**Vancomycin pre-treatment impairs tissue healing in experimental colitis:
importance of innate lymphoid cells**

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Abstract

Background and aims: The interplay between luminal microbes and innate immunity during colonic epithelial repair has been well noted. At the same time, antibiotic has widely been used during flare-ups of ulcerative colitis. The possible effects of luminal microbiota disruption caused by antibiotics usage on epithelial repairing have been scarcely discussed. Innate lymphoid cells (ILCs) embedded in the lamina propria can be modulated by gut microbes, resulting in altered colonic IL-22/pSTAT3 levels, which is considered a prominent molecular axis in tissue repairing after epithelium damage. This study aimed to investigate whether antibiotics could interfere with ILCs-dependent tissue repair.

Methods: Dextran sodium sulfate (DSS)-induced colitis was established in mice pre-treated with reagent of different antibiotic spectrum. Both morphological and molecular markers of tissue repair after DSS cessation were detected. ILCs population and function status were also recorded. Further attention was paid to the response of dendritic cells after antibiotics treatment, which were claimed to regulate colonic ILC3s in an IL-23 dependent way.

Results: Using of vancomycin resulted in delayed tissue repairing after experimental colitis. Both colonic IL-22/pSTAT3 axis and ILC3s population were abated in this situation. Vancomycin treatment diminished the upstream IL-23 and producer dendritic cell population. The reduced dendritic cell number may due to inadequate chemokines and colony-stimulating factors supply.

Conclusion: Presence of vancomycin-sensitive microbiota is required for the

maturation of ILC3-activating dendritic cells hence maintain the sufficient IL-22/pSTAT3 level in the colon during tissue healing. Manipulation of colonic microbiota may help achieve colonic mucosal healing post inflammation and injury.

Key word

inflammatory bowel disease; commensal; innate lymphoid cells; mucosal healing.

1. Introduction

Inflammatory bowel diseases, including Crohn's Disease (CD) and Ulcerative Colitis (UC), identified to be resulted from a dysregulated response of the intestinal immune system to bacteria related antigens present in the lumen. The recurring of inflammation, tissue injury and repairing is a notorious feature of UC. Interaction of body immune system and microbiotas can dictate this natural history of UC [1, 2].

One molecular axis involved in tissue repairing is IL-22/STAT3. After breach of epithelial barrier, colonic IL-22 level increased instantly and STAT3 activated in a tyrosine-kinase-2-dependent manner [3]. Phosphorylated STAT3 translocates to the nucleus and regulates genes involved in apoptosis, proliferation, migration, and survival in a cell-specific manner. Specific deletion of STAT3 in intestinal epithelial cells (IEC) disrupts IL-22-induced mucosal healing and aggravates the course of experimental colitis [4, 5]. However, the modulation of IL-22/STAT3 axis during tissue healing in IBD remains unclear.

Tissue-resident immune cells are the major producers of IL-22. This recent characterized emerging family of innate immune cells termed innate lymphoid cells (ILC), play an essential role in the initiation, regulation and resolution of inflammation. They are relatively rare in comparison to adaptive lymphocytes in lymphoid tissue, but widely found in barrier surface of the mammalian body, such as the skin, lung and intestine, as well as adipose and some mucosal-associated lymphoid tissue [6, 7]. One group of ILCs that featured by expression of transcription factor ROR γ t are noted for their potency of IL-22 production and are crucial for maintaining

gastrointestinal epithelial integrity and barrier function in several experimental models of intestinal injury [8]. Recent studies have proposed possible interactions between ILC3s and intestinal microbiota. On one hand, cytokines produced by ILC3s help maintain the homeostasis of microbes, on the other hand, microbiota can influence the proliferation and activation of ILCs both in cell-surface-receptor-dependent direct ways and phagocytes-involving indirect ways [9-13]. However, the mechanisms of microbes inducing and regulating epithelial regeneration through ILC3s after tissue damage remain poorly understood.

In previous researches, IL-22 expression in immune cells increased significantly in patients with UC during the recovery phase. This augmentation is associated with activation of epithelial STAT3 and downstream anti-apoptosis genes, which indicates the essentiality of IL-22 in epithelial recovery after inflammation [14]. However, the mechanism by which ILC3s modulated the expression of IL-22 during tissue repair is still unclear. Here, by using dextran sodium sulfate (DSS)-induced colitis in mice pre-treated with different antibiotics, we demonstrated that vancomycin-sensitive bacteria played a crucial role in the process of tissue healing after experimental colitis. We further showed that the presence of those bacteria was required for the migration of ILC3-activating monocytes/macrophages into the colon. Together, those results reveal a novel role of antibiotics usage in epithelium healing by affecting IL-22/STAT3-dependent responses.

2. Materials and methods

2.1 Experimental animals

4-week-old C57BL/6 male mice were obtained from the Shanghai SLAC Laboratory Animal CO. LTD (Shanghai Laboratory Animal Center, Chinese Academy Sciences). They were housed in environmentally-controlled conditions (temperature 22°C, with light cycle from 06:00 am to 6:00 pm). Ampicillin 1 g/l (A), vancomycin 500 mg/ml (V), neomycin 1 g/l (N), metronidazole 1 g/L (M) or combined antibiotics (AVNM) was added to drinking water and let the mice drink ad lib for 2 to 3 weeks. After that, DSS colitis model was established as described before [15]. MDP (Sigma–Aldrich) was given intraperitoneally at a dose of 15 µg/mice. All animal experimentations were approved by the Institutional Animal Use and Care Committee of Shanghai University of Traditional Chinese Medicine.

2.2 Isolation of RNA and quantitative RT–PCR

Total cellular RNA from cells or tissues was extracted using TRIzol (Life Technologies). We reverse transcribed the total amount of RNA and quantify the cDNA content following the manufacturer’s instructions (RR037, RR820, both from Takara). RNA samples were treated with RNase-free DNase I (Invitrogen) before reverse transcription to eliminate contaminating genomic DNA. Primer used was listed in table 1. Expressions were normalized to the levels of β-actin in each sample.

2.3 Colonic lamina propria cell isolation and culture

Bulk colonic lamina propria cells were obtained after collagenase treatment as described before [25]. Products were subjected to density-gradient centrifugation 66.7% Percoll (GE Healthcare). The cells at the interface were collected and washed in PBS

for two times. Cells were plated into 24-well plates at the concentration of 7.5×10^5 cells/ml and incubated in the presence of plate-bound anti-CD3 ϵ (10 μ g/ml, BD) for 48 h before detection. The supernatants were collected and cytokine levels were measured by ELISA assay.

2.4 Cytokine quantification by ELISA assay

As for examination of explants, the colons of mice were flushed with RPMI and open along a longitudinal axis. Thereafter, 3 mm² punch biopsies were obtained from the distal colon and incubated for 24 h in RPMI supplemented with 10% fetal bovine serum and antibiotics. Supernatants from explants or cell culture were collected and kept frozen until assessed. Paired antibodies and recombinant standards were used to quantify factors present in supernatants of colon tissue or cell cultures as the kit manufacturer instructed.

2.5 Immunohistochemistry

Dissected samples were fixed in 10% paraformaldehyde overnight and cut into 5 to 10 mm thick slides. After microwave repair and intensive wash with PBS, samples were blocked with normal goat serum (Maxim, Fuzhou, China) to reduce unspecific binding. Slides were then incubated at 4 °C with primary antibodies. On the second day, slides were washed extensively with PBS supplemented with 1% tween-20, and incubated for 1 h with HRP conjugated secondary antibodies (Maxim, Fuzhou, China) diluted 1:200 in PBS. After cover slides mounting, samples were analyzed using an Olympus BX51 microscope.

2.6 Flow cytometry detection

As for cytokine detection, on the last day of culture, cells were stimulated for 4-6 h with PMA (10 ng/ml) and ionomycin (1 mg/ml; both from peprotech) to enhance cytokine expression unless otherwise instructed by the antibody producer. Thoroughly suspended cells were fixed and permeabilized (554714 BD) and stained for target protein. Flow cytometry analyses were performed on an LSR II flow cytometer (BD Pharmingen).

2.7 Western blot

Tissue was lysed in RIPA buffer (Beyotime) supplemented with protease and phosphatase inhibitors (Beyotime). Equal amounts of protein was loaded on SDS PAGE and blotted on nitrocellulose (Millipore). Membranes were blocked and probed with Abs for pSTAT3 (1:2000, Cell signaling Technology) and β -actin (1:2000, Santa Cruz). Finally, proteins were visualized using ECL system (Pierce).

2.8 Statistical analysis

Data were presented as mean values $\pm$ SEM, unless otherwise indicated. Statistical significance between 2 sets of data was assessed with the two-tailed unpaired Student t test.

3. Results

3.1 Vancomycin prolonged epithelium repairing in DSS-induced colitis

To study the effect of oral administering of antibiotics on intestinal wound healing process, five antibiotic regimens were used: Ampicillin (A) that targets both Gram+ and Gram- bacteria, Vancomycin (V) that mainly targets Gram+ bacteria, Neomycin (N) that targets Gram- bacteria, Metronidazole (M) that targets anaerobic bacteria and broad-spectrum combination (AVNM). Body weight recovery, vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) levels were examined consecutively during the 7-14 days post DSS treatment. Histological analysis was performed on day 12. Compared with other groups, vancomycin resulted losing less body weight and colon length in mice during the acute colitis phase; however, there was notably delayed body weight recovery (fig. 1A, B). In consistent with this observation, pathological changes, such as epithelial cell mitosis and crypts rebuilding were reduced in vancomycin treated mice compared with otherwise treated mice or SPF mice that did not receive any antibiotics before DSS (fig. 1C). Colonic growth cytokine EGF and VEGF also were found to express in a relatively low level (fig. 1D, E). IL-22/STAT3 signaling was inhibited in vancomycin treated mice as evidenced by decreased IL-22 and phosphorylation of STAT3 in recovery stage (fig. 1F to H).

We noted that of 10 mice treated with ampicillin, 7 died during 8 to 12 days post DSS treatment (fig. 1I). Autopsy revealed notably enlarged spleen in those moribund mice, suggesting systemic sepsis because of expansion of drug-resistant pathogenic *Escherichia coli*. Combined broad-spectrum antibiotics (AVNM) treated mice

displayed the least severe symptoms hence a more swift recovery, which coincides with previous reports, which demonstrated that luminal microbiota contents are indispensable in the development of DSS, induced colitis [15]. Collectively, these findings suggest that vancomycin-sensitive bacteria is required during the recovery from DSS-induced experimental colitis.

3.2 Treatment of vancomycin leads to decreased tissue IL-22 levels accompanied by reduced ROR γ ^t ILC3s

To validate the role of ILCs in the regulation of IL-22/STAT3 axis, first we determined the proportion of ROR γ ^tILC3 among intestinal IL-22 expressing immune cells. Mice on the fifth day after finishing DSS treatment were examined. In SPF group, a considerable amount of IL-22⁺ cells expressed ROR γ ^t and ILC marker CD127 were observed but not lineage marker (fig. 2A), indicating they were ROR γ ^tILC3s. Those results were compatible with the finding from another study that ROR γ ^tILCs are the main producers of IL-22 in the murine intestine under homeostatic states [8].

To investigate whether discrepancies of tissue repairing among groups pretreated with different antibiotics were due to ILC's altered ability to secrete IL-22, we extracted mononuclear cells from colonic lamina propria and analyzed markers of interest using flow cytometry. Cell surface markers revealed two subsets of ILCs in this fraction: Tbet⁺ ILC1s and ROR γ ^t ILC3s (fig. 2B). The absolute number and proportion of ILC3s as well as the expression level of IL-22 were measured among

antibiotics groups and SPF mice 5 days after cease of DSS treatment. The absolute number of ILC3s significantly decreased in vancomycin group (fig 2C). The IL-22 expression by ILC3 ex vivo was also impaired in vancomycin treated mice compared with that in SPF mice and neomycin treated mice (fig. 2D, E). These results were in consistent with the IL-22 levels in colon tissue. In contrast, expression of IL-17, a representative cytokine that also expressed by ILC3 under the regulation of transcription factor ROR γ t was less affected (fig. 2F, G). We also noted an increase of ILC1s subsets and IFN γ production by ILC1s after vancomycin treatment (fig. s1). Above findings suggested that the decreased IL-22 production is a more specific response of ROR γ t⁺ ILC3 to G⁺ microbe elimination rather than muffled epithelial immunity caused by decreased luminal microbiome richness.

Commensals have been reported to facilitate tissue repairing via type II inflammatory response in a mice model of hepatic infection. Those processes were epitomized by IL-4 expressing T-cell in a macrophages dependent manner [16]. However, 7 days of vancomycin treatment did not affect the frequencies of IL-4 expressing Th2 cells in the colon (fig. s2), suggesting immune system regulates repairing process in a tissue specific manner.

3.3 Decreased colonic IL-22 level was related to undersupply of IL-23 expressing dendritic cell

IL-23 is the most potent inducer of colonic ILC3 production of IL-22 along with other less effective pro-inflammatory cytokines such as IL-1 β [17]. To determine

whether microorganisms can affect cytokines, we measured their mRNA expressions in the lamina propria of colon 5 days after the termination of DSS treatment. The fold change of expression of IL-23 heterodimer IL-12p40 and IL-23p19 during the recovery phase were much higher in SPF and neomycin treated mice comparing to vancomycin treated mice (fig. 3A, B). The differences between IL-1 β level was less noticeable (fig. [s3](#)).

The recruitment of DC is an integral part of colonic inflammation. The MHCII⁺ CD11c⁺ lamina propria DC population is composed of two major groups, CD103⁺ DC that derived from classic DC precursors and CX₃CR1⁺DC derived from monocyte precursors [18]. The latter is considered as the major source of IL-23 based on a *C.rodentium*-infection model [17]. To make certain if decreased colonic IL-23 expression is related to the shrink of CX₃CR1⁺ DC population, we analyzed the lamina propria DC composition after DSS termination (fig [3C](#)). We found that the absolute number of CX₃CR1⁺ DC in vancomycin group was significantly less than neomycin treated and SPF mice. In comparison, there was no obvious difference of CD103⁺ DC population among those groups. These results were in consistent with previous reports found that the recruitment and expansion of CD103⁺ DC was dependent on flagellin-TLR5 pathway [19] and application of vancomycin ought to have no evident effects on this group of DC (fig. [3D, E](#)).

We also examined the aryl hydrocarbon receptor (AhR) expression on the cell surface of ILC3s, and no significant difference were observed among groups, which suggested the depletion of G⁺ commensals by vancomycin was less likely acted

through cell surface markers to orchestrate ILC3s proliferation and activation (fig. [s4](#)).

3.4 Depletion of vancomycin-sensitive microbiota hindered the recruitment of CX₃CR1⁺DC

The circulation precursors of CX₃CR1⁺DC were identified as the CCR2⁺ monocytes. They migrate to the lamina propria of colon driven by CCR2-CCL2/CCL7 chemokine pair after breach of epithelial barrier [20]. The CCR2 receptors on the surface of monocytes migrated to the colon remained intact after antibiotics treatment, while qPCR analysis revealed that the mucosal CCL2 and CCL7 were lower in vancomycin group (fig. 4A, B). The differentiation of CCR2⁺ monocytes into CX₃CR1⁺DC is instructed by mucosal colony stimulating factor 2 (CSF2) and we found it decreased in vancomycin treated mice (fig 4C). The impaired migration and differentiation of CCR2⁺ monocytes could explain the diminished CX₃CR1⁺DC discovered during recovery phase. Some study brought forward the facts that activated ILC3s are strong producers of CSF2 [20], which suggests a defect feedback loop of CX₃CR1⁺DC-ILC interaction resulted from the absence of G⁺ bacteria. The expression of mucosal Flt3L which is the inducer for the proliferation and maturation of CD103⁺ DC was less affected (fig. [s5](#)). These results suggest vancomycin-sensitive commensal may contribute to the homeostasis of IL-22 producing ILC3 in a CX₃CR1⁺DC dependent way.

As vancomycin mainly targets gram+ bacteria, these observations encouraged us

to investigate whether substitute of gram⁺ bacteria derivatives could rescue the recovery defect of vancomycin treated mice. Muramyl dipeptide (MDP), an immune-reactive derivative of peptidoglycan from all Gram⁻ and Gram⁺ bacteria was chosen as a substitution. MDP was given intraperitoneally after completing DSS treatment to mice in the vancomycin group. Judging from body weight recovery and histology, MDP failed to promote tissue healing after DSS challenge, though it did not affect the survival (fig. [4D](#), [E](#) and [F](#)). No visible alterations of colonic IL-22 and pSTAT3 level were observed (fig [4G](#), [H](#)). Nonetheless, mice received MDP expressed significantly higher mucosal CCL2 and CCL7 (fig [4I](#)), the absolute number of CX₃CR1⁺DC also increased by day 12 (fig [4J](#)), though the RORγt⁺ILC3s subsets did not expand as expected (fig [4K](#)). Those results indicated that stimulation of NOD2 by MDP alone during the acute inflammation was enough to recruit CX₃CR1⁺DC, however, there were other driven force (s) missing for the complete recovery of the population of IL-22 expressing RORγt⁺ILC3s.

4. Discussion

An extraordinarily large number of commensal microbes colonize the gastrointestinal tract especially the large intestine. Those luminal contents are critical for damage-induced intestinal regeneration, partly through their ability to modulate inflammatory responses of innate immune cells [21-23]. Previous studies have established the role of microbial commensals that colonize the large bowel to interfere with the proliferation and activation of ILCs. However, the cellular cues that promote

ILCs derived IL-22 accumulation in response to gut commensals have only recently started to be unraveled.

Our data identified a mechanism by which the vancomycin-sensitive microbiota promote intestinal tissue healing by supporting an IL-23 dependent IL-22/pSTAT3 axis in the intestinal mucosa. Elimination of Gram⁺ bacteria by vancomycin hindered the mucosal repair after DSS treatment accompanied by reduced mucosal IL-22 and pSTAT3 level. We also discovered that ROR γ ⁺ILC3s are responsible for most IL-22 production during recovery phase. Decreased expression of IL-22 may be due to the impaired production of IL-23 by CX₃CR1⁺DC in the colon. Lack of Gram⁺ stimulus led to the reduced expression of CCR2 ligand CCL2 and CCL7 of the IEC, which drew the CX₃CR1⁺DC precursor CCR2⁺ monocytes to the colon in the first place. Replacement of MDP rescues the ability to attract CCR2⁺ monocytes after interruption of epithelium, however, neither the number of ROR γ ⁺ILC3s nor the IL-22 level was rescued in this setting. These observations suggest there ought to be factors other than bioactive peptidoglycan stimulus of NOD receptor that regulate the proper responses of ILC3s during tissue healing.

Our findings are in agreement with previous reports suggesting that IL-22 is a central regulator of tissue healing in inflammatory conditions, and several studies have previously suggested a protective role for IL-22 in the regulation of gut inflammation. In experimental infection models, knock out IL-22 or its downstream STAT3 in mice led to greatly diminished expression of Reg proteins accompanied by increased intestinal epithelial damage and systemic bacterial burden [4].

Accordingly, IL-22 gene delivery has been shown to ameliorate DSS-induced colitis [24]. IL-22 induced STAT3 activation in the injured gut may not only promote IEC proliferation and protection against apoptosis but also regulate bacterial growth in the lamina propria adjacent to the wound. In conclusion, IL-22/STAT3 signaling emerges as an important protective pathway for the mucosal immune system.

Our data represent an important advance in the understanding of how commensal microbes can regulate innate intestinal immunity. However, more work needs to be done as to elucidate the specific species that present in the Gram⁺ group or their metabolites that could promote tissue healing. Moreover, it implies the manipulation of colonic microbiota, combined with anti-TNF α antibody, a proven therapeutic for IBD patients, may meet the ever increasing demand of mucosal healing post colitis and injury.

Competing interests

The authors have no competing interests.

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Figure Legends

Fig 1. Pretreatment of antibiotics can affect tissue healing post inflammation. (A) Body weight changes compared to the base line after DSS challenge. (B) Length of colon in each group (n=four/group) on day 7. (C) Hematoxylin and eosin staining of the distal colon from experimental mouse model. Original magnification: $\times 40$. Colonic growth cytokine EGF (D) and VEGF (E) levels. The mRNA levels of cytokine EGF and VEGF of colon homogenates 12 days after DSS challenge were assayed by q-PCR. Data were normalized to GAPDH, the fold induction was compared with as that on day 0. (F) IL-22 levels of distal colon on day 14. (G) IL-22 levels in colon explant culture supernatants extracted on day 12. (H) pSTAT3 levels in colon homogenate on day 12. (I) Survival curves for six groups after DSS challenge.

Fig 2. Role of IL-22 expressing ROR γ ⁺ ILC3 cells in colon tissue healing. (A) Flow cytometry analysis of the origin of IL-22 in the colon lamina propria. (B) Flow cytometry analysis of colon lamina propria cells on day: ROR γ ⁺ ILC3s (lower left) and Tbet⁺ ILC1s (lower right) within a CD45.2⁺ LIN⁻ CD127⁺ gate. (C) Absolute number of ROR γ ⁺ ILC3s within colon lamina propria on day 12. Representative analysis of IL-22 expression of ROR γ ⁺ ILC3 after IL-23 stimulation (D) and the percents of IL-22 expressing cells (E) were presented. Representative analysis of IL-17 expression of ROR γ ⁺ ILC3s after phorbol 12-myristate 13 acetate stimulation

(F) and the percent of IL-17 expressing cells (G) were presented. The data were expressed as mean $\pm$ s.e.m. (n=4 mice per group). *** $P < 0.005$. Data were compared to SPF group until otherwise indicated.

Fig 3. Recruitment of IL-23 expressing dendritic cells was modulated by antibiotics usage. (A-B) The mRNA levels of heterodimer IL-23 of colon homogenates on day 12 were assayed by q-PCR. Data were normalized to GAPDH, the fold induction was compared with as that on day 0. (C) Flow cytometry analysis of dendritic cells with in a MHCII⁺ CD11c⁺ gate from colon lamina propria on day 12. Absolute number of CX₃CR1⁺DC (D) or CD103⁺DC (E) in each group within colon lamina propria on day 12. *** $P < 0.005$, compared to SPF group until otherwise indicated.

Fig 4. Antibiotics treatment regulates chemokines related to DC recruitment and maturation. (A) Flow cytometry of CD11b⁺ CD11c^{int-neg} cells isolated from the colon lamina propria on day 12. (B-C) The mRNA levels of indicated cytokines of colon homogenates on day 12 were assayed by q-PCR. Data were normalized to GAPDH, and the fold induction was compared with as that on day 0. (D) Body weight changes compared to base line after DSS. (E) Survival curves for the three groups of the mice after DSS challenge. (F) Hematoxylin and eosin staining of the distal colon from experimental mouse model. Original magnification: $\times 40$. (G) IL-22 levels of distal colon on day 14. (H) pSTAT3 levels in colon homogenate from each experimental mouse models on day 12. (I) The mRNA levels of indicated cytokines of colon homogenates on day 12 were detected by q-PCR. Data were normalized to GAPDH, and the fold induction was compared with as that on day 0. *** $P < 0.005$, compared to

SPF group until otherwise indicated.

Table 1. Primer pairs used in this study.

GENE	Forward (5'-3')	Backward (5'-3')
VEGF	ACAGCAGATGTGAATGCAGACCAA	ATGGCGTGGTGGTGACATGGTT
EGF	AGCCTTCACAGAGCACACCTCA	AAACTGTGCCGTGCTTGATGCC
IL-22	AGCCATACATCGTCAACCGCAC	TGAGTTTGGTCAGGAAAGGCACC
IL-12P40	ACGTGCTCATGGCTGGTGCAAA	TGGGCGGGTCTGGTTTGATGATGT
IL-23P19	AGCAGCAACTCTGACTGAGCCCTT	GTGTCCTTGCCCTTCACGCAAA
IL-6	CCTACCCCAATTTCCAATGCTCTCC	GCATAACGCACTAGGTTTGCCGA
IL-18	AGAAAGCCGCCTCAAACCTTCCA	TGGTCTGGGGTTCACTGGCACTTT
IL-1B	ACGGACCCCAAAAGATGAAGGGCT	ATGGGAACGTCACACACCAGCA
CCL2	ATGCTTCTGGGCCTGCTGTTCA	ACCCATTCTTCTTGGGGTCAGCA
CCL7	AGAAGCAAGGCCAGCACAGAGT	GCGCAGACTTCCATGCCCTTCTTT
CSF2	AGCTCTTTTGCAGTGAGCCCAG	ACCTCTTCATTCAACGTGACAGGC
FLT3L	GGCTGTCATCCAGCTTTGGCTT	TGCAGTGCTTCTCGTCCTGAAGAT
β -actin	TCCAGCCTTCCTTCTTGGGTATGGA	AGCCACCGATCCACACAGAGTA

Figure 1

Figure 2

Figure3

Figure 4