

# Expression of developmental genes in brown fat cells grown in vitro is linked with lipid accumulation

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**Abstract** *En1*, *Nr2f1*, *Gpc4*, *Sfrp2*, *Shox2*, *Tbx15* and *Thbd* are among the genes involved in development process of an organism in a number of tissues, in particular adipose tissue. Considering the involvement of isobutyl-methyl-xanthine (IBMX), indomethacin, dexamethasone (DEX), triiodothyronine (T3), and insulin in adipocyte differentiation, we propose that these differentiation-inducing agents may regulate differentiation in brown adipose tissue through a developmental process. Stromavascular cells isolated from interscapular brown fat of mice were cultured in DMEM-LG medium. Proliferating brown preadipocytes were differentiated in the presence of IBMX, indomethacin, DEX, T3 and insulin. *Pref1* (marker of proliferation stage) and uncoupling protein 1 (*UCP1*, marker for differentiation stage) were upregulated during proliferation and differentiation, respectively. Relative expression of *Pref1*, *UCP1* and developmental genes was determined in different stages of adipogenesis. *En1*, *Gpc4*, *Nr2f1*, *Sfrp2* and *Shox2* were upregulated during differentiation. Differentiation of preadipocytes in the absence of IBMX, indomethacin, and DEX resulted in drastic reduction in fat accumulation in differentiated adipocytes with simultaneous decrease in *En1*, *Gpc4*, *Nr2f1*, *Sfrp2*, *Shox2* and *Tbx15* gene expression. T3 upregulated the expression of *En1*, *Gpc4*, *Sfrp2* and *Tbx15* genes during differentiation and

downregulated *Shox2* expression as compared to proliferated state. Insulin upregulated the expression of *Shox2*.

**Keywords** Brown adipose tissue · Developmental gene · Proliferation · Adipocyte differentiation · Induction medium

## Introduction

Brown as well as white adipose tissues (WAT) store fat during energy excess and release triglycerides under low-energy condition. Brown adipose tissue (BAT) also generates heat by oxidizing lipids during non-shivering thermogenesis where uncoupling protein 1 (UCP1), unique protein present in inner mitochondrial membrane, prohibits formation of ATP (Canon and Nedergaard 2010). As opposed to WAT, BAT is not considered as unhealthy fat. Mesenchymal stem cells present in adipose tissue and committed to become preadipocytes are first converted to preadipocytes which finally differentiate into mature adipocytes. This occurs both in vitro and in vivo (Dani *et al.* 1997). Proliferation and differentiation stages are morphologically distinct stages which appear during adipogenesis. The former stage results in fibroblast-like cells while the latter is identified by accumulation of fat. Both the stages can be easily ascertained microscopically. The marker *Pref1* predominantly appears when preadipocytes are proliferating while UCP1 is abundantly noted when brown preadipocytes are differentiated into mature adipocytes (Villena *et al.* 2002). The fat synthesis in mature cells is regulated by transcription factor PPAR $\gamma$ , its co-activator PGC1 $\alpha$  and other molecules, viz. CCAAT/enhancer-binding proteins, specifically C/EBP $\alpha/\beta/\delta$ , sirtuin 1 and early B cell factor 2 (Tontonoz *et al.* 1994; Wu *et al.* 1999; Qiang *et al.* 2012; Rajakumari *et al.* 2013).

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**Table 1.** Scheme of methodology used for treatment of T3 or insulin on differentiation

Scheme of methodology used for treatment of T3 or insulin on differentiation											
Components of medium	Days of culture										
	1	2	3	4	5	6	7	8	9	10	11
	Proliferation							Differentiation			
<b>Effect of T3 on differentiation</b>											
DMEM-LG medium				+				+		+	
Charcoal-dextran treated FBS (10 % V/V)				-				+		+	
Charcoal-dextran treated FBS (15 % V/V)				+				-		-	
Ascorbic acid (15 µM)				+				-		-	
T3 (1 nM/ 2 nM/ 4 nM)				-				-		+	
<b>Effect of insulin on differentiation</b>											
DMEM-LG medium				+				+		+	
FBS (10 % V/V)				-				+		+	
FBS (15 % V/V)				+				-		-	
Ascorbic acid (15 µM)				+				-		-	
Insulin (0.5 nM/ 3 nM/ 18 nM)				-				-		+	

*En1* (mouse homolog of a drosophila patterning gene), *Gpc4* (gene involved in cell division and growth regulation), *Nr2f1* (gene involved in organogenesis), *Sfrp2* (modulator of Wnt signaling), *Shox2* (transcription factor expressed during embryonic development), *Tbx15* (transcription factor involved in craniofacial and limb development) and *Thbd* (a surface glycoprotein of endothelial and placental cells) are differentially expressed in subcutaneous and intra-abdominal white fat depots (Hanks *et al.* 1998; de Cat and David 2001; Singh *et al.* 2005; Gesta *et al.* 2006; Nakajima *et al.* 2009; Schneider *et al.* 2011; Li *et al.* 2012; Bosch *et al.* 2014). Ever since, developmental genes are reported in adipose tissue; precise role of developmental genes is still difficult to

understand (Cowherd *et al.* 1997; Cantile *et al.* 2002; Gesta *et al.* 2006; Gesta *et al.* 2007; Yamamoto *et al.* 2009; Waldén *et al.* 2012). The expression of developmental genes varies between white and brown adipose tissues (Gesta *et al.* 2006; Gesta *et al.* 2007; Yamamoto *et al.* 2009; Waldén *et al.* 2012). Although preadipocytes and adipocytes constitutes more than 80% of adipose tissue, the presence of other cells such as stem cells, fibroblasts, smooth muscle cells, endothelial cells, macrophages, and pericytes can also account for expression of developmental genes (Katz 2002). In vitro culture system allows preadipocytes and differentiated adipocytes in purified form and therefore, expression of developmental genes at these stages can be studied to understand the role of

**Table 2.** Primer of developmental genes used in quantitative PCR

S. no.	Gene	Primer sequences (5'-3')	
1	<i>En1</i>	Forward	5'-ACACAACCCTGCGATCCTACTC-3'
		Reverse	5'-CGCTTGCTTCCTTCTCGTTCT-3'
2	<i>Gpc4</i>	Forward	5'-GGCAGCTGGCACTAGTTTG-3'
		Reverse	5'-AACGGTGCTTGGGAGAGAG-3'
3	<i>Nr2f1</i>	Forward	5'-ACATCCGCATCTTTCAGGAAC-3'
		Reverse	5'-ACAAGCATCTGACGTGAATAGC-3'
4	<i>Sfrp2</i>	Forward	5'-AGGACAACGACCTCTGCATC-3'
		Reverse	5'-TGTCGTCTCATTCTTGTTTT-3'
5	<i>Shox2</i>	Forward	5'-TGGAACAACCAACGAGCTGGAGA-3'
		Reverse	5'-TTCAAACCTGGCTAGCGGCTCCTAT-3'
6	<i>Tbx15</i>	Forward	5'-TGTTGCGACACTGACCTTTG-3'
		Reverse	5'-CCAGTGCTGGAGGTGGTT-3'
7	<i>Thbd</i>	Forward	5'-TCCCAAGTTTCCATGTTTCC-3'
		Reverse	5'-GCATGAGTTGTGTGCTTCGT-3'
8	<i>Pref1</i>	Forward	5'-GTGTCAATGGAGTCTGCAAG-3'
		Reverse	5'-AAGCCCGAACGTCTATTT C-3'
9	<i>UCP1</i>	Forward	5'-CAAAATACTGGCAGATGACG-3'
		Reverse	5'-TTTACCACATCCACTGGAGA-3'
10	<i>β-actin</i>	Forward	5'-AGAGGGAAATCGTGCCTGAC-3'
		Reverse	5'-CAATAGTGATGACCTGGCCGT-3'

developmental genes in adipogenesis. The adipogenesis is regulated by isobutyl-methyl-xanthine (IBMX), indomethacin, dexamethasone (DEX), triiodothyronine (T3) and insulin. However, whether these differentiation-inducing agents influence expression of developmental genes is not known. In this paper, the effect of these agents on expression of *En1*, *Gpc4*, *Nr2f1*, *Sfrp2*, *Shox2*, *Tbx15* and *Thbd* is reported.

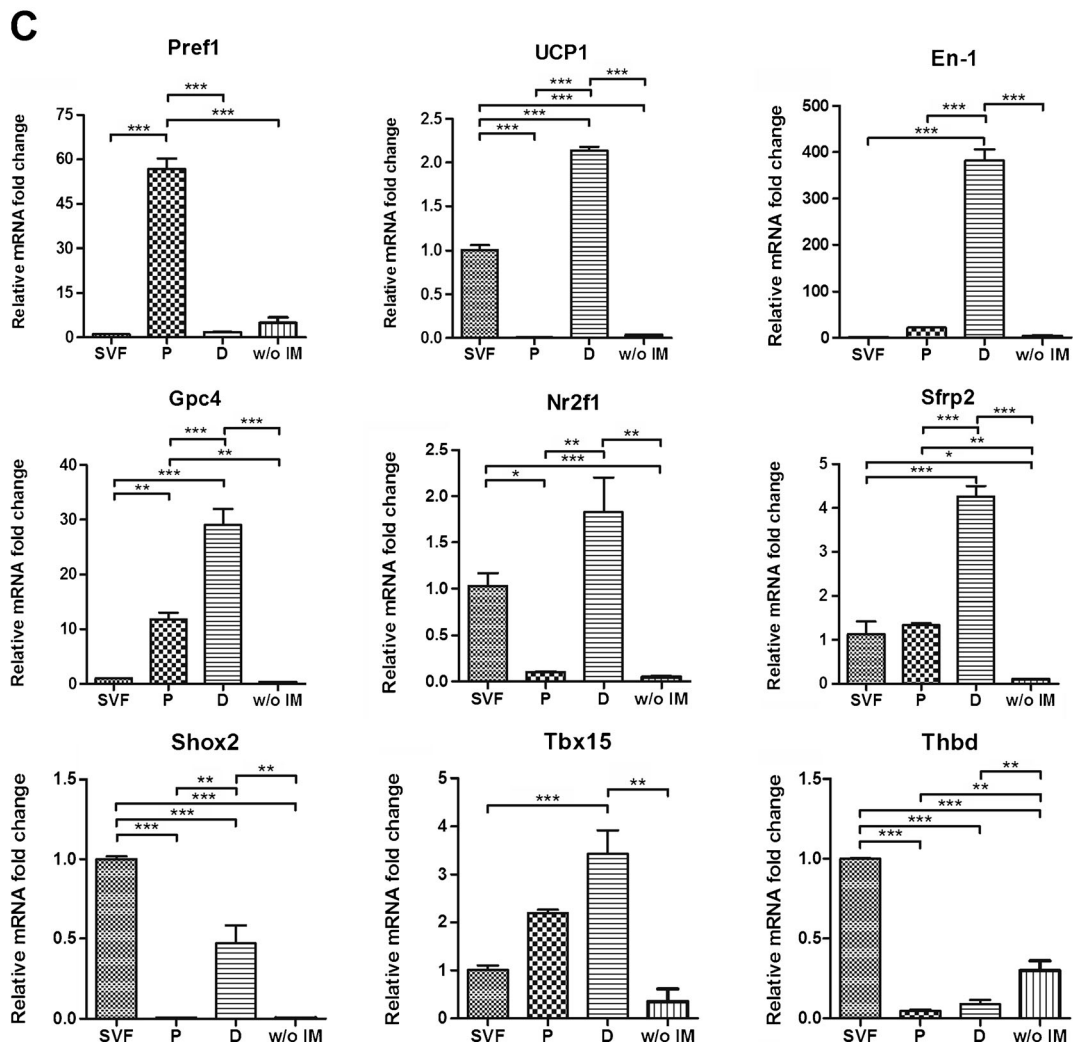
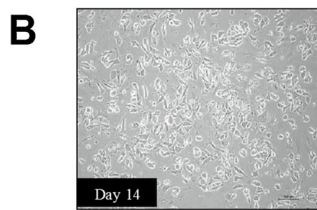
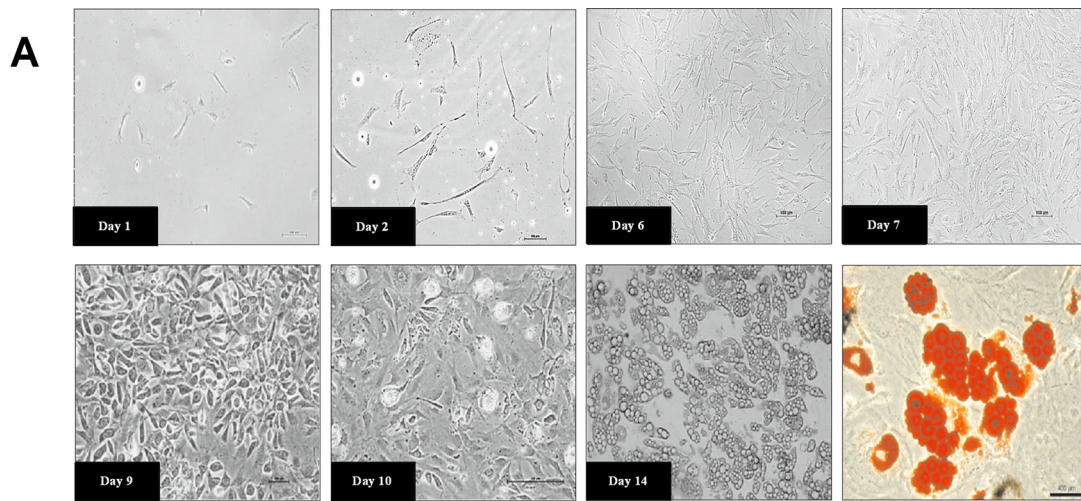
## Materials and Methods

**Subjects.** Swiss albino mice in random fed groups were allowed ad libitum access to water and food and were maintained in a temperature-controlled room (25±3°C) for 12 h light–dark cycle at small animal house facilities of the National Dairy Research Institute, Karnal. Experiments were performed on 4–6-wk-old male mice in compliance with the Institute Animal Ethics Committee (IAEC) guidelines.

**Isolation of stromavascular fraction and proliferation of preadipocytes.** BAT (200–300 mg) was collected aseptically from the interscapular region of mice. The methodology described by Fasshauer *et al.* (2000) was used for isolation of stromavascular fraction (SVF). BAT was excised from three animals and treated with a digestion medium containing 2 ml 0.2% collagenase (type II) in HEPES buffer (100 mM HEPES,

123 mM NaCl, 5 mM KCl, 1.3 mM CaCl<sub>2</sub>, 5 mM glucose, 1.5% BSA, pH 7.4) for 10–15 min at 37°C on an orbital shaker (90 rpm). The digested contents were filtered through a 100-μm nylon filter and centrifuged at 450×g for 10 min. The pellet comprising SVF was suspended in Dulbecco's modified Eagle's medium-low glucose (DMEM-LG) proliferation medium containing 15% fetal bovine serum (FBS), 10 mM HEPES, 50 IU penicillin/ml, 50 μg streptomycin/ml, 3.7 g/l NaHCO<sub>3</sub>, 3 nM insulin and 15 μM ascorbic acid and then plated in a 25<sup>cm</sup><sup>2</sup> tissue culture flask and cultured in 5% CO<sub>2</sub> atmosphere at 37°C. After 24 h of incubation, non-adherent cells were removed by repeatedly washing the cells with proliferation medium without FBS. Adherent cells were further maintained in DMEM-LG proliferation medium, and spent medium was replenished with fresh medium until the cells reached 70–80% confluency.

**Adipocyte differentiation.** Differentiation of brown preadipocytes was achieved as per method described by Klein *et al.* (1999) with some modifications. Proliferative preadipocytes were cultured in an induction medium (DMEM-LG medium (pH 7.4) containing 10% FBS, 500 μM IBMX, 125 μM indomethacin, 2 μg/ml DEX, 18 nM insulin and 1 nM T3) for 2 d and then cultured in a differentiation medium (induction medium devoid of IBMX, indomethacin and DEX) for 5 d. The medium was changed in every alternate day.



◀ **Figure 1** Proliferated and differentiated brown adipocytes and expression of stage-specific marker and developmental genes (A); brown adipocytes at different days of culture (B); brown adipocytes cultured without induction medium at day 14 (C); expression pattern of marker genes and developmental genes. SVF stroma vascular fraction, P proliferation stage, D differentiation stage, w/o IM without induction medium. Bars represent relative mRNA expression fold change and error bars represent standard error of the mean. The number of technical and biological replicates was three each. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

*Differentiation in absence of induction medium.* Proliferative preadipocytes were also cultured in a differentiation medium for 7 d without pre-culturing in an induction medium.

*Effect of T3.* Effect of three different levels of T3, viz. 1, 2 and 4 nM on expression pattern of developmental genes during differentiation of brown adipocytes was studied, and the scheme of culture is shown in Table 1 (Cousin *et al.* 1992). SVF was isolated from three Swiss albino mice and were cultured in a proliferation medium containing 15% charcoal–dextran-treated FBS and 15  $\mu$ M ascorbic acid for 7 d to induce preadipocytes in proliferation stage. At day 8, the spent medium was replaced with DMEM-LG medium containing 10% charcoal–dextran-treated FBS (devoid of IBMX, indomethacin, DEX, T3 and insulin) for 48 h. Further, cells were treated with different concentration of T3 for 2 d when cultured in DMEM-LG medium containing 10% charcoal–dextran-treated FBS and 1 nM/2 nM/4 nM concentration of T3.

*Effect of insulin concentration.* Effect of insulin was studied similar to T3 except insulin at three different levels, viz. 0.5, 3 and 18 nM was added in cultured medium at day 10 instead of T3 (Table 1).

*RNA extraction and gene expression analysis.* Using TRI reagent (Sigma Chemicals Co., St. Louis, MO) total RNA from SVF, proliferated adipocytes, and differentiated adipocytes was isolated and quantified by NanoPhotometer (IMPLEN, Munich, Germany). RNA was treated with DNase (Fermentas Life Sciences, Ontario, Canada). Subsequently, RNA (1  $\mu$ g) was reverse transcribed with RevertAid™ first-strand complementary DNA (cDNA) synthesis kit (Fermentas Life Sciences, Canada). Quantitative PCR was done on Mx3005 apparatus (Stratagene, La Jolla, CA) using SYBR Green, double-stranded DNA-specific fluorescent dye (Roche Diagnostics, Mannheim, Germany) using comparative quantitation method and  $\beta$ -actin normalization. cDNA samples were prepared in three biological replicates from SVF and proliferated and differentiated adipocytes. Primers of genes *Pref1*, *UCP1* and  $\beta$ -actin were designed using the web-based software PRIMER-3. Mouse sequences for genes *Pref1* and *UCP1* were obtained from NCBI ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and  $\beta$ -actin from ENSEMBL genome ([www.ensembl.org](http://www.ensembl.org)) browser.

The primers for genes *En1*, *Gpc4*, *Nr2f1*, *Sfrp2*, *Shox2*, *Tbx15* and *Thbd* were used as reported by Gesta *et al.* (2006). Details of primers are given in Table 2.

Critical threshold (CT) of SVF value was considered as a calibrator except in case of hormonal studies where CT value in the absence of hormone was used as a calibrator. Relative mRNA fold change was calculated in comparison to  $\beta$ -actin. The data were analyzed using  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen 2001).

*Statistical analysis.* The gene expression was studied in three biological replicates, and each value was determined in triplicates. Comparison between groups was performed using one-way analysis of variance (ANOVA) followed by Tukey: Compare all pairs of columns test.  $P$  value of  $< 0.05$  was considered as significant. Statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software Inc., San Diego, CA).

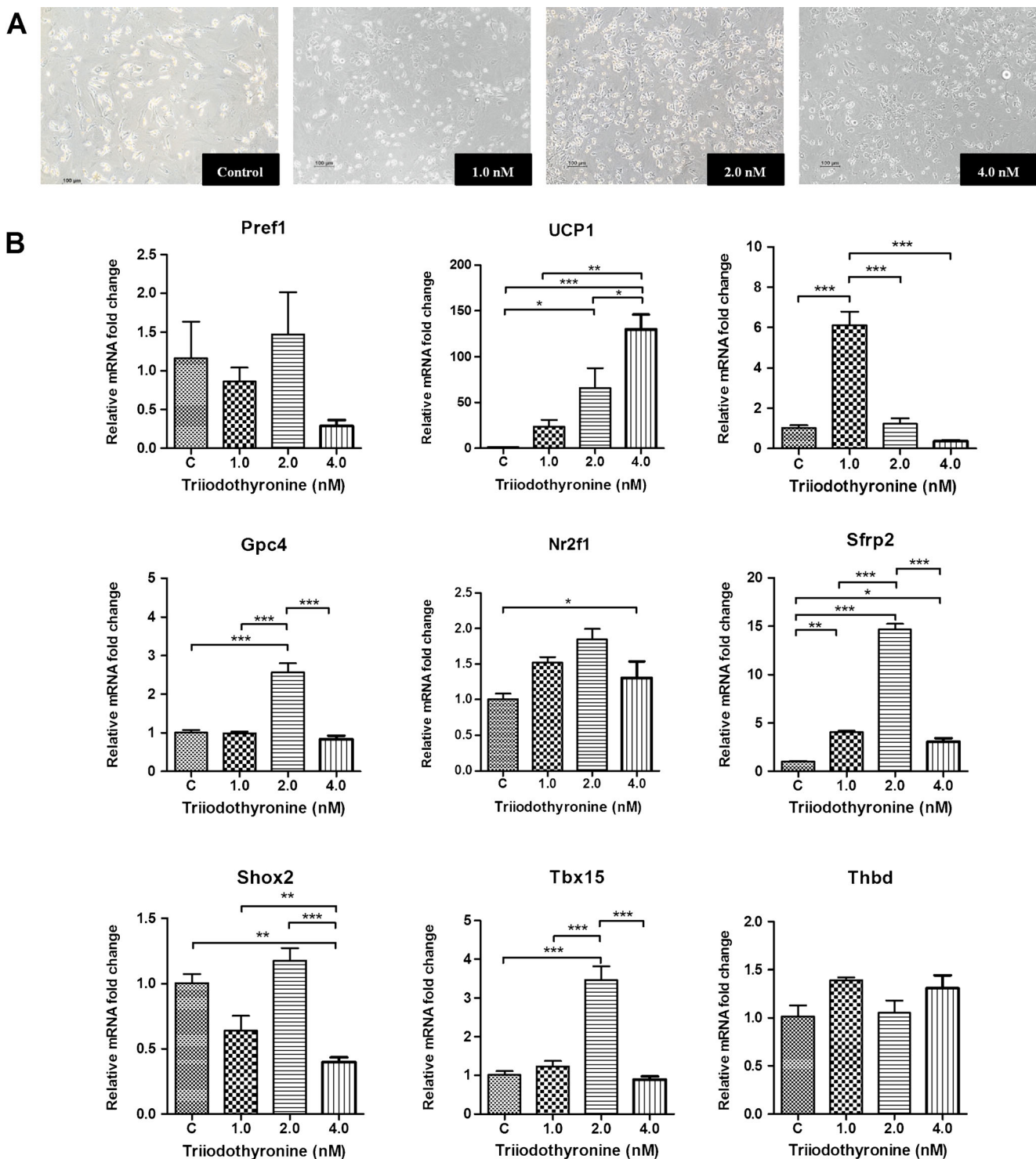
## Results

*Adipogenesis.* When stromavascular cells were plated in DMEM-LG proliferation medium, fibroblast-like cells were visible after 24 h of plating. The fibroblast-like brown fat cells multiplied rapidly and at day 6, 70–80% confluency is usually reached. At day 7, proliferated cells (70–80% confluency) were treated with induction medium containing IBMX, DEX, indomethacin, T3 and insulin for 2 d. At day 9, distinct changes in cell morphology were noted. Cells acquired almost a round shape. The cells were then cultured in differentiation medium containing T3 and insulin for 6 d. At day 10, cytoplasm of these cells became enlarged and cells contained numerous small-sized lipid droplets. At day 14 of cell culture, lipid incorporation increased and small-sized multilocular

**Table 3.** Relative mRNA fold change (mean $\pm$ SD) in marker and developmental genes at different stages of adipogenesis

Genes	SVF	P	D	w/o IM
<i>Pref1</i>	1.018 $\pm$ 0.19	56.64 $\pm$ 6.3	1.76 $\pm$ 0.098	4.880 $\pm$ 3.2
<i>UCP1</i>	1.000 $\pm$ 0.094	0.010 $\pm$ 0.0036	2.14 $\pm$ 0.076	0.034 $\pm$ 0.0026
<i>En1</i>	1.299 $\pm$ 0.68	22.09 $\pm$ 0.54	382.1 $\pm$ 42	3.287 $\pm$ 1.1
<i>Gpc4</i>	1.007 $\pm$ 0.12	11.80 $\pm$ 2.2	29.01 $\pm$ 5.2	0.357 $\pm$ 0.022
<i>Nr2f1</i>	1.030 $\pm$ 0.24	0.101 $\pm$ 0.019	1.83 $\pm$ 0.65	0.049 $\pm$ 0.0097
<i>Sfrp2</i>	1.125 $\pm$ 0.52	1.341 $\pm$ 0.077	4.265 $\pm$ 0.41	0.102 $\pm$ 0.015
<i>Shox2</i>	1.000 $\pm$ 0.028	0.007 $\pm$ 0.0024	0.471 $\pm$ 0.19	0.001 $\pm$ 0.0008
<i>Tbx15</i>	1.013 $\pm$ 0.16	2.188 $\pm$ 0.12	3.433 $\pm$ 0.85	0.353 $\pm$ 0.46
<i>Thbd</i>	1.000 $\pm$ 0.007	0.048 $\pm$ 0.012	0.092 $\pm$ 0.04	0.300 $\pm$ 0.11

SVF stroma vascular fraction, P proliferation stage, D differentiation stage, w/o IM differentiation without induction medium



**Figure 2** Effect of T3 on differentiation of brown adipocytes and expression of stage-specific marker and developmental genes. Proliferative adipocytes were cultured in the absence (C control) or in the presence of T3 at indicated concentrations. (A) Cells at day 12; (B)

expression pattern of marker genes and developmental genes. Bars represent relative mRNA expression fold change and error bars represent standard error of the mean. The number of technical and biological replicates was three each. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

lipid droplets were observed in differentiated cells. Differentiated cells on staining with Oil Red O: [1-(2, 5-dimethyl-4-(2-5-dimethylphenyl) phenyldiazene) azonaphthalen-2-ol] dye, appeared red (Fig. 1A) (Peng *et al.* 2013).

*Pref1* and *UCP1* levels changed as expected, according to their specificity in proliferation and differentiation stage, respectively (Fig. 1C). The expression of seven developmental genes, viz. *En1*, *Gpc4*, *Nr2f1*, *Sfrp2*, *Shox2*, *Tbx15* and *Thbd*

**Table 4.** Effect of T3 and insulin on relative mRNA fold change (mean±SD) in marker and developmental genes during differentiation

Genes	T3 (nM)				Insulin (nM)			
	Control	1.0	2.0	4.0	Control	0.5	3.0	18.0
<i>Pref1</i>	1.163±0.81	0.861±0.31	1.474±0.94	0.287±0.13	1.040±0.37	0.428±0.036	0.276±0.027	0.276±0.027
<i>UCP1</i>	1.084±0.52	23.570±13	65.930±37	129.6±28	1.014±0.20	7.384±1.3	0.414±0.31	0.336±0.14
<i>En1</i>	1.018±0.24	6.108±1.2	1.228±0.48	0.365±0.048	1.024±0.26	1.607±0.70	0.131±0.047	0.610±0.45
<i>Gpc4</i>	1.005±0.11	0.986±0.079	2.564±0.41	0.837±0.15	1.001±0.057	1.250±0.16	0.605±0.14	0.811±0.17
<i>Nr2f1</i>	1.006±0.13	1.520±0.14	1.845±0.26	1.306±0.40	1.053±0.40	0.767±0.59	0.230±0.15	1.402±1.4
<i>Sfrp2</i>	1.003±0.094	4.070±0.25	14.679±1.0	3.096±0.56	1.015±0.22	1.334±0.67	0.816±0.57	0.447±0.14
<i>Shox2</i>	1.005±0.12	0.640±0.20	1.175±0.17	0.398±0.060	1.032±0.30	3.689±0.44	2.158±0.45	1.655±0.99
<i>Tbx15</i>	1.010±0.16	1.222±0.25	3.464±0.61	0.888±0.14	1.048±0.38	1.105±0.43	0.200±0.021	0.654±0.44
<i>Thbd</i>	1.014±0.20	1.392±0.049	1.052±0.22	1.307±0.24	1.016±0.21	1.020±0.13	1.154±0.24	1.133±0.30

in SVF and proliferated and differentiated brown adipocytes indicated (Table 3) that there was about 16.4-fold, 2.5-fold, 18-fold, 3.1-fold and 67.9-fold higher expression of *En1*, *Gpc4*, *Nr2f1*, *Sfrp2* and *Shox2* in differentiated over proliferated adipocytes (Fig. 1C), respectively. However, expression of *Tbx15* and *Thbd* was not changed significantly on differentiation of proliferated cells. Also, the expression of *UCP1*, *Shox2* and *Thbd* decreased in proliferated cells over SVF while that of *Pref1* and *Gpc4* increased.

**Effect of induction medium on differentiation.** Cells grown in the absence of induction medium components do not fully differentiate, and few tiny droplets of fat were visible at day 14 of culture (Fig. 1B). Expression of *UCP1*, *En1*, *Gpc4*, *Nr2f1*, *Sfrp2*, *Shox2* and *Tbx15* were downregulated approximately 62, 116, 81, 38, 42, 294 and 10 times (Fig. 1C, Table 3) respectively compared to differentiation achieved in the presence of induction medium components. However, *Thbd* expression increases three times (Fig. 1C).

**Effect of T3 on differentiation.** Cells grown in the presence of T3 (at all the concentrations) but in the absence of insulin had swollen cytoplasm in comparison to cell grown in the absence of T3. Lipid droplets were absent both in the presence and absence of T3 in medium (Fig. 2A). Significantly higher expression of *UCP1* was noted at 2 and 4 nM T3 (Fig. 2B). Expression of *Pref1* was not altered at all the three levels of T3 (Fig. 2B).

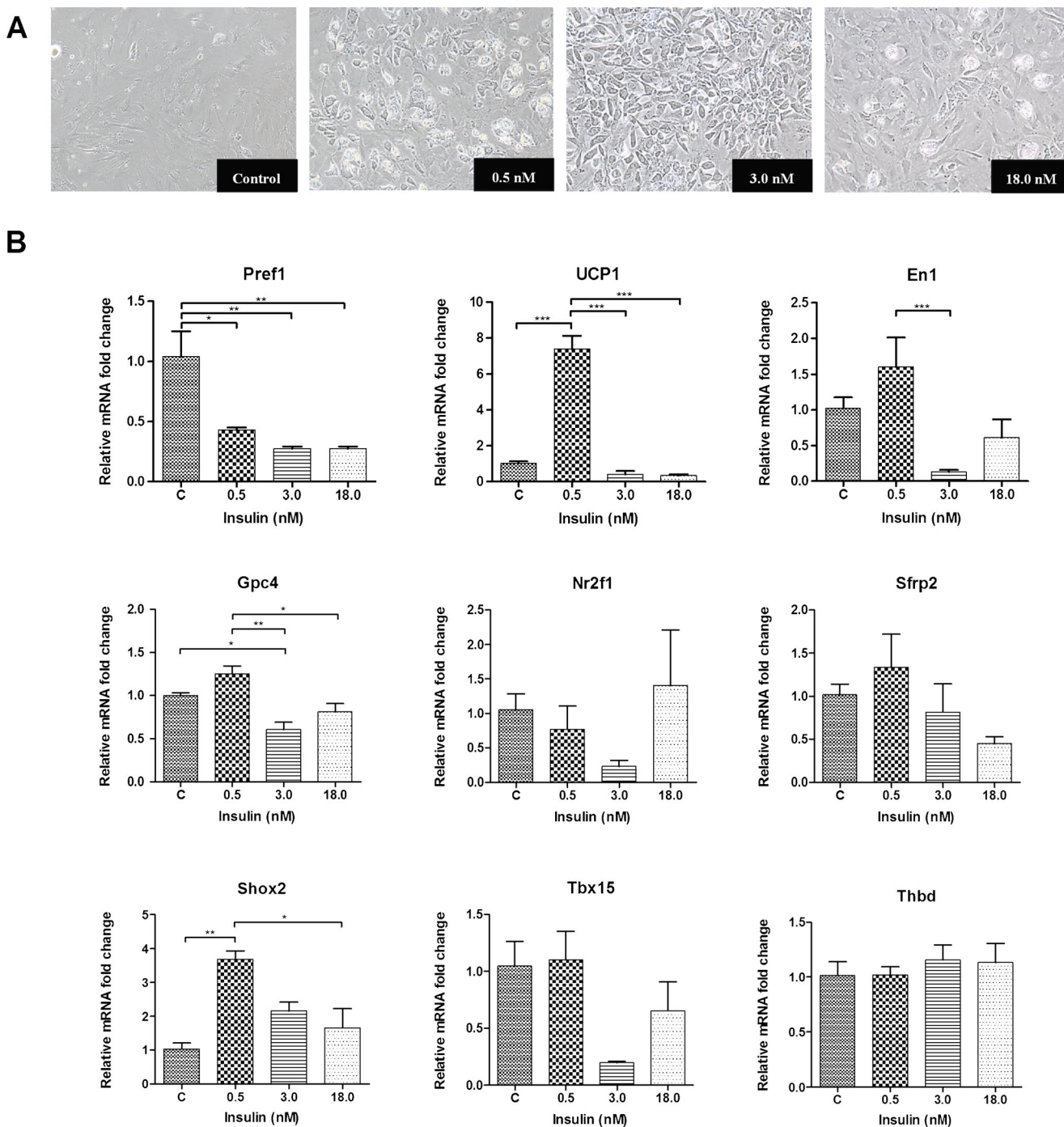
Effect of different levels of T3 on expression *En1*, *Gpc4*, *Nr2f1*, *Sfrp2*, *Shox2*, *Tbx15* and *Thbd* is shown in Fig. 2 and Table 4. The expression of *En1*, *Gpc4*, *Nr2f1*, *Sfrp2*, *Shox2* and *Tbx15* was dependent on concentration of T3. *Gpc4*, *Sfrp2* and *Tbx15* were maximally produced at 2 nM T3 and difference in expression was statistically significant when compared with control. At other concentrations of T3 (1 and 4 nM), the expression was lower. The *En1* expression was significantly higher at 1 nM T3 in comparison to the control

group. At 2 or 4 nM T3, there was no significance difference in expression of *En1* between the treatment group and control group. Although the expression of *Nr2f1* was significantly higher ( $P<0.05$ ) at 2 nM T3 in comparison to the control group, the fold difference in expression was less than 2 and therefore, the results should be taken as indicative in nature. On the other hand, expression of *Shox2* was significantly lower at 4 nM in comparison to the control group. There was no effect on the expression of *Thbd* at all the three levels of T3. At 1 nM T3, the expression of *En1* and *Sfrp2* enhanced while other genes remained unaffected.

**Effect of insulin on differentiation.** Cells grown in the presence of insulin (at all the concentrations) but in the absence of T3 in the differentiation medium had a distinct morphology. Differentiated cells were spindle in shape with a swollen cytoplasm and little lipid droplets (Fig. 3A). Insulin did not alter expression of *En1*, *Nr2f1*, *Sfrp2*, *Tbx15* and *Thbd* (Fig. 3B; Table 4). Expression of *Shox2* was significantly higher at 0.5 nM, whereas *Gpc4* decreased at 3 nM (Fig. 3B). At all the concentrations of insulin, *Pref1* was expressed at a reduced level. *UCP1* expressed was enhanced only at 0.5 nM insulin (Fig. 3B).

## Discussion

*En1*, *Gpc4*, *Nr2f1*, *Sfrp2*, *Shox2*, *Tbx15*, *Thbd* and a number of *Hox* genes including *HoxA1*, *HoxA5*, *HoxC4*, *HoxC8*, *HoxC9* and *HoxA10* are present in adipose tissue or preadipocytes and mature adipocytes derived from adipose tissue (Cowherd *et al.* 1997; Cantile *et al.* 2002; Gesta *et al.* 2006; Gesta *et al.* 2007; Yamamoto *et al.* 2009; Waldén *et al.* 2012). Adipose tissue comprises stem cells, preadipocytes and mature adipocytes and these cells types accounts for >80% (Katz 2002). The other cells such as macrophages, pericytes, fibroblast and endothelial cells are also present in adipose tissue and the possibility exists that these can also account of



**Figure 3** Effect of insulin on differentiation of brown adipocytes and expression of stage-specific marker and developmental genes. Proliferative adipocytes were cultured in the absence (C control) or in the presence of insulin at indicated concentrations. (A) Cells at day 12; (B)

expression pattern of marker genes and developmental genes. Bars represent relative mRNA expression fold change and error bars represent standard error of the mean. The number of technical and biological replicates was three each. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

developmental genes identified so far (Katz 2002). In vitro culture system provides purified preparation of preadipocytes which on appropriate culture condition can be differentiated. Large fat globule(s) are present in differentiated white adipocyte, whereas several fat globules of small size are observed in differentiated brown adipocytes (Seale *et al.* 2009). Our

studies on differentiation of brown adipocytes indicate that either insulin or T3 alone in the absence of differentiation-inducing agents (IBMX, indomethacin and DEX) cannot lead to accumulation of fat in brown adipocytes in a significant way. In the presence of T3 alone, even proliferative preadipocytes were not healthy.



The involvement of developmental genes in adipogenesis is further supported from the present study. Treating preadipocytes with induction medium results in the upregulation of *En1*, *Gpc4*, *Nr2f1*, *Sfrp2* and *Shox2*, whereas these genes are downregulated in the absence of an induction medium. Further, significant lipid accumulation is observed only when preadipocytes are pretreated with a cocktail of IBMX, indomethacin and DEX during differentiation. The results therefore support that role of IBMX, indomethacin and DEX in lipid accumulation is linked with regulation of developmental genes. *Tbx15* and *Thbd* expressions remains unaltered during differentiation in the presence of induction medium suggesting that these genes are not of much consequence in differentiation (Fig. 1C).

Effect of T3 on UCP1 (marker for brown adipocytes differentiation) was dose dependent and therefore, T3 affects differentiation. However, except *Thbd*, the effects of T3 on other developmental genes were dose specific (Fig. 2). The results presented in the paper suggest that *Nr2f1*, *Sfrp2*, *Tbx15* and *Thbd* are not regulated through insulin (Fig. 3).

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**Authors' contributions** Y.S.R. developed the concept. S.S. and A.K.B. performed the experiments, and collected and analyzed the data. Y.S.R., S.S., and A.K.B. wrote the manuscript. R.S. and S.G. gave the conceptual advice.

**Conflict of interest** The authors declare that they have no competing interests.

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