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Role of Wnt10b and C/EBPα in spontaneous adipogenesis of 243 cells[☆,☆☆]

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Abstract

This report examines the balance of positive and negative adipogenic factors in a line of immortalized 243 embryonic fibroblasts that undergo spontaneous preadipocyte differentiation. Control of adipogenesis reflects the interplay of factors that promote or inhibit expression of C/EBPα and PPARγ. The 243 cells express C/EBPα early and at elevated levels compared to 3T3-F442A preadipocytes or adipocytes. Cell clones were derived from the heterogeneous 243 population for ability or inability to differentiate into adipocytes. Wnt10b, a secreted protein that inhibits adipogenesis, is expressed at high levels in cells with low adipogenic potential and is undetectable in preadipocytes that spontaneously differentiate. In contrast, C/EBPα is expressed at reduced levels in cells with low adipogenic potential, and is expressed at high levels in preadipocytes that spontaneously differentiate. These data are consistent with a model in which decreased Wnt10b, coupled with increased C/EBPα, results in induction of PPARγ and spontaneous adipogenesis of 243 cells.
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The program of adipogenesis has been extensively analyzed over the past 30 years [1–4]. Treatment of confluent preadipocytes with adipogenic media promotes clonal expansion, which is characterized by one or two rounds of cell division, and then terminal differentiation into adipocytes. During clonal expansion, transient expression of C/EBPβ and C/EBPδ initiates a second phase of gene expression necessary for adipogenesis. Within this second wave, C/EBPα and PPARγ, the essential transcriptional activators of adipogenesis and two markers of terminal differentiation, are expressed. An emerging model of adipogenesis places C/EBPα as a critical factor that maintains expression of PPARγ during adipogenesis and confers insulin sensitivity in adipocytes, while PPARγ maintains expression of C/EBPα and promotes adipocyte gene expression and lipid accumulation [5].

Whether preadipocytes undergo differentiation reflects a balance between factors that promote or inhibit expression of C/EBPα and PPARγ. Wnt10b is a secreted factor that acts through an autocrine and paracrine mechanism to inhibit adipogenesis [6]. Expression of Wnt10b is high in 3T3-L1 preadipocytes and is rapidly suppressed by the adipogenic agent, methylisobutylxanthine [7]. While activation of Wnt signaling inhibits preadipocyte differentiation, disruption of endogenous

Wnt signaling results in spontaneous adipogenesis [6,7]. Wnt10b inhibits differentiation by blocking induction of C/EBPα and PPARγ. Consistent with this model, differentiation in the presence of Wnt is partially rescued by enforced expression of either of these adipogenic transcription factors. In the present study, spontaneous adipogenesis of 243 immortalized embryonic fibroblasts is the result of decreased expression of Wnt10b and elevated expression of C/EBPα early in the adipogenic program.

Materials and methods

Cells and cell culture. 243 cells, provided by Drs. B.M. Spiegelman and E. Hu (Harvard University), were generated by immortalization of primary embryonic fibroblasts from embryonic day 15 *c-fos* null mouse embryos [8,9]. The 243 cells were grown to confluence and maintained in Dulbecco's Modified Eagle's Medium and 10% fetal calf serum (FCS). Clonal cell lines were derived from 243 cells by limiting dilution. 3T3-F442A cells were provided by Dr. H. Green (Harvard University) and Dr. M. Sonenberg (Sloan-Kettering). 3T3-F442A cells were cultured as previously described [10]. At confluence (243 cells) or two days post-confluence (3T3-F442A cells), adipogenesis was induced with methylisobutylxanthine, dexamethasone, and insulin (MDI) in a series of media as described previously [10,11]. Degree of adipogenesis was estimated by phase contrast microscopy in cells stained with Oil Red-O [12]. Clone 4 cells were infected with control (pLNCX) or Wnt10b-expressing retroviruses as described [12].

Analyses of mRNA and protein. For Northern blot analysis, total RNA was isolated as described [10]. Prehybridization was performed at 55 °C and hybridization was performed at 68 °C with ExpressHyb (Clontech). Membranes were probed with cDNAs labeled with [α-^{32}P]dCTP using Rediprime (Amersham). The cDNA probe for C/EBPα was received from Dr. M.D. Lane (John Hopkins University) [13], glyceraldehyde phosphate dehydrogenase (GAPDH) was from Dr. M. Alexander-Bridges (Harvard University), and PPARγ was from Dr. B.M. Spiegelman (Harvard University) [14]. RNase protection assays for Wnt10b and GAPDH were as described [7]. Immunoblotting was performed essentially as described [15] with antibody to C/EBPα [16], PPARγ (Santa Cruz Biotechnology), or 422/aP2 (Dr. D.A. Bernlohr; University Minnesota).

Results

Spontaneous adipogenesis of 243 cells

While 3T3-F442A preadipocytes do not differentiate into adipocytes when incubated in the presence of FCS (Fig. 1A), treatment with standard adipogenic media containing MDI causes 3T3-F442A cells to undergo adipogenesis (Fig. 1B). In contrast, as many as 70% of 243 cells accumulate lipid droplets when incubated with FCS (Fig. 1C), and the proportion of cells that differentiate is increased further by treatment with MDI (Fig. 1D). Adipogenesis of 243 cells results in a more unilocular phenotype than that observed with many other in vitro models, including 3T3-F442A cells.
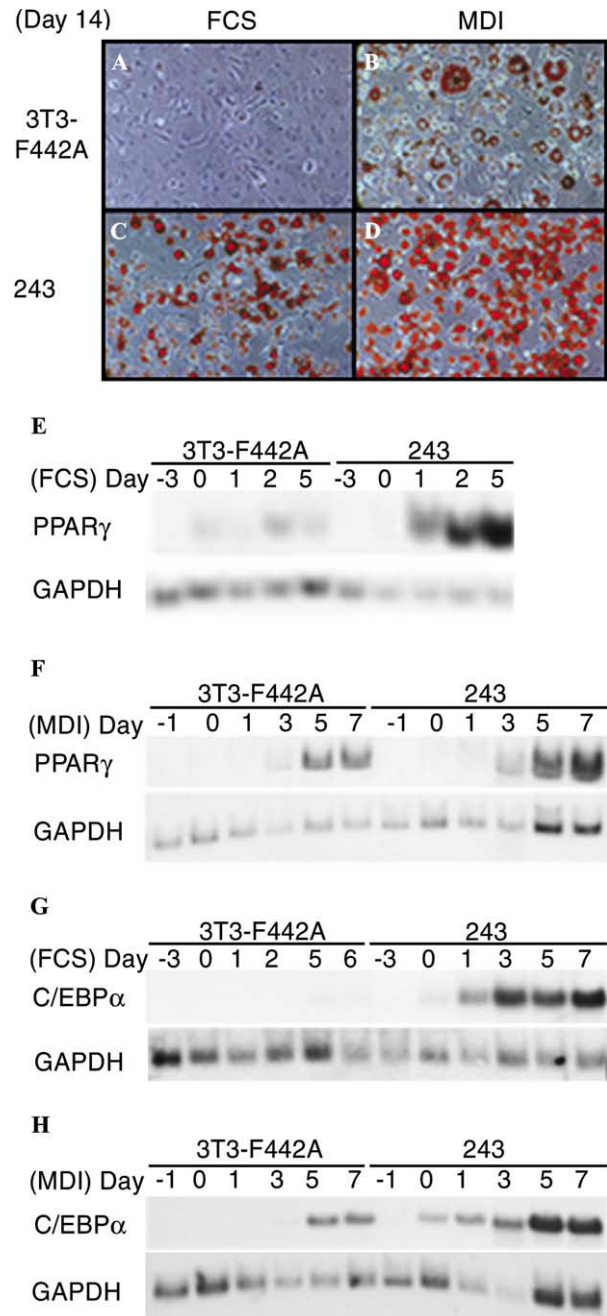

Fig. 1. Spontaneous adipogenesis of 243 cells is accompanied by early expression of C/EBPα. 3T3-F442A cells were cultured in DMEM containing 10% calf serum until confluence. At two days post-confluence, cells were switched to media containing 10% FCS (A) or MDI (B). 243 cells were cultured in 10% FCS until confluence and then were maintained in 10% FCS (C) or induced to differentiate with MDI (D). To visualize the degree of adipogenesis, cells were stained with Oil Red-O 16 days after confluence. Total RNA was prepared from 3T3-F442A or 243 cells that were maintained in 10% FCS (E,G) or treated with MDI (F,H), on the indicated days. Amount of mRNAs (15 μg) was analyzed by northern blotting with cDNA probes for PPARγ (E,F) and C/EBPα (G,H). Amount of GAPDH is shown as a control for RNA quality and loading. Results are representative of at least three-independent experiments.

Adipogenesis of 243 cells is accompanied by early expression of C/EBPα

The observation that 243 cells differentiate spontaneously made it important to investigate the adipogenic program in these cells. As expected, PPARγ is not induced in 3T3-F442A cells incubated in FCS (Fig. 1E), but PPARγ is induced concurrently with adipogenesis in response to MDI (Fig. 1F). PPARγ is also induced during adipogenesis of 243 cells, with expression observed as early as one day after confluence (Fig. 1E). Likewise, C/EBPα is not induced in 3T3-F442A cells incubated in FCS (Fig. 1G), but it is induced during adipogenesis of 3T3-F442A in response to MDI (Fig. 1H). It is notable that C/EBPα protein (not shown) and mRNA are expressed at confluence (Day 0) in 243 cells. While both PPARγ and C/EBPα undoubtedly play important roles in adipogenesis of 243 cells, it is likely that the early expression of C/EBPα predisposes these cells to undergo spontaneous differentiation.

Expression of C/EBPα correlates with adipogenesis in clones from 243 cells

The 243 cells used for these experiments were derived from a pool of embryonic cells and are heterogeneous with respect to adipogenesis. To explore more fully the possibility that early C/EBPα expression contributes to spontaneous adipogenesis of 243 cells, clonal cell lines with low or high susceptibility to adipogenesis were derived from the 243 cell population. Clone 9 does not differentiate when cells are maintained in medium containing 10% FCS (Fig. 2A), and differentiates poorly in response to treatment with adipogenic media (Fig. 2B). In contrast, 40–60% of Clone 4 cells differentiate within 7 days when maintained in medium containing FCS (Fig. 2C), and at least 90% of these cells differentiate within 7 days when treated with MDI (Fig. 2D). Expression of C/EBPα mRNA (Fig. 2E) and protein (Fig. 2F) is very low in Clone 9 cells incubated with adipogenic media, correlating well with lack of differentiation. In contrast, C/EBPα mRNA (Fig. 2E) and protein (Fig. 2F) are expressed at detectable levels in confluent 243 cells and are increased dramatically after induction of differentiation. Early and elevated expression of C/EBPα is also observed in two other adipogenic clones (data not shown). There is no evidence to suggest that absence of *c-fos* contributes to adipogenic events in 243 cells. These data suggest that in confluent 243 cells, elevated expression of C/EBPα stimulates the program of adipogenesis.

Wnt 10b suppresses adipogenesis in Clone 4 cells

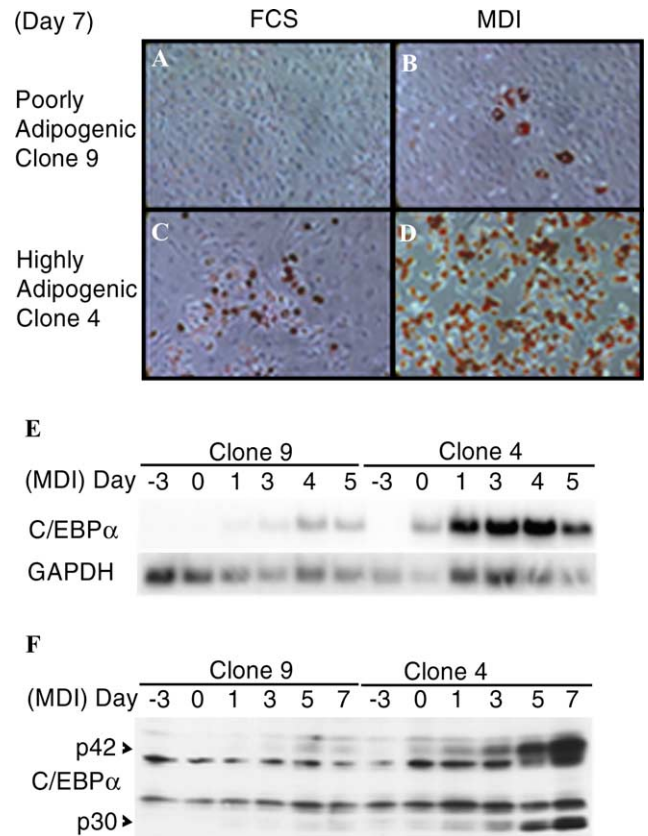A well-characterized inhibitor of C/EBPα and adipogenesis is Wnt10b [6,7]. To determine if expression

Fig. 2. Expression of C/EBPα correlates with adipogenesis in clones from 243 cells. Clonal lines were derived from the heterogeneous 243 cell population for the ability (Clone 4) or inability (Clone 9) to differentiate into adipocytes. Both clones were maintained in 10% FCS (A and C) or transferred into adipogenic media (MDI) at confluence (B and D). To visualize the degree of adipogenesis, cells were stained with Oil Red-O 7 days after confluence. Total RNA and whole cell lysates were prepared on the indicated days and assayed for C/EBPα mRNA (E) and protein (F). Results in (E) and (F) are representative of at least two-independent experiments.

of Wnt10b corresponds with adipogenic potential of 243 cell clones, RNA was isolated from confluent cells and subjected to RNase protection analyses for Wnt10b and GAPDH. Wnt10b is expressed in Clone 9 cells, which do not differentiate, and is undetectable in Clone 4 cells, which undergo adipogenesis in response to FCS (Fig. 3A). If the lack of Wnt10b in Clone 4 cells permits subsequent adipogenesis, then expression of Wnt10b in Clone 4 cells should mimic the lack of adipogenesis observed with Clone 9 cells. Control or Wnt10b-expressing retroviruses were infected into Clone 4 cells and effects on differentiation were evaluated two weeks after confluence. Expression of Wnt10b strongly inhibits spontaneous adipogenesis of Clone 4 cells (Fig. 3B) and also suppresses expression of C/EBPα, PPARγ, and 422/aP2 (Fig. 3C). This combination of low Wnt10b and high C/EBPα is believed to increase expression of PPARγ and stimulate adipogenesis.
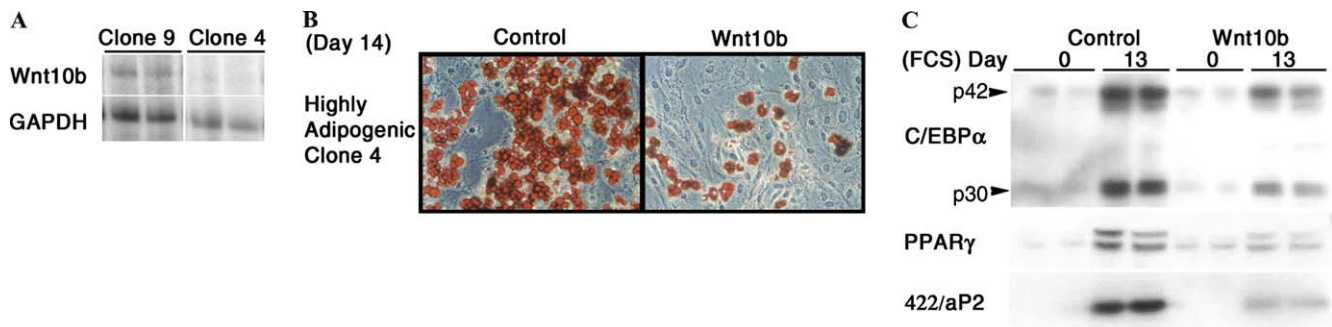
Fig. 3. Wnt 10b suppresses adipogenesis in Clone 4 cells. Expression of Wnt10b in confluent cultures of non-adipogenic Clone 9 cells and adipogenic Clone 4 cells was evaluated by RNase protection assay (A). Clone 4 cells were infected with control retrovirus (pLNCX) or a Wnt10b-expressing retrovirus and maintained under antibiotic selection until confluence. Two weeks after confluence, cells were stained with Oil Red-O to visualize lipid accumulation by phase-contrast microscopy (B). Control or Wnt10b-expressing Clone 4 cells were lysed at Day 0 and Day 13 and expression of C/EBPα (p42 and p30), PPARγ, and 422/aP2 was evaluated by immunoblot analyses (C).

Discussion

The genetic program of adipogenesis has been extensively studied in vitro, with embryonic fibroblasts and preadipocyte lines, and in vivo, with transgenic and knockout mouse models [1–4]. A paradigm for the cascade of genetic events has emerged: After induction of differentiation, there is a rapid and transient induction of C/EBPβ and C/EBPδ. These transcription factors then activate expression of both C/EBPα and PPARγ, which then induce each other's expression through a positive feedback loop. Interestingly, C/EBPβ mRNA is barely detectable in highly adipogenic 243 or Clone 4 cells relative to 3T3-F442A cells (data not shown). The lack of C/EBPβ suggests that this transcription factor is not required to induce C/EBPα and/or PPARγ expression during adipogenesis of these cells. Although C/EBPα is required for acquisition of insulin sensitivity in adipocytes, the ability of C/EBPα to stimulate adipogenesis appears to be through induction of PPARγ. A large body of evidence indicates that PPARγ is necessary and sufficient for the activation of many adipocyte genes, as well as accumulation of lipid. Thus, it is likely that the high levels of C/EBPα observed in heterogeneous 243 cells and highly adipogenic Clone 4 cells drive spontaneous adipogenesis through induction of PPARγ.

Preadipocytes that express Wnt10b are unable to differentiate in the presence of MDI [6]. Analysis of the mechanism whereby Wnt signaling inhibits adipogenesis indicates that induction of C/EBPβ and C/EBPδ is unaltered. However, induction of the master adipogenic transcription factors, C/EBPα and PPARγ, is completely blocked. In the current work, highly adipogenic Clone 4 cells show precocious expression of C/EBPα, while minimal expression of C/EBPα is observed in the poorly adipogenic Clone 9 cells. Consistent with the idea that there is a loss of an inhibitory factor that would otherwise block adipogenesis, Wnt10b mRNA is absent in the highly adipogenic Clone 4 cells but is expressed in Clone 9 cells. Further, Wnt10b-expressing Clone 4 cells do not differentiate as well as controls. Likewise, Wnt10b reduced expression of adipocyte proteins C/EBPα, PPARγ, and 422/aP2 in Clone 4 cells. These data demonstrate how altering the presence of positive and/or negative factors can shift the delicate balance of inhibitory and stimulatory factors that regulate adipogenesis.

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