Calreticulin inhibits commitment to adipocyte differentiation

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The editors of The Journal of Cell Biology have been notified by Dr. Michal Opas that he and the other authors of the paper referenced above retract the paper. As a result of this retraction, no data in this paper should be cited in the scientific literature. University of Toronto supports this retraction.

The authors provided the following statement:

Due to errors in image placement and data presentation during figure preparation, four figure parts (Fig. 1 C, Fig. 3 C, Fig. 7 A, and Fig. 7 E) contain incorrect data and/or presentation errors, as articulated below. Due to the inability of the authors to locate the correct data for Fig. 7 E, the quantification in Fig. 7 F cannot be validated. Due to an error in experimental design, the Western blot data in Fig. 4 D are not sufficient to support the quantification in Fig. 4 D. The other figures in the paper and the other parts of the figures listed above (Fig. 1, A, B, and D–F; Fig. 2; Fig. 3, A, B, D, and E; Fig. 4, A–C and E–H; Fig. 5; Fig. 6; Fig. 7, B–D and G–I; Fig. S1; and Fig. S2) were not affected by these errors.

Authors Eva Szabo and Michal Opas take full responsibility for these errors. Authors Yuanyuan Qiu, Shairaz Baksh, and Marek Michalak did not participate in data collection or figure preparation for any of the figures for which errors have been identified.

The errors identified were the following:

(1) The text fails to note the intentional duplication of the top GAPDH panel in Fig. 1 C with the top GAPDH panel in Fig. 7 A.

(2) Two panels in Fig. 1 C do not accurately represent the original data. Specifically, the PPARγ2 panel and the corresponding GAPDH panel do not properly show that lanes 1 and 2 are derived from a different gel than lanes 3 and 4. In addition, the image for the left two GAPDH lanes is flipped horizontally in the figure relative to the original data.

(3) Two panels in Fig. 3 C do not accurately represent the original data. Specifically, lanes 1 and 2 of the bottom GAPDH panel in the untreated dataset, which are identical to lanes 3 and 4 of the top GAPDH panel in the untreated dataset, are incorrectly presented with a splice line between the lanes. Lanes 1 and 2 of the C/EBPα panel in the untreated dataset, lanes 3 and 4 of the C/EBPα panel in the untreated dataset, and lanes 3 and 4 of the bottom GAPDH panel in the untreated data also are incorrectly presented with splice lines between the lanes. Lastly, lanes 1 and 2 of the bottom GAPDH panel should be presented as lanes 3 and 4 and vice versa.

(4) The text fails to note the intentional duplication in Fig. 3 C of the WT and G45crt−/− data in the top and bottom GAPDH panels for the untreated dataset, once corrected as per point 3 above.

(5) Three panels in Fig. 3 C do not contain the correct data. Specifically, all three L32 panels are placeholder images that were not replaced with the corresponding experimental data before publication.

(6) The +BAPTA-AM C/EBPα panel in Fig. 4 D is a duplicate of the +BAPTA-AM PPARγ2 panel in the same figure. The correct C/EBPα data were derived from a separate gel than the untreated C/EBPα data and thus cannot be used to support the quantification in Fig. 4 D.

(7) Three panels in Fig. 7 A do not properly represent the original data. Both GAPDH panels and the CaMK II Thr286 panel fail to contain the necessary splice lines between lanes 2 and 3. In addition, the data in lanes 3 and 4 of the top GAPDH panel are flipped horizontally relative to the original data.

(8) Five panels in Fig. 7 E do not contain the correct data. Specifically, all four L32 panels are placeholder images that were not replaced with the corresponding experimental data before publication. In addition, the data shown in the top GAPDH panel in the untreated dataset, which is identical but flipped 180 degrees relative to the bottom GAPDH panel
in the KN-93–treated sample, are not the correct data. The correct GAPDH data corresponding to the PPARγ2 panel in the untreated dataset could not be located. As a result, the quantification in Fig. 7 F cannot be validated.

(9) The text incorrectly states that quantification of aP2 levels is shown in the graph in Fig. 7 F.

As a result of these errors, the conclusion that BAPTA-AM treatment increased C/EBPα expression in all cell lines and was indicative of restored adipogenic potential in G45[P+C] cells (Fig. 4 D) cannot be validated. In addition, the conclusions that inhibition of CaMK II in CGR8+/+, G45−/−, L7+/−, or L7−/− cells decreased PPARγ2 expression (Fig. 7, E and F); that treatment with KN-92 had no effect on PPARγ2 expression in CGR8+/+, G45−/−, L7−/−, or L7−/− cells (Fig. 7, E and F); and that these findings were indicative of an important role for the calmodulin–CaMK II pathway during adipogenesis in embryonic stem cells cannot be validated.

The authors apologize for any confusion these errors may have caused to the research community.
Calreticulin inhibits commitment to adipocyte differentiation

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Calreticulin, an endoplasmic reticulum (ER) resident protein, affects many critical cellular functions, including protein folding and calcium homeostasis. Using embryonic stem cells and 3T3-L1 preadipocytes, we show that calreticulin modulates adipogenesis. We find that calreticulin-deficient cells show increased potency for adipogenesis when compared with wild-type or calreticulin-overexpressing cells. In the highly adipogenic crtmice, the ER luminal calcium concentration was reduced. Increasing the ER luminal calcium concentration led to a decrease in adipogenesis. In calreticulin-deficient cells, the calmodulin–Ca2+/calmodulin-dependent protein kinase II (CaMKII) pathway was up-regulated, and inhibition of CaMKII reduced adipogenesis. Calreticulin inhibits adipogenesis via a negative feedback mechanism whereby the expression of calreticulin is initially up-regulated by peroxisome proliferator–activated receptor γ (PPARγ). This abundance of calreticulin subsequently negatively regulates the expression of other key adipogenic proteins, CCAAT enhancer–binding protein α (C/EBPα), and aP2. Thus, calreticulin appears to be an important Ca2+-dependent molecular switch that regulates commitment to adipocyte differentiation by modulating the expression and transcriptional activation of critical proadipogenic transcription factors.

Introduction

Obesity is rapidly becoming a worldwide epidemic, leading to numerous medical conditions, including cardiovascular disease. The development of obesity is marked by both an increase in adipocyte size and number, a consequence of differentiation of the stem cells or preadipocytes into mature adipocytes (for review see Gesta et al., 2007). Spontaneous differentiation of embryonic stem (ES) cells into adipocytes in culture is a rare event; thus, for ES cells to commit toward the adipocyte lineage, it is necessary to pretreat cells with retinoic acid (RA), a ligand of the nuclear receptor retinoid X receptor (RXR), during the first phase of differentiation (Dani et al., 1997). RXR in response to RA heterodimerizes with peroxisome proliferator–activated receptor (PPAR), resulting in activation of genes involved in terminal differentiation and lipid metabolism (Gregoire et al., 1998). PPARγ2, CCAAT enhancer–binding protein α (C/EBPα), and aP2 are crucial for the development of adipose cells (Rosen et al., 2000). The process of adipogenesis may also be influenced by different extrinsic factor and intracellular signaling pathways (Rosen and MacDougald, 2006), yet very little is known about the role of Ca2+ homeostasis in adipogenesis. Increased Ca2+ levels lead to inhibition of adipocyte differentiation, which is accompanied by the decrease or, in some cases, the loss of PPARγ2, C/EBPα, and aP2 expression (Jensen et al., 2004; Serlachius and Andersson, 2004; Zhang et al., 2007). Thapsigargin, an inhibitor of the sarco/ER Ca2+-ATPase, inhibits adipocyte differentiation in 3T3-L1 preadipocytes (Ntambi and Takova, 1996; Shi et al., 2000), suggesting a role for ER stores and ER Ca2+-binding proteins in the modulation of adipogenesis.

Calreticulin is a major Ca2+-buffering protein in the lumen of the ER, which also acts as a molecular chaperone and modulator of gene expression (Bedard et al., 2005). The two major functions of calreticulin, chaperoning and Ca2+ buffering, are confined to specific protein domains. The N+P domain of calreticulin forms a folding module, and the acidic C-terminal C domain binds and buffers Ca2+ with high capacity (Baksh and Michalak, 1991; Nakamura et al., 2001). Calreticulin deficiency is embryonic lethal (Mesaeli et al., 1999), and calreticulin-deficient cells have a reduced capacity for Ca2+ storage.

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Results

Calreticulin deficiency promotes adipogenesis

Fig. 1 A shows that the adipogenic potential of ES cells, as measured by oil red O staining at differentiation day 20 (D20), was ~30-fold higher in the absence of calreticulin. The number of adipocyte colonies in calreticulin-deficient embryoid bodies (EBs) was approximately ninefold higher than that in the calreticulin-containing EBs at D20 (Fig. 1 B). This was irrespective of whether the calreticulin gene was removed by homologous recombination (G45crt−/− cells) or by Cre
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Calreticulin-deficient and calreticulin-containing cells, which makes it difficult to visualize all of them when equally loaded. Modulation of the expression of calreticulin also impacted adipogenesis of 3T3-L1 preadipocytes, a commonly used model for adipogenesis (Otto and Lane, 2005). Increased expression of calreticulin in 3T3-L1 preadipocytes inhibited their adipogenesis, as indicated by oil red O staining (Fig. 1 E). In agreement with ES cell results, molecular markers of adipogenesis (lipoprotein lipase, aP2, PPARγ2, C/EBPα, and C/EBPβ) were all down-regulated in 3T3-L1 cells overexpressing calreticulin (Fig. 1 F).

Upon induction of adipogenesis with RA, the abundance of calreticulin increased dramatically in the WT ES cells (Fig. 2 A), whereas the abundance of PPARγ2 and C/EBPα remained persistently low (Fig. 2, B and C). In contrast, in calreticulin-deficient (G45crt−/−) cells after induction of adipogenesis with RA, PPARγ2 and C/EBPα levels steadily increased over 20 d of differentiation (Fig. 2, B and C). Similar to the WT ES cells, the 3T3-L1 preadipocytes and 3T3-L1 cells overexpressing calreticulin showed an increase in calreticulin abundance and reduced adipogenic potential upon RA treatment (Fig. 2, D and E). We concluded that the increased expression of calreticulin plays a negative regulatory role during adipogenesis.

Adipogenesis in ES cells was RA and insulin dependent and required an initial 3-d exposure to RA followed by insulin treatment (unpublished data). Microscopically, by Nile red staining, lipid droplets in the wild-type (WT) adipocytes were indistinguishable from those in calreticulin-deficient cells (Fig. S1 B), indicating that the gross morphology of lipid stores was not affected in the absence of calreticulin. Nile red is a red-emitting fluorescent lysochrome (Greenspan et al., 1985) that can be used as a fluorescent alternative to oil red O lipid visualization (Fowler and Greenspan, 1985). Fig. S2 shows the nucleotide sequence of the calreticulin promoter.

Next, we examined the expression of both early (C/EBPα and PPARγ2) and late (aP2) adipogenic markers. The abundance of PPARγ2 and C/EBPα adipogenesis markers was markedly increased at D20 in G45crt−/− and L7crt−/− cells compared with WT (Fig. 1, C and D). The aP2 mRNA levels were undetectable in extracts from the WT and L7 cells, whereas they were high at D20 in extracts from calreticulin-deficient cells (Fig. 1, C and D). It should be stressed that in Fig. 1 C as well as in subsequent figures, adipogenic markers in calreticulin-containing cells are barely discernible. This is caused by a large disparity in the markers’ abundance between calreticulin-deficient and calreticulin-containing cells, which makes it difficult to visualize all of them when equally loaded. Modulation of the expression of calreticulin also impacted adipogenesis of 3T3-L1 preadipocytes, a commonly used model for adipogenesis (Otto and Lane, 2005). Increased expression of calreticulin in 3T3-L1 preadipocytes inhibited their adipogenesis, as indicated by oil red O staining (Fig. 1 E). In agreement with ES cell results, molecular markers of adipogenesis (lipoprotein lipase, aP2, PPARγ2, C/EBPα, and C/EBPβ) were all down-regulated in 3T3-L1 cells overexpressing calreticulin (Fig. 1 F).

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Figure 2. Calreticulin, PPARγ2, and C/EBPα levels increase over the 20-d differentiation time line, whereas overexpression of calreticulin inhibits adipogenesis. (A–C) Calreticulin (CRT), PPARγ2, and C/EBPα expression was examined in WT and G45crt−/− cells by Western blotting over 20 d of adipocyte differentiation as described in Materials and methods. The arrows represent the addition of retinoic acid (RA) or insulin (Ins) and triiodothyronine (TIT). (A) 10−7 M RA treatment of cells during the first 3 d of differentiation induced calreticulin expression in WT cells. (B and C) Expression of PPARγ2 and C/EBPα in RA-treated cr−/− cells gradually increased, whereas their levels in WT cells remained low over the 20-d differentiation. Standard error bars in A–C are smaller than the symbols. RA treatment up-regulates calreticulin in 3T3-L1 cells. (D) Western blot analysis with anti-HA antibodies of 3T3-L1 preadipocytes (3T3-L1 control) and 3T3-L1 cells overexpressing HA-tagged calreticulin (3T3-L1 + CRT) show a marked increase in calreticulin levels after RA treatment. (E) 3T3-L1 cells treated with RA show a decrease in oil red O staining, whereas overexpression of calreticulin reduced the staining further. Error bars represent SD.
or L7crt^{−/−}; Fig. 3, A and B). In contrast, after treatment with ionomycin, there was no detectable oil red O–positive adipocytes derived from WT and L7 cells, whereas differentiation of both calreticulin-deficient cell lines (G45crt^{−/−} and L7crt^{−/−}). Ionomycin treatment decreased oil red O staining in calreticulin-deficient ES cells (G45crt^{−/−} and L7crt^{−/−}). Conversely, ionomycin treatment decreased the adipogenic marker expression in calreticulin-deficient cells (G45crt^{−/−} and L7crt^{−/−}) cells. (D) Quantitative analysis of relative adipogenic marker expression after BAPTA-AM treatment (P < 0.01; n = 6). (E) Quantitative analysis of relative adipogenic marker expression after ionomycin treatment (P < 0.01; n = 6). Error bars represent SD.

Changes in the intracellular Ca^{2+} concentration affect adipogenesis

One important function of calreticulin is modulation of Ca^{2+} homeostasis (Nakamura et al., 2001; Bedard et al., 2005). Thus, we next examined whether calreticulin-dependent changes in Ca^{2+} homeostasis might be responsible for modulation of adipogenesis. We used BAPTA-AM, a membrane-permeable Ca^{2+} chelator (Tsien, 1980), to lower cytosolic Ca^{2+} concentration ([Ca^{2+}]_{cyt}) as well as ionomycin, a Ca^{2+} ionophore (Bergling et al., 1998), to increase [Ca^{2+}]_{cyt}. Treatment of cells with BAPTA from 20 min to 2 h produced exactly the same results (unpublished data), which implies that BAPTA was not secreted from the cells. BAPTA-AM-treated WT and L7 cells showed a dramatic increase in oil red O staining, indicating that chelation of cytoplasmic Ca^{2+} promoted adipogenesis in these cells (Fig. 3, A and B). There was no significant increase in oil red O staining in BAPTA-AM-treated calreticulin-deficient ES cells (G45crt^{−/−} or L7crt^{−/−}; Fig. 3, A and B). In contrast, treatment with ionomycin, there was no detectable oil red O–positive adipocytes derived from WT and L7 cells, whereas differentiation of both calreticulin-deficient cell lines (G45crt^{−/−} and L7crt^{−/−}) produced an extremely low number of oil red O–positive cells (Fig. 3, A and B).

In agreement with oil red O staining results, BAPTA-AM treatment significantly increased the abundance of all three adipogenic markers in adipocytes derived from WT and L7 cells containing calreticulin but did not have a significant effect on adipocytes derived from calreticulin-deficient (G45crt^{−/−} and L7crt^{−/−}) ES cells (Fig. 3, C and D). In the presence of ionomycin, the levels of PPARγ2, C/EBPα, and aP2 remained nearly undetectable in extracts from WT and L7 cells (Fig. 3, C and E). However, ionomycin significantly decreased the abundance of PPARγ2, C/EBPα, and aP2 levels in extracts from calreticulin-deficient cell lines (G45crt^{−/−} and L7crt^{−/−}) at D20 (Fig. 3, C and E).
Figure 4. Expression of the P+C domains of calreticulin reduces adipogenesis. [A] A schematic of the structural and functional calreticulin domains as well as Western blot analysis (with anti-HA antibodies) of G45crt\(^{-/-}\) ES cells expressing the N+P and P+C domain of calreticulin. [B] Oil red O staining in WT, G45crt\(^{-/-}\), and G45crt\(^{-/-}\) ES cells expressing P+C and N+P domains. G45crt\(^{-/-}\) cells expressing the P+C domain had decreased staining compared with the G45crt\(^{-/-}\) cells. When the cells were treated with 50 nM BAPTA-AM, both the P+C domain-expressing and WT ES cells showed a dramatic increase in oil red O staining. The N+P domain–expressing ES cells had oil red O staining similar to that of the G45crt\(^{-/-}\) ES cells. When the cells were treated with 500 nM ionomycin, both the N+P domain–containing and G45crt\(^{-/-}\) ES cells showed a decrease in oil red O staining. [C] Expression of the P+C domain in 3T3-L1 cells (3T3-L1 + [P+C]) reduced oil red O staining. Because 3T3-L1 is used as a control, it is always counted as 1. Expression of the P+C domain was confirmed using HA antibody. [D] Adipogenic marker expression was decreased in the P+C domain–containing G45crt\(^{-/-}\) cells when compared with the G45crt\(^{-/-}\) cells (P < 0.01; n = 6) and was comparable to that in WT cell levels (P > 0.05). BAPTA-AM treatment increased adipogenic marker expression in all cell lines (n = 6). [E] Ionomycin treatment reduced adipogenic marker expression in all cell lines (P > 0.05). Ionomycin treatment reduced adipogenic marker expression in all cell lines (n = 6). [F] P2Y2R-loaded ES cells were treated with thapsigargin or ionomycin to measure ER-releasable and total \([Ca^{2+}]_\text{ER}\) and \([Ca^{2+}]_\text{Tot}\) levels. 3T3-L1 + CRT cells (overexpressing calreticulin) and 3T3-L1 expressing the P+C domain had higher \([Ca^{2+}]_\text{ER}\) and \([Ca^{2+}]_\text{Tot}\) than the control 3T3-L1 cells (P < 0.01; n = 3). Error bars represent SD.
Modulation of adipogenesis by functional modules of calreticulin

Calreticulin has two structural and functional domains (Nakamura et al., 2001); one responsible for chaperoning, and another for Ca\textsuperscript{2+} buffering (Fig. 4 A). To determine which of calreticulin’s functions (domains) may be involved in the modulation of adipogenesis, two ES cell lines expressing single functional modules of calreticulin in calreticulin-deficient ES cells (G45crt\textsuperscript{-/-}) were created and tested for adipogenic potential. Because the C domain of calreticulin cannot be stably maintained, it was fused to the P domain of calreticulin (Nakamura et al., 2001). Expression of the domains was tracked using anti-HA antibodies (Fig. 4 A).

Fig. 4 B shows that reexpression of the P+C domain in calreticulin-deficient cells inhibited adipogenesis (Fig. 4, B and D). Overexpression of the P+C domain in 3T3-L1 preadipocytes also resulted in reduced adipogenesis (Fig. 4 C). Treatment of the P+C domain–expressing crt\textsuperscript{-/-} ES cells with BAPTA-AM restored their adipogenic potential (Fig. 4, B and D). In contrast, expression of the N+P domain in crt\textsuperscript{-/-} ES cells had no significant effect on their ability to differentiate into adipocytes, and they maintained a phenotype identical to calreticulin-deficient...
cells (Fig. 4 B). This was further supported by BATPA-AM and ionomycin experiments (Fig. 4), indicating that the chaperone function of calreticulin was not involved in the modulation of adipogenesis. Therefore, crt−/− cells expressing the N+P domain of calreticulin displayed features resembling those of WT ES cells, whereas crt−/− cells expressing the Ca2+ buffering region of calreticulin, we used thapsigargin, an inhibitor of sarco/ER Ca2+-ATPase, to measure the amount of Ca2+ associated with ER-exchangeable intracellular Ca2+ stores in either ES (Fig. 4 F) or 3T3-L1 (Fig. 4 H) cells. To assess the residual amount of Ca2+ contained within thapsigargin-insensitive luminal Ca2+ stores, we used the Ca2+ ionophore ionomycin (Fig. 4, F and H). Measurement of ionomycin- and thapsigargin-induced Ca2+ discharge from the ER indicated that the WT ES cells and calreticulin-deficient ES cells expressing the P+C domain had higher [Ca2+]ER and [Ca2+]P+c, respectively, compared with the crt−/− ES cells (Fig. 4 F). To provide yet another measure of ER-releasable Ca2+, we measured thapsigargin-induced Ca2+ discharge from the ER versus cytosolic [Ca2+]cyt using the Ca2+-sensitive fluorescent dye fura-2-AM under conditions preventing dye sequestration into the ER (Mery et al., 1996). Fig. 4 G shows that calreticulin-containing WT ES cells and calreticulin-deficient ES cells expressing the P+C domain had higher [Ca2+]ER and [Ca2+]cyt, respectively, in comparison to the crt−/− ES cells. Similar to the ES cells, overexpression of calreticulin in the WT ES cells expressing the P+C domain had higher [Ca2+]ER and [Ca2+]P+c compared with the ES cells expressing the N+P domain. In the calreticulin-deficient cells, PPARγ2 (green) colocalized with propidium iodide (red). (B) In the calreticulin-containing cells, labeling for PPARγ2 was perinuclear and perinuclear. Bar, 25 μm.

Figure 5. PPARγ2 localization in calreticulin-deficient G45 and calreticulin-expressing GCR8 ES cells. At D20 of differentiation, the cells were labeled for PPARγ2 and propidium iodide (PI) to identify the nuclei as described in Materials and methods. [A] In the calreticulin-deficient cells, PPARγ2 (green) colocalized with propidium iodide (red). [B] In the calreticulin-containing cells, labeling for PPARγ2 was perinuclear and perinuclear. Bar, 25 μm.

**Functional relationship between calreticulin and PPARγ2**

The results so far suggested that calreticulin plays a modulatory role during adipogenesis. We next wanted to determine whether there was a functional relationship between calreticulin and the PPARγ transcriptional complex. Upon RA-dependent induction of adipogenesis, RXR and PPARγ form a transcriptionally active complex. The calreticulin promoter contains two PPARγ-binding sites termed peroxisome proliferator responsive elements (PPREs); one is found at −1,944 bp (designated PPRE1), and the other is found at −590 bp (designated PPRE2; Fig. 5 A), suggesting that the PPARγ transcription factor may regulate the calreticulin gene. To test this, NIH3T3 fibroblasts were cotransfected with PPARγ expression vector and RXRα expression vector (pLC0, pLC1, pLC2, pLC0mt1, and pLC0mt2) or luciferase reporter gene vectors (pCL2 and pCL3; Fig. 5 A) under control of the calreticulin promoter. pSVβ-galactosidase was used as an internal control. In the pLC0 vector, luciferase was controlled by the calreticulin promoter containing 590 bp 5′ of the calreticulin start site, pCL2 by 1,944 bp 5′ of the calreticulin start site, pCL3 by 1,944 bp 5′ of the calreticulin start site, and pCL0mt2 by 2.1 kb of the calreticulin promoter containing both PPRE sites (Fig. 5 A and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200712078/DC1). Fig. 5 B shows that PPARγ induced luciferase activity in NIH3T3 cells. Cells transfected with calreticulin into the WT ES cells in the 3T3-L1 preadipocytes that contained intracellular [Ca2+]ER and [Ca2+]P+c compared with control 3T3-L1 cells (Fig. 4 H). Finally, as ionomycin may cause in release of Ca2+ from acidic intracellular compartments, we added the sodium proton ionophore monensin after the experiment (Fig. 4 F). The ionophore-sensitive Ca2+ release was very small (unpublished data), suggesting that WT, crt−/− cells, and cells expressing the P+C domain did not contain substantial quantities of Ca2+ within acidic compartments. Thus, we conclude that the regulation of calreticulin on adipogenesis may be mediated by calreticulin-dependent changes in intracellular Ca2+.
promoterless control plasmids showed no detectable luciferase activity (unpublished data). This finding indicates that PPARγ activates transcription of the calreticulin gene.

To identify the PPARγ-binding site on the calreticulin promoter, we performed reporter gene assay with calreticulin promoter deletions and mutations (Fig. 5, B and C). Deletion of PRE1 and PRE2 sites (pLC2 vector) completely abolished PPARγ-dependent activation of the calreticulin promoter (Fig. 5 B). Deletion of PRE site 2 at −1,944 bp (Fig. 5 A) or mutation of PRE site 1 or 2 (Fig. 5 A) had no effect on PPARγ activation of the promoter (Fig. 5, B and C). Thus, upon induction of adipogenesis with RA, RXR and PPARγ form a complex, which binds PRE sites 1 and 2 on the calreticulin promoter and transcriptionally activates the calreticulin gene.

To further elucidate a physical interaction between the calreticulin promoter and PPARγ, an electrophoretic mobility shift assay (EMSA) was performed (Fig. 5 C). Synthetic oligodeoxynucleotides corresponding to PRE sites 1 and 2 were used along with a positive control probe, an ideal PPARγ site. Fig. 5 C shows that the PPARγ-RXRα complex bound to PRE1 and PRE2. This DNA–protein interaction was only observed in the presence of RXRα, indicating that PPARγ is only functional once complexed with RXRs (Fig. 5 C). The specificity of the PRE and PPARγ–RXRα binding was confirmed by reduced signal intensity after the addition of 30-fold excess cold probe (Fig. 5 C). The lesser intensity in PRE1 complexes (Fig. 5 C, lanes 4 and 6) when compared with the complexes containing the ideal PRE site (Fig. 5 C, lane 10) is likely the result of the difference in the sequences of these probes. Specificity of the bands was further confirmed by supershift EMSA and with no shift of PRE sites (Fig. 5 C, lanes 7 and 8). Chromatin immunoprecipitation (ChIP) was also performed to determine whether there was a direct interaction between the calreticulin promoter and PPARγ. ChIP analysis indicated that PPARγ bound to both PRE1 sites 1 and 2 on the calreticulin promoter (Fig. 5 E). We concluded that the PPARγ–RXRα complex binds to calreticulin PRE1 and PRE2 sites and activates the calreticulin gene.

PPARγ2, along with C/EBPα, directly affect gene transcription in the nucleus (Rosen, 2005; Rosen and MacDougall, 2006). Given that PPARγ2 is both necessary and sufficient for adipogenesis (Rosen et al., 2000), we determined its spatial expression during adipogenesis in our ES cell system (Fig. 6). On D20 of differentiation in the crt−/− ES cells (which exhibit increased adipogenesis), PPARγ2 was distinctly nuclear (Fig. 6 A). However, in the WT ES cells, PPARγ2 was barely detectable and appeared cytosolic (Fig. 6 B). Given that the WT ES cells show reduced adipogenesis and, thus, only sparse adipocyte colonies, Fig. 6 B represents a region of low adipogenic potential that predominates in WT outgrowths. Localization of C/EBPα was also investigated in the ES cells, and it showed an essentially identical pattern to that observed for PPARγ2 (unpublished data).

Calmodulin/CaMKII, C/EBPα, and calcineurin during adipogenesis

Calmodulin–Ca2+/calmodulin-dependent protein kinase II (CaMKII) and cAMP response element binding (CREB) pathways affect adipogenesis (Wang et al., 1997; Reusch et al., 2000). Western blot analysis with anti-Thr286–phosphorylated CaMKII, anticalmodulin, and anti-Ser133–phosphorylated CREB antibodies showed that their expression was higher in calreticulin-deficient G45crt−/− and L7crt−/− cells than in WT and L7 cells at the end of the 20-d differentiation (Fig. 7, A and B). These results indicate that CaMKII and CREB play a positive role during adipogenesis from ES cells and that they are up-regulated in the presence of the calreticulin gene.

To elucidate the role of the CaMKII pathway, we used specific inhibitors of CaMKII–RN-62 and KN-93 (Hidaka and Kobayashi, 1994). Treatment of G45crt−/− and CaMKII in G45crt−/− and calreticulin-deficient ES cells (Fig. 7, C and D) showed that KN-92, an inactive analogue of KN-92, did not alter oil red O staining (Fig. 7, C and D) or adipogenic marker expression in either ctn−/− or calreticulin-containing ES cells (Fig. 7, E and F). These data suggest that the calmodulin–CaMKII pathway plays an important role during adipogenesis from ES cells.

Calcineurin is a negative regulator of adipogenesis (Neal and Clipstone, 2002; Kennell and MacDougall, 2005) and is known to affect calcineurin activity (Guo et al., 2002; Lynch et al., 2005). On D20, calcineurin activity was significantly higher in the WT ES cells than in the calreticulin-deficient cells (Fig. 7 G). In the 3T3-L1 preadipocytes, inhibition of calcineurin with cyclosporin A promoted adipogenesis (Fig. 7, H and I), whereas constitutive expression of activated calcineurin decreased adipogenesis (Fig. 7, H and I). These data give support for the
role of calcineurin in adipogenesis of both ES cells and 3T3-L1 preadipocytes. The present findings are consistent with earlier studies relating activation of calcineurin to the presence of calreticulin (Lynch and Michalak, 2003; Lynch et al., 2005).

**Discussion**

In this study, we show that calreticulin affects adipocyte differentiation from either ES cells or 3T3-L1 preadipocytes. The absence of calreticulin sways ES cell differentiation toward the adipocyte lineage. This choice of fate is reversed to the WT phenotype by overexpression of calreticulin or by the Ca$^{2+}$-buffering domain of calreticulin but not by expression of the chaperoning domain of the protein. These effects of calreticulin are likely mediated through its Ca$^{2+}$ regulatory function. In comparison to WT cells, calreticulin-deficient ES cells have reduced ER Ca$^{2+}$ storage capacity, resulting in reduced ER Ca$^{2+}$ release upon stimulation. Expression of full-length calreticulin or the calreticulin Ca$^{2+}$-buffering domain in ES cells or 3T3-L1 cells increases ER Ca$^{2+}$ content and reduces the commitment of these cells to adipogenesis. In WT cells, chelating cytoplasmic [Ca$^{2+}$]$\equiv$1,000 nM at days 4–7 with the same effects. Moreover, as calreticulin-deficient ES cells have reduced ER Ca$^{2+}$, all pointing out the importance of Ca$^{2+}$ homeostasis. Ca$^{2+}$-buffering P+C domain, adipogenesis was halted, whereas expression of the N+P domain had no effect on the progression of adipogenesis.

Our findings that calreticulin affects the commitment to adipocyte differentiation are in line with reports that calreticulin-deficient Ca$^{2+}$ signaling also affects several aspects of the differentiation of cardiomyocytes (Li et al., 2002; Grey et al., 2005; Puceat and Jaconi, 2005) and human myeloid cells (Clark et al., 2002). Interestingly, diminished intracellular Ca$^{2+}$ stores attenuate cardiomyogenesis but promote adipogenesis and differentiation of myeloid cells. Combined, these findings imply that calreticulin and ER Ca$^{2+}$ must play important roles in a variety of differentiation pathways. Moreover, our data regarding timing of the Ca$^{2+}$-buffering molecules indicate that in adipogenesis, as in cardiomyogenesis (Li et al., 2002), there is a calreticulin-dependent Ca$^{2+}$ buffering step referred to as a checkpoint by Li et al. (2002).

**Ca$^{2+}$-handling module of calreticulin inhibits adipogenesis**

An important finding of this study is that calreticulin, at least in part, exerts its effects on adipogenesis via its role as a modulator of Ca$^{2+}$ homeostasis. Ca$^{2+}$ has previously been shown to affect adipogenesis of 3T3-L1 preadipocytes (Shi et al., 2000; Jensen et al., 2004). For example, 3T3-L1 cells exposed to elevated external Ca$^{2+}$ levels accumulated little or no cytoplasmic lipids and showed the diminished expression of PPAR$\gamma$2, C/EBP$\alpha$, and aP2 (Jensen et al., 2004). Increasing cytoplasmic Ca$^{2+}$ levels by thapsigargin also inhibited early stages of adipogenesis (Shi et al., 2000), all pointing out the importance of Ca$^{2+}$ on adipogenesis. Indeed, we show here that an increase in intracellular Ca$^{2+}$ concentration leads to a decrease in adipocyte differentiation. Conversely, decreasing intracellular Ca$^{2+}$ concentrations promotes adipogenesis of calreticulin-deficient ES cells. The relationship between calreticulin expression, intracellular Ca$^{2+}$ concentration, and adipogenesis implies that calreticulin exerts its effects on adipogenesis via its Ca$^{2+}$ homeostatic function. However, the formal proof comes from experiments in which functional modules of calreticulin were expressed in calreticulin-deficient ES cells. Upon expression of calreticulin’s Ca$^{2+}$-buffering P+C domain, adipogenesis was halted, whereas expression of the N+P domain had no effect on the progression of adipogenesis.

Given that [Ca$^{2+}$]$_{\text{ER}}$ is lower in calreticulin-deficient cells, the question arises as to how CaMKII activity could be increased in these cells. In calreticulin-deficient cells, the level of tyrosine phosphorylation, including that of c-Src (Papp et al., 2007), is higher than in WT cells (Fadel et al., 1999; 2001; Szabo et al., 2007). Activated c-Src phosphorylates calmodulin on tyrosine 99, thereby increasing the affinity of calmodulin for CaMKII (Abdel-Ghany et al., 1990; Benaim and Villalobo, 2002). This phosphorylation event is inhibited by high Ca$^{2+}$.
concentration (Fukami et al., 1986). Tyrosine-phosphorylated calmodulin effectively activates CaMKII (Corti et al., 1999). In addition, after initial activation, CaMKII becomes auto-phosphorylated, remaining active even though its activators are removed (Meyer et al., 1992). Therefore, CaMKII may remain active and promote adipogenesis in calreticulin-deficient cells even under reduced intracellular Ca\(^{2+}\) concentration.

We have found that endogenous calcineurin activity was significantly higher in calreticulin-expressing cells that have reduced adipogenic potential compared with the calreticulin-deficient cells that are highly adipogenic. This is in agreement with previous studies on calcineurin-dependent inhibition of adipogenesis (Neal and Clipstone, 2002; Kennell and MacDougald, 2005). Thus, we propose that although the calcineurin pathway inhibits adipogenesis, the Ca\(^{2+}\)-independent CaMKII pathway might be responsible for the promotion of adipogenesis in the absence of calreticulin observed here.

Calreticulin modulates PPAR\(\gamma\) activity through a negative feedback mechanism

PPAR\(\gamma\) and C/EBP\(\alpha\) are crucial transcription factors during adipogenesis (Rosen, 2005); however, although PPAR\(\gamma\) is necessary for adipogenesis, it does not play a crucial role during this process (Rosen et al., 2002). We showed here that PPAR\(\gamma\) activity and adipogenic differentiation, the calcineurin pathway may be involved in the absence of calreticulin observed here.

Cell culture and adipocyte differentiation

G45 CRT\(^{+/+}\) ES cells and the WT ES cell line (CGR8) were derived from J1 129/Sv mice. To generate the L7 ES cell line, J1 129/Sv ES cells were electroporated with a targeting vector containing the calreticulin gene with the lox\(^P\) site inserted in introns 1 and 2 (Fig. S1 A) and maintained on mitomycin C-treated G418-resistant mouse embryonic fibroblast feeder cells (Mesaeli et al., 1999). Recombinant clones were selected with 0.2 mg/ml G418 and 2 mM gancyclovir. Several hundred colonies were picked after 10 d in selection medium and expanded. The L7 clone was selected based on Southern blot analysis and Western blot analysis with anticalreticulin antibodies (Mesaeli et al., 1999). Cre recombinase with additional mutant estrogen receptor ligand-binding domains [Cre-ER and MerCreMer] was used to excise exons 2–4 from the calreticulin gene (Fig. S1 A). In brief, L7 ES cells were stably transfected with Cre recombinase mutator expression vector PAMER and Cre-ER (gift from J.D. Molkentin, University of Cincinnati, Cincinnati, OH) to generate the L7-Cre cell line. To generate L7\(^{crt-}\), 10 \(\mu\)M 4-hydroxytamoxifen was added to promote nuclear translocation of Cre recombinase. L7\(^{crt-}\) controls expression of the calreticulin gene with interrupted reading frame and no expression of the protein (Fig. 1 C). Calreticulin-deficient 3T3-L1 adipocytes expressing N\(^{+}P\) and P\(^{+}C\) domains of G45\(^{crt-}\) were created by hanging drop method. This method was used to isolate ES cells co-transfected with Cre recombinase and calreticulin expression vectors. The 3T3-L1 adipocytes expressing N\(^{+}P\) and P\(^{+}C\) domains of G45\(^{crt-}\) were isolated by hanging drop method. The isolated clones were then cultured in differentiation medium containing high glucose DME supplemented with 15% FBS (Multicell, Wisent), 5\(\mu\)M sodium pyruvate, 0.5 mM methylisobutylxanthine, 5\(\mu\)M insulin, and 0.5 \(\mu\)M mosiglitazone (Cayman Chemical). After 2 d, the aggregated cells were maintained in differentiation medium supplemented with 1 \(\mu\)g/ml insulin and 2 nM triiodothyronine. The differentiation medium was changed every 2 d for the duration of differentiation (15 d). The EBs were then collected for Western blot analysis, RNA isolation, or staining with oil red O.

Plasmid DNA and transfections

pSG5\(r\)-RAR\(\alpha\) and pSG5-mPPAR\(\gamma\) were gifts from R. Racubinski (University of Alberta, Edmonton, Alberta, Canada). Plasmid DNA was purified using Mega plasmid preparation and columns (Qiagen) as recommended by the manufacturer. Expression vectors encoding the HA-tagged N\(^{+}P\) domain (pcDNA3.1 Zeo-CRTNPd-1) and P\(^{+}C\) domain (pcDNA3.1 Zeo-CRTCPCd-3) of calreticulin were previously described (Nakamura et al., 2001). ES cells were electroporated with 30 \(\mu\)g/cuvette of pcDNA3.1 Zeo-CRTNPd-I or pcDNA3.1 Zeo-CRTCPCd-3 expression vector by electroporation (1,500 V/cm; 25 \(\mu\)F). Zeocin (30 \(\mu\)g/ml)-resistant clones were isolated. Expression of the recombinant N\(^{+}P\) and P\(^{+}C\) domains was monitored with anti-HA antibodies (Nakamura et al., 2001). 3T3-L1 cells were stably transfected with pcDNA3.1-CRT (encoding HA-tagged full-length calreticulin), pcDNA3.1 Zeo-CRTNPd-I, or pcDNA3.1 Zeo-CRTCPCd-3 using Lipofectamine reagent (Invitrogen) according to the manufacturer’s instructions. 3T3-L1 clones expressing full-length calreticulin or calreticulin mutants were selected with 50 \(\mu\)g/ml Zeocin. Expression of recombinant proteins was monitored with anti-HA antibodies (Nakamura et al., 2001).
Reporter gene assay
3T3-NIH cells were cotransfected with reporter plasmids containing the calreticulin promoter, deletion of the calreticulin promoter, or the calreticulin promoter with mutations of PPRE1 or PPRE2 [pLC0, pLC1, pLC2, pLC0mt1, and pLC0mt2], PPARγ, and RXRα expression vectors. pLC1 and pLC2 plasmids encode the luciferase reporter gene under the control of 1.74-kb and 0.4-kb calreticulin promoters, respectively (Waser et al., 1997). pLC0 encoded the luciferase reporter gene under control of the 2.1-kb calreticulin promoter (Waser et al., 1997). To generate pLC0mt1 and pLC0mt2 plasmids, site-directed mutagenesis of PPRE1 and PPRE2 of the calreticulin promoter, respectively, was performed using the QuickChange Site-Directed Mutagenesis kit (Stratagene). Specifically, PPRE1 [AGGTCAGAGGAC] was mutated to AGGCGAGGGAC, whereas PPRE2 (TG GCCCTGACCC) was mutated to GCCGCTGCCCC (lowercase letters are mutated nucleotides). After 48 h, cells were harvested in a lysate buffer containing 100 mM Tris, pH 7.8, 0.5% NP-40, and 0.5 mM DTT. Luciferase and β-galactosidase activity were measured as described previously (Waser et al., 1997).

Inhibitor studies
At the floating stage (days 3–5 of differentiation), EBs were incubated with KN-62 CaMK inhibitor or its inactive analogue (KN-93; Hidaka and Kobayashi, 1994) at concentrations of 10 μM for 2 h during the 3 d of EB flotation stage. The optimal inhibitory concentrations of KN-62 and KN-93 were determined to be in the range of 10 to 15 μM. Under these conditions, the drugs had no effect on cell survival/proliferation. For inhibition of calcineurin, 3.34 μM preapredesines were incubated with 1 μg/ml cyclosporin-A. The cells were then allowed to differentiate for an additional 15 d and were harvested for Western blot analysis, RNA isolation, or staining with oil red O.

Ca2+ studies and measurements
The 45Ca2+ measurements as well as [Ca2+]i measurements using the Ca2+-sensitive fluorescent dye fura-2-AM were performed as previously described in detail by Merry et al. (1996). The ionomycin and BAPTA-AM regimen was performed as previously described by Li et al. (1999) and Grey et al. (2005) with the following modifications: to ensure functional output, three consecutive pulse treatments with 1 μM ionomycin or 10 μM BAPTA-AM. To chelate cytoplasmic Ca2+, cells were incubated with 50 nM BAPTA-AM for 30 min at day 3 of differentiation. The ionomycin and BAPTA-AM regimen was performed as described previously (Lynch et al., 2005). KN-62 cells were transiently transfected with HA-PPARγ and stained in 1% formaldehyde at room temperature for 20 min. Cells were then lysed with the Extract-N-Amp kit (Sigma-Aldrich) according to the manufacturer’s instructions. Chromatin was sheared by sonication followed by centrifugation for 10 min. Supernatants were precleared with protein A–Sepharose beads for 1 h at 4°C. Immunoprecipitation was performed with mouse anti-PPARγ antibodies at 4°C overnight. DNA was purified and analyzed by PCR using the following primers: PPRE site 1 forward primer 5′-TGGTCAGAGGACCCCGCTCC-3′ and reverse primer 5′-CACGGGATCCATCTGCAGGTC-3′; for pLC0mt2, reverse primer 5′-GGCAGGATGATGACCCCTGAGAGGC-3′ and forward primer 5′-GCTCTGATG-3′ for pAP2, reverse primer 5′-CATACGCCGATGGGAGATG-3′ and forward primer 5′-TGACTTTTCCATCACTCACC-3′; and for L32, reverse primer 5′-CATGGTGCCCCTGCCGCTCTC-3′ and forward primer 5′-CATTCTCTGCTGCGTACAGCC-3′. PCR products were separated in 1.5% agarose gel. The mRNA levels were normalized using H2 as the housekeeping gene, and relative mRNA levels were quantified using ImageJ software.

Oil red O staining
Before staining with oil red O, cells were washed twice with PBS, fixed with 10% formaldehyde for 15 min at room temperature, and washed twice with distilled water and once with 70% isopropanol. Next, cells were stained for 1 h at room temperature with filtered oil red O at a ratio of 60% oil red O stock solution (0.5% wt/vol in isopropanol) to 40% distilled water. The cells were washed twice with distilled water, twice with PBS, and examined under a light microscope. An invertoscope (Diaphot; Nikon) equipped with a 10/0.25 DL dry plan Apochromatic objective (Nikon) was used for imaging at room temperature. A camera (Coolpix 4500; Nikon) was used for image acquisition. For quantitative analysis, oil red O was extracted with 5 ml isopropanol for 2 min, and optical density of each sample was determined at 540 nm.

EMSA
Full-length RXRs and PPARγlesser control proteins were synthesized using a coupled transcription and translation reticulocyte system (Promega; Guo et al., 2001) with oligonucleotides corresponding to PPAR site 1 (5′-GCTCTGAGAGGACCCCGCTCC-3′) and PPAR site 2 (5′-GGCAGGATGATGACCCCTGAGAGGC-3′). An ideal PPRE oligodeoxynucleotide with the consensus sequence (H11032)5′-CATGGCTGCCCTTCGGCCCTC-3′, was used as cold competitor to correspond to the PPAR-binding oligodeoxynucleotide [125I]ATP (GE Healthcare) and was performed as described previously (Lynch et al., 2005). KN-62 cells were transiently transfected with HA-PPARγ and stained in 1% formaldehyde at room temperature for 20 min. Cells were then lysed with the Extract-N-Amp kit (Sigma-Aldrich) according to the manufacturer’s instructions. Chromatin was sheared by sonication followed by centrifugation for 10 min. Supernatants were precleared with protein A–Sepharose beads for 1 h at 4°C. Immunoprecipitation was performed with mouse anti-PPARγ antibodies at 4°C overnight. DNA was purified and analyzed by PCR using the following primers: PPRE site 1, forward primer 5′-TGTGTCGAGAGGACCCCGCTCC-3′ and reverse primer 5′-GACAGGAGAGGAAGAAGAGAG-3′; PPRE site 2, forward primer 5′-CTCTGAGAGGACCCCGCTCC-3′ and reverse primer 5′-CTGTGCGCAGGAAGAAGAGAG-3′.

Calcineurin activity assay
Calcineurin activity assay was performed as previously described (Fruman et al., 1992). In brief, a peptide corresponding to the regulatory domain of protein kinase A (Sigma-Aldrich) was used as the substrate in an in vitro dephosphorylation assay (RIP peptide). 1.0 × 106 cells were lysed in 30 ml hypotonic lysis buffer containing 50 mM Tris, pH 7.5, 0.1 mM EGTA, 1 mM EDTA, 250 mM DTT, and protease inhibitors. 20 ml of lysate was added to 5 mM γ-[32P]ATP (GE Healthcare) and was performed as described previously (Lynch et al., 2005). KN-62 cells were transiently transfected with HA-PPARγ and stained in 1% formaldehyde at room temperature for 20 min. Cells were then lysed with the Extract-N-Amp kit (Sigma-Aldrich) according to the manufacturer’s instructions. Chromatin was sheared by sonication followed by centrifugation for 10 min. Supernatants were precleared with protein A–Sepharose beads for 1 h at 4°C. Immunoprecipitation was performed with mouse anti-PPARγ antibodies at 4°C overnight. DNA was purified and analyzed by PCR using the following primers: PPRE site 1, forward primer 5′-TGTGTCGAGAGGACCCCGCTCC-3′ and reverse primer 5′-GACAGGAGAGGAAGAAGAGAG-3′; PPRE site 2, forward primer 5′-CTCTGAGAGGACCCCGCTCC-3′ and reverse primer 5′-CTGTGCGCAGGAAGAAGAGAG-3′.

Immunofluorescence and Nile red staining
Cells on coverslips were fixed in 3.7% formaldehyde in PBS for 10 min. After washing three times for 5 min in PBS, the cells were permeabilized with 1% Triton X-100 in buffer containing 100 mM Pipes, pH 6.9, 1 mM EGTA, and 4% (wt/vol) polyethylene glycol 8000 for 2 min, washed three times for 5 min in PBS, and incubated with goat polyclonal anti-PPARγ antibody (diluted 1:50 in PBS; Santa Cruz Biotechnology, Inc.) for 30 min at room temperature. After washing three times for 5 min in PBS, the cells were incubated with the secondary antibody for 30 min at room temperature. The secondary antibody was FITC-conjugated donkey anti-goat IgG(1+2) diluted 1:50 in PBS. The cells were then incubated with 0.2 mM...


