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STAT3-Induced Wnt5a Provides Chronic Lymphocytic Leukemia Cells with Survival Advantage

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The wingless and integration site growth factor-5a (Wnt5a) is a ligand of the receptor tyrosine kinase-like orphan receptor-1 (ROR1). Because both Wnt5a and ROR1 are expressed in circulating chronic lymphocytic leukemia (CLL) cells, and because in other cell types, STAT3, which is constitutively activated in CLL, induces Wnt5a signaling, we wondered whether STAT3 induces the expression of Wnt5a in CLL cells. Sequence analysis detected four putative STAT3 binding sites in close proximity to the Wnt5a gene promoter's start codon. Chromatin immunoprecipitation and EMSA revealed that STAT3 binds to the Wnt5a gene promoter, and a luciferase assay showed that STAT3 activates the Wnt5a gene. Additionally, transfection of peripheral blood CLL cells with STAT3 short hairpin RNA downregulated Wnt5a mRNA and protein levels, suggesting that STAT3 binds to the Wnt5a gene promoter and induces the expression of Wnt5a in CLL cells. Flow cytometry and confocal microscopy determined that both Wnt5a and its receptor ROR1 are coexpressed on the surface of CLL cells, and Western immunoblotting showed an inverse correlation between Wnt5a and ROR1 protein levels, implying that, regardless of CLL cells' ROR1 levels, blocking the interaction between Wnt5a and ROR1 might be beneficial to patients with CLL. Indeed, transfection of CLL cells with Wnt5a small interfering RNA reduced Wnt5a mRNA and protein levels and significantly increased the spontaneous apoptotic rate of CLL cells. Taken together, our data unravel an autonomous STAT3-driven prosurvival circuit that provides circulating CLL cells with a microenvironment-independent survival advantage. ***The Journal of Immunology*****, 2019, 203: 3078–3085.**

Receptor tyrosine kinase-like orphan receptor-1 (ROR1) is an evolutionary conserved type I surface membrane protein that is commonly expressed during embryogenesis but repressed in most adult tissues (1). Like most adult cells, normal B cells do not express ROR1. Conversely, B cell–chronic lymphocytic leukemia (CLL) cells express high levels of ROR1 (2), whose activation provides the cells with survival advantage (3) and induces cellular proliferation (4).

The ligand that binds to and activates ROR1 is Wnt5a. Wnt5a is a member of the wingless and integration site growth factor (WNT) family of secreted glycoproteins known to modulate several biological processes, including embryogenesis, organogenesis, and tumorigenesis (5). Wnt5a binds to ROR1 and induces activation of β-catenin–dependent and –independent pathways (4). Bone marrow mesenchymal stromal cells are the only cells known to produce Wnt5a in human bone marrow, where Wnt5a also binds ROR2 and participates in the induction of osteogenesis (6, 7). Recent studies demonstrated that in CLL cells, Wnt5a-induced signaling depends on the interaction between ROR1 and ROR2 (8) and that Wnt5a induces ROR1 to recruit DOCK2 and activate Rac1/2 to enhance CLL cell proliferation (4). Like ROR1 transcripts (3), Wnt5a transcripts have been detected in CLL cells of nearly all patients (9). However, what factor(s) activate the transcription of Wnt5a in CLL cells is currently unknown.

Previously, we found that STAT-3 induces the expression of ROR1 in CLL cells (3). STAT3 is constitutively phosphorylated and activated in CLL cells (10). Phosphorylated STAT3 forms dimers, shuttles to the nucleus, and induces the expression of several STAT3 target genes (10–13). Because Wnt5a is constitutively expressed in CLL cells (9) and phosphorylated STAT3 was found to induce Wnt5a signaling in various cell types (14), we sought to determine whether, in addition to inducing the expression of ROR1 (3), STAT3 also induces the expression Wnt5a in CLL cells.

Materials and Methods

Fractionation of CLL cells and normal B cells

Peripheral blood (PB) cells were obtained from previously untreated CLL patients who were followed at The University of Texas MD Anderson

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U.R. and Z.E. planned the study and wrote the manuscript. D.M.H., P.L., and Z.L. conducted the experiments and analyzed the data. P.J. and O.U. analyzed the data. A.F., J.A.B., P.B., P.A.T., N.J., W.G.W., and M.J.K. treated the patients and provided the patients' blood samples and clinical characteristics. All authors read and approved the manuscript.

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; CLL, chronic lymphocytic leukemia; GAS, IFN-γ activation site; LPL, lipoprotein lipase; PB, peripheral blood; PI, propidium iodide; ROR1, receptor tyrosine kinase-like orphan receptor-1; shRNA, short hairpin RNA; siRNA, small interfering RNA; WNT, wingless and integration site growth factor.

Cancer Center leukemia clinic after obtaining an Institutional Review Board–approved informed consent. The patients' clinical characteristics are depicted in Supplemental Table I. To isolate low-density cells, PB cells were fractionated using Ficoll-Hypaque 1077 (Sigma-Aldrich, St. Louis, MO). More than 90% of the PB lymphocytes obtained from the low-density fraction of the studied CLL patients coexpressed CD19 and CD5, as assessed by flow cytometry using an upgraded FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). To isolate normal $CD19^+$ B cells, normal donors' PB low-density cells were fractionated using microimmunomagnetic beads and Miltenyi Columns (Miltenyi Biotec, San Diego, CA) in accordance with the manufacturer's instructions. More than 97% of the fractionated cells were $CD19^+$ cells as assessed by flow cytometry.

Western immunoblotting

Western blot analysis was performed as previously described (10). Briefly, cell lysates were assayed for their protein concentrations using the BCA protein assay reagent (Pierce Chemical, Rockford, IL). Each set of paired lysates were adjusted for the same protein concentration. A lysate of CLL cell extract was mixed with 4× Laemmli sample buffer and was then denatured by boiling for 5 min. Forty micrograms of each lysate were separated using 8% SDS-PAGE and then transferred to a nitrocellulose membrane. The transfer was done overnight at 30 V in a cooled (4°C) reservoir. The nitrocellulose membrane was then placed in Ponceaus S stain to verify equal loading of protein. The membranes were blocked with 5% dried milk dissolved in 50 ml of PBS with Tween-20. After blocking, the membrane was incubated with the following primary Abs: monoclonal mouse anti-human Wnt5a (Invitrogen, Waltham, MA), monoclonal mouse anti-human ROR1 (Invitrogen), monoclonal mouse anti-human STAT3 Abs (BD Biosciences, San Jose, CA), and mouse anti-human β-actin (Sigma-Aldrich). After incubation with HRP–conjugated secondary Abs (GE Healthcare, Buckinghamshire, U.K.) for 1 h, blots were visualized with an ECL detection system (GE Healthcare).

Immunoprecipitation studies

Immunoprecipitation studies were done as previously described (15). Briefly, CLL cell lysates were incubated with rabbit anti-Wnt5a Abs (Invitrogen) for 16 h at 4°C. Protein A agarose beads (Cell Signaling Technologies, Danvers, MA) were added for 2 h at 4°C. For negative controls, the cytoplasmic lysates were incubated either with rabbit serum plus protein A agarose beads or with protein A agarose beads alone. After three washes with radioimmunoprecipitation assay buffer, the beads were suspended in SDS sample buffer, boiled for 5 min, the beads removed by centrifugation, and the supernatant proteins were separated by SDS-PAGE. Normal B cells from two healthy volunteers and HeLa cells were used as controls.

Flow cytometry

Live, unpermeabilized cells were used for flow cytometry. Before staining, cells were washed three times in PBS with 2% FBS (Invitrogen). Cells were then stained with Wnt5a (Invitrogen) and ROR1 (Invitrogen) Abs and their corresponding isotypic Ab. Cells were analyzed using an upgraded FACSCalibur flow cytometer (Becton Dickinson) and data analysis was performed using CellQuest software (BD Biosciences). Graphics were created with CellQuest (BD Biosciences) and WinList (Verity Software House, Topsham, ME) software.

Confocal microscopy

CLL cells were fixed in 2% paraformaldehyde for 10 min at 37°C and permeabilized overnight at −20°C. Before staining, cells were washed three times in PBS with 2% FBS (Invitrogen), then the cells were incubated in microtubes in PBS supplemented with 5% FBS. After 1 h of incubation, the cells were washed three times with PBS and then incubated with rabbit anti-Wnt5a Abs (Invitrogen) and mouse anti-ROR1 Abs (Invitrogen) for 1 h. After being washed three times with PBS, the cells were incubated with Alexa Fluor 488–labeled anti-rabbit and Alexa Fluor 647–labeled anti-mouse Abs. After being washed three times with PBS, the cells were suspended in 5 mg/ml solution of DAPI dye (Invitrogen) for 15 min and then washed in PBS to remove the unbound dye. The cells were then placed into μ-Slide VI chamber slides (ibidi) for microscopic analysis. The slides were viewed using an Olympus FluoView 500 confocal laser-scanning microscope (Olympus America, Waltham, MA), and images were analyzed using the FluoView software (Olympus America).

Transfection of MM1 cells with Wnt5a promoter fragments and luciferase assay

Four different Wnt5a promoter fragments were transfected into MM1 cells via electroporation using the Gene Pulser Xcell Electroporation System (Bio-Rad Laboratories, Hercules, CA). Each construct included a luciferase reporter gene and a Wnt5a promoter fragment that included between 1 and 4 IFN-γ activation sites (GAS)–like elements. The luciferase activity of unstimulated or IL-6–stimulated MM1 cells was assessed 24 h after transfection using a Dual-Luciferase Reporter Assay System (Promega) and a BD MonolightTM and 3010 Luminometer (BD Biosciences). The luciferase activity of each of the human Wnt5a promoter constructs was determined by calculating the constructs' luciferase activity relative to the activity of the *Renilla* luciferase produced by the pRL-SV40 control vector.

Chromatin immunoprecipitation assay

A chromatin immunoprecipitation (ChIP) assay was performed using a SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology, Boston, MA) according to the manufacturer's instructions. Briefly, cells were cross-linked with 1% formaldehyde for 10 min at room temperature and then harvested and incubated on ice for 10 min in lysis buffer. Nuclei were pelleted and digested with micrococcal nuclease. Following sonication and centrifugation, sheared chromatin was incubated with anti-STAT3 or rabbit serum (negative control) overnight at 4°C. Next, protein G beads were added, and the chromatin was incubated for 2 h in rotation. Ab-bound protein–DNA complexes were eluted and subjected to PCR analysis. The primers to amplify the human c-Myc promoter were as follows: forward, 5′-TGA GTA TAA AAG CCG GTT TTC-3′ and reverse, 5′-AGT AAT TCC AGC GAG AGG CAG-3′, which generated a 63-bp product; the primers to amplify the Wnt5a promoter were as follows: forward, 5′-CAG AGA GGA GGA GCT GGA GAT-3′ and reverse, 5′-CCC AGT TCA TTC ACA CCA CAG-3′, which generated a 74-bp product; the primers to amplify the ROR1 promoter were as follows: forward, 5′-TTT GAG GAG TGT GGG GGA GGG-3′ and reverse, 5′-GTT GAG AGG CTG CAG CAG AGG−3′, which generated a 110-bp product; the primers to amplify the STAT3 promoter were as follows: forward, 5′-CCG AAC GAG CTG GCC TTT CAT-3′ and reverse, 5′-GGA TTG GCT GAA GGG GCT GTA-3′, which generated an 86-bp product; the primers to amplify the VEGF promoter were as follows: forward, 5′-CTT CTC CAG GCT CAC AGC TT-3′ and reverse, 5′-CCT GGA AAT AGC CAG GTC AG-3′, which generated a 181-bp product. The primers to amplify the human RPL30 gene were provided by Cell Signaling Technologies. The primers used to amplify fragments of the human Wnt5a gene promoter were as follows: forward, +56 and reverse, +130, encompassing a 74-bp product that covers the IFN-γ activation sequence (GAS) binding sites from 100 to 108 bp upstream of the *Wnt5a* start codon; forward, +110 and reverse, +254, a 144-bp product that covers the upstream GAS binding sites from 208 to 216 bp; forward, +234 and reverse, +294, a 60-bp product that covers the upstream GAS binding sites from 234 to 243 bp; forward, +244 and reverse, +348, a 104-bp product that covers the upstream GAS binding sites from 249 to 257 bp.

EMSA

Nondenatured cellular nuclear extracts were prepared using an NE-PER extraction kit (Thermo Scientific Pierce). Nuclear protein extracts were incubated with 5″ biotin-labeled DNA probes derived from the Wnt5a promoter sequence. Each probe was synthesized by Sigma-Genosys (The Woodlands, TX) and designed to include one of the above-described four GAS-like elements in the Wnt5a promoter. The probes were incubated for 30 min on ice. Following incubation, the samples were separated on a 5% polyacrylamide gel, transferred onto a nylon membrane, and fixed on the membrane via UV cross-linking. The biotin-labeled probe was detected with streptavidin–HRP (Gel Shift Kit; Panomics, Fremont, CA). The control consisted of a 7-fold excess of unlabeled cold probe. To test the effect of STAT3, anti-STAT3 Abs (BD Biosciences) or mouse IgG1 (BD Biosciences) were added to the nuclear extracts, as previously described (10).

Generation of GFP-conjugated lentiviral STAT3 short hairpin RNA and infection of CLL cells

293T cells were cotransfected with GFP-conjugated lentiviral STAT3 short hairpin RNA (shRNA) or a GFP-conjugated empty lentiviral vector and with packaging vectors (pCMV δ R8.2 and pMDG, generously provided by Dr. G. Inghirami, (Department of Pathology, University of Torino, Italy) using the SuperFect Transfection Reagent (QIAGEN), as previously described (10). The 293T cell culture medium was replaced after 16 h and collected after 48 h. Next, the culture medium was filtered through a 45-μm syringe filter to remove floating cells, the lentivirus was concentrated by filtration through an Amicon Ultra centrifugal filter device (EMD; Millipore, Burlington, MA), and the concentrated supernatant was used to infect CLL cells. CLL cells (5×10^6/ml) were incubated in six-well plates (Becton Dickinson) in 2 ml of DMEM (Thermo Fisher Scientific, Grand Island, NY) supplemented with 10% FBS and were transfected with 100 μl of viral

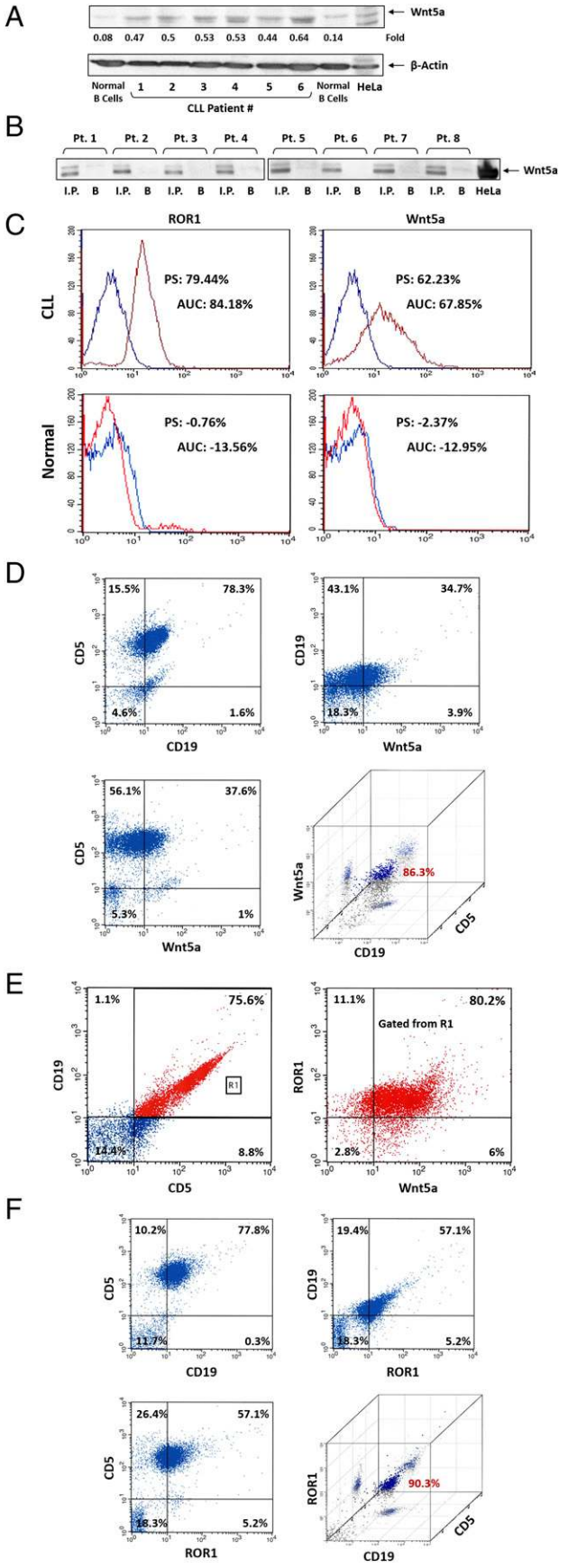

FIGURE 1. CLL cells coexpress cell surface Wnt5A and ROR1 protein. (**A**) Western blot analysis of CLL lysates of PB low-density cells obtained from six patients. As shown, Wnt5A was detected in all patients but not in normal B cells. β-actin was used for loading control. (**B**) Immunoprecipitation of CLL cell lysates with Wnt5a Abs. CLL cells from six additional CLL patients were lysed and immunoprecipitated with Wnt5a Abs. The immune complexes were separated using SDS-PAGE and, as shown, Wnt5a was detected in all patient samples. B represents beads coated with the Wnt5a Ab isotype control. (**C**) Flow cytometry analysis of live, unfixed CLL and normal $CD19^+$ cells using anti-ROR1 (left panel) and anti-Wnt5a (right panel) Abs is depicted. The Kolmogorov–Smirnov test was used to assess the difference in expression between ROR1, Wnt5a, and their corresponding isotopic controls. As shown, ROR1 and Wnt5a were detected on the surface of CLL but not normal B cells. The percentages of differences in peak shifts (PS) and the areas under the curve (AUC) are depicted. Blue: isotype; red: specific Ab. In CLL cells the difference of the AUC was 84.18% ($p < 0.001$) for ROR1 and 67.85% ($p < 0.001$) for Wnt5a. (**D**) Flow cytometry analysis of PB cells from another patient with CLL shows that 86.3% of the cells that coexpress CD5 and CD19 express Wnt5a. (**E**) Flow cytometry analysis of PB low-density cells from another patient with CLL. In this sample only 75.6% of the cells coexpressed CD5 and CD19 (left panel) and, of the gated cells (R1), 80.2% coexpressed ROR1 and Wnt5a (right panel). (**F**) Flow cytometry analysis of PB cells from an additional CLL patient show that 90.3% of the cells coexpress CD5, CD19, and ROR1.

supernatant. Polybrene (10 ng/ml) was added to the viral supernatant at a ratio of 1:1000 (v/v) and allowed to complex for 10 min before infection. Transfection efficiency was measured after 48 h and ranged between 40 and 50% (calculated based on the ratio of propidium iodide $[PI]^-$ and GFP^+ cells). These experiments were conducted using an upgraded FACSCalibur flow cytometer (Becton Dickinson).

Quantitative RT-PCR analysis

We used 500 ng of total RNA in one-step quantitative RT-PCR; Applied Biosystems, Foster City, CA) analysis with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using a TaqMan gene expression assay for STAT3, Bcl2, cyclin D1, c-Myc, p21, caspase 3, CSF2R-α, lipoprotein lipase (LPL), ROR1, and Wnt5a genes according to the manufacturers' instructions. Samples were run in triplicate, and relative quantification was performed by using the comparative C_T method.

Transfection of CLL cells with Wnt5a small interfering RNA

Twenty micromolars human Wnt5a–small interfering RNA (siRNA) or scrambled siRNA, 20 μM FAM-labeled human GAPDH or reagent control (Applied Biosystems) were added to 10 μl siPORT NeoFX Transfection Reagent diluted in 50 μl Opti-MEM I Reduced Serum Medium (Thermo Fisher Scientific) and incubated at room temperature for 10 min. Next, the reagents were incubated at room temperature with 1×10^7 CLL cells suspended in 0.2 ml of Opti-MEM I. After 1 h of incubation, electroporation was performed using the Gene Pulser Xcell Electroporation System (Bio-Rad Laboratories), and the cells were incubated in RPMI 1640 (Thermo Fisher Scientific) supplemented with 10% FBS for 24 h. Transfection efficiency of the FAM-conjugated siRNA was assessed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences).

Annexin V/PI assay

The rate of cellular apoptosis was analyzed using double staining with a Cy5-conjugated annexin V kit and PI (BD Biosciences) according to the manufacturer's instructions. Briefly, 1×10^6 cells were washed once with PBS and resuspended in 200 μl of binding buffer with 0.5 μg/ml annexin V–Cy5 and 2 μg/ml PI. After incubation for 10 min in the dark at room temperature, the samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Cell viability was calculated as the percentage of annexin V^+ cells.

Results

CLL cells coexpress Wnt5a and ROR1

Previous studies demonstrated that CLL cells express ROR1 (2, 3). To assess whether CLL cells also express the ROR1 ligand Wnt5a, we performed Western immunoblotting of PB samples of six randomly selected patients. As shown in Fig. 1A, we detected Wnt5a

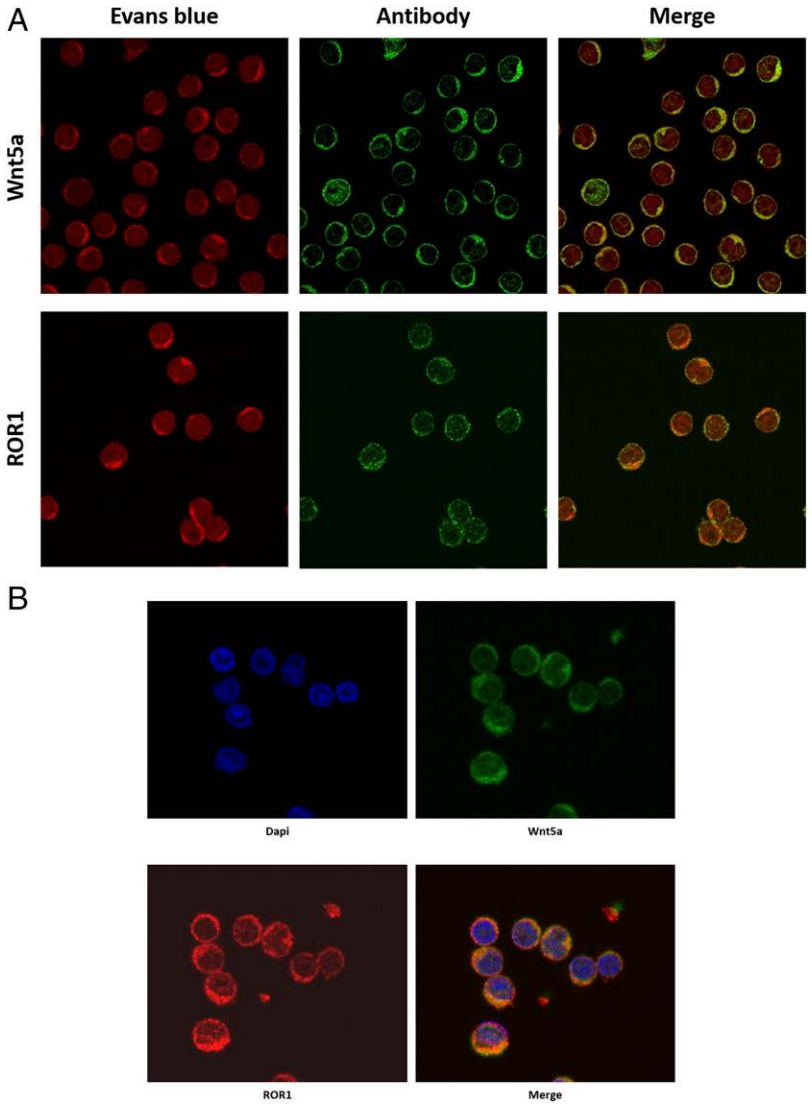

FIGURE 2. ROR1 and Wnt5a are detected in CLL cells. (**A**) Confocal microscopy images of fixed and permeabilized CLL cells costained with either anti-Wnt5A or ROR1 Abs show that Wnt5A and ROR1 are detected in CLL cells. (**B**) Confocal microscopy analysis of fixed and permeabilized CLL cells from another patient show that both Wnt5a and ROR1 are present in the same CLL cells. DAPI staining (blue) was used to detect the cell nuclei. Original magnification ×100.

protein in CLL cell extracts of all patient samples. To confirm these findings, we obtained CLL cells from eight additional patients, immunoprecipitated the cell lysates with anti-Wnt5a Abs, and by using Western immunoblotting, detected Wnt5a protein in all samples (Fig. 1B). To further confirm these data, we used flow cytometry and detected cell surface Wnt5a and ROR1 on the majority of CLL cells but not on normal $CD19^+$ B lymphocytes (Fig. 1C–E). To determine whether Wnt5a and ROR1 are present in CLL cells, we used confocal microscopy. We found that Wnt5a and ROR1 are expressed in all CLL cells (Fig. 2A). Next, to assess whether Wnt5a and ROR1 are expressed in the same cell, we used dual staining and confirmed that CLL cells coexpress Wnt5a and ROR1 (Fig. 2B).

A recent study suggested that high levels of ROR1 are associated with an accelerated disease progression (16). Because the expression of both ROR1 and Wnt5a is induced by STAT3, we wondered whether, in patients with high ROR1 levels, Wnt5a levels would be high as well. Using Western immunoblotting, we assessed the levels of ROR1 and Wnt5a on the same CLL patients' CLL cell samples. We found that CLL cells with high ROR1 levels expressed low Wnt5a levels, whereas most samples with low ROR1 levels expressed high levels of Wnt5a (Fig. 3), excluding the possibility that we detected receptor-bound ligands and suggesting that low levels of the ROR1 receptor are "compensated" by increased levels of the Wnt5a ligand.

STAT3 binds to the Wnt5a promoter and activates the Wnt5a gene

Because we previously found that ROR1 expression in CLL cells is driven by STAT3 (3) and STAT3 was found to induce Wnt5a signaling in various cell types (14), we sought to assess the effect of STAT3 on the expression of Wnt5a. Using the TFSEARCH database (http://diyhpl.us/~bryan/irc/protocol-online/protocol-cache/TFSEARCH.html), we found four GAS-like elements, known as putative STAT3 binding sites (17), within the Wnt5a promoter upstream and in close proximity to the Wnt5a start codon (Fig. 4A,

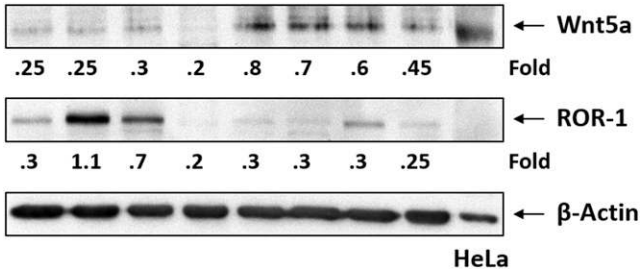

FIGURE 3. Both Wnt5a and ROR1 protein are detected in cell extracts obtained from the same CLL patients. As shown, an inverse correlation between ROR1 and Wnt5a levels was detected in most patient samples.

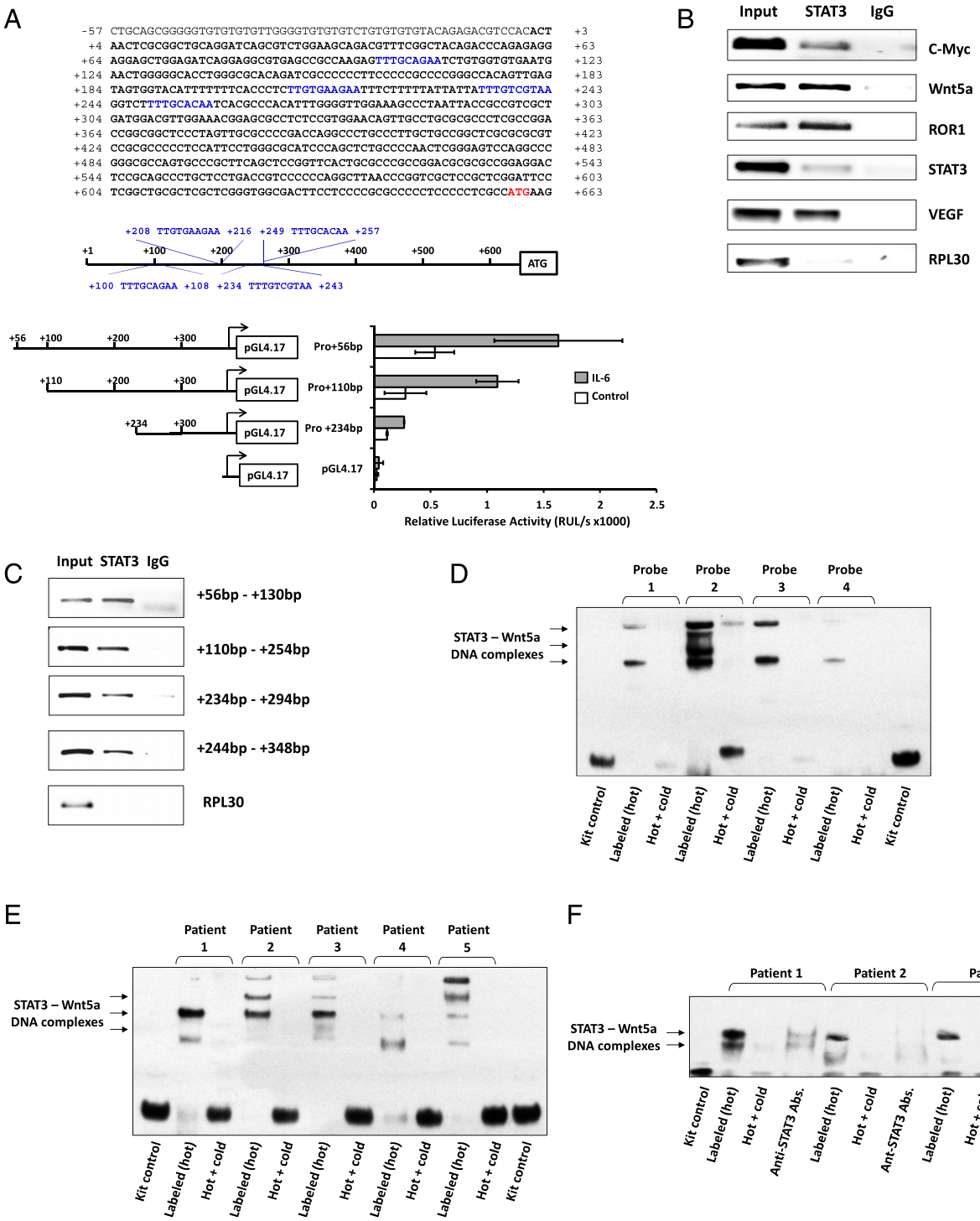

FIGURE 4. STAT3 binds to and activates the Wnt5a gene promoter. (**A**) Sequence analysis of the Wnt5a gene promoter, performed by using the NCBI Web site https://www.ncbi.nlm.nih.gov/nucleotide/NG_031992.1?report=genbank&log$=nuclalign&blast_rank=1&RID=RJ0BMXJ8014 detected four putative STAT3 binding sites (shown in blue) located in close proximity to the Wnt5a gene start codon outlined (shown in red in the upper panel). Three truncated forms of those putative STAT3 binding sites (middle panel) were attached to the luciferase reporter gene (pGL4.17) and transfected into MM1 cells (lower panel). Each horizontal bar in the lower panel shows the mean luciferase activity ± SE in MM1-transfected cells incubated without (control) or with 20 ng/ml of IL-6, which activates STAT3. As shown, the luciferase activity was increased with the addition of another STAT3 binding site in IL-6–stimulated but not in unstimulated MM1 cells (p = 0.02, 0.22, and 0.001, respectively). ATG (shown in red) is the start codon and +1 denotes the first nucleotide of the first exon as well as the first nucleotide of the Wnt5a promoter. Arrowheads indicate start codon. (**B**) ChIP shows that Wnt5a DNA coimmunoprecipitates with STAT3 protein. Protein extracts from CLL cells were incubated with or without anti-STAT3 Abs, and DNA was extracted from the immunoprecipitated chromatin fragments. As shown, anti-STAT3 Abs coimmunoprecipitated with DNA of Wnt5a promoter region and of several other known STAT3 target genes, including c-Myc, ROR1, STAT3, and VEGF. RPL30 was used as negative control. "Input" denotes extracted DNA obtained from chromatin fragments of nonimmunoprecipitated CLL cell (negative control). (**C**) Four primers for each of the above described STAT3 putative binding sites were prepared, and their binding to STAT3 protein was assessed using ChIP. Protein extract of CLL cells was incubated with anti-STAT3 Abs, and each of these four primers was used to amplify the coimmunoprecipitated DNA. As shown, DNA was amplified with all four primers. RPL30 was used as negative control, and IgG was used as the isotype of the anti-STAT3 Abs. (**D**) EMSA confirmed that CLL nuclear *(Figure legend continues)*

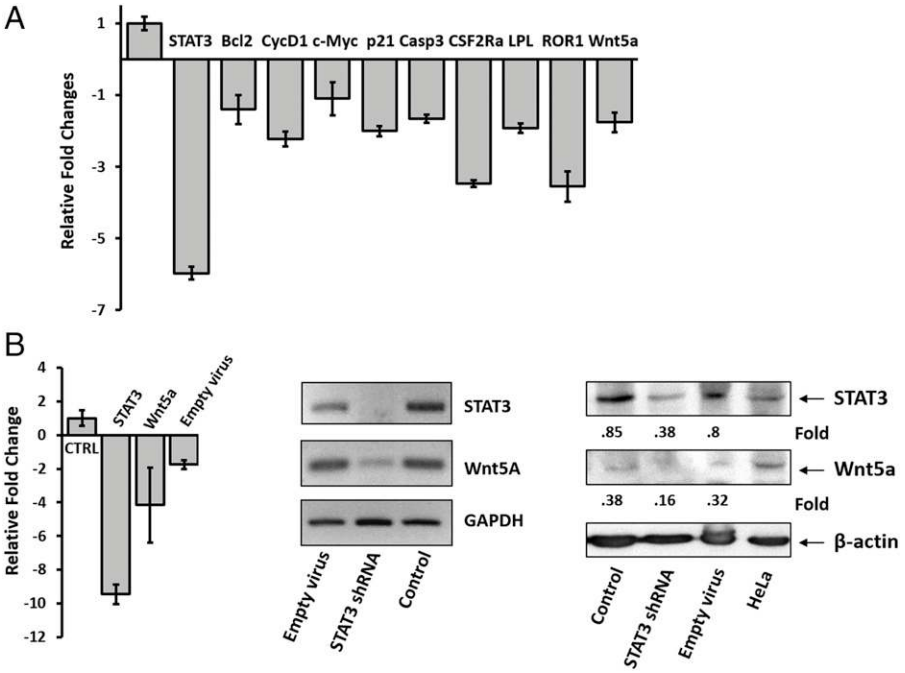

FIGURE 5. STAT3 induces the expression of Wnt5a in CLL cells. (**A**) CLL cells were infected with STAT3 shRNA or with an empty vector. As shown, infection of CLL cells with STAT3 shRNA significantly downregulated mRNA levels of STAT3, Wnt5a, and several STAT3-regulated genes, including Bcl2, cyclin D1, c-Myc, p21, caspase 3, CSF2Rα, LPL, and ROR1. $p < 0.0001$, Student t test (left panel). (**B**) Infection of CLL cells with STAT3 shRNA but not the empty lentiviral vector significantly downregulated STAT3 and Wnt5a mRNA as assessed by quantitative RT-PCR. $p < 0.0001$, Student t test (left panel) and conventional PCR (middle panel) and protein levels (right panel). Ctrl., transfection with empty virus (control).

upper panel). Next, to test whether STAT3 binds to any of those GAS-like elements, we constructed four DNA fragments derived from the Wnt5a promoter, containing one, two, three, and four STAT3 putative binding sites attached to a luciferase reporter gene (Fig. 4A, middle panel). These constructs were transfected into MM1 cells to which IL-6 was added to phosphorylate and activate STAT3. As shown in the lower panel of Fig. 4A, each of the putative STAT3 binding sites induced luciferase activity, and with each added STAT3 binding site, an increased luciferase activity was observed, suggesting that STAT3 binds to each of these GAS-like elements and activates the Wnt5a promoter. To validate these data and test whether STAT3 binds to the Wnt5a promoter in CLL cells, we used ChIP. Using the 104-bp product that covers the upstream GAS binding sites from 249 to 257 bp, we found that, like the STAT3 target genes c-Myc, ROR1, STAT3, and VEGF, Wnt5a DNA coimmunoprecipitated with STAT3 (Fig. 4B). Next, using the same assay, we confirmed that all four GAS-like elements bind STAT3 in CLL cells (Fig. 4C). To further confirm these data, we used an EMSA and found that, like in MM1 cells, all four GAS-like elements bind to the Wnt5a promoter in CLL cells (Fig. 4D). Furthermore, we found that in nuclear extracts from five randomly chosen patients, probe 1, previously found to bind to GAS-like elements at +100–+108 bp, formed STAT3–Wnt5a DNA complexes (Fig. 4E) and that the binding of nuclear extract to the Wnt5a DNA probe was significantly attenuated by anti-STAT3 Abs but not by their isotype (IgG1) (Fig. 4F).

STAT3 shRNA downregulates Wnt5a mRNA and protein levels

To confirm that STAT3 induces the expression of Wnt5a, we infected CLL cells with a lentiviral STAT3 shRNA or with an empty vector. As shown in Fig. 5A, infection of CLL cells with STAT3 shRNA downregulated mRNA levels of STAT3, Wnt5a, and several STAT3-regulated genes, including STAT3, Bcl2, cyclin D1, c-Myc, p21, caspase 3, CSF2R-α, LPL, and ROR1. Furthermore, STAT3 shRNA downregulated STAT3 and Wnt5a mRNA (Fig. 5B, left and middle panels) and protein levels (Fig. 5B, right panel), confirming that STAT3 induces the expression of Wnt5a in CLL cells.

Wnt5a siRNA induces apoptosis of CLL cells

Because ROR1 protects CLL cells from apoptosis (3, 18) and CLL cells express both ROR1 and its ligand Wnt5a, we wondered how knockdown of Wnt5a would affect the survival of CLL cells. To answer this question, we transfected CLL cells with Wnt5a siRNA. We found that Wnt5a siRNA but not scrambled siRNA downregulated Wnt5a mRNA and protein levels (Fig. 6A) and significantly increased the apoptosis rate of CLL cells (Fig. 6B), suggesting that endogenously produced Wnt5a provides CLL cells with a survival advantage.

Discussion

In this study, we show that both ROR1 and its ligand Wnt5a are coexpressed on the surface of CLL cells, that the interaction between Wnt5a and ROR1 provides CLL cells with survival advantage, and that the expression of Wnt5a is driven by the transcriptional activity of STAT3.

Several types of neoplastic cells secrete soluble ligands that bind to their corresponding cell surface receptor and activate signaling in an autocrine fashion (19). For example, in breast cancer, cells autocrine activation of the canonical WNT signaling pathway by secreted frizzled-related protein-1 induces β-catenin–driven

protein extract binds to the putative STAT3 binding sites in the Wnt5a gene promoter. CLL cell nuclear protein extract was incubated with each of the four labeled Wnt5a promoter fragments harboring one putative STAT3 binding site. As shown, STAT3–Wnt5a promoter complexes were detected with each labeled fragment, and the binding was attenuated with the addition of unlabeled (cold) fragments. (**E**) A labeled Wnt5a promoter fragment harboring a single putative STAT3 binding site (probe 1) was added to nuclear protein extracts of CLL cells from five different patients. As shown, STAT3–Wnt5a complexes were detected in samples of all patients, and STAT3–Wnt5a binding was significantly attenuated with the addition of unlabeled (cold) Wnt5a promoter fragment. (**F**) Anti-STAT3 Abs but not their isotype (IgG1) significantly attenuated STAT3–Wnt5a binding in nuclear extract from three CLL patient cells, further confirming that STAT3 binds to and activates the Wnt5a promoter.

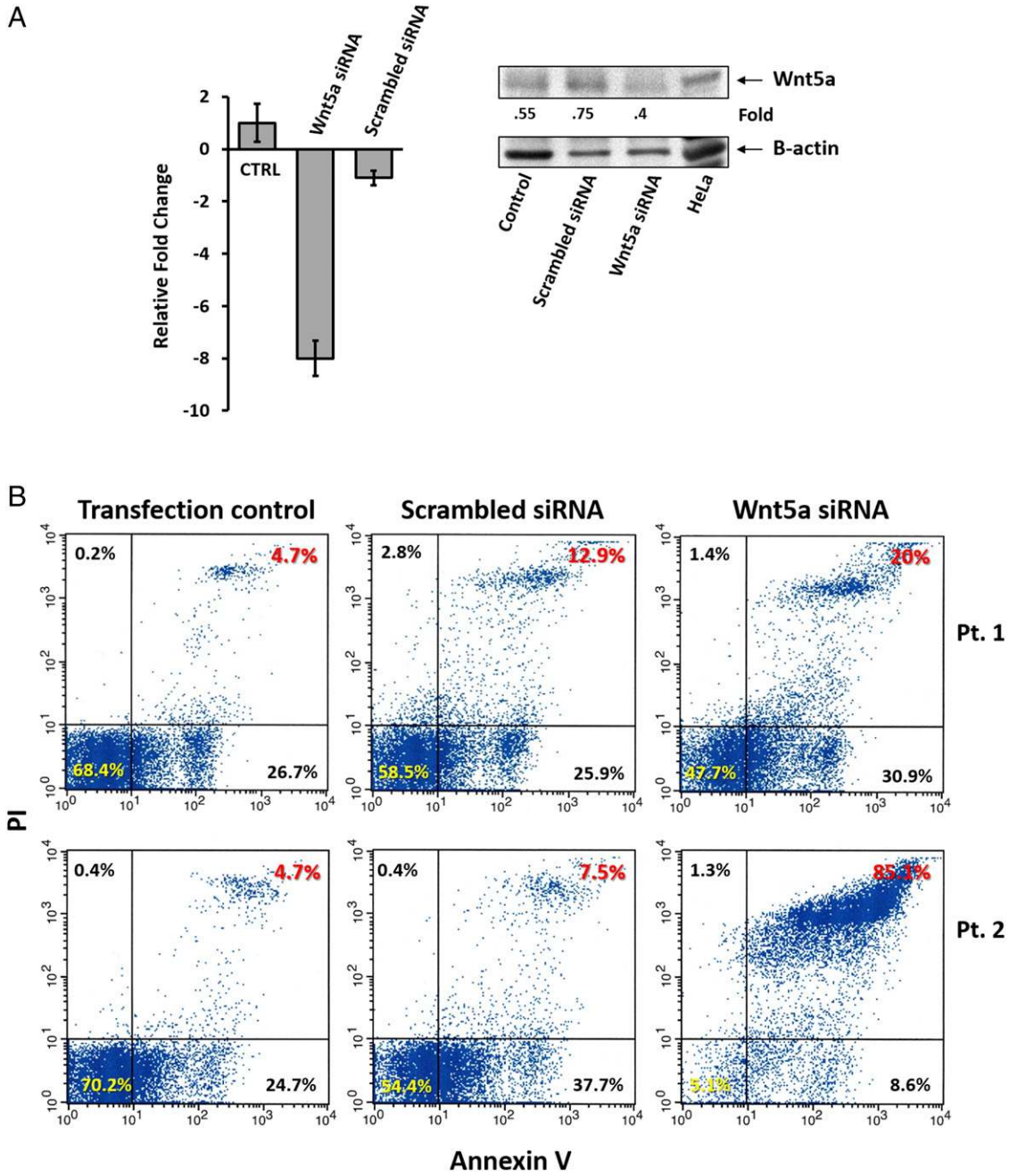

FIGURE 6. Wnt5a provides CLL cells with a survival advantage. (**A**) Transfection of CLL cells with Wnt5a siRNA but not with scrambled siRNA (transfection efficiency 73%) induced an 8-fold reduction in Wnt5a RNA levels (left panel). $p < 0.0001$, Student t test. It also significantly downregulated Wnt5a protein levels (right panel). (**B**) Flow cytometry of annexin/PI–stained cells was used to assess the effect of Wnt5a expression on CLL cell spontaneous apoptosis rate. As shown, CLL cells transfected with Wnt5a siRNA had a significantly higher spontaneous apoptosis rate than that of CLL cells transfected with scrambled siRNA or untransfected cells incubated in the transfection reagents alone (transfection control). In one patient (Pt. 1; upper panel) the transfection efficiency was 73%, whereas in a different patient (Pt. 2; lower panel) it was 88%. The rate of cells in active early and late apoptosis (right lower and upper corner) was 38.8% in CLL cells transfected with scrambled siRNA and 50.9% in CLL cells transfected with Wnt5a siRNA in Pt. 1 and 45.2% in CLL cells transfected with scrambled siRNA and 93.7% in CLL cells transfected with scrambled siRNA in Pt.2.

transcriptional activity of genes that activate cell proliferation (20). Similarly, in glioblastoma-endogenous secreted platelet-derived growth factors bind to and activate platelet-derived growth factors receptors-α and -β in an autocrine manner (21). Our data suggest that in CLL cells endogenously produced Wnt5a activates endogenous ROR1.

We found that in CLL cells STAT3 induces the expression of both ROR1 (3) and its ligand Wnt5a and that the ROR1 ligand Wnt5a provides the cells with survival advantage. Because ROR1 and Wnt5a are coexpressed on the surface of CLL cells, Wnt5a might stimulate ROR1 in an autocrine, paracrine, or acrine manner through attachment to adjacent CLL cells. Indeed, overexpression of Wnt5a was found to increase cell motility (9) and high Wnt5a levels were shown to correlate with unfavorable prognostic indicators, such as mutated *TP53, SF3B1*, or unmutated *IgHV*, and with short time to first treatment (9).

We have recently found that CLL cells harbor a unique protein complex that enables serine creatine kinase 2 to phosphorylate STAT3 on serine 727 residues (22). As a result, constitutively phosphorylated STAT3 induces STAT3-driven transcriptional

activity in unstimulated CLL cells (10). STAT3 activates a metabolic program which provides the cells with adequate means to meet their metabolic requirements (22), regulate translational activity (12, 23), and induce STAT3 target gene expression (3, 24). We have previously shown that STAT3 induces aberrant expression of ROR1 in CLL cells (3). In this study, we show that STAT3 binds to the Wnt5a gene promoter and transcription. By driving the expression of both the ROR1 receptor and its ligand Wnt5a, STAT3 activates a potent autonomous prosurvival signaling circuit. Inhibition of the expression of either ROR1 or Wnt5a induced a marked increase in the spontaneous apoptosis rate of CLL cells, suggesting that this circuit provides CLL cells with survival advantage.

Remarkably, we found an inverse correlation between ROR1 and Wnt5a protein levels in CLL cells. CLL cells with high ROR1 levels expressed low levels of Wnt5a, and cells with low ROR1 levels express high levels of Wnt5a. Although ROR1 levels correlate with poor clinical outcome (16) and inhibition of ROR1 might be most beneficial in these patients, our data suggest that inhibiting the interaction between ROR1 and its ligand Wnt5a might be therapeutically beneficial, even in CLL patients in whom the neoplastic cells express low levels of ROR1.

Whereas circulating CLL cells are quiescent, CLL cells proliferate and progressively expand in lymph nodes, bone marrow, and spleen (25). We demonstrated that endogenously produced Wnt5a protects CLL from apoptosis, an effect that is further enhanced by CLL cell-to-cell interaction and/or direct attachment with mesenchymal stromal cells (26), endothelial cells (27, 28), and monocyte-derived cells (29). Yet, circulating CLL cells detached from their microenvironment benefit from both endogenously produced Wnt5a and ROR1 that enact autonomous prosurvival pathways that enable CLL cells to survive, even in the presence of unfavorable environmental pressures. Therefore, inhibiting the interaction between Wnt5a and ROR1 might prove to be beneficial in patients with CLL.

Disclosures

The authors have no financial conflicts of interest.

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