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Linifanib exerts dual anti-obesity effect by regulating adipocyte browning and formation

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ABSTRACT

Obesity is caused by energy imbalance and accompanied by adipocyte hypertrophy and hyperplasia. Therefore, both enhancement of adipocyte energy expenditure and inhibition of adipogenesis are viable ways to combat obesity. Using the *Ucp1*-2A-luciferase reporter animal model previously reported by us as a screening platform, a chemical compound Linifanib was identified as a potent inducer of UCP1 expression in primary inguinal adipocytes *in vitro* and *in vivo*. Signal pathway analyses showed that Linifanib promoted adipocyte browning by attenuating STAT3 phosphorylation. The effects of Linifanib on adipocyte browning were blocked by the compound, SD19, which activates the STAT3 signaling cascade. Linifanib also inhibited adipocyte differentiation, by blocking mitotic clonal expansion, which could be rescued by STAT3 activator. Taken together, our results indicate that Linifanib might serve as a potential drug for the treatment of obesity.

1. Introduction

Obesity is a significant health threat and has become a global epidemic. It is a complex disease which is fundamentally caused by a positive imbalance between energy intake and energy expenditure [1]. Recruitment of beige adipocytes in WAT, described as adipocyte browning, has emerged as a potential effective strategy to combat obesity by increasing energy expenditure [2]. Beige adipocytes can be induced within white adipose tissue upon cold exposure or other pharmacological conditions [3]. Like classical brown adipocytes, beige adipocytes highly express uncoupling protein 1 (UCP1), which is localized to the inner mitochondrial membrane where it converts energy into heat for adaptive thermogenesis [4]. Beige adipocytes arise either from adipocyte precursor cells or by trans-differentiation of white adipocytes and several cytokines and compounds have been demonstrated to treat obesity in animal models by increasing adipocyte browning [5–11]. However, there is still a need to develop new drugs since there is no effective therapeutics for the treatment of obesity.

Obesity is accompanied with an increase of adipocyte size and number. Hyperactive adipogenesis leads to central obesity [12,13]. 3T3-L1 preadipocytes have been used extensively as an *in vitro* model system to study adipogenesis and can be readily differentiated into adipocytes by the established mixture of methylisobutylxanthine (IBMX), dexamethasone (Dex), and insulin (MDI). Pre-adipocytes first undergo one or two rounds of division in the process of so-called mitotic clonal expansion to reach growth arrest at confluence, before they continue with subsequent differentiation steps. Adipogenesis is governed by a battery of transcription factors. Among them, peroxisome proliferator-activated receptor gamma (PPARγ) is the master regulator of adipogenesis in the activation of many genes characteristically transcribed in mature adipocytes, for example, adipocyte protein 2 (aP2) and so on [14,15]. Clearly, agents that inhibit or interfere with

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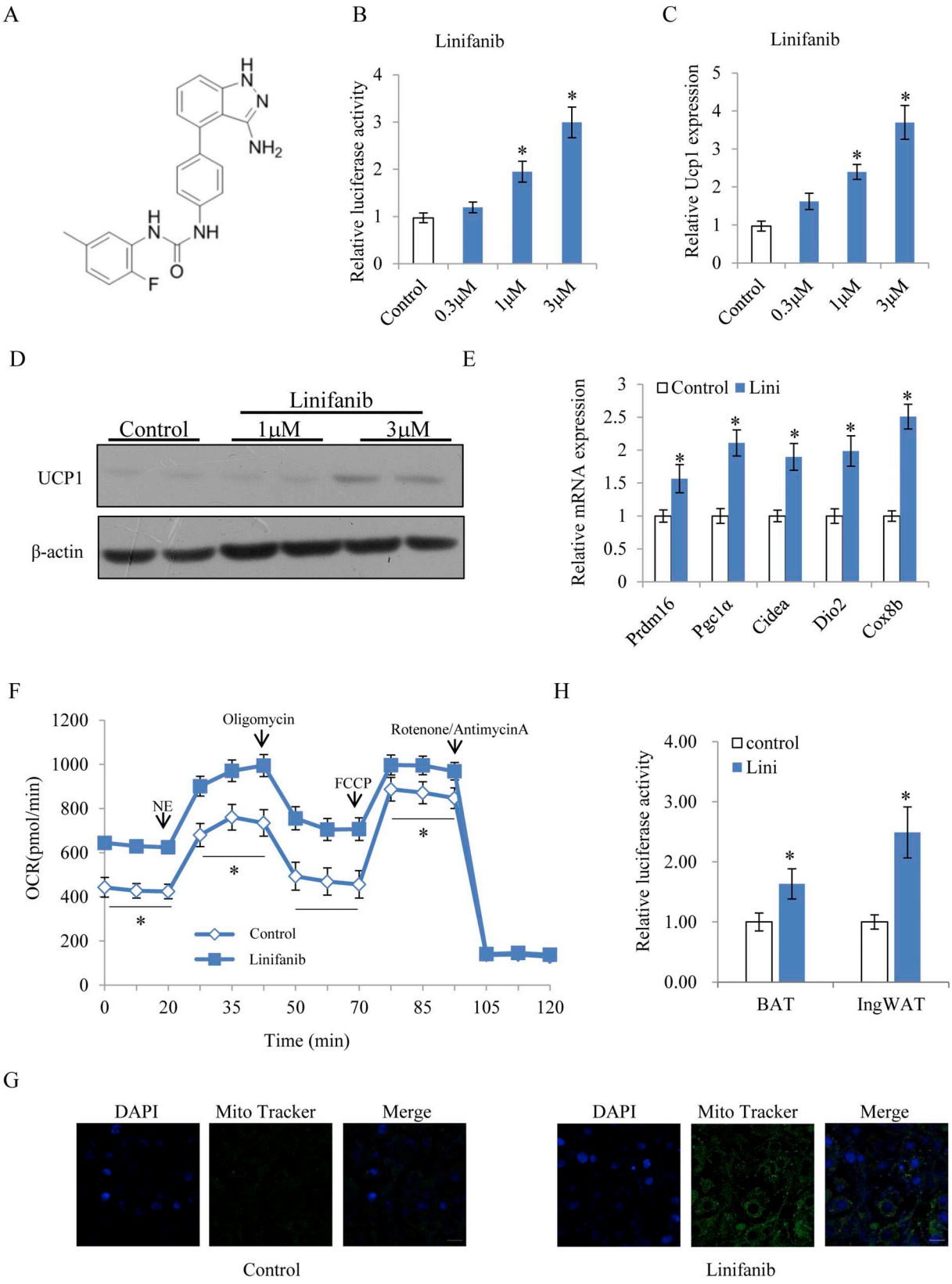

Fig. 1. Linifanib promotes adipocyte browning *in vitro* and *in vivo*. (A) Molecular structure of Linifanib. (B) Luciferase activity assay of Linifanib on primary inguinal adipocytes derived from *Ucp1*-2A-luciferase mice ($n = 5$). *Ucp1* expression as analyzed by qPCR analysis (C) and Western blot analysis (D) in inguinal primary differentiated adipocytes treated with different doses of Linifanib ($n = 5$). (E) The expression of known thermogenic genes was higher in inguinal primary differentiated adipocytes treated with Linifanib ($n = 5$). (F) OCR in primary inguinal adipocytes treated with DMSO (control) or Linifanib ($n = 5$). (G) Inguinal primary differentiated adipocytes were treated with Linifanib (3 μM) for 24 h and stained with Mitotracker green. Scale bars, 20 μm. (H) The expression of *Ucp1* in brown and inguinal white adipose tissue of *Ucp1*-luciferase reporter mice treated with saline or Linifanib respectively *in vivo* ($n = 6$). * means $p < 0.05$ *versus* control.

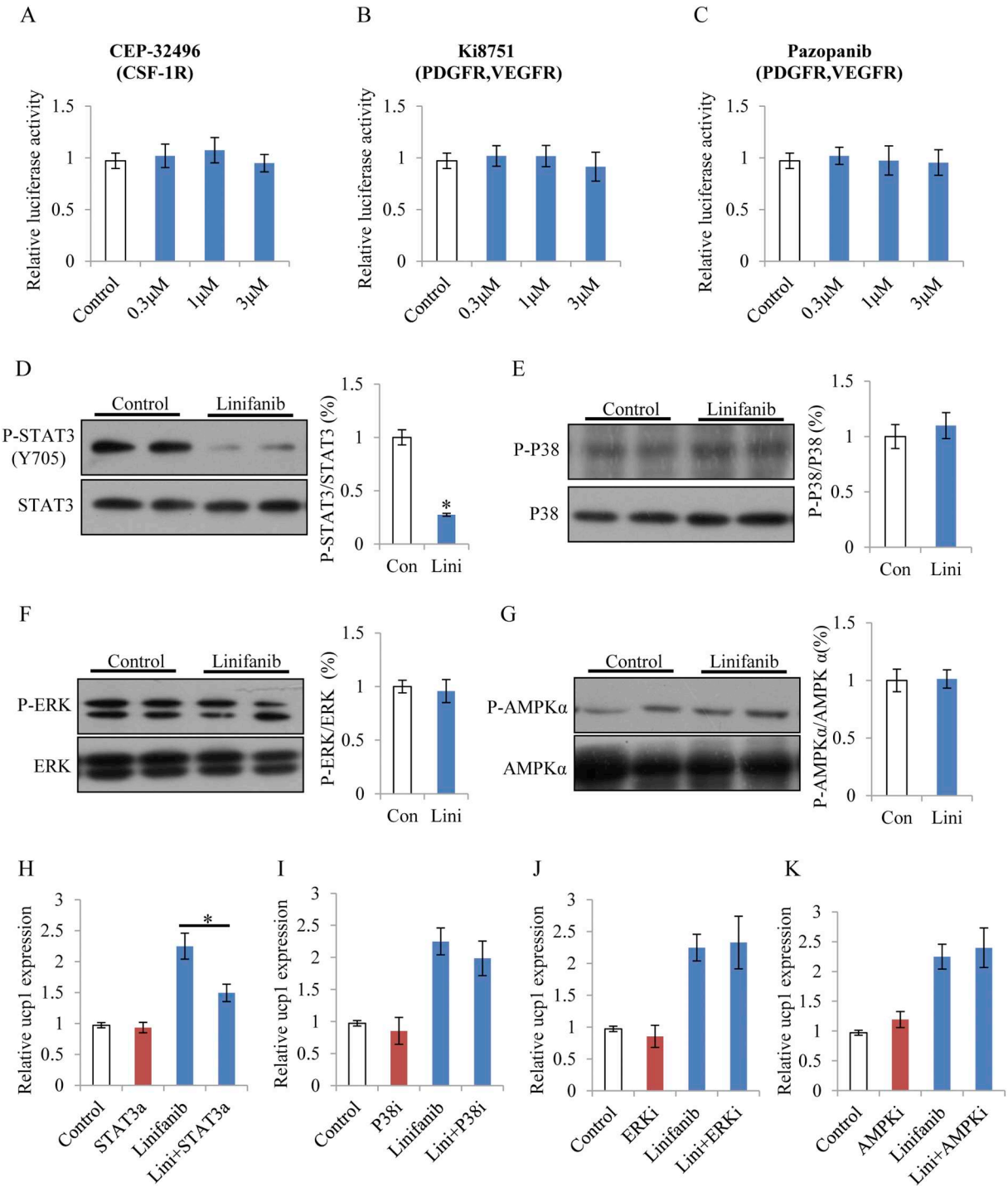

Fig. 2. The STAT3 pathway mediates the browning effect of Linifanib. Luciferase activity assay of CEP-32496 (A), Ki8751 (B) and Pazopanib (C) on inguinal primary differentiated adipocytes derived from *Ucp1*-2A-luciferase mice ($n = 5$). Western blot result (left) and quantitative analysis (right) of STAT3 (D), P38 (E), ERK (F) and AMPK (G) signaling pathways in inguinal primary differentiated adipocytes treated with either DMSO or Linifanib. qPCR analysis of *Ucp1*expression in inguinal primary differentiated adipocytes treated with DMSO (control), Linifanib or Linifanib with STAT3 activator (H), or P38 inhibitor (I) or ERK inhibitor (J) or AMPK inhibitor (K) ($n = 3$). * means $p < 0.05$ *versus* control.

adipogenesis would be beneficial for the treatment of obesity.

Linifanib (ABT-869) is an orally bioavailable, small-molecular receptor tyrosine kinase (RTK) inhibitor with anti-neoplastic activity. Linifanib inhibits members of the receptor families for vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) [16,17]. A broad spectrum of tumor types is inhibited by Linifanib, including small cell lung cancer, colon carcinoma (CRC), breast carcinoma (BC), and gastric cancer [18]. Furthermore, potent anti-angiogenic and anti-tumor effects have been reported in preclinical studies and currently Linifanib is undergoing phase III trials [19,20]. Interestingly, we found that this agent has a promising anti-obesity effect by promoting the adipocyte browning and inhibiting adipogenesis.

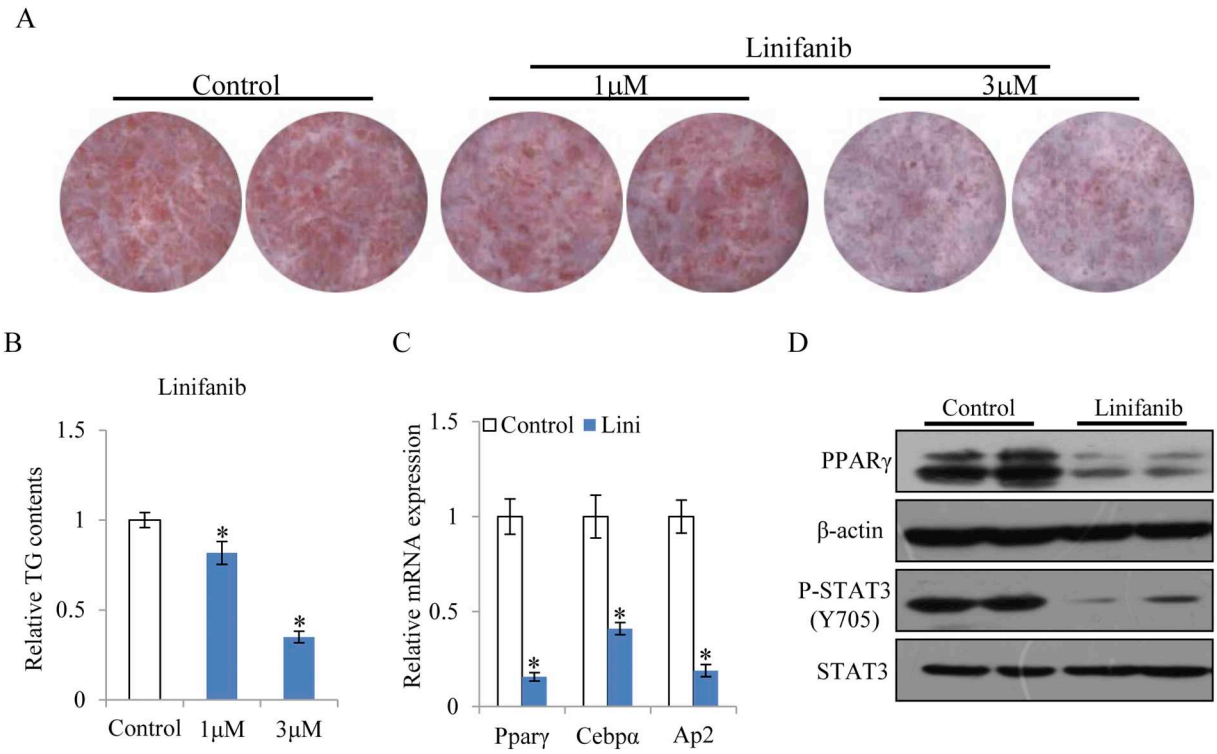

Fig. 3. Differentiation of 3T3-L1 preadipocytes into adipocyte is blocked by Linifanib. (A) Linifanib dose dependently downregulates the differentiation of 3T3-L1 preadipocytes into adipocytes ($n = 5$). (B) Quantitative analysis of TG contents in 3T3-L1 preadipocytes treated with either DMSO or Linifanib ($n = 5$). (C) qPCR analysis of adipocyte specific gene expression in differentiated 3T3-L1 adipocytes treated with either DMSO or Linifanib ($n = 5$). (D) Western blot analysis of PPARγ, β-actin, P-STAT3 and STAT3 levels in differentiated 3T3-L1 preadipocytes treated with either DMSO or Linifanib ($n = 3$). * means $p < 0.05$ *versus* control.

2. Materials and methods

2.1. Isolation of adipose stromal cells from adipose tissues

Adipose tissues were dissected from *Ucp1*-2A-luciferase mice as described previously [21], rinsed in phosphate-buffered saline (PBS), minced, and digested for 40 min at 37 °C in 0.1% (w/v) type I collagenase solution (Sigma) with D-Hanks buffer. Digested tissue was filtered through a 250-μm nylon mesh and centrifuged at 800 ×*g* for 3 min. The sediment was resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% fetal bovine serum (HyClone). Two days after reaching confluence (day 0), the cells were induced to differentiate into adipocytes in a medium containing 5 μg/mL insulin (Sigma), 1 μM of dexamethasone (Sigma), 0.5 mM of isobutylmethylxanthine (Calbiochem), and 1 μM of rosiglitazone (Sigma). Two days later, the medium was replaced with DMEM supplemented with 10% fetal bovine serum, 5 μg/mL of insulin, and 1 μM of rosiglitazone, and the cells were cultured for 6 days. For the *in vivo* study, 10-week-old *Ucp1*-2A-luciferase reporter mice were randomly divided into two groups and gavaged for the following 7 days with either Linifanib (5 mg/kg) or vehicle (control). Mice were sacrificed by cervical dislocation. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Guangzhou Institute of Biomedicine and Health (GIBH), Chinese Academy of Sciences.

2.2. In vitro analysis of luciferase activity

In vitro luciferase activity was measured using the Steady-Glo Luciferase Assay System (E2510; Promega) according to the manufacturer's instructions. Cells and mouse tissues were washed twice with ice-cold PBS and lysed with lysis buffer (Promega) for 30 min on ice. Cell lysates were centrifuged at 12,000 ×*g* for 15 min at 4 °C, and 20 mL of supernatant and 20 mL of DMEM were mixed with 40 mL of Steady-Glo reagent in the wells of 96-well solid-bottom white plates (CulturPlate-96, PerkinElmer, North Billerica, MA). The signal was measured with a Veritas Microplate Luminometer (Turner BioSystems, Sunnyvale, CA) and normalized with protein concentrations.

2.3. Culture of 3T3-L1 cell and induction of differentiation

Murine 3T3-L1 pre-adipocytes were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 10% (v/v) fetal bovine serum (FBS, Hyclone). Briefly, two days after cells reached confluence (day 0), culture medium was changed to MDI differentiation medium containing 5 μg/mL insulin (Sigma), 1 μM Dexamethasone (Sigma), and 0.5 mM IBMX (Calbiochem). Two days later, medium was replaced with DMEM supplemented with 10% FBS and 5 μg/mL insulin. Cells were subsequently re-fed every two days until day 8.

2.4. RNA extraction and quantitative PCR

Total RNA was isolated with Trizol (Invitrogen), and first-strand cDNA was synthesized with Superscript III Reverse Transcriptase (Invitrogen) with 0.5 μg of RNA as the template for each reaction. mRNA levels were quantified under optimized conditions with SYBR Premix Ex Taq (Takara Bio) following the manufacturer's instructions. The reference gene was 18S ribosomal RNA. The sequences of primers are listed in Supplementary Table S1.

2.5. Seahorse analysis

The oxygen consumption rate (OCR) of cultured adipocytes was analyzed using the XFe24 Seahorse bioanalyzer. One day prior to the analysis, primary inguinal adipocytes were treated with Linifanib

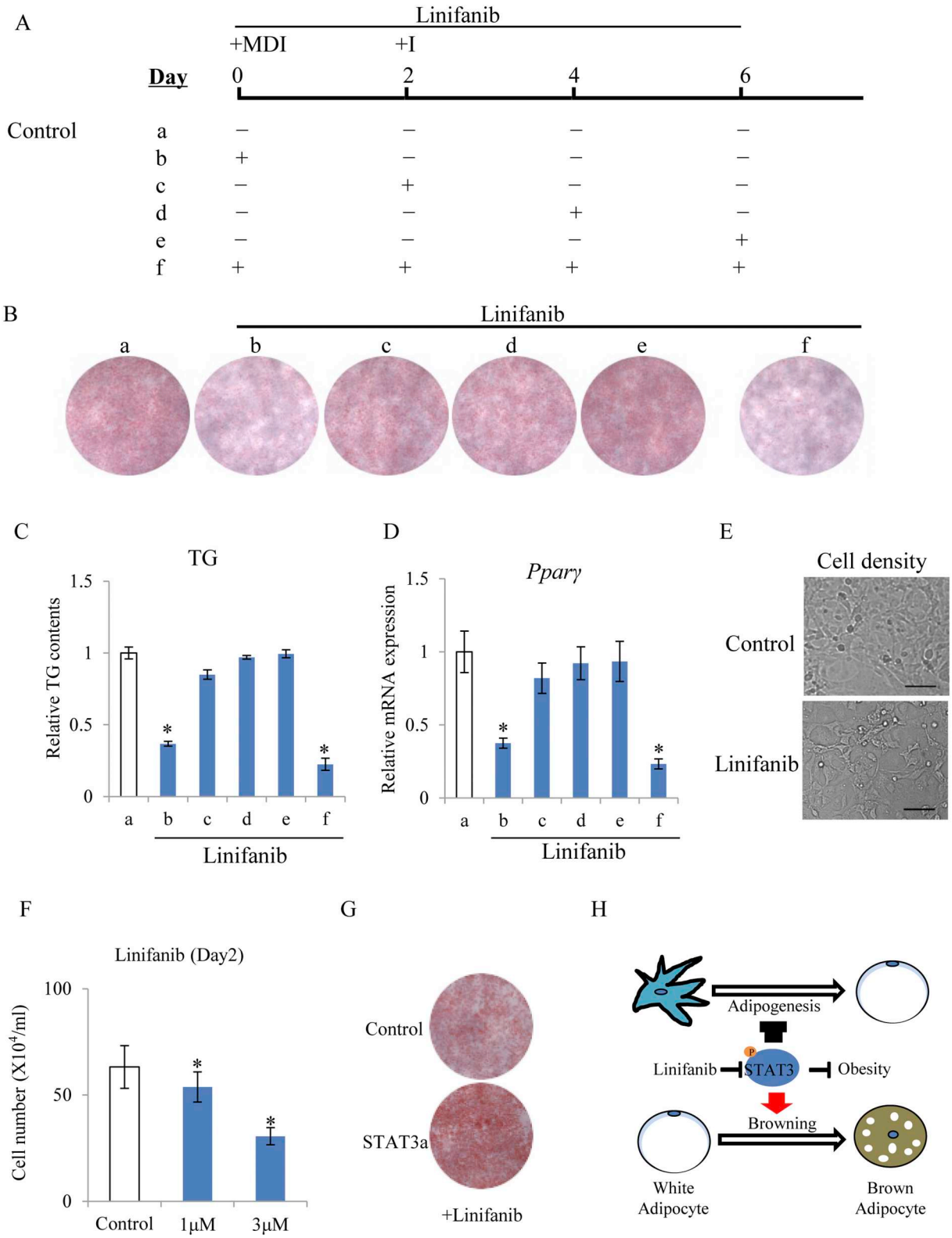

Fig. 4. Linifanib attenuates adipogenesis of 3T3-L1 preadipocytes at an early stage through inhibition of the STAT3 signaling pathway. (A) Summary of 3T3-L1 preadipocytes treatments with either DMSO or Linifanib at the indicated time points. (B) Oil red O staining of differentiated 3T3-L1 preadipocytes treated with either DMSO or Linifanib at the different indicated days. (C) Quantitative analysis of adipocyte TG content ($n = 5$). (D) qPCR analysis of *Pparγ* expression in differentiated 3T3-L1 preadipocytes treated with either DMSO or Linifanib at the indicated days ($n = 5$). (E) Morphology of 3T3-L1 cells following treatment with either DMSO or Linifanib for 2 days at the beginning of adipogenesis (scale bar: 100 μm). (F) Cell number of 3T3-L1 preadipocytes following treatment with either DMSO or Linifanib for 2 days at the beginning of adipogenesis. (G) Oil red O staining results showing that the STAT3 activator, SD19, rescues the inhibitory effect of Linifanib on adipogenesis. (H) A diagram showing the effect of Linifanib on adipocyte formation and browning. * means $p < 0.05$ *versus* control.

(3 μM) or DMSO. After 24 h, cells were equilibrated in sodium carbon dioxide-free DMEM for 1 h in CO_2 free incubator. After measuring basal levels of OCR, the following drugs were sequentially loaded to each well: Norepinephrine (NE, 1 μM), oligomycin (5 μM), FCCP (5 μM), rotenone (3 μM) + antimycin (5 μM).

2.6. Oil red O staining and TG analysis

For Oil red O staining, 3T3-L1 adipocytes were fixed with 10% formalin for 5 min, and then incubated in fresh formalin for 1 h. After washing with 60% (v/v) isopropanol, cells were stained for 10 min in freshly diluted Oil red O. For relative TG quantitative analysis, Oil red O was eluted with 100% isopropanol for 10 min, after which the optical density was determined at 500 nm with a spectrophotometer as relative TG content (Beckman Coulter).

2.7. Glycerol release assay

The glycerol release of primary inguinal differentiated adipocytes was quantitated using a glycerol detection kit (Applygen). Briefly, adipocytes were treated with DMSO or Linifanib (3 μM) in DMEM with 1% FBS. After 16 h, media was removed and replaced with incubation media that consisted of 2% fatty acid free bovine serum albumin (BSA; Sigma) and 0.1% glucose in glucose-free phenol red-free DMEM, and the adipocytes were treated again with DMSO or Linifanib (3 μM). After 2 h, conditioned media was collected and used for enzymatic determination.

2.8. Cell counting

After collected by digestion and centrifugation, cells were added 5 mL medium to suspend. The cells were added to the Counting Chamber for analysis.

2.9. Western blot analysis

Cells were lysed in a buffer containing 1% Nonidet-P 40, 150 mM NaCl, 10 mM Tris-Cl (pH 7.5), and 1 mM EDTA. Lysates were resolved by 10% SDS-PAGE and transferred onto a PVDF membrane, which was then blotted with antibodies to AMPKα (Cell Signaling), ERK (Cell Signaling), STAT3 (Cell Signaling), P38 (Cell Signaling), UCP1 (Abcam), AKT (Cell Signaling), β-actin (Cell Signaling), PPARγ (Cell Signaling), phosphor-AMPKα (Cell Signaling), phosphor-ERK (Cell Signaling), phosphor-STAT3 (Cell Signaling), phosphor-P38(Cell Signaling), or phosphor-AKT (Cell Signaling).

2.10. Mito Tracker staining and confocal microscopy

Mito Tracker® Green FM (CAT#M7514 Invitrogen Molecular Probes, Inc., Eugene, OR, USA), contain a mildly thiol-reactive chloromethyl moiety for labeling mitochondria. The adipocytes grown on BD Flacon culture slides were treated with DMSO or Linifanib (3 μM) for 24 h, then stained with 100 nmol/L Mito Tracker® Green FM in serum-free DMEM for 30 min at 37 °C according to the manufacturer's instructions. ZEISS LSM 800 confocal laser scanning microscope oil-immersion objective lens was used to characterize the optical properties of these samples.

2.11. Mitochondrial DNA analysis

Mitochondrial content was determined by the ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) as, measured by RT-qPCR. For each DNA extract, nuclear gene ribosomal protein large p0 and mitochondrial gene cytochrome *c* oxidase subunit I (CoxI) were quantified individually by RT-qPCR. Data were normalized to the nuclear gene p0 DNA (DDCT analysis).

2.12. Statistical analysis

Data are expressed as means ± SEM. All comparisons were analyzed by unpaired, two-tailed Student's *t*-test, and *p*-values < 0.05 are considered significantly.

3. Results

3.1. Linifanib induces adipocyte browning in a dose dependent manner

To screen for new compounds targeting adipocyte browning, a UCP1 reporter mouse model [21] was utilized. Briefly, expression of *Ucp1* can be detected and measured by determining the activity of luciferase since the luciferase is coupled at the transcriptional and translational level with *Ucp1*. The inguinal adipose tissue is the main adipose depot for browning, so inguinal adipose stromal cells from *Ucp1*-2A-luciferase mice were isolated and differentiated into adipocytes *in vitro*. Next, the primary differentiated inguinal adipocytes were treated with various compounds and lysed to measure luciferase activity. Linifanib, an anti-tumor drug currently in clinical trials, significantly increased luciferase activity in a dose dependent manner (Fig. 1A and B). To further confirm this observation, qPCR and Western blot analysis were carried out to demonstrate that both the mRNA expression and protein expression of *Ucp1* were elevated (Fig. 1C and D). Additionally, the expressions of other thermogenic genes were upregulated by Linifanib in primary inguinal adipocytes (Fig. 1E). The effect of Linifanib on adipocyte thermogenesis was further verified using a Seahorse bioanalyzer, which indicated that Linifanib increased both basal and uncoupled mitochondrial respiration (Figs. 1F and S1A). Consistent with our oxygen consumption rate data, Linifanib also increased the mitochondrial content and glycerol release respectively in primary inguinal adipocytes (Figs. 1G and S1B–C). To examine whether Linifanib promoted adipocyte browning *in vivo*, *Ucp1*-luciferase reporter mice were treated with saline or Linifanib for 7 days and then used for detecting luciferase activity in brown and inguinal white adipose tissue. As shown in Fig. 1H, UCP1 expression was elevated in both brown and inguinal white adipose tissues by Linifanib. These results show that Linifanib significantly promotes adipocyte browning *in vitro* and *in vivo*.

Linifanib promotes adipocyte browning by inhibiting STAT3 signaling pathway.

Linifanib is an antagonist for CSF-1, PDGF and VEGF receptors. Therefore, other antagonists targeting these receptors, including CEP-32496, Ki8751 and Pazopanib, were also examined for their ability in adipocyte browning. Interestingly, these drugs showed no effect on adipocyte browning (Fig. 2A–C), suggesting that Linifanib induces beige cell biogenesis by acting on targets other than these known receptors. Next, we examined the effect of Linifanib on signaling pathways for various protein kinases such as STAT3, P38, ERK and AMPK, which are previously reported to enhance *Ucp1* expression [22–25]. The results showed that phosphorylation of STAT3 was dramatically attenuated by Linifanib, while the other three signaling pathways were not affected (Fig. 2D–G). To further examine whether these signaling pathways were responsible for Linifanib-induced adipocyte browning, additional agonists or antagonists for these kinases (STAT3 activator: SD19; MEK inhibitor: AZD6244; P38 inhibitor: SB202190 and AMPK inhibitor: compound C) were used to examine their effects on adipocyte browning induced by Linifanib. We found that only the STAT3 activator, SD19, blocked Linifanib's effects. This finding is consistent with the inhibition of STAT3 phosphorylation by Linifanib. Taken together, these results show that Linifanib enhances adipocyte browning through inhibition of the STAT3 signaling pathway.

3.2. Linifanib inhibits differentiation of 3T3-L1 into adipocytes at an early stage of adipogenesis

Considering that STAT3 also regulates adipocyte differentiation, the effect of Linifanib on adipogenesis was examined. 3T3-L1 cells, a murine preadipocyte cell line, were treated with Linifanib during the differentiation process. Oil red O (a dye for neutral lipids) staining showed that Linifanib potently blocked adipocyte differentiation in a dose dependent manner (Fig. 3A). Quantification of triglyceride content further confirmed the inhibitory effect of Linifanib on adipogenesis (Fig. 3B). As shown in Fig. 3C, expressions of the adipogenic genes, including *Pparγ*, *Cebpα* and *Ap2*, were reduced by Linifanib (3 μM). Western blot analysis also showed that the protein levels of PPARγ, the master regulator of adipogenesis, was significantly reduced in 3T3-L1 cells treated with Linifanib, along with phosphorylation of STAT3 (Fig. 3D).

To further investigate the mechanism by which Linifanib inhibits adipogenesis, 3T3-L1 adipocytes were treated with Linifanib (3 μM) at different time points as shown in Fig. 4A. Oil red O staining showed that Linifanib blocked adipocyte differentiation only when it was added to cells during the first two days of differentiation (Fig. 4B). TG quantification and qPCR analysis for *Pparγ* and *ap2* mRNA expression further supported this conclusion (Figs. 4C–D and S1). When 3T3-L1 cells are grown to confluency and then stimulated with an adipogenic cocktail, a round of mitotic cell expansion is induced, which is essential for successive adipogenesis. We observed that under these experimental conditions the cell density of 3T3-L1 cells was reduced after treatment with Linifanib (Fig. 4E) and postulated that Linifanib may influence mitotic cell expansion. Quantitative analysis of the cell numbers confirmed that Linifanib inhibited cell expansion (Fig. 4F). Similar to that observed for adipocyte browning, addition of SD19 mitigated the inhibitory effect of Linifanib on adipocyte differentiation (Fig. 4G). Based on these observations, we conclude that Linifanib confers metabolic benefits by suppressing STAT3 signaling pathway, which simultaneously leads to enhanced adipocyte browning and inhibition of adipogenesis (Fig. 4H). Given that obesity is accompanied with insulin resistance, we also examined the effect of Linifanib on AKT signaling pathway, which is predominantly activated by insulin. The results suggested that Linifanib did not alter the phosphorylation of AKT (Fig. S3). Taken together, our data suggests that Linifanib may be a potential drug for treating obesity.

4. Discussion

Enhancement of adipocyte browning or thermogenesis contributes to energy expenditure and weight loss. Consequently, new drugs that augment adipocyte browning open up a promising strategy to treat and prevent obesity [26]. In this study, we found that Linifanib, a compound currently in clinical trials for cancer, induced adipocyte browning and inhibited adipocyte formation from 3T3-L1 cells. Although Linifanib is a known inhibitor for VEGFR and PDGFR, other inhibitors for these targets did not show similar effects, indicating that Linifanib has an off-target mode of action for adipocyte browning. Our findings showed the STAT3 signaling pathway most likely mediates Linifanib's browning effect since STAT3 phosphorylation was attenuated by Linifanib and the STAT3 activator, SD19, blocked its browning effect.

Inhibition of the JAK-STAT3 pathway has been shown to induce adipocyte browning [27]. STAT3 is abundantly expressed in the adipose tissue, and contributes to the regulation of cellular differentiation, proliferation, survival and metabolism [28,29]. Emerging evidence suggests STAT3 function of is correlated with mitochondrial metabolism [30] and is involved together with the JAK tyrosine kinase member, TYK2, in regulating the differentiation of brown adipocytes [31]. However, the detailed mechanism as to how Linifanib decreases phosphorylation of STAT3 is still unclear and requires further investigation.

Our study also provides evidence that Linifanib has an inhibitory effect on adipogenesis *in vitro* for 3T3-L1 adipocytes, which are a well-established and characterized adipogenic system [32]. Previous studies have demonstrated that STAT3 plays a role during the early stage of adipogenesis. In particular, STAT3 is crucial for adipogenesis through its interaction with other factors such as PPARγ and high-mobility-group protein AT-hook 2 (HMGA2) [33,34]. Based on our analyses on 3T3-L1 adipocytes with Linifanib at different designated time points and stages during adipogenesis, it is evident that Linifanib inhibits the early process of adipocyte formation.

In summary, our study suggests that Linifanib has a significant effect on inducing the browning of adipocyte and inhibiting adipogenesis *via* suppression of the STAT3 signaling pathway. Future studies are needed to delineate the biological target of Linifanib on browning and explore its potential as a possible drug for the treatment of obesity and its related metabolic disorders.

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Declaration of interest

The authors declare no competing financial interests.

Author contribution statement

S. Z., Y. C., Y. Zhang, Y. Zhou, W. C., Z. J., Z., W., L. M., K. L. and W. S. performed experiments. P. L., S. J., C. W., A. X., K. L., S. T., D. W., X. H. and T. N. analyzed and interpreted the data. X. H. and T. N. conceived and designed the experiments. S. Z., K. L., D. W., X. H. and T. N. wrote and revised the paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.01.047.

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