Differentiation of rat brown adipocytes during late foetal development: role of insulin-like growth factor I

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Rat brown adipocytes at day 22 of foetal development showed greater size, higher mitochondria content and larger amounts of lipids, as determined by flow cytometry, than 20-day foetal cells. Simultaneously, an inhibition on the percentage of brown adipocytes into S+G₂/M phases of the cell cycle was observed between days 20 and 22 of foetal development. The expression of several adipogenesis-related genes, such as fatty acid synthase, malic enzyme, glucose-6-phosphate dehydrogenase and insulin-regulated glucose transporter, increased at the end of foetal life in brown adipose tissue. In addition, the lipogenic enzyme activities and the lipogenic flux increased during late foetal development, resulting in mature brown adipocytes showing a

multilocular fat droplet phenotype. Concurrently, brown adipocytes induced the expression of the uncoupling protein (UP) mRNA and UP protein, as visualized by immunofluorescence. The three isoforms of CCAAT enhancer-binding proteins (C/EBPs) were expressed at the mRNA level in brown adipose tissue at day 20. C/EBP α decreased and C/EBP β and δ increased their expression between days 20 and 22 of foetal development, respectively. Brown adipose tissue constitutively expressed insulin-like growth factor I (IGF-I) and IGF-I receptor (IGF-IR) mRNAs. Moreover, IGF-IR mRNA content increased between days 20 and 22 in parallel with the occurrence of tissue differentiation.

INTRODUCTION

Brown adipose tissue is a major site for lipid metabolism specialized in the non-shivering thermogenesis required to address the physiological hypothermia, in newborn mammals. The development of this tissue in the rat occurs mainly during the perinatal period and relies on two differentiation programmes; the adipogenic programme related to lipid synthesis and multilocular fat droplet accumulation, and the thermogenic programme related to the induction of the tissue-specific uncoupling protein (UP) gene, in order to yield an identifiable and functional tissue at birth [1]. White adipogenesis has been extensively investigated in the 3T3L1 pre-adipocyte differentiation model [2], where confluent cells express early markers such as lipoprotein lipase, but do not yet accumulate triacylglycerol. At a further differentiation stage these cells expressed acetyl-CoA carboxylase, fatty acid synthase (FAS) or malic enzyme (ME) genes and accumulated triacylglycerol droplets. Terminal differentiation leads to the expression of genes such as phosphoenolpyruvate carboxykinase (PEPCK) [3]. However, brown cell adipogenesis is still poorly understood. Thus, early *in vivo* studies on foetal brown adipose tissue of FAS, ME and glucose-6-phosphate dehydrogenase (G6PD) enzymic activities, and on the lipogenic flux were reported [4,5]. Glucose transport in adipose tissues is maintained mainly by the activity of insulin-regulated glucose transporter (Glut4). However, Glut4 mRNA was detected at low levels in brown adipose tissue at day 19 of foetal life [6]. The thermogenic activity of brown adipose tissue relies on the presence of UP, a gene expressed prenatally [7,8]. In addition, foetal brown adipocyte primary cultures have been successfully used in differentiation studies over the last few years [9–16]. However, the changes in transcription of lipogenic and thermo-

genic genes in foetal brown adipose tissue in the final 3 days of *in utero* life has not yet been reported.

Potential candidates as regulatory factors for the adipogenic differentiation programme, such as CCAAT enhancer-binding proteins (C/EBPs) α , β and δ , which are sequence-specific DNA-binding proteins, have been identified [17]. These transcription factors may play a general role in establishing and maintaining the differentiated state in liver and white adipose tissue [18], the transcription of these genes being induced directly by adipogenic hormones (as reviewed in [19]). The C/EBP proteins cause expression of several adipocyte-specific genes, such as the 422 adipose P2 protein and Glut4 [20]. Moreover, the expression of C/EBP α and β during late foetal brown adipose tissue development runs parallel to the onset of UP transcription, this gene bearing two binding sites for C/EBP in its promoter [21,22].

Although the nature of the circulating hormones and/or paracrine/autocrine factors involved in the foetal development of brown adipose tissue remains unknown, we have recently described that tri-iodothyronine induces directly the expression of UP in the absence of noradrenergic stimulation [15], and also that insulin-like growth factor I (IGF-I) induces the expression of UP in a dose- and time-dependent manner [14,16], suggesting that both signals may be major players in the differentiation of this tissue during late foetal life. In addition, we reported that brown adipocytes highly expressed IGF-I receptor (IGF-IR) mRNA, bearing a high number of binding sites per cell of high affinity for IGF-I [14].

Accordingly, the aim of this work was to investigate the adipogenic- and thermogenic-differentiation of brown adipose tissue during late foetal development by studies of flow cytometry, gene expression and lipogenesis, that resulted in the tissue characteristic multilocular fat droplet phenotype. The possible

Abbreviations used: FAS, fatty acid synthase; ME, malic enzyme; G6PD, glucose-6-phosphate dehydrogenase; G3PDH, glycerol-3-phosphate dehydrogenase; UP, uncoupling protein; PEPCK, phosphoenolpyruvate carboxykinase; C/EBP, CCAAT enhancer-binding protein; IGF-I, insulin-like growth factor I; IGF-IR, insulin-like growth factor I receptor; Glut4, insulin-regulated glucose transporter.

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role of IGF-I as a paracrine/autocrine factor involved in brown adipocytes differentiation is also discussed.

MATERIALS AND METHODS

Isolation of brown adipocytes

Albino Wistar rats on days 20, 21 and 22 of pregnancy were supplied by the Animal House (Universidad Complutense, Madrid, Spain). Conception was assumed by the presence of spermatozoa in the vagina, and gestational age was verified by the foetal weight, as previously described [5]. Foetuses were delivered by rapid hysterectomy, and pooled interscapular brown adipose tissue was collected. Brown adipocytes were isolated by collagenase dispersion as described elsewhere [9], and used as source for flow cytometer determination, enzymic activities and RNA isolation. Although the amount of brown adipose tissue from 22-day-old foetuses (30 mg/animal) was higher than from 20-day-old foetuses (10 mg/animal), the isolated brown adipocytes yield was 1×10^6 cells/animal in both 22- or 20-day-old foetuses, indicating that there is an increase in cell size, but not in cell number, in the transition of brown adipose tissue between days 20 and 22 of foetal development. All the determinations were performed from three independent experiments, each one with four pooled sets of litters of similar numbers of animals, size and gestational age.

Flow cytometric analysis

Several cellular parameters were determined by flow cytometric analysis of individual cells, such as size, endogenous fluorescence, cytoplasmic lipid content and analysis of cell cycle. Aliquots of 2×10^5 cells were used for each flow cytometer determination. All measurements were performed in a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, U.S.A.). Excitation light wavelength was 488 nm. Size signals (FSC-H) were measured through a filter BP 488/10 nm. Endogenous flavin green fluorescence (FL1-H) was measured through a filter BP 530/30 nm. Cytoplasmic lipid content was determined by Nile Red fluorescence emission 530 (BP 530/30 nm). Nile Red (0.1 $\mu\text{g}/\text{ml}$) was added 5–10 min before flow cytometer measurements were taken. Results represent mean intensities of fluorescence (obtained from the histograms of number of cells versus intensity of fluorescence) and are expressed as arbitrary units. Cell cycle analysis was done after staining nuclei with propidium iodide (0.005%) by using the Cycle test DNA reagent kit (Becton-Dickinson, San Jose, CA, U.S.A.). For cell cycle analysis Double Discriminator Module and Cellfit software were used (Becton-Dickinson). Flow cytometry figures were done using Lysys II software (Becton-Dickinson).

Microscope characterization of brown adipocytes

Isolated adipocytes were plated at 1.5×10^6 cells/60-mm-diam. tissue-culture plates in 2.5 ml of Eagle's medium modified with Earle's salts supplemented with 10% (v/v) foetal-calf serum (Imperial Laboratories, Hants., U.K.) and cultured for 4 h at 37 °C prior to treatments for microscope observation. The presence of typical multilocular brown adipocyte fat droplets was confirmed by Oil-Red O staining of cells [23] and microscopy. The presence of UP in brown adipocytes was detected by immunofluorescence with rabbit anti-(UP) serum and microscopy.

RNA extraction and analysis

Isolated adipocytes were lysed directly with RNazol B (Biotec

Lab, Dallas, TX, U.S.A.) following the protocol supplied by the manufacturer for total RNA isolation [24]. Poly(A)⁺ RNA was isolated by affinity chromatography on oligo(dT)cellulose columns from 1 mg of total RNA as previously described [14].

Total cellular RNA (20 μg) and poly(A)⁺ RNA (10 μg) were subjected to Northern-blot analysis, being electrophoresed on 0.9% agarose gels containing 0.66 M formaldehyde, transferred to GeneScreen membranes (New England Nuclear Research Products, Boston, MA, U.S.A.) using a VacuGene blotting apparatus (LKB, Pharmacia, Uppsala, Sweden) and cross-linked to the membranes by UV light. Hybridization was in 0.25 M NaHPO₄, pH 7.2, 0.25 M NaCl, 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA, 7% SDS and 50% deionized formamide, containing denatured ³²P-labelled cDNA (10^6 c.p.m./ml) for 40 h at 42 °C as described [25]. cDNA labelling was carried out with a [³²P]dCTP to a specific activity of 10^9 c.p.m./ μg of DNA by using a multiprimer DNA-labelling system kit (Amersham, Bucks., U.K.). For serial hybridization with different probes, the blots were stripped and rehybridized sequentially as needed in each case. Probes utilized included UP [26], FAS [27], glycerol 3-phosphate dehydrogenase (G3PDH) [28], ME [29], G6PD [30], Glut4 [31], PEPCK [32], C/EBPs [33], IGF-I and IGF-IR [34]. The resulting membranes were subjected to autoradiography for 1–3 days. Relative densities of the hybridization signals were determined by densitometric scanning of the autoradiograms in a laser densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Enzyme activities

Isolated brown adipocytes were sonicated for 30 s at 1.5 mA in 1 mM EDTA/1 mM dithiothreitol/0.25 M sucrose/25 mM Tris/HCl (pH 7.5) for ME and G6PD activity determinations and in 1 mM dithiothreitol/1 mM MgCl₂/0.1 mM EDTA/10% (v/v) glycerol/0.1 mM NaH₂PO₄ (pH 7.2) for FAS activity determination, and centrifuged at 12000 g for 4 min. Cytosolic supernatant was used for the spectrophotometrical determination at 37 °C of ME and G6PD, and at 25 °C of FAS activities [35–37] respectively. Proteins were determined as in [38] with γ -globulin as standard. Enzyme activities were expressed as m-units/mg of protein. A m-unit is a nmol of NADPH formed/consumed per min.

Determination of lipid synthesis *in vivo*

Rats on days 20 and 22 of gestation were injected intraperitoneally with 5 mCi of ³H₂O, 55 min later they were anaesthetized with Nembutal (50 mg/kg body wt) and 5 min later the abdomen was opened and maternal blood was collected from the aorta for determination of specific radioactivity of plasma water. Foetuses were delivered by rapid hysterectomy, decapitated and exsanguinated. Similar specific activities of plasma water were found in the maternal and foetal compartments. Samples of pooled brown adipose tissue (0.1–0.3 g) were added to 1–3 ml of 30% (w/v) KOH in 95% (v/v) ethanol, and the lipids were saponified and extracted [39]. Lipogenesis was expressed in terms of μmol of ³H₂O incorporated into fatty acids/h per g wet weight.

RESULTS

Characterization by flow cytometry of brown adipocytes during late foetal development: size, endogenous fluorescence, lipid content and cell cycle

Foetal brown adipocytes were characterized by flow cytometric determinations of their size, endogenous fluorescence, lipid

Figure 1 Characterization by flow cytometry of brown adipocytes during late foetal development: size, endogenous fluorescence and lipid content

Brown adipocytes were isolated from 20-, 21- and 22-day foetuses and were characterized by flow cytometry for (a) size, (b) endogenous fluorescence, and (c) cytoplasmic lipid content determined by Nile Red fluorescence. Mean intensities of Nile Red fluorescence from a representative experiment are expressed as arbitrary units.

Table 1 Cell cycle analysis by flow cytometry in brown adipocytes during late foetal development

Brown adipocytes were isolated from 20-, 21- and 22-day foetuses. Cell cycle study was performed after propidium iodide staining of nuclei and flow cytometric analysis. Results from a representative experiment are expressed as percentage of cells in the G_0/G_1 and $S + G_2/M$ phases of the cell cycle.

Phase of cell cycle	Foetal age (days) ...	Cells in phase (%)		
		20	21	22
G_0/G_1		83.2	90.3	94.8
S		10.1	5.0	2.8
G_2/M		6.7	4.7	2.4

content (Figure 1) and cell cycle (Table 1). Cell size signals gave a broad peak of small size for 20-day foetal brown adipocytes, meanwhile 22-day foetal brown adipocytes showed a greater size (Figure 1a). The measurement of endogenous fluorescence (flavin fluorescence) reveals the metabolic state of living cells, its detection being possible due to the high mitochondria content of these cells. Brown adipocytes from 20-day foetuses showed a single sharp peak of low fluorescence, meanwhile we observed the presence of one peak of higher fluorescence in 22-day foetal

cells (Figure 1b). The peak of high fluorescence observed in 22-day fetal cells could indicate mature mitochondria with flavins in the oxidized state. Nile Red fluorescence serves as a sensitive detector of cytoplasmic lipid content and allows us to monitor the adipocytic differentiation programme [40]. Brown adipocytes from 20-day foetuses showed a peak of low Nile Red fluorescence with a long tail. Mean intensity of Nile Red fluorescence increased gradually in adipocytes on days 21 and 22 of foetal life. Double the amount of Nile Red fluorescence was observed on 22-day cells as compared with 20-day brown adipocytes (Figure 1c). Cell cycle analysis of 20-day foetal brown adipocytes indicated that 16.8% of cells were into the $S + G_2/M$ phases of the cell cycle. This percentage decreased to 9.7 and to 5.2 in 21- and 22-day foetal brown adipocytes respectively (Table 1).

Adipogenic differentiation of brown adipocytes during late foetal development

Since the data of Nile Red fluorescence indicated an increase in total lipid content in foetal brown adipocytes at the end of gestation, we studied the expression of several genes involved in adipogenesis during this period. Among them, the expression of FAS, G3PDH, ME, G6PD and Glut4 genes was studied by Northern blot analysis (Figure 2) and the corresponding densitometric analysis is shown in Table 2. FAS mRNA abundance was low in 20-day foetal brown adipocytes, increasing between days

Figure 2 Gene expression in brown adipocytes during late foetal development

Total RNA (20 µg) from 20-, 21- and 22-day foetal brown adipocytes was submitted to Northern-blot analysis and hybridized with labelled FAS, G3PDH, ME, G6PD, Glut4, UP, PEPCK, and C/EBPα/β/δ cDNAs. A final hybridization with the 18 S rRNA was done for normalization. A representative autoradiogram of three independent experiments is shown.

Table 2 Densitometric analysis of mRNA expression in brown adipocytes during late foetal development

Autoradiograms from Figure 2 were submitted to densitometric analysis and normalized with the amount of 18 S rRNA. mRNA content is expressed as arbitrary units (density of band/density of 18 S rRNA × 100), and results are means ± S.E.M. from three independent experiments. Statistical analysis by Student's paired *t*-test between values at day 21/22 versus day 20 (*) and between day 22 versus day 21 (†) *P* < 0.05.

Enzyme	Foetal age (days) ...	mRNA expression (arbitrary units)		
		20	21	22
FAS		27 ± 5	151 ± 17*	149 ± 25*
G3PDH		40 ± 9	66 ± 12	49 ± 9
ME		15 ± 4	71 ± 10*	201 ± 30*†
G6PD		9 ± 3	28 ± 7*	319 ± 40*†
GLUT4		36 ± 10	38 ± 9	435 ± 70*†
UP		60 ± 13	98 ± 17*	374 ± 36*†
PEPCK		4 ± 1	6 ± 2	7 ± 3
C/EBPα		48 ± 5	34 ± 3*	22 ± 3*†
C/EBPβ		35 ± 6	30 ± 5	98 ± 13*†
C/EBPδ		30 ± 8	79 ± 9*	196 ± 23*†

20 and 21 by 5-fold, and remaining high on day 22. The expression of G3PDH was constitutive in 20-day foetal cells and did not change significantly during late gestation. The expression of NADPH-providing enzymes, ME and G6PD, was very low in 20-day brown adipocytes. ME mRNA accumulated between days 20 and 22, with an overall increase of 13-fold. A similar profile of expression was found for G6PD mRNA at the end of gestation in brown adipocytes, increasing its expression by 35-fold between day 20 and day 22 of foetal life. The expression of the glucose transporter Glut4 gene was very low in foetal brown adipocytes either at day 20 or 21. However, its expression increased significantly (by 11-fold) in 22-day brown adipocytes.

Table 3 Rates of lipogenesis and lipogenic enzyme specific activities in brown adipocytes during late foetal development

Rates of lipogenesis were determined *in vivo* as described in the Materials and methods section. Enzymic activities were determined in brown adipocytes isolated from 20- and 22-day foetuses. Results are means ± S.E.M. from three independent experiments. Statistical analysis by Student's *t*-test between values at day 22 versus 20 is stated; *P < 0.001.

	Foetal age (days) ...	20	22
Lipogenesis (µmol of ³ H ₂ O/h per g wet wt.)		10 ± 2	40 ± 5*
FAS activity (nmol/min per mg of protein)		1.2 ± 0.2	10.0 ± 0.5*
ME activity (nmol/min per mg of protein)		15.3 ± 2.4	70.2 ± 5.0*
G6PD activity (nmol/min per mg of protein)		32.5 ± 2.7	140 ± 15*

Accordingly, 22-day foetal brown adipocytes showed a high abundance of FAS, ME, G6PD and Glut4 mRNAs, in parallel to an increased lipid content. We determined the specific activities of FAS, ME and G6PD on 22-day as compared with 20-day foetal cells, as well as studying the rates of lipogenesis in brown adipose tissue at the end of the foetal life (Table 3). FAS activity was very low in 20-day and increased by 8-fold in 22-day foetal brown adipocytes, in parallel with the accumulation of FAS mRNA described above (Figure 2, Table 2). ME and G6PD specific activities were also very low in 20-day foetal brown adipocytes but increased by more than 4-fold in 22-day foetal cells, in parallel with their mRNA accumulation. Concurrently, the rates of lipogenesis *in vivo* increased 4-fold between days 20 and 22 of foetal life in brown adipose tissue (Table 3).

Thermogenic differentiation of brown adipocytes during late foetal development

Simultaneously to the ongoing adipogenic programme, we studied the expression of the tissue-specific gene UP, a marker of the thermogenic programme, in brown adipocytes during late foetal development. Accordingly, the UP mRNA was present in 20-day foetal brown adipocytes, increasing its abundance between days 20 and 22 of gestation by 6-fold (Figure 2, Table 2). The expression of PEPCK, a very late differentiation marker in white adipocytes [3], remained in brown adipocytes at very low levels throughout late foetal life.

Microscope characterization of differentiated brown adipocytes

Brown adipocytes from 22-day foetuses highly expressing lipogenic-related genes at mRNA and protein level, showing a high lipogenic rate and a high lipid content by Nile Red fluorescence, were microscopically characterized by fat staining with Oil-Red O. Mature adipocytes showed their typical multilocular fat droplet distribution phenotype (Figure 3a). In addition, these cells also showed a high expression of the tissue-specific marker UP, as revealed by the immunofluorescence assay shown in Figure 3(b).

Expression of C/EBPs in brown adipocytes during late foetal development

The expression of nuclear factors during late foetal life could be involved in the onset of differentiation-specific genes. Among them, a family of nuclear factors, the C/EBP isoforms α/β/δ, seems to be involved in adipogenic differentiation in 3T3-L1 preadipocytes [17] as well as in the thermogenic differentiation of

Figure 3 Microscope characterization of mature foetal brown adipocytes

Brown adipocytes obtained from 22-day foetuses were stained with Oil-Red O for fat droplet visualization; light-field microphotograph (a). The expression of UP was detected by immunofluorescence with a specific anti-(UP) serum (b). Bar, 20 μ m.

brown adipose tissue [21]. Accordingly, we have studied the pattern of C/EBPs expression during late foetal development in brown adipocytes, as shown in Figure 2 and Table 2. The three C/EBP isoforms reported (α , β and δ) were expressed at day 20 of foetal development, in brown adipocytes. The expression of C/EBP α decreased between days 20 and 22. The expression of C/EBP β remained unmodified between days 20 and 21, but significantly increased during the last day of foetal development. C/EBP δ mRNA abundance was very low at day 20 of foetal life, increasing significantly during the last two days of gestation. Then, the pattern of expression of these genes in mature foetal brown adipocytes was C/EBP δ > C/EBP β > C/EBP α (Figure 2, Table 2).

Expression of IGF-I and IGF-IR mRNAs in brown adipocytes during late foetal development

Although the nature of the signal(s) involved in the onset of adipogenic/thermogenic differentiation processes remains unknown, insulin and/or IGF-I could be potential candidates [14,16]. We have studied in brown adipocytes the expression of IGF-I and its receptor (IGF-IR), during late gestation. Brown adipocytes highly expressed IGF-I and IGF-IR constitutively at the end of the foetal life, as shown in the poly(A)⁺-Northern blot depicted in Figure 4. Since the IGF-I gene is expressed as primary transcripts, which are processed in a complex manner to yield a number of distinct mRNA species [41], we found in brown adipocytes three IGF-I mRNA isoforms of 7.5, 1.8 and 0.8–1.2 kb respectively, as well as a 7–7.5 kb mRNA isoform for IGF-IR. Rat foetal brown adipocytes showed that the IGF-I mRNA isoforms of 1.8 and 0.8–1.2 kb were predominant, meanwhile in rat liver and fibroblast the most abundant isoforms were 7.5 and 0.8–1.2 kb [42,43]. The expression of the two predominant IGF-

Figure 4 Expression of IGF-I and IGF-IR mRNAs in brown adipocytes during late foetal development

Poly(A)⁺ RNA (10 μ g) was isolated from total RNA obtained from 20- and 22-day foetal brown adipocytes and submitted to Northern-blot analysis. Hybridization was performed with labelled IGF-I and IGF-IR cDNAs. A representative autoradiogram is shown. The arrows on the right represent the positions of the three IGF-I mRNA isoforms of 7.5, 1.8 and 0.8–1.2 kb, respectively.

I mRNA shorter species remained essentially unmodified between days 20 and 22 of foetal development, meanwhile the expression of the larger species dramatically decreased. Moreover, this decrease in the larger IGF-I mRNA isoform abundance did not affect the amount of IGF-I peptide produced by brown adipocytes, since only the shorter species have been found in polysomes [41]. However, a higher expression (3-fold) of the IGF-IR gene was found on 22-day foetal brown adipocytes as compared with its expression on day 20 (Figure 4).

DISCUSSION

Brown adipocytes differentiate at the end of the foetal life on the basis of two programmes: adipogenesis and thermogenesis. Accordingly, 22-day foetal brown adipocytes show greater size, higher mitochondria content and larger amounts of lipids compared with 20-day foetal cells, monitoring a process of cell differentiation. Simultaneously, the proliferative capacity of foetal brown adipocytes decreases between days 20 and 22, as revealed by the cell cycle analysis, indicating that cell arrest is required for cell differentiation. The expression of the adipogenic-related genes occurred as a multistep process: FAS gene is expressed as early as day 20 of foetal life, but its mRNA abundance significantly increases between days 20 and 22. The expression of the NADPH-generating enzymes is initiated mainly at day 21 of foetal life, increasing significantly at day 22. The Glut4 mRNA is expressed later, on day 22 of foetal development. The induction observed on lipogenic enzymes at the mRNA level between days 20 and 22 of foetal life correlates to the increase in the enzymic activities and the lipogenic flux, producing an accumulation of lipids that results in a multilocular fat droplet phenotype.

In parallel to adipogenesis, brown adipocytes induce the tissue-specific gene UP, a protein marker of thermogenesis. This mRNA accumulates gradually during the last days of foetal development, being detectable as high levels of UP mRNA by Northern-blot and UP protein by immunofluorescence assay on day 22. Thus, brown adipocytes prior to birth are differentiated cells that accumulate lipids and show high levels of UP. However, these cells are not terminally differentiated since they do not yet

express PEPCK [3], and *in vitro* are rescued for mitogenesis in response to specific mitogens [12,14].

C/EBP transcriptional factors recruitment may determine the differentiated phenotype of brown adipose tissue, transactivating genes whose expression marks the differentiated state of these cells. The presence of C/EBP α , β and δ mRNAs in 20-day brown adipocytes concurs with the initial expression of UP gene, suggesting that C/EBP proteins could be involved in the transcriptional regulation of UP gene, as recently reported [22]. Moreover, C/EBP β and C/EBP δ increase their expression between days 20 and 22, when UP mRNA is fairly abundant. An increased expression of C/EBP β parallel to UP induction has been reported in brown adipose tissue after cold exposure [21]. With regard to adipogenesis, a sequential pattern of early expression of C/EBP β and δ and late expression of C/EBP α genes throughout the late phase of 3T3-L1 adipose conversion has been reported [17]. However, in brown adipose tissue *in vivo* we observed an early expression of C/EBP α on day 20 of fetal development decreasing later on, with an increased expression of C/EBP β and δ between days 20 and 22. Whether or not this pattern of C/EBPs expression could be directly involved in the commitment to adipogenesis of brown cells, remains to be established.

Since the noradrenergic stimulus, induced by hypothermia after birth, is not working in brown adipose tissue during foetal development, other potential candidates involved in the onset of differentiation-related gene expression, such as tri-iodothyronine or IGF-I, could be implicated. Thus, tri-iodothyronine induces directly the expression of the UP, in the absence of noradrenergic stimulation, in foetal brown adipocytes in primary cultures [15]. In addition, we have recently reported that brown adipocytes constitutively expressed IGF-IR mRNA and bear IGF-I receptors, showing a great number of binding sites per cell of high affinity [14], and that IGF-I induces the expression of UP in a dose- and time-dependent manner [16]. Since we primarily describe that brown adipose tissue expresses IGF-I mRNA throughout late foetal development, predominantly the shorter translatable species [41], an autocrine or paracrine effect of IGF-I on brown adipocytes may occur. Moreover, the increase in the IGF-IR mRNA content between days 20 and 22 of foetal development runs parallel to the differentiation of the tissue. Thus, IGF-I, in an autocrine/paracrine fashion, may be a major signal involved in driving brown adipose tissue to differentiate prior to birth.

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