-2
GLI1
Hedgehog signaling
Feedback loop
Tumor cell viability

ABSTRACT

Kindlin-2 is engaged in tumor progression. However, the mechanism accounting for Kindlin-2 regulation in tumor cells remained largely unknown. Here, we report a regulatory loop between Kindlin-2 and GLI1, an effector of Hedgehog signaling pathway. We show that Kindlin-2 is transcriptionally downregulated via GLI1 occupancy on the Kindlin-2 promoter. Adversely, we found that Kindlin-2 promotes GLI1 expression through a mechanism involving GSK3β inactivation and is independent of Smoothened. Functionally, knockdown of Kindlin-2 cooperates with cyclopamine, a Smoothened antagonist, to decrease the viability of prostate cancer cells. Taken together, targeting the Kindlin-2–GLI1 feedback loop may facilitate the killing of prostate cancer cells.

1. Introduction

Kindlin-2 belongs to a novel family of focal adhesion proteins consisting of three members, Kindlin-1, Kindlin-2 and Kindlin-3 (also known as FERMT1, FERMT2 and FERMT3). Kindlin-2 was initially identified as an integrin-interacting focal adhesion molecule and was found to be essential for the regulation of integrin signaling through direct interaction with the cytoplasmic tail of integrin β3. Kindlin-2 cooperated with Talin to activate integrin αIIbβ3 [1–5]. Loss of Kindlin-2 caused early embryonic lethality in mice [3], whereas knockdown of Kindlin-2 homolog in *Zebrafish* resulted in severe abnormalities of heart development [6]. Furthermore, loss of UNC-112, the homolog of Kindin-2 in *Caenorhabditis elegans* leads to an embryonic lethal Pat (paralyzed, arrested elongation at twofold) phenotype [7,8]. These findings indicate that Kindlin-2 is a biologically important molecule.

Recently, we and others found that Kindlin-2 was expressed differentially in a variety of cancers including malignant mesothelioma (MM) [9], breast cancer [10], uterine leiomyosarcoma [11] and gastric cancer [12]. We found that Kindlin-2 was highly expressed in MM, and knockdown of Kindlin-2 with small interfering RNA (siRNA) impaired MM cell spreading, adhesion and migration [9]. Gozgit et al. [10] also obtained similar results in breast cancer cells. Kindlin-2 was found highly expressed in androgen-insensitive prostate cancer cells PC-3 and DU-145, and knockdown of Kindlin-2 enhanced the sensitivity of PC-3 cells to cisplatin [13]. In addition, Kato et al. [11] reported that the expression of Kindlin-2 was increased in leiomyomas but not in leiomyosarcomas. Furthermore, knockdown of Kindlin-2 considerably increased the invasiveness of mesenchymal cancer cells by inhibiting the secretion of urokinase-type plasminogen activator (uPA) [14]. Our recent findings demonstrated that Kindlin-2 is a Wnt signaling regulator by forming a molecular complex with β-catenin and TCF4 [15]. Taken together, these findings suggested that Kindlin-2 plays a diverse role in cancer progression. However, little is known about the regulation of Kindlin-2 itself. Therefore,

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0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.12.028

elucidating the molecular mechanisms accounting for how Kindlin-2 is regulated will help to understand the complex role of Kindlin-2 in human cancers.

GLI1, as a member of the Krüppel family of zinc finger proteins, is an effecter of Hedgehog signaling that plays an important role in cancer development. GLI1 regulates proliferation and differentiation in various cell types as well as the morphogenesis of a variety of tissues and organs during embryo development [16]. GLI1 was upregulated in several types of human cancers including malignant glioma [17], prostate cancer [18] and pancreatic cancer [19]. Albeit GLI1 was known to be a transcriptional activator in most cases, the N terminus of Gli1 was found to recruit components of histone deacetylase complexes [20], suggesting that GLI1 might inhibit transcription under certain circumstances that were rarely known.

In the current investigation, we aimed to understand the mechanism of how Kindlin-2 is regulated. To this end, we identified that Kindlin-2 is a novel target of GLI1 by demonstrating that GLI1 inhibits the transcription of Kindlin-2 in both normal and cancer cells through GLI1 occupancy onto the promoter of Kindlin-2; whereas Kindlin-2 upregulated GLI1 expression in a HH receptor Smoothened independent manner. These data indicated a regulatory loop between GLI1 and Kindlin-2 that regulates cancer cell viability, which suggests that targeting Kindlin-2 might be a new choice for cancer therapeutics.

2. Materials and methods

2.1. Cell culture

HKC cells were maintained in H-DMEM: F12 medium (Gibco), DU-145 and PC-3 cells were maintained in RPMI 1640 supplemented with 10% FBS (Hyclone), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen). PC-3 and DU-145 cells were treated with cyclopamine (Sigma) for various time points in different experiments.

2.2. Plasmids and antibodies

Human Kindlin-2 full length cDNA was cloned from prostate cancer cDNA library (Clontech) into pCMV 3× Flag vector as reported [21] Flag-GLI1 plasmid was prepared as described in [22]. Kindlin-2 promoter full length sequence (−1565 to +1334) was prepared as described in [21], and the different truncation forms of Kindlin-2 were cloned into pGL4.21-null vector (Promega) using the following primers: fragment −241 to +293: Forward primer: 5′-CTCGAGCTTCATTTCCATAA, Reverse primer: 5′-AAGCTTTAGCG GACCGCGGAGGGCTTCAC-3′; fragment −207 to +293: Forward primer: 5′-CTCGAGGCCGCGCCCCCCGCCACC-3′, Reverse primer: 5′-AAGCTTTAGCGGACCGCGGAGGGCTTCAC-3′; fragment −195 to +293: Forward primer: 5′-CTCGAGCCGCCACCCCCGCGCCGC-3′, Reverse primer: 5′-AAGCTTTAGCGGACCGCGGAGGGCTTCAC-3′; fragment −77 to +293: Forward primer: 5′-CTCGAGAGGGCAGCTCT GCGGGCGGCGAA-3′, Reverse primer: 5′-AAGCTTTAGCGGACCGC GGAGGGCTTCAC-3′; Kindlin-2 polyclonal antibody was prepared as described in [9], mouse monoclonal antibodies for GLI1(D-1), Bcl-2(N-19) and Bax(B-9) were purchased from Santa Cruz Biotechnology Inc. (CA, USA). Rabbit polyclonal antibody for PCNA was the product of Lab Vision (Fremont, CA, USA), and mouse monoclonal antibody for Actin was purchased from Beijing Zhongshan Inc. (China).

2.3. Small interference RNAs

The sequences of the siRNA used in this research were: Kindlin-2 siRNA: 5′-AAGCUGGUGGAGAAACUCG-3′; GLI1 siRNA: 5′-AACUC CACAGGCAUACAGGAUTT-3′ Negative control siRNA: 5′-UUCUCCG AACGUGUCACG-3′ (Qiagen). The siRNA was transfected using Lipofectamine 2000 (Invitrogen) at a concentration of 10 μM in serum-free medium.

2.4. Generation of PC-3 stable cells expressing Kindlin-2 shRNA

The generation of Kindlin-2 shRNA stably expressing PC-3 cells was described in [13].

2.5. Luciferase reporter assays

PC-3 and DU-145 cells were seeded into 24-well plates one day before transfection, 200 ng of different Kindlin-2 promoter truncation plasmids and 4 ng of *Renilla* plasmid were transfected with Lipofectamine 2000. 200, 400 or 600 ng of Flag-GLI1 plasmids were co-transfected as indicated per well. The reporter activity was measured at 24 h post transfection using a Dual-luciferase Reporter Assay System (Promega).

2.6. Quantitative real-time PCR

RNA was prepared using Trizol Reagent (Invitrogen) and reversely transcribed with TaqMan (Applied Biosystems) using random primers as described by the manufacturer. cDNA was prepared for quantitative real-time PCR reactions using SYBR® Green Realtime PCR Master Mix (Toyobo) and 0.5 μM of each primer. Triplicate reactions were analyzed using the iCycler iQ (Bio-Rad Laboratories) with melt curve analysis. Relative expression was calculated by correcting for Ct value differences for human actin loading. Empty vector and siRNA control were set to 1 and the relative fold changes of indicated mRNA were graphed. The primers used were: human Kindlin-2 Forward primer: 5′-TGTCCCCGCTATCTAAAAAA GT-3′, Reverse primer: 5′-TGATGGGCCTCCAAGATTCT-3′; human GLI1 Forward primer: 5′-GGGATGATCCCACATCCTCAGTC-3′, Reverse primer: 5′-CTGGAGCAGCCCCCCCAGT-3′.

2.7. Chromatin immunoprecipitation (ChIP)

Chip was performed using EZ-ChIP™ kit (Millipore) as described by the manufacturer. Briefly, approximately 1×10^7 PC-3 cells were crosslinked in PBS containing 1% formaldehyde for 10 min at room temperature followed by 0.125 M glycine treatment for 5 min at room temperature. Nuclei extract was isolated and DNA was sonicated to an average length of 0.2–1 kb. Soluble chromatin was diluted to 1 ml with dilution buffer and precleared using 60 μl Protein G Agarose. Immunoprecipitations were performed using 10 μg mouse monoclonal anti-GLI1 antibody (Santa Cruz) or mouse IgG control (Sigma) at 4 °C overnight. Immunoprecipitates were washed sequentially by low salt wash buffer, high salt wash buffer, LiCl wash buffer and TE wash buffer. Crosslinks were reversed by incubation at 65 °C with rotation for 4 h. And then the DNA was elucidated into collection tubes. The DNA samples were analyzed by real-time PCR using the iCycler iQ (Bio-Rad Laboratories). The primers used in ChIP assay were: Kindlin-2: Primer1: Forward: 5′-AGTCCAGCCTGAGCTCTAG-3'; Reverse: 5′-CTAATGG AGTCCCGTCGTC-3′. Primer2: Forward: 5′-AGACTGCATTG TTTGG CTCCCT-3′; Reverse: 5′-TCAGTCACCTTCAACAGCTCCT-3′. PTCH1: Forward: 5′-CCTTAATGGAAGTATTGCATGCG-3'; Reverse: 5'- CTG TCAGATGGCTTGGGTTTCTG -3'.

2.8. WST-1 cell viability assay

Kindlin-2 shRNA or control shRNA stably expressing PC-3 cells were seeded into 96 wells plate with 4000 cells in each well, the