Up-regulated NRIP2 in colorectal cancer initiating cells modulates the Wnt pathway by targeting RORβ

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Abstract

Background: Colorectal cancer remains one of the most common malignant tumors worldwide. Colorectal cancer initiating cells (CCICs) are a small subpopulation responsible for malignant behaviors of colorectal cancer. Aberrant activation of the Wnt pathways regulates the self-renewal of CCIC. However, the underlying mechanism(s) remain poorly understood.

Methods: Via retroviral library screening, we identified Nuclear Receptor-Interacting Protein 2 (NRIP2) as a novel interactor of the Wnt pathway from enriched colorectal cancer colosphere cells. The expression levels of NRIP2 and retinoic acid-related orphan receptor β (RORβ) were further examined by FISH, qRT-PCR, IHC and Western blot. NRIP2 overexpressed and knockdown colorectal cancer cells were produced to study the role of NRIP2 in Wnt pathway. We also verified the binding between NRIP2 and RORβ and investigated the effect of RORβ on CCICs both in vitro and in vivo. Genechip-scanning speculated downstream target HBP1. Western blot, ChIP and luciferase reporter were carried to investigate the interaction between NRIP2, RORβ, and HBP1.

Results: NRIP2 was significantly up-regulated in CCICs from both cell lines and primary colorectal cancer tissues. Reinforced expression of NRIP2 increased Wnt activity, while silencing of NRIP2 attenuated Wnt activity. The transcription factor RORβ was a key target through which NRIP2 regulated Wnt pathway activity. RORβ was a transcriptional enhancer of inhibitor HBP1 of the Wnt pathway. NRIP2 prevented RORβ to bind with downstream HBP1 promoter regions and reduced the transcription of HBP1. This, in turn, attenuated the HBP1-dependent inhibition of TCF4-mediated transcription.

Conclusions: NRIP2 is a novel interactor of the Wnt pathway in colorectal cancer initiating cells. interactions between NRIP2, RORβ, and HBP1 mediate a new mechanism for CCIC self-renewal via the Wnt activity.

Keywords: Colorectal cancer initiating cells, Self-renewal, Non-canonical Wnt pathway, NRIP2, RORβ, HBP1

Background

Colorectal cancer remains one of the most common malignant tumors in the world [1]. Studies have indicated that colorectal cancer consists of heterogeneous populations of cells differing in gene expression and growth capacities [2, 3]. CCICs are a small subpopulation of cells within colorectal tumors that can self-renew, differentiate into multiple lineages, and drive tumor growth [4, 5]. Among the CCIC properties, the self-renewal ability, which allows the cells to replicate is a crucial step for CCICs that is responsible for maintaining their homeostasis and malignant behaviors [6–8]. It is therefore of particular importance to clarify which molecules are abnormally activated during CCIC self-renewal.

There are several pathways participating in the regulation of the self-renewal of CCICs. Notch signaling plays an important role in promoting CCIC self-renewal. The Notch effector Hes1 up-regulates the expression of the

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stem-related molecules CD133, ABCG2, Nanog, and ALDH1 and increases the amount of CD133+ and stem-like SP cells within colorectal cancer cells [9]. miR-34a targeting Notch1 promotes the differentiation of CCICs [10]; The BMP pathway maintains a stem cell self-renewal balance by inhibiting the Wnt pathway. The zinc-finger transcription factor GATA6 is a crucial regulation factor connecting the Wnt and BMP pathways. Competing with β-catenin/TCF4, GATA6 binds to a distal regulatory region of BMP4, decreases the threshold of the BMP pathway and enables the self-renewal of CCICs [11]. Colorectal cancer cells also have a high level of activity of HedgeHog (HH)-GLI signaling, and the self-renewal of CCICs relies on the direct function of HH-GLI activity in xenograft tumors [12]; Akt can activate 14-3-3eta in the beta-catenin complex, which contributes to the stabilization and nuclear translocation of β-catenin, thus facilitating CCSC self-renewal by activating Wnt [13]. Akt also phosphorylates Oct4 to promote iPS factor transcription [14]. Among these pathways, the abnormal activation of the Wnt pathway is one of the most critical events in the tumorigenesis and development of colorectal cancer and plays a key role in maintaining the self-renewal of CCICs [15–18]. Aberrant activation of the Wnt pathway occurs in >90% of colorectal cancers [19]. Strong Wnt activation is found in cancerous intestinal epithelial ALDH+ initiating cells in ulcerative colitis in addition to CD133+ initiating cells in animal tumorigenesis models [20, 21]. Hence, Wnt activity is an important target for inhibition of the self-renewal of CCICs.

The Wnt pathways include canonical (β-catenin-dependent) and non-canonical (β-catenin-independent) pathways. In the canonical Wnt pathway, the ligands Wnt1, Wnt3a, and Wnt8 can bind the Frizzled receptor and LRP5/6 co-receptor on the cell surface, promoting the recruitment of Disheveled (Dvl) from the cytoplasm to the membrane. This, in turn, induces GSK-3β phosphorylation, which suppresses β-catenin degradation. Free β-catenin then accumulates in the cytoplasm and translocates to the nucleus, where it interacts with LEF and TCF to activate the transcription of downstream targets, including MYC, CCND1, AXIN2, and LECT2, etc. [22, 23]. The non-canonical pathways mainly include the PCP pathway and Wnt/Ca2+ pathway. In the PCP pathway, Wnt5a and other ligands bind to Frizzled and ROR2/PTK7 co-receptors, inducing a signaling cascade involving RhoA, Rac, Cdc42, and JNK, which act on the cytoskeleton [24, 25]. In the Wnt/Ca2+ pathway, Wnt ligands combine with Frizzled, leading to PKC and CamKII activation, which regulate target transcription [26, 27]. Thus, the non-canonical Wnt pathways maintain the self-renewal capacity of tumor cells and promote their tumorigenic ability by influencing canonical Wnt pathway activation at different levels [19, 26]. However, the mechanism of Wnt pathway activation in CCICs is still unclear; especially in terms of how non-canonical Wnt signaling molecules affect the canonical pathway.

Here, we identified NRIP2 as a novel molecule that collaborates with RORβ and HMG box-containing protein 1 (HBP1) to modulate Wnt activity.

Methods
Cancer tissues and cDNA database
All fresh primary colorectal cancer tissues were collected in the Second Affiliated Hospital of the Zhejiang University School of Medicine, with Institutional Review Board approval and informed consent provided by the patients (Reference number: R2014-041). All 565 cDNA genechip databases derived from patients with colorectal cancer were from the City of Hope National Medical Center of USA.

Cell culture
Colorectal cancer SW620, HT29, and LoVo cells; gastric cancer SGC7901 cells; and 293 T cells were purchased from The Cell Bank of Chinese Academy of Sciences at the Shanghai Institute of Cell Biology. Colorectal cancer cells which were derived from primary colorectal cancer tissue were cultured in DMEM/F12 medium (Gibco, Gaithersburg, MD, USA) [28]; SW620 cells were cultured in L-15 medium (Gibco); HT29 cells were cultured in Macoy’s 5A medium (Gibco); Lovo cells were cultured in F-12 medium (Gibco); SGC7901 and P1 cells were cultured in RPMI-1640 medium (Celsgro, Manassas, USA); and 293 T cells were cultured in DMEM high-glucose medium (Gibco). All media were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (Gibco). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO2.

Culturing and counting spheres
Colorectal cancer cell lines and primary colorectal cancer cells were seeded into 24-well low-adhesion plates (Corning, NY, USA) at 200 cells/well and cultured in serum-free sphere medium (containing 1× B27, 20 μg/L EGF, 20 μg/L bFGF, 4 mg/L insulin, 0.4% BSA, and 200 IU/mL streptomycin). These cells were grown in the presence or absence of Wnt Pathway Inhibitor XI, Wnt/β-catenin Inhibitor, Cardamonin (Merck, Germany), or the Wnt activator recombinant Wnt3a (R&D Systems, MN, USA) for 7–14 days at 37 °C in a humidified atmosphere containing 5% CO2. After the incubation period, the spheres were dilute passaged for an additional 1 week and the number of spheres was counted manually.
Organoid culture
The above colosphere cells were digested with 0.25% trypsin and produced single cells using a 40 μM cell strainer (BD, USA). One-hundred cells in 40 μL of medium were mixed well with 70 μl of growth factor-deficient Matrigel (Biocat, USA) and inoculated on the rim of a 24-well plate at 37 °C for 1 h. Subsequently, 1 mL of serum-free sphere medium was added for 5–7 days in a humidified atmosphere containing 5% CO₂.

Creation and screening of a retroviral cDNA library
Total RNA was extracted from SW620 colosphere cells using an RNase Kit (Qiagen, Germany). A ZAP cDNA Library Preparation Kit (Stratagene, CA, USA) was used to prepare cDNA, according to the manufacturer’s instructions. Briefly, RNA was reverse transcribed using a ZAP hemi-methylation primer. Next, double-stranded cDNA was synthesized in vitro, digested with Xhol/ EcoRI endonucleases, cloned into the modified pLXSN vector (Clontech Laboratories, CA, USA), and transformed into *Escherichia coli* DH5α cells (Stratagene). The plasmids were then extracted and transfected into PT67 packaging cells (Clontech) to produce the recombinant retroviral particles. Before infection, CD133+ and CD44+ SGC7901 cells were removed by magnetic-activated cell sorting (Miltenyi, Germany), and the remaining SGC7901 cells were infected (multiplicity of infection is 20) and cultured in a serum-free low adhesion culture system for 7 days. Colospheres were then collected and digested into a single cell suspension and cloned by limiting dilution, and the clonal cells were further propagated. The Top/Fop flash reporter assay was used to determine Wnt activity. Genomic DNA from cells with obvious changes in Wnt activity was extracted using a DNA extraction kit (Qiagen, Germany), and PCR was used to amplify the inserted DNA fragment with primers from pLXSN plasmids. Finally, DNA sequencing was performed to verify the clones.

mRNA hybridization
A QuantiGene @ ViewRNA ISH Tissue Assay Kit (Affymetrix, USA) was used for RNA hybridization according to the kit instructions. Briefly, RORB and NRIP2 were used as TYPE1 probes, and GAPDH was used as a control probe. After staining the nucleus with DAPI or Hoechst 33342 dye (Invitrogen, Carlsbad, CA), the distribution and expression of RORB and NRIP2 were observed under a confocal microscope (Carl Zeiss Jena, Germany).

Assessing tumor sizes in mice
With approval from a local animal protection association, NOD/SCID and naked Balb/c mice were purchased from the Shanghai Laboratory Animal Center (Chinese Academy of Sciences) and bred in specific pathogen-free animal housing at the Laboratory Animal Research Center (Zhejiang Traditional Chinese Medical University). The mice were randomized into groups (5 mice/group for each tumor cell dose) and subcutaneously inoculated in their backs with 0.3 mL of different numbers of SW620 cells. The formation and growth of the transplanted tumors were observed, and the tumor sizes were recorded. The tumor volumes were calculated as 4/3π [(long diameter/2 + short diameter/2)²].

Measuring Wnt pathway activity
Wild-type and mutant plasmids were co-transfected with Top/Fop flash reporters (Millipore, Germany) and the pRL plasmid as an internal reference (ratio of 3:1:0.1). The cells were harvested after 24–48 h, washed twice with phosphate-buffered saline (PBS), lysed in lysis buffer (Promega, Madison, WI, USA), and centrifuged at 13,000 rpm for 1 min. The luciferase activities were measured in the lysate supernatants using the Dual-Luciferase Reporter Assay System (Promega).

Co-immunoprecipitation and western blot analysis
For Co-IP studies, cells were harvested; incubated on ice for 15 min with 200 μl of RIPA lysis buffer containing 1% NP-40, 0.25% deoxycholic acid, 5 mM Dithiothreitol (DTT), and 1× protease inhibitor cocktail (Merck, NJ, USA); and centrifuged for 10 min at 13,000 rpm. The supernatants were collected and incubated with 5 μg of primary antibody for 2 h at 4 °C, then incubated overnight at 4 °C with 50 μl protein A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and centrifuged at 13,000 rpm for 10 min. The beads were washed with lysis buffer and centrifuged 5 times. Subsequently, 50 μl of loading buffer was added to the beads and the samples were heated for 3 min in a water bath at 100 °C, cooled to room temperature (RT), and centrifuged for 1 min at 13,000 rpm. The resulting supernatants were collected for western blot analysis. For western blot analysis, the cells were harvested and incubated on ice for 15 min with 200 μl of RIPA lysis buffer containing 1% NP-40, 0.25% deoxycholic acid, 5 mM DTT, and 1× protease inhibitor cocktail (Merck, NJ, USA). The lysates were centrifuged for 10 min at 13,000 rpm, and the supernatants were collected. The samples were mixed with 2× loading buffer, heated for 3 min in a 100 °C water bath, cooled to RT, and subjected to SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane (Whatman, Dassel, Germany), blocked for 1 h at RT with Tris-base buffer saline +0.05% Tween 20 (TBST) buffer containing 5% skim milk, and then incubated with a primary antibody for 1 h at RT or overnight at 4 °C. Primary antibodies against the following target proteins were used in this study: NRIP2, HBP1 (1:1,000; Novus, USA), cyclin D1, c-Myc, RARα, RORβ.
ORF DNA sequences were subcloned into the NRIP2 vector (Fulengen Co. Ltd, Guangzhou, China). Subsequently, the pReceiver plasmid was purchased from Fulengen Co. Ltd and cloned into the pUC57 vector (Thermoscientific, MA USA). The RORB ORF DNA sequence (Genbank access: AL136557) was synthesized by the Shanghai Xuguan Biotechnology Co. Ltd and AL136557) was synthesized by the Shanghai Xuguan Biotechnology Co. Ltd and cloned into the pReceiver plasmids (Fulengen) using Lipofectamine 2000 reagent (Invitrogen, USA) for 48 h. The cells were lysed by the addition of 1 mL of Trizol reagent (BBI, Canada), and total RNA was extracted. A GeneChip® PrimeView™ WT Kit (Affymetrix, USA) was used to detect global mRNA expression profiles. Differences in mRNA expression were verified by RT-qPCR.

**Immunohistochemical staining**
Following approval by the Ethics Committee of The Second Affiliated Hospital of the Zhejiang University School of Medicine, histological sections of colorectal cancer tissues were incubated overnight at 60 °C, fully hydrated with xylene and gradient alcohol, placed in antigen retrieval solution (pH 8.0, 100 mM EDTA), and heated for 15 min. Subsequently, the sections were cooled to RT, washed 3 times in TBST, blocked for 20 min at RT with TBST containing 10% goat serum, and washed 3 times in TBST. Sections were incubated overnight at 4 °C with an anti-NRIP2 antibody (1:1000, Novus, CO, USA), an anti-RORβ antibody (1:250, Novus, USA). After incubation with the primary antibody, the sections were washed 3 times in TBST and incubated for 1 h at RT with a secondary rabbit antibody (1:200; Dako, Denmark). The sections were developed with 3,3′-diaminobenzidine, counter-stained with hematoxylin, and examined by microscopy.

**Lentivirus infection**
For NRIP2 or RORβ over-expression, colorectal cancer cells were infected for 24 h with recombinant lentivirus encoding human NRIP2 or RORB (Shanghai Innovation Biotechnology Co. for NRIP2 and Shanghai Ruisai Biotechnology Co. for RORB); meanwhile, the cells were infected with blank vector lentivirus as a control. For target gene knockdown, colorectal cancer cells were infected for 72 h with lentivirus encoding shRNAs against NRIP2, RORB, or HBP1 or with scrambled shRNA as a control (Santa Cruz Biotechnology). Subsequently, cells were selected in puromycin (5 μg/ml) for 2 weeks. Clonal cells stably expressing shRNAs were cultured by limiting dilution, and the efficiency of target gene knockdown was verified by western blot analysis.

**Construction of NRIP2 and RORB plasmids**
The NRIP2 ORF DNA sequence (Genbank access: AL136557) was synthesized by the Shanghai Xuguan Biotechnology Development Co. Ltd. and cloned into the pUC57 vector (Thermoscientific, MA USA). The RORB/pReceiver plasmid was purchased from Fulengen Co. Ltd (Guangzhou, China). Subsequently, the NRIP2 and RORB ORF DNA sequences were subcloned into the pEGFP-C1 vector at the Xhol and BamHI sites, respectively. Constructs were confirmed by DNA sequencing.

**RT-PCR and RT-qPCR**
Total RNA was extracted from cells receiving different treatments using a RNA mini kit (Qiagen, Germany). After the quantity of RNA was checked, the RNA was reversed transcribed to cDNA by PrimeScript™ reverse transcriptase with a gDNA eraser kit (TaKara, Japan). RT-PCR and Taqman RT-qPCR were carried out using the Premix EX Taq™ hot start version PCR and Perfect Real Time PCR kits following the manufacturer’s instructions. The primers used are as below: NRIP2: 5′-cacaggcacccaatacact-3′ (Forward), 5′-ttagctcacaactgc cacc-3′ (Reverse), 5′Fam-cacagggccgtgagacatcca-3′ Tamra (Probe); RORB: 5′-gccctttgcaggacatggat-3′ (Forward), 5′-ctgccacactctccaaact-3′ (Reverse), 5′Fam-aacgggtggaaacactgc-3′ Tamra (Probe); GAPDH: 5′-atcatccctgcc tct actgg-3′ (Forward), 5′-gtcaggtcaccagctac-3′ (Reverse), 5′Fam-acttgcc cacagcctgcc-3′ Tamra (Probe).

**Gene chip detection**
SGC7901 cells were transiently transfected with RORB/pReceiver plasmids (Fulengen) using Lipofectamine 2000 reagent (Invitrogen, USA) for 48 h. The cells were lysed by the addition of 1 mL of Trizol reagent (BBI, Canada), and total RNA was extracted. A GeneChip® PrimeView™ Human Gene Expression Array (Affymetrix, USA) was used to detect global mRNA expression profiles. Differences in mRNA expression were verified by RT-qPCR.

**Chromatin immunoprecipitation**
A commercial kit (Upstate, Millipore, USA) was used to perform ChIP assays according to the manufacturer’s instructions. In brief, SGC7901 cells were seeded in a 100-mm dish at 70% confluence overnight and subsequently transiently transfected with RORB/pReceiver plasmids for an additional 48 h. Transfected cells were fixed by a final concentration of 1% formaldehyde for 10 min; the reaction was terminated by adding 0.5 mL of 1 M glycine solution. The cells were collected and lysed by SDS reagent. The DNA fragments were co-immunoprecipitated by anti-myc tag antibodies in agarose at 4 °C overnight after sonication. The immunoprecipitated DNA fragments were purified and eluted by the spin filter method. PCR was used for the detection of the HBP1 upstream DNA fragments with the primers: Forward:5′-gtctttgagacactgat-3′; Reverse: 5′-gtccagaggtttgct-3′. Blank, normal mouse IgG was used as a negative control, and anti-RNA polymerase II was used as a positive control.

**EMSA**
An Electrophoretic Mobility Shift Assay (EMSA) kit (Pierce, Thermo Scientific, USA) was used to perform EMSA assays according to the manufacturer’s instructions. DNA sequences of the wild-type and mutant hormone response elements were chemically synthesized. Upstream
and downstream DNA primers (100 μM) were mixed and incubated at 94 °C in vitro for 5 min and allowed to cool to RT. The probe was incubated with recombinant RORβ for 30 min and resolved on a 6% PAGE gel. The DNA was then transferred to a nylon membrane and crosslinked for 1 min, after which HRP-labeled streptavidin was added for 30 min and an enhanced ECL reagent was used for detection. WT: 5′-Bio-gatcagagctgatagcatcctgctcctg-3′ (Forward), 5′-Bio-caggatgtcctgtgcgatc-3′ (Reverse); Mut1: 5′-Bio-gatcagaggtcagagatcgtcctg-3′ (Forward), 5′-Bio-caggatgtcctgctgatc-3′ (Reverse); Mut2: 5′-Bio-gatcagaggtcagagctctgagcatcctg -3′ (Forward), 5′-Bio-caggatgtcctgctgatc-3′ (Reverse); Mut3: 5′-Bio-gatcagaggtcagagctctgagcatcctg -3′ (Reverse).

Results

Wnt activity is important for the self-renewal of CCICs

To evaluate the effect of Wnt activity on CCIC self-renewal, we first enriched colospheres in vitro from primary colorectal cancer tissues and colorectal cancer cell lines and identified their stem-like properties. Using serum-free, low-adhesion culture conditions, colospheres were successfully enriched and dilute passaged from 3 primary colorectal cancer cells as well as colorectal cancer-derived HT29 and SW620 cells. Furthermore, single cells from these spheres were able to form organoids in the conditioned Matrigel medium (Fig. 1a). These colospheres were inoculated into NOD/SCID mice, and they exhibited significantly increased tumorigenicity (Fig. 1b). These results suggested that the enriched colospheres possessed cancer-initiating cell properties. Next, we assayed for Wnt activity in the colosphere cells. Both Top/Fop flash reporter assays and western blots indicated that these colosphere cells had relatively strong Wnt activity compared with their parental cells (Fig. 1c and d). Finally, we evaluated the effect of Wnt pathway activation on the self-renewal capacity of CCICs. The number of colospheres was obviously increased after Wnt signaling was activated by the addition of recombinant Wnt3a (Fig. 1e). However, the number of colospheres significantly decreased in primary colorectal cancer P1, HT-29, and SW620 cells after CTRNB1 was knocked down by RNA interference (Fig. 1f). Treatment with Wnt and β-catenin chemical inhibitors showed similar results (Fig. 1g). Collectively, these results suggested that activation of the Wnt pathway plays an important role in the self-renewal capacity of CCICs.

NRIP2 is significantly up-regulated in CCICs

The above results demonstrated that Wnt pathway activation plays an important role in maintaining the self-renewal of CCICs; however, the molecular mechanisms whereby Wnt pathway activation occurs in CCICs remain unclear. To screen molecular activators of the Wnt pathway, we constructed a retroviral cDNA library from SW620 colosphere cells and screened it by colosphere formation and Top/Fop flash reporter assays as well as DNA sequencing (Fig. 2a). Based on these screening strategies, there were 13 candidates from SW620 colosphere cells identified by DNA sequencing (Additional file 1: Figure S2a). We found that among these candidates,
only the function of NRIP2 was undefined for promoting the self-renewal of colosphere cells.

Western blot analysis showed that NRIP2 was expressed in primary colorectal P1, HT29, and SW620 cells (Fig. 2b, Additional file 1: Figure S2b). The presence of NRIP2 in primary tumor cells was confirmed by mRNA fluorescence in situ hybridization (FISH) and immunohistochemical (IHC) staining (Fig. 2c and d).

To verify that NRIP2 is expressed at a higher level in CCICs, we isolated CD44^+CD24^+ colorectal cancer-initiating cells from primary colorectal P1 and SW620 cells by fluorescence-activated cell sorting (FACS) and colospheres from colorectal cancer cell lines and primary colorectal cancer tissues. RT-qPCR analysis showed that NRIP2 expression was significantly higher in CD44^+CD24^+ cells and colosphere cells (Fig. 2e-g). Similar results were observed in CD133^+ and Aldefluor^+ colorectal cells (Additional file 1: Figure S2c and S2d). On the other hand, we analyzed the relationship between NRIP2 expression and colorectal cancer molecular typing in 565 cases of colorectal cancer from a global cDNA expression genechip database and found that high NRIP2 expression was closely related to the C4-cancer stem cell (CSC) subtype of colorectal cancer (Fig. 2h) [29].

Geneset enrichment analysis (GSEA) also showed that in colorectal cancer cells expressing high levels
of NRIP2, the alteration of the mRNA expression profiles was similar to that observed with low- and intermediate-CpG-density promoters bearing the histone H3 trimethylation marker at K4 and K27 (H3K4me3 and H3K27me3) in embryonic stem cells (ES) and induced pluripotent cells (iPS) (Fig. 2i). These results suggested that NRIP2 may play an important role in the self-renewal of CCICs.

NRIP2 up-regulates Wnt pathway activity
To characterize the relationship between NRIP2 and Wnt activity, we determined the impact of NRIP2 on
Wnt activity in HT29, P1 and SW620 cells. Western blots showed that the Wnt pathway downstream targets were significantly increased in cells overexpressing of NRIP2, while it was obviously decreased after silencing of NRIP2 in these cells (Fig. 3a and b, Additional file 1: Figure S3). Furthermore, the colosphere numbers were significantly attenuated in NRIP2 overexpressing HT29 and P1 cells after silencing of NRIP2 (Fig. 3c). Finally, we evaluated whether NRIP2 promoted the self-renewal of CCICs dependent on Wnt activation. To this end, we observed a change in the colosphere number in NRIP2-overexpressing cells after inactivation of Wnt. The results showed that NRIP2 overexpression did not reverse the inhibition caused by Wnt- and β-catenin chemical inhibitors (Fig. 3d). NRIP2 overexpressing cells were inoculated into NOD/SCID mice, and they exhibited significantly increased tumorigenicity (Fig. 3e). Together, these findings suggested that NRIP2 involves in the self-renewal of colorectal cancer cells by activating the Wnt pathway.

NRIP2 regulates the activity of the Wnt pathway via RORβ

To discover downstream target molecules by which NRIP2 regulates the Wnt pathway, we performed a literature review and target prediction (www.genecards.org) and found that NRIP2 can interact with RORβ [30]. Thus, we performed Co-IP and western blot analysis with cells overexpressing NRIP2 and RORβ. The results confirmed that both exogenous and endogenous NRIP2 could be co-immunoprecipitated with RORβ (Fig. 4a and b), but NRIP2 could not bind to RARα (Additional file 1: Figure S4). These results suggest that NRIP2 may be involved in the Wnt pathway.

Although NRIP2 can interact with RORβ, it is not clear whether RORβ affects Wnt pathway activity. Therefore, we transiently expressed RORβ in SGC7901 cells, which had the highest transfection efficiency. The results from Top/Fop flash assays and western blots showed that the activity of the Wnt pathway was significantly inhibited by RORβ overexpression (Fig. 4c and d).

To determine whether NRIP2 activates the Wnt pathway dependent on RORβ, we established RORB-knockdown cells and checked the effect of NRIP2 on these cells. The results showed that NRIP2 could not activate the Wnt pathway after knockdown of RORB (Fig. 4e and f). Together, these results suggested that RORβ is an inhibitor of the Wnt pathway and that NRIP2 may affect Wnt pathway activity via RORβ.
ROR\(\beta\) inhibits tumorigenesis and the self-renewal of CCICs

Previous studies have demonstrated that ROR\(\beta\) is mainly distributed in the central nervous system [31, 32], however, whether ROR\(\beta\) is also expressed in intestinal epithelial cells is unverified. To confirm that ROR\(\beta\) is expressed in intestinal epithelial tissue, we evaluated ROR\(\beta\) expression in colorectal cancer cells by RT-qPCR and western blotting. Immunostaining of ROR\(\beta\) was also carried out in human primary colorectal cancer tissues. We also examined RORB mRNA expression in primary colorectal cancer tissues by RT-qPCR and mRNA FISH. We found that ROR\(\beta\) was expressed in colorectal cancer cells, but at a lower level in colorectal cancer tissue than in matched para-carcinoma tissues (Fig. 5a-c, Additional file 1: Figure S5). The level of ROR\(\beta\) in CCICs was not significantly different from their parental cells (data not shown). To further clarify the effect of ROR\(\beta\) on CCICs, we observed the tumorigenicity in vivo, the change in the colosphere number and the ratios of CD44+CD24+ cancer-initiating cells in cells with overexpression of ROR\(\beta\). The results showed that the tumorigenic capacity was significantly reduced (Fig. 5d), the sphere-forming efficiency was decreased and the ratio of CD44+CD24+ cells and the number of colospheres were also obviously reduced in colorectal cancer cells after overexpression of ROR\(\beta\) (Fig. 5e and f). Inversely, RORB knockdown led to increased colosphere formation (Fig. 5g and h). These results suggested that ROR\(\beta\) negatively regulates the self-renewal of CCICs as a suppressor.

HBP1 is a crucial target of ROR\(\beta\) in regulation of the Wnt pathway

To investigate how ROR\(\beta\) inhibits Wnt activation, we first analyzed changes in mRNA expression in cells overexpressing ROR\(\beta\). Genechip-scanning experiments showed that HBP1, a protein that blocks TCF binding to DNA [33], was significantly increased in cells overexpressing ROR\(\beta\) (Fig. 6a). This result was subsequently confirmed by western blot analysis (Fig. 6b). However, HBP1 obviously reduced following RORB silencing (Fig. 6c). Similarly, HBP1 was also reduced in the CCICs and crypts of intestinal mucosa from Rorb\(^{-/-}\) mice (Additional file 1: Figures S6 and S7a). These results suggest that HBP1 is a downstream target of ROR\(\beta\). Furthermore, Chromatin Immunoprecipitation (ChIP) experiments showed that ROR\(\beta\) could bind to HBP1 upstream DNA sequences (Fig. 6d). Next, the upstream promoter region sequences of HBP1...
were analyzed, and several RORβ binding sequences were identified (Additional file 1: Figure S7b), EMSA detection confirmed that the HRE sequence AGGTCA is essential for RORβ binding to the HBP1 promoter region (Fig. 6e). By co-transfecting a RORB plasmid and a reporter encoding luciferase under the control of the HBP1 promoter region sequences, we found that RORβ obviously enhanced downstream luciferase activity, while co-transfection with NRIP2 significantly weakened its transcription activity (Fig. 6f and g). NRIP2 could not activate the Wnt activity in HBP1-silencing cells (Fig. 6h). Western blots revealed that the Wnt pathway was activated in HBP1 knockdown cells (Fig. 6i and j). These HBP1 knockdown cells were inoculated into NOD/SCID mice, and they exhibited significantly increased tumorigenicity (Fig. 6k). The in vitro colosphere-formation potential was also enhanced in these cells (Fig. 6l and m). However, both Wnt activation and the number of colospheres decreased in cells with
HBP1 plasmids were used as a control. That HRE increased the luciferase activity of the HBP1 promoter. The results showed that NRIP2 could not activate Wnt activity in cells after silencing HBP1. HBP1 expression was significantly decreased after knockdown of RORβ. P1 cells infected with blank lentivirus and SGC7901 cells transfected with pReceiver plasmids were used as controls. ChIP analysis of the interaction between RORβ and HBP1 upstream DNA. DNA fragments were immunoprecipitated by anti-myc-tag antibodies agarose in RORβ-overexpressing SGC7901 cells after sonication. PCR was used for the detection of the HBP1 upstream DNA sequence. The results showed that RORβ bound to the region upstream of HBP1 DNA. Blank, normal mouse IgG was used as a negative control, and anti-RNA polymerase II was used as a positive control. RORβ binds to hormone response elements (HRE) upstream of the HBP1 promoter region. An EMSA assay was used to identify the seed region for RORβ binding within upstream hormone response elements of the HBP1 promoter region. Three mutants containing different potential binding sequences were constructed. The results showed that the hormone response element sequence AGGTCA is essential for RORβ binding with the HBP1 promoter region. HRE increased the activity of the promoter. Plasmids containing HRE or the promoter of HBP1 were co-transfected into 239 T cells for 24 h. Luciferase activity was evaluated by the dual-luciferase reporter assay system. The results showed that HRE increased the HBP1 promoter activity. **p < 0.001 (ANOVA). pRL3 plasmids were used as a control. pRL2 attenuated RORβ transactivation. The luciferase activity of the HBP1 promoter was determined in 293 T cells at 24 h after co-transfection of RORβ and/or pRL2 as well as in pRL3 plasmids containing HRE and the HBP1 promoter. The results showed that NRIP2 attenuated RORβ transactivation. ***p < 0.001 (ANOVA). Blank pRL3 plasmids were used as a control. NRIP2 could not activate Wnt activity in HBP1-silenced cells. Wnt activity was evaluated by a luciferase activity assay in HBP1-silenced cells and scrambled P1 and SGC7901 cells (control) 24 h after co-transfection with Top/Fop flash reporters and NRIP2 plasmids. The results showed that NRIP2 could not activate Wnt activity in cells after silencing HBP1. ***p < 0.001 (ANOVA). Detection of HBP1 in HBP1-knockdown cells. HBP1 was detected by western blotting in the SGC7901 cells with knockdown of HBP1 by shRNAs. SGC7901 cells transfected with scrambled shRNAs as a control. HBP1 expression was significantly increased in HBP1-knockdown colorectal cancer cells. Colorectal cancer cells were infected with HBP1 shRNA lentivirus for knockdown of HBP1. HBP1 was detected by western blotting in these HBP1-knockdown colorectal cancer cells (control). Quantification of colospheres in HBP1-knockdown cells. Colospheres were counted in the above HBP1-knockdown colorectal cancer cells. The number of colospheres was significantly increased in HBP1-knockdown cells. **p < 0.01 (ANOVA). Detection of the Wnt downstream targets in HBP1-overexpressing cells. c-Myc and cyclin D1 were analyzed by western blotting in HBP1-overexpressing and control (transfected with pCMV-XL4 plasmids) P1 cells. The results showed that HBP1 significantly inhibited colosphere formation. *p < 0.05 (ANOVA)
reinforced HBPI expression (Fig. 6n and o). In summary, these data suggested that the interactions between NRIP2, RORB, and HBPI regulated Wnt pathway activation and the self-renewal of CCICs.

Discussion

Using a retroviral library screening strategy, we demonstrated increased expression of NRIP2 in CCICs. NRIP2 was shown to be a novel interactor with the Wnt pathway. RORB was identified as a key target of NRIP2, through which NRIP2 regulates the activity of the Wnt pathway. The NRIP2-RORB interaction reduces HBPI transcription, thereby attenuating HBPI-dependent inhibition of the TCF4-DNA complex, finally promoting the self-renewal of CCICs by up-regulating Wnt pathways (Fig. 7).

NRIP2 belongs to the aspartic protease family [34]. Unlike other members of this family, such as Ddi1, both the ubiquitin-associated domain and the ubiquitin-like binding domain are absent from the NRIP2 sequence, suggesting that NRIP2 has a different function from the other aspartic protease family members [35]. NIX1, a murine NRIP2 homolog, can bind directly to the C-terminal ligand-binding domain (LBD) of mouse RORB independently of retinoic acid and thyroid hormone T3 to inhibit the transcriptional activity of RORB. NIX1 was found to participate in transcriptional repression in yeast cells [30], but the mechanism whereby NRIP2 regulates the Wnt activity and the self-renewal of cancer initiating cells has been rarely reported.

Previously, RORB was primarily detected by Northern blotting, the expression of which was restricted to the central nervous system, in particular, to regions involved in modulating circadian rhythms, such as the suprachiasmatic nucleus, the pineal gland, and the retina [31, 32]. Recently, RORB was detected in tissues outside of the nervous system, such as normal bone tissue, the endometrium and pancreatic cancer [36–38]. RORB, which had a high expression level in the endometrium in healthy pre- or post-menopausal women, was significantly down-regulated in endometrial cancer cells [38]. We detected RORB expression in normal intestinal epithelial cells and intestinal tumors; moreover, RORB was also decreased in cancer tissues, suggesting that the distribution of RORB may be more widespread than is currently known and that RORB may play a role as a tumor suppressor. Similar to RORa and RORe, RORc contains 4 functional domains, including an amino-terminal A/B domain, a DNA-binding domain (DBD), a hinge region, and a carboxy-terminal LBD [39, 40]. The DBD is highly homologous between RORβ and RORγ (92%), but the LBD is not well conserved among RORs [41]. RORB includes RORb1 and RORb2 isoforms. RORb1 and RORb2 are characterized by different A/B domains that contain 2 and 13 amino acids, respectively. The N-terminal 2nd–13th amino acids of RORb1 are replaced by an arginine in RORb2 [42]. The molecular function of RORB needs to be further clarified. RORB is considered to be a critical transcription factor regulating rod differentiation [43, 44]. RORB1 induces the expression of the early key transcription factors Ptf1a and Foxn4 and promotes the differentiation of amacrine and horizontal cells [45]. RORB also regulates bone formation by inhibiting Runx2 activity [36]. There is relatively little evidence supporting a functional relationship between the RORB and Wnt activities related to the self-renewal of CCICs. It has been reported that RORa binds to the promoter region of CTNNB1 to inhibit Wnt activity [46], which is involved in a non-canonical Wnt pathway. Among the target molecules of NRIP2, RORB is homologous to RORa [42, 47], but whether RORB affects the Wnt pathway remains unclear. Here, we show that RORB suppresses the Wnt pathway and unlike RORa, RORB neither binds with β-catenin nor affects its transcription.

RORB is a DNA transcription enhancer. Thus, we screened for target genes at the transcriptional level, enabling the discovery of HBPI as an interaction partner. RORB enhances the transcription of HBPI by binding to its HRE region upstream promoter. HBPI belongs to the sequence-specific, HMG family of transcription factors [48]. As a putative suppressor of the Wnt pathway, HBPI may also inhibit the transcription of TCF4 targets by directly blocking the binding of TCF4 with DNA [33, 49]. Therefore, we speculate that RORB may affect the activity of the Wnt pathway by regulating HBPI transcription.
and NRIP2 up-regulates Wnt activity by attenuating RORβ transcriptional activity. Due to the critical role of the Wnt pathway in CCIC self-renewal, HBP1 also participated in the regulation of CCIC self-renewal. These results indicate that the NRIP2/RORβ/HBP1 pathway is a beneficial supplement to the Wnt pathway. In addition to the activation of the Wnt pathway by NRIP2/RORβ/HBP1, NRIP2 is also associated with DNA mismatch repair in colorectal cancer cells, and RORβ may be correlated with tumorigenesis and tumor stages (Additional file 2), suggesting that the NRIP2/RORβ/HBP1 pathway is also involved in other biological processes.

**Conclusion**

In this study, we identified NRIP2 as a novel molecule acting in Wnt pathway. The interaction between NRIP2 and RORβ activates downstream target HBP1 and is probably involved in CCIC self-renewal. For the positive role in Wnt, NRIP2 may be a potential alternative target for inhibiting CCIC self-renewal.

**Additional files**

Additional file 1: Determination of NRIP2 and HBP1 in the colorectal cancer initiating cells and detection of RORβ in the colorectal epithelia. (DOC 3098 kb)

Additional file 2: Analysis of the relationship between NRIP2, RORβ and clinical parameters. (DOCX 1238 kb)

**Abbreviations**

CCICs: Colorectal cancer initiating cells; ChIP: Chromatin immunoprecipitation; Co-IP: Co-immunoprecipitation; CSC: cancer stem cell; Dvl: Disheveled; EMSA: Electrophoretic mobility shift assay; ES: Embryonic stem cells; FACS: Fluorescence-activated cell sorting; FISH: Fluorescence in situ hybridization; GSEA: Gene set enrichment analysis; GSK-3β: Glycogen synthase kinase-3β; HBP1: HMGB box-containing protein 1; HMG: High-mobility-group; HRE: Hormone response elements; IHC: Immunohistochemical; IPS: Induced pluripotent cells; LEF: Lymphoid enhancer factor; LRRP6: Low-density lipoprotein receptor-related protein 5/6; NRIP2: Nuclear receptor-interacting protein 2; ROR2/PTK7: Receptor tyrosine kinase-like orphan receptor 2/protein tyrosine kinase 7; RORβ: Retinoic acid-related orphan receptor β; SP: Side population; TCF: T-cell factor.

**Acknowledgments**

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**Received: 6 October 2016 Accepted: 17 January 2017**

**Published online: 31 January 2017**

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