Thiazolidinediones Cause Compaction of Nuclear Heterochromatin in the Pluripotent Mesenchymal Cell Line C3H10T1/2 when Inducing an Adipogenic Phenotype

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OBJECTIVE: To characterize the nuclear changes induced in vitro by thiazolidinediones (TZDs) in a murine pluripotent mesenchymal cell line.

STUDY DESIGN: The C3H10T1/2 cell line, which can differentiate either in osteoblast or in adipocyte, was cultured in the presence of pioglitazone (5 μM) or rosiglitazone (0.5 μM) for 6, 8 and 9 days (D). Quantitative real-time polymerase chain reaction analysis evaluated the expression of key genes of the adipocytic or osteoblastic differentiation (PPARγ [peroxisome proliferator-activated receptor γ], Runx2 [runt-related transcription factor 2] and alkaline phosphatase). Cells were stained with Oil Red O for lipids, and chromatin was counterstained with hematoxylin. Cells were photographed at ×1,000 magnification and analyzed with texture analysis software. Nuclear area, mean gray level and run-length parameters were calculated.

RESULTS: PPARγ was significantly expressed from D6 (normalized ratio > 7) in TZD groups (ratio > 27 at D9). No significant differences were found for either Runx2 or alkaline phosphatase expression versus control at D6 or D9. Cells cultured with TZDs began to differentiate into adipocytes with numerous lipid droplets which appeared at D6. Nuclear area decreased suddenly at D6 for both TZDs, and the mean gray level increased. Run-length parameters changed significantly due to chromatin compaction.

CONCLUSION: TZDs provoked differentiation of C3H10T1/2 into adipocytes, leading to inactivation of genes that were highly compacted into heterochromatin.

Keywords: adipocytes, chromatin, osteoblasts, texture analysis, thiazolidinediones.

Mesenchymal stem cells (MSCs) are multipotent cells able to differentiate into different distinct cell types (osteoblasts, adipocytes, myoblasts and chondrocytes). This can be achieved by the expression of different activation pathways. A reciprocal relationship exists between the differentiation of MSCs into osteoblasts and adipocytes. Experimental data suggest that mature osteoblasts can differentiate...
into adipocytes and conversely.\textsuperscript{2-4} For example, the activation of nuclear nicotinamide adenine dinucleotide (NAD)--dependent protein deacetylase Sirt1, osterix and Runx2 (runt-related transcription factor 2) reduces adipocyte formation and promotes osteoblast differentiation.\textsuperscript{5-7} In contrast, the peroxisome proliferator-activated receptor \( \gamma \) (PPAR\( \gamma \)—a nuclear receptor protein with a regulator action on metabolism, differentiation, cell growth and apoptosis of different cell types) was reported to induce in vitro adipogenesis in 3T3-L1 preadipocytes and C3H10T1/2 pluripotent stem cells.\textsuperscript{8-10} PPAR\( \gamma \) is a key element for the differentiation of MSCs into adipocytes.\textsuperscript{11-13} The best-known PPAR\( \gamma \)-ligands are the insulin-sensitizing thiazolidinedione (TZD) drugs.\textsuperscript{14-16} TZDs represent a class of oral antidiabetic agents currently used for treatment of diabetes mellitus in humans.\textsuperscript{17} TZDs regulate the expression of many genes including PPAR\( \gamma \).\textsuperscript{18-22} Activation of PPAR\( \gamma \) by TZDs was found to cause an increase in bone marrow adiposity and a decrease in osteoblastogenesis associated with osteocyte apoptosis, resulting in bone mass reduction and an increase in fracture risk.\textsuperscript{23-29}

C3H10T1/2s are well-characterized pluripotent mesenchymal stem cells that have been extensively used as a model of adipogenic differentiation.\textsuperscript{30} They are capable of differentiating either into osteoblasts or adipocytes, with a preferential differentiation into adipocytes during the proliferation phase when they are treated by insulin or TZDs.\textsuperscript{31} These cells undergo growth arrest and initiate the adipogenic program of differentiation characterized by accumulation of little and large lipid droplets in their cytoplasm.\textsuperscript{32} It is speculated that changes in chromatin structure, which occur during cell differentiation, may be required for the binding of PPAR\( \gamma \) to its respective response elements.

In this study we have evaluated changes in the nuclear chromatin pattern distribution of C3H10T1/2 cells in the presence of 2 different TZDs: pioglitazone (PGZ) and rosiglitazone (RGZ). RT-PCR analysis was used to analyze the pattern of expression of selected candidate genes of the adipogenic and osteoblastic lineage. Chromatin changes were measured by texture analysis, a powerful tool to study homogeneity and pattern repartition.\textsuperscript{33}

\textbf{Materials and Methods}

\textbf{Reagents}

Dulbecco’s modified Eagle’s medium (DMEM), L-glutamine, penicillin-streptomycin and fetal calf serum were obtained from Eurobio (Eurobio, Les Ulis, France). PGZ hydrochloride (CAS number 112529-15-4) and RGZ (CAS number 122320-73-4) were obtained from Molekula (Shaftesbury, U.K.). TZDs were first diluted in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/mL (stock solution). Aqueous TZD solutions were then prepared by addition of the appropriate volume of stock solution to reach a final concentration of 10\textsuperscript{-5} M. The final concentration of DMSO in the culture medium never exceeded 0.2%.

\textbf{Cell Culture}

The murine pluripotent mesenchymal cell line C3H10T1/2 clone 8 was obtained from American Type Culture Collection (ATCC catalog #CCL-226). Cells were cultured in DMEM containing 1 g/L glucose and supplemented with 10% fetal calf serum, 2% L-glutamine, 100 UI/mL of penicillin and 100 \( \mu \)g/mL streptomycin at 37\textdegree C in a 5% CO\textsubscript{2} humidified atmosphere. The medium was changed every 2 days. Degreased glass coverslips, rinsed in distilled water and sterilized with ultraviolet light for 3 hours were placed in 24-well plates. A total of 2,500 cells were seeded in each well. Twenty-four hours after cell seeding (referred to as day 0 [D0]), PGZ (final concentration 5 \( \mu \)M) or RGZ (final concentration 0.5 mM) were added in the culture medium. Control cell cultures were supplemented with an equal volume of DMSO. Cells were cultured for 6, 8 and 9 days.

\textbf{Quantitative RT-PCR Analysis (qPCR)}

C3H10T1/2 cells were lysed and processed for RNA extraction using the RNeasy mini kit (Qiagen, Courtaboeuf, France) following the manufacturer’s procedure. Cells were obtained at days 6 and 9. After elution, total RNA was aliquoted and stored at ~80\textdegree C until use. The quality of RNA samples was examined on a denaturing agarose gel, and tRNA concentrations were determined by spectrometry. 1 \( \mu \)g of total RNA sample was reverse transcribed using random hexamer primers and SuperScript II reverse transcriptase (SSII, Invitrogen, France). Primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 .www.cgi). The following primers were used: alkaline phosphatase (forward 5'-TGCCAGAGAAGAGAAGAGACC-3' and reverse 5'-AGCGGGTTACTGTGGAGACG-3'), PPAR\( \gamma \) (forward 5'-CTGATGCACTGCCTATGAGC-3' and reverse...
5'-GGGTCAGCTCTTGTGAATGG-3'), and Runx2
(forward 5'-GTGGCCACTTACCACAGAGC-3'
and reverse 5'-GTTCTGAGGCGGGACACC-3').
Amplification was performed in duplicates with iQ
SYBR Supermix (BioRad, Courtaboeuf, France) and
using a Chromo4 real-time system (BioRad). Ther-
mal cycling was initiated with 10 minutes incuba-
tion at 95°C followed by 40 cycles of 15 seconds at
95°C, 11 seconds at 55°C and 22 seconds at 72°C.
Gene expression was calculated using the compara-
tive CT method with the normalization of the target
genes to 3 housekeeping genes (G3PDH, HPRT1
and RPS18).

Cytochemical Staining with Oil Red O
Cells were washed twice with phosphate buffered
saline and fixed with 10% formalin for 10 minutes
at room temperature. They were rinsed twice with
distilled water and in 60% 2-propanol. Cells were
then stained for 1 hour at room temperature with a
freshly prepared and filtered solution of Oil Red O
(C.I. #26125) 0.3 g in a 60% aqueous 2-propanol
solution. Cells were washed twice with 2-propanol,
counterstained with Harris’ hematoxylin (2 min-
utes). Bluing was done with a saturated lithium car-
bonate aqueous solution. Slides were mounted with
Apathy’s syrup, a lab-made mounting medium that
does not contain solvents and allows permanent
preparations of solvent-sensitive stains.34

Image Analysis
Cells were photographed at a 1000× magnification
with an Olympus BX51 equipped with a DP71
digital camera (Cell A software, Olympus, France).
Image acquisition was done the same day under the
same illumination parameters: full light power, no
neutral gray filter, and automatic shadings for
white and black were performed by the software.
The cells were chosen when overlapping was mod-
erate so that the cell boundaries could be easily
identified and manually redrawn during the fur-
ther steps. For cells cultured in the presence of
TZD, only cells containing Oil Red O–positive
droplets were considered. Images were then trans-
ferrred to Photoshop CS3 (Adobe, San Jose, Califor-
nia, U.S.A.) and converted into gray images (coded
on in 8 bits, gray level ranging from 0 [black] to 255
[white]). Pictures were then analyzed with the
MAZDA texture analysis software (Institute of
Electronics, Technical University of Lodz, Poland)
for calculation of texture parameters with the run-
length distribution method.35,36 In an image, con-
secutive pixels of the same gray value in a given
direction constitute a run. The following param-
eters were calculated:
- Nuclear area.
- Mean gray level.
- Long run emphasis (LRE). It is highly depend-
ent on the occurrence of long runs and is
expected to be large for coarse structural tex-
tures.
- Gray level nonuniformity (GLN). It measures
the similarity of gray level values throughout
the image. The GLN is expected to be large if
the number of runs of same gray level values
increases throughout the image.
- Run-length nonuniformity (RLN). It measures
the similarities of the length of the runs
throughout the image. The RLN is expected to
be large if the number of runs of same lengths
increases throughout the image.
- Fraction of image in runs (FIRs). It measures
the homogeneity and the distribution of runs
of an image. FIR is a measure of the percentage
of image pixels that are part of any of the runs.
FIR is expected to be large for an image with a
homogeneous texture.

For a detailed mathematical description of these
parameters, see Szczypinski et al35 and Chappard et
al.37 For each type of culture condition, a minimum
of 40 cells was analyzed. For measuring the amount
of lipid droplets, the ImageJ software (National In-
html) was used.

Statistical Analysis
Statistical analysis was performed using the Systat
statistical software release 13.0 (Systat Software
Inc., San Jose, California, U.S.A.). All data were
expressed as mean ± standard deviation. Group
differences were searched by analysis of variance
(ANOVA), Scheffé’s method, and Fisher’s least sig-
nificant difference post hoc tests. Differences were
considered as significant when at p < 0.05.

Results
qPCR
PPARγ was significantly higher at day 6 for the
2 treatments. Normalized ratios of 7.7 and 7.1, re-
spectively, were obtained for PGZ versus control
and RGZ versus control (Figure 1). At day 9 the
ratios were significantly higher, with a value of
27.6 for PGZ versus control and 29.3 for RGZ. No
significant differences were found for Runx2 and
alkaline phosphatase expression versus control either at day 6 or day 9.

**Nuclear Morphology and Texture Analysis**

Between 1 and 5 days in culture, cells were dividing and appeared confluent after 5 days (data not shown). Treatment with PGZ or RGZ began during culture proliferation. Morphological observations showed no detectable Oil Red O–positive droplet at the beginning of the culture (e.g., from D0 to D4, data not shown), independent of the culture medium. Oil Red O–positive droplets appeared in the cytoplasm at D6 in cells cultured with RGZ or PGZ. During the following days, fine droplets appeared in the cytoplasm of some cells, and the number of cells engaged in the adipogenic differentiation increased rapidly (Figure 2). The droplets became larger at D8–D9 and occupied a larger place in the cell cytoplasm (Figure 3A). However, a number of cells were not engaged in the adipogenic differentiation at D9 and did not exhibit any detectable Oil Red O–stained droplets. The cells containing lipids droplets were predominantly situated in areas with the great cell density.

The nuclei were well stained in blue by Harris’ hematoxylin, and the mottled heterochromatin was well identifiable. Nuclear area decreased suddenly at D6 for cells cultured with RGZ or PGZ when the first lipid droplets were detectable. The decrease in nuclear area was initially rapid for PGZ and then continued slowly until D9. The decrease appeared less intense on the first days for cells cultured with RGZ and, here again, the nuclear area continued to decrease regularly until D9 and the mean gray level also decreased (i.e., the nuclei became darker) (Figure 3B–C).

Texture analysis exhibited variations in the heterochromatin disposition. RLN run-length nonuniformity decreased and characterized the compaction of euchromatin and the increase of dark run number in the heterochromatin (Figure 4A). The minimum RLN run-length nonuniformity seems to be observed at D8 for RGZ. LRE long run emphasis increased at D6 for cells cultured with PGZ and RGZ with apparition of large dark runs in a compacted chromatin texture (Figure 4B). GLN gray level nonuniformity measures the similarity of gray level values throughout the image; it decreased with the presence of very dark runs in the heterochromatin (Figure 4C). Fraction of image in runs percentage, which measures the homogeneity and the distribution of runs, decreased due to the pres-

![Figure 1](image-url)  
**Figure 1** qPCR results of PPARγ expression (marker of adipocyte differentiation) and alkaline phosphatase and Runx2 (markers of osteoblast differentiation) in C3H10T1/2 cells treated with pioglitazone or rosiglitazone.
ence of dense packets of heterochromatin (Figure 4D).

**Discussion**

Chromatin compaction modifies the DNA density in the nucleus by densely packing parts of the chromosomes with few or no active genes. Heterochromatin is thus formed and contains inactive and nontranscribed genes. They exhibit an increased methylation in the CG islands of their promoters. In addition, histones in the nucleosomes of heterochromatin present a number of changes that favor compaction of the DNA: reduced acetylation of histones and increased methylation of LYS in the H3 domain leading to a high condensation of the genetic material. Several papers have focused on the interrelationships between chromatin compaction and histone acetylation or methylation.

Heterochromatin comprises constitutive heterochromatin (all genes that are poorly expressed in most cells, at the interphase, e.g., chromosome centromeres, region of the Y chromosome) and facultative heterochromatin. Facultative heterochromatin contains regions of DNA that are replicated in a given cell type and which are also densely packed. This compaction may be visible after staining with classical nuclear stains such as hematoxylin and may represent a cytological characteristic of the cell: plasma cell is a typical example in which heterochromatin has a cartwheel arrange-
ment. In fat tissues, mature white adipocytes have a dense and flattened nucleus at the periphery of the cell; it is margined by the large intracytoplasmic lipid droplet. Previous studies have shown that TZDs can induce an adipocytic differentiation of mesenchymal cells. In the present study, the C3H10T1/2 cell line, which presents the ability to differentiate into adipocyte or osteoblast, was treated by 2 different TZDs, which provoked the engagement into fat cell differentiation. Fat was evaluated by the appearance of Oil Red O–positive droplets in the cytoplasm, and the differentiation toward the adipocytic pathway was confirmed by overexpression of PPARγ and the absence of change in the expression of ALP and Runx2. Stained cells were predominant in areas with a great density of cells, suggesting that cell-to-cell contact may be critical for differentiation. The increase in PPARγ expression appeared to be significantly increased at D6 and was associated with a decrease in the size of the nucleus and concomitant accumulation of lipid droplets. Texture analysis of nuclei confirmed the compaction of the chromatin, which further increased until D9 when nuclei contained blocks of

Figure 3  (A) Accumulation of lipid droplets in the cytoplasm of C3H10T1/2 cells treated with pioglitazone or rosiglitazone. (B) Nuclear area and (C) mean gray level of the nuclei of cells containing lipid droplets.
highly condensed heterochromatin. Independent of the run-length parameter considered, the values did not differ at the end of the experiment between the 2 TZD molecules. Texture analysis applied to images is a powerful tool to study the nonuniform variations in intensity that form repeated patterns within an image (often called *primitives*). The technique has been found interesting for detecting microcalcifications on X-ray mammograms and characterizing variations in roughness of X-ray and MRI images. The software used here was developed in the European COST B11 project. The run-length method applied on gray images was first introduced by Galloway and can provide information according to the direction of pixels in an anisotropic image. Because cell nuclei have not such a preferential orientation, we used the average of the vertical and horizontal values provided by

**Figure 4** Texture analysis of the nucleus of C3H10T1/2 cells treated with pioglitazone or rosiglitazone (A) run-length nonuniformity, (B) long run emphasis, (C) gray level nonuniformity and (D) fraction of image in runs.
the software. The run-length descriptors identified the appearance of blocks of heterochromatin (LRE increased regularly) associated with an inhomogeneous repartition of the run-lengths (FIR, RLN and GLN decreased). Evolution of these parameters parallels the increased expression of PPARγ in cells. Texture analysis of cell nuclei has also been used in a variety of cells and cytological conditions, and different algorithms have been used.41,46–54 Run-length texture analysis has previously been used to grade nuclei of renal carcinoma cells and thyroid tumors.55,56 In the present study the method was used to follow dynamic changes induced in the chromatin compaction due to differentiation of C3H10T1/2 cells toward the adipocytic phenotype. Texture analysis parameters clearly identified a reduction in the mean size of the nuclei and a compaction of the nuclear material. These 2 characteristics are typical of the findings observed in fully mature adipocytes on histological sections. The progressive evolution of the nuclear parameters observed here is parallel with the progressive changes observed in molecular biology and also with the progressive accumulation of lipid droplets in the cell cytoplasm. The differentiation of the C3H10T1/2 cells toward an adipocyte phenotype is accompanied by changes in the nuclei together with cytoplasmic modifications with the storage of lipid droplets.

Conclusion

TZDs are able to modify the cellular metabolism of the C3H10T1/2 cells which differentiate into adipocytes. It is likely that they induce inactivation of a large number of genes, leading to a decrease of the nucleus size and an increase in chromatin condensation.

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References


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