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Wnt5a Regulates Odontoblast Inflammation by Promoting CCL2 Expression

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Abstract

Objective

Wnt5a is involved inflammation, including pulpitis, by upregulating cytokine/chemokine expression. Odontoblasts are the first layer cells in dental pulp and respond to inflammatory stimuli. However, whether Wnt5a is involved in odontoblast inflammation is unclear. This study aimed to investigate the role of Wnt5a in odontoblast inflammation.

Methods

We measured and compared Wnt5a- or TNF- α -induced cytokine/chemokine expression in mouse odontoblast-like (17IIA11) cells by real-time PCR and Western blotting. Transwell assays were used to examine the effect of Wnt5a on RAW264.7 macrophage migration. We examined whether the nuclear factor-kappa B (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways were involved in the molecular mechanism of TNF- α or Wnt5a in 17IIA11 cells by Western blotting.

Results

TNF- α upregulated Wnt5a in odontoblasts. Wnt5a upregulated CCL2 expression in odontoblasts and enhanced RAW264.7 cell migration. We also found that TNF- α -induced Wnt5a expression was abrogated by inhibiting the MAPK pathway or NF- κ B activity and that inhibiting MAPK activity could lead to decreased NF- κ B activity.

Conclusions

Wnt5a is involved in the TNF- α -induced inflammatory response in odontoblasts. TNF- α upregulates Wnt5a expression via MAPK-dependent NF- κ B activation in odontoblasts. Wnt5a upregulates CCL2 expression in odontoblasts and enhances RAW264.7 macrophage migration.

Key Words

odontoblasts; Wnt5a; pulp inflammation; TNF- α

Introduction

The Wnt signaling pathway is an evolutionarily conserved signaling pathway that regulates embryonic development, tissue regeneration, and various types of inflammation [1-5]. The β -catenin-dependent Wnt signaling pathway is the canonical Wnt signaling pathway, while the β -catenin-independent pathway is the noncanonical Wnt signaling pathway [6].

Wnt5a is a member of the Wnt signaling family and is involved in the activation of noncanonical Wnt signaling; Wnt5a is also involved in regulating various type of inflammation, including rheumatoid arthritis, periodontitis, and pulpitis [7-11]. In addition, Wnt5a induces the expression of multiple cytokines/chemokines, including IL-6, IL-8, IL-17, CXCL1, and CCL2, and indirectly activates the migration of immune cells in human dental pulp cell (hDPC) populations [9, 10].

Pulp inflammation, also called pulpitis, is a major oral health problem caused by the invasion of microorganisms and their components via dentinal tubules in the pulp [12] that frequently leads to persistent and referred pain. Pulpitis often results in root canal therapy, whose disadvantages include the loss of living pulp and increased tooth brittleness. Therefore, we hope to provide a theoretical basis for the development of new treatments for pulpitis by

1 understanding pulpitis pathogenesis.

2 Odontoblasts are the first layer cells in dental pulp, and the main function of odontoblasts is
3 dentin formation. During pulpitis, odontoblasts are the first cells encountered by invading
4 bacteria, and the products of these bacteria are located at the pulp-dentin interface [13, 14].
5 Numerous studies have shown that odontoblasts are involved in pulp immune and inflammatory
6 responses to bacteria and their components [15, 16]. In addition, odontoblasts produce Tumor
7 necrosis factors- α (TNF- α), IL-6, IL-8, CCL2, CXCL10 and IL-10 in response to
8 inflammatory stimuli [17, 18]. However, it is unclear whether Wnt5a is involved in regulating
9 the inflammatory response of odontoblasts.

10 The mitogen-activated protein kinase (MAPK) pathway, including its three main members p38
11 MAP kinase (p38 MAPK), c-Jun N-terminal kinase (JNK), and extracellular signal-regulated
12 protein kinase 1/2 (ERK 1/2), is an important signaling pathway related to inflammation. A number
13 of studies have shown that MAPK is involved in the inflammatory response, and inflammatory
14 stimuli such as proinflammatory chemokines and cytokines can activate the MAPK pathway [19-
15 21]. The nuclear factor-kappa B (NF- κ B) pathway is another important signaling pathway
16 involved in regulating inflammation [22]. Furthermore, P65 phosphorylation is required for the
17 optimal activation of NF- κ B-dependent gene transcription [23-25].

18 TNF- α is a well-known inflammatory cytokine that can cause dental pulp inflammation [26].
19 Both NF- κ B and MAPK are critical for inducing the expression of genes involved in inflammation
20 [13, 27] and are linked to Wnt5a-stimulated signaling [10, 28]. In previous experiments, we
21 demonstrated that TNF- α upregulates the expression of Wnt5a in hDPC through the MAPK and
22 NF- κ B pathways [9, 10]. However, whether Wnt5a regulates odontoblast inflammation and the
23 crosstalk between NF- κ B and MAPK is poorly understood.

24 In this study, we investigated the role of Wnt5a in odontoblast inflammation and the
25 potential signaling pathways by which TNF- α upregulates Wnt5a expression in odontoblasts.

26 **Materials and Methods**

27 **Cell Lines and Culture**

28 The 17IIA11 cell line was grown and maintained in Alpha Modification of Minimum
29 Essential Medium Eagle (α -MEM) supplemented with 10% fetal bovine serum (FBS) in a
30 humidified atmosphere containing 5% CO₂ at 37 °C. 17IIA11 cells were treated with
31 recombinant human TNF- α (rhTNF- α , 10 ng/ml, Invivogen, San Diego, CA) or recombinant
32 human Wnt5a (rhWnt5a, 500 ng/ml, R&D Systems) for the indicated times. To examine the
33 signaling pathways, cells were pretreated with one of the following specific pathway inhibitors:
34 BAY11-7082 (an NF- κ B inhibitor, 10 mM, Beyotime Institute of Biotechnology, Shanghai,
35 China), SB203580 (a p38 MAPK inhibitor, 20 μ M, Cell Signaling Technology, Danvers, MA),
36 SP600125 (a JNK inhibitor, 10 μ M, Merck), or U0126 (an ERK inhibitor, 10 μ M, Merck).

37 The RAW 264.7 macrophage cell line was purchased from the American Type Culture
38 Collection (ATCC). These cells were cultured in RPMI 1640 with 10% FBS, 2 mM L-
39 glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin in a humidified atmosphere
40 containing 5% CO₂ at 37 °C.

42 **Real-time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**

43 Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's

instructions. One microgram of mRNA was reverse transcribed using the RT reagent kit (Takara Biotechnology, Dalian, Liaoning, China), and real-time PCR was performed using Sso Advanced™ SYBR® Green Super Mix. The primer sequences used for real-time RT-PCR were as follows: Wnt5a, 5'-CAGTTCAAGACCGTGCAGAC-3' (forward) and 5'-GCACCCACTACTTGCACACA-3' (reverse); IL-6, 5'-ACCTTCCAAAGATGGCTGAA-3' (forward) and 5'-GCTCTGGCTTGTTCCTCACT-3' (reverse); IL-8, 5'-CACTCCATAAGGCACAACTTTC-3' (forward) and 5'-GCCAGCTTGGAAGTCATGTT-3' (reverse); GAPDH, 5'-TCAACAGCGACACCCACTC-3' (forward) and 5'-GCTGTAGCCAAATTCGTTGTC-3' (reverse); CXCL1, 5'-ACTGGTGGCTGTTTCCTGAAG-3' (forward) and 5'-CTTCTCCTAAGCGATGCTCAA-3' (reverse); and CCL2, 5'-CTGCTCATAGCAGCCACCTT-3' (forward) and 5'-CAGGTGACTGGGGCATTGAT-3' (reverse). The results were analyzed with the $\Delta\Delta CT$ method, and the relative amount or fold change in the target gene was normalized to the level of GAPDH. All real-time RT-PCR analyses were performed in triplicate.

Western Blot Analysis

Western blot analysis was performed as previously described [10]. In brief, 17IIA11 cells were treated with TNF- α (10 ng/ml) or Wnt5a (500 ng/ml) for different times. The cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, and 2% SDS) supplemented with a proteinase inhibitor mixture (Roche R&D Center China, Shanghai, China). Cell protein samples were subjected to 10% SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes by a semidry transfer apparatus. To examine signaling pathways, the following antibodies from Cell Signaling Technology were used: phospho-P65 (catalog no. 3033), P65 (catalog no. 8242), phospho-ERK (catalog no. 4370), ERK (catalog no. 4695), phospho-p38 (catalog no. 4511), p38 (catalog no. 9212), phospho-JNK (catalog no. 4668), and JNK (catalog no. 9258).

Cytokine and Chemokine Analyses

IL-6, IL-8 and CCL2 in Wnt5a-treated or TNF- α -stimulated cells were measured using RT-PCR.

Transwell Migration Assay

The migratory capacity of macrophages was examined using Transwell chambers (3- μ m pore, Corning) in triplicate. 17IIA11 cells were cultured with or without rhWnt5a treatment for 48 h before supernatants were collected. Then, 200 μ l of macrophages (2×10^6 /ml) was seeded in the upper chamber in serum-free RPMI 1640, and 600 μ l of culture supernatant from untreated or Wnt5a-treated 17IIA11 cells was added to the lower well. rhWnt5a was also added to the bottom well. After 4 h of incubation, the membranes were removed, and the underside of the polycarbonate membrane was stained with 1% crystal violet for 30 min. Then, the number of migrating cells was counted in five randomly selected fields under light microscopy.

Statistical Analysis

Each experiment was repeated at least three times, and the results are expressed as the means $\pm$ standard deviation (SD). The statistical significance of differences was determined

using ANOVA. A value of $p < 0.05$ was considered statistically significant.

Results

1. TNF- α upregulated Wnt5a expression in 17H1A11 cells through MAPK and NF- κ B activation

We treated 17H1A11 cells with TNF- α and found that TNF- α not only promoted the upregulation of Wnt5a expression but also significantly promoted the phosphorylation of ERK1/2, P38, JNK and P65 (Fig. 1 a-e).

TNF- α has been shown to activate the NF- κ B [23, 29] and MAPK signaling pathways [30]. To explore whether the NF- κ B and MAPK pathways are involved in the process of TNF- α upregulation of Wnt5a expression, we used inhibitors of the MAPK and NF- κ B pathways. Western blot results showed that inhibition of the MAPK and NF- κ B pathways significantly inhibited the expression of Wnt5a induced by TNF- α (Fig. 1 f-i).

Figure 1. TNF- α induces the upregulation of Wnt5a through the MAPK and NF- κ B pathways (these results are from different parts of the same gel). a, c: Western blot showing the protein expression of Wnt5a after odontoblasts were stimulated with TNF- α for the indicated time. b: Wnt5a mRNA expression in odontoblasts stimulated by TNF- α for the indicated time. d and e: Protein expression of total and phosphorylated ERK, P38, JNK, and P65 after odontoblasts were stimulated with TNF- α for of the indicated time. f and g: Effect of inhibiting the phosphorylation of ERK, P38, JNK, and P65 on Wnt5a expression. SP600125 (a JNK inhibitor), SB203580 (a p38 inhibitor), and U0126 (an ERK inhibitor). p-p38, phospho-p38; pJNK, phospho-JNK; pERK, phospho-ERK. GAPDH was used as a control. *, $p < 0.05$ versus the control (CO).

2. TNF- α upregulated the expression of Wnt5a through the MAPK-dependent NF- κ B pathway

TNF- α -induced Wnt5a expression was markedly decreased in the presence of specific MAPK or NF- κ B pathway inhibitors. However, the crosstalk between NF- κ B and MAPK is poorly understood. Therefore, we examined whether the phosphorylation of NF- κ B and MAPK were

1 affected by each other in the presence of specific inhibitors. At the indicated concentration, these
 2 inhibitors did not influence cell viability as assessed by the MTT assay (data not shown).
 3 Interestingly, we found that inhibiting MAPK activity significantly abrogated TNF- α -induced NF- κ B
 4 activation. However, inhibiting NF- κ B activity did not affect TNF- α -induced MAPK
 5 activation (Fig. 2).

6
 7 Figure 2. Western blot detected the protein expression of NF- κ B and MAPK pathways (these
 8 results are from different parts of the same gel). GAPDH was used as a control. PP65, phospho-65;
 9 PP38, phospho-P38; PJNK, phospho-JNK; PERK, phospho-ERK. BAY11-7082 (an NF- κ B
 10 inhibitor); SP600125 (a JNK inhibitor), SB203580 (a p38 inhibitor), or U0126 (an ERK inhibitor).
 11

12 3. Wnt5a induced CCL2 expression and enhanced RAW264.7 cell migration

13 We investigated how Wnt5a responds to inflammatory stimuli and whether Wnt5a activates
 14 the MAPK and NF- κ B pathways in odontoblasts. First, we measured the cytokines and chemokines
 15 that were induced by TNF- α and found that TNF- α upregulated the expression of IL-8, CXCL1, and
 16 CCL2 (Fig. 3 a-c). Then, we treated 17IIA11 cells with Wnt5a and found that CCL2 was obviously
 17 upregulated by Wnt5a (Fig. 3 d-e). Because Wnt5a induces CCL2 expression, we examined whether
 18 macrophages were attracted to Wnt5a. Therefore, RAW264.7 cells were subjected to a Transwell
 19 assay. We found that the supernatant of 17IIA11 cells treated with Wnt5a enhanced the migration
 20 of RAW264.7 cells (Fig. 3 f-g). Moreover, Wnt5a activated the NF- κ B and MAPK pathways in
 21 odontoblasts (Fig. 3)

Figure 3. Wnt5a induces CCL2 expression and enhances macrophage migration. In addition, Wnt5a activates the MAPK and NF- κ B signaling pathways. a-e: The mRNA expression of cytokines and chemokines. f, g: Transwell detected the migration of RAW 264.7 cells. h: On the left side is the result that Western blot detected the protein expression of NF- κ B and MAPK pathways (these results are from different parts of the same gel); on the right is the ratio of phosphorylated protein to non-phosphorylated protein of MAPK and NF- κ B pathway related proteins. **, $p < 0.01$ versus the control. GAPDH was used as a control.

4. Summary of results

In this study, we first verified whether TNF- α induces the upregulation of Wnt5a and its pathway in odontoblasts and then explored how Wnt5a responds to inflammatory stimuli in odontoblasts. We found that TNF- α -induced Wnt5a upregulation occurs through MAPK-dependent NF- κ B activation and that Wnt5a upregulates CCL2 expression. In addition, Wnt5a enhanced macrophage migration (Fig. 4).

Figure 4. a: TNF- α promotes Wnt5a expression through MAPK and NF- κ B activation in 17IIA11 cells and significantly upregulates IL-8, CXCL1, and CCL2 expression. b: Wnt5a activates the NF- κ B and MAPK pathways and upregulates the expression of CCL2. c, d: The supernatant of 17IIA11 cells treated with Wnt5a promotes the migration of RAW264.7 cells.

Discussion

Studies have confirmed that Wnt5a can regulate a variety of inflammatory responses including the response to pulpitis [9, 10, 31, 32]. In hDPC, Wnt5a can not only promote the upregulation of the expression of cytokines and chemokines induced by TNF- α but can also itself increase the levels of cytokines and chemokines in DPCs [10]. Therefore, Wnt5a itself is an inflammatory factor. Odontoblasts are the outermost cells of the dental pulp tissue and are the first line of defense for this tissue against various external stimuli. However, whether Wnt5a is involved in odontoblast inflammation is unclear. We found that TNF- α upregulated the expression of Wnt5a in odontoblasts, and Wnt5a in turn upregulated the expression of CCL2 in odontoblasts. In addition, the supernatant of 17IIA11 cells treated with Wnt5a enhanced the migration of RAW264.7 cells. Therefore, the main role of Wnt5a in odontoblasts is to chemoattract immune cells to migrate to inflammation sites by promoting the expression of chemokines.

Both NF- κ B and MAPK are important signaling pathways in inflammation [13, 27]. In hDPC, TNF- α upregulates the expression of Wnt5a through the MAPK and NF- κ B pathways [10]. However, the crosstalk between the NF- κ B and MAPK pathways and whether Wnt5a activates the MAPK and NF- κ B pathways in odontoblasts is unclear. In this study, we showed that TNF- α upregulates the expression of Wnt5a through the MAPK-dependent NF- κ B pathway. When Wnt5a is involved in the inflammatory process, it also activates the MAPK pathway (including ERK1/2, P38, JNK) and the NF- κ B pathway [10, 33]. Both the MAPK and NF- κ B pathways are also involved in the upregulation of cytokine and chemokine expression [9, 34]. In this study, we demonstrated that

1 Wnt5a not only activates the MAPK and NF- κ B pathways but also upregulates the expression of
2 CCL2 in odontoblasts. Therefore, Wnt5a may upregulate the expression of CCL2 via the MAPK
3 and NF- κ B pathways.

4 In addition, Wnt5a is involved in the development of teeth and the differentiation of
5 odontoblasts [35, 36]. Under inflammatory conditions, the expression of mineralization-related
6 genes is also upregulated through Wnt5a in hDPC [37, 38]. In this study, we did not explore the
7 effects of inflammatory stimuli on the mineralization ability of odontoblasts. We intend to
8 investigate whether Wnt5a involved in the mineralization process of odontoblasts induced by
9 inflammation in subsequent experiments.

10 In summary, we have demonstrated that Wnt5a is induced by TNF- α via MAPK-dependent NF-
11 κ B activation in odontoblasts and that Wnt5a regulates the expression of CCL2. In addition, Wnt5a
12 indirectly promotes macrophage migration.

14 Conclusion

15 Wnt5a is involved in the TNF- α -induced inflammatory response in odontoblasts. TNF- α
16 upregulates Wnt5a expression via MAPK-dependent NF- κ B activation in odontoblasts. Wnt5a
17 upregulates CCL2 expression in odontoblasts and enhances RAW264.7 macrophage migration.

19 Declarations

20 **Ethics approval and consent to participate:** Not applicable.

21 **Consent for publication:** Not applicable.

22 **Availability of data and materials:** All data generated or analysed during this study are
23 included in this published article.

24 **Competing interests:** The authors declare that they have no competing interests.

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26 **Authors' contributions:** RZ and WG completed experiments and data analysis, and wrote
27 articles. RH and ZM participated in the production of the pictures and the writing of the captions.
28 XX and XL participated in the revision of the article, including spelling and grammar. YZ
29 designed the experiment, guided the implementation of the experiment and the writing of the
30 article, and received funding support.

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32 List of abbreviations

Full spelling	Abbreviations
mitogen-activated protein kinase	MAPK
c-Jun N-terminal kinase	JNK
extracellular signal-regulated protein kinase 1/2	ERK 1/2
nuclear factor-kappa B	NF- κ B
tumor necrosis factor-alpha	TNF- α
human dental pulp cell	hDPC
fetal bovine serum	FBS
Real-time Reverse Transcriptase-Polymerase Chain Reaction	RT-PCR

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Figures

Figure 1

TNF- α induces the upregulation of Wnt5a through the MAPK and NF- κ B pathways (these results are from different parts of the same gel). a, c: Western blot showing the protein expression of Wnt5a after odontoblasts were stimulated with TNF- α for the indicated time. b: Wnt5a mRNA expression in odontoblasts stimulated by TNF- α for the indicated time. d and e: Protein expression of total and phosphorylated ERK, P38, JNK, and P65 after odontoblasts were stimulated with TNF- α for of the indicated time. f and g: Effect of inhibiting the phosphorylation of ERK, P38, JNK, and P65 on Wnt5a expression. SP600125 (a JNK inhibitor), SB203580 (a p38 inhibitor), and U0126 (an ERK inhibitor). p-p38, phospho-p38; pJNK, phospho-JNK; pERK, phospho-ERK. GAPDH was used as a control. *, p < 0.05 versus the control (CO).

Figure 2

PP65

P65

PP38

P38

P-JNK

JNK

P-ERK

ERK

GAPDH

TNF- α

BAY11-7082

SB203580

SP600125

U0126

TNF- α	-	+	+	+	+	+
BAY11-7082	-	-	+	-	-	-
SB203580	-	-	-	+	-	-
SP600125	-	-	-	-	+	-
U0126	-	-	-	-	-	+

Figure 2

Western blot detected the protein expression of NF- κ B and MAPK pathways (these results are from different parts of the same gel). GAPDH was used as a control. PP65, phospho-65;PP38, phospho-P38; PJNK, phospho-JNK; PERK, phospho-ERK. BAY11-7082 (an NF- κ B inhibitor); SP600125 (a JNK inhibitor), SB203580 (a p38 inhibitor), or U0126 (an ERK inhibitor).

Figure 3

Figure 3

Wnt5a induces CCL2 expression and enhances macrophage migration. In addition, Wnt5a activates the MAPK and NF-κB signaling pathways. a-e: The mRNA expression of cytokines and chemokines. f, g: Transwell detected the migration of RAW 264.7 cells. h: On the left side is the result that Western blot detected the protein expression of NF-κB and MAPK pathways (these results are from different parts of the same gel); on the right is the ratio of phosphorylated protein to non-phosphorylated protein of MAPK and NF-κB pathway related proteins. **, $p < 0.01$ versus the control. GAPDH was used as a control.

Figure 4

Figure 4

TNF-α promotes Wnt5a expression through MAPK and NF-κB activation in 17IIA11 cells and significantly upregulates IL-8, CXCL1, and CCL2 expression. b: Wnt5a activates the NF-κB and MAPK pathways and upregulates the expression of CCL2. c, d: The supernatant of 17IIA11 cells treated with Wnt5a promotes the migration of RAW264.7 cells.