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Interleukin-6/STAT pathway is responsible for the induction of gene expression of REG Iα, a new auto-antigen in Sjögren's syndrome patients, in salivary duct epithelial cells

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ABSTRACT

The regenerating gene, *Reg*, was originally isolated from a rat regenerating islet cDNA library, and its human homolog was named *REG Iα*. Recently, we reported that *REG Iα* mRNA as well as its product were overexpressed in ductal epithelial cells in the minor salivary glands of Sjögren's syndrome (SS) patients. This study was undertaken to elucidate the role of cytokines and the subsequent intracellular mechanism for induction of *REG Iα* in the salivary glands of SS patients. We prepared a reporter plasmid containing *REG Iα* promoter (−1190/+26) upstream of a luciferase reporter gene. The promoter plasmid was introduced by lipofection into human NS-SV-DC and rat A5 salivary ductal cells. The cells were treated with interleukin (IL)-6, IL-8, and a combination of the two. Thereafter transcriptional activity of *REG Iα* was measured by luciferase assay. We found that IL-6 stimulation, but not IL-8, significantly enhanced the *REG Iα* promoter activity in salivary ductal cells. Deletion analysis revealed that the region of −141 to −117 of the *REG Iα* gene was responsible for the promoter activation by IL-6, which contains a consensus sequence for signal transduction and activation of transcription (STAT). The introduction of siRNA for human STAT3 abolished IL-6-induced *REG Iα* transcription. These results showed that IL-6 stimulation induced *REG Iα* transcription through STAT3 activation and binding to the consensus sequence of *REG Iα* promoter in salivary ductal cells. This IL-6/STAT dependent *REG Iα* induction might play a role in the pathogenesis of SS.

1. Introduction

The regenerating gene, *Reg*, was originally isolated from a rat regenerating islet cDNA library [1,2]. The *Reg* and *Reg*-related genes were isolated and revealed to constitute a multigene family, the *Reg* family, which consists of four subtypes (types I, II, III, and IV) based on the primary structures of the encoded proteins of the genes [2,3]. In humans, five functional *REG* family genes (*REG Iα*, *REG Iβ*, *REG III*, *HIP/PAP*, and *REG IV*) have been isolated [2,3]. *Reg* family gene products act as growth factors and promote cell proliferation and regeneration; therefore, they are considered to be important in various inflammatory diseases [2,3].

It has been reported that *REG* family gene expression was regulated by several cytokines or chemokines, such as interleukin (IL)-6, IL-8, IL-11, IL-22, interferon (IFN)β, IFNγ, and cytokine-induced neutrophil chemoattractant-2β [4–10]. IL-6, a pleiotropic proinflammatory cytokine, fulfills its functions by activating Janus kinase (JAK) and subsequent signal transducer and activator of transcription (STAT) [11,12]. STAT plays a key role in transmitting cytokine signals as a transcription factor and in promoting cell proliferation and anti-apoptosis [13–16]. The involvement of STAT signaling in the *REG* gene family expression in gastrointestinal epithelial cells has also been reported [8,9,17].

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized by inflammation of the salivary and lacrimal glands; local or systemic overexpression of proinflammatory cytokines is involved in its pathogenesis [11,18–21]. It has been reported that proinflammatory cytokines such as IFNα, IFNγ, tumor necrosis factor (TNF)α, IL-12 and IL-18, along with other cytokines important in T and B cell activation and auto-antibody production, such

Abbreviations: IFN, interferon; IL, interleukin; JAK, Janus kinase; MSG, minor salivary glands; SS, Sjögren's syndrome; STAT, signal transducer and activator of transcription

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as IL-6 and B cell activating factor (BAFF), are overexpressed in exocrine glands (such as salivary and lacrimal glands). Recently, we reported that *REG Iα* mRNA and its product (REG Iα protein) were overexpressed in ductal epithelial cells in the minor salivary glands (MSGs) of SS patients [22]. Furthermore, auto-antibodies against REG Iα were found in SS patients and the anti-REG Iα auto-antibody positive patients exhibited significantly lower saliva secretion than the auto-antibody negative patients [22]. We also showed that the mRNA levels of IL-6 and IL-8 were significantly higher in the SS MSGs than those in normal MSGs [22], suggesting that those cytokines might be involved in the overexpression of *REG Iα* mRNA in the SS MSGs. However, the precise mechanism by which *REG Iα* gene activation occurs has been elusive. This study was undertaken to elucidate the role of cytokines and the subsequent intracellular mechanism of the induction of *REG Iα* mRNA in the salivary ductal cells of SS patients.

2. Materials and methods

2.1. Cell culture

To investigate why/how *REG Iα* expression is induced in the salivary duct epithelial cells of SS patients [7], we searched salivary duct epithelial cells and found two available cell lines: NS-SV-DC and A5 cells. The NS-SV-DC cells, simian virus 40-immortalized cells derived from human salivary ducts, were kindly provided by Dr. M. Azuma (Department of Oral Medicine, Tokushima University School of Dentistry, Tokushima, Japan) and maintained in Keratinocyte SFM (Life Technologies, Carlsbad, CA) [23]. The A5 cells, derived from the salivary ducts of male Fischer 344 weanling rats by treating explanted tissue clumps with 3-methylcholanthrene, were kindly provided by Dr. B.J. Baum (National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD) and maintained in DMEM supplemented with 10% FCS [24,25]. For the stimulation experiments, the cells were treated with 20 ng/mL human IL-6 (Roche, Mannheim, Germany), 200 ng/mL rat IL-6 (Wako Pure Chemical, Osaka, Japan), 100 nM IL-8 (Wako Pure Chemical, Osaka, Japan), 100 nM dexamethasone (Dx; MP Biomedicals, Santa Ana, CA) or combinations thereof.

2.2. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the NS-SV-DC and A5 cells with an RNAprotect Cell Mini Kit (Qiagen, Hilden, Germany) as described previously [22,26]. The isolated RNA was reverse transcribed to the cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) for the real-time PCR template, as described previously [26,27]. The cDNA was subjected to PCR with the following primers: β-actin (NM_001101) sense primer, 5'-GCGAGAAGATGACCCAGA-3' and anti-sense primer, 5'-CAGAGGCGTACAGGGATA-3'; *REG Iα* (NM_002909) sense primer 5'-AGGAGAGTGGCACTGATGACTT-3' and anti-sense primer 5'-TAGGAGACCAGGGACCCACTG-3'; *STAT3* (NM_213662) sense primer, 5'-CAGGATGGCCCAATGGAATC-3' and anti-sense primer 5'-CCCAGGAGATTATGAAACACC-3'. All the PCR primers were synthesized by NGRL (Sendai, Japan). Real-time PCR was performed using KAPA SYBR Fast qPCR Master Mix (Kapa Biosystems, Boston, MA) and a Thermal Cycler Dice Real Time System (Takara, Otsu, Japan), as described previously [22,26]. PCR was performed with an initial step of 3 min at 95 °C followed by 40 cycles of 3 s at 95 °C and 20 s at 60 °C for *β-actin*, and 40 cycles of 3 s at 95 °C and 20 s at 64 °C for *REG Iα*. The level of *REG Iα* mRNA level was normalized to the mRNA level of β-actin as an internal standard.

2.3. Construction of reporter plasmid and luciferase assay

Reporter plasmids were prepared by inserting fragments of human *REG Iα* promoter gene (−1190/+26, −508/+26, -508/+26, −468/+26, −402/+26, −204/+26, −141/+26, and −117/+26) into pGL3-Basic vector (Promega, Madison, WI). NS-SV-DC and A5 cells were seeded in a 24-well plate at 1×10^5 cells per well, and promoter plasmids were transfected into those cells using Lipofectamine™ 2000 (Life Technologies) [26,28]. Six hours after transfection, the medium of each well was replaced with fresh medium containing various stimulants, such as IL-6 or IL-8, and incubated for an additional 24 h. The cells were harvested and extracts were prepared in Extraction Buffer (0.1 M potassium phosphate, pH 8.8/0.2% Triton X-100; Life Technologies). To monitor transfection efficiency, pCMV–SPORT–βgal plasmid (Life Technologies) was co-transfected in all experiments at a 1:10 dilution. Luciferase activity was measured using a PicaGene luciferase assay system (Toyo-ink, Tokyo, Japan) and was normalized by the β-galactosidase activity as described previously [4,29,30].

2.4. RNA interference (RNAi)

Small interfering RNA (siRNA) directed against human STAT3 was synthesized by NGRL. The sense sequence of siRNA for human *STAT3* was 5′-GCACCUUCCUGCUAAGAUUtt-3′. The Silencer® Select human scrambled siRNA was purchased from Ambion® and used as a control. The transfection of siRNA into NS-SV-DC cells was carried out using Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies). The cells were transfected with 5 pmol each of siRNA in a 24-well culture dish as described previously [31].

2.5. Data analysis

All values were expressed as the mean ± SEM. The data were analyzed by unpaired two-tailed *t*-test using GraphPad Prism (GraphPad Software, La Jolla, CA). *P* value of < 0.05 was considered to be statistically significant.

3. Results

3.1. Induction of REG Iα mRNA by IL-6

We reported previously that the mRNA levels of *REG Iα*, *IL-6* and *IL-8* in the MSGs of SS patients increased significantly [22]. To investigate whether IL-6 or IL-8 up-regulate REG Iα, we by real-time RT-PCR to analyze the *REG Iα* mRNA expression in human NS-SV-DC salivary ductal cells. Treatment with IL-6 but not IL-8 or Dx,

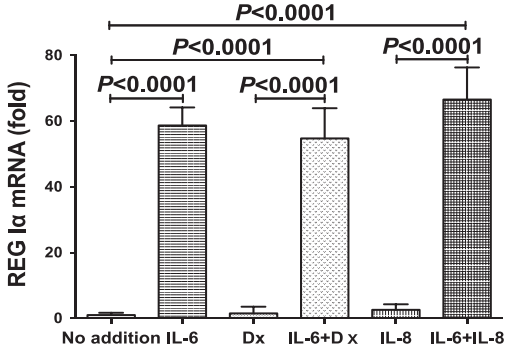

Fig. 1. The mRNA levels of *REG Iα* gene in NS-SV-DC human salivary ductal cells treated with IL-6 (20 ng/mL), Dx (100 nM) or IL-8 (100 nM). The levels of *REG Iα* mRNA were measured by real-time RT-PCR using β-actin as an endogenous control. Data are expressed as mean ± SEM of the samples ($n=4$). The statistical analyses were performed using Student's *t* test.

induced *REG Iα* mRNA expression (Fig. 1). The combination of IL-6+Dx or IL-6+IL-8 had no additional effect compared to IL-6 alone. These results indicate that salivary ductal cells express *REG Iα* mRNA in response to IL-6 stimulation.

3.2. Activation of REG Iα gene promoter by IL-6

To determine whether the increase in *REG Iα* mRNA was caused by the activation of transcription, a 1216-bp fragment containing 1190-bp of the promoter region of the human *REG Iα* gene was fused to the luciferase gene and transfected into human NS-SV-DC and A5 rat salivary ductal cells. We found that IL-6 stimulation significantly enhanced *REG Iα* promoter activity in both the NS-SV-DC and A5 cells. However, treatment with IL-8 did not change the transcriptional activity of *REG Iα* (Fig. 2). These results suggest that *REG Iα* mRNA was induced by IL-6 in salivary ductal cells at the transcriptional level.

3.3. Localization of IL-6-responsible region in the REG Iα gene promoter

In order to identify the region essential for the transcription of the *REG Iα* mRNA by IL-6, progressive deletions of the *REG Iα* promoter were performed. Down to position −141, the deletions did not attenuate IL-6-induced *REG Iα* promoter activity; however, an additional deletion to −117 caused a remarkable decrease in IL-6-induced promoter activity of *REG Iα* (Fig. 3). These results indicated that the −141 to −117 promoter region of the *REG Iα* gene is responsible for *REG Iα* promoter activation by IL-6.

3.4. STAT3 is a key factor for REG1a gene transcription

A computer-aided search for sequences similar to known *cis*-acting elements revealed that the −141 to −117 region of the *REG Iα* gene contains consensus binding sequences for STAT. In order to verify the role of STAT3 in IL-6-induced *REG Iα* induction, siRNA for human *STAT3* mRNA was introduced into NS-SV-DC cells, and IL-6-induced *REG Iα* mRNA expression was analyzed by real-time RT-PCR. As shown in Fig. 4, the introduction of siRNA for human *STAT3* abolished both IL-6-induced *STAT3* up-regulation and IL-6-induced *REG Iα* up-regulation.

4. Discussion

In our previous study, we reported that *REG Iα* protein was overexpressed in the ductal epithelial cells of MSGs of SS patients, and that the saliva secretion was attenuated with auto-antibodies to REG Iα [22]. We also showed that mRNA levels of *IL-6* and *IL-8* were significantly higher in the SS MSGs than in normal MSGs [22,32]. In the present study we showed that *REG Iα* overexpression in salivary ductal cells was induced by IL-6 but not by IL-8 at the transcriptional level.

IL-6, a potent proinflammatory cytokine, is involved in acute phase response, B cell proliferation and plasma cell formation, and T cell stimulation and recruitment [11]. High serum concentrations of IL-6 were found in primary SS (pSS) patients, and the levels of IL-6 concentration correlated with the degree of lymphocyte

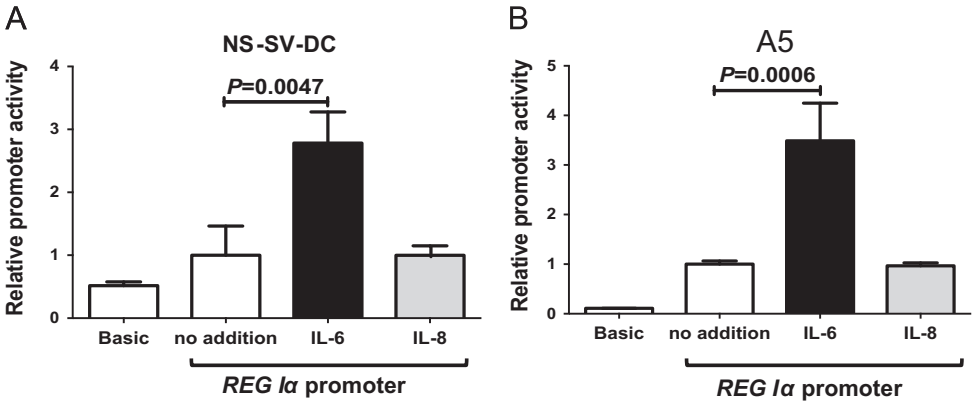

Fig. 2. Luciferase assays in salivary ductal cells. Human NS-SV-DC cells (A) and rat A5 cells (B) were transfected with constructs containing *REG Iα* promoter. After transfection, the cells were stimulated with IL-6 (20 ng/mL human IL-6 in NS-SV-DC cells or 200 ng/mL rat IL-6 in A5 cells) or IL-8 (100 nM human IL-8 in NS-SV-DC cells or 100 nM rat IL-8 in A5 cells); and then the luciferase activities were measured. The diagram represents relative luciferase activities to the untreated group. "Basic" represents a promoterless construct, pGL3-Basic. All data are represented as the mean ± SEM of the samples ($n=3-4$). The statistical analyses were performed using Student's *t* test.

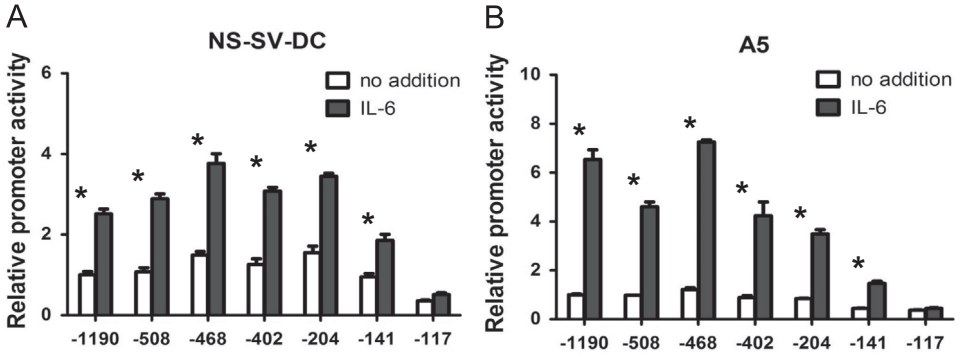

Fig. 3. Deletion analysis of *REG Iα* promoter. Human NS-SV-DC cells (A) and rat A5 cells (B) were transfected with constructs containing various deletion mutants of *REG Iα* promoter. Constructs listed on ordinate are numbered according to their 5' terminus in the *REG Iα* promoter. The transfected cells were stimulated with IL-6 (20 ng/mL human IL-6 in NS-SV-DC cells or 200 ng/mL rat IL-6 in A5 cells), after which the luciferase activities were measured. The diagram represents relative luciferase activities to the untreated group of "−1190". All data are represented as the mean ± SEM of the samples ($n=3-4$). The statistical analyses were performed using Student's *t* test against no addition. $*P<0.01$ vs no addition.

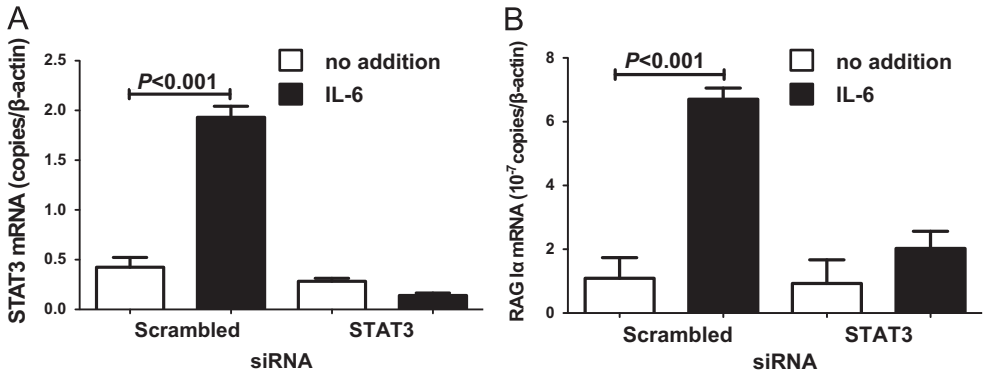

Fig. 4. Effects of *STAT3*-siRNA transfection on IL-6-induced *REG Iα* promoter activity in NS-SV-DC cells. After siRNA was introduced, NS-SV-DC human salivary ductal cells were stimulated with IL-6 (20 ng/mL). The expression of (A) *STAT3* and (B) *REG Iα* mRNA was measured by real-time RT-PCR using β-actin as an endogenous control. Data are expressed as means ± SEM for each group ($n=4$). The statistical analyses were performed using Student's *t* test.

infiltration in the salivary gland [19,33,34]. Binding of IL-6 to the receptor leads to homodimerization of IL-6 receptor component gp130, which results in the activation of JAK and the subsequent phosphorylation of STAT3 [12]. STAT3 plays a central role in transmitting cytokine signals to the nucleus and promoting cell proliferation and anti-apoptosis [13–16]. Thus, the JAK/STAT pathway has been shown to be involved in carcinogenesis under a background of inflammation. Furthermore, accumulating evidence indicates that the JAK/STAT pathway might be involved in multiple immune functions: STAT1 and STAT4 mainly induce IFNγ expression in Th1 cells, STAT6 induces IL-4 expression in Th2 cells, and STAT3 induces IL-17 expression in Th17 cells [35].

Our results revealed that IL-6 stimulation enhanced *REG Iα* gene expression through STAT3 activation in salivary ductal cells. The involvement of STAT signaling in *REG* family gene expression has been reported by other groups in different cell systems. Lee et al. reported that the IL-11/STAT3 signaling pathway was important in *Helicobacter pylori* CagA-directed *REG 3γ* (*HIP*/*PAP*) expression in gastric epithelial cells [8]. Sekikawa et al. showed that *REG Iα* gene expression was regulated by the IL-22/STAT3 pathway in colon cancer cells and by the IL-6/STAT3 pathway in gastric cancer cells [9,17]. Most recently, we showed that *REG Iα* gene expression was activated in human pancreatic β-cells by the combined stimulation of IL-6+Dx via JAK/STAT3 signaling [36]. These studies were implemented in gastrointestinal cell lines and pancreatic β-cells, and to the best of our knowledge, this is the first study to report an association between the IL-6/STAT pathway and *REG Iα* expression in salivary ductal epithelial cells.

IL-8, also known as CXCL8, is a proinflammatory chemokine associated with the promotion of neutrophil chemotaxis and degranulation [37]. Several reports have indicated the involvement of IL-8 in the pathogenesis of SS. Cuello et al. reported that the MSG ductal epithelial cells of SS patients highly expressed IL-8 [38]. As mentioned above, IL-8 levels were also reported to be correlated with *REG* gene expression [7]. In our previous study, we showed that *IL-8* mRNA was highly expressed in SS MSGs [22]. In the present study, however, we found that IL-8 did not induce *REG Iα* expression in salivary ductal cells, suggesting that high levels of IL-8 in SS MSGs are not involved in *REG Iα* up-regulation in the MSGs.

The *Reg I* gene was originally found to be expressed in rat regenerating islets, but not in normal pancreatic islets [1]. RINm5F, a rat insulinoma-derived cell line, displayed a significant increase in cell numbers in the presence of Reg I/REG Iα protein [2,4,26,39]. Human REG Iα protein administration ameliorated diabetes in NOD mice, with an increase in the β-cell mass [40]. In addition, anti-REG Iα auto-antibodies, found in the sera of diabetic patients, retarded the proliferation of pancreatic β-cells in vitro [39]. These results suggest that Reg I/REG Iα protein stimulates the proliferation of pancreatic β-cells. We previously reported that pSS patients with anti-REG antibodies exhibited significantly lower salivary secretion [22], suggesting that IL-6-induced *REG Iα* protein in salivary ductal cells is associated with the regeneration of damaged MSG ductal epithelial cells and that anti-REG auto-antibodies attenuate the proliferation/regeneration.

In the present study, we showed that the *REG Iα* gene was activated by IL-6/STAT3 signaling in salivary duct epithelial cells. The STAT binding element was reported not only in the *REG Iα* promoter but also in other *REG* family promoters, such as *REG Iβ*, *HIP*/*PAP*, and *REG III* promoters [36,41], suggesting possible activation of the genes in the salivary ducts of SS patients through the IL-6/STAT3 axis. In our previous study, however, no *REG Iβ* mRNA was detected either in either the control or the SS salivary glands. The mRNA levels of *REG III* and *HIP*/*PAP* were not different between the control and SS salivary glands. In contrast, *REG Iα* mRNA levels were significantly higher in the salivary glands of SS patients than in those of controls [22]. In SS, the involvement of several cytokines other than IL-6, such as BAFF, IL-12/IL-23, and IFNα, has also been reported [11]. Combination of these cytokines with IL-6 might contribute to *REG Iα*-specific overexpression in the salivary duct epithelial cells of SS patients. As a result, anti-REG Iα auto-antibody levels are elevated in SS patients [22] and salivary functions might be affected in SS patients.

Xerostomia, which is the chief manifestation of SS, is caused by salivary gland dysfunction resulting from immune-mediated inflammation. To alleviate xerostomia, treatment with conventional systemic immunosuppressive drugs has been tried as with other immune-mediated connective tissue diseases; however, the therapeutic effects were doubtful [42–45]. Recently, treatments using with monoclonal antibodies that target inflammatory cytokines or cell surface antigens were developed to treat several autoimmune diseases [46]; a monoclonal antibody against the IL-6 receptor exhibited its efficacy in rheumatoid arthritis [47]. As IL-6 has been shown to be highly expressed in the salivary glands of SS patients, blocking IL-6 and/or its receptor might have beneficial effects [19]. Our results, however, suggested that IL-6 was associated with regeneration of ductal epithelial cells via REG Iα protein expression. Therefore, blocking IL-6 and/or its receptor might induce unwanted effects. Several trials have shown that rituximab, a chimeric anti-CD20 monoclonal antibody that binds to the B-cell surface antigen CD20, has beneficial effects in treating xerostomia in SS patients [48,49]. Rituximab therapy has also been shown to be effective in rheumatoid arthritis, reducing disease activity and decreasing auto-antibody production [50–52]. It is possible that the beneficial effects of rituximab in the SS patients were associated with B-cell depletion and the subsequent

decrease in the production of pathogenic auto-antibodies, including anti-REG Iα auto-antibodies.

In conclusion, the present study showed that *REG Iα* transcription in salivary ductal cells was stimulated by IL-6. Our study also demonstrated that STAT3 bound the consensus sequence of *REG Iα* promoter and regulated transcription in ductal epithelial cells in response to IL-6 stimulation. It is suggested that overexpression of REG Iα protein in salivary ductal cells is dependent on the IL-6/STAT pathway and might play a role in the pathogenesis of SS.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.05.006.

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