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***In vitro* Effects of RU486 on Proliferation and Differentiation Capabilities of Human Bone Marrow Mesenchymal Stromal Cells**

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Abstract

Although exogenous glucocorticoids (GC) play a role in the regulation of bone marrow mesenchymal stem/stromal cells (MSCs) proliferation and differentiation, the function of endogenous GC is not well understood. The purpose of this study was to investigate the effect of the blockage of endogenous GC using RU486, an antagonist of the glucocorticoid receptor, on the *in vitro* proliferation and differentiation capabilities of human MSCs. We quantitatively measured cell proliferation of human MSCs after treatment with increasing concentrations of RU486. We also evaluated multiple MSC differentiation capabilities, as well as the expression of stemness and senescence genes after proliferation of these human cells *in vitro* in the presence of RU486 at 10⁻⁸M. It was observed that RU486 treatment significantly increases the proliferation of human MSCs, although the optimal dose of RU486 for this increase in proliferation differs depending on the gender of the MSC donor. This improvement in MSC proliferation with RU486 treatment was higher in MSCs from male donors than that from females. No effect of RU486 on MSC proliferation was observed in a steroid-free medium. RU486 pretreatment significantly increased the expression of mRNA for alkaline phosphatase in human MSCs and the mRNA expression of osteocalcin of these cells up-regulated earlier after their exposure to osteogenic differentiation medium. Although no statistical significance in terms of chondrogenic differentiation markers was detected, mRNA expression for aggrecan and collagen type 2 were higher in a majority of the RU486-pretreated donor MSCs than their untreated controls. No significant difference in terms of MSC adipogenic differentiation capabilities were observed after RU486 treatment. RU486 treatment up-regulated the expressions of FGF-2 and Sox-11 in human MSCs. These results indicate that blockage of endogenous GCs may be developed as a novel approach to effectively improve the proliferation and osteochondral differentiation capabilities of human MSCs for potential clinical applications. Additional studies will be required to determine the potential long-term effects of RU486 treatment on these bone marrow cells.

Keywords

Glucocorticoids; Antagonist; Bone marrow mesenchymal stem/stromal cells; Proliferation; Differentiation; Senescence

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Introduction

Adult stem cell-based tissue regeneration has emerged as a promising approach to substitute current clinical treatment of tissue defects and malfunction caused by trauma, tumor dissection, and congenital deficiencies. Autologous bone marrow mesenchymal stem/stromal cells (MSCs) are considered a potential cell source for this approach due to their high proliferation capacity, multiple differentiations, and immune tolerance [1–2]. However, the natural population of MSCs for feasible isolation is relatively small [3]. Pathophysiological factors, including age, estrogen depletion, and arthritis further reduce the numbers and capacities of MSCs [4–8]. While typical characteristics of MSCs include self-renewal and multiple differentiations, MSCs exhibit replicative senescence and lose proliferative and osteogenic differentiation capacity after cell expansion using current *in vitro* culture systems [9]. Therefore, the development of therapeutic interventions to improve the stem cell characteristics of MSCs and prevent their senescence is necessary for potential clinical application of these cells.

While the mechanisms regulating the stem cell characteristics of MSC are unclear, it is recognized that MSCs lose their capability for self-renewal and multiple differentiations once they differentiate into a specific cell lineage or become senescent. Glucocorticoids (GC) have been demonstrated to effectively stimulate multiple pathways of MSC differentiation, but inhibit their proliferation [10–11]. Response mediated by the ligand-activated glucocorticoid receptor (GR) determine the shift of hematopoietic stem cells from self-renewal into the differentiated state [12]. GC have been demonstrated to down-regulate telomerase activity and accelerate senescence [13–14]. The elevated levels of endogenous GC that are observed during aging and in estrogen depleted states are responsible for the reduced populations and accelerated senescence of MSCs [15–16]. Collectively, this previous evidence indicates that naturally occurring endogenous GC impact MSCs and cause them to lose stem cell characteristics in terms of their self-renewal and multiple differentiations and also hasten their senescence. Therefore, blockage of endogenous GC may potentially be used to protect the stem cell characteristics of MSCs and improve their capabilities.

Although GC appear to mediate some rapid, non-genomic cellular responses by interacting with membrane receptors, many GC responses are mediated by classical intracellular GR that function as ligand-activated transcription factors. In the present study we investigated the *in vitro* effects of mifepristone (RU486), an antagonist of GR, on the capacities of human MSCs in terms of proliferation and multiple differentiations. We also investigated the effects of RU486 on the expression of fibroblast growth factor (FGF)-2, which is a stemness gene of MSCs, as well as Sox-11, which is a gene that participates in MSC self-renewal and functions as a senescence marker [17–18]. Our data demonstrate that RU486 treatment *in vitro* effectively enhances the proliferation and osteochondral differentiation capabilities of human MSCs. This enhancement may be mediated by up-regulation of MSC capabilities and prevention of MSC senescence.

Materials and Methods

Preparation of Human MSCs

Fresh human bone marrows, donated by healthy donors from 21 to 47 years of age, were purchased from Allcells (Emeryville, CA). Mononuclear cells were isolated using density gradient centrifugation (Histopaque-1.077; Sigma, St. Louis, MO). These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA)

and 1% antibiotic-antimycotic (Sigma, St. Louis, MO). On day 5, non-adherent cells were removed after medium exchange. The adherent cells continued to proliferate and were collected when they reached 70%–80% confluence. The cells were then cultured in 100mm/ petri dishes (passage-1) with an initial seeding density of 10^5 cells/per dish. Passage-1 cells were then used to test the potential effects of RU486 treatment on the proliferation and differentiation of human MSCs.

Proliferation of MSCs treated with RU486

The effect of RU486 on the proliferation of human MSCs was tested in both conventional cell culture medium consisting of DMEM containing 10% FBS, which contains endogenous steroids, and in steroid-free medium consisting of phenol red-free DMEM (Invitrogen, Carlsbad, CA) containing 10% charcoal-stripped FBS, which is devoid of endogenous steroids (Gemini Bio-product, West Sacramento CA). 1000 MSCs from each donor were placed into each well of 96-well plates and cultured in either conventional medium (plus or minus RU486) or steroid-free medium (plus or minus RU486) (Sigma, St. Louis, MO). The RU486 concentrations ranged from 10^{-6} to 10^{-10} M. MSCs proliferated in medium lacking RU486 served as controls. We also measured the effects of exogenous dexamethasone (10^{-6} M) on MSC proliferation in conventional medium as well as the potential effects of dexamethasone on the enhanced proliferation of those cells mediated by RU486 (10^{-8} M). The cell proliferation was measured using a MTS-based colorimetric method according to the manufacture's manual (CellTiter 96®, Promega, Madison, WI). Each measurement for each donor was performed in triplicate. The proliferation of MSCs treated with RU486 was normalized to the control (minus RU486) for each donor.

Differentiation capabilities of MSCs proliferated in vitro with RU486 treatment

To investigate the differentiation capabilities of MSCs proliferated *in vitro* in the presence of RU486, human MSCs from each donor were cultured in DMEM medium supplemented with 10% FBS and containing RU486 at a concentration of 10^{-8} M. MSCs not treated with RU486 served as controls. After one week the MSCs were collected and analyzed for differentiation capacities, including osteogenesis, chondrogenesis and adipogenesis. To test osteogenic and adipogenic differentiation capabilities, MSCs were placed in 6-well plates at a density of 5×10^4 cells per well and exposed to osteogenic or adipogenic differentiation medium for up to two weeks. The differentiation medium for osteogenesis consisted of the basic medium supplemented with 100 nM dexamethasone, 10 mM β -glycerophosphate, and 0.05 mM ascorbic acid-2-phosphate [19]. For adipogenesis, the medium was supplemented with 500 nM dexamethasone, 10 μ M insulin, and 0.5 μ M isobutyl-methylxanthine [19]. For chondrogenic differentiation, pellets of MSCs (10^5 cells/pellet) were generated by centrifugation and subsequently exposed to chondrogenic medium consisting of high glucose DMEM medium supplemented with 10 ng/ml TGF- β 1, 6.25 μ g/ml of insulin, 6.25 μ g/ml of transferrine, and 100 nM dexamethasone for up to 2 weeks [20]. Real-time polymerase chain reactions (PCR) were used to quantify the expression of specific markers of MSC differentiations. Alkaline phosphatase (ALP), core binding factor alpha (Cbfa)-1, and osteocalcin (OCN) were used to indicate osteogenic differentiation of MSCs. Aggrecan (ACAN) and collagen type II (COL2) were used as markers for chondrogenic differentiation, and peroxisome proliferator-activated receptors (PPAR)- γ 2 and lipoprotein lipase (LPL) were used as markers for adipogenic differentiation. Fold changes in the expression of osteogenic and chondrogenic markers were normalized to control MSCs that were not stimulated to differentiate. Since the adipogenic marker was not detected in MSCs without stimulation of differentiation, fold changes in the expression of adipogenic markers of MSCs pretreated with RU486 were normalized to untreated controls with differentiation stimulation. Measurements for each donor were performed in triplicate.

Effects of RU486 on the regulation of stemness gene and senescence of human MSCs

To evaluate the effects of RU486 on the regulation of expression of a stemness gene as well as senescence of human MSCs, cells were cultured for one week in DMEM medium supplemented with 10% FBS and containing 10^{-8} M RU486 or 10^{-6} M dexamethasone. Untreated MSCs served as controls. The expression of fibroblast growth factor (FGF)-2 and Sox-11 were measured using real-time PCR. Fold changes in the expressions were normalized to untreated controls. Each measurement of MSCs from each donor was performed in triplicate.

Real-time PCR analysis

Cellular RNA was isolated using the Aqua Pure RNA Isolation Kit (Bio-Rad, Hercules, CA) as recommended by the manufacturer's manual. Real-time PCR amplifications were performed using the iCycler iQ detection system, and the data were collected and analyzed using iCycler iQ version 3.0 software (Bio-Rad Laboratories Inc). Table 1 lists the real-time PCR primers and probes that were utilized for multiple MSC differentiation markers. The Syber Green Real-time PCR primers and probes for human GAPDH, FGF-2, and Sox-11 were purchased from Applied Biosystems (Foster City, CA). Each measurement was performed in triplicate.

Statistics

All quantitative data were expressed as means $\pm$ standard deviations. All statistical analyses were measured using commercially available statistical software (SPSS Inc., Chicago, IL) and *p* values less than 0.05 were considered significant. The proliferation of MSCs from each donor following RU486 treatment was normalized to the controls. The differentiation markers in each group following different treatments were normalized to those of control MSCs without differentiation stimulation. One-way ANOVA with Tukey post hoc test was used to analyze the differences between treatment groups (different concentrations of RU486) on MSC proliferation and differentiation capabilities. Students' *t* test was used to analyze potential gender differences in terms of responses of MSCs to RU486. FGF-2 and Sox-11 in each treatment group were compared to the controls using paired Students' *t* test.

Results

Treatment with RU486 enhances the proliferation of human MSCs

After 4 and 6 days of treatment with RU486 (10^{-6} to 10^{-10} M) significantly increased MSC proliferation was detected as compared to that in untreated controls. This enhanced proliferation was increased further with the more prolonged exposure to RU486 (*n*=10, 5 males and 5 females) (Fig. 1A). No statistical differences were observed among the treatment groups with different concentrations of RU486 (Fig. 1A). In contrast, no effect of RU486 in the same concentration range was detected on human MSC proliferation in steroid-free medium (*n*=5) (Fig. 1B). Treatment with dexamethasone at a concentration of 10^{-6} M reduced MSC proliferation and inhibited the RU486-mediated enhancement of proliferation (*n*=6) (Fig. 1C). Gender differences regarding the effect of RU486 on the proliferation of human MSCs were observed. After 4 days, RU486 at concentrations of 10^{-6} to 10^{-8} M significantly increased the proliferation of MSCs from male donors, while no significant increase in the proliferation of MSCs from female donors was detected (Fig. 2A). After 6 days, a significant increase in the proliferation of MSCs from male donors occurred at 10^{-6} to 10^{-10} M, while a significant increase in the proliferation of MSCs from female donors was only detected at an RU486 concentration of 10^{-8} M. Furthermore, the ability of RU486 (10^{-8} and 10^{-10} M) to enhance proliferation of MSCs from male donors was

significantly higher than the ability of this GR antagonist to enhance the proliferation of MSCs from female donors (Fig. 2B).

Treatment with RU486 modulates expression of differentiation markers in human MSCs

Figure 3 shows that, after 1 week of osteogenic differentiation, expression of Cbfa-1, ALP, and OCN in RU486-pretreated MSC were significantly up-regulated compared with the MSCs without differentiation. In contrast, the expression of ALP and Cbfa-1 were up-regulated in non-RU486-pretreated MSCs after differentiation. The up-regulation of ALP in MSCs treated with RU486 was significantly higher than that of control MSCs. After 2 weeks, although ALP, Cbfa-1, and OCN of MSCs were significantly up-regulated with differentiation stimulation, no significant changes between MSCs with or without pretreatment with RU486 were detected (Fig. 3). For chondrogenic differentiation, ACAN expression by MSCs from 5 donors and Col2 from 4 donors (total of 6 donors) following treatment with RU486 were higher than the untreated controls after one week. After 2 weeks, ACAN expression by MSCs from 4 donors and Col2 from 5 donors (total of 6 donors) were higher than the controls. However, due to individual variations within donors, no statistical significance in the fold change of differentiation markers was detected ($n=6$, Fig. 4). No significant difference in the expression of adipogenic markers, PPAR γ -2 and LPL, was observed between RU486-pretreated MSCs compared to untreated controls ($n=8$, Fig. 5). No gender differences were observed regarding the enhancement of osteochondral differentiation capabilities of MSCs after RU486 treatment.

Treatment with RU486 modulates expression of MSC stemness and senescence genes

The effects of RU486 treatment on the expression of a stemness gene (FGF-2) and senescence gene (Sox-11) of MSCs were evaluated after MSCs were proliferated in the presence of RU486 (10^{-8} M) or dexamethasone (10^{-6} M). After one week, the expression of FGF-2 was significantly up-regulated in MSCs treated with RU486, while treatment with dexamethasone (10^{-6} M) down-regulated FGF-2 (Fig. 6, $p<0.05$, $n=5$). Expression of Sox-11 was significantly up-regulated by RU486 treatment (Fig 6, $p<0.05$, $n=5$).

Discussion

This study demonstrated for the first-time that blockage of endogenous GC using a GR antagonist effectively enhances the proliferation and osteochondral differentiation capabilities of human MSCs. RU486 treatment effectively up-regulates the expressions of FGF-2 and Sox-11 during *in vitro* expansion of MSCs. Since MSC senescence is characterized by reduced proliferation, decreased osteochondral differentiation capability, as well as decreased Sox-11 expression [9, 21], these data strongly suggest that blocking the effects mediated by endogenous GC using RU486 preserves the stem cells characteristics of MSCs and prevents their replicative senescence.

Although self-renewal of MSCs is a key characteristic required to maintain a high proliferative capacity of these cells, the effects of GC on the self-renewal of MSCs is unclear and their effects on proliferation are controversial [22–23]. However, the ability of GC to stimulate the differentiation of MSCs indicates that GC may impact the stem cell characteristics of these cells in terms of self-renewal and multiple differentiations, since MSCs lose their stem cell characteristics once they commit to differentiate into specific lineages. Therefore, endogenous GC may act to stimulate and force MSCs into specific differentiations. In addition, other known effects of GC, including onset of apoptosis and down-regulation of telomerase activity, suggest that endogenous GC may reduce the stem cell characteristics of MSCs during *in vitro* expansion by accelerating senescence. Therefore, the blockage of endogenous GR during *in vitro* expansion may protect, and

actually improve, the stem cell characteristics of MSCs from the impact of endogenous GC. The present study revealed that RU486 significantly enhances the proliferative capability of MSCs *in vitro*, but only when it is added to cell culture medium containing serum, which contains endogenous GC. These results thus demonstrate that blocking endogenous GC with a GR antagonist enhances the proliferative capability of MSCs during *in vitro* expansion. Although GC have been demonstrated to play critical roles in the differentiation of MSCs and pre-exposure to GC has been shown to increase the bone formation capability of MSCs, we observed that the osteochondral differentiation capabilities of MSCs were actually increased if endogenous GC were blocked during *in vitro* proliferation. These results suggested that blocking endogenous MSCs may also protect osteochondral differentiation capacity of MSCs and potentially enhance their capabilities during *in vitro* expansion.

Although the mechanism(s) underlying the effects of RU486 on enhancing MSC proliferation and osteochondral differentiation need to be further investigated, the up-regulation of FGF-2 and Sox-11 indicates that blocking endogenous GC may function to maintain the stemness of MSCs and prevent senescence. FGF-2 is a well-known stemness gene that participates in self-renewal of MSCs. Treatment with FGF-2 has been demonstrated to increase proliferation and osteochondral differentiation capabilities of MSCs and also inhibit their cellular senescence [24–25]. The present study showed that the expression of FGF-2 is up-regulated in MSCs treated with RU486 during *in vitro* expansion. This suggests that blocking endogenous GCs may enhance MSC proliferation and osteochondral differentiation by up-regulating FGF synthesis. Up-regulated FGF-2 may further inhibit MSC senescence and improve stem cell characteristics of MSCs by autocrine or paracrine mechanisms. Sox-11 has also been demonstrated to participate in the self-renewal of MSCs. A recent study demonstrated that Sox-11 is expressed in the early passages of MSCs and that its expression decreases, and is ultimately eliminated, following increased passage of MSCs *in vitro*. This protein can thus function as a marker for replicative senescence for MSCs during *in vitro* expansion [18]. Up-regulation of the expression of Sox-11 following treatment with RU486 supports the conclusion that blocking endogenous GC prevents replicative senescence of MSCs *in vitro*.

Gender differences regarding the differentiation and regenerative capabilities of adult stem cells have been recently reported [26–27]. In the present study, we observed that the effective dose of RU486 required to enhance MSC proliferation depends on the gender of the MSC donor. Although the mechanism(s) underlying this apparent gender difference are unknown, one possibility is that the GR expression level and/or the GR binding affinity may vary with the gender of the MSC donor. Sex differences regarding GC functions have been reported in the brain, cardiovascular tissues, muscle and bone. Once again, these sex-specific responses may reflect differences in the expression and/or binding affinity of GR in these tissues and cells. For example, treatment with a low dose of glucocorticoid is more likely to induce osteoporosis in females than males [28]. In the present study, we also found that the enhancement of MSC proliferation with RU486 treatment in cells from male donors is higher than in cells from female donors. These results suggested that the inhibitory effect of endogenous GC on the stem cell characteristics of MSCs from male donors is higher than on MSCs from female donors. These data also suggest that MSCs from male donors may express higher levels of an intracellular factor or factors that act to oppose the effects mediated by endogenous GC. Alternatively, MSCs from female donors may express higher levels of an intracellular factor or factors that act to facilitate, or potentiate, the effects mediated by endogenous GC. In addition, the uptake of GC by a number of cells is modulated by several species of membrane receptors that could themselves be expressed at different levels based on the gender of the MSC donor [29–30]. Future studies are needed to investigate these possibilities in human MSCs.

Although bone marrow MSCs are a potentially attractive source of cells for stem cell-based tissue regeneration, their relatively small numbers and limited capacities for *in vitro* cellular expansion serve as rate-limiting factors in capitalizing upon such therapeutic possibilities. Although the present study demonstrated the effects of blocking endogenous GC using the GR antagonist RU486 on the enhancement of MSC proliferation and differentiation capabilities, further investigation would be required in order to develop this approach for clinical utility for MSC-based tissue regeneration and cell therapy. We predict that the responses seen after blocking the effects of endogenous GC will be more significant following increased passages of MSCs *in vitro* and with the development of MSC senescence. The significance of these results generated *in vitro* with a GR antagonist is that they may relate to *in vivo* situations, including aging and estrogen-depletion, where endogenous GC levels are known to be elevated.

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- > We investigated effects of RU486 on human MSC characteristics.
- > RU486 increase proliferation and osteochondral capabilities.
- > RU486 up-regulate FGF-2 and Sox-11 of human MSCs.
- > RU486's effect on proliferation is gender dependent.

Fig. 1.

Effects of RU486 on proliferation (% of control) of human MSCs. **A.** MSCs grown in DMEM medium containing serum (endogenous GCs) and different concentrations of RU486 (n=10, 5 for male and female respectively) **B.** MSCs grown in medium containing charcoal-stripped serum (free of endogenous GCs) and different concentrations of RU486 (n=5, 3 male and 2 female). **C.** MSCs grown in medium containing serum (endogenous GCs) and RU486 (10^{-8} M) and/or Dexamethasone (Dex) (10^{-6} M). n=6 (3 for male and female respectively); Mean $\pm$ SD; *: $p < 0.05$, **: $p < 0.01$ versus controls indicated by dotted lines

Fig. 2. Gender-specific effects of RU486 on proliferation (% of control) of human MSCs. **A.** MSCs from male and female donors grown in medium containing serum (endogenous GCs) and different concentrations of RU486 for 4 days (n=5). **B.** MSCs from male and female donors grown in medium containing serum (endogenous GCs) and different concentrations of RU486 for 6 days. n=5; Mean $\pm$ SD; *: $p < 0.05$, **: $p < 0.01$ versus controls indicated by dotted lines; # : $p < 0.05$ versus females at the same concentration of RU486.

Fig. 3.

Effects of RU486 treatment on osteogenic differentiation capacity of human MSCs. Fold changes in mRNA expression for Cbfa-1 (**A**), ALP (**B**), and OCN (**C**) in control MSCs and MSCs with RU486 treatment (10^{-8} M) after osteogenic differentiation stimulation for 1 and 2 weeks. Cont-OS: osteogenic stimulation of control MSCs without RU486 treatment; RU486-OS: osteogenic stimulation of MSCs with RU486 treatment. n=8 (4 for male and female respectively); Mean $\pm$ SD; *: p<0.05, **:p<0.01 versus non-osteogenic stimulation indicated by dotted lines; #:p<0.05 versus control MSCs with osteogenic stimulation.

Fig. 4. Effects of RU486 treatment on chondrogenic differentiation capacity of human MSCs. Fold changes of mRNA expressions of ACAN (**A**) and Col2 (**B**) in control MSCs and MSCs with RU486 treatment after chondrogenic differentiation stimulation for 1 and 2 weeks. Cont-CS: chondrogenic stimulation of control MSCs without RU486 treatment; RU486-CS: chondrogenic stimulation of MSCs with RU486 treatment. n=6 (3 for male and female respectively); Mean $\pm$ SD; **:p<0.01 versus non-chondrogenic stimulation.

Fig. 5. Effects of RU486 treatment on adipogenic differentiation capacity of human MSCs. Fold changes of mRNA expressions of PPAR and LPL of MSCs with RU486 treatment versus control MSCs after adipogenic differentiation stimulation for one week. n=8 (4 for male and female respectively); Mean $\pm$ SD.

Fig. 6. Effects of RU486 on stemness gene (FGF-2) and senescence gene (Sox-11) in human MSCs. Fold change of mRNA expression of FGF-2 and Sox-11 in MSCs treated with RU486 or dexamethasone (Dex) supplement for 1 week, compared to controls. n=5 (3 male and 2 female); Mean $\pm$ SD; * p <0.05 versus controls indicated by a dotted line.

Table 1

Real-time PCR primers and probes for multiple differentiation of human MSCs.

Markers	5' to 3'	3' to 5'	probe
CBFA-1	CAA CAA GACCCT GCC CGT	TCC CAT CTG GTA CCT CTC CG	CTT CAA GGT GGT AGC CC
OCN	TAG TGA AGA GAC CCA GGC GC	CAC AGT CCG GAT TGA GCT CA	TGT ATC AAT GGC TGG GAG CCC CAG
ALP	GGG AAC GAG GTC ACC TCC AT	TCG TGG TGG TCA CAA TGC C	TGG GCC AAG GAC GCT GGG AAA T
ACAN	ACC CCG ACC CCT CAT	TGG CAG CTC CAT GTC A	AAA GTC TTC ACC TGT GTA GCA GAT GGC
COL2	TCC TGG CAT TGC TGG T	CCT TGG GGC CTT GTT CA	TCA GAC GGG TGA ACC TGG TATTGC
LPL	ACA CTT GCC ACC TCA TTC C	ACC CAA CTC TCA TAC ATT CCT G	AGT CCG TGG CTA CCT GTC ATT TCA ATC
PPAR γ 2	CTC CTA TTG ACC CAG AAA GCG	GAG TGG TCT TCC ATT ACG GAG	ATC CAC GGA GCT GAT CCC AAA GTT