Biochemistry and Biophysics Reports 6 (2016) 179–184

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

# Noncanonical Wnt signaling in stromal cells regulates B-lymphogenesis through interleukin-7 expression

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ARTICLE INFO

*Article history:*
Received 27 December 2015
Received in revised form
28 March 2016
Accepted 31 March 2016
Available online 1 April 2016

*KeyWords:*
IL-7
Noncanonical Wnt signaling
Bone marrow stromal cells
B-lymphogenesis

ABSTRACT

The regulation of early B cell development and the interaction of hematopoietic precursors with stromal cells in the bone marrow (BM) are controlled by various secreted signaling molecules. Several recent studies showed Wnt signaling involved in B-lymphogenesis through stromal cells. However, the molecules modulated by Wnt signaling in stromal cells regulating B-lymphogenesis have not been identified yet. Interleukin (IL)-7 and CXC chemokine ligand (CXCL) 12 are known to be express in stromal cells, and both molecules are essential for B-lymphogenesis. In the present study, we examined the role of Wnt signaling in regulating IL-7 and CXCL12 expression and in affecting B-lymphogenesis. In mouse stromal ST2 cells, expression of IL-7 and CXCL12 mRNA was augmented by noncanonical Wnt5a. When mouse BM-derived cells were cultured on Wnt5a-overexpressing ST2 cells, an increased number of B220+/IgM- B-lymphoid precursor cells was observed. These results show that Wnt5a regulates IL-7 gene expression in stromal cells and suggest the possibility that noncanonical Wnt regulates B-lymphogenesis via IL-7 expression in stromal cells.



## 1. Introduction

B cells are generated from hematopoietic stem cells (HSCs) in the bone marrow (BM) after birth, and they migrate into the blood to reach secondary lymphoid tissues, such as the spleen and lymph nodes in mammals. The early development of B cells in the BM is dependent on supportive microenvironment consisting of stromal cells known as niches that maintain blood cells and supply factors for their development [1,2]. Regulation of early B cell development and stromal interactions of hematopoietic precursors are thought to be controlled by various secreted signaling molecules, particularly, cytokines, chemokines, and growth factors [3].

Wnt secreted proteins are powerful regulators of embryonic development, cell differentiation, and proliferation [4,5], and they can activate two pathways: the β-catenin-dependent canonical and the β-catenin-independent noncanonical pathway. Noncanonical Wnt ligands activate the Wnt/$Ca^{2+}$ pathway and Wnt/planar cell polarity (PCP) pathway [6]. It has been reported that receptor tyrosine kinase-like orphan receptor 2 (Ror2), a member of the Ror-family of receptor tyrosine-protein kinases, acts as a receptor or coreceptor for Wnt5a [7]. Ror2 by itself or in combination with Frizzled protein through its Frizzled-like cysteine-rich domain mediates diverse Wnt5a signaling by activating the Wnt–c-Jun N-terminal kinase PCP pathway [8].

Several studies have shown that Wnt signaling pathway is involved in the regulation of B-lymphogenesis in the hematopoietic microenvironment, the BM [9]. Canonical Wnt3a-stimulated stromal cells negatively regulated hematopoiesis, including early B-lymphogenesis. In contrast, noncanonical Wnt5a-producing stromal cells enhanced B-lymphopogenesis in culture [10,11]. However, the molecules modulated by the Wnt signaling in stromal cells and involved in early B-lymphogenesis have not been characterized yet.

Interleukin (IL)-7 and CXC chemokine ligand (CXCL) 12 (stromal cell-derived factor-1/pre-B-cell-growth-stimulating factor), which are supplied by stromal cells, are well known to play crucial and essential roles in B-lymphogenesis [12,13]. Both molecules were identified and characterized by *in vitro* coculture system using several stromal cell lines, such as ST2 and PA6 cells [14]. The *in vivo* studies using mutant mice with targeted gene disruption have revealed that CXCL12 and IL-7 expression on stromal cells are essential for B-lymphogenesis [15]. Also, IL-7 and IL-7 receptor α-chain (IL-7Rα)–deficient mice revealed impaired Bcell development due to early B-cell progenitors [16,17]. To date, little is known on the regulation of IL-7 production, especially in stromal cells that are considered the main source of this cytokine.

Several growth factors and cytokines are known to modulate B-lymphogenesis via the regulation of IL-7 and CXCL12 expression on stromal cells. Tang et al. [18] showed that transforming growth

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http://dx.doi.org/10.1016/j.bbrep.2016.03.017

factor (TGF)-β downregulates IL-7 secretion in stromal cells and inhibits proliferation of Bcell precursors [18]. Also, TGF-β1 downregulates CXCL12 expression in the stromal cell line MS-5 [19]. Mice deficient of G protein α subunit (GSα), which is a major downstream activator of the parathyroid hormone- related peptide receptor signaling in osterix-expressing stromal cells, specifically showed a failure of B-lymphopogenesis through the reduction of IL-7 production in stromal cells [20]. Our previous study demonstrated that canonical Wnt3a regulates CXCL12 expression in ST2 cells [21]. However, the role of Wnt signaling in the regulation of IL-7 expression in stromal cells and in the development of B cells remains unclear.

In this study, we examined the effects of Wnt signaling on the regulation of IL-7 expression in ST2 cells, and then on B-lymphogenesis using an *in vitro* coculture system. Wnt5a enhanced IL-7 expression in ST2 cells and increased the number of Bcell progenitors. These findings demonstrate that noncanonical Wnt signaling in stromal cells regulates B-lymphogenesis partially through IL-7 expression.

## 2. Materials and methods

### 2.1. Murine BM cells

Murine adherent cell-depleted BM cells were isolated from seven-week-old C57BL/6J mice from Nippon Clea (Tokyo, Japan). The experiments were performed in accordance with the guidelines on the care and use of laboratory animals and have been approved by Hokkaido University.

### 2.2. Cell cultures

ST2 cells were obtained as described previously [21]. Wnt3a-ST2 and Wnt5a-ST2 cells were established as described previously [21]. Cells were grown to semiconfluence in alpha minimum essential medium (α-MEM) (Sigma-Aldrich, St. Louis, MO, USA) containing 100 μg/mL kanamycin (Meiji, Tokyo, Japan) and supplemented with 10% fetal bovine serum (FBS; PAA Laboratories; Pasching, Austria) at 37 °C (Corning, Corning, NY, USA) in a humidified atmosphere of 5% $CO_2$. The medium was removed, and $1 \times 10^6$ adherent cell-depleted BM cells were cultured with or without ST2, Wnte3a-ST2, or Wnt5a-ST2 cell layer in RPMI1640 medium (Sigma-Aldrich) supplemented with 5% FBS and 50 μM 2-mercaptoethanol at 37 °C for 4, 5, or 7 days. Floating cells were analyzed by flow cytometry.

### 2.3. Reagents

Mouse recombinant Wnt5a was obtained from R&D Systems Inc. (Minneapolis, MN, USA).

### 2.4. Flow cytometry

Flow cytometry analysis was carried out using the following antibodies: PE-anti-B220, PE-anti-CD3ε and PE-anti-CD11b from BD Bioscience (BD Bioscience, San Jose, CA). Stained cells were analyzed for surface expression using a flow cytometer (FACSCalibur; BD Biosciences) and analyzed with CellQuest software (BD Biosciences) as described previously [22].

### 2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells using Isogen (Nippongene, Toyama, Japan), and RT-PCR was performed as previously described [23]. All the primers were synthesized by Hokkaido System Science (Sapporo, Japan). The primer sequences were described previously [24].

### 2.6. Quantification of gene expression by quantitative RT-PCR (qRT-PCR)

Total RNA was reverse transcribed using first-strand cDNA synthesis with random primers (Promega, Madison, WI, USA). The PCR was performed using SYBER Green (Invitrogen Life Technologies Carlsbad, CA, USA) and ABI StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). Primer sequences for each gene were the following: CXCL12, 5′-CAACACTCCAAACTGTGCCCTTCA-3′ (forward), 5′-TCCTTTGGGCTGTTGTGCTTACT-3′ (reverse); IL-7, 5′-TGGGAGTGATTATGGGTGGT-3′ (forward), 5′-GCGAGCAGCACGATTTAGAAAAGC-3′ (reverse); thymic stromal lymphopoietin (TSLP), 5′-AGGCTACCCTGAAACTGAG-3′ (forward), 5′-GGAGATTGCATGAAGGAATACC-3′ (reverse); β-actin; 5′-CTTCTTTGCAGCTCCTTCGTTG-3′ (forward), 5′-CGACCAGCGCAGCGATATC-3′ (reverse). The relative level of gene expression was quantified using the comparative Ct method with β-actin as the endogenous control.

### 2.7. Cell proliferation assay

To quantify cell proliferation, the tetrazolium-based colorimetric CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) was used. A 20 μL aliquot of the substrate WST-8 (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt) was added to each well. After incubation for 2 h at 37 °C, the optical density was measured at a wavelength of 450 nm using a microplate reader (iMark, Bio-Rad Laboratories, Richmond, CA, USA).

### 2.8. Statistical analysis

All experiments were repeated three to four times and representative results are shown. The data are reported as the mean ± standard deviation (SD) and were analyzed by Student's *t*-test, where p values $< 0.05$ were considered significant.

## 3. Results

### 3.1. Wnt signaling regulates IL-7 and CXCL12 expression in ST2 cells

To evaluate the role of Wnt signaling on IL-7 and CXCL12 regulation, which is essential for B-lymphogenesis in stromal cells, we examined their mRNA expression using qRT-PCR. We had previously established stromal ST2 cells expressing either Wnt3a (Wnt3a-ST2 cells), which stimulates canonical Wnt signaling, or Wnt5a (Wnt5a-ST2 cells), which stimulates noncanonical Wnt signaling [21]. IL-7 mRNA expression level was upregulated in both Wnt3a-ST2 and Wnt5a-ST2 cells, particularly in Wnt5a-ST2 cells, compared with vehicle-transfected ST2 cells (Fig. 1A). CXCL12 mRNA level was increased in Wnt5a-ST2 cells, whereas it was reduced in Wnt3a-ST2 cells. However, mRNA expression of the IL-7-related cytokine TSLP, the receptor formed by a heterodimer of IL-7Rα and another receptor subunit, was altered neither by Wnt3a nor by Wnt5a overexpression in ST2 cells (Fig. 1A). Treatment with the recombinant Wnt5a protein (200 ng/mL) also significantly increased IL-7 mRNA expression in ST2 cells (Fig. 1B). To confirm the increment of IL-7 mRNA expression by Wnt5a in another cell line, we examined Wnt5a-C2C12 cells, previously established [23]. In Wnt5a-C2C12 cells, IL-7 mRNA expression level was 1.75 times higher than that in C2C12 cells (data not shown; relative average value, n=6). These results indicate that