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R-spondin3-LGR4 signaling protects hepatocytes against DMOG-induced hypoxia/reoxygenation injury through activating β -catenin

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Abstract

Background & aims: Leucine-rich repeat G-protein-coupled receptor 4 (LGR4) and its ligands R-spondin1-4 (Rspos) have been vastly investigated in embryonic development. The biological functions of Rspos-LGR4 system in liver remains largely unknown. Here, we explored whether it protects hepatocytes against hypoxia/reoxygenation (H/R) induced damage.

Methods: H/R injury was induced by dimethylxalylglycine (DMOG) in AML12 cells and the effects of Rspo3 on cell proliferation and apoptosis were assessed. Specific shRNAs were used to interfere LGR4 or β -catenin.

Results: DMOG caused hepatocytes damage evidenced by increase in HIF-1 α , cell death and apoptosis genes *p27* and *Bax*, with concurrent decrease of cell proliferation genes *PCNA* and *CyclinD1*. Of all the Rspos, Rspo3 is predominantly expressed in AML12 hepatocytes. Importantly, Rspo3 demonstrated an alteration in a manner similar to proliferation-related genes during H/R injury. Rspo3 pretreatment rendered hepatocytes less vulnerable to DMOG induced H/R injury. Ablation of LGR4 using shRNA attenuated the protective effects of Rspo3. Wnt3a also protected AML12 cells from damages caused by H/R, showing enhanced proliferation activity. Notably, knockdown of β -catenin in hepatocytes completely abolished the effect of Rspo3 pretreatment on the expression levels of *PCNA* and *CyclinD1*.

Conclusion: Rspo3-LGR4 axis protects hepatocytes from H/R injury via activating β -catenin.

Keywords

R-spondin3; LGR4; β -catenin; Hypoxia/reoxygenation injury; Hepatocytes

1. Introduction

Developed countries are experiencing an epidemic of obesity. Hepatic steatosis affects almost 30% of adults. Steatotic livers are particularly vulnerable to damage in the settings of

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traumatic injury and operative stress, responding with both hepatocytes apoptosis and necrosis [1,2]. Apoptosis and necrotic cell death of hepatocytes following trauma involves complex crosstalk between endoplasmic reticulum (ER) stress, oxidative processes, and inflammation initiated by ischemia [3]. Hypoxia induces generation of reactive oxygen species (ROS), which in turn (i) stimulate NF κ B-dependent transactivation of pro-inflammatory mediators and (ii) promote protein unfolding to trigger the ER stress response. Levels of ROS are increased significantly in steatotic livers during ischemia and reperfusion, which may partially explain the poor tolerance of steatotic livers to this form of injury [4]. Current therapy for liver injury in the setting of steatosis is limited. Pharmacological agents such as antioxidants, inflammatory regulators and microcirculatory mediators have been investigated in attempts to protect steatotic livers from injury in animal models, with mixed success. Defining key endogenous pathways that connect steatosis to hepatic injury is crucial if we are to provide safe and effective therapy in the setting of hepatic steatosis.

Leucine-rich repeat G-protein-coupled receptor 4 (LGR4), also known as GPR48, is highly conserved in mammals [5]. LGR4 is a 7-transmembrane protein comprised of 951 amino acids characterized by its large extracellular amino terminus [6]. Notably, the extracellular domain of LGR4 reveals a twisted horseshoe-like architecture [7], which is responsible for its interaction with its functional ligands-R-spondins (Rspos) [7]. Four homologs of Rspos have been identified so far, named as Rspo1~Rspo4. The interplay between Rspos and LGR4 coordinates multiple physiological and pathophysiological processes, including embryogenesis [8], energy and lipid homeostasis [9], tumorigenesis [10] and several other disorders [11]. The physiological function of Rspos-LGRs in the liver remains largely unexplored. A recent study by Planas-Paz et al. also demonstrate that Rspo1-LGR4/5 signaling modulates liver zonation and hepatic growth/size during development, homeostasis and regeneration. Whether Rspos-LGRs signaling impacts the cytoprotection and survival of hepatocytes in response to injury insults remains unknown. Numerous studies have uncovered that Rspos-LGR4 signaling can strongly potentiate Wnt- β -catenin activities [12]. Of note, Wnt signaling plays a crucial role in hepatic I/R injury. Pharmacological and genetic activation of Wnt- β -catenin signaling renders hepatocytes resistant to injury induced by ischemia/ reperfusion, while promoting the survival of hepatocytes under hypoxic conditions. We thus hypothesized that Rspos-LGR4 signaling protects hepatocytes from damages initiated by hypoxia. To test this concept, we induced hypoxic conditions of AML12 mouse hepatocytes using dimethylxallylglycine (DMOG), a well-characterized hypoxia-mimetic agent that inhibits prolyl hydroxylases, leading to the subsequent stabilization of hypoxia-inducible factor 1- α (HIF-1 α). The combination of clinical relevance and experimental standardization is the reason for the use of DMOG-induced hypoxia model in this study. We found that Rspo3 reinforced cell proliferation following H/R injury. Knockdown of LGR4 abolished the effects of Rspo3. Further, deficiency of β -catenin attenuated the function of Rspo3, indicating Rspo3-LGR4 signaling accelerates cell proliferation after H/R via β -catenin.

2. Materials and methods

2.1. Materials

Rabbit anti-PCNA and CyclinD1 were purchased from Abcam (Cambridge, MA). Rabbit anti-HIF-1 α , mouse anti- β -actin and GAPDH were obtained from Proteintech (Chicago, US). In situ cell death detection POD kit (Cat. No. 11684817910) were from Roche (Penzberg, Germany). RNATrip and BCA protein quantitative assay kit were purchase from Applied Gene (Beijing, China). *Lgr4* shRNA, β -catenin shRNA and NC control vectors were obtained from Vigene Biosciences (Shandong, China). RIPA lysis buffer was from Beyotime Biotechnology (Shanghai, China). Neofect™ DNA transfection re-agent was purchased from Neofect (Beijing, China). DMOG was obtained from Cayman Chemical (Michigan, USA). R-spondin3 and Wnt3a were purchased from R&D Systems (Minneapolis, MN, USA).

2.2. Cell culture and shRNA transfection

AML12 cells were cultured in a humid atmosphere (5% CO₂) with high glucose DMEM supplemented with 10% FBS at 37 °C. Cells were seeded in a 12-well plate until 60%e80% confluent. Cell culture medium was replaced with 950 μ l/well 2h before transfection. The transfection mixture was prepared following the manufacturer's instructions. The mixture was then incubated for 18 min at 37 °C and added 50 μ l/well. After 36h of incubation, culture medium was changed into high glucose DMEM supplemented with 10% FBS. Cells were stimulated with 100 ng/ml Rspo3 for 1h before treatment with 0.4 mM DMOG for 24h. The cell culture medium was then changed to drug-free high glucose DMEM supplemented with 10% FBS for another 6h. shRNA sequences were as follows:

shLgr4.

5'-GGGTGTGACTCTTATGCAAATTTAATTCAAGAGATTAAATTTGCATAAGAGTCACACCCTTTTTT-3';

sh β -catenin.

5'-GCACGCAAGAGCAAGTAGCTGATATTTTCAAGAGAAATATCAGC TACTTGCTCTTGCGTGTTTTT-3';

NC control shRNA.

5'-UUCUCCGAACGUGUCACGUTT-3'.

2.3. DMOG-induced hypoxia/reperfusion model

AML12 cells cultured in a humid atmosphere (5% CO₂) with high glucose DMEM supplemented with 10% FBS at 37 °C were treated with 0.4 mM DMOG for 24h to induce hypoxia. The medium was than replaced with drug-free high glucose DMEM with 10% FBS. After reoxygenation for indicated times, the cells were harvested for protein or RNA extraction. As for R-spondin3 or Wnt3a pre-treatment, 100 ng/ml R-spondin3 or 20 ng/ml Wnt3a were added to cultured AML12 cells 1h before DMOG treatment.

2.4. In suite cell death detection

Cell death assay was performed in accordance with the manufacture's instruction. AML12 cells were seeded on the round coverslips placed in 12-well plate. After rinsed for 3 times with PBS, cells were fixed with 4% paraformaldehyde for 30min, followed by incubating in 3% H₂O₂ in methanol to block endogenous peroxidase (POD) and permeabilize with 20 µg/ml proteinase K. TUNEL reaction mixture was then added on the coverslips and incubated for 60 min at 37 °C in a humidified atmosphere in dark. After incubating with Hoechst for 15min, fluorescent signals were observed and captured under a fluorescence microscope (Nikon).

2.5. Gene expression analysis

RNA was extracted from AML12 cells using RNATrip (Applied Gene, Beijing, China) and reverse-transcribed into cDNAs using reverse transcription system (PromegaCat#3500). Quantitative real time-PCR based on SYBER Green was performed using the Agilent AriaMx real-time PCR system. Sequences for the primer pairs used in this study are listed below:

2.6. Western blot analysis

AML12 cells seeded in 6-well plates were harvested using RIPA lysis buffer. Protein samples were subjected to 10% SDS-PAGE running gel, and then transferred onto a nitrocellulose (NC) membrane. Membranes were incubated in 4% fat-free milk in Tris buffered saline containing Tween 20 at room temperature for 1h. Subsequently, membranes were incubated overnight at 4 °C with primary antibodies. Fluorescent secondary antibodies were used for detection of combination between primary antibodies and relevant proteins. Odyssey infrared imaging systems (LI-COR Biosciences, Lincoln, NE, USA) were used to visualize the fluorescence signals.

2.7. Statistics

All data were expressed as mean ± SEM. Statistic difference was determined by two-way ANOVA and Student's T test. P value < 0.05 was considered significant.

3. Results

3.1. DMOG-induced hypoxia/reperfusion injury of hepatocytes

HIF-1α is one of the most dominating factors mediating cell responses upon hypoxia. Under normoxia conditions, HIF-1α undergoes hydroxylation modification conducted by O₂-dependent prolyl hydroxylase, which leads to ubiquitination and degradation of HIF-1α. DMOG, a synthetic analogue of α-ketoglutarate acting to effectively repress the activity of O₂-dependent prolyl hydroxylase, could stabilize HIF-1α and transactivate hypoxia-related genes in normoxic states [13]. DMOG has been used to induce hypoxia/ reoxygenation injury in cultured cells or in rodents. In our study, treatment of AML12 hepatocytes with DMOG for 24h dramatically induced the expression of HIF-1α protein (Fig. 1A). The alteration in HIF-1α was associated with a significant increase in cell death measured by TUNEL staining (Fig. 1B), as well as the expression level of *VEGFA* (Fig. 1C), a well-

established target gene of HIF-1 α . During the early phase of reoxygenation, *VEGFA* maintained low expression, followed with a time-dependent increment (Fig. 1C). DMOG treatment notably suppressed the expression of proliferation genes *PCNA* and *CyclinD1* (Fig. 1D-E), with concurrent up-regulation of apoptotic genes *p27* and *Bax* (Fig. 1G-H). Importantly, after reoxygenation for 6h, levels of cell proliferation-related gene including *PCNA*, *CyclinD1* and *CyclinE1* started to elevate gradually (Fig. 1D-F), indicating the beginning of cell repair after hypoxia/reoxygenation injury. Our results reveal that DMOG could induce hypoxia/reoxygenation injury in hepatocytes.

3.2. Rspo3 protects hepatocytes from H/R injury

To illustrate the role of Rspos in hepatic H/R injury, we first determined the expression of Rspos in hepatocytes. Among 4 homologs, Rspo3 was the only Rspo with abundant expression in AML12 cells (Fig. 2A). Importantly, Rspo3 displayed an expression pattern similar to proliferation related genes after H/R injury. Hypoxia significantly downregulated Rspo3. After reoxygenation for 6h, the expression level of Rspo3 steadily increased (Fig. 2B). Hepatic recovery after injury is characterized by the proliferation of hepatocytes. We next examined the proliferation related genes in cultured AML12 cells. Cells were treated with Rspo3 or DMSO control 1h before DMOG stimulation. After 24h of DMOG treatment, DMOG was withdrawn for 6h and expressions of relative genes or proteins were examined. Remarkably, despite no significant differences were observed in the expression of *p27* or *Bax* (Fig. 2C), Rspo3 pretreatment prominently reversed the suppression of H/R injury on the mRNA and/or protein expression of *PCNA*, *CyclinD1* and *CyclinE1* (Fig. 2D-F). Thus, our data demonstrates that Rspo3 enhance the proliferation activity of hepatocytes after H/R injury.

3.3. LGR4 mediates the protective effects of Rspo3 during H/R injury

Rspo3 has been reported to function through the B type LGRs, i.e. LGR4-6 [14]. We then examined the expression of each B type LGRs in AML12 cell. As shown in Fig. 3A, LGR4 was most abundantly expressed in hepatocytes. We thus proposed that Rspo3 exerts its protective functions during H/R injury via LGR4. To confirm our hypothesis, we employed *Lgr4* shRNA to silence its expression in AML12 cells. Upon treatment with *Lgr4* shRNA for 36h, significant reduction of *Lgr4* expression was observed (Fig. 3B). Deficiency of *Lgr4* completely abolished the Rspo3-mediated protection of hepatocytes against the H/R injury. Rspo3-induced elevations of *PCNA*, *CyclinD1*, *CyclinE1* mRNA and/or protein in hepatocytes were both blunted by LGR4 ablation (Fig. 3C and D). Nevertheless, *p27* and *Bax* were barely affected (Fig. 3E). Together, our data indicates that LGR4 mediates the pro-proliferation effects of Rspo3 during hepatic H/R injury.

3.4. The pro-proliferation function of Rspo3 in hepatocytes is β -catenin-dependent

To test the functions of Wnt signaling in hepatocytes proliferation following H/R injury, we pretreated AML12 cells with Wnt3a before DMOG stimulation. Treatment of AML12 cells with Wnt3a significantly increased mRNA and/or protein levels of *PCNA*, *CyclinD1* and *CyclinE1* (Fig. 4A and B), whereas demonstrated no effect on *p27* and *Bax* (Fig. 4C). These results confirm that Wnt3a signaling could improve hepatocytes proliferation following H/R injury.

We then examined whether Rspo3 promotes hepatocytes proliferation during H/R via activating β -catenin. Expression of β -catenin was interfered by specific shRNA. Relative to the control, β -catenin shRNA substantially decreased the expression of β -catenin (Fig. 4D). β -catenin deficiency completely reversed Rspo3-induced elevation of mRNA and/or protein levels of PCNA, CyclinD1 and CyclinE1 (Fig. 4E and F), while demonstrating little effects on p21 and Bax (Fig. 4G).

4. Discussion

Our present study demonstrates that Rspo3-LGR4 signaling may accelerate the repair process of hepatocytes following H/R injury by activating β -catenin. This conclusion is supported by following observations: (1) By treating AML12 hepatocytes with DMOG for 24h followed by its withdrawal, we established a cellular model mimicking the H/R injury. (2) Rspo3 increases the expression of proliferation related genes PCNA, CyclinD1 and CyclinE1 in hepatocytes during H/R injury. (3) Silence of *Lgr4* attenuates the effects of Rspo3. (4) Deficiency of β -catenin abolishes the up-regulation of PCNA, CyclinD1 and CyclinE1 induced by Rspo3.

Although most studies concerning Rspo3-LGR signaling focused on embryogenesis, several recent researches have shed light on the crucial roles of this signaling pathway in regeneration following tissue injury. Graft-versus-host disease (GVHD) is a major complication caused by allogeneic bone marrow transplantation, which may result in impairment of gastrointestinal tract. Injection of Rspo1 ameliorates GVHD-related intestinal stem cells (ISCs) damages, enhances proliferation of intestinal crypt cells, accelerates restoration of injured intestinal epithelium and restrains activation of inflammatory cytokine cascades [15]. In the model of chemotherapy-induced intestinal mucositis, Rspo1 also displays therapeutic efficacy via promoting amplification of stem cells [16]. Another study conducted in mesenteric I/R model has suggested Rspo3 as a promising treatment to alleviate I/R-induced inflammatory response by tightening endothelium and preventing vascular leakage [17]. Taken together, previous studies have confirmed that Rspo3 could act through enhancing stem cell amplification and differentiation as well as blunting immune response to potentiate restoration after tissue injury. Our present study extends the action site of Rspo3 to the liver. Rspo3 may function in an autocrine/paracrine manner to directly interact with LGR4 on parenchymal hepatic cells to trigger β -catenin signaling, which ultimately reinforces self-replication of hepatocytes following H/R injury.

Self-replication of hepatocytes is controlled by a group of proteins regulating cell cycle progression. Consistently, our studies detected the upregulation of PCNA, CyclinD1 and CyclinE1 in hepatocytes treated with Rspo3 during H/R. Our studies also suggest that activation of β -catenin is responsible for the Rspo3-mediated modulation of these genes. Indeed, a number of studies have verified CyclinD1 as one of the downstream targets of β -catenin [18]. β -catenin-CyclinD1 axis is involved in enhancing proliferation of both osteoclasts and Schwann cells [19,20]. Moreover, this signaling pathway is closely related to the onset of colon cancer, ovarian carcinomas, as well as breast cancer [21–23]. In addition to the β -catenin-CyclinD1 pathway, our data also indicates that PCNA and CyclinE1 are the

direct targets of β -catenin. Further examination is required to identify the underlying mechanism by which β -catenin regulates PCNA and CyclinE1.

In conclusion, our studies suggest that Rspo3 could enhance proliferation activity of hepatocytes during H/R injury. The beneficial effect of Rspo3 is mediated by its interacting with LGR4 and subsequent activation of Wnt- β -catenin signaling. Rspo3 may provide an alternative strategy for the intervention of hepatic dysfunctions following ischemia/reperfusion injury.

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Fig. 1.
DMOG-induced hypoxia/reperfusion injury.

A. DMOG time-dependently increases the expression of HIF-1α. AML12 cells were treated with DMOG for indicated time. Protein level of HIF-1α was detected by Western blotting. β-actin was used as internal control. Signal intensity was analyzed using Image J software and expressed as mean ± SEM. Shown is the representative of at least 3 repeat experiments. *P < 0.05 v. s. DMSO group.

B. DMOG treatment aggravated cell death. AML12 cells were incubated with DMOG (0.4 mM) for 24h to induce hypoxia. Cell injury was evaluated by in situ cell death assay (TUNEL).

C. Expression of VEGFA following H/R injury. AML12 cell were treated with DMOG (0.4 mM) for 24h, followed by withdrawal of DMOG for indicated times. Hepatic mRNAs were extracted and analyzed by RT-qPCR. n = 4. *P < 0.05 v. s. DMSO group.

D-F. Expression of proliferation-related genes following H/R injury. AML12 cell were treated with DMOG (0.4 mM) for 24h, followed by withdrawal of DMOG for indicated

times. Hepatic mRNAs were extracted and analyzed by RT-qPCR. $n = 4$. $*P < 0.05$ v. s. DMSO group.

G-H. Expression of apoptosis-related genes following H/R injury. AML12 cell were treated with DMOG (0.4 mM) for 24h, followed by withdrawal of DMOG for indicated times. Hepatic mRNAs were extracted and analyzed by RT-qPCR. $n = 4$. $*P < 0.05$ v. s. DMSO group.

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Fig. 2.

Rspo3 potentiates proliferation of hepatocytes after H/R injury.

A. Expression of Rspos in AML12 cells. mRNAs of AML12 were extracted and analyzed by RT-qPCR. $n = 6$. * $P < 0.05$ v. s. Rspo3 expression level.

B. Expression of Rspo3 following H/R injury. AML12 cell were treated with DMOG (0.4 mM) for 24h, followed by withdrawal of DMOG for indicated times. mRNAs were extracted and analyzed by RT-qPCR. $n = 4$. * $P < 0.05$ v. s. DMSO group.

C. Rspo3 upregulates the expression of proliferation-related genes during H/R. AML12 cells were treated with DMOG (0.4 mM) for 24h to induce hypoxia, followed by reoxygenation for 6h. mRNAs were then extracted and proliferation-related genes such as PCNA, CyclinD1 and CyclinE1 were analyzed by RT-qPCR. $n = 4$. * $P < 0.05$ v. s. DMOG treated group.

D. Rspo3 upregulates the expression of proliferation-related proteins during H/R. The protein levels of PCNA and CyclinD1 were examined by Western blot. GAPDH was used as internal control. The relative expression levels of PCNA and CyclinD1 were calculated using Image J software and expressed as mean $\pm$ SEM. Shown is the representative of at least 3 repeat experiments. * $P < 0.05$ v. s. DMOG treated group.

E. Rspo3 demonstrates no effect on apoptosis genes during H/R. AML12 cells were treated with DMOG (0.4 mM) for 24h to induce hypoxia, followed by reoxygenation for 6h. mRNAs were then extracted and genes related to apoptosis were analyzed by RT-qPCR. $n = 4$.

Fig. 3.

LGR4 mediates the protective effects of Rspo3.

A. Expression of LGRs in AML12 cells. mRNAs of AML12 were extracted and analyzed by RT-qPCR. n = 6. *P < 0.05 v. s. *Lgr4* expression level.

B. Lgr4 shRNA attenuates Lgr4 expression in AML12 cells. mRNAs from AML12 cells were extracted following transfection of *Lgr4* shRNA for 36h. The expression level of *Lgr4* was determined by RT-qPCR. n = 6. *P < 0.05 v. s. NC group.

C. LGR4 deficiency blocks Rspo3-induced upregulation of proliferation-related genes. Expression of *PCNA*, *CyclinD1* and *Iin* in AML12 cells were examined following different treatments using RT-qPCR. n = 6. *P < 0.05 v. s. DMOG treated group. #P < 0.05 v. s. Rspo3+DMOG treated group.

D. LGR4 deficiency blocks Rspo3-induced upregulation of proliferation-related proteins. Western blotting was used to detect expression of PCNA and CyclinD1 protein after different treatments with β-actin as internal control. The expression of corresponding proteins was quantified using Image J software and expressed as mean ± SEM. Each experiment was repeated for at least 3 times. *P < 0.05 v. s. DMOG treated group. #P < 0.05 v. s. Rspo3+DMOG treated group.

E. LGR4 deficiency demonstrate no effect on the down-regulation of apoptosis genes triggered by Rspo3. Expression of *p27* and *Bax* in AML12 cells were examined following different treatments using RT-qPCR. n = 6.

Fig. 4.

The pro-proliferation function of Rspo3 in hepatocytes is b-catenin-dependent.

AML12 cells were exposed to Wnt3a 1h before DMOG treatment. After 24h of incubation, DMOG was withdrawn for 6h.

A. Wnt3a attenuates the decrease of proliferation genes caused by H/R. Expressions of relevant mRNAs in AML12 cells were analyzed by RT-qPCR. n = 6. *P < 0.05 v. s. DMOG treated group.

B. Increase of PCNA and CyclinD1 protein levels in Wnt3a-pretreated AML12 cells during H/R. Western blotting was performed to determine the level of PCNA and CyclinD1. β-actin was used as control. The expression of PCNA and CyclinD1 was quantified as the gray value using Image J software and expressed as mean ± SEM. Each experiment was repeated for at least 3 times. *P < 0.05 v. s. DMOG treated group.

C. Unchanged expression of p27 and Bax after Wnt3a treatment during H/R.

Expressions of relevant mRNAs in AML12 cells were analyzed by RT-qPCR. n = 6.

D. Decreased expression of β -catenin induced by β -catenin shRNA. mRNAs from AML12 cells were extracted following transfection with β -catenin shRNA for 36h. The expression levels of β -catenin were determined by RT-qPCR. n = 6. *P < 0.05 v. s. NC group.

E-F. β -catenin silencing abolishes the protective effects of Rspo3 during H/R.

(B) Expression of *PCNA*, *CyclinD1* and *CyclinE1* in AML12 cells were examined following different treatments using RT-qPCR. n = 6. *P < 0.05 v. s. DMOG treated group. #P < 0.05 v. s. Rspo3+DMOG treated group. (C) Western blotting was performed to detect protein expression of PCNA and CyclinD1. Results were further quantified using Image J software and expressed as mean $\pm$ SEM. At least 3 repeat experiments were performed. *P < 0.05 v. s. DMOG treated group. #P < 0.05 v. s. Rspo3+DMOG treated group.

G. No effect on apoptosis genes after H/R. Expression of *p27* and *Bax* in AML12 cells were examined following different treatments using RT-qPCR. n = 6.

	Forward	Reverse
VEGFA	CTGGCTTTGGTGAGGTTTGAT	GGAGTACCCCGACGAGATAGA
PCNA	AGATGCCGTCGGGTGAATTTGC	TCCCATTGCCAAGCTCTCCA
CyclinD1	TGTGCTGCGAAGTGGAGACCAT	ACTTCTGCTCCTCACAGACCTCCA
CyclinE1	TCTTCGTGCAGATCGCAGAGCT	AACCATGGCGAACGGAACCAT
p27	AGCGACCTGCTGCAGAAGAT	TTACGTCTGGCGTCGAAGGC
Bax	TGAGCGAGTGTCTCCGGCGAAT	GCACTTTAGTGACAGGGCCTTG
Lgr4	CCATTCGTGGACTGAGTGCT	GTTGGTGAATGCGAAGTCGG
Lgr5	TCAAGCCCTGTGAGCACCTAT	ATGTCCACTACCGCGATTACC
Lgr6	ACTCCAACCTCCATAGCAAGA	GCCAAGACAAAGCAAGAC
R-spondin1	GCTGGAGAGGAACGACATCC	CAGCGGCCCTTGTAAGTA
R-spondin2	CAAGCATGGACTCAGCGTTA	TCTACAGCCTTGTCCTCCT
R-spondin3	GTACACTGTGAGGCCAGTGAA	ATGGCTAGAACACCTGTCCTG
R-spondin4	TTTCCCGTCTGTCTGCTCTT	ACACGGGGAGAAGGAAAGTT
β-catenin	CACAATGCACGTTTCAGACACT	ACTCCAGAAAGACTTGGTAGGG
β-actin	ATCTGGCACCACACCTTC	AGCCAGGTCCAGACGCA