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ORIGINAL ARTICLE

Fat mass- and obesity-associated (FTO) gene promoted myoblast differentiation through the focal adhesion pathway in chicken

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Abstract

The action of *FTO* on myoblasts proliferation and differentiation and molecular mechanism underlying it were investigated by transfecting with *FTO* lentiviral overexpression vector and gene expression profile sequencing. Compared with the control group, myoblasts with *FTO* transfection was significantly enhanced proliferation; the expression of *MYOG* and *MYOD mRNA* was significantly increased. In cells transfected with *FTO*, 129 differentially expressed genes were determined compared with control group, with 104 up-regulated and 25 down-regulated genes. Twelve pathways (Phagosome, Focal adhesion, Adrenergic signaling in cardiomyocytes, Endocytosis, Cardiac muscle contraction, Toll-like receptor, Ribosome, Tight junction, Regulation of actin cytoskeleton, Cytokine–cytokine receptor interaction, Adrenergic signaling in cardiomyocytes and MAPK) were significantly enriched. Eight genes known to be directly or indirectly related to skeletal muscle development (*LAMA5*, *SPP1*, *CAV3*, *RASGRF1*, *FAK*, *PDGFB*, *PDGFRα,* and *RAC2*) were enriched in the focal adhesion and expressed differentially. Altogether, these data suggested that *FTO* stimulated differentiation of myoblasts through regulation of eight genes enriched in the focal adhesion.

Keywords *FTO* · Chicken · Myoblast proliferation · Myoblast differentiation · Focal adhesion

Introduction

Fat mass- and obesity-associated (FTO) gene is highly conserved and widely exits in vertebrate. However, it did not attract much attention until in 2007 when a cluster of SNPs (single nucleotide polymorphisms) located in the first intron of *FTO* was found to be closely associated with obesity-related traits in human (Dina et al. 2007; Frayling et al. 2007; Scuteri et al. 2007). In addition, studies had indicated

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that *FTO* played an essential role in body development, metabolic homeostasis, and hypothalamus-governed food intake in mice (Caruso et al. 2011; Church et al. 2010). The mice lacking Fto completely, compared to control mice, showed immediate postnatal growth retardation with shorter body length, lower body weight, and lower bone mineral density (Gao et al. 2010). More importantly, skeletal muscle development was impaired in FTO-deficient mice (Wang et al. 2017). These findings suggested that *FTO* not only had been found to be important role in adipose deposition but also in skeletal muscle development in mammals.

In birds, *FTO*-regulated gluconeogenesis in DF-1 cells (Guo et al. 2015). The expression of *FTO* mRNA was identified in muscle, liver, visceral fat, hypothalamus, cerebellum and telencephalon tissues, and there was significant difference in liver and visceral fat in broilers and layer chickens (Wang et al. 2012). However, studies of our research team have showed that *FTO* mRNA was expressed in breast and leg muscle tissues, and exhibited tissue- and breed-specific patterns in the Recessive White Plymouth Rock and the Qingyuan partridge chicken from embryo to postnatal period (Song et al. 2015). These findings indicated that *FTO* was likely to play an essential role in skeletal muscle development in chickens.

Skeletal muscle development is closely associated with myogenesis but the physiological significance of its connection with *FTO* needs to be clarified. Thus, our present study was undertaken to examine the effect of *FTO* and its underlying molecular mechanisms on the differentiation of chicken myoblasts, which could lay a foundation on further elucidating the molecular mechanism of skeletal muscle development in chicken.

Material and methods

Ethical approval of the study protocol

The study project was conducted in accordance with the Guidelines for Experimental Animals formulated by the Ministry of Science and Technology (Beijing, China). All experimental programs were approved by the Science Research Department (in charge of animal welfare) of the Institute of Animal Sciences, CAAS (Beijing, China).

Culture of myoblasts

The hatching eggs of S3 strain, which has characteristic by yellow feather, good flavor and excellent growth performance, were used in this study. More than three female embryo were selected for breast muscle separation at 13 embryo age (Luo et al. 2014). Cells were seeded in 6- and 96-well culture dishes (culture medium: DMEM/F12 (HyClone, USA) + 20% FBS (Gibco, USA) + 1% penicillin/streptomycin (HyClone, USA) at 37 ℃ in a humidified atmosphere of 5% CO_2 in air. When cells reached 70 ~ 80% confluence, myoblast differentiation was induced with 2% horse serum (BI, Israel).

Construction and transfection of *FTO* lentiviral vectors

The vectors containing cDNA of *FTO* (HM050377.1) was synthesized by GenePharma (GenePharma, Shanghai, China). Primers including *NotI* and *NsiI* sites were used to generate PCR fragments that were subcloned into LV5(EF-1α/GFP/Puro) lentiviral expression vector. Positive clones were identified by sequencing. Virus drops is 10^8 TU/mL.

Transfection

Transfections were undertaken at 40–50% confluence using polybrene (5 μg/mL). Myoblasts cultured in six-well plates were transfected with *FTO* lentiviral vector and negative vector to detect gene expression and cell differentiation using qPCR in three replicate wells, respectively. Cells of the same group in 96-well plates were transfected with *FTO* lentiviral vector to monitored cell proliferation using Cell Counting Kit-8 (CCK-8; Dojindo, Fukuoka, Japan) and Edu staining (RiboBio, Guangdong, China) in eight replicate wells.

RNA extraction

Total RNA was isolated from myoblasts using the Total RNA kit (TIANGEN, Beijing, China) following the manufacturer's instructions. An ND-1000 spectrophotometer (Agilent Technologies, Palo Alto, CA, USA) was used to measure the concentration and purity of RNA ($A_{260/280} \geq 1.8$ and ≤ 2.0). RNA integrity (RIN ≥ 7) was evaluated by a 2100 Bioanalyzer Lab-on-chip system (Agilent Technologies). RNA samples were stored at − 80 ℃ until use.

Gene expression profile

The gene expression profile was undertaken by LC (Hangzhou, China) using total RNA from three replicate differentiated myoblasts treated with *FTO* lentiviral and negative control vector for 9 days, respectively. Raw data were determined by FASTQC (v0.11.2), and Clean data were mapped to the reference genome and genes of chickens (Gallus_gallus-5.0) using Tophat2 (v2.0.12) (Huang et al. 2015). Fragments per kilobase of exon per million mapped reads (FPKM) was employed to quantifying gene expression. *P* values ≤ 0.05 and fold changes ≥ 1.5 were assumed

to indicate differentially expressed genes (DEGs). Pathways were analyzed by Kobas v3.0 (kobas.cbi.pku.edu.cn/help.do) (Mao et al. 2005; Wu et al. 2006). Pathways with < 3 known chicken genes were discarded.

qPCR of genes

Abundance of *MYOD* and *MYOG* and differentially expressed genes from three replicate cells treated with *FTO* lentiviral and negative control vector for 9 days were quantified by qPCR (Qiagen, Düsseldorf, Germany). The final concentration of each primer was 10 μmol/μL. The primers are detailed in Table 1.

Statistical analyses

Statistical differences between treatment groups were determined by *t* tests using Excel software. The data are the mean ± standard deviation (SD), and $P < 0.05$ was considered significant.

Results

FTO promoted the proliferation of myoblast

FTO lentiviral and negative control vector were successfully transfected with myoblast (Fig. 1a), respectively. The expression of *FTO* mRNA, compared with the control group, was increased significantly 72 h after transfection with *FTO* (Fig. 1b, $P < 0.001$).

The effect of *FTO* on the proliferation of myoblast-was showed in Fig. 2a, b, respectively. Cell numbers were enhanced significantly at 72 and 96 h after transfection with *FTO* (Fig. 2a, $P < 0.05$). EdU staining also indicated that the proliferation rate of *FTO* lentiviral cells was increased compared to control cells. Altogether, the data suggested that *FTO* increased myoblast proliferation.

FTO-enhanced myoblast differentiation

The myotubes were longer and more complete compared with those of control myoblasts after transfection *FTO* (Fig. 3a). Two major myoblast differentiation marker genes (*MYOG* and *MYOD*) were detected at 9d after transfection with *FTO* lentiviral using qPCR. The results showed that *MYOG* and *MYOD* expression transfection *FTO* were significant higher that of control group (Fig. 3b).

Table 1 qPCR primers

Gene name	Sequence (5′ to 3′)	Fragments/bp	Nnnealing temperature/℃
SPP1	S: 5′ CAAAGCTGCCAGGAAGCTCAT 3’ A: 5′ TCCACCTCAGGGCTGTCAAA 3’	171	60
CAV3	S:5′ CTTCCTCTTCGCCCTCATCT 3’ A:5′ CCTTCCGCAGCATAACCCT 3’	182	60
PTK2	S: 5′ GCAGTCTGAGGAGGTTCACT 3’ A: 5′ TTGGGCAGGTACCGAATTCT 3’	123	60
PDGFB	S: 5′ TGGACAGCACCAATGCCAA 3’ A: 5′ GGTCCTCCAAAGGCACGAT 3’	192	60
PDGFRα	S: 5′ CTTGGCAGCTCGTAATGTCC 3’ A: 5′ TTACTGGGAGGAAGGTGCTG 3’	125	60
RAC2	S: 5′ TGCTGGACAAGAGGATTACGA 3’ A: 5′ ACAAGGATGATGGGAGTGCTG 3’	165	60
MYOD	S: 5′GCTACTACACGGAATCACCAAAT 3’ A: 5′ CTGGGCTCCACTGTCACTCA 3’	21	60
MYOG	S: 5′ CGGAGGCTGAAGAAGGTGAA 3’ A: 5′CGGTCCTCTGCCTGGTCAT 3’	314	60
RASGRF1	S: 5′ TTTGACATCCTGCTGCCCAT 3’ A: 5′ AGCTTGTCGAAGTCACGGTT 3’	110	60
ß-actin	S: 5 ’GAGAAATTGTGCCTGACATCA 3’ A: 5 ’CCTGAACCTCTCATTGCCA 3’	152	60

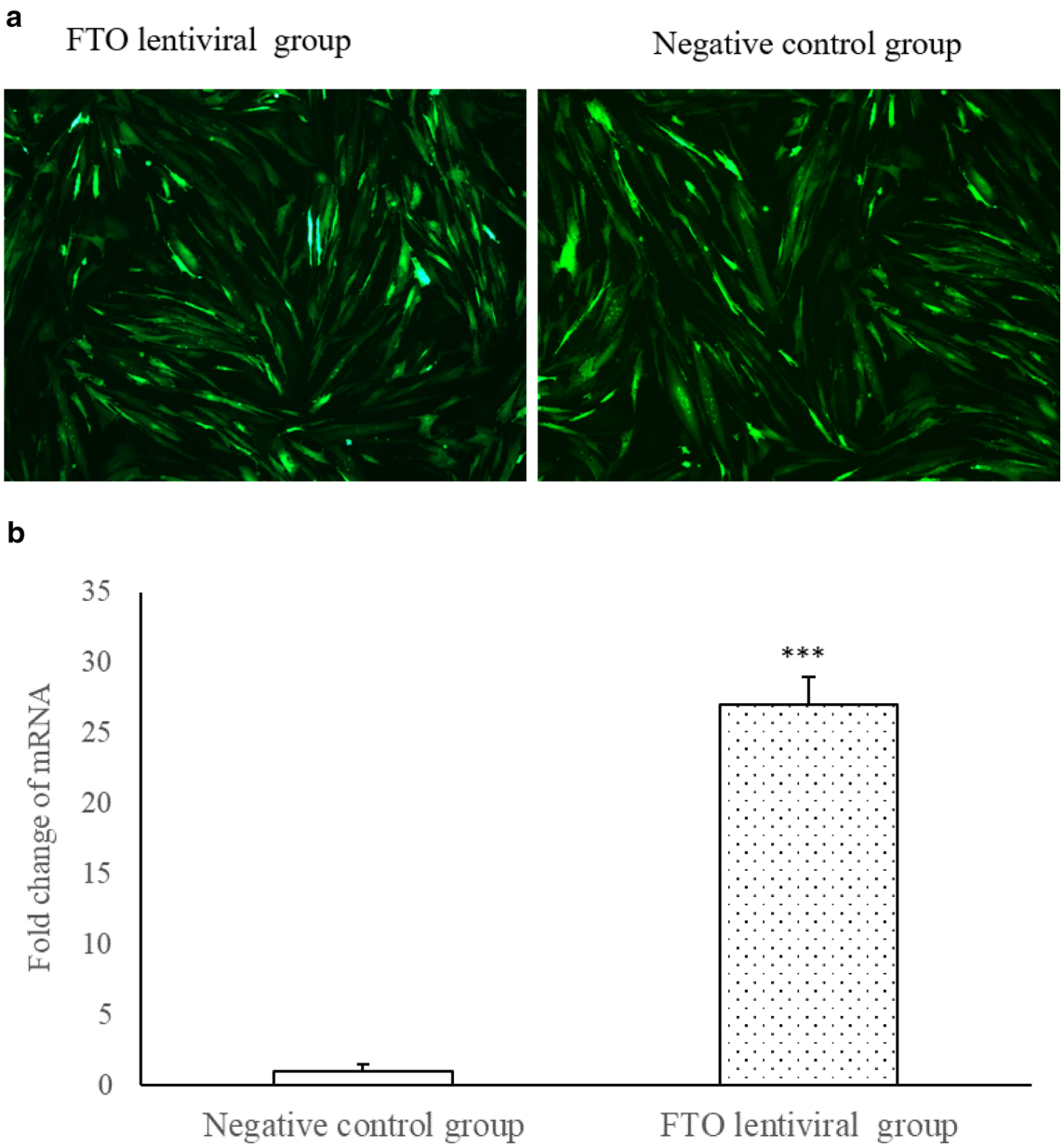

Fig. 1 **a** Transfection of *FTO* lentiviral overexpression and negative control vectors (inverted microscope, × 100 magnification, $n = 3$); Green fluorescence denotes successful transfection. **b** The expression of *FTO* mRNA was increased significantly 72 h after transfection with *FTO* lentiviral overexpression vectors, ***$P < 0.001$

FTO-regulated myoblast differentiation through a focal adhesion pathway

The gene expression profile was used to explore the key genes and pathways by which *FTO* influenced myoblast differentiation. In the present study, three cDNA libraries were constructed using total RNA from transfection *FTO* lentiviral vector and negative control vector, respectively (for details of quality control, see table S1). A total of 129 DEGs ($P < 0.05$, $\log 1.5 > 0.67$ or < -0.67) were identified, with 104 up-regulated and 25 down-regulated genes (Table S2).

94 GO terms (Gene number ≥ 10, $P < 0.05$) were significantly enriched based on 129 DEGs, including regulation of cellular process, cytoplasm, regulation of biological process, biological regulation, cellular metabolic process, cellular developmental process, cell differentiation, developmental process, etc. As shown in Fig. 4, the top of twenty GO terms related to cell differentiation were showed (Fig. 4). Using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway to analyze the DEGs, twelve pathways were significantly enriched: Phagosome, Focal adhesion, Adrenergic signaling in cardiomyocytes, Endocytosis, Cardiac muscle

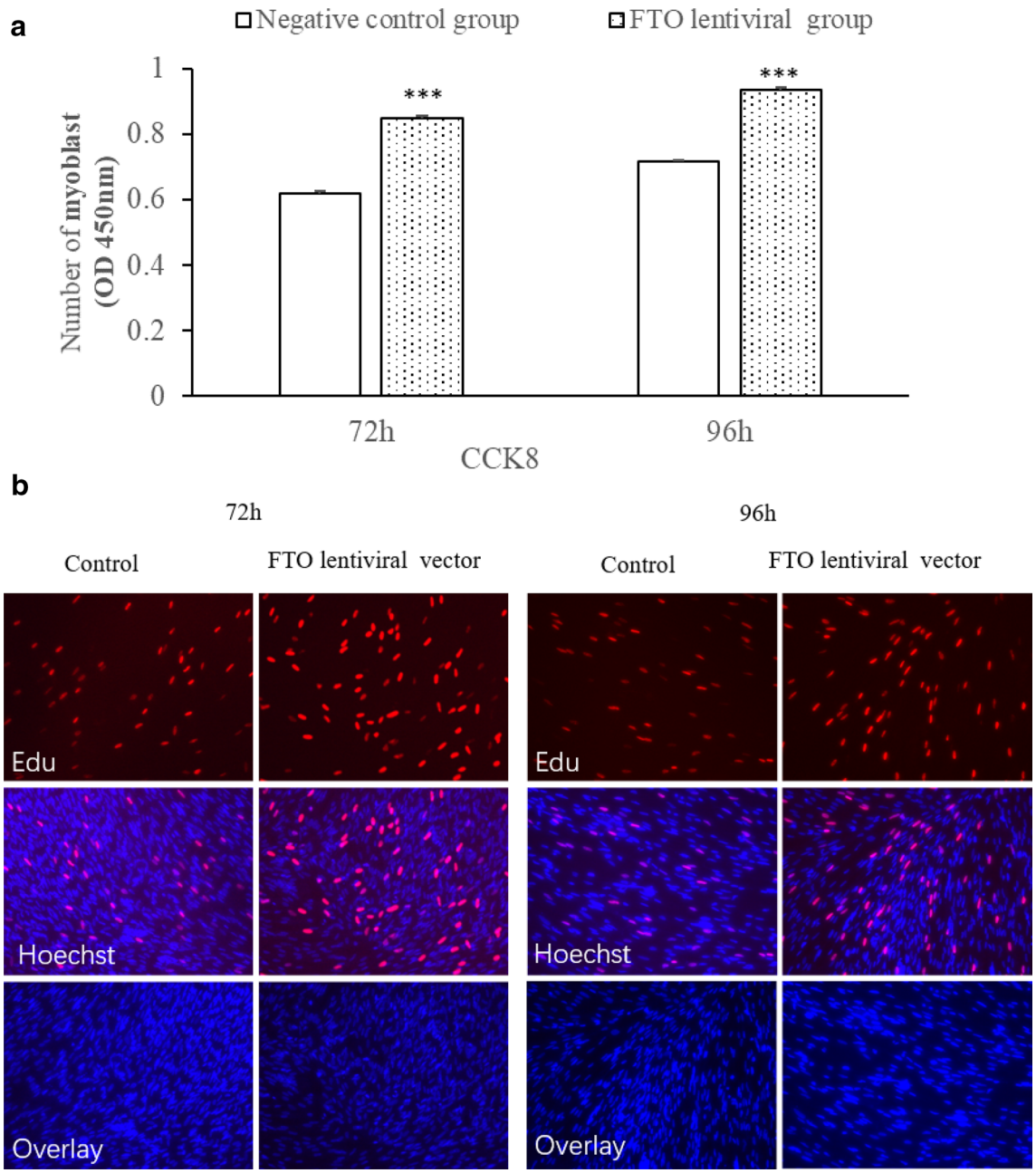

Fig. 2 Effect of *FTO* on proliferation of cells. **a** The number of myoblasts was significantly enhanced after transfection with *FTO* lentiviral overexpression vectors ($n=8$, $^{***}P<0.001$ vs. control). **b** The number of EdU-stained cells was significantly enhanced after transfection with *FTO* lentiviral overexpression vectors ($n=3$, ×200 magnification)

contraction, Toll-like receptor signaling pathway, Ribosome, Tight junction, Regulation of actin cytoskeleton, Cytokine–cytokine receptor interaction, Adrenergic signaling in cardiomyocytes and MAPK (Table 2, $P \leq 0.05$). Most noticeably of all, six genes (*LAMA2*, *RASGRF1, RAC2, SPP1, CAV3,* and *PDGFB)* enriched in focal adhesion pathway were reported to be closely related to the morphology, metabolism, insulin sensitivity and myoblast differentiation in skeletal muscle. The six genes showed consistency between the qPCR assays and the deep sequencing analysis in terms of the direction of regulation and statistical significance (Fig. 5). The two genes, *PDGFRα* and *FAK* located in focal adhesion pathway and related to skeletal muscle development, were also detected using Q-PCR and differentially expression (Fig. 5). As shown in Fig. 6, *FAK* may play an important pivotal role in the response of the *FTO* regulation of myoblast differentiation. Therefore, it was hypothesized that *FTO* could contribute to myoblast differentiation

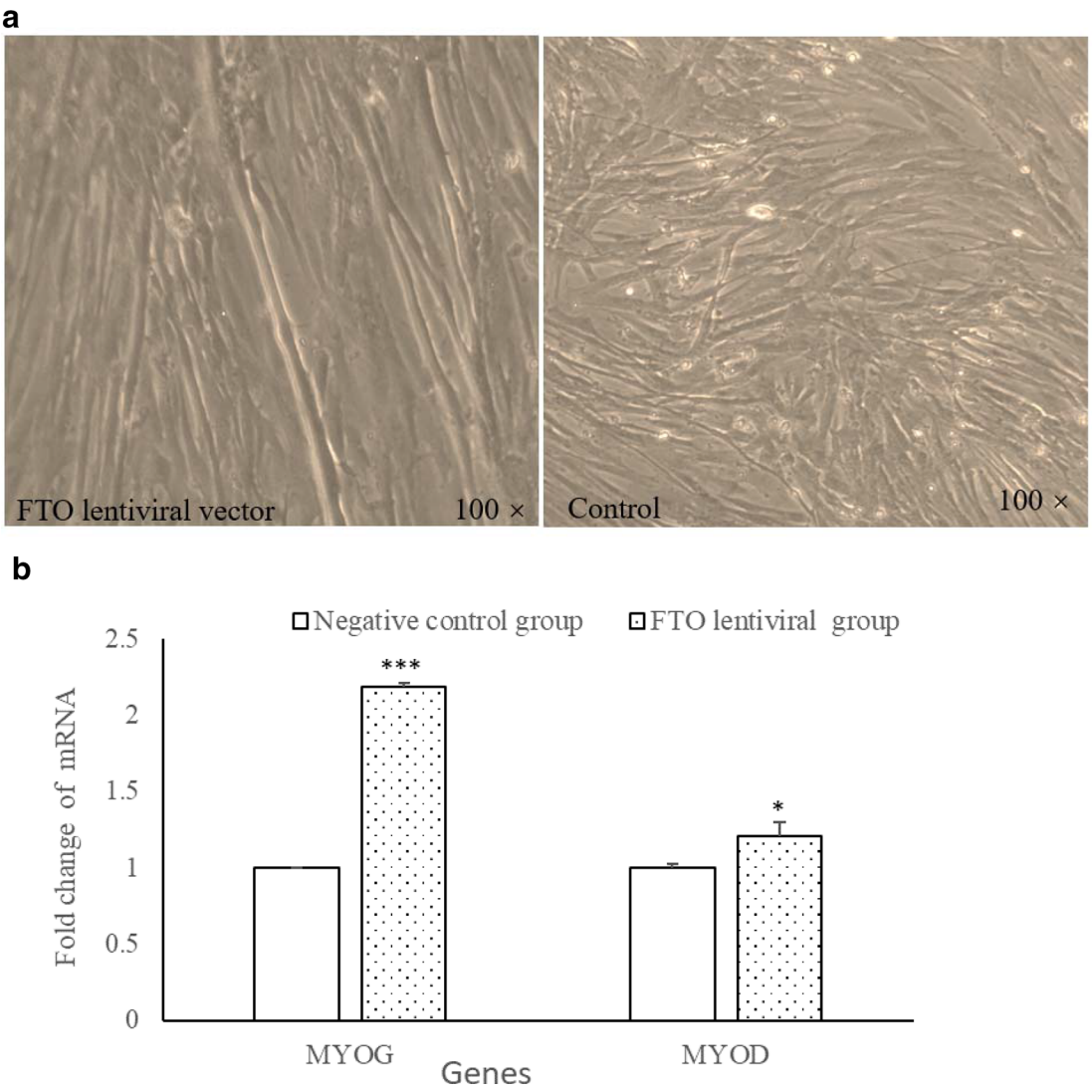

Fig. 3 Effect of *FTO* on myoblast differentiation. **a** Microscopic images of myoblasts at 144 h (×100 magnification). The myotubes were observed to be longer and more complete in the *FTO* lentiviral overexpression vectors than that of control group. **b** *MYOG* and *MYOD* mRNA were significantly enhanced at 9d after transfection with *FTO* lentiviral overexpression vectors ($n=3$, $^{*}P<0.05$, $^{***}P<0.001$ vs. control)

Table 2 Significantly enriched pathways for differentially expressed gene

Pathway	*P* value	Genes
Phagosome	7.75E-05	CYBB, MARCO, TLR4, CTSS, NCF1, ITGB2
Focal adhesion	5 E-05	RASFRF, PDGFB, RAC2, SPP1, LAMA5,CAV3
Regulation of actin cytoskeleton	0.0003	CXCR4, LPAR5, RAC2, ITGB2, PDGFB
Cytokine-cytokine receptor interaction	0.0042	CXCR4, INHA, IL2RG, CCL4, CSF1R
Adrenergic signaling in cardiomyocytes	0.0047	ACTC1, TNNC1, TNNT2, PIK3R5L
Calcium signaling pathway	0.0147	CXCR4, TNNC2, TNNC1, PTK2B
Endocytosis	0.0312	CXCR4, DNAJC6, IL2RG, CAV3
MAPK signaling pathway	0.0470	RAC2, RASGRF1, CSF1R, PDGFB
Cardiac muscle contraction	0.0053	ACTC1, TNNC1, TNNT2
Toll-like receptor signaling pathway	0.0100	TLR4, SPP1, CCL4
Ribosome	0.0252	RPS28, RPS29, RPL3L
Tight junction	0.0480	CD1B, MYH1C, HCLS1

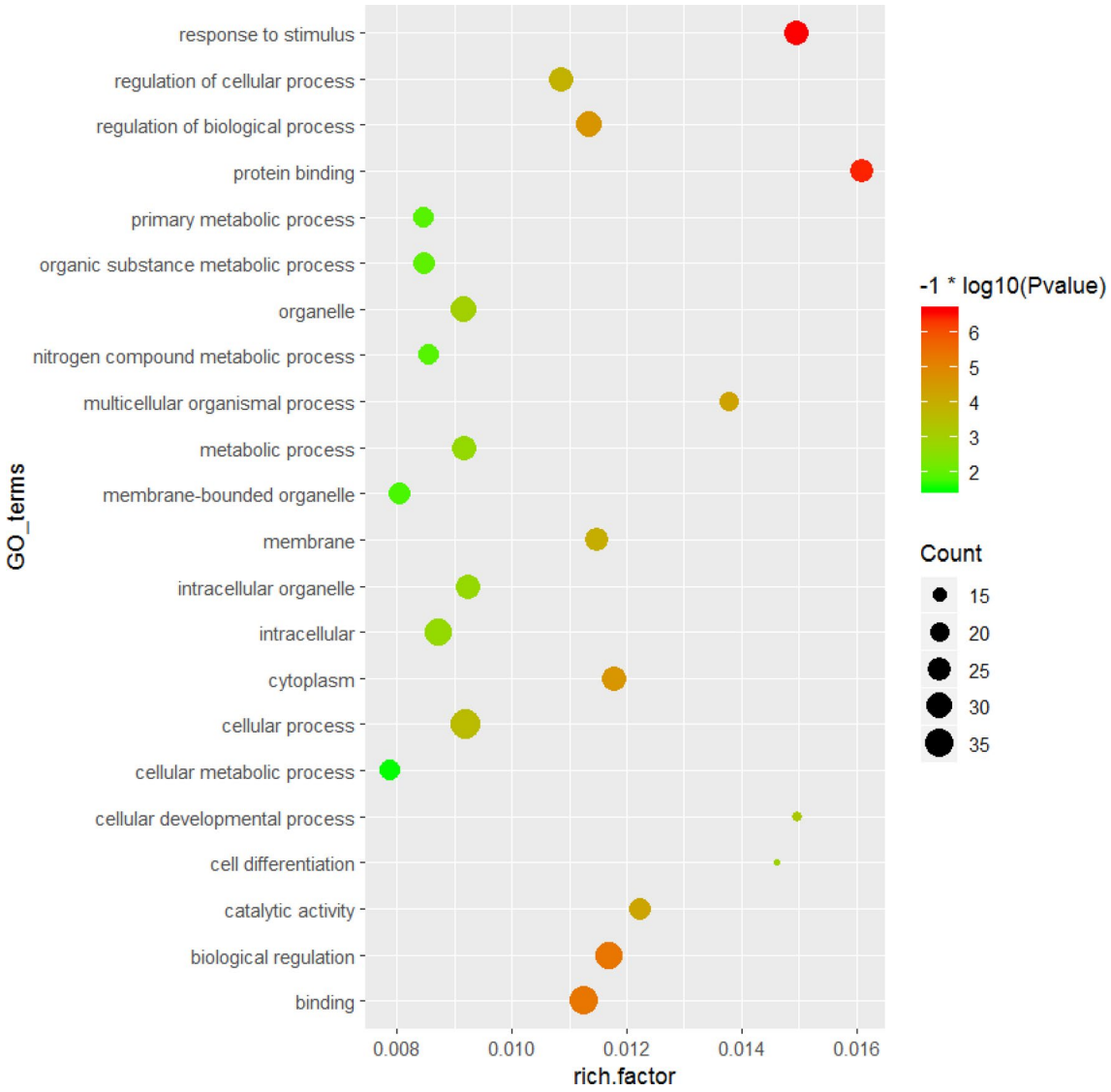

Fig. 4 GO analysis of the differentially expressed genes between *FTO* lentiviral overexpression and negative vectors groups. Significantly enriched GO terms of biological process in the top 20 were used ($P \leq 0.05$)

through regulation of the expression of genes (e.g., *LAMA5*, *SPP1, CAV3, RASGRF1, FAK, PDGFB, PDGFRα,* and *RAC2*) enriched in focal adhesion (Fig. 6).

Discussion

Skeletal muscle constitutes the largest proportion and most valuable component of meat mass in chicken, and its development is a complex physiological process (Bloise et al. 2018). Myoblast differentiation plays a decisive role in number of the muscle fibers after birth in animals. Hence, it is essential to clarify the molecular mechanisms of myoblast differentiation. Many genes and pathways were involved in the process of skeletal muscle development. Focal adhesion is one of the important molecular signaling pathways and its play pivotal role in cell proliferation, differentiation, motility, migration, and survival (Block et al. 2009; Cebrian-Serrano et al. 2014; Saeed-Zidane et al. 2019; Wu 2007). Of the special interest, eight genes (*LAMA5*, *SPP1, CAV3, RASGRF, FAK, PDGFB, PDGFRα,* and *RAC2*) known to be directly or indirectly related to skeletal muscle, were enriched in the focal adhesion pathway and showed differential expression. Based on the previous studies, these genes have been found to be important role in focal adhesion pathway. Mutation of *LAMA2* was associated with merosin-deficient congenital muscular dystrophy (Ge et al. 2018; Hashemi-Gorji et al. 2018); *SPP1* may contribute to regulating the myogenic in the early stages of muscle regeneration (Uaesoontrachoon et al. 2008) and interact with AKT1/MSTN/FoxO1 to

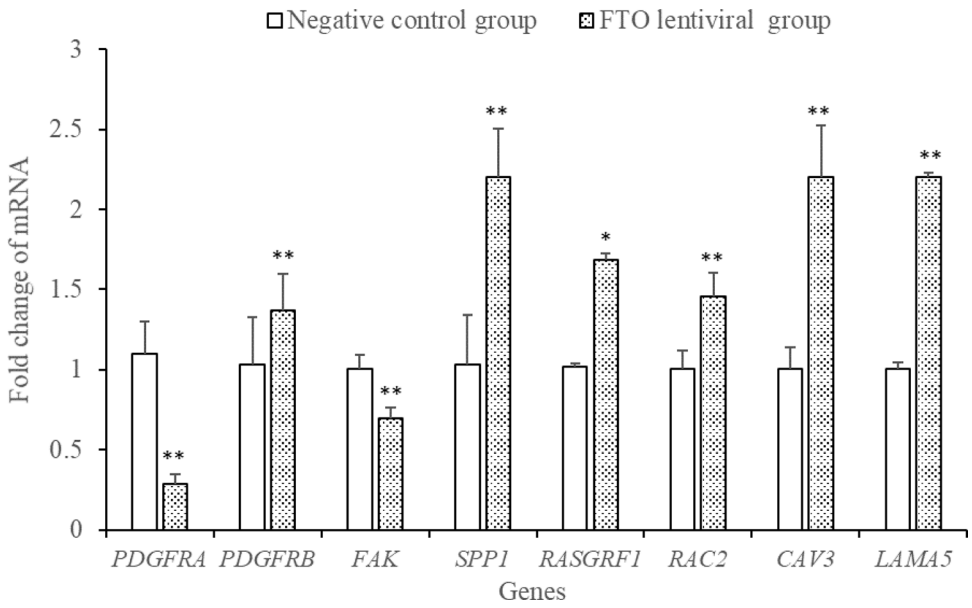

Fig. 5 Validation of differentially expressed genes by qPCR ($n=3$). $^{*}p < 0.05$, $^{**}P < 0.01$ vs. control

modify normal and dystrophic muscle (Nghiem et al. 2017); Cav3 is the main structural component of caveolae in skeletal muscle (Galbiati et al. 2001) and its deficiency resulted in disturbances of the mitochondrial respiration and energy status (Shah et al. 2020); FAK, a mechanosensitive/exercise responsive protein, regulated myoblast development and myofibrogenesis, skeletal muscle morphology, and insulin sensitivity (Bisht et al. 2008; Graham et al. 2015); palmitate oxidation and reduced glycogen synthesis enhanced after silencing FAK in primary human skeletal muscle cells (Lassiter et al. 2018). The function of *PDGF* member is mediated by two receptors (*PDGFRα* and *PDGFRβ*). *PDGFRα* signaling was associated with muscle pathology (Ito et al. 2013; Olson and Soriano 2009; Tidball et al. 1992; Zhao et al. 2003), and its promoted muscle development in growing embryos and angiogenesis in regenerating adult muscle (Orr-Urtreger et al. 1992; Soriano 1997). Focal adhesion mainly included ECM–receptor intersection and cytokine–cytokine receptor intersection signaling. As shown in Fig. 6a, eight genes mentioned above were located in the important position in each branches of the focal adhesion pathway. *Spp1* is located at the beginning of ECM–receptor intersection, it was hypothesized that *SPP1* firstly respond to *FTO* regulation, then *CAV3, FAK* simultaneously receive the signaling of *SPP1*, and inspire of the *PDGFB, PDGFRα* located in the beginning of cytokine–cytokine receptor intersection; *FAK* receives the feedback from *PDGFB, PDGFRα,* and deliver the signaling to *RAC2*, ultimately resulting in altered myoblast proliferation and differentiation. *FAK* may be an important node factor and is likely to play a key role in myoblast differentiation respond to *FTO* regulation.

In summary, our data showed that *FTO* promotes the differentiation of myoblast through regulation of gene expression (*LAMA5*, *SPP1*, *CAV3*, *RASGRF1*, *FAK*, *PDGFB*, *PDGFRα* and *RAC2*) enriched in focal adhesion pathway.

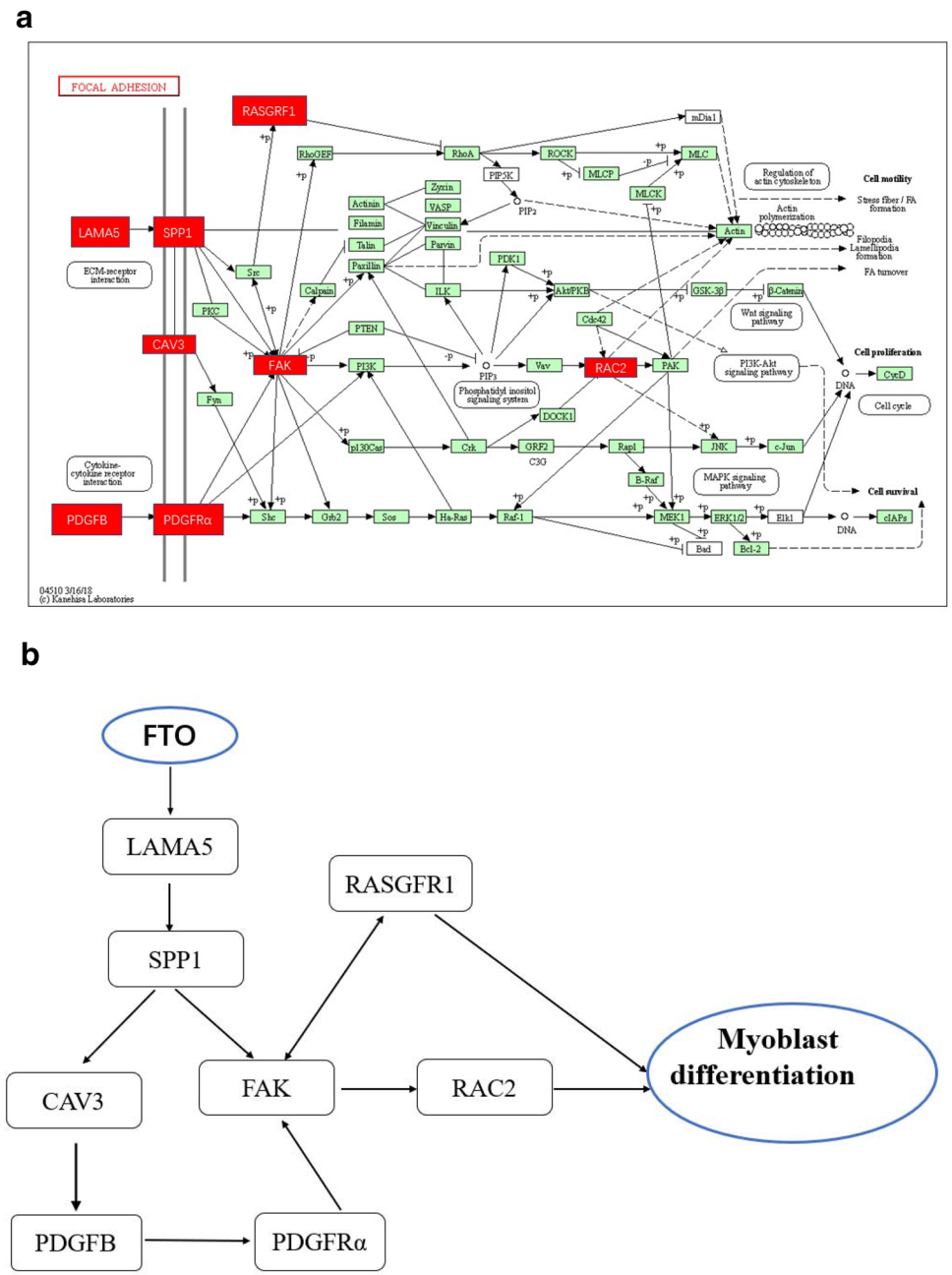

Fig. 6 Exploration of pathways by which *FTO* regulates chicken myoblast differentiation using analyses of gene expression profile. **a** Focal adhesion pathway from the KEGG database. Genes marked in red show differential expression between the *FTO* lentiviral overexpression vectors and control group. **b** *FTO* promotes the proliferation, differentiation of myoblast through regulation of gene expression (*LAMA2*, *SPP1, CAV3, RASGRF1, PTK2, PDGFB, PDGFRα,* and *RAC2*) enriched in focal adhesion pathway

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Author contributions Conceptualization: HH and SL; validation: CL and LL; data curation: ZL; formal analysis: QW and ZH; writing the original draft: HH; supervision: SL.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval The study project was conducted in accordance with the Guidelines for Experimental Animals formulated by the Ministry of Science and Technology (Beijing, China). All experimental programs were approved by the Science Research Department (in charge of animal welfare) of the Institute of Animal Sciences, CAAS (Beijing, China).

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