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RESEARCH ARTICLE

Investigating the Involvement of Fibroblast Growth Factors in Adipose Tissue Thermogenesis

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ABSTRACT

Objective: Thermogenesis in white and brown adipose tissues can be induced by various stimuli, including cold exposure, β-adrenergic stimulation, and tumor growth. Fibroblast growth factor (FGF) 21 has emerged as an important mediator of thermogenesis. This study investigated the involvement of other FGF family members in the regulation of adipose tissue thermogenesis.

Materials and Methods: Mice were exposed to cold and administrated a β-adrenergic agonist (CL-316,243) to stimulate a thermogenic response in adipose tissues. Stromavascular fractions isolated from white and brown adipose tissues were cultured and differentiated into primary adipocytes. These cells were treated with recombinant FGFs. Changes in the expression levels of thermogenic genes and FGFs were determined by real-time quantitative PCR.

Results: Cold exposure stimulated thermogenic gene expression in the adipose tissue, which was accompanied by the upregulation of certain FGFs. *Ffg9* and *Fgf21* were prominently induced in white and brown adipose tissues. β-adrenergic stimulation also upregulated thermogenic genes in adipocytes. *Fgf21* was identified as the main responder to the β-adrenergic pathway. The administration of recombinant FGFs to cultured primary white and brown adipocytes induced thermogenic genes, including *uncoupling protein-1 (Ucp1)*. FGF2, FGF9, and FGF21 triggered the most significant *Ucp1*-inducing effects in these cells.

Conclusion: FGF21 is as a prominent inducer of thermogenesis in adipose tissue and a promising therapeutic target against cardiovascular and metabolic diseases. FGF2 and FGF9 potently promote thermogenic gene expression in adipocytes. Therefore, their therapeutic targeting should be considered to enhance energy metabolism in adipose tissues.

Keywords: Adipose tissue thermogenesis, Browning, Fibroblast growth factors, FGF2, FGF9, FGF21

INTRODUCTION

Nonshivering thermogenesis involves the generation of heat by brown adipose tissue and is an important response to cold exposure. Brown fat contributes to the maintenance of body temperature in rodents and human infants.[1,2] Recently, the activation of adipose depots by cold exposure has been described in human adults.[3-5] In addition, white adipose tissue has been shown to acquire thermogenic capacity in response to various stimuli, including cold exposure, β-adrenergic stimulation, and tumor growth.[1,6,7] This process is referred to as "browning" and is associated with the increased expression of Uncoupling Protein 1 (UCP1). This protein is localized to the mitochondrial inner membrane and facilitates proton leakage. UCP1 uncouples ATP generation from the proton gradient, which results in enhanced oxidative metabolism and heat generation.[8] The upregulation of *Ucp1* expression is an important marker of adipose tissue browning and thermogenic activity.[9] In addition, the thermogenic program in adipocytes involves increased levels of genes such as *Cidea, Dio2,* and *Pgc1a*.[10] Importantly, *Pgc1a* is a transcriptional regulator, which is responsible for inducing the expression of thermogenic genes.[9]

Fibroblast growth factor (FGF) 21 has previously been described as a prominent inducer of browning in the white adipose tissue of rodents.[11-13] The expression of this protein is stimulated in adipose tissues in response to cold exposure or through β-adrenergic stimulation.[14] Treatment of adipocytes with recombinant FGF21 protein upregulated *UCP1* and other thermogenesis-related genes.[15] The genetic deletion of FGF21 in mice impaired heat generation in response to cold exposure and β-adrenergic stimulation.[15,16] However, the long-term cold adaptation in mice did not require FGF21, which suggests the potential contribution of other factors.[17] FGF21 is

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a member of the FGF family that is made up of 18 proteins, which exhibit varying expression profiles in different tissues. These include FGF1–FGF10 and FGF16–FGF23. FGF homologous factors, FGF11- FGF14, are not considered FGF family members because these proteins do not activate FGF receptors (FGFRs).[18] FGF proteins play diverse roles in pathophysiology, ranging from angiogenesis and wound healing to embryonic development.[19] Some of these factors have been associated with the regulation of various aspects of glucose and energy metabolism.[19,20]

In this study, we explored the involvement of different FGF family members in the regulation of adipose tissue thermogenesis. We examined the expression levels of FGFs in the brown and white adipose tissue of mice and their regulation in response to cold exposure and β-adrenergic stimulation. The FGFs that were expressed in adipose tissues at detectable levels (FGF1, FGF2, FGF7, FGF9, FGF10, FGF16, FGF17, FGF18, FGF21, and FGF22) were also tested in the primary white and brown adipocytes for their ability to stimulate thermogenic gene expression.

MATERIALS AND METHODS

Reagents

Recombinant proteins FGF1, FGF2, FGF7, FGF9, FGF10, FGF16, FGF17, FGF18, FGF21, and FGF22 were purchased from R&D Systems, and the β-adrenergic agonist CL-316,243 (C5976) was obtained from Sigma.

Mice

C57BL/6 male mice were used for all experiments, which were performed at the KUTTAM Animal Research Facility of Koç University. These 8–12-week-old mice were provided ad libitum access to standard rodent chow diet and water. For temperature-controlled experiments, the mice were placed under thermoneutral conditions (30°C) for 24 h to acclimate. The cold exposure group was transferred to 4°C for 6 h. The mice were individually housed to prevent huddling under the cold conditions. Mice were administered CL-316,243 intraperitoneally (1 mg/kg body weight) and sacrificed after 6 h. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Koç University (permit number: 2024, HADYEK.003). All experiments were conducted in accordance with institutional policies and animal care ethics guidelines.

Primary White Adipocyte Culture

Stromavascular fractions of inguinal fat depots were isolated from mice that were 30–35 days old. The detailed procedure has been previously described.[6] Cells were maintained in an adipocyte culture medium composed of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12)-glutamax (Invitrogen), penicillin/streptomycin (Invitrogen), and 10% fetal bovine serum (FBS). The cells were differentiated into primary white adipocytes by the addition of a differentiation cocktail containing 0.5 mM isobutylmethylxanthine (Sigma), 5 µg/mL insulin (Sigma), 1 µM dexamethasone (Sigma), and 1 µM rosiglitazone (Sigma) for 2 days. In the 4 days that followed, the adipocyte culture medium was supplemented with 5 µg/mL insulin and 1 µM rosiglitazone. During the next 2 days, the cells were maintained in the adipocyte culture medium, treated with the recombinant FGF proteins for 24 h, and then harvested 8 days after the initiation of differentiation.

Primary Brown Adipocyte Culture

Stromavascular fractions of interscapular brown fat depots were isolated from newborn mice that were 2–4 days old. The detailed procedure thereof has been previously described.[6] Cells were maintained in an adipocyte culture medium composed of DMEM/F12-glutamax (Invitrogen), penicillin/streptomycin (Invitrogen), and 10% FBS. The cells were differentiated into primary brown adipocytes by the addition of a differentiation cocktail containing 0.5 mM isobutylmethylxanthine (Sigma), 125 µM indomethacin (Sigma), 5 µM dexamethasone (Sigma), 0.02 µM insulin (Sigma), 1 nM T3 (Sigma), and 1 µM rosiglitazone (Sigma) for 2 days. The adipocyte culture medium was then supplemented with 0.02 µM insulin, 1 nM T3, and 1 µM rosiglitazone for a further 2 days. Cells maintained in the adipocyte culture medium were treated with recombinant FGF proteins for 24 h and then harvested 8 days after the initiation of differentiation.

Gene Expression Analysis (RT-qPCR)

Total RNA was isolated and used to perform reverse transcription reactions and real-time quantitative PCR (RT-qPCR) reactions, as previously described.[21] The ΔCt method was used to calculate the relative mRNA expression levels, which were normalized to cyclophilin. The RT-qPCR primer sets used in the study are presented in Table 1.

Statistical Analysis

Statistical analysis was conducted using a two-tailed and unpaired *t*-test for the comparison of two groups and a one-way analysis of variance (ANOVA) for the comparison of multiple groups. ANOVA comparisons were corrected using Tukey's post-hoc test. Values were presented as the mean ± standard error of the mean. Error bars represent deviation between biological replicates. Differences with a *P* value less than 0.05 were regarded as statistically significant.

Table 1. A list of RT-qPCR primer sets used in the study.

Gene	Forward (5'-3')	Reverse (5'-3')
Cyclo	GGAGATGGCACAGGAGGAA	GCCCGTAGTGCTTCAGCTT
Ucp1	AAGCTGTGCGATGTCCATGT	AAGCCACAAACCCTTTGAAAA
Dio2	TCCTAGATGCCTACAAACAGGTTA	CGGTCTTCTCCGAGGCATAA
Cidea	GGTTCAAGGCCGTGTTAAGG	CGTCATCTGTGCAGCATAGG
Pgc1a	AGACAAATGTGCTTCGAAAAAGAA	GAAGAGATAAAGTTGTTGGTTTGGC
Ap2	AGTGAAAACTTCGATGATTACATGAA	GCCTGCCACTTTCCTTGTG
Fgf1	ACACCGAAGGGCTTTTATACG	GTGTAAGTGTTATAATGGTTTTCTTCCA
Fgf2	CAACCGGTACCTTGCTATGA	TCCGTGACCGGTAAGTATTG
Fgf7	AAGGGACCCAGGAGATGAAG	ACTGCCACGGTCCTGATTT
Fgf9	CTATCCAGGGAACCAGGAAAGA	CAGGCCCACTGCTATACTGATAAA
Fgf10	GCGGGACCAAGAATGAAGA	AGTTGCTGTTGATGGCTTTGA
Fgf16	GGCCTGTACCTAGGAATGAATGA	TTCCCGGAAAACACATTCAC
Fgf17	GGCAAATCCGTGAATACCA	CTGCTGCCGAATGTATCTGT
Fgf18	TGCTGTGCTTCCAGGTTCA	GGATGCGGAAGTCCACATT
Fgf21	CCTCTAGGTTTCTTTGCCAACAG	AAGCTGCAGGCCTCAGGAT
Fgf22	GTGGGCACTGTGGTGATCA	GCGATTCATGGCCACATAGA

RESULTS

Cold Exposure Stimulated the Expression of Thermogenic Genes and FGFs, Including *Fgf9* and *Fgf21*, in Adipose Tissues

We investigated the transcriptional changes that occurred in the adipose tissue in response to cold exposure and assessed the FGF expression levels. For this purpose, mice were exposed to thermoneutral conditions (30°C) for 24 h, and a group of them was transferred to 4°C for 6 h as the cold exposure group. Epididymal white adipose tissue (eWAT) and inguinal white adipose tissue (iWAT), which represent visceral and subcutaneous fat depots, respectively, were collected, as well as the interscapular brown adipose tissue (iBAT). The thermogenesis-inducing effect of cold exposure was confirmed by the increased expression of *Ucp1* and other thermogenic genes, including *Dio2, Cidea*, and *Pgc1a*, while *Ap2* expression was evaluated as an adipose tissue-specific marker gene. The up-and down-regulation of the FGFs was investigated in these tissues.

We found that adipose tissue expressed the following FGFs: F*gf1, Fgf2, Fgf7, Fgf9, Fgf10, Fgf16, Fgf17, Fgf18, Fgf21*, and *Fgf22*. In the eWAT depots, mRNA levels of *Ucp1, Dio2*, and *Cidea* were induced by cold exposure compared to thermoneutrality (Figure 1A). *Fgf1* and *Fgf16* expression were stimulated in this tissue, whereas *Fgf2* and *Fgf7* were downregulated (Figure 1B). iWAT tissue responded to cold exposure robustly as the expression of all thermogenic genes was significantly stimulated (Figure 1C). In this tissue, *Fgf9* and *Fgf21* were upregulated, whereas *Fgf2, Fgf17*, and *Fgf18* were downregulated (Figure 1D). *Ucp1* and *Dio2* were induced by cold exposure in iBAT, and the expression of *Fgf1, Fgf9, Fgf18*, and *Fgf21* was also stimulated (Figures 1E and 1F). All other FGFs were downregulated in this tissue upon cold exposure (Figure 1F). These results demonstrate that cold exposure stimulated thermogenic activity in adipose tissues, which was accompanied by the upregulation of certain FGFs. In the iWAT and iBAT depots, the expression of *Ffg9* and *Fgf21* was prominently induced.

β-Adrenergic Activation Upregulated Thermogenic Genes and *Fgf21* in Adipose Tissues

We next investigated the influence of β-adrenergic stimulation on adipose tissue gene expression. Mice that were housed at room temperature were administered the β-adrenergic agonist CL-316,243 and sacrificed after 6 h. We assessed the expression of thermogenic genes and FGFs in the adipose tissues. *Ucp1, Dio2*, and *Pgc1a* were robustly upregulated in eWAT upon β-adrenergic stimulation (Figure 2A). This was accompanied by a mild effect on *Fgf7* expression and a marked increase in *Fgf21* mRNA levels (Figure 2B); however, the expression of *Fgf1* and *Fgf7* was reduced (Figure 2B). The same thermogenic genes were also induced in iWAT, where *Fgf21* was dramatically upregulated (Figures 2C and 2D), and *Fgf1, Fgf2*, and *Fgf7* were downregulated (Figure 2D). iBAT responded to the β-adrenergic agonist with significantly increased levels of *Dio2* and *Pgc1a* (Figure 2E). The expression of *Fgf21* was robustly induced with milder increases in *Fgf17, Fgf18*, and *Fgf22* (Figure 2E). These results indicate that β-adrenergic stimulation upregulates the thermogenic gene expression in adipose tissues, particularly in eWAT and iWAT. *Fgf21* was identified as the main responder to the β-adrenergic pathway.

FGF2, FGF9, and FGF21 were the Most Prominent Inducers of Thermogenic Gene Expression in Adipocytes

To investigate the potential impact of FGFs on the regulation of thermogenic gene expression, we utilized primary white and brown adipocytes isolated from mice. Briefly, stromavascular

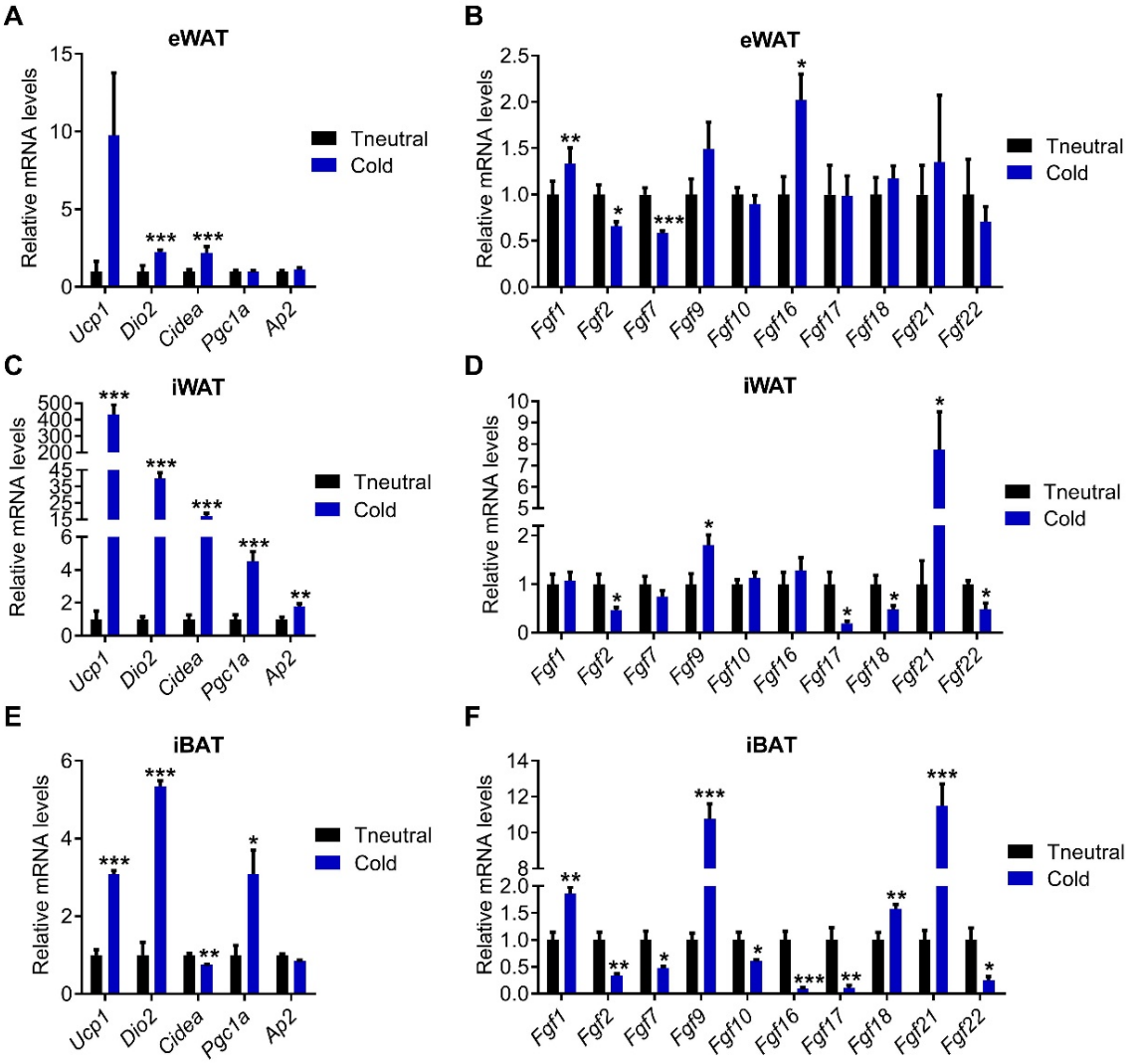

Figure 1. Cold exposure stimulates the expression of thermogenic genes and FGFs, including *Fgf9* and *Fgf21*. Mice were placed under thermoneutrality (30°C; Tneutral) for 24 h. One group was exposed to cold (4°C) for 6 h. eWAT (**A** and **B**), iWAT (**C** and **D**) and iBAT (**E** and **F**) tissues were collected and gene expression was tested by RT-qPCR (n = 5). Values are mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus Tneutral.

fractions prepared from iWAT and iBAT depots were cultured and differentiated into white and brown adipocytes, respectively, through the administration of a differentiation-inducing cocktail. After 7 days, the fully differentiated adipocytes were treated with recombinant FGFs for 24 h, and changes in their gene expression were studied. In the white adipocytes, *Ucp1* mRNA was significantly upregulated by FGF2, FGF9, and FGF21 treatment (Figure 3A). *Dio2* was upregulated by FGF9 and FGF17 treatment, while *Cidea* was induced by FGF16 and FGF21 treatment (Figure 3A). In the brown adipocytes, *Ucp1* mRNA was significantly upregulated by FGF2, FGF9, FGF17, and FGF21 treatment, while *Cidea* was induced by FGF9, FGF16, and FGF21 treatment (Figure 3B). These results suggest that FGF2, FGF9, and FGF21 triggered the most significant *Ucp1*-inducing effects in the primary adipocytes.

DISCUSSION

FGF21 has emerged as an important therapeutic target in cardiovascular and metabolic diseases.[22] In addition to FGF19 and FGF23, FGF21 is a member of the FGF subfamily, whose members function as hormones because of their reduced affinity toward heparan sulfate that is found in the extracellular matrix.[23] Heparan sulfate is required for the binding of FGFs to FGFRs.[24] However, for the endocrine FGFs, this function is performed by the Klotho and β-klotho coreceptors.[20] Previous studies have demonstrated that FGF21 is highly induced upon cold exposure or through β-adrenergic stimulation and that FGF21 stimulated adaptive thermogenesis in adipocytes by the upregulation of UCP1 and other thermogenic genes.[14,15] The findings presented in this study confirmed the cold exposure and β-adrenergic

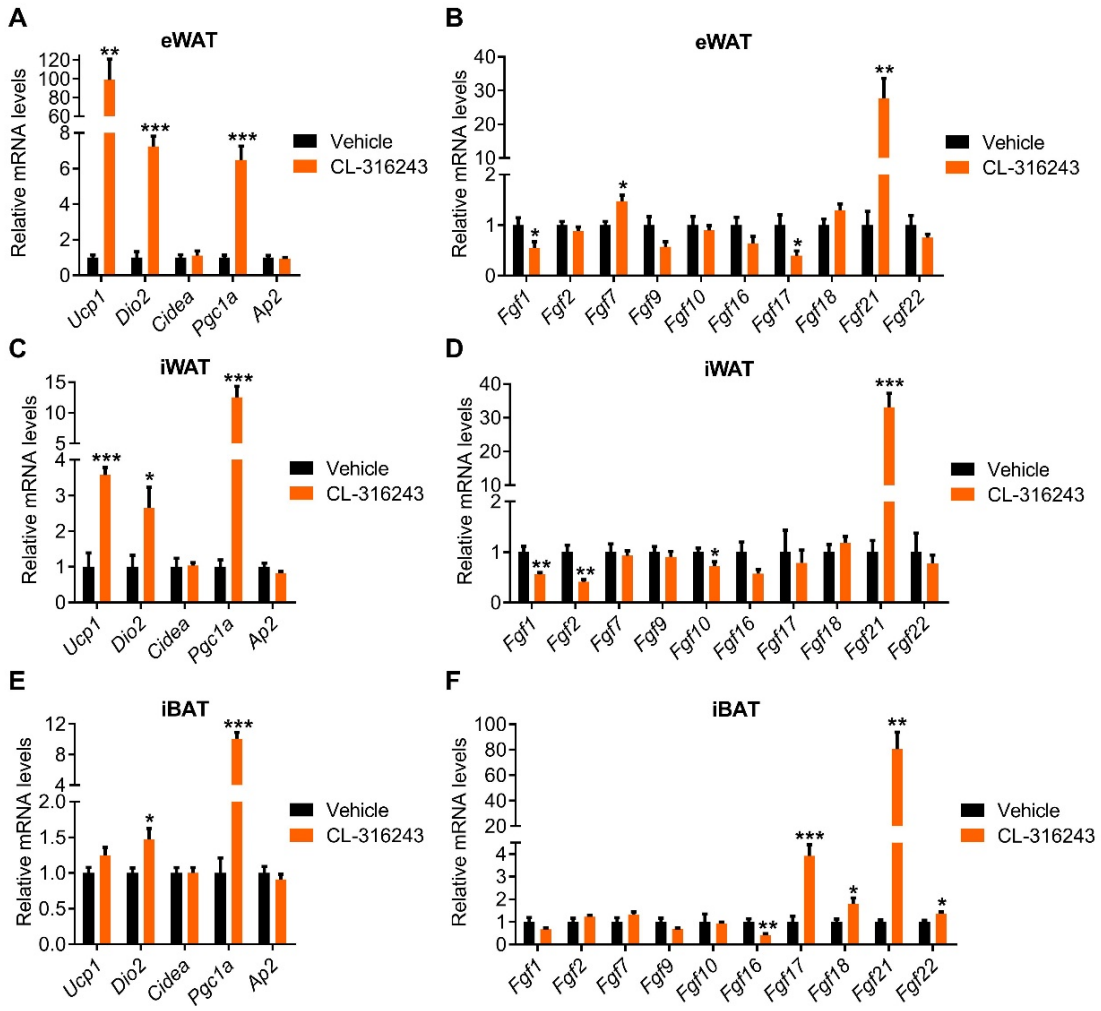

Figure 2. β -adrenergic stimulation induces the expression of thermogenic genes and FGFs, including *Fgf17* and *Fgf21*. Mice received intraperitoneal injections of CL-316,243 (1 mg/kg body weight) and sacrificed 6 h later. eWAT (**A** and **B**), iWAT (**C** and **D**) and iBAT (**E** and **F**) tissues were harvested and gene expression was tested by RT-qPCR (n = 5). Values are mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus vehicle.

pathway-dependent regulation of FGF21 and further confirmed the potency of this protein to upregulate thermogenic genes. Previously, FGF21-related improvements in glucose and energy metabolism were partly attributed to the thermogenic and browning-inducing effects of this hormone.[12,25] The autocrine functions of FGF21 in adipose tissue have been found to be relevant for the induction of thermogenic gene expression.[16] However, FGF21 likely stimulates energy metabolism via mechanisms that are independent of UCP1-mediated thermogenesis, and the long-term adaptation to cold exposure may not require FGF21.[17,26] Various therapeutic agents targeting FGF21 have been developed and tested in preclinical and clinical studies to treat hepatic lipid accumulation and systemic insulin resistance.[12] Particularly, FGF21 analogs have demonstrated efficacy in reversing the fat content in the liver. However, side effects are associated with these drugs, which have been observed in clinical trials, and FGF21-induced bone loss has been reported in rodent models, thus raising safety concerns regarding the use of these therapeutics.[12,27]

Previous studies have indicated that FGF2 and FGF9 negatively influence the browning program of adipocytes.[28,29] FGF9 expression was reported to be suppressed in adipose tissue in response to cold stress.[28] In contrast, our study detected significantly upregulated levels of *Fgf9* in iWAT and iBAT tissues upon cold exposure, and FGF9 treatment of fully differentiated adipocytes activated the thermogenic gene expression. In agreement with the results presented here, a recent study demonstrated that FGF9 expression in adipose tissue was upregulated by exposure to cold conditions, and FGF9 promoted UCP1 expression in adipocytes. FGF9 expression is positively correlated with UCP1 levels in human neck fat biopsies. BAT-specific deletion of FGF9 caused impaired cold tolerance, which suggests an important role of this protein in BAT thermogenesis.[30] Previously, whole-body FGF2 disruption was

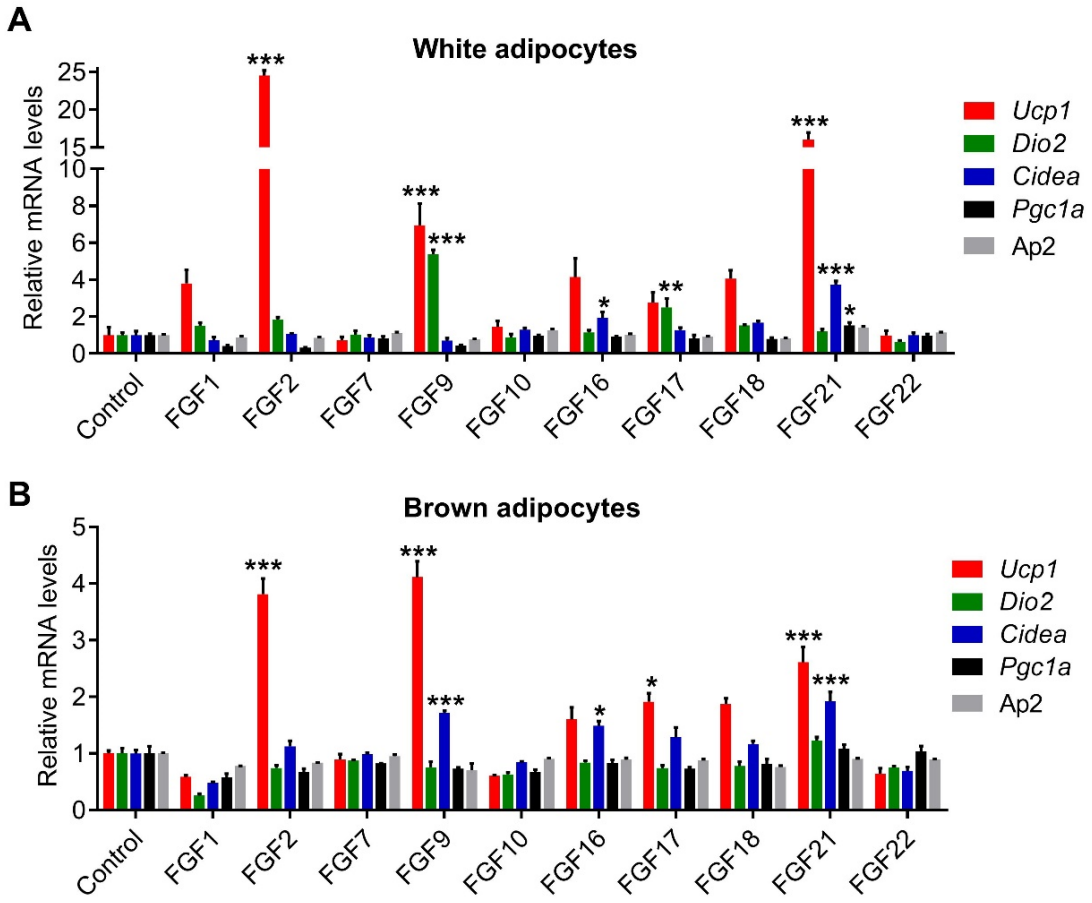

Figure 3. Thermogenic gene expression in adipocytes is induced by FGFs, including FGF2, FGF9 and FGF21. White (**A**) and brown (**B**) adipocytes were fully differentiated and treated with various FGFs (250 ng/ml) for 24 h. Gene expression was tested by RT-qPCR (n = 3). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control.

revealed to enhance thermogenic capacity in the adipose tissue of mice, whereas the administration of exogenous FGF2 suppressed UCP1 expression in the adipocytes.[29] However, we found that FGF2 administration robustly induced UCP1 expression in the adipocytes, and FGF2 triggered a more potent effect in white adipocytes compared with that of FGF9 and FGF21. Adipocyte-specific FGF2 deletion studies are required to ascertain the function of this protein in adipose tissue thermogenesis.

Unlike FGF21, FGF2 and FGF9 are nonendocrine FGFs that induce cell proliferation.[20] Therefore, any therapeutic strategy utilizing FGF2 and FGF9 to promote adipose tissue thermogenesis and energy metabolism is limited by undesirable mitogenic effects and reduced tissue penetration. However, paracrine FGFs have previously been converted into hormonal factors by substituting the C-terminal tails with the tails of endocrine FGFs.[31,32] These chimeric factors can be generated as pharmacological agents to overcome the limitations associated with the therapeutic use of FGF2, FGF9, and FGF21 and utilize the remarkable thermogenesis-inducing effects of these FGFs.

CONCLUSION

In this study, we investigated the transcriptional regulation of FGFs in response to cold exposure and through β-adrenergic stimulation and assessed the potential of these proteins to stimulate the expression of thermogenic genes in primary adipocytes. Most FGFs were differentially expressed upon these stimuli, and FGF9 and FGF21 exhibited the greatest changes. FGF21 has been recognized as a prominent inducer of adipose tissue thermogenesis, and the findings presented here support this notion. Moreover, FGF21 is a promising target for the development of therapeutics against cardiovascular and metabolic diseases. Our results further reveal that FGF2 and FGF9 potently promote thermogenic gene expression in adipocytes, and therefore, their therapeutic targeting should be considered to enhance energy metabolism in adipose tissue.

Ethics Committee Approval: This study was approved by the ethics committee of the Institutional Animal Care and Use Committee of Koç University (permit number: 2024, HADYEK.003).

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