Gene Expression Patterns 20 (2016) 99–105

Contents lists available at ScienceDirect

Gene Expression Patterns

journal homepage: http://www.elsevier.com/locate/gep

Fat accumulation in differentiated brown adipocytes is linked with expression of *Hox* genes

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ARTICLE INFO

Article history:
Received 8 September 2015
Received in revised form
23 December 2015
Accepted 23 January 2016
Available online 26 January 2016

Keywords:
Hox genes
Brown fat differentiation
Adipogenesis
Insulin
Triiodothyronine
Induction medium

ABSTRACT

Homeobox (Hox) genes are involved in body plan of embryo along the anterior–posterior axis. Presence of several *Hox* genes in white adipose tissue (WAT) and brown adipose tissue (BAT) is indicative of involvement of *Hox* genes in adipogenesis. We propose that differentiation inducing agents *viz.* isobutyl-methyl-xanthine (IBMX), indomethacin, dexamethasone (DEX), triiodothyronine (T3) and insulin may regulate differentiation in brown adipose tissue through *Hox* genes. *In vitro* culture of brown fat stromalvascular fraction (SVF) in presence or absence of differentiation inducing agents was used for establishing relationship between fat accumulation in differentiated adipocytes and expression of *Hox* genes. Relative expression of *Pref1, UCP1* and *Hox* genes was determined in different stages of adipogenesis. Presence or absence of IBMX, indomethacin and DEX during differentiation of proliferated preadipocytes resulted in marked differences in expression of *Hox* genes and lipid accumulation. In presence of these inducing agents, lipid accumulation as well as expression of *HoxA1, HoxA5, HoxC4 & HoxC8* markedly enhanced. Irrespective of presence or absence of T3, insulin down regulates *HoxA10*. T3 results in over expression of *HoxA5, HoxC4* and *HoxC8* genes, whereas insulin up regulates expression of only *Hox*C8. Findings suggest that accumulation of fat in differentiated adipocytes is linked with expression of *Hox* genes.

1. Introduction

In vitro differentiation of pre-adipocytes requires culturing of cells in presence of isobutyl-methyl-xanthine (IBMX), indomethacin and dexamethasone (DEX) besides triiodothyronine (T3) and insulin (Fasshauer et al., 2000, 2002; Klein et al., 1999). Differentiated adipocytes are morphologically distinct from pre-adipocytes and acquires lipids droplets. Differentiated brown adipocytes have multilocular lipid droplets while cytoplasm of differentiated white adipocytes is virtually filled up with unilocular fat. The role of white and brown adipose tissue is distinctly different. The expression of uncoupling protein 1 (UCP1), protein involved in non-shivering thermogenesis is only noted in brown adipocytes and thereby is of more significance in early stage of life.

In mice (*Hox* genes) and Human (*HOX* genes), there are at least 39 genes organized in four genomic clusters, each localized in different chromosomes (Apiou et al., 1996). *Hox* genes are associated in angiogenesis, wound repair (Uyeno et al., 2001), functions of female reproductive tract, endometrial development (Taylor, 2000), acute myeloid leukemia, acute lymphoid leukemia (Alharbi et al., 2013), hematopoiesislimbs and genitalia development (Dolle et al., 1989; Yokouchi et al., 1991). The presence of *HoxA1, HoxA5, HoxA10, HoxC4* and *HoxC8* is established in brown adipose tissue (BAT). Except *HoxA1* and *HoxC4*, these genes are also expressed in white adipose tissue (WAT). In adipose tissue, major cells are pre-adipocytes and adipocytes which constitutes more than 80% of cell mass while stem cells, fibroblasts, smooth muscle cells, endothelial cells, macrophages and pericytes account for other cell types (Katz, 2002). Preadipocytes are plastic adherent cells and therefore *in vitro* culture of stromal vascular fraction is a convenient way for removal of cells other than adipocytes (Ripoll and Bunnell, 2009; Colter et al., 2000). The adipogenesis is regulated by isobutyl-methyl-xanthine (IBMX), indomethacin, dexamethasone (DEX),

List of abbreviations: BAT, Brown adipose tissue; CT, Critical threshold; DEX, Dexamethasone; IBMX, Isobutyl-methyl-xanthine; SVF, Stromalvascular fraction; T3, Triiodothyronine; UCP1, Uncoupling protein 1; WAT, White adipose tissue.

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http://dx.doi.org/10.1016/j.gep.2016.01.002
1567-133X/

T3 and insulin. However, whether fat accumulation in differentiated brown adipocytes and expression of hox genes is linked or not, is not known. In this paper, expression pattern of *HoxA1, HoxA5, HoxA10, HoxC4, HoxC8 & HoxC9* during brown fat differentiation under the influence of differentiation inducing agents is reported.

2. Results

2.1. Proliferation and differentiated adipocytes

When stromalvascular cells were plated in DMEM-LG proliferation medium in tissue culture flask, cells attached to surface after 24 h of culturing. At this stage, some cells with fibroblast like morphology were observed on the surfaces of culture flask. At day 6 of culture, 60–70% confluency was noted (Fig. 1A). Next day cells were treated with induction medium containing IBMX, DEX, indomethacin, T3 and insulin for 2 days. At day 9, cells acquired oval or round shape. Next day numerous small-size lipid droplets in cytoplasm of these cells were observed. At day 14 of cell culture, size of the lipid droplets increased and thereby enhancing lipid accumulation (Fig. 1A). When cells were stained with Oil Red O dye, triglycerides appeared as red droplets (Fig. 1A). This dye binds with neutral triglycerides and lipid (Peng et al., 2013).

2.2. Quantification of Hox genes in different stages of adipogenesis

There was 56 folds increase in *Pref1* (marker of preadipocyte) and 97 folds decrease in expression of *UCP1* (marker of mature adipocytes) in proliferated cells over SVF expression. The expression of *Pref1* and *UCP1* in differentiated cells over proliferated cells was 32 folds lower & 207 folds higher respectively (Fig. 1C). The pattern of expression of *Pref1* and *UCP1* indicates that culture conditions for proliferation and differentiation were optimum and isolated RNA from cells was from proliferation and differentiation stages of adipogenesis. There were about 13 folds, 22 folds, 12 folds, 78 folds and 7 folds higher expression of genes *HoxA1*, *HoxA5*, *HoxC4*, *HoxC8* and *HoxC9* in differentiated over proliferated adipocytes (Fig. 1C). However, expression of *HoxA10* gene decreased in differentiated cells in comparison to proliferated cells and the decrease was 32 folds (Fig. 1C).

2.3. Effect of induction medium on differentiation

In absence of induction medium, adipocytes do not differentiate nicely and have only few small fat droplets at day 14 of culture (Fig. 1B*). UCP1, HoxA1, HoxA5, HoxC4 & HoxC8* genes were expressed in lower amounts ranging from 12 fold to 3000 folds (Fig. 1C) compared to differentiation achieved in presence of induction medium components. However, *HoxC9* expression increased by 4 folds (Fig. 1C).

2.4. Effect of T3

In presence of T3 at all the three levels (1 nM, 2 nM and 4 nM), the density of cells was higher and fat droplets were absent in cytoplasm which was relatively more swollen as compared to cell differentiated in absence of T3 (Fig. 2A). Effect of different levels of T3 on markers of proliferation *(Pref1)* and differentiation *(UCP1)* stages was shown in Fig. 2B. There was dose effect of T3 on *UCP1* expression. Expression of *UCP1* was significantly higher at 2 nM and 4 nM T3 in comparison to in its absence.

Effect of different levels of T3 on expression of Hox genes *viz. HoxA1, HoxA5, HoxA10, HoxC4, HoxC8* and *HoxC9* are shown in Fig. 2B. There was no effect of T3 on expression of *HoxA1* and *HoxA10*. However, the expression of *HoxA5, HoxC4, HoxC8* and *HoxC9* was dependent on the level of T3 in the culture medium. The expression of *HoxA5* and *HoxC4* was optimum at 2 nM T3. There is significance increase ($p < 0.001$) in *HoxC8* gene expression between 1 nM T3 treatment and control (Fig. 2B).

2.5. Effect of insulin

In presence of insulin at all the three levels (0.5 nM, 3 nM and 18 nM), cells acquired spindle shape with swollen cytoplasm. Small size fat droplets and distinct cell membranes in differentiated adipocytes were visible. In absence of insulin, the cells were elongated and looks like fibroblast cells (Fig. 3A).

Expression of *Pref1* was significantly lower at all the three levels of insulin in comparison to its absence. However, effect of insulin on *UCP1* expression was dose dependent (Fig. 3B). At 0.5 nM insulin, there was significant increase in expression of *UCP1* in comparison to that in absence of insulin. At higher level of insulin (3 nM and 18 nM), UCP1 was expressed in lower amounts.

The effect of insulin on expression of *HoxA5* and *HoxC8* was dose dependent. In presence of insulin, expression of *HoxA5* ($\geq$3 nM insulin) decreased while that of *HoxC8* (0.5 nM insulin) increased. At all three levels of insulin, expression of *HoxA10* was significantly lower ($p < 0.001$) than in its absence. Thus, at physiological concentration (0.5 nM) of insulin, *HoxA10* is down regulated whereas *HoxC8* is up regulated and there is no effect on expression of *HoxA5. HoxA1, HoxC4* and *HoxC9* expression was not influenced by levels of insulin used in the study.

3. Discussions

Numerous developmental genes *viz. HoxA1, HoxA5, HoxC4, HoxC8, HoxC9 HoxA10, En1, Gpc4, Nr2f1, Sfrp2, Shox2, Tbx15 & Thbd* are present in preadipocytes, mature adipocytes and adipose tissue (Cowherd et al., 1997; Cantile et al., 2002; Gesta et al., 2006, 2007; Singh et al., 2015; Walden et al., 2012; Yamamoto et al., 2010). Present and other studies establish that Hox genes are differentially expressed in preadipocytes, differentiated adipocytes and adipose tissue and therefore are involved in process of adipogenesis. Also, expression of *Hox* genes in WAT and BAT differs. Even the pattern of expression is different in WAT present in various locations. *HoxA1* and *HoxC4* genes are expressed in brown adipose tissue, not in white adipose tissue (Cantile et al., 2002). Our studies clearly demonstrate that these genes are only expressed in differentiated brown adipocytes, not during their proliferation. Thus, *HoxA1* and *HoxC4* can be regarded as markers for differentiation in BAT and may be used for monitoring the progress of differentiation under *in vitro* culture condition. *HoxA1* contains retinoic acid response elements (Seifert et al., 2015) which in presence of retinoic acid induces UCP1 expression (Murholm et al., 2013). In stem cells, *HoxC4* appears to be involved in self-renewal as well as in differentiation (Seifert et al., 2015). However, its role in brown adipocytes might be restricted to differentiation.

Low and unaltered expression of *HoxA10* during differentiation suggests that *HoxA10* is of no significance in brown fat adipogenesis (Fig. 1C). The reported presence of *HoxA10* in mouse and human subcutaneous WAT as well as in brown fat (Cantile et al., 2002; Gesta et al., 2006, 2007; Yamamoto et al., 2010) might be linked with other cell types present in adipose tissue.

Treatment of preadipocytes with induction medium containing IBMX, DEX and indomethacin are essential for differentiation into mature adipocytes (Fig. 1A). Further in absence of IBMX, DEX and indomethacin, *HoxA1, HoxA5, HoxC4* and *HoxC8* as well as *UCP1* genes, a marker for brown fat differentiation are down regulated. However, expression of *HoxC9* is up regulated. Thus, it appears that differentiation inducing agents regulate expression of these *Hox*