

Wnt7b is Upregulated in Macrophages during Thymic Regeneration and Negatively Regulated by RANKL

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Thymus can regenerate to its normal mass within 14 days after acute involution induced by cyclophosphamide (CY) in adult rat. Despite the established role of Wnt pathways in the process of thymus development, they have not yet been associated with the regeneration of adult thymus. The purpose of this study was to investigate whether Wnt7b, which is expressed in developing thymic epithelial cells rather than in thymocytes, is modulated during thymic regeneration in adult rat. Here, we show that Wnt7b expression was up-regulated in the regenerating thymus. Cells immunolabeled for the Wnt7b were identified as macrophages. Furthermore, Wnt7b gene expression was decreased by the treatment of receptor activator of NF-kappaB ligand (RANKL). Taken together, our results demonstrate that Wnt7b gene expression was increased in macrophages during thymic regeneration and negatively regulated by RANKL.

Key words – Wnt7b, RANKL, Thymus, Regeneration, Macrophage

Introduction

Thymus has a central role in the immune system, providing the optimal microenvironment required for the development of bone marrow-derived precursor cells into mature, functional T cells with self-restriction and self-tolerant mechanism [10]. With advancing age or under any of stressful conditions, the thymus rapidly diminishes in size due to the massive thymocyte death, and consequently host immunity may be suppressed and susceptibility to disease increased [17,21]. Recently, it has been well documented that acute involution, induced in experimental animals, is followed by intensive thymic regeneration after the removal of the causative stimuli [9,25]. Thus, it is a matter of considerable importance that we understand the mechanism of thymic regeneration and develop methods of normalizing or improving host immunity when the immune function is depressed due to thymic involution.

The Wnt family of at least 19 secreted glycoproteins plays important roles during a variety of developmental and homeostatic processes including cell fate specification, proliferation, polarity, migration differentiation, maturation

and death [3,8]. In thymus, the Wnt signaling is required for normal thymocyte development [20]. In the process of thymus development, several Wnt proteins including Wnt 4, Wnt 7a and 7b, Wnt 10a and 10b, are expressed by thymic stromal cells rather than by thymocytes, while thymocytes demonstrate a developmentally regulated pattern of Frizzled receptor expression [15]. Overall these observations suggest that Wnt signaling has a functional role in thymus development. However, whether the Wnt signaling is also involved during thymic regeneration in adult is actually not known.

The purpose of this study was to investigate the expression patterns of Wnt family, especially Wnt7b, and its regulation by RANKL signaling during thymic regeneration in adult rat.

Materials and methods

Experimental acute thymic involution and regeneration model

Adult male-specific pathogen-free Sprague-Dawley rats were purchased from Dae Han Bio Link (Seoul, Korea). All rats were housed three to four per cage and maintained under a 12 hr light/dark cycle at 24°C in a specific pathogen-free and humidity-controlled facility. The animals were provided with standard sterile food and water and were

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allowed to adjust to their environment for 1 week. The animals were used at 8-10 weeks of age by giving a single intraperitoneal dose of cyclophosphamide (150 mg/kg body weight ; Sigma) in normal saline [25], and were killed in groups of four at 3, 7 and 14 day after injection. Rats given the same amount of normal saline were used as controls. Three independent experiments were performed, with 16 animals in each experiment (four animals in each group). Animal care and all experimental procedures were conducted in accordance with the "Guide for Animal Experiments" edited by the Korean Academy of Medical Sciences.

Immunohistochemistry

After cryosections, immunostaining was performed using the streptavidin-biotin complex (ABC) method, as described in previous studies [26]. In brief, sections were incubated for 20 min in a solution of phosphate-buffered saline (PBS) containing 0.3% H₂O₂ to eliminate endogenous peroxidase activities. After washing in PBS, sections were incubated with 2% normal donkey serum (Vector Laboratories, Burlingame, CA). The excess solution was shaken off and sections were incubated for 16-18 hr at 4°C with an affinity-purified goat polyclonal anti-Wnt7b antibody (sc-26363, diluted 1:100, Santa Cruz Biotechnology, Santa Cruz, CA). Following incubation with the primary antibody, sections were washed three times for 5 min with PBS and incubated for 2 hr at room temperature with an affinity-purified F(ab')₂ fragment donkey anti-goat biotinylated antibody diluted 1:100 (Jackson ImmunoResearch Laboratories, West Grove, PA). They were then rinsed in PBS and incubated for 60 min at room temperature with an ABC reagent (Vectastain Elite Kit; Vector Laboratories) according to the manufacturer's instructions. Sections were developed in 0.025% 3,3'-diaminobenzidine and 0.003% H₂O₂ medium under microscopic control at room temperature to visualize peroxidase activity. Sections were rinsed in distilled water, counterstained with Mayer's hematoxylin, and mounted in a xylene-based mounting medium (Entellan, Darmstadt, Germany). Slides were observed and photographed using an Olympus BX50 microscope. All photomicrographs were taken with an Olympus C-3030 digital camera.

Double Immunofluorescence staining

Two-color double immunohistochemical analysis was

performed in order to confirm whether the cells that express Wnt7b are macrophages. After the sections were rinsed in PBS and incubated with 2 % normal donkey serum for 60 min, they were incubated with the first primary antibody (goat polyclonal anti-Wnt7b antibody, sc-26363, Santa Cruz Biotechnology) for 16-18 hr at 4°C. Following incubation with the primary antibody, the sections were incubated with an affinity-purified F(ab')₂ fragment of donkey anti-goat Texas Red-conjugated antibody at a dilution of 1:100 (Jackson ImmunoResearch Laboratories). After the sections were rinsed in PBS, they were further incubated with the second primary antibody. For the second primary antibody, we used affinity-purified mouse anti-ED1 antibody (Jackson ImmunoResearch Lab.) at a dilution of 1:100 to identify for the reactivity with the macrophages of the rat thymus. The sections were incubated with an affinity-purified F(ab')₂ fragment of donkey anti-mouse FITC-conjugated antibody diluted 1:100 (Jackson Immuno Research Laboratories). The samples were examined under a fluorescence microscope (Axiphot, Zeiss).

Thymocyte and thymic stromal cell isolation

Two to three thymi were dissected from freshly killed rats and trimmed of fat and connective tissue. Small cuts (2-3 mm) were made into the capsules with a pair of razors, and thymi were gently agitated in 30 ml of RPMI-1640 using a magnetic stirrer at 4°C for 40 min. The resulting thymic fragments and supernatant were transferred into separate tubes. For the isolation of thymocytes, the supernatant was passed three times through 70- μ m mesh and centrifuged. The cell pellet was resuspended in 20 ml of ACK lysis solution (0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂EDTA) to remove red blood cells. The thymocytes suspension was washed three times with HBSS buffer. The thymocytes were resuspended in HBSS buffer, and viable cells were counted using the hemocytometer after trypan blue staining. For the isolation of thymic stromal cells, the thymic fragments were transferred into 5 ml of RPMI-1640 containing 0.125% (w/v) collagenase D and 0.1% (w/v) DNase I (both from Roche) and then incubated for 15 min with gentle shaking in a water bath at 37°C. The thymic fragments in the enzyme mixtures were carefully dispersed with a Pasteur pipette several times, and the supernatant was removed after fragments had settled and was replaced with fresh enzyme mixture. Gentle mechanical agitation was provided using a 5 ml syringe

and 18G needle, then a 21G needle, followed by a 23G needle. Tissue fragments were allowed to settle, and the supernatant was discarded. This digestion process was repeated four more times until the tissue was fully digested. Cells liberated by the fourth, fifth, and sixth digests were saved, filtered through a 100 µm mesh to remove undigested particles, and washed with HBSS buffer. They were then resuspended in HBSS buffer, and viable cells were counted using the hemocytometer after trypan blue staining.

Western blot analysis.

Harvested cells were lysed in a lysis buffer (0.1% SDS, 1.0% Triton X-100 and 1.0% Deoxycholate in PBS containing 1 mM DTT, 1% protease inhibitor cocktail (Sigma), 1 mM PMSF, 1 mM Na₃VO₄, 1 mM NaF and 1 mM β-glycerophosphate) and protein concentration was measured with BCA assay. A constant protein concentration (30 µg /lane) was used. The protein extracts were boiled for 5 min, loaded, separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were then probed with appropriate antibodies. The signal was developed with the enhanced chemiluminescence (ECL) detection system (Amersham-Pharmacia Biotech.).

Cell culture

Mouse macrophage cell line, RAW 264.7, was maintained in Dulbecco's modified Eagle medium (Gibco-BRL) supplemented with 10% Donor calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% CO₂ in 95% air.

RT-PCR

The mRNA levels of Wnt family genes were analyzed by RT-PCR. Total RNA was isolated from each sample using Trizol RNA Extraction Reagent (Bio Rad) following the manufacturer's protocol. First-strand cDNA synthesis was carried out on 3 µg of total RNA with a reverse transcription kit (Invitrogen). One-tenth of the reaction mixture was used as template for PCR amplification. The PCR reaction condition and the sequences of primers are available upon request.

Reporter plasmid

A genomic DNA fragment of the mouse Wnt7b gene containing 1,005 promoter region was prepared by PCR

amplification of mouse genomic DNA. Primers for mouse Wnt7b promoter are 5'-CACGGTACCCCATTTGATGCTGCTGTCCGG-3' and 5'-CACCTCGAGGGCACGCGTTGCCACCATGGTGAG-3'. The resulting PCR fragments were cloned into the *Kpn*I and *Xho*I sites of the pGL3basic luciferase reporter plasmid (Promega) to generate the luciferase reporter plasmid pGL3/Wnt7b-1.0. The construct was confirmed by automatic DNA sequencing analysis.

Transient transfection and luciferase assay

RAW 264.7 cells were plated on 24-well plates (Nunc) and transfected the next day with mouse Wnt7b promoter-luciferase constructs (pGL3/Wnt7b-1.0). Control transfections were made using a lacZ gene that is driven by an RSV promoter. Transfections were performed using Lipofectamin (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were treated with sRANKL (0.5 µg/ml, CRR101, Cellsciences) for 24 hr and then solubilized. Luciferase activity was measured by using a luminometer (Turner Designs, Inc.) and β-galactosidase activity was measured by using the Galactolight kit (Promega) as recommended by the manufacturer. The relative level of promoter activation in each experiment was calculated by dividing the luciferase activity by β-galactosidase activity.

Results

Differential expressions of Wnt family members in thymocyte and thymic stromal cells during thymic regeneration

Since Wnt 4, Wnt 7a and 7b, Wnt 10a and 10b, are expressed by thymic stromal cells during thymus development [15], we investigated their expression patterns in regenerating thymus of adult rats. As shown in Figure 1, the mRNA levels of Wnt 4, Wnt7b and Wnt 10a were higher in both thymocyte and thymic stromal cells of regenerating thymus than in the normal thymus. The levels of Wnt 7a and Wnt 10b mRNAs were rather decreased during thymic regeneration (Fig. 1).

Upregulation of Wnt7b expression during thymic regeneration

As shown in Figure 1, the highest induction of Wnt7b mRNA at 3 day after CY treatment in both thymocyte and thymic stromal cells, was further confirmed at both mRNA

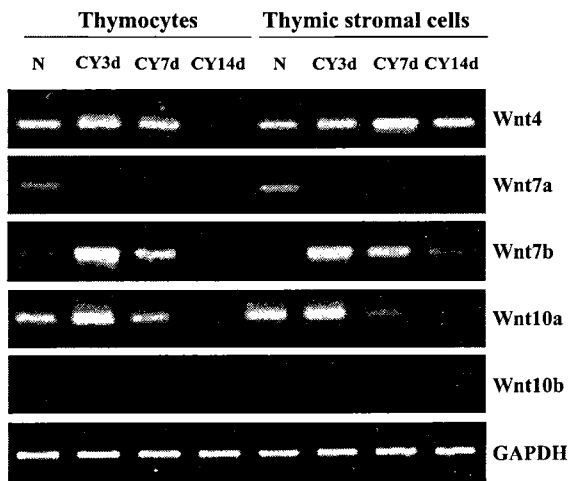


Fig. 1. Differential expression of Wnt family members in thymocytes and thymic stromal cells during thymic regeneration. RT-PCR analysis of Wnt family members and GAPDH mRNA expression in thymocytes and thymic stromal cells isolated from normal thymus (N) and regenerating thymus at 3 (CY3d), 7 (C7d) and 14 (CY14d) days after cyclophosphamide (CY) treatment.

and protein levels using whole thymic extracts. RT-PCR analysis showed that the Wnt7b mRNA levels were dramatically increased at 3 day after CY treatment by 5.0-fold over the normal control (Fig. 2A). Western blot analysis also showed that reactive bands of Wnt7b in whole thymic extracts from rats at 3 and 7 day after CY treatment exhibited increased expression compared with those from the normal rat thymus (Fig. 2B). Immunostaining for Wnt7b protein revealed that while weak signals for Wnt7b protein were observed in both cortex (C) and medulla (M) of the normal thymus (Fig. 2C: a and b), the levels of Wnt7b protein were highest at 3 day after CY treatment (Fig. 2C: c and d) and then decreased gradually to basal level by 14 day (Fig. 2C: e to h).

Expression of Wnt7b in ED1-positive macrophages

We next examined what types of cells are responsible for the Wnt7b expression in regenerating thymus following involution after CY treatment. Double-immunohistochemistry for Wnt7b and cytokeratin (CK) revealed that no Wnt7b signal was detected in the thymic epithelial cells displaying immunoreactivity for CK during thymic regeneration (data not shown). Instead, as shown in Figure 3, double-immunohistochemistry revealed that strong Wnt7b signals were detected in the ED1-positive macrophages in the regenerating thymus.

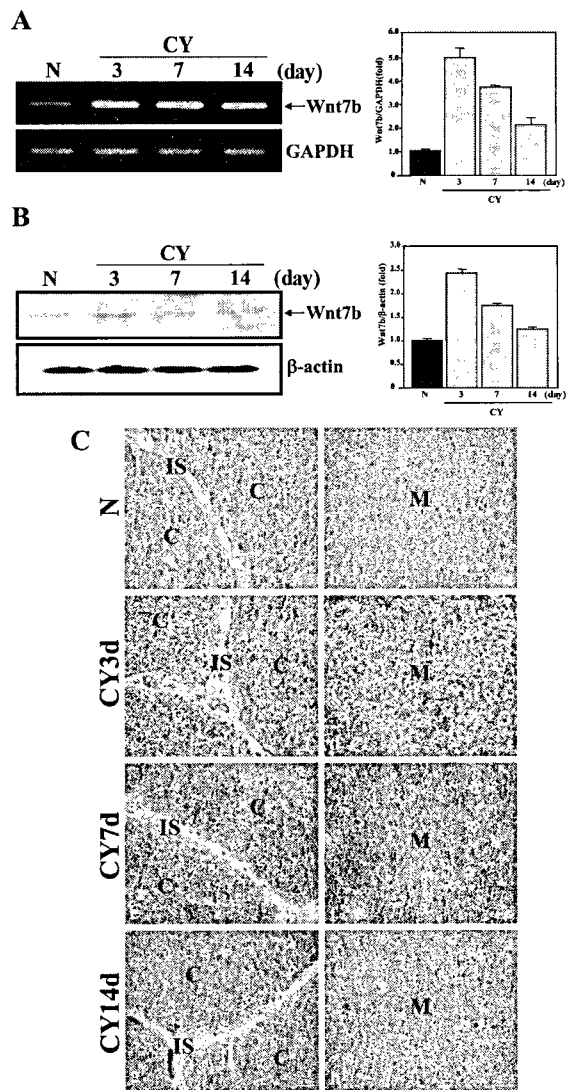


Fig. 2. Upregulation of Wnt7b expression during thymic regeneration. (A) RT-PCR analysis showing mRNA levels of Wnt7b in the normal thymus (N) and regenerating thymus at 3, 7, and 14 day after CY treatment (left). Relative Wnt7b mRNA levels normalized with GAPDH mRNA (right). The density of each band in each lane from three independent experiments was quantified by scanning densitometry and then expressed as mean \pm SD. (B) Western blot analysis showing protein levels of Wnt7b in the normal thymus (N) and regenerating thymus at 3, 7, and 14 day after CY treatment (left). Relative Wnt7b protein levels normalized with β -actin protein (right). The density of each band in each lane from three independent experiments was quantified by scanning densitometry and then expressed as mean \pm SD. (C) Expression of Wnt7b in the normal thymus (a,b) and regenerating thymus at 3 (c,d), 7 (e,f) and 14 (g,h) days after CY treatment was determined by immunohistochemical staining. C cortex. IS Interlobular septum. M medulla. Magnification is 400X.

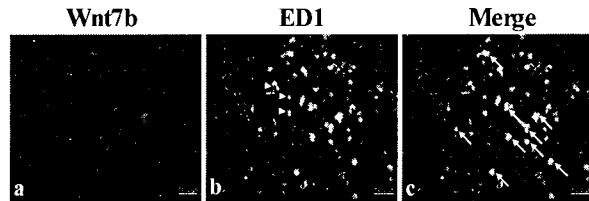


Fig. 3. Expression of Wnt7b in ED1-positive macrophages. Two-color, double labeled immunofluorescent staining of Wnt7b (a: red) and ED1 (b: green) on thymic macrophages in rat thymus at 3 day after CY treatment. The individual recordings were used to create the merged images (c: yellow) showing simultaneous detection of Wnt7b and ED1. Cells expressing Wnt7b are ED1-positive macrophages (arrows). Arrowheads indicate ED1-positive macrophages that are Wnt7b-negative. Magnification is 400X.

Down-regulation of Wnt7b expression by RANKL in RAW 264.7 cells

Recent study reported a closed correlation between Wnt and RANKL signals [19]. To investigate whether RANKL affects Wnt7b mRNA levels, RT-PCR analysis was performed. Since Wnt7b is expressed in macrophages during thymic regeneration, mouse macrophage cell line, RAW 264.7, was used in this study. As shown in Figure 4A, sRANKL (0.5 μ g/ml) decreased the level of Wnt7b mRNA in a time-dependent manner by $\sim 25\%$, suggesting that RANKL/RANK signaling may negatively affect the transcription of Wnt7b gene. To confirm the notion, a luciferase reporter vector containing 1.0kb Wnt7b promoter (pGL3/Wnt7b-1.0) was constructed and the activity of Wnt7b promoter was analyzed in sRANKL-treated RAW 264.7 cells using the transient expression system. Consistent with the RT-PCR results (Fig. 4A), when the construct containing 1.0 kb of Wnt7b promoter was introduced into RAW 264.7 cells and treated with sRANKL, the promoter activity was declined by $\sim 32\%$ when compared to that of untreated control (Fig. 4B), suggesting the inhibitory effects of RANKL signaling on Wnt7b gene expression in macrophage.

Discussion

The Wnt genes/proteins play important roles during embryonic development and in homeostatic mechanisms in adult tissues [3,8]. Recent studies demonstrated that Wnt proteins, Fz receptors, and Wnt signaling antagonist Kremen1, known to be important regulators of Wnt signaling pathway, are expressed in stromal cell and thymocyte

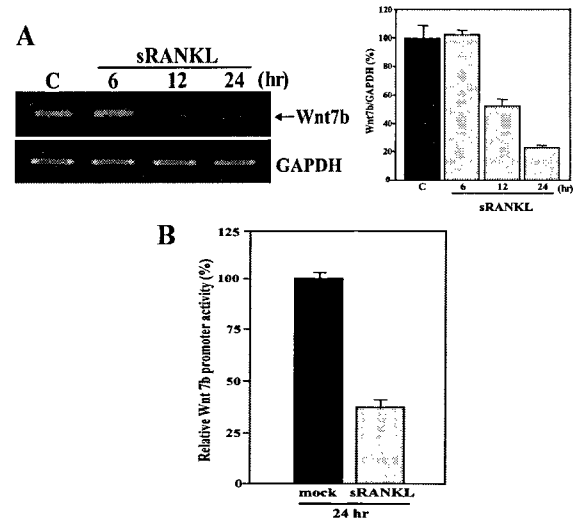


Fig. 4. Down-regulation of Wnt7b expression by RANKL in RAW 264.7 cells. (A) RT-PCR analysis showing mRNA levels of Wnt7b in mouse macrophage cells (RAW 264.7) (left). The cells were incubated for 6, 12, and 24 hr after RANKL treatment (0.5 μ g/ml). Relative Wnt7b mRNA levels normalized with GAPDH mRNA (right). The density of each band in each lane from three independent experiments was quantified by scanning densitometry and then expressed as mean \pm SD. (B) RANKL represses Wnt7b promoter activity in RAW 264.7 cells. RAW 264.7 cells were transfected with Wnt7b 1.0 kb promoter-luciferase reporter construct (pGL3/Wnt7b-1.0). Twenty-four hours after transfection, cells were either unstimulated or stimulated with RANKL for a further 24 hr. Results were normalized using β -galactosidase activity and expressed as a percentage of the pGL3/Wnt7b-1.0-transfectant without treatment with RANKL. Data are the mean \pm SD from three independent experiments.

populations of the developing thymus during embryogenesis [11,15]. However, the expression patterns or roles of Wnt proteins during thymic regeneration in adult remain poorly understood.

The present results showed for the first time that the levels of Wnt7b mRNA and protein were significantly up-regulated in thymic macrophages during thymic regeneration in adult rat. Among the Wnt genes analyzed, Wnt7b was the most interesting one because dramatic induction of Wnt7b expression was observed particularly in thymic stromal cells as well as in thymocytes during thymic regeneration (Fig. 1 and 2A). The increase in the level of Wnt7b protein during thymic regeneration was confirmed by Western blot analysis (Fig. 2B and C). Most interestingly, cells immunolabeled for the Wnt7b protein

were identifiable as macrophages by their coexpression of Wnt7b and ED1, as demonstrated by double-labeled immunofluorescence staining (Fig. 3). Considering that thymic stromal cells cross-talk with thymocytes within the thymus [13], thus contributing to the generation of mature T-cells, it is likely that Wnt7b protein produced by stromal cells, particularly macrophages, may play an important role in the cross-talk between stromal cells and thymocytes during thymic regeneration.

In most systems, it has largely been assumed that macrophage respond to 'eat-me' signals from dead and dying cells and involved in programmed cell death comes after the apoptotic event [16]. However, in some circumstances phagocytes actively induce programmed cell death [1,6]. Recently it has been reported that macrophages initiate a cell-death program in target cells by activating the canonical Wnt pathway [7]. Interestingly, macrophage WNT7b is a short-range paracrine signal required for WNT-pathway responses and programmed cell death in the vascular endothelial cells of the temporary hyaloid vessels of the developing eye [7]. Therefore, since the levels of Wnt7b were up-regulated in thymic macrophage during thymic regeneration (Fig. 2C), thymic macrophages may use Wnt ligands such as Wnt7b to influence cell-fate decisions--including cell death--in adjacent cells, for example, damaged cells including thymocytes and endothelial cells.

On the other hand, macrophages may be more generally involved in regulating vascularity through the activation of the Wnt pathway in vascular structures [4,24]. In addition, tumor-associated macrophages are also known to express Wnt genes [18] and can extrinsically regulate the growth of the tumor vasculature [14]. The role of Wnt/ β -catenin signaling in vasculature is becoming apparent [2]. Interestingly, our recent results showed dramatic induction of reparative angiogenesis during adult thymic regeneration [12]. Therefore, considering that Wnt7b signaling is required for proper lung mesenchymal growth and vascular development [22,23], it is likely that Wnt7b may play a role in regulating vascular remodeling or neoangiogenesis that is accompanied by adult thymic regeneration. Of course, further studies need to clarify our hypothesis underlying the functional contribution of Wnt7b to the thymic regeneration.

RANKL is related to Wnt canonical signaling pathway for the regulation of bone mass [5]. Wnt signalling in osteoblasts down-regulates expression of RANKL and inhibits osteoclastogenesis in vitro [3,19]. In the case of mac-

rophage, the treatment of RANKL reduced the Wnt7b gene expression (Fig. 4). So far, several transcription factors have been reported to contribute to Wnt7b gene induction. For example, TTF-1, GATA6, and Foxa2 have been shown to induce Wnt7b gene expression in the lung epithelium [23]. Since one putative NF- κ B binding site was found in 5' Wnt7b promoter using by TRANSFAC database, it was expected that NF- κ B signaling activates Wnt7b transcription through the NF- κ B binding site. However, as shown in Figure 4A and B, the treatment of RANKL, which activates NF- κ B transcription factor, rather reduced the Wnt7b gene expression in macrophages. Thus, it is less likely that NF- κ B activated by RANKL is directly involved in the regulation of Wnt7b gene expression. Instead, the reduction in Wnt7b expression is likely due to unknown factor(s) that may be upregulated by RANKL-mediated NF- κ B activation and acts as a repressor for Wnt7b gene expression in macrophages.

In conclusion, our study provides evidence for the first time that Wnt7b expression was increased in thymic macrophages during thymic regeneration and negatively regulated by RANKL. Our findings may help to identify the roles of Wnt7b signaling in regenerating adult tissue such as thymus and provide new insights into the regenerating mechanism of adult tissue/organ.

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초록 : 흰쥐 가슴샘 재생과정 동안 대식세포에서 Wnt 7b의 발현증가 및 RANKL에 의한 발현조절

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성체흰쥐의 경우 항암제인 싸이클로포스파마이드 (CY)치리로 퇴축된 가슴샘은 2주 후에 정상조직으로 재생된다. 가슴샘 발생과정에서 이미 알려진 Wnt 신호전달의 중요성과는 달리 성체의 가슴샘 재생과정에서 그 역할에 관해서는 알려진 바 전혀 없다. 본 연구의 목적은 발생중인 가슴샘 상피세포에서 발현이 증가된다고 이미 알려져 있는 Wnt7b가 성체의 가슴샘재생과정에서 어떤 발현 양상을 보이는지를 조사하는 것이다. Wnt7b는 가슴샘의 급성 퇴축 이후 3일째 되는 시기에 mRNA와 단백질의 양이 급격히 증가 하였으며, 이중 면역 염색 형광법을 통해 큰포식 세포와 위치적 분포가 일치함을 확인하였다. 또한, Wnt7b유전자의 발현 조절 기전을 밝히기 위해 Wnt7b의 Reporter Vector를 제작하여 Luciferase assay를 이용하여 상위의 신호를 분석하였고, 그 결과 Wnt7b는 RANKL에 의해 그 발현이 감소된다는 사실을 처음으로 밝혔다. 따라서, 본 연구 결과들을 통해 Wnt 7b는 가슴샘의 급성 퇴축 초기 과정에서 나타나는 손상된 세포를 처리하는 큰포식 세포의 기능 조절에 관여할 것으로 생각된다.