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GPR120 promotes adipogenesis through intracellular calcium and extracellular signal-regulated kinase 1/2 signal pathway

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Abstract

Numerous researches have demonstrated that GPR120 (also called FFAR4) exerts novel functions in insulin resistance and adipogenesis. However, the molecular mechanism of GPR120-mediated adipogenic differentiation is still unclear. This study was aimed to interpret the relevant function mechanism of GPR120 in the differentiation of 3T3-L1 adipocytes. The results showed that *GPR120* expression was dramatically increased along with the adipogenic differentiation of 3T3-L1 adipocytes and the adipogenic ability was significantly inhibited in shGPR120-transfected cells. TUG-891, a selective agonist of GPR120, promoted the intracellular triglyceride accumulation in a dose-dependent manner and did not enhance adipogenesis in shGPR120-transfected cells. Markedly, TUG-891 increased the activation of PPARγ in a GPR120-dependent pathway as assessed by luciferase reporter assay. Furthermore, in the adipogenic differentiation process of 3T3-L1 adipocytes, TUG-891 increased the $[Ca^{2+}]i$ and phosphorylation level of ERK1/2. Pretreatment with inhibitors of either ERK1/2 (U0126) or $[Ca^{2+}]i$ (BAPTA-AM) notably attenuated the GPR120-mediated adipogenesis. These results show that GPR120 promotes adipogenesis by increasing PPARγ expression via $[Ca^{2+}]i$ and ERK1/2 signal pathway in 3T3-L1 adipocytes.

Keywords GPR120. 3T3-L1. Adipogenesis. $[Ca^{2+}]i$, ERK1/2.

1. Introduction

White adipose tissue (WAT), which is considered as the main reservoir to store energy, plays an important role as an endocrine organ in glucose metabolism and immune functions by secreting a vast range of regulatory factors (Kershaw and Flier, 2004). Given the vital role of WAT in the health implications and energy homeostasis, it is essential to investigate the molecular mechanisms of adipogenesis. Until now, a variety of transcription factors, including CCAAT enhancer binding protein α (C/EBPα), C/EBPβ, C/EBPδ and peroxisome proliferator activated receptor γ (PPARγ), have been demonstrated to be involved in this process (Otto and Lane, 2005; Tang and Lane, 2012). Among them, PPARγ is considered as the master regulator of the complex transcriptional cascade in adipogenic differentiation and the terminal differentiation does not occur without PPARγ (Cristancho and Lazar, 2011; Rosen et al., 1999).

Recently, the novel fatty acid receptor, GPR120 (also called FFAR4), has been shown to be implicated in diverse physiological homeostasis, such as insulin sensitization, anti-inflammation and regulation of appetite (Hirasawa et al., 2005; Oh et al., 2010). Notably, it is abundantly expressed in WAT and mature adipocytes, whereas it is undetectable in preadipocytes (Gotoh et al., 2007; Ichimura et al., 2012; Miyauchi et al., 2009; Oh et al., 2010). *GPR120* knockdown reduces the expression of *PPARγ* and *fatty acid binding protein 4* (FABP4, also known as aP2) in 3T3-L1 adipocytes (Gotoh et al., 2007). Consistently, suppression of these adipogenic marker genes was detected in the mouse-embryonic fibroblast (MEF) derived adipocytes isolated from the GPR120-deficient mouse (Ichimura et al., 2012). These evidences indicate that GPR120 contributes to the process of adipogenesis. However, its precise molecular function in the regulation of adipogenic processes remains unclear.

As a G protein-coupled receptor (GPCR), GPR120 is located in the cell surface and induces a wide range of cellular responses when responding to appropriate ligand binding. Several experimental observations showed that GPR120 stimulated by fatty acids or synthetic agonists increases intracellular calcium concentration ($[Ca^{2+}]i$) and elevates the phosphorylation level of extracellular signal-regulated kinase1/2 (ERK1/2) cascade (Hirasawa et al., 2005; Hudson et al., 2013; Ichimura et al., 2012; Katsuma et al., 2005). ERK1/2 facilitates the early stage of adipogenic differentiation but needs to be turned off at the adipocyte maturation phase, suggesting a dual role of ERK1/2 in adipogenesis (Bost et al., 2005b; Prusty et al., 2002). On the other hand,

$[Ca^{2+}]i$ also plays a time-dependent role in adipogenesis. Improving intracellular calcium concentration inhibits the early stage but accelerates the maturation stage of adipogenic differentiation in both murine and human adipocyte (Neal and Clipstone, 2002; Shi et al., 2000). Hence, it would be interesting to understand the roles of $[Ca^{2+}]i$ and ERK1/2 signaling in GPR120-induced adipogenesis. In this study, we determined the expression of GPR120 during the differentiation progress of 3T3-L1 adipocytes and found that GPR120 facilitates 3T3-L1 adipogenesis. Moreover, we for the first time showed that $[Ca^{2+}]i$ and ERK1/2 signaling contributes to GPR120-induced adipogenesis.

2. Materials and methods

2.1 Materials

Oil Red O, paraformaldehyde, insulin, dexamethasone, isobutylmethylxanthine, collagenase I, BAPTA-AM, U0126, Docosahexoenoic acid (DHA, 22:6n-3), α-linolenic acid (ALA, 18:3n-3), linoleic acid (LA, 18:2n-6), Dimethylsulfoxidewere (DMSO) were purchased from Sigma Aldrich. TUG-891 was obtained from R&D systems. Rabbit anti-PPARγ and anti-p-ERK1/2 (T202/Y204) were purchased from Affinity biosciences Inc. DHA, ALA and LA were dissolved in DMSO. The stock solutions (500 mM) were aliquoted and stored at -20℃.

2.2 Cell culture and DNA transfection

The 3T3-L1 preadipocyte and HEK293T cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco, San Diego, CA, USA), penicillin (100 IU/mL) and streptomycin (100 pg/mL) at 37℃ and 5% CO_2. The cells were transfected at 70% confluence in 24-well plates by using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. Briefly, both Lipofectamine™ 2000 (1 μL) and plasmid (0.5 μg) were diluted using Opti-MEM (Invitrogen, Carlsbad, CA, USA) to a final volume of 50 μL per well and then mixed together for 15 min at room temperature. Then the lipo-DNA complex was added into the wells. Cells were assayed at 48 h post transfection.

2.3 Adipogenic differentiation

The 3T3-L1 preadipocyte was induced to differentiate as described previously (Gotoh et al., 2007). Briefly, the adipose differentiation was initiated after 2-day post-confluence and the medium was replaced with the differentiation medium containing isobutylmethylxanthine, dexamethasone, and insulin (MDI) supplemented with 95% DMEM, 10% FBS, 10 μg/mL insulin, 1 μM

dexamethasone, and 0.5 μM isobutylmethylxanthine. Two days later, the medium was replaced with DMEM containing 10% FBS and 10 μg/mL insulin for the next 2 days. Then, cells were maintained in DMEM containing 10% FBS only to the end of differentiation for 4 days. The fresh medium was changed every 48 hours. For the fatty acid treatment, 2-day MDI induced 3T3L1 cells were then maintained in DMEM with 10% FBS containing each fatty acid and the fresh fatty acid media was changed every 24 h. All the fatty acids were freshly prepared from the stock solution and diluted with growth medium at the indicated concentrations every 24 h.

2.4 Oil Red O staining

After 48 h of the MDI differentiation, 3T3-L1 cells were treated with the agonists or inhibitors for 4 d. Then, the triglyceride accumulation was measured by Oil Red O (ORO) staining. The cells were washed twice with phosphate buffer saline (PBS) gently and fixed in 4% paraformaldehyde for 20 min. Then, cells were washed twice with PBS again and stained with 0.5% Oil Red O diluted in ddH_2O for 15 min. After staining, cells were washed thrice with PBS and photographed by using the microscope (ECLIPSE Ti, Nikon, Japan).

2.5 Triglyceride assay

The cells were rinsed twice with PBS and lysed in tissue lysis buffer (Applygen, Beijing, China). The triglyceride was quantified according to the manufacturer's instructions using a triglyceride measure kit (Applygen, Beijing, China) and normalized to the total protein with the bicinchoninic acid protein assay kit (Beyotime, Nanjing, China).

2.6 Western blotting

After 48 h of the MDI differentiation, 3T3-L1 cells were treated with the agonists or inhibitors for 2 d. Then, the cells were collected for western blotting. Western blotting analysis was used to measure the phosphorylation of ERK1/2 and protein abundance of PPARγ. Cells were washed with cold PBS and lysed in buffer (Beyotime, Nanjing, China). After 30 min extraction at 4°C, samples were then centrifuged at 10000 g for 10 min at 4°C, and the supernatant containing the total proteins was collected. The supernatant was subjected to SDS-PAGE and immunoblotting. Twenty mg of proteins/lane was separated on a 10% polyacrylamide, precast SDS gel (Bio-Rad) followed by transferring onto PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked for 2 h with 5% skim milk powder (Sigma) and incubated overnight with the anti-PPARγ, anti-phosphorylated ERK1/2, anti-β actin and anti-tubulin at a 1:2000 dilution. After

3 washes, the secondary antibody was added at a 1:10000 dilution and incubated at room temperature for 1 h. After 3 washes, the membrane was exposed by using WesternBrigh™ Peroxide (Advansta, California, USA) in imaging system (Carestream, New York, USA). The protein amount was normalized with the amount of tubulin or β-actin as internal control.

2.7 Real-time quantitative PCR

Total RNA was isolated and purified using RNA extraction kit (GeneMark, Taiwan, China) and transcribed into cDNA by the first strand cDNA synthesis kit (TOYOBO, Japan). The cDNA was diluted into 20 times for Real-time quantitative PCR (qPCR) and the samples were run in 10 μL reaction system with SYBR GREEN qPCR mix (BIO-RAD, USA). The data were detected using a Bio-Rad CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Richmond, CA, USA). Gene expression levels were calculated after normalization to the standard housekeeping gene *β-actin* using the ΔΔCT method. In brief, the mean of the triplicate cycle thresholds (CT) of the target gene (*GPR120, PPARγ* and *aP2*) was normalized to the mean of triplicate CT of the reference *β-actin* using the calculation formula "$2^{CT_{\beta\text{-actin}}-CT_{\text{target gene}}}$", which indicated a relative value as a fraction of the target gene. The following primers for *GPR120, PPARγ* and *aP2* of mouse and pig (shown in table1) were synthesized from Sangon (China).

2.8 Lentivirus generation and 3T3-L1 transduction

Lentivirus generation was performed as described in previous report (Aguilo et al., 2015). In brief, lentiviruses were generated in HEK293T cells by Lipofectamine™ 2000-mediated co-transfection of pLKO.1-based shRNAs targeting GPR120 or scramble plasmids, psPAX and pMD2G. Lentvirus supernatants were filtered through 0.45 μM filter (Millipore). For infection, 3T3-L1 preadipocytes were transduced with lentiviruses in 5% FBS growth media supplemented with polybrene (8μg/mL). Cells were incubated overnight with lentiviruses and cultured in fresh 5% FBS growth media for another 3 days. Subsequently, cells were cultured in the 10% FBS growth media supplemented with 2 μg/mL puromycin for 4 days to establish the shGPR120 or shScramble stable cells. The culture medium was replaced every 2 days. All the shRNAs used in the present study are listed in Table1.

Table 1 Primers used for Polymerase Chain Reaction and shRNA sequences for lentiviruses

Gene	Primer sequences(5' to 3')	Product size(bp)	Annealing temperature(℃)

mouse GPR120	*CCATCCCTCTAGTGCTCGTC* *TGCGGAAGAGTCGGTAGTCT*	*522*	*60*
mouse PPARγ2	*TGGGTGAAACTCTGGGAGATTC* *AGAGGTCCACAGAGCTGATTCC*	*150*	*60*
mouse aP2	*GATGCCTTTGTGGGAACCTG* *TCCTGTCGTCTGCGGTGATT*	*232*	*60*
mouse β-actin	*CCAGGTCATCACCATCGG* *CCGTGTTGGCGTAGAGGT*	*133*	*60*
pigGPR120	*AAGGAGGAGGCTCACGATG* *TGACAAATAGATGCCGATAGAC*	*407*	*59*
pigPPARγ	*AGAGTATGCCAAGAACATCC* *AGGTCGCTGTCATCTAATTC*	*261*	*60*
pigaP2	*AAGTCAAGAGCACCATAACC* *GATACATTCCACCACCAACT*	*119*	*60*
pigβ-actin	*CCAGGTCATCACCATCGG* *CCGTGTTGGCGTAGAGGT*	*158*	*60*
pLKO.1-puro-GPR120-shRNA	*GCACCCACTTCCCTTTCTTCT*	*–*	*–*
pLKO.1-puro-Scramble-shRNA	*TTCTCCGAACGTGTCACGT*	*–*	*–*

149

150 **2.9 Luciferase Reporter Assays**

151 For luciferase reporter assays, the HEK293T cells were plated into 24-well plates. Then the
152 peroxisome proliferator activated receptor γ response element (PPRE) luciferase vector combined
153 with renilla luciferase expression plasmid (pTK) were co-transfected with the expression vectors
154 for GPR120 or pcDNA3.1 control plasmid. Then, 24 h after the transfection, the cells were
155 washed twice with DMEM without FBS and maintained in DMEM for at least 18 h for luciferase
156 analysis. Then cells were treated with the appropriate ligand for 6 h. After treatment, cells were
157 harvested and luciferase activity in cell extracts was determined using a luciferase assay system
158 according to standard methods in a Dual-GLO® reporter assay system (Progema, Madison, USA).
159 Luciferase values were normalized by the Renilla values. Transfection experiments were
160 performed in duplicate and repeated for at least three times.

161 **2.10 Real-time measurement of intracellular calcium**

162 Intracellular calcium level was measured by using a Synergy2 microplate reader (BioTek, Gene

Company Limited, USA) as described previously (Mora et al., 2002). Briefly, 2-day differentiated 3T3-L1 cells were seeded into 96 well assay plate with black side and clear bottom and incubated for 24 h. Then, the culture medium was replaced by DMEM supplemented with 1% endotoxin-free bovine serum albumin and maintained for 2 h. 4 μM Fluo8-AM (AAT Bioquest, Sunnyvale, CA, USA) was added into cells for 30 min in the incubator and the cells were washed twice with preheated DMEM following by balancing in 37℃ for another 30 min before measurement. After the stimulation with the positive agonist of intracellular calcium, namely thapsigargin (TG), or three concentration gradients of TUG-891(10, 25 and 100 μM), the data were recorded at 37℃ every 5 s over a 60 s time range.

2.11 Statistical analysis

Data were presented as means ± standard deviation and analyzed by GLM procedures followed by Tukey's post-hoc test. All statistical analyses were completed using the SAS statistical package (v 8.2, SAS Inst., Inc., Cary, NC). *P* values <0.05 were indicated as significant difference.

3. Results

3.1 GPR120 knockdown decreased adipogenesis in 3T3-L1 cells

To test whether the fatty acid receptor GPR120 plays a role in adipogenesis, the mRNA expression profile of *GPR120* was detected using qPCR at the indicated time points (0, 0.5, 1, 2, 4, 5, 6 day) during the adipogenesis of the 3T3-L1 cells. As shown in Fig. 1A, the results indicated that *GPR120* was weakly expressed at the beginning of adipogenic differentiation. Upon the induction of differentiation, the expression of *GPR120* and the adipogenic marker genes (*PPARγ2* and *aP2*) was dramatically up-regulated at 2 days and was high in mature 3T3-L1 cells. Besides, similar expression patterns of *GPR120*, *PPARγ* and *aP2* were also observed in the differentiating porcine stromal vascular fraction (SV) cells (supplemental Fig. 1A).

Compared with in the lentivirus-mediated shControl 3T3-L1 cells, the adipogenic ability was significantly inhibited in the lentivirus-mediated shGPR120 cells. After 6 days of differentiation, the mRNA levels of *PPARγ2* and *aP2* were lower in shGPR120 transfected cells than in shControl transfected cells. The protein level of PPARγ was also lower in the shGPR120 transfected cells (Fig.1B to D).

3.2 Effect of natural polyunsaturated fatty acids on adipogenesis in 3T3-L1 cells

Based on the expression pattern of *GPR120* in adipogenic progress of 3T3L1 cells, the 2-day MDI induced 3T3L1 cells were treated with several natural polyunsaturated fatty acids which are the ligands of GPR120. As shown in Fig. 2A, C and E, DHA, ALA and LA were tested for their abilities to influence adipogenesis in 3T3-L1 cells. Compared with the control group, DHA dose-dependently decreased the percentage of lipid droplets at day 8, while ALA treatments resulted in more abundant lipid accumulation with the increase of concentration. However, the treatment with LA did not show any effect. The protein level of PPARγ was measured synchronously, which was shown to be closely correlated with the phenotype of Oil Red O straining (Fig. 2 B, D and F). The 3×PPRE luciferase reporter assay was used to further clarify the activity of PPARγ in HEK293T cells. As shown in supplemental Fig. 2, DHA and ALA, but not LA, could well activate PPARγ.

3.3 GPR120 was related to the pro-adipogenic function of ALA

To determine the involvement of GPR120 in mediating the pro-adipogenic effect of ALA, lentivirus-mediated shGPR120 3T3L1 cells were established to knock down the expression of

GPR120 in 3T3-L1 adipocytes. As seen in ORO staining cells, ALA did not affect lipid accumulation of 3T3L1 adipocytes at any concentrations. There was also no different effect on the expression of PPARγ at either mRNA or protein level (Fig. 3). These results implied the function of GPR120 in the pro-adipogenic function of ALA.

3.4 Synthetic agonist TUG-891 promoted adipogenesis in a GPR120-dependant manner

To verify the precise function of GPR120 on adipogenesis, the potent and selective agonist TUG-891 was used to activate GPR120 in the 2-day differentiated 3T3-L1 cells, and then triglyceride accumulation and expression of PPARγ and aP2 were determined in 6-day differentiated 3T3-L1 cells. As shown by the oil red O staining images, the triglyceride accumulation in the 6-day differentiated 3T3-L1 cells was significantly improved with the increase of TUG-891 concentration (0 to 100 μM) (Fig. 4A). In addition, 10 and 100 μM TUG-891 treatment significantly increased mRNA and protein expression of PPARγ in 6-day differentiated 3T3-L1 cells (Fig. 4B and C). Similar effect of TUG-891 on triglyceride accumulation was also observed in the differentiated porcine SV cells (supplemental Fig. 1B).

In the differentiated shGPR120 3T3-L1 cells, TUG-891 did not enhance the triglyceride accumulation and the mRNA levels of *PPARγ2* and *aP2* (Fig 4D). Furthermore, luciferase reporter assay was performed to investigate whether TUG-891 and ALA increased the transcriptional activity of PPARγ. The 3×PPRE-luc and porcine GPR120 overexpression plasmid or empty pcDNA3.1 plasmid were co-transfected into HEK293T cells, and then the luciferase activity was measured after agonist stimulation. Fig. 4E showed that in both GPR120 and empty plasmid transfected cells, the luciferase activity in 10 μM rosiglitazone (Rosi) or 100 μM ALA treatment was greater than that in DMSO treatment (control). As shown in Fig. 4F, TUG-891 significantly increased the luciferase activity in the cells transfected with porcine GPR120 in a dose-dependent manner. However, TUG-891 failed to increase luciferase activity in the cells transfected with empty plasmid.

3.5 Effect of GPR120 on the activation of intracellular calcium [Ca^{2+}]i and ERK1/2 signaling

To test whether GPR120 activation induced intracellular calcium mobilization and ERK1/2 activation, 2-day differentiated 3T3-L1 cells were treated with TUG-891, and the [Ca^{2+}]i and phosphorylation level of ERK1/2 were determined. As expected, thapsigargin (TG) treatment

253 significantly increased the $[Ca^{2+}]i$ level as a positive control, whereas the basal group treated with
254 basic media did not show any change in $[Ca^{2+}]i$ level. Moreover, stimulation of 10, 25 and 100
255 μM TUG-891 transiently increased the $[Ca^{2+}]i$ levels in the 2-day differentiated 3T3-L1 cells in a
256 concentration-dependent manner (Fig. 5A). In addition, the 2-day differentiated 3T3-L1 cells
257 stimulated with 100 μM TUG-891 for 5-15 min induced a pronounced increase of ERK1/2
258 phosphorylation (Fig. 5B).

259 Furthermore, a membrane-permeable intracellular calcium chelator (BAPTA-AM) was used to
260 clarify whether the ERK1/2 signaling was in the downstream of $[Ca^{2+}]i$ signaling. As shown in Fig.
261 5C, compared with the untreated cells, the cells treated with BAPTA-AM had lower
262 phosphorylation level of ERK1/2. Additionally, cells pretreated with BAPTA-AM prior to
263 TUG-891 stimulation had a lower phosphorylation level of ERK1/2 compared with those
264 stimulated with TUG-891 alone. In contrast, in the GPR120 knockdown cells, TUG-891 did not
265 increase the phosphorylation level of ERK1/2 (Fig. 5D).
266

267 **3.6 Effect of intracellular calcium on TUG-891-stimulated adipogenesis in 3T3-L1 cells**

268 To clarify whether $[Ca^{2+}]i$ contributes to the adipogenesis induced by GPR120 activation, the
269 2-day differentiated 3T3-L1 cells were treated with TUG-891 and BAPTA-AM respectively or
270 TUG-891 combined with BAPTA-AM for 4 days, and then triglyceride accumulation was
271 measured. As shown in Fig. 6A, compared with untreated cell, the cells treated with TUG-891 had
272 an increased triglyceride accumulation, whereas the cells pretreated with BAPTA-AM showed
273 decreased TUG-891-induced triglyceride accumulation. Compared with the basal treatment, the
274 TUG-891 treatment significantly elevated the mRNA expression levels of *PPARγ2* and *aP2* (Fig.
275 6B) as well as the protein level of PPARγ (Fig. 6C). Moreover, this elevating effect of TUG-891
276 was attenuated by pre-treatment with BAPTA-AM. In addition, incubation of BAPTA-AM alone
277 notably decreased the luciferase activity in both GPR120 and empty plasmid transfected
278 HEK293T cells. Moreover, pre-treatment of GPR120 transfected cells with BAPTA-AM
279 abolished the increase of luciferase activity induced by TUG-891 (Fig. 6D).
280

281 **3.7 Effect of ERK1/2 on TUG-891-stimulated adipogenesis in 3T3-L1 cells**

282 ERK1/2 signal might be responsible for the adipogenic effect in the downstream of $[Ca^{2+}]i$.

To identify the role of ERK1/2 in GPR120-induced adipogenesis, the 2-day differentiated 3T3-L1 cells were treated with TUG-891 and U0126 respectively or TUG-891 combined with U0126 for 2 days to measure PPARγ expression and for 4 days to determine triglyceride accumulation. As shown in Fig. 7A, pretreatment with U0126 reduced triglyceride accumulation and declined the acceleration of TUG-891 on adipogenesis. Pre-incubated with U0126 abolished TUG-891-induced increase in ERK1/2 phosphorylation. TUG-891 did not promote the phosphorylation level of ERK1/2 in the GPR120 knockdown cells (Fig. 7B). Moreover, U0126 pretreatment also dramatically restored the increased mRNA levels of *PPARγ2* and *aP2* and the protein expression of PPARγ due to TUG-891 stimulation (Fig. 7C and D). In addition, pre-treatment of GPR120 transfected cells with U0126 attenuated the increase of luciferase activity induced by TUG-891 (Fig. 7E).

4. Discussion

Until now, there have been increasing evidences demonstrating that the adipose tissue has profound effects on glucose and energy homeostasis (Rosen and Spiegelman, 2014). The adipogenesis process is regulated by a large body of factors ranging from the extracellular space to the nuclear depot and involves a complex and orchestrated program of adiposity-related gene expression (Otto and Lane, 2005; Tang and Lane, 2012). Several studies have demonstrated that GPR120 may function as a novel fatty acid sensor/receptor and play a notable role in regulating obesity both in vivo and in vitro (Gotoh et al., 2007; Ichimura et al., 2012; Oh et al., 2010). Understanding the role and underlying mechanism of GPR120 in adipogenesis will undoubtedly facilitate the understanding of the relationship between selective fatty acids and obesity and obesity-related disease.

The 3T3-L1 preadipocyte is a good model in vitro for characterizing preadipocyte differentiation and faithfully recapitulating the adipogenic step from preadipocytes to adipocytes (Tang and Lane, 2012). By using RNAi, Gotoh et al. reported that GPR120 knock-down in 3T3-L1 cells inhibited adipogenesis. In the present study, the adipogenic ability was significantly inhibited in shGPR120 transfected cells, indicating that GPR120 may play a role in adipogenesis. An earlier study has shown that high expression of GPR120 was detected in four kinds of mouse fat tissues and the differentiated adipocytes but not in preadipocytes (Gotoh et al., 2007; Miyauchi et al., 2009). In this study, we found that the mRNA level of GPR120 was undetectable in the initial stage of differentiation but was dramatically increased after 2-day induction of 3T3-L1 cells. Therefore, the 2 day-differentiated 3T3-L1 cells were incubated with natural ligands and a synthetic agonist (TUG-891) (Shimpukade et al., 2012) to clarify the role of GPR120 in the adipogenesis.

As natural ligands of GPR120, several long chain polyunsaturated fatty acids (Lc-PUFAs) have been demonstrated to modulate the 3T3-L1 adipogenic differentiation. However, the effects of the Lc-PUFAs are inconclusive. DHA is shown to inhibit, promote or even not affect the differentiation of preadipocytes to adipocytes like other fatty acids (Barber et al., 2013; Casado-Díaz et al., 2013; Kim et al., 2006; Murali et al., 2014; Wang et al., 2016). In our experiment, similar to the study of Kim et al. (2006) (Kim et al., 2006), DHA could significantly

inhibit the lipid accumulation and the protein level of PPARγ in a dose-dependent manner from the 2 days after MDI-induction to the termination of differentiation. On the other hand, DHA may suppress lipid droplet formation and increase lipolysis by up-regulating adipose triglyceride lipase (ATGL) (Barber et al., 2013). Unlike the results of Kim et al. (2006), 3T3-L1 cells pretreated with DHA for 24 h before the MDI-induction showed pro-adipogenic phenotype due to the DHA treatment (Murali et al., 2014). In addition, by using the luciferase reporter system, DHA was demonstrated as a natural ligand of PPARγ in HEK293T cells in the present study. Wang et al. (2016) reported that the enhancement of adipogenesis in mouse adipose tissue-derived stromal cells (ADSC) was observed in both ALA and LA treatments (Wang et al., 2016). In our results, only ALA could promote adipogenesis by activating PPARγ. The luciferase reporter experiments showed that the ALA, not LA, can well activate PPARγ as reported by others (Lecka-Czernik et al., 2002). Furthermore, ALA cannot improve adipogenesis in the GPR120-knockdown cells, suggesting that GPR120 may be related to the improvement of adipogenesis by ALA. Based on the different effects of fatty acids, we therefore chose the synthetic agonist TUG-891 (Shimpukade et al., 2012) to study the signaling mediated by GPR120 in adipogenesis to avoid other potential GPR120-independent mechanisms which are involved in the role of natural ligands in the present study.

We showed that TUG-891 promoted adipogenic progress in both 3T3-L1 and porcine SV cells. Although GPR40 may be also activated by TUG-891 in murine cells, it is not expressed in the murine preadipocytes and adipocytes from the adipose tissues (Gotoh et al., 2007). Both in 3T3-L1 preadipocytes and adipocytes, the mRNA expression level of GPR40 was low but the expression of GPR120 was shown to be significantly increased during the differentiation of predipocytes to mature adipocytes (Oh et al., 2010). Additionally, we have previously reported that porcine GPR120 shares a higher homology with human GPR120 compared with mouse GPR120, and TUG-891 is a potent agonist for porcine GPR120 (Song et al., 2015).

TUG-891 increased both mRNA and protein expression of PPARγ, indicating that GPR120 might promote adipogenesis via PPARγ. However, it should be noted that PPARγ and GPR120 have similar ligand binding pockets and share common natural agonists including eicosapentaenoic acid and DHA (Gim et al., 2013; Hudson et al., 2014; Suzuki et al., 2008). It has been demonstrated that the agonist of PPARγ also increases the expression of GPR120 in 3T3-L1

cells (Gotoh et al., 2007). Although TUG-891 is a potent agonist for GPR120, no evidence is available for the relationship between TUG-891 and PPARγ. Therefore, it is interesting to clarify whether the dose of TUG-891 used in the present study can activate PPARγ directly. By using 3× PPRE-luc reporter assay, we showed that TUG-891 increased the activation of PPARγ in HEK293T cell transfected with GPR120-pcDNA3.1 but not with empty plasmid. The expression of GPR120 in HEK293T cells was negligible and the synthetic agonists cannot activate GPR120 signaling in HEK293T cells (Briscoe et al., 2006; Song et al., 2015). Thus, these results suggested that the responses observed were indeed mediated by GPR120, which excluded the possibility of TUG-891 to activate PPARγ directly.

The intracellular signaling that mediates the adipogenesis induced by GPR120 still remains largely unknown. In the present study we showed that TUG-891 induced the activation of $[Ca^{2+}]i$ and ERK1/2 signaling in 3T3-L1 cells. Moreover, incubation of TUG-891 combined with BAPTA-AM or U0126 abolished TUG-891-induced adipogenesis. From our results, we speculate that $[Ca^{2+}]i$ and ERK1/2 signaling are involved in GPR120-induced adipogenesis. Activation of ERK1/2 is likely to facilitate adipogenesis at the mitotic clonal expansion (MCE) stage, but block adipogenic gene expression in later stage of adipogenesis (Bost et al., 2005a; Burgermeister and Seger, 2007; Prusty et al., 2002). MCE is a required phase in the adipogenic program (Otto and Lane, 2005; Tang and Lane, 2012). The differentiation of 3T3-L1 cells has two rounds of MCE. The first round is finished in 24-36 h after adipogenic induction and another round is completed in 48-60 h, which results in an increase >2-fold in cell number at day 2 (Kim et al., 2007; Tang et al., 2003). In addition, there is evidence suggesting that the $[Ca^{2+}]i$ appears to exert a biphasic regulatory function in the preadipocyte differentiation. Both in mice and human preadipocyte cells, the increase of $[Ca^{2+}]i$ in early stages before 2 days suppresses the adipogenic differentiation, whereas it promotes adipogenic differentiation in the later stage (Liu and Clipstone, 2007; Mora et al., 2002; Neal and Clipstone, 2002; Ntambi and Takova, 1996). In 3T3-L1 cells, GPR120 was expressed from 2 days after adipogenic induction, which is the beginning of the second round of MCE. These evidences indicate that the activation of $[Ca^{2+}]i$ and ERK1/2 by the TUG-891 is favorable for the following terminal differentiation. Recently, a research group reported that GPR120 is activated by low concentration of TUG-891 to promote the adipogenesis of bone marrow mesenchymal stem cells (BMMSC), while higher concentration of TUG-891 fails to

facilitate BMMSC adipogenesis (Gao et al., 2015). In this study, 3T3-L1 preadipocyte is known as a determined adipogenic cell line, which is different from BMMSC. Moreover, GPR120 was only slightly expressed at the beginning of the differentiation of 3T3-L1 preadipocytes. These phenomena may result in diverse mechanisms of GPR120 in regulating the adipogenesis of stem cells and preadipocytes.

Both our results and those from previous studies of other groups suggest that GPR120 is required for normal adipogenesis (Gotoh et al., 2007; Ichimura et al., 2012). The expression of GPR120 was significantly increased in both subcutaneous (SAT) and visceral (VAT) adipose tissues in the obese individual (Ichimura et al., 2012). However, another group reported that morbidly obese individuals have lower expression of GPR120 protein and mRNA in VAT than lean humans (Rodriguez-Pacheco et al., 2014). That is the discrepancy between studies (Ulven and Christiansen, 2015). In fact, GPR120 plays an important role in systemic homeostasis (Oh et al., 2010; Oh et al., 2014). The GPR120-deficient mice are easy to develop obesity and other obesity metabolic problems with decreased adipocyte adipogenesis and promoted hepatic lipogenesis than the wild type mice (Ichimura et al., 2012). Besides, it seems that the GPR120 expressed in mature adipocytes might induce the production of C16:1n7 palmitoleate, which has been proposed to be a lipid hormone (Cao et al., 2008), to regulate systemic metabolic homeostasis (Ichimura et al., 2012). Chen et al. have reported that C/EBPβ plays a critical role in the regulation of GPR120 expression in 3T3-L1 cells (Chen et al., 2016). It is interesting to know whether C/EBPβ mediates the regulation of fatty acids on GPR120 expression. These results suggest the association between GPR120 and obesity and imply that the role of GPR120 in metabolic regulation is still not fully clarified (Ulven and Christiansen, 2015).

In summary, our data demonstrate that synthetic agonist TUG-891-induced GPR120 activation in 2-day differentiated 3T3-L1 adipocytes increases the expression of adipogenic master regulator PPARγ, resulting in the increased expression of lipogenic marker genes and stimulated triglyceride accumulation. GPR120-mediated adipogenesis is transduced via at least one vital $[Ca^{2+}]$i-ERK1/2 pathway. Our results for the first time demonstrate the signaling involved in GPR120-induced adipogenesis in 3T3-L1 adipocytes. However, further studies are still needed to elucidate the precise mechanism. GPR120 has been proposed as a potential target to treat obesity-related inflammation and diabetes. For the abundant expression of GPR120 in fat tissues

(Gotoh et al., 2007), studying the role and mechanism of GPR120 in adipogenesis, interaction between adipocytes and macrophage and production of adiponectin will improve the understanding of the physiological processes manipulated by selective fatty acids and help the development of GPR120-targeted compound to treat obesity related disease.

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Conflict of interest

The corresponding author states that there is no conflict of interest.

References

Aguilo, F., Zhang, F., Sancho, A., Fidalgo, M., Di Cecilia, S., Vashisht, A., Lee, D.F., Chen, C.H., Rengasamy, M., Andino, B.*, et al.* (2015). Coordination of m(6)A mRNA Methylation and Gene Transcription by ZFP217 Regulates Pluripotency and Reprogramming. Cell Stem Cell *17*, 689-704.

Barber, E., Sinclair, A.J., and Cameron-Smith, D. (2013). Comparative actions of omega-3 fatty acids on in-vitro lipid droplet formation. Prostaglandins, leukotrienes, and essential fatty acids *89*, 359-366.

Bost, F., Aouadi, M., Caron, L., and Binetruy, B. (2005a). The role of MAPKs in adipocyte differentiation and obesity. Biochimie *87*, 51-56.

Bost, F., Aouadi, M., Caron, L., Even, P., Belmonte, N., Prot, M., Dani, C., Hofman, P., Pages, G., Pouyssegur, J.*, et al.* (2005b). The extracellular signal-regulated kinase isoform ERK1 is specifically required for in vitro and in vivo adipogenesis. Diabetes *54*, 402-411.

Briscoe, C.P., Peat, A.J., McKeown, S.C., Corbett, D.F., Goetz, A.S., Littleton, T.R., McCoy, D.C., Kenakin, T.P., Andrews, J.L., and Ammala, C. (2006). Pharmacological regulation of insulin secretion in MIN6 cells through the fatty acid receptor GPR40: identification of agonist and antagonist small molecules. Brit J Pharmacol *148*, 619-628.

Burgermeister, E., and Seger, R. (2007). MAPK kinases as nucleo-cytoplasmic shuttles for PPAR gamma. Cell Cycle *6*, 1539-1548.

Cao, H.M., Gerhold, K., Mayers, J.R., Wiest, M.M., Watkins, S.M., and Hotamisligil, G.S. (2008). Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism. Cell *134*, 933-944.

Casado-Díaz, A., Santiago-Mora, R., Dorado, G., and Quesada-Gómez, J. (2013). The omega-6 arachidonic fatty acid, but not the omega-3 fatty acids, inhibits osteoblastogenesis and induces adipogenesis of human mesenchymal stem cells: potential implication in osteoporosis. Osteoporosis International *24*, 1647-1661.

Chen, K., Zhou, J.D., Zhang, F., Zhang, F., Zhang, R.R., Zhan, M.S., Tang, X.Y., Deng, B., Lei, M.G., and Xiong, Y.Z. (2016). Transcription factor C/EBPbeta promotes the transcription of the porcine GPR120 gene. J Mol Endocrinol *56*, 91-100.

Cristancho, A.G., and Lazar, M.A. (2011). Forming functional fat: a growing understanding of adipocyte differentiation. Nat Rev Mol Cell Bio *12*, 722-734.

Gao, B., Huang, Q., Jie, Q., Lu, W.G., Wang, L., Li, X.J., Sun, Z., Hu, Y.Q., Chen, L., Liu, B.H.*, et al.* (2015). GPR120: A bi-potential mediator to modulate the osteogenic and adipogenic differentiation of BMMSCs. Sci Rep *5*, 14080.

Gim, H.J., Li, H., Lee, E., Ryu, J.H., and Jeon, R. (2013). Design and synthesis of alkoxyindolyl-3-acetic acid analogs as peroxisome proliferator-activated receptor-gamma/delta agonists. Bioorg Med Chem Lett *23*, 513-517.

Gotoh, C., Hong, Y.H., Iga, T., Hishikawa, D., Suzuki, Y., Song, S.H., Choi, K.C., Adachi, T., Hirasawa, A., Tsujimoto, G.*, et al.* (2007). The regulation of adipogenesis through GPR120. Biochem Bioph Res Co *354*, 591-597.

Hirasawa, A., Tsumaya, K., Awaji, T., Katsuma, S., Adachi, T., Yamada, M., Sugimoto, Y., Miyazaki, S., and Tsujimoto, G. (2005). Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nat Med *11*, 90-94.

Hudson, B.D., Shimpukade, B., Mackenzie, A.E., Butcher, A.J., Pediani, J.D., Christiansen, E., Heathcote, H., Tobin, A.B., Ulven, T., and Milligan, G. (2013). The Pharmacology of TUG-891, a Potent and Selective Agonist of the Free Fatty Acid Receptor 4 (FFA4/GPR120), Demonstrates Both Potential Opportunity and Possible Challenges to Therapeutic Agonism. Mol Pharmacol *84*, 710-725.

Hudson, B.D., Shimpukade, B., Milligan, G., and Ulven, T. (2014). The Molecular Basis of Ligand Interaction at Free Fatty Acid Receptor 4 (FFA4/GPR120). J Biol Chem *289*, 20345-20358.

Ichimura, A., Hirasawa, A., Poulain-Godefroy, O., Bonnefond, A., Hara, T., Yengo, L., Kimura, I., Leloire, A., Liu, N., Iida, K.*, et al.* (2012). Dysfunction of lipid sensor GPR120 leads to obesity in both mouse and human. Nature *483*, 350-U149.

Katsuma, S., Hatae, N., Yano, T., Ruike, Y., Kimura, M., Hirasawa, A., and Tsujimoto, G. (2005). Free fatty acids inhibit serum deprivation-induced apoptosis through GPR120 in a murine enteroendocrine cell line STC-1. J Biol Chem *280*, 19507-19515.

Kershaw, E.E., and Flier, J.S. (2004). Adipose tissue as an endocrine organ. J Clin Endocr Metab *89*, 2548-2556.

Kim, H.-K., Della-Fera, M., Lin, J., and Baile, C.A. (2006). Docosahexaenoic acid inhibits adipocyte differentiation and induces apoptosis in 3T3-L1 preadipocytes. The Journal of nutrition *136*, 2965-2969.

Kim, K.A., Kim, J.H., Wang, Y., and Sul, H.S. (2007). Pref-1 (Preadipocyte factor 1) activates the MEK/extracellular signal-regulated kinase pathway to inhibit adipocyte differentiation. Mol Cell Biol *27*, 2294-2308.

Lecka-Czernik, B., Moerman, E.J., Grant, D.F., Lehmann, J.M., Manolagas, S.C., and Jilka, R.L. (2002). Divergent effects of selective peroxisome proliferator-activated receptor-gamma 2 ligands on adipocyte versus osteoblast differentiation. Endocrinology *143*, 2376-2384.

Liu, L., and Clipstone, N.A. (2007). Prostaglandin F2 alpha inhibits adipocyte differentiation via a G alpha q-calcium-calcineurin-dependent signaling pathway. J Cell Biochem *100*, 161-173.

Miyauchi, S., Hirasawa, A., Iga, T., Liu, N., Itsubo, C., Sadakane, K., Hara, T., and Tsujimoto, G. (2009). Distribution and regulation of protein expression of the free fatty acid receptor GPR120. N-S Arch Pharmacol *379*, 427-434.

Mora, S., Durham, P.L., Smith, J.R., Russo, A.F., Jeromin, A., and Pessin, J.E. (2002). NCS-1 inhibits insulin-stimulated GLUT4 translocation in 3T3L1 adipocytes through a phosphatidylinositol 4-kinase-dependent pathway. J Biol Chem *277*, 27494-27500.

Murali, G., Desouza, C.V., Clevenger, M.E., Ramalingam, R., and Saraswathi, V. (2014). Differential effects of eicosapentaenoic acid and docosahexaenoic acid in promoting the differentiation of 3T3-L1 preadipocytes. Prostaglandins, leukotrienes, and essential fatty acids *90*, 13-21.

Neal, J.W., and Clipstone, N.A. (2002). Calcineurin mediates the calcium-dependent inhibition of adipocyte differentiation in 3T3-L1 cells. J Biol Chem *277*, 49776-49781.

Ntambi, J.M., and Takova, T. (1996). Role of Ca2+ in the early stages of murine adipocyte differentiation as evidenced by calcium mobilizing agents. Differentiation; research in biological diversity *60*, 151-158.

Oh, D.Y., Talukdar, S., Bae, E.J., Imamura, T., Morinaga, H., Fan, W.Q., Li, P.P., Lu, W.J., Watkins, S.M., and Olefsky, J.M. (2010). GPR120 Is an Omega-3 Fatty Acid Receptor Mediating Potent Anti-inflammatory and Insulin-Sensitizing Effects. Cell *142*, 687-698.

Oh, D.Y., Walenta, E., Akiyama, T.E., Lagakos, W.S., Lackey, D., Pessentheiner, A.R., Sasik, R., Hah, N., Chi, T.J., Cox, J.M.*, et al.* (2014). A Gpr120-selective agonist improves insulin resistance and chronic inflammation in obese mice. Nat Med *20*, 942-947.

Otto, T.C., and Lane, M.D. (2005). Adipose development: From stem cell to adipocyte. Crit Rev Biochem Mol *40*, 229-242.

Prusty, D., Park, B.H., Davis, K.E., and Farmer, S.R. (2002). Activation of MEK/ERK signaling promotes adipogenesis by enhancing peroxisome proliferator-activated receptor gamma (PPAR gamma) and C/EBP alpha gene expression during the differentiation of 3T3-L1 preadipocytes. J Biol Chem *277*, 46226-46232.

Rodriguez-Pacheco, F., Garcia-Serrano, S., Garcia-Escobar, E., Gutierrez-Repiso, C., Garcia-Arnes, J., Valdes, S., Gonzalo, M., Soriguer, F., Moreno-Ruiz, F.J., Rodriguez-Canete, A.*, et al.* (2014). Effects of obesity/fatty acids on the expression of GPR120. Mol Nutr Food Res *58*, 1852-1860.

Rosen, E.D., Sarraf, P., Troy, A.E., Bradwin, G., Moore, K., Milstone, D.S., Spiegelman, B.M., and Mortensen, R.M. (1999). PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. Molecular cell *4*, 611-617.

Rosen, E.D., and Spiegelman, B.M. (2014). What We Talk About When We Talk About Fat. Cell *156*, 20-44.

Shi, H., Halvorsen, Y.D., Ellis, P.N., Wilkison, W.O., and Zemel, M.B. (2000). Role of intracellular calcium in human adipocyte differentiation. Physiological genomics *3*, 75-82.

Shimpukade, B., Hudson, B.D., Hovgaard, C.K., Milligan, G., and Ulven, T. (2012). Discovery of a potent and selective GPR120 agonist. Journal of medicinal chemistry *55*, 4511-4515.

Song, T., Peng, J., Ren, J., Wei, H.K., and Peng, J. (2015). Cloning and characterization of spliced variants of the porcine G protein coupled receptor 120. BioMed research international *2015*, 813816.

Suzuki, T., Igari, S.I., Hirasawa, A., Hata, M., Ishiguro, M., Fujieda, H., Itoh, Y., Hirano, T., Nakagawa, H., Ogura, M.*, et al.* (2008). Identification of G protein-coupled receptor 120-selective agonists derived

from PPAR gamma agonists. Journal of medicinal chemistry *51*, 7640-7644.
Tang, Q.Q., and Lane, M.D. (2012). Adipogenesis: From Stem Cell to Adipocyte. Annu Rev Biochem *81*, 715-736.
Tang, Q.Q., Otto, T.C., and Lane, M.D. (2003). Mitotic clonal expansion: A synchronous process required for adipogenesis. P Natl Acad Sci USA *100*, 44-49.
Ulven, T., and Christiansen, E. (2015). Dietary fatty acids and their potential for controlling metabolic diseases through activation of FFA4/GPR120. Annual review of nutrition *35*, 239-263.
Wang, J., Liang, Y., Jian, L., Zhang, J., Liang, S., Xiao, S., Liu, B., and Wang, H. (2016). Linoelaidic acid enhances adipogenic differentiation in adipose tissue-derived stromal cells through suppression of Wnt/β-catenin signaling pathway in vitro. Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA) *110*, 1-7.

Figures

Fig.1 GPR120 knockdown decreased adipogenesis in 3T3-L1 cells.

The 3T3-L1 cells were induced with normal adipogenic MDI cocktail for 6 days and the cells were harvested at indicated time points. The expression levels of *GPR120* and adipogenic marker genes, *PPARγ2* and *aP2*, were detected by qPCR using mouse β-actin as an internal control (A). The lentivirus-mediated GPR120 knockdown cells were established and MDI-differentiated for 6 days. The triglyceride accumulation was measured by ORO staining with 200× magnification (B). The levels of *GPR120*, *PPARγ2* and *aP2* mRNA were tested by qPCR (C) and the protein level of PPARγ was measured by western blot (D). The results represent mean ± SD of three separate experiments and **P*<0.05, ***P*<0.01, *** *P*<0.001 compared with the control.

Fig.2 Effect of polyunsaturated fatty acids on adipogenesis in 3T3L1 cells.

After 48 h treatment with MDI, 3T3-L1 cells were treated with 10, 100 and 200 μM DHA, ALA, LA for 2 or 4 days. Control cells treated with DMSO alone after 48 h treatment with MDI were included as control. After 4-day treatment, the triglyceride accumulation in cells was measured by ORO staining with 200× magnification (A, C, E). After 2-day treatment, the cells were collected for protein extraction. Proteins were subjected to western blot analysis of PPARγ (B, D, F). The results represent mean ± SD of 2 separate experiments and *P<0.05, **P<0.01, *** P<0.001 compared with the control.

Fig.3 Role of GPR120 in the pro-adipogenic function of ALA

After 48 h treatment with MDI, 3T3-L1 cells were treated with 10, 100 and 200 μM ALA for 2 or 4 days. Control cells treated with DMSO alone after 48 h treatment with MDI were included as

control. After 4-day treatment, the triglyceride accumulation in cells was measured by ORO staining with 200× magnification (A). After 2-day treatment, the cells were collected for total RNA and protein extraction. The gene expression of *PPARγ2* and *aP2* in cells was quantified by qPCR analysis (B). Proteins were subjected to western blot analysis of PPARγ (C). The results represent mean ± SD of 3 separate experiments and *P<0.05, **P<0.01, *** P<0.001 compared with the control.

Fig.4 TUG-891 promoted adipogenesis in a GPR120-dependant manner

After 48 h treatment with MDI, 3T3-L1 cells were treated with 1, 10 and 100 μM TUG-891 or DHA for 2 or 4 days. Control cells treated with DMSO alone after 48 h treatment with MDI were included as control. After 4-day treatment, the triglyceride accumulation in cells treated with TUG-891 (A) was measured by ORO staining with 200× magnification. After 2-day treatment, the cells were collected for total RNA and protein extraction. The gene expression of *PPARγ2* and *aP2* in cells treated with TUG-891 (B, C) was quantified by qPCR analysis. Proteins were subjected to western blot analysis of PPARγ. Pig GPR120 overexpression plasmid or pcDNA3.1 empty plasmid with 3×PPRE luciferase vector was transfected into HEK293T cells. After 18 h serum free starvation, the transfected cells were treated with DMSO (control), 100 μM TUG-891, 100 μM ALA and 10 μM Rosi (D) or 1-100 μM TUG-891(E) for 6h. The luciferase activity was measured from the cell lysis and adjusted by the Renilla values. The results represent mean ± SD of three separate experiments and *P<0.05, **P<0.01, *** P<0.001 compared with the control.

Fig.5 Effect of GPR120 on the activation of intracellular calcium [Ca^{2+}]i and ERK1/2 signaling.

The 3T3-L1 cells at day 2 of differentiation were reseeded from 6-well plate to 96 well assay plate with black side and clear bottom and incubated for 24 h. The cells were loaded with Fluo8-AM as described in methods. Then, cells were stimulated with TG (1.5 μM) or TUG-891(10, 25 and 100 μM) or DMSO (basal) at the time indicated with the red arrow. Cellular basal calcium level was measured for 20 s before the stimulation. The results represent mean ± SEM of three separate experiments (A). The 2-day differentiated 3T3-L1 cells were treated with 100 μM TUG-891 from 0 to 60 min. The phosphorylated ERK1/2 was measured by western blot (B). The 3T3-L1 cells (C) or GPR120 knockdown 3T3-L1 cells (D) at day 2 of differentiation were pre-incubated with 20

μM BAPTA-AM for 30 min, and then TUG-891 was added for an additional 15 min before measuring the expression level of pERK1/2 by western blot. The results are representatives of three separate experiments.

Fig.6 Effect of intracellular calcium [Ca^{2+}]i on the TUG-891-stimulated adipogenesis in 3T3-L1 cells

The 3T3-L1 cells at day 2 of differentiation were pre-incubated with 10 μM BAPTA-AM for 30 min, and then TUG-891 was added for an additional 4 days for ORO staining analysis of triglyceride accumulation (200× magnification), or for an additional 2 days for qPCR analysis of *PPARγ* and *aP2* expression (B) and western blot analysis of PPARγ (C). Pig GPR120 overexpression plasmid or pcDNA3.1 empty plasmid with 3×PPRE luciferase vector was transfected into HEK293T cells and the transfected cells were treated with 100 μM TUG-891, 10 μM BAPTA-AM, or combination of TUG-891 and BAPTA-AM (D) for 6 h after 18 h serum free starvation. The luciferase activity was measured from the cell lysis and adjusted by the Renilla values. The results represent mean ± SD of three separate experiments and $^{**}P<0.01$ compared with the control.

Fig.7 Effect of ERK1/2 on TUG-891-stimulated adipogenesis in 3T3-L1 cells

The 2-day differentiated 3T3-L1 cells were pre-incubated for 30 min with 10 μM U0126, and then TUG-891 was added for an additional 4 days for ORO staining analysis of triglyceride accumulation (200× magnification), or for an additional 2 days for western blot analysis of phosphorylation level of ERK1/2 (B), qPCR analysis of *PPARγ2* and *aP2* expression (C) and western blot analysis of PPARγ (D). Pig GPR120 overexpression plasmid or pcDNA3.1 empty plasmid with 3×PPRE luciferase vector was transfected into HEK293T cells and the transfected cells were treated with 100 μM TUG-891, 10 μM U0126, or combination of TUG-891 and U0126 (D) for 6 h after 18 h serum free starvation. The luciferase activity was measured from the cell lysis and adjusted by the Renilla values. The results represent mean ± SD of three separate experiments and $^{**}P<0.01$ compared with the control.

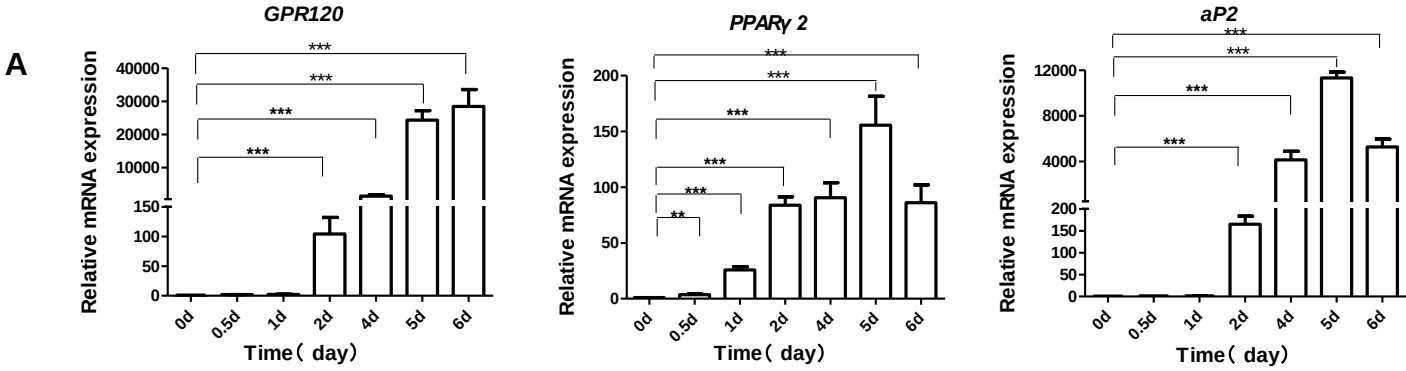
Supplemental Fig. 1 Effect of GPR120 activation by TUG-891 on adipogenesis in pig

primary SV cells.

SV cells were isolated and differentiated as described in methods. Total RNA was extracted at the indicated time points of differentiation and reversed into cDNA. The expression of *GPR120*, *PPARγ* and *aP2* was measured by Q-PCR (A). After 48 h differentiation in MDI cocktail, the SV cells were incubated with 0-100 μM TUG-891 for 6 d and stained by ORO with 200× magnification (B).

Supplemental Fig. 2 Activation of fatty acids on PPARγ by luciferase reporter system.

3×PPRE luciferase vector were transfected into HEK293T cells and the cells were treated with DHA, ALA and LA in the indicated concentrations for 6 h after 18 h serum free starvation. The luciferase activity was measured from the cell lysis and adjusted by the Renilla values. The results represent mean ± SD of three separate experiments and $^{**}P<0.01$ compared with the control.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Relative mRNA expression
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4d
5d
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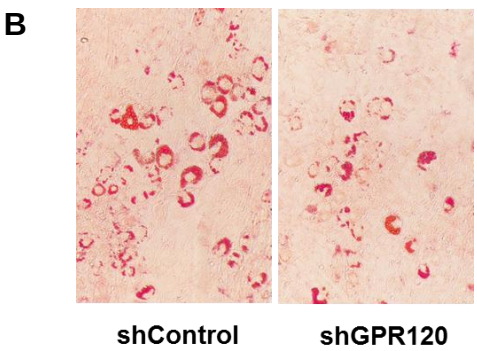
B
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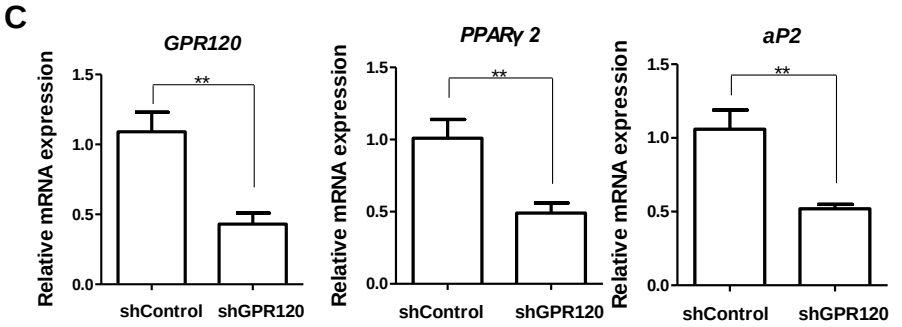
shGPR120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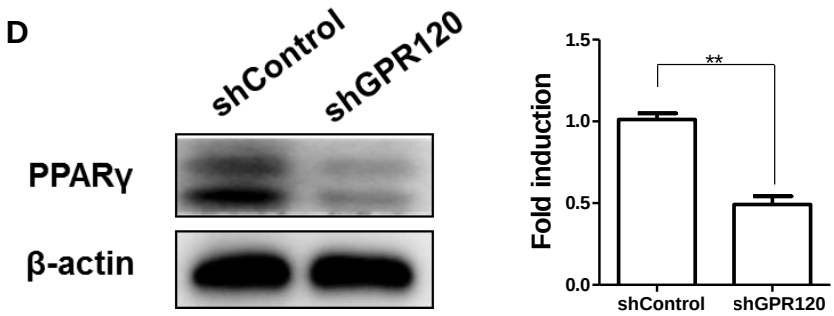
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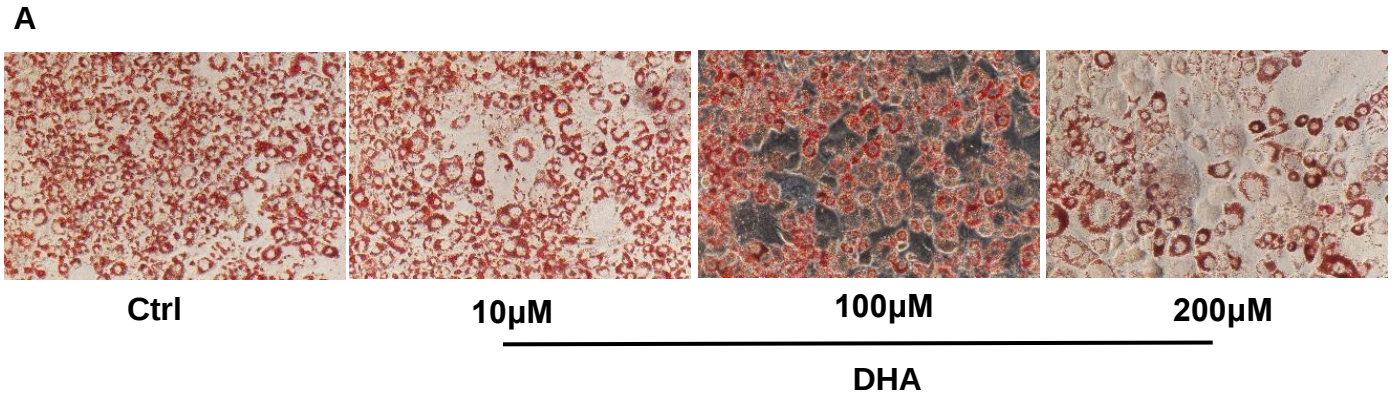
PPARγ
β-actin
Fold induction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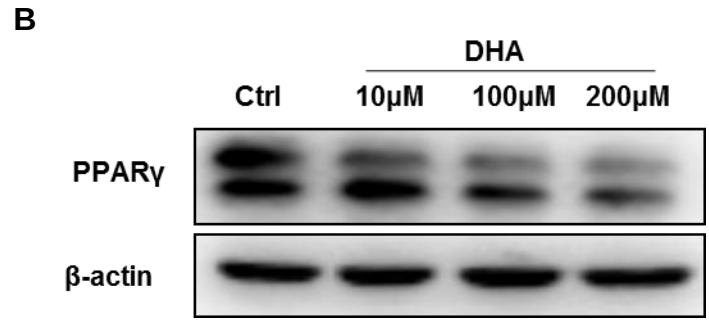
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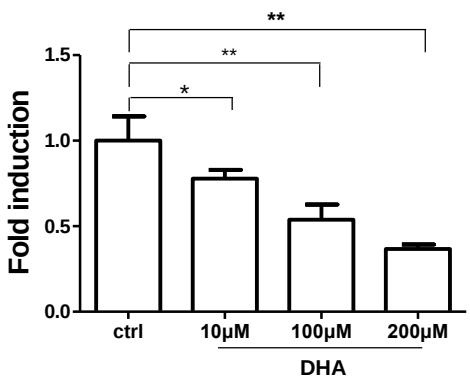
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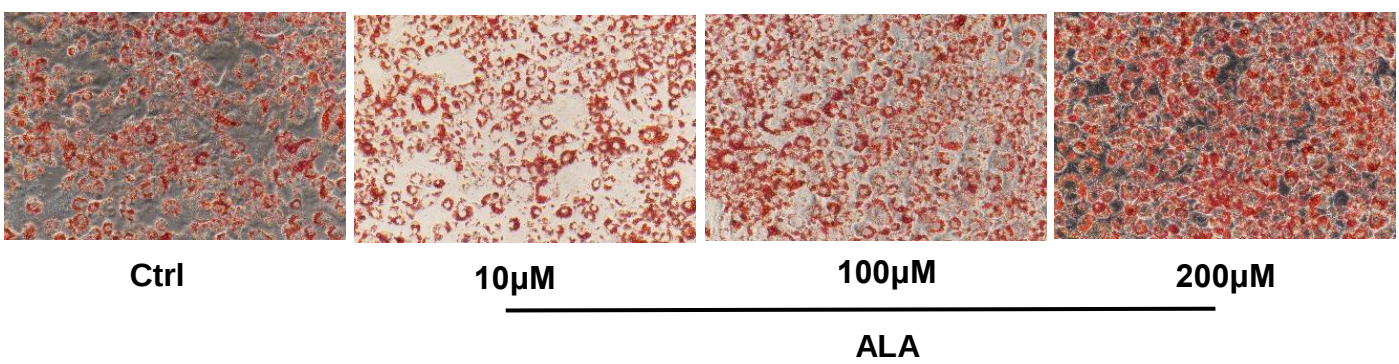
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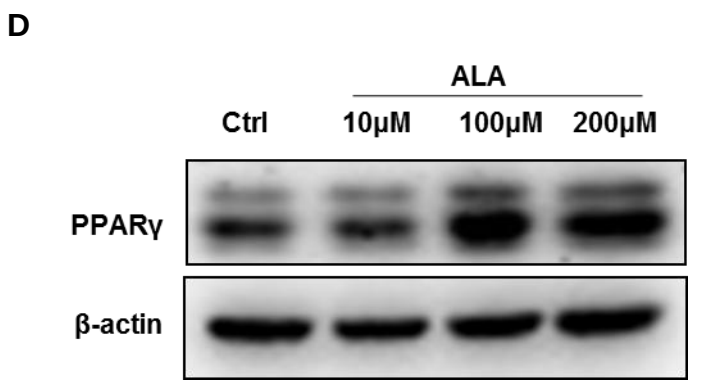
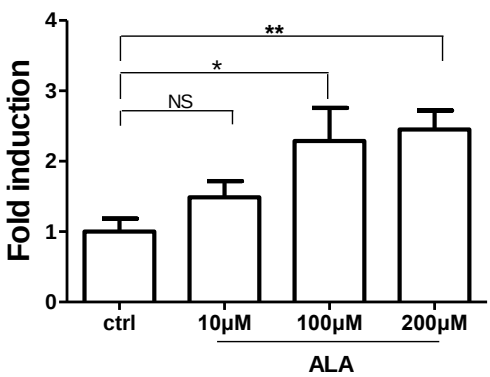
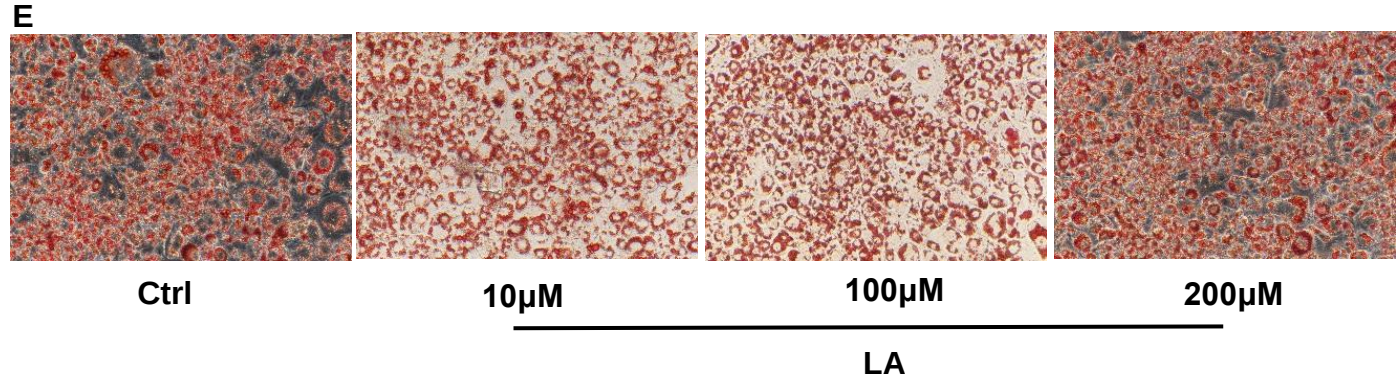
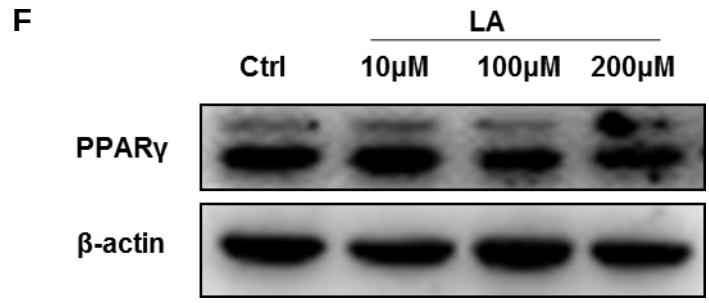
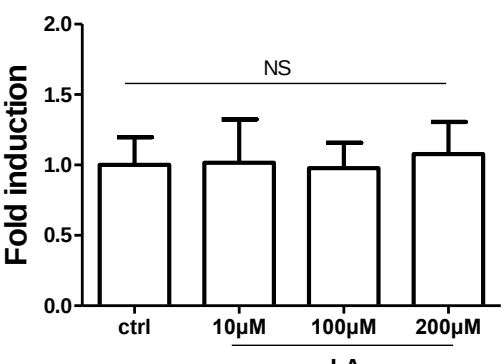
200μM
ALA


D
ALA
Ctrl
10μM
100μM
200μM
PPARγ
β-actin

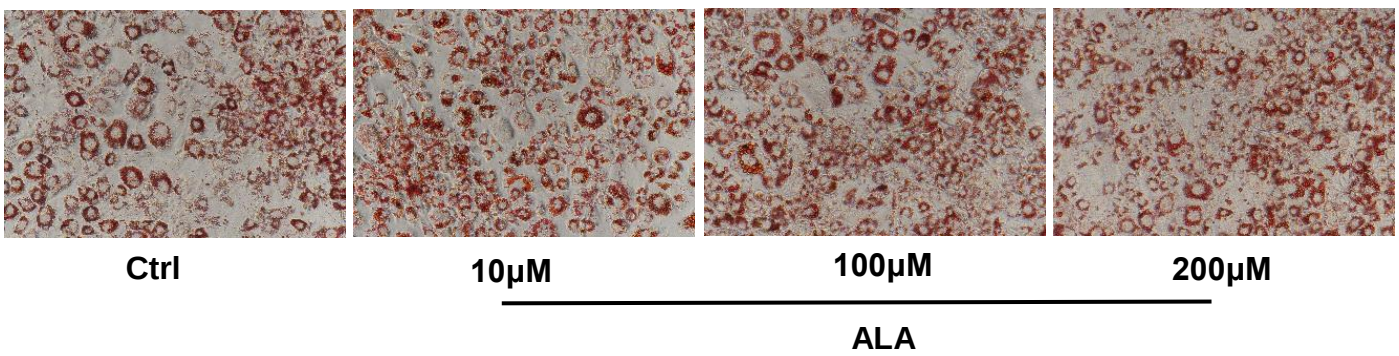

**
*
NS
4
3
2
1
0
Fold induction
ctrl
10μM
100μM
200μM
ALA


E
Ctrl
10μM
100μM
200μM
LA

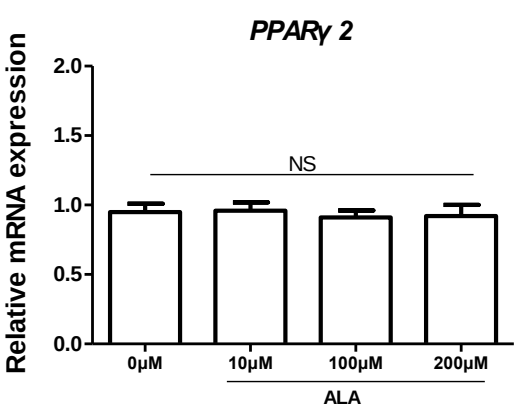

F
LA
Ctrl
10μM
100μM
200μM
PPARγ
β-actin

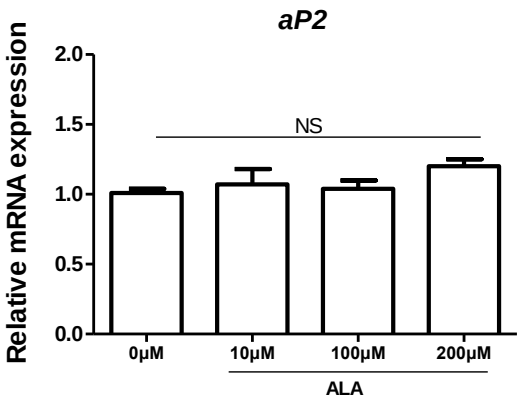

NS
2.0
1.5
1.0
0.5
0.0
Fold induction
ctrl
10μM
100μM
200μM

A

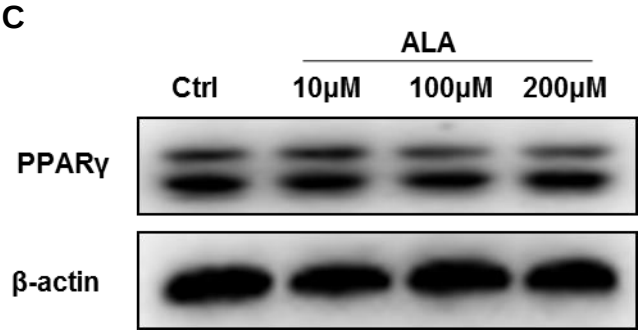

Ctrl
10μM
100μM
200μM
ALA

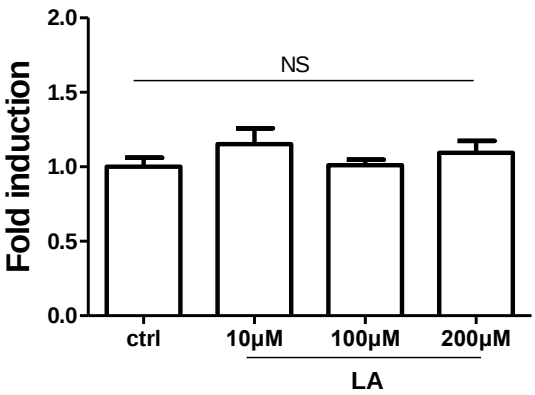
B

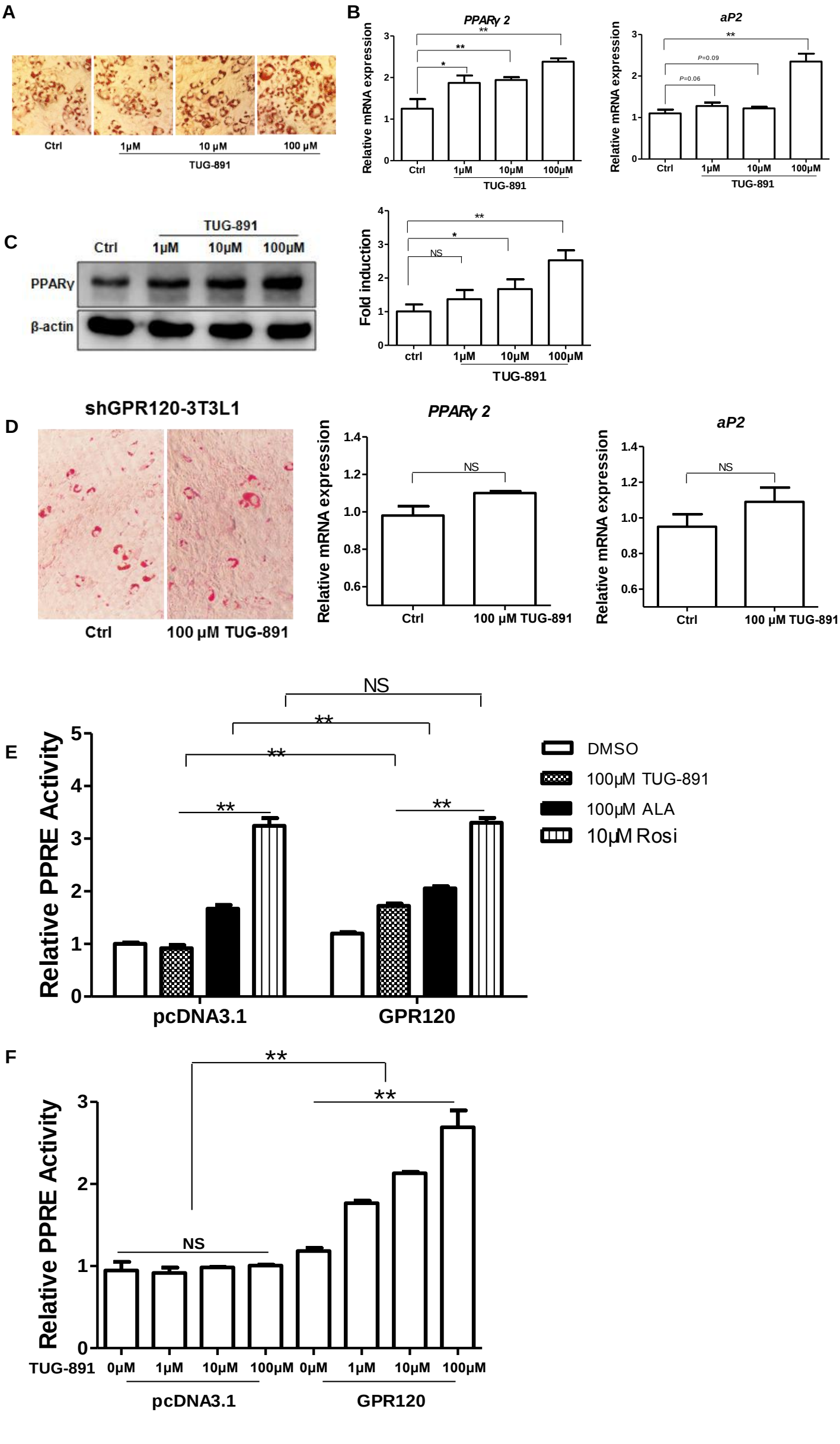

PPARγ 2
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
NS
0μM
10μM
100μM
200μM
ALA

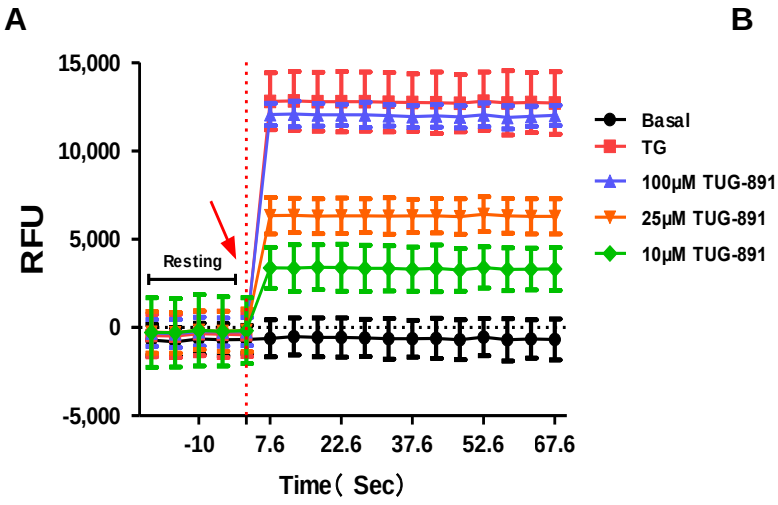

aP2
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
NS
0μM
10μM
100μM
200μM
ALA

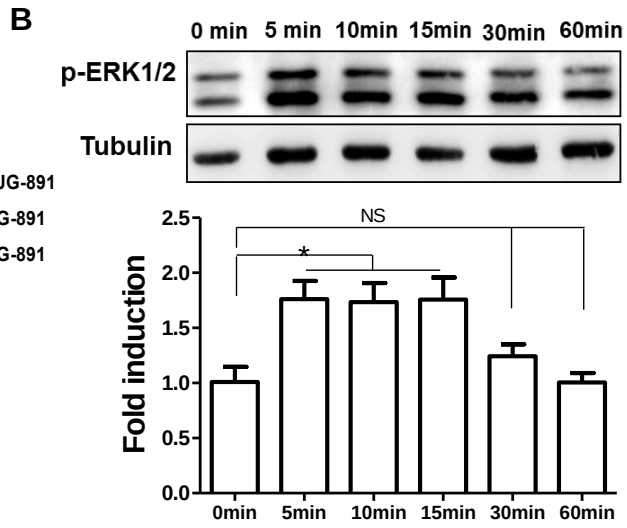
C

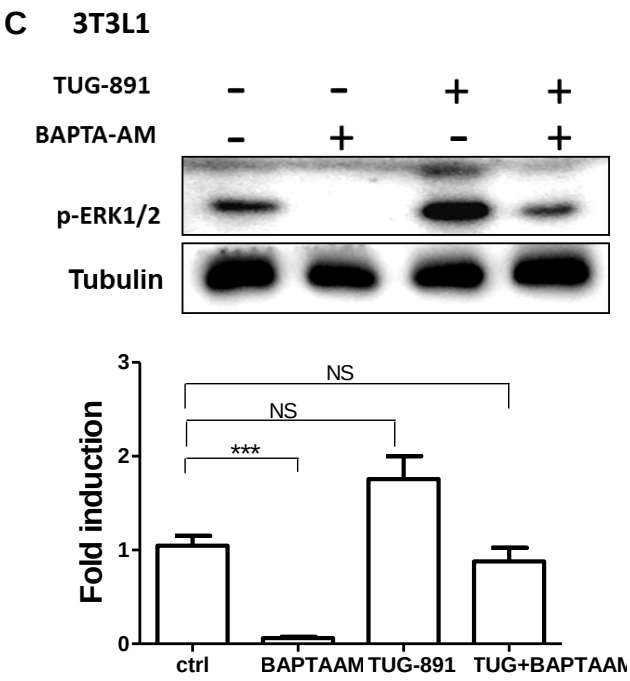

ALA
Ctrl
10μM
100μM
200μM
PPARγ
β-actin

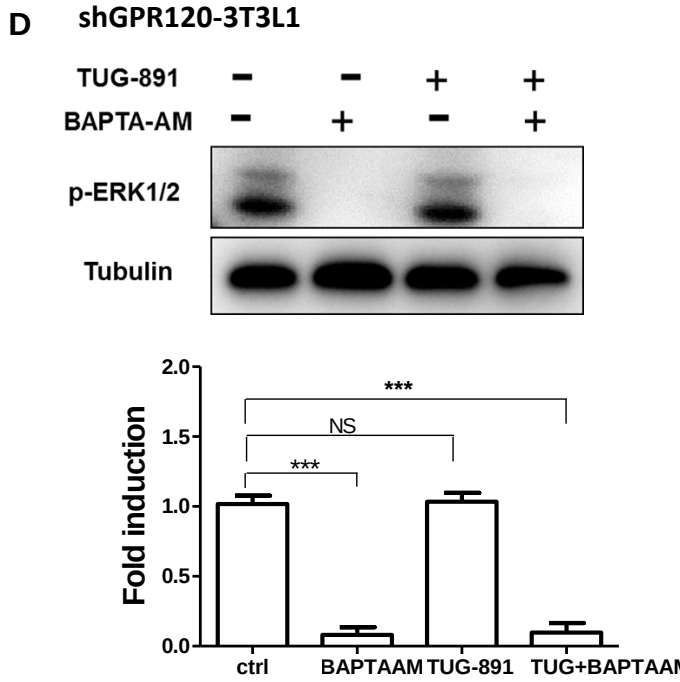

Fold induction
2.0
1.5
1.0
0.5
0.0
NS
ctrl
10μM
100μM
200μM
LA

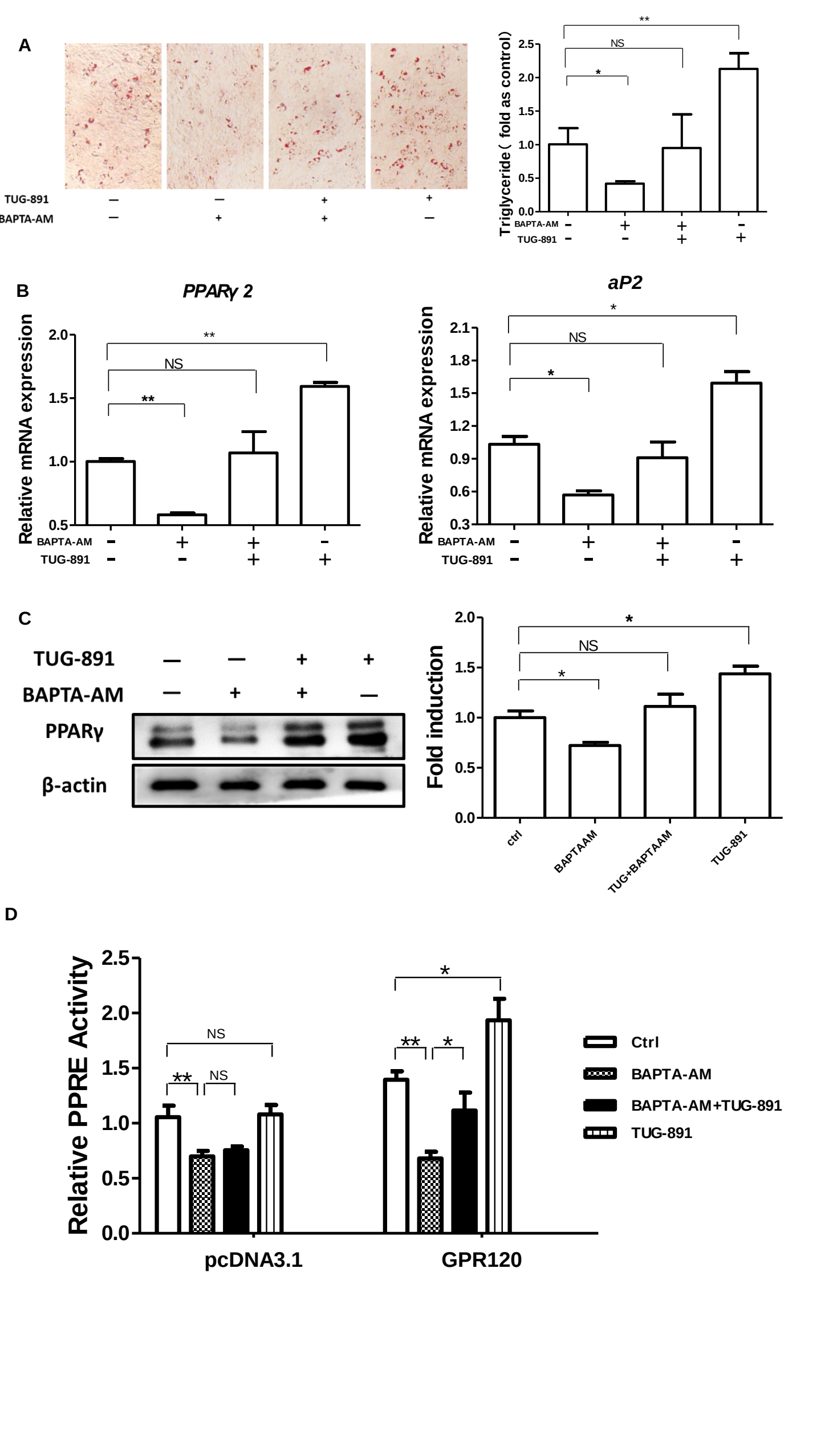

A
Ctrl
1μM
10 μM
100 μM
TUG-891
B
PPARγ 2
Relative mRNA expression
Ctrl
1μM
10μM
100μM
TUG-891
aP2
P=0.09
P=0.06
C
TUG-891
Ctrl
1μM
10μM
100μM
PPARγ
β-actin
Fold induction
NS
ctrl
D
shGPR120-3T3L1
Ctrl
100 μM TUG-891
E
Relative PPRE Activity
DMSO
100μM TUG-891
100μM ALA
10μM Rosi
pcDNA3.1
GPR120
F
Relative PPRE Activity
TUG-891
0μM
1μM
10μM
100μM
pcDNA3.1
GPR120

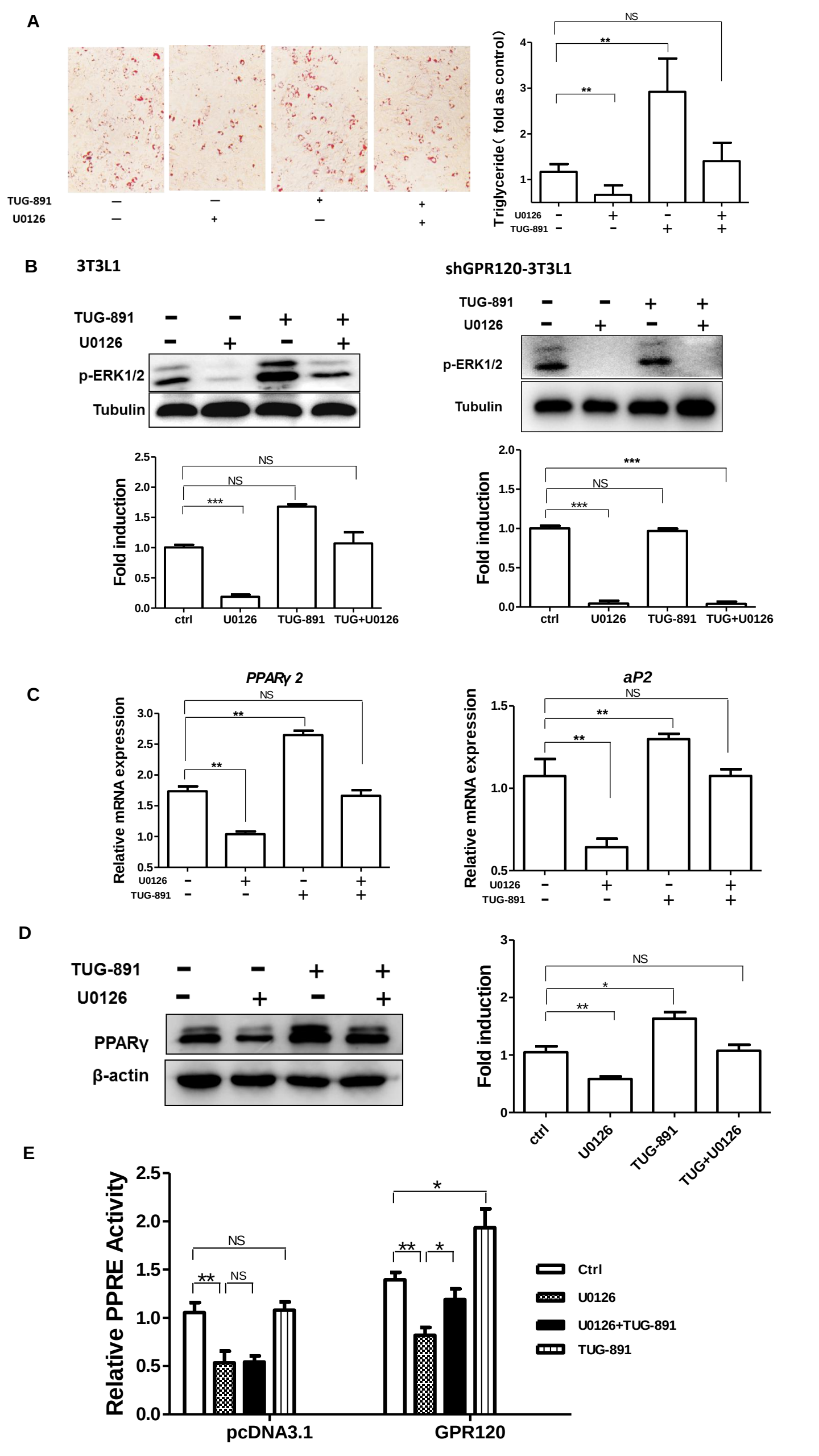

A
15,000
10,000
5,000
0
-5,000
RFU
Resting
-10
7.6
22.6
37.6
52.6
67.6
Time(Sec)
Basal
TG
100μM TUG-891
25μM TUG-891
10μM TUG-891


B
0 min
5 min
10min
15min
30min
60min
p-ERK1/2
Tubulin
Fold induction
NS
*
0min
5min
10min
15min
30min
60min


C
3T3L1
TUG-891
BAPTA-AM
p-ERK1/2
Tubulin
Fold induction
NS
NS
***
ctrl
BAPTAAM
TUG-891
TUG+BAPTAAM


D
shGPR120-3T3L1
TUG-891
BAPTA-AM
p-ERK1/2
Tubulin
Fold induction
***
NS
***
ctrl
BAPTAAM
TUG-891
TUG+BAPTAAM


A
TUG-891
BAPTA-AM
Triglyceride (fold as control)
B
PPARγ 2
aP2
Relative mRNA expression
BAPTA-AM
TUG-891
C
TUG-891
BAPTA-AM
PPARγ
β-actin
Fold induction
ctrl
BAPTAAM
TUG+BAPTAAM
TUG-891
D
Relative PPRE Activity
pcDNA3.1
GPR120
Ctrl
BAPTA-AM
BAPTA-AM+TUG-891
TUG-891


A
TUG-891
U0126
Triglyceride (fold as control)
NS
**
**
U0126
TUG-891
B
3T3L1
shGPR120-3T3L1
TUG-891
U0126
p-ERK1/2
Tubulin
Fold induction
NS
***
ctrl
U0126
TUG-891
TUG+U0126
C
PPARγ 2
aP2
Relative mRNA expression
NS
**
D
PPARγ
β-actin
Fold induction
*
E
Relative PPRE Activity
pcDNA3.1
GPR120
Ctrl
U0126
U0126+TUG-891
TUG-891

1, DHA, ALA, LA showed the different effects on adipogenesis and GPR120 was related to the pro-adipogenic function of ALA

2, TUG-891, a selective agonist of GPR120, promoted adipogenesis in a dose-dependent manner and did not enhance adipogenesis in shGPR120 transfected cells.

3, TUG-891 increased expression of PPARγ in a GPR120-dependent manner.

4, Ca^{2+}-ERK1/2 signaling contributed to the GPR120-induced lipogenesis.