

The spatial repositioning of adipogenesis genes is correlated with their expression status in a porcine mesenchymal stem cell adipogenesis model system

Izabela Szczerbal · Helen A. Foster · Joanna M. Bridger

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Abstract Alterations in the nuclear positioning of chromosomes and specific genes during differentiation and development have suggested strongly the existence of a relationship between non-random organization of the genome and its function. In this study, we have examined the genome organization in interphase nuclei during adipogenesis, using the pig as a model organism. We hypothesized that changes in the gene expression profile and chromatin remodeling which occur during cellular differentiation would elicit repositioning of whole chromosomes, moving specific genes on them to different regions of the nucleus. We established an *in vitro* adipogenesis differentiation system using mesenchymal stem cells, derived from porcine bone marrow. The nuclear position of seven adipogenesis genes (*PPARG*, *SREBF1*, *FABP4*, *CEBPA*, *CEBPB*, *CREB*, and *GATA2*), two control genes (*SOX9* and *MYL1*), and six chromosomes carrying these gene loci

(*SSC4*, *SSC6*, *SSC12*, *SSC13*, *SSC15*, and *SSC17*) was determined. We found that during adipogenesis, using the *in vitro* stem cell model system, in contrast to our original hypothesis, the nuclear position of genes involved in adipogenesis was altered radically with the up-regulation of gene expression correlating with these genes becoming more internally located within nuclei. Chromosome territories, containing these genes, were also found to alter their nuclear position during the *in vitro* adipogenesis model, with the most dramatic repositioning being *SSC4* that moved from the nuclear periphery towards the nuclear interior. We found that during *in vitro* adipogenesis chromosome territories decondensed and the genes were found on loops and projections of chromatin, away from the main body of the chromosomes. From our data, it appears that the temporal repositioning of genes, emanating away from chromosomes, during adipogenesis is correlated with gene activity, supporting models of the involvement of spatial genome repositioning in regulating gene expression and the nuclear interior being an important region of the nucleus for transcription.

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I. Szczerbal
Department of Genetics and Animal Breeding,
Poznan University of Life Sciences,
Wolynska 33,
60-637 Poznań, Poland

I. Szczerbal (✉) · H. A. Foster · J. M. Bridger (✉)
Laboratory of Nuclear and Genomic Health,
Centre for Cell & Chromosome Biology, Biosciences,
School of Health Sciences and Social Care, Brunel University,
West London UB8 3PH, UK
e-mail: izabel@jay.au.poznan.pl
e-mail: joanna.bridger@brunel.ac.uk

Introduction

Genome positioning within the interphase nuclei is non-random, with each chromosome territory occupying a specific radial location (Boyle et al. 2001; Cremer et al. 2006; Meaburn and Misteli 2007). Importantly, for the field of genome biology, the arrangements of chromosomes within the nuclei are being well characterized in a number of different species and cell types (Foster and Bridger 2005). Sub-chromosomal regions and individual genes also display preferential nuclear positions. Recent studies have

demonstrated that chromatin domains are organized in nuclei according to local GC content (Heppenger et al. 2008; Saccone et al. 2002), local gene density (Federico et al. 2008; Goetze et al. 2007; Küpper et al. 2007), or replication timing (Grasser et al. 2008) rather than by gene expression level. However, emerging evidence indicates that placing genes in specific nuclear space has a regulatory effect (Hu et al. 2008; Lanctôt et al. 2007; Misteli 2007; Takizawa et al. 2008b). For example, positioning of loci relative to the nuclear periphery or heterochromatin domains has been linked to gene repression (Brown et al. 1999; Finlan et al. 2008; Reddy et al. 2008; Somech et al. 2005), and conversely, repositioning of loci from nuclear periphery to nuclear interior or away from heterochromatin domains is correlated with gene activation (Ballabio et al. 2009; Brown et al. 2001; Kosak et al. 2002; Williams et al. 2006; Zink et al. 2004). Formation of chromatin loops away from chromosome territories has been suggested as a mechanism of genome regulation (Chambeyron and Bickmore 2004; Chambeyron et al. 2005; Volpi et al. 2000; Williams et al. 2002), but genes looping out from chromosome territories may not always be linked to up-regulation of gene expression (Morey et al. 2009). Some genes can migrate to share the same nuclear sub-compartments or transcription factories (Osborne et al. 2004, 2007; Xu and Cook 2008). Recently, it has been shown that distinct alleles of monoallelically expressed genes occupied different nuclear positions and can be taken as direct evidence for correlation between nuclear position and activity status of a gene (Takizawa et al. 2008a).

Several important findings concerning spatial organization of the genome in relation to function have been made during cellular differentiation and development. Among many examples, nuclear organization has been studied during lymphoid differentiation (Brown et al. 1999, 2001; Kosak et al. 2002), erythroid differentiation (Brown et al. 2001, 2008; Ragozy et al. 2006), epidermal differentiation (Williams et al. 2002), neuronal differentiation (Williams et al. 2006), granulocyte differentiation (Bártová et al. 2002), adipocyte differentiation (Kuroda et al. 2004), embryonic development (Chambeyron and Bickmore 2004; Chambeyron et al. 2005; Morey et al. 2007), and spermatogenesis (Foster et al. 2005; Zalenskaya and Zalensky 2004). Differentiation can be characterized by dynamic changes in cellular morphology, the gene expression profile, and chromatin remodeling, and therefore can be considered as an ideal system for studies correlating nuclear position and gene expression. *In vitro* differentiation systems using progenitor cells (embryonic or adult stem cells) can be used for understanding epigenetic processes regulating cell lineage commitment.

In this study, we established an *in vitro* system of adipogenesis differentiation for porcine mesenchymal stem

cells. Adipogenesis is a complex process composed of two phases known as determination and terminal differentiation. In the first phase, the mesenchymal precursor cells commit to an adipocyte lineage and traverse to preadipocyte cells. In the second phase, preadipocytes take on characteristics of mature adipocytes (Rosen and MacDougald 2006). Formation of fat cells is regulated by a complex network of transcription factors among which the most important are peroxisome proliferator-activated receptor γ and members of CCAAT/enhancer-binding proteins (C/EBP) family (Farmer 2006). Adipogenesis has been studied extensively *in vitro* using mainly established mouse cell lines (3T3-L1, 3T3-F442A); however, there are a number of reports on isolating mesenchymal stem cells derived from bone marrow and differentiating them into adipocytes for different species, including pig (Zeng et al. 2006; Zou et al. 2008). Different aspects of adipogenesis have been investigated extensively since excess of adipose mass, i.e., obesity is one of the major health problems, contributing to pathogenesis of obesity-related disorders such as diabetes, hypertension, and coronary heart disease (Formiguera and Cantón 2004). The pig is considered as a model organism for the study of human obesity (Lunney 2007; Schook et al. 2005). Additionally, this species has a central position in agricultural sciences, and its whole genome is now being sequenced and is soon to be completed (Chen et al. 2007). Therefore, detailed knowledge about molecular and cellular events during adipogenesis is important for biomedical research as well as in animal breeding. With respect to the genome behavior and the pig's usefulness as a model organism for humans, it may be more pertinent to investigate than mouse since it has a range of differently sized chromosomes with differences in centromeric positioning along a chromosome and many expansive syntenic genomic regions.

In the present study, we have examined genome organization in porcine interphase nuclei during adipocyte differentiation. We hypothesized that dynamic changes in gene expression profile, and chromatin remodeling that occur during cellular differentiation would induce changes in the nuclear positions of genes and chromosomes. Therefore, we investigated the nuclear position of nine genes involved in adipogenesis and the chromosomes they are located on, in progenitor mesenchymal stem cells and during *in vitro* induced adipogenesis. Here, we show that the radial position of chromosome territories was altered during adipocyte differentiation, apart from one chromosome, but most evident were large long-range changes in gene positioning. This repositioning of genes was related to gene activity, with up-regulation correlating with chromatin looping into the nuclear interior and down-regulation with movement towards the nuclear periphery.

Materials and methods

Stem cell culture and induction of differentiation

Mesenchymal stem cells were isolated from porcine bone marrow samples. The bone marrow was submerged for 1 h in a 1:5 dilution of antibiotics (10 U/ml penicillin, 50 µg/ml streptomycin) and 1:20 dilution of amphotericin B (250 µg/ml) in MesenCult® basal medium for human mesenchymal stem cells, with mesenchymal stem cells stimulatory supplements (StemCell Technologies, The Cell Experts™, USA). The bone marrow was then washed in fresh medium before being finely cut with a sterile scalpel in a 90-mm sterile culturing dish. The mesenchymal stem cells (MSCs) were cultured in complete MesenCult® basal medium with mesenchymal stem cells stimulatory supplements and antibiotics (10 U/ml penicillin, 50 µg/ml streptomycin) at 37°C with 5% CO₂. Cells were expanded by passage using standard procedures until cell differentiation was to be induced. To confirm that cells isolated from porcine bone marrow were MSCs, indirect immunofluorescence was performed using antibodies directed against CD34 (monoclonal mouse anti-human CD34 QBEnd 10 antibody diluted 1:25; Dako, USA), CD45 (monoclonal mouse anti-human CD45 2B11+ PD7/26 antibody diluted 1:50; Dako, USA), and Thy 1.2 (monoclonal rat anti-mouse Thy 1.2 FF-10 antibody diluted 1:5, Serotec) diluted with 1% (v/v) NCS in phosphate-buffered saline (PBS). Cells were incubated in the primary antibody at 4°C for 24 h. The cells were washed with PBS before application of the appropriate fluorochrome-conjugated secondary antibody for 30 min at 37°C (donkey anti-mouse fluorescein isothiocyanate (FITC) diluted 1:60, Jackson Laboratory; rabbit anti-rat FITC diluted 1:40, Dako). To stimulate adipogenesis, MSCs were grown to confluency and cultured with MesenCult® basal medium with adipogenic stimulatory supplements (StemCell Technologies, The Cell Experts™, USA). The cells were cultured for 14 days in adipogenic complete medium. The adipocyte differentiation was confirmed by phase contrast microscopy and indirect immunofluorescence with anti-SREBP-1 antibodies, polyclonal anti-SREBP-1 H-160 (a kind gift from Dr. Sue Shackleton, Leicester and Santa Cruz diluted 1:100), and SREBP-1 2A4 (monoclonal anti-mouse SREBP-1 2A4 antibody, diluted 1:10, BD Pharmingen™). The primary antibodies were incubated for 24 h at 4°C, washed with PBS, and then incubated with the appropriate fluorochrome-conjugated secondary antibody (donkey anti-mouse Cy3 diluted 1:100, Jackson Laboratory or swine anti-rabbit TRITC diluted 1:30, Sigma-Aldrich) for 30 min at 37°C. MSCs undergoing adipogenesis were also monitored for lipid accumulation before differentiation and during differentiation at days 3, 7, 11, and 14 using oil red O staining.

Briefly, cells were fixed with 3.7% formaldehyde for 10 min at room temperature. The cells were then rinsed with double distilled water before incubating with 0.3% oil red O (Sigma-Aldrich) diluted in water (3:2 v/v) at room temperature for 1 h. The oil red O was decanted, rinsed with water, and left in fresh double distilled water for examination using a phase contrast microscope.

Indirect immunofluorescence

Cells were fixed in 4% paraformaldehyde in PBS (w/v) for 10 min at room temperature, followed by washing in PBS. The cells were then treated with 1% Triton-X 100 in PBS (v/v) for 15 min. Primary mouse monoclonal antibody for fibrillarlin (a kind gift from Dr. John Aris dilution 1:1,000) was applied to the cells and incubated for 1 h at room temperature. The cells were washed three times in PBS and then incubated for 1 h at room temperature with secondary antibodies—donkey anti-mouse FITC, dilution 1:70 (Jackson Immunoresearch Laboratories). After further washing in PBS, slides were mounted with Vectashield (Vector Laboratories) containing 4',6-diamidino-2-phenylindole (DAPI).

DNA probes

Whole porcine chromosome painting probes (SSC4, SSC6, SSC13, SSC15, SSC17) isolated by flow sorting were kindly provided by Prof. M.A. Ferguson-Smith (Cambridge Resource Centre for Comparative Genomics, Cambridge University, UK). The chromosome templates underwent primary and secondary amplification by performing degenerate-oligonucleotide-primed polymerase chain reaction (DOP-PCR) and were subsequently labeled with biotin-16-dUTP (Roche). Labeled probe for SSC12 was kindly provided by Prof. F. Yang (Wellcome Trust Sanger Institute, Cambridge, UK).

Bacterial artificial chromosomes (BACs) clones were obtained from available porcine libraries: the RPCI-44 Male Porcine BAC Library, the CHORI-242 Porcine BAC Library (<http://bacpac.chori.org/libraries.php>), and the PigE BAC library (Anderson et al. 2000; <http://www.ark-genomics.org/clonesVectors/> Table 1). BAC probes for adipogenic genes were selected based on previous information about the gene localization (Szczerbal and Chmurzynska 2008; Szczerbal et al. 2007a, b). BAC probes for control genes (*MYLI*, *SOX9*) were used for the first time in this study and were verified by PCR using specific primers (primer sequences available on request) and by fluorescence in situ hybridization (FISH) on metaphase chromosomes. BAC DNA was labeled using random-priming method with biotin-16-dUTP (Roche) or digoxigenin-11-dUTP (Roche).

Table 1 Characterization of the selected genes

Gene symbol	Gene name	BAC clone number	Chromosomal localization	Function ^a
<i>PPARG</i>	Peroxisome proliferator-activated receptor gamma	PigE-86P19	13q24-31	Nuclear receptor
<i>CEBPA</i>	CCAAT/enhancer-binding protein (C/EBP), alpha	RP44-367A16	6q12	Late transcription factor
<i>CEBPB</i>	CCAAT/enhancer-binding protein (C/EBP), beta	CH242-263E16	17q23	Early transcription factor
<i>SREBF1</i>	Sterol regulatory element-binding transcription factor 1	CH242-409P19	12q15	Early transcription factor
<i>CREB</i>	cAMP-responsive element-binding protein	RP44-312K3	15q24	Transcription factor
<i>GATA2</i>	GATA-binding protein 2	PigE-7C8	13q24	Preadipocyte transcription factor
<i>FABP4</i>	Fatty acid-binding protein 4, adipocyte	CH242-243J15	4q12	Binding protein specific to adipocytes
<i>SOX9</i>	SRY (sex-determining region Y)-box 9	CH242-191B18	12p14	Chondrogenic transcription factor
<i>MYL1</i>	Myosin, light chain 1, alkali; skeletal, fast	CH242-137K5	15q25	Myosin alkali light chain

^a According to Entrez Gene database

Labeled BAC probes and paint probes (150–300 ng), with excess of porcine genomic competitor DNA (3 µg) and salmon sperm DNA (10 µg) were ethanol-precipitated and dissolved in 10 µl hybridization mixture (10% dextran sulfate, 50% formamide/2× SSC (0.3 M NaCl, 0.03 M sodium citrate), and 1% Tween20).

2D FISH

Cells were subjected to hypotonic buffer and fixed in 3:1 (v/v) methanol–acetic acid (Szczerbal et al. 2007b) and prepared for FISH by incubation in 100 µg/ml RNase/2× SSC at 37°C for 1 h, followed by washing in 2× SSC and further treated with a pepsin solution (0.005% pepsin/0.01 N HCl {w/v}) at 37°C for 10 min, followed by a wash in PBS/1 M MgCl₂. Nuclei were subjected to standard 2D FISH as described in Meaburn et al. (2007). Probes were denatured at 70°C for 10 min and then were preannealed at 37°C for 20 min. For hybridization, slides were incubated in a humid environment at 37°C overnight. After hybridization, slides were washed three times in 50% formamide/2× SSC at 43°C for 5 min and three times in 2× SSC at 43°C. The slides were placed in 0.05% Tween20/4× SSC at room temperature (RT) for 10 min and blocked in 3% BSA/4× SSC at RT for 30 min. Biotin-labeled probes were detected using streptavidin Cy3 (Amersham) diluted 1:200 and digoxigenin-labeled probes were detected with antidigoxigenin-fluorescein Fab fragments (Roche) diluted 1:200. Cells were mounted in Vectashield (Vector Laboratories) with DAPI counterstain.

3D FISH

Cells grown directly onto glass slides were fixed in 4% PFA/PBS (w/v) for 10 min, washed in PBS, and then permeabilized for 20 min in 0.5% saponin/0.5% TritonX-100/PBS, followed by further washing in PBS (Bridger et

al. 1998). The 3D preserved cells were then incubated in 20% glycerol/PBS (v/v) for 1 h and subjected to six repeat freeze–thaw cycles in liquid N₂. Before hybridization, cells were treated with 0.1 N HCl for 5 min at room temperature and a pepsin solution (0.002%/0.01 N HCl) for 5 min at 37°C, followed by washing in PBS with 1 M MgCl₂ and equilibration in 50%formamide/2× SSC apart from when indirect immunofluorescence was performed. Probes were denatured at 70°C for 10 min and then were preannealed at 37°C for 20 min. Nuclei were denatured at 76°C for 3 min in 70% formamide/2× SSC followed by 50% formamide/2× SSC for 1 min. Hybridization was performed over two nights at 37°C. Post-hybridization washes and signal detection were performed as for 2D FISH.

Image analysis

Flattened nuclei were examined with an Olympus epifluorescence microscope and observed under ×100 oil immersion objective. Images of randomly selected nuclei were acquired with a CCD camera and were pseudocolored using Smart Capture VP v1.4. The nuclear position of whole chromosome paint probes and gene-specific probes were analyzed using a script developed by Dr. Paul Perry in IPLab software (Croft et al. 1999) and was a kind gift from Prof. Wendy Bickmore (MRC HGU, Edinburgh). This analysis was performed by ascertaining the distribution of mean proportion (percent) of hybridization signals, normalized by percent of DAPI stain, over five concentric shells (shell 1—periphery, shell 5—center of the nucleus). Approximately, 60 nuclei for positioning of chromosome territories and 100 for gene position were analyzed.

For 3D preserved cells, subjected to 3D FISH, stacks of images through the z-axis of cells were captured with a Nikon Eclipse confocal microscope TE 2000-S with a ×100 oil immersion lens and C1 (EZ-C1) software version 3.00. Stacks of optical sections with an axial distance of 0.2 µm

were collected. Stacks of 8-bit gray-scale 2D images were obtained with a pixel dwell of 4.56 and 8 averages were taken for each optical image. The measurements and 3D reconstructions were performed using Imaris 5.7.0 software (Bitplane). The relative distance of hybridization signal from the center of the nucleus to the center of hybridization signal was calculated as a ratio of the nuclear radius (r = total distance from the center of the nucleus to the nuclear periphery through the FISH signal). At least 60 FISH signals were scored per experiment. In Fig. 8, 0% represents the geometric center of the nucleus, and 100% was considered as the nuclear border.

Statistical analysis

Before statistical analysis, the mean proportion of hybridization signals already normalized by percent DAPI stain was added together and the mean for the signals was calculated from five concentric shells for each nucleus. After this adjustment, the signals from five concentric shells within a nucleus add up to 1. Within each concentric shell, the median of adjusted hybridization signals in three cell types/time points (MSC, AD7, AD14) were compared using the Kruskal–Wallis rank sum test. This test was used because the gene signal distribution strongly deviated from a normal distribution, and this test allowed us to compare the three different data sets (i.e., MSC, day 7 and day 14) with each other in each of the five shells. In total, 75 tests were performed.

Student's *t* test was used to compare the number of signals co-localized with nucleoli in mesenchymal stem cells and in cells from day 14 of differentiation. *P* value of <0.05 was considered significant.

Reverse transcription polymerase chain reaction

Total RNA from undifferentiated porcine MSCs and from differentiated cells at days 7 and 14 was isolated using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma Aldrich) according to manufacturer's specification. RNA samples were treated with DNaseI (Sigma Aldrich). The quality of RNA samples was verified by agarose electrophoresis and measured at A260/280 nm absorbance. The complementary DNA (cDNA) was synthesized using SuperScript™ III reverse transcriptase (Invitrogen). The synthesized cDNA was amplified by PCR in a 50- μ l reaction containing 5 U Platinum® *Taq* polymerase (Invitrogen) and 10 μ M of each primer (Table S 1). The β -actin gene was used as an internal control. The following cycling conditions were used: 94°C for 5 min, then 28–30 cycles of 94°C for 30 s, 54–60°C for 30 s, and 72°C for 1 min, finishing with 72°C for 7 min. The annealing temperature and number of PCR cycles were optimized in each case to ensure that the intensity of the product corresponded with

the linear phase of amplification. The PCR product was separated by electrophoresis in 1.5% agarose gel, stained, and photographed under UV illumination. Each cDNA was analyzed in duplicate.

Results

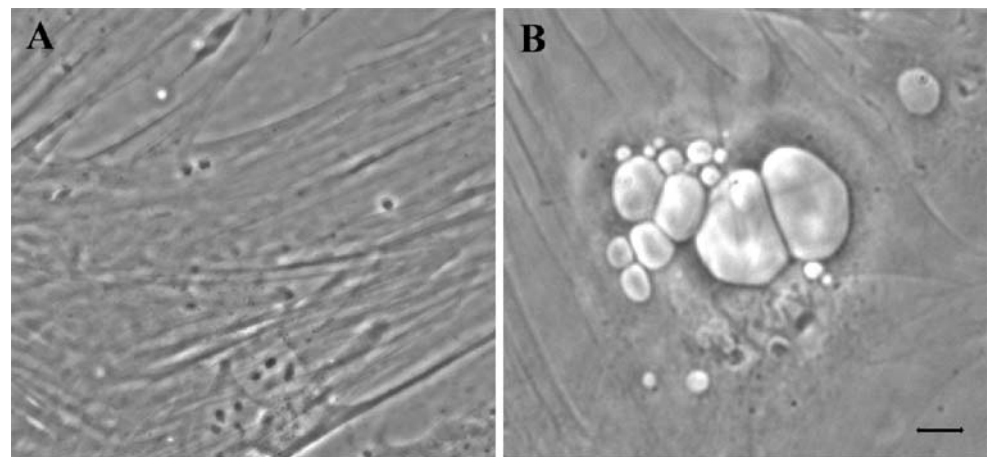
Establishment of an in vitro model system for differentiation of porcine mesenchymal stem cells into adipocytes

To assess spatial genome organization during adipogenesis, we first established an in vitro system of differentiation of mesenchymal stem cells into adipocytes. Porcine mesenchymal stem cells were successfully isolated from bone marrow (Fig. 1a). The presence of porcine MSCs was verified by indirect immunofluorescence by the lack of anti-CD34 and anti-CD45 staining and the presence of anti-Thy-1 staining (data not shown). Adipogenesis was induced with complete adipogenic medium and cells were cultured for 14 days. Single lipid vesicles were observed from the third day after stimulation and then the size and number of the lipid droplets increased up to day 14 (Fig. 1b). Oil red O staining revealed that ~40% of the cells contained lipid by day 14. Further, anti-SREBP1 staining was evident in the culture after 7 days in adipogenic medium and >80% of cells displayed nuclear foci of SREBP1 by day 14.

Chromosome territory behavior during in vitro porcine adipogenesis from mesenchymal stem cells

In order to determine if adipogenesis genes and the chromosomes they are housed upon undergo nuclear repositioning during differentiation, we investigated the nuclear positioning of a set of adipogenic genes and controls, along with their associated chromosomes. We selected nine genes—seven of which are involved in adipogenesis (*PPARG*, *SREBF1*, *CEBPA*, *CEBPB*, *CREB*, *FABP4*, *GATA2*) and two that were used as control genes (*SOX9*, *MYL1*; Table 1). The genes were mapped by FISH to different porcine chromosomes, such as SSC4, SSC6, SSC12, SSC13, SSC15, and SSC17 (Szczerbal and Chmurzynska 2008; Szczerbal et al. 2007a, b). We selected the genes so that adipogenic and control genes or pro- and anti-adipogenic genes were located on the same chromosome (Fig. S 1). Radial positioning of the nine genes and six chromosomes was determined in nuclei derived from MSC and from cells induced to differentiate for 7 days (AD7) and for 14 days (AD14; Table 2). This was performed by preparing cells for 2D FISH whereby standard hypotonic treatment and fixation with methanol/acetic acid were used. Digital images were captured of random cells containing the chromosome

Fig. 1 In vitro culture of adipogenesis using porcine mesenchymal cells. Representative images of porcine mesenchymal stem cells stimulated to undergo adipogenesis. **a** MSCs prior to differentiation; **b** cells induced to undergo adipogenesis, showing the presence of lipid droplets. Cell cultures were imaged using phase contrast optics, with $\times 40$ lens, on a Zeiss Cell Observer. Scale bar, 10 μ m



territories (Fig. 2) and gene signals (Fig. 4). These images were subjected to erosion analysis using a bespoke script in IPLab, such that the nucleus is delineated and divided into five shells of equal area, and the fluorescent intensity of the FISH signal (gene or chromosome territory) is calculated. This value is normalized by the amount of DAPI signal found in each radial shell (see Boyle et al. 2001; Croft et al. 1999). These radial distribution data are plotted as histograms for chromosomes (Fig. 3) and genes (Fig. 5). Figure 3 displays the distributions of whole chromosome territories during induced adipogenesis. Shell 1 represents the periphery of nuclei and shell 5 the nuclear interior. To explain the location of chromosomes (or genes) in the nucleus, we have selected four terms: peripheral (P) to refer to a situation where signal was located

preferentially at the nuclear periphery, interior (I) where the signal was preferentially observed towards the nuclear interior, intermediate (IM) to define a situation where signal was positioned between the nuclear periphery and the nuclear interior, and equally distributed (EQ) to describe a random distribution of signal. Throughout adipogenesis, chromosome territories demonstrated non-random positioning within the nuclei, i.e., in MSC SSC4, 13, and 15 are located at the nuclear periphery, while SSC6 is located in an intermediate location with chromosomes 12 and 17 being found in the nuclear interior. In MSC, SSC4 territories were located at the nuclear periphery and relocated to the nuclear interior at day 7 of adipogenesis. An equal distribution of chromosome signal for SSC4 was observed at day 14. This distribution indicates a more random positioning of this chromosome. SSC13 also appears to be relocated during adipogenesis since day 0, and day 14 SSC13 displays a more peripheral distribution, but at day 7, the shape of the graph indicates a more intermediate nuclear location (Fig. 3, Table 2). All the other chromosomes demonstrate lesser changes in overall nuclear location. Table S 2 reveals the results of a Kruskal–Wallis test, a non-parametric analysis of variance, where all data sets for the normalized chromosome position have been analyzed for the normalized signal within each individual shell for all three time points, i.e., days 0, 7, and 14. The data for SSC4 display significant differences between shells 1, 2, 4, and 5 for chromosome position at all three time points. SSC13 displays significant differences in shells 1, 2, and 5, whereas SSC17 only displays a significant difference for shells 1 and 2, SSC12 and 15 only demonstrate significant difference in shell 1, and SSC6 displays no significant differences at all.

Table 2 Position of chromosomes and genes during adipocyte differentiation

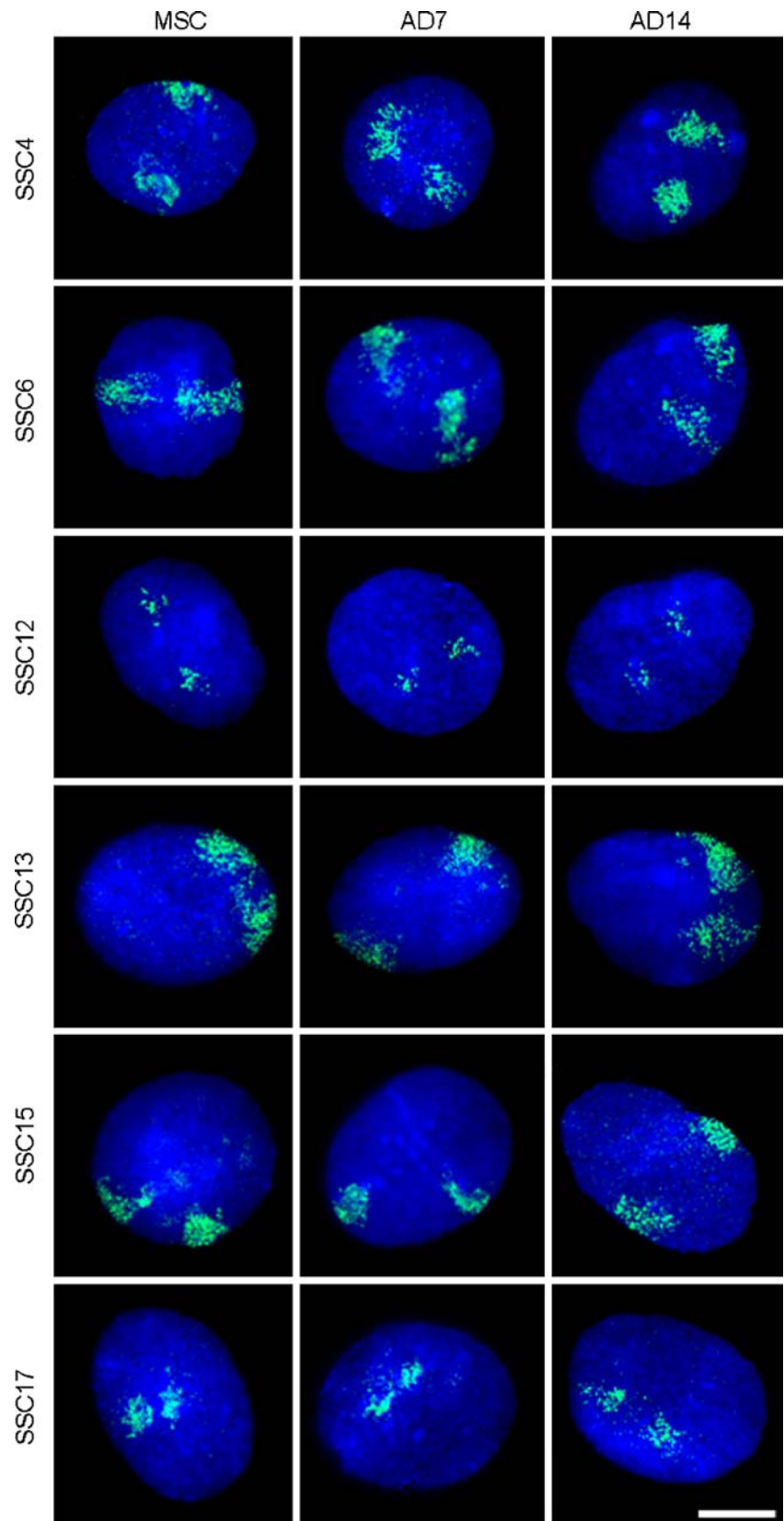
Chromosome/gene	MSC Position	AD7 Position	AD14 Position
SSC4	P	I	EQ
<i>FABP4</i>	P	I	I
SSC6	IM	IM	IM
<i>CEBPA</i>	I	I	I
SSC12	I	I	I
<i>SREBF1</i>	I	I	I
<i>SOX9</i>	I	I	I
SSC13	P	IM	P
<i>PPARG</i>	P	IM	I
<i>GATA2</i>	P	I	P
SSC15	P	P	P
<i>CREB</i>	P	IM	IM
<i>MYL1</i>	P	P	P
SSC17	I	I	I
<i>CEBPB</i>	IM	I	I

P peripheral, I interior, IM intermediate, EQ equally distributed

Major nuclear repositioning of adipogenic specific genes during differentiation

When we analyzed gene loci position in nuclei during adipogenesis, we found much more dramatic differences

Fig. 2 Nuclear distribution of whole chromosomes in interphase nuclei of porcine mesenchymal stem cells induced to differentiate into preadipocytes and adipocytes. Representative images of nuclear positioning of whole chromosome territories in mesenchymal stem cells (*MSC*) and in cells induced to become adipocytes from day 7 (*AD7*) and day 14 (*AD14*). Cells were fixed with methanol/acetic acid and subjected to 2D-fluorescence in situ hybridization. Chromosome territories were delineated using flow-sorted porcine whole chromosome painting probes for chromosomes SSC4, SSC6, SSC12, SSC13, SSC15, and SSC17. Digital images were collected on an Olympus epifluorescence microscope via Smart Capture VP v1.4 software. Chromosome territories are *green*; DNA in the nuclei is counterstained with DAPI (*blue*). Scale bar, 10 μ m



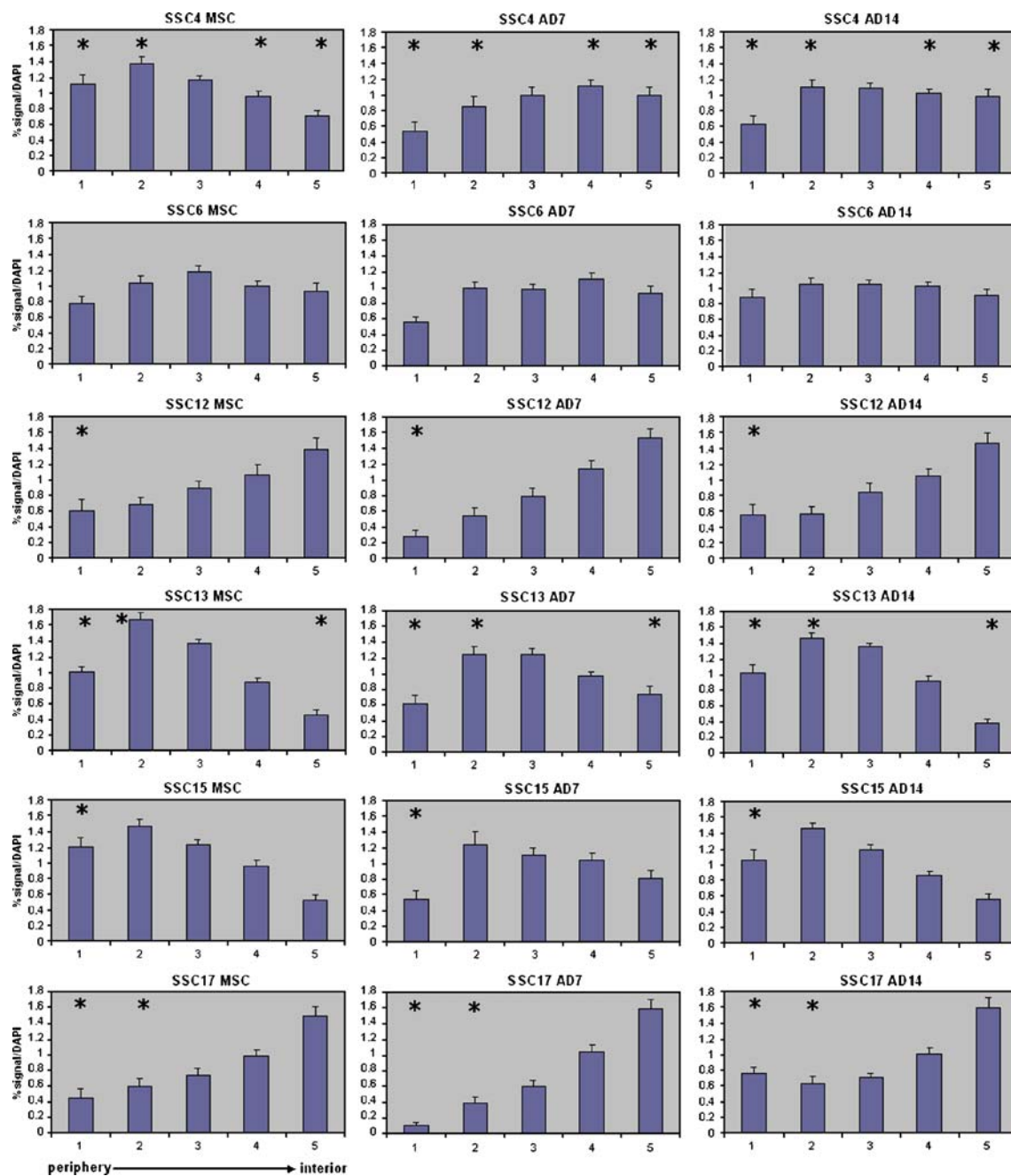


Fig. 3 Nuclear positioning of chromosome territories in interphase nuclei of porcine mesenchymal stem cells induced to differentiate into preadipocytes and adipocytes. The distribution of whole chromosome territories within the interphase nuclei was analyzed by erosion analysis of at least 50 digital images of cells subjected to 2D FISH. The script divides the nuclei into five shells of equal area and measures the intensity of the DNA and FISH signal in each shell. Each

measurement for the FISH signal is normalized by the DAPI measurement for that shell. The histograms represent the mean proportion of the normalized chromosome signal (y -axis), across the five concentric shells of the nuclei (x -axis), from the nuclear periphery (shell 1) to the nuclear interior (shell 5). Statistical difference ($P < 0.05$) was assessed by the non-parametric Kruskal–Wallis rank sum test and indicated by asterisks (for details, see Table S 2)

with major relocations of adipogenesis genes than for the chromosomes (Fig. 4), revealing genes moving from the nuclear periphery to the nuclear interior (*PPARG*, *FABP4*, *GATA2*), from an intermediate position to the nuclear interior (*SREBF1*, *CEBPB*) and from a peripheral to an intermediate location (*CREB*). Thus, six out of the seven

adipogenesis genes have significantly changed their nuclear location during adipogenesis. Of the two control genes, *SOX9* changes location from an interior location to a more random distribution at day 7 and then returns to a more interior location by day 14. The other control gene *MYL1* does not change its nuclear distribution in 14 days. Again,

we have performed a Kruskal–Wallis test for all three data sets for each time point for the individual shells (Table S 3) and reveal significance difference in gene loci positioning for four out of the five shells in four genes (*PPARG1*, *SREBF1*, *FABP4*, and *GATA2*); two genes (*CEBPA* and *CREB*) display significant difference in two shells for the three time points, and one gene (*CEBPB*) displays a significant difference in shell 4 (Fig. 5).

From the skew of the histograms displayed in Fig. 5, it can be seen that a number of the adipogenesis genes do indeed alter their nuclear location, quite dramatically and are located in disparate nuclear compartments over the time period of the experiments.

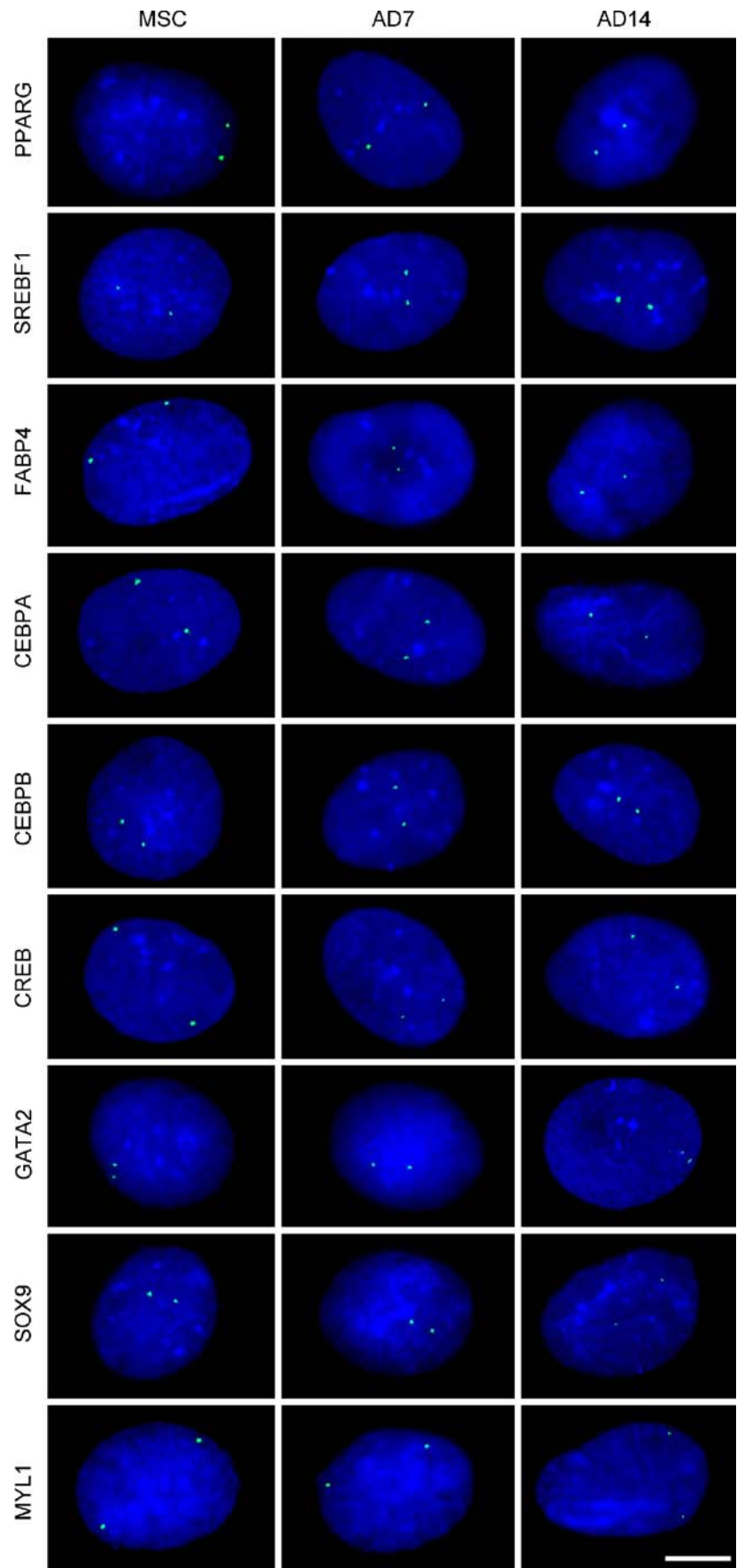
Genes associated with adipogenesis loop out from their chromosome territory during differentiation and this is associated with gene expression. Out of the seven adipogenesis genes analyzed, two of them were deemed to reside in different nuclear regions to their parent chromosomes (see Table 2 for comparisons) in MSC at time 0, and these were *CEBPA* on SSC6 and *CEBPB* on SSC17. By day 7 (AD7), there are four genes that were deemed to reside in a different nuclear compartment to their parent chromosome, and by day 14, there were still four with *SREBF1* being significantly more interiorly located at day 14 than day 7 with no change in its parent chromosome. These data imply that there is a reorganization of the adipogenesis genes studied, to the edge or away from the main bulk of the chromosome territory. In other studies, this behavior of gene loci, i.e., moving away from the chromosome territory, towards the nuclear interior, can be associated with an increase in gene expression. In order to test this postulation, we measured the amount of gene expression by reverse transcriptase (RT)-PCR for each of the nine genes at the three time points, and we have demonstrated that all the genes associated with adipogenesis increase their expression significantly (Fig. 5 and Table S 4) from day 0. The RT-PCR results were in agreement with previous studies with respect to the pattern of expression of the studied genes for human, mouse, and pig (Guo and Liao 2000; Kouadjo et al. 2007; Nakamura et al. 2003; Zou et al. 2008). We found that messenger RNA (mRNA) encoding the adipogenic transcription factors *PPARG*, *CEBPA*, and *SREBF1* increased significantly during the course of adipogenic differentiation. *CEBPB*'s expression was up-regulated at day 7 and then down-regulated at day 14. The expression of the *CREB* gene was present in all the analyzed time points. Expression levels of *FABP4*, a late marker of adipogenesis, increased during differentiation with the highest level at day 14. The *GATA2* gene was highly expressed in cells from day 7 of differentiation and down-regulated at day 14 and is known to be involved in preadipogenesis (Tong et al. 2000). Expression of *SOX9*, a key regulator of chondrogenic differentiation, was sup-

pressed during adipogenesis. This was expected since it is high in MSC and is switched off during adipogenesis. We did not detect transcripts for *MYL1* gene during adipogenesis since this gene is characteristic for skeletal muscle and not expressed in either MSCs or adipocytes.

One of the most interesting gene behaviors belongs to *GATA2*. This gene is an anti-adipogenic transcription factor and its expression is restricted to preadipocytes. In our study, *GATA2*'s expression is at its highest at day 7, and by day 7, it is relocated from a peripheral nuclear location to one in the nuclear interior. By day 14, its expression has fallen and the gene is relocated to the nuclear periphery again. However, for the other gene, *CEBPB*, that has an up-regulation of expression at day 7, which has diminished by day 14, the loci remain in the nuclear interior. *CEBPB* is located on porcine chromosome 17q, which is syntenic to human 20q. There are many different genes on 20q in human whose function are associated with adipogenesis, obesity, and diabetes. It is possible that this region remains within the nuclear interior for co-regulation of other important genes for adipogenesis.

From these analyses and data, it seems that the nuclear radial location of whole chromosome territories (see Tables S 2 and 3) is not quite as dramatic, in response to adipogenic stimulants, as the gene loci. This implies that genes may be looping out away from their chromosome territories. This is confirmed in 2D RE-FISH experiments that delineate both the gene loci probes and the parent chromosome territories using whole chromosome painting probes where the gene loci are observed at some distance from the chromosome territories (Fig. 6). Indeed, in MSC (day 0), 14%, 6%, and 10% of chromosome territories have a gene locus at a distance not associated with them for *SREBP1*:SSC12, *PPARG*:SSC13, and *CREB*:SSC15, respectively. Conversely, for AD14 (day 14) cells, these numbers had increased dramatically to 32%, 45%, and 42%, respectively. Further, using 3D FISH analysis, we revealed that during the course of differentiation the architecture of chromosome territories was altered (Fig. 7a–d). In MSCs, more condensed chromosome territories with gene signals buried inside the chromosome territories were observed (Fig. 7a, c), while in differentiated cells, chromosome territories studied were more decondensed and were approximately twice the size (Figs. 7b, d and 9). These decondensed chromosomes displayed loops of chromatin emanating from the chromosome territory with the gene signal on the end of the loop, oriented towards the nuclear interior (Fig. 7b, d). This was confirmed by 3D measurement analyses, plotted as frequency distributions with 0% on the *x*-axis representing the nuclear interior and 100% the nuclear periphery, for the following gene and chromosome combinations: *FABP4*:SSC4 (Fig. 8a, b), *PPARG*:SSC13 (Fig. 8c, d), and *CEBPB*:

Fig. 4 Nuclear distribution of gene loci in interphase nuclei of porcine mesenchymal stem cells induced to differentiate into preadipocytes and adipocytes. Representative images of the fluorescent gene signals of the seven adipogenesis genes and two controls in mesenchymal stem cells (*MSC*) and preadipocytes and adipocytes from day 7 (*AD7*) and day 14 (*AD14*) after induction of adipogenesis. Cells were fixed with methanol/acetic acid and subjected to 2D FISH. Gene loci were delineated using BAC probes. Digital images were collected on an Olympus epifluorescence microscope via Smart Capture VP v1.4 software. Gene signals are *green*; DNA in the nuclei is counterstained with DAPI (*blue*). *Scale bar*, 10 μm



SSC17 (Fig. 8e, f). The frequency distribution of the nuclear location of the gene loci and chromosome territories in Fig. 8a, c reveals the co-positioning in MSC (day 0) of the *FABP4* and *PPARG* gene signals with their chromosome territories, SSC4 and SSC13, respectively. *CEBPB* is not so well co-positioned with the territories of SSC17 and is slightly more peripheral than the chromosome. After 14 days of differentiation, the movement of all three genes to the nuclear interior is confirmed (Fig. 8b, d, f). The chromosome positioning is also confirmed with SSC4 moving towards the nuclear interior during course of differentiation (Fig. 8b) and SSC13 and SSC17 not changing their position very much at all.

For most of the genes, we observed a strong correlation between gene repositioning and a change in expression levels. The significance of these movements has been tested by Kruskal–Wallis rank sum test (Table S 2), and if the movement is significant, it is signified by asterisks on the graphs in Fig. 5. For down-regulated genes, we observed movement to a more peripheral nuclear position. The *GATA2* gene was up-regulated at day 7 of adipogenesis and moved to the nuclear interior and down-regulated at day 14 and became positioned peripherally. The *SOX9* genes were down-regulated during the course of differentiation and their position became more peripheral when compared to day 0. The control gene *MYL1* did not show expression in our system of differentiation and the position of loci was constant, as was the chromosome containing *MYL1*. However, for the other three genes, the correlation between gene activity and gene position was not so clear. The *CEBPA* gene was up-regulated during differentiation but the location changed only slightly, more towards the interior. *CEBPB* was up-regulated at day 7 and moved to a more interior position; however, at day 14, it was down-regulated, but its position remained unchanged. Although the *CREB* gene showed the same level of transcriptional activity throughout differentiation, the nuclear position of the gene loci changed from peripheral to intermediate during induction of adipogenesis.

Discussion

In order to investigate the role of genome organization in differentiation, we have established an in vitro differentiation mesenchymal stem cell model system using porcine stem cells. The pig is an important model organism used to understand adipogenesis, obesity, and diabetes, and its genome is organized in a similar way to humans, with different sized chromosomes with a range of chromosome positions, where the centromere is located. Thus, we investigated the behavior of a panel of important adipogenesis genes and the chromosomes they are housed on

in porcine mesenchymal stem cells, preadipocytes, and adipocytes.

There are a number of studies that support the importance of genome/chromosome/gene positioning within interphase nuclei in regulating gene expression (Foster and Bridger 2005; Lanctôt et al. 2007; Takizawa et al. 2008b). There have been examples where chromosomes alter their nuclear location as cells differentiate (Schöfer and Weipoltshammer 2008), for example, in human adipogenesis (Kuroda et al. 2004). Further, other studies have revealed genes being relocated as they are switched on or off. Therefore, it does appear that where a sequence is located within the nucleus, it could affect its expression. It is not clear for a number of genes that are relocated whether it is the gene by itself or whether it is the whole chromosome territory including the gene that moves. Indeed, it appears that both situations may be true. There have been examples of genes being relocated without the chromosome territory moving. These genes come away from the main body of the chromosome territory on chromatin loops (Chambeyron and Bickmore 2004; Chambeyron et al. 2005; Volpi et al. 2000). Recent studies on gene repositioning during erythropoiesis indicate that local chromatin environment plays a crucial role in spatial associations of genes located on different chromosomes (Brown et al. 2008).

Although the higher-order chromatin organization in the nucleus is conserved in vertebrates (Habermann et al. 2001; Tanabe et al. 2002a, b), there is also an increasing number of examples demonstrating differential chromosome behavior in different species. Studies on human and murine α -globin and β -globin genes revealed a different nuclear position in these two species, in relation to their transcriptional activity (Brown et al. 2006). A similar situation was observed in the case of *CFTR* locus and adjacent loci (Sadoni et al. 2008; Zink et al. 2004). The nuclear positioning of these highly conserved loci in human and mouse is therefore species-specific, the murine loci were localized, irrespective of their transcriptional status within the nucleus interior, while the human orthologs became repositioned, depending on their transcription activity. These findings indicate the importance of investigating the nuclear and genome organization and their role in gene regulation in different species. We demonstrate such an example here with our study on porcine adipogenesis. We found that whole chromosomes relocated to different extents over the three time points assessed. One chromosome in particular, SSC4, relocated from the nuclear periphery more towards the nuclear interior, as yet we do not know the reason for this. We do know, however, that SSC4 demonstrates synteny with HSA8q, where a number of genes associated with adipogenesis such as *SLUG* (*SNAI2*; Pérez-Mancera et al. 2007), *DDEF1* (Ehlers et al. 2005), as well as *FABP4* are found. In another study, and to date, the only one on chromosome

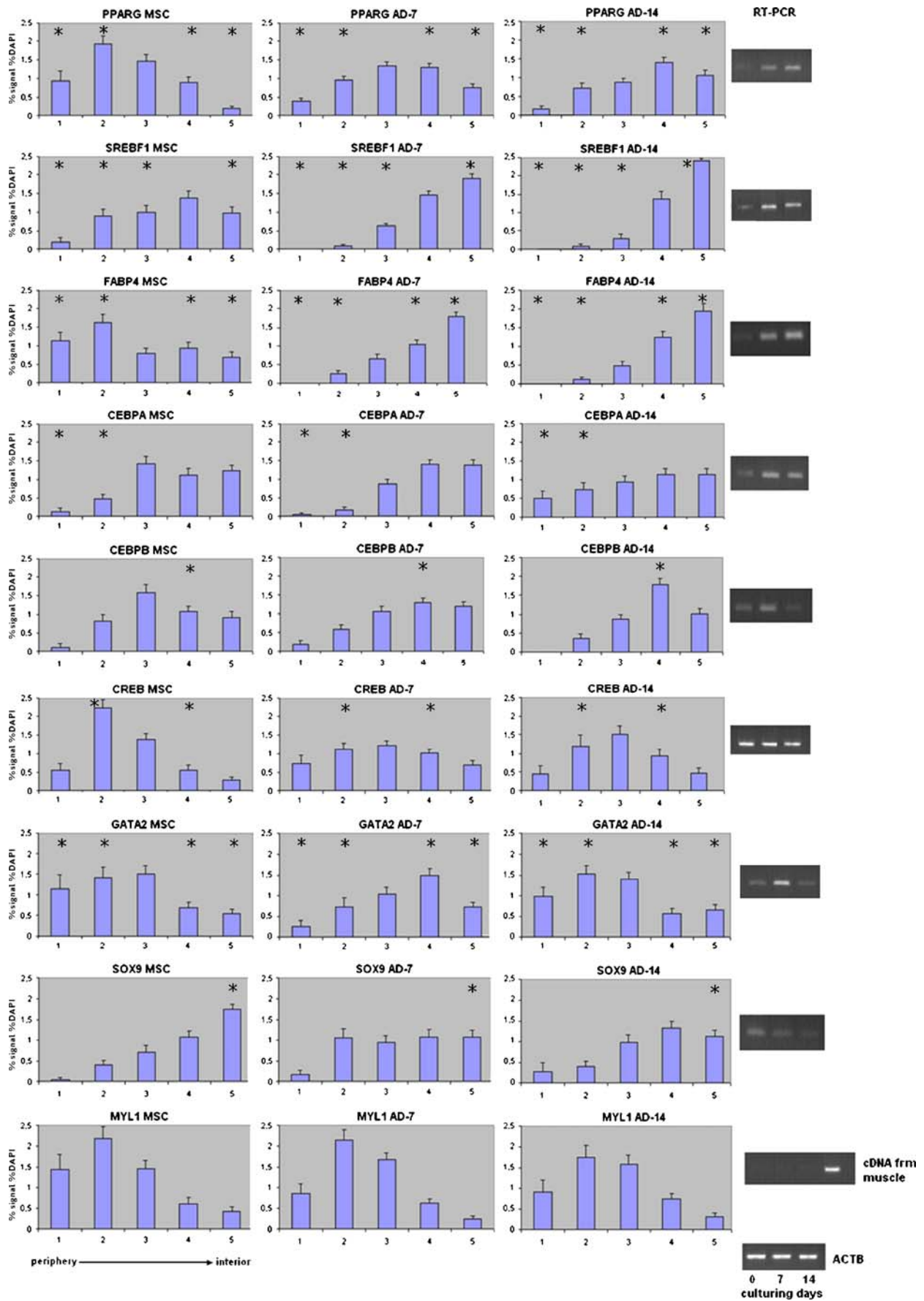


Fig. 5 Nuclear positioning of gene loci in interphase nuclei of porcine mesenchymal stem cells induced to differentiate into preadipocytes and adipocytes. The distribution of specific gene loci within the interphase nuclei was analyzed by erosion analysis of at least 50 digital images of cells subjected to 2D FISH. The script divides the nuclei into five shells of equal area and measures the intensity of the DNA and FISH signal in each shell. Each measurement for the FISH signal is normalized by the DAPI measurement for that shell. The *histograms* represent the mean proportion of the normalized chromosome signal (*y*-axis), across the five concentric shells of the nuclei (*x*-axis), from the nuclear periphery (shell 1) to the nuclear interior (shell 5). Statistical difference ($P < 0.05$) was assessed by the non-parametric Kruskal–Wallis rank sum test and indicated by *asterisks* (for details, see Table S 3). Reverse transcriptase-PCR was performed on total RNA extracted from the cells cultured in vitro using gene-specific primers. β -Actin was used as a control for cDNA synthesis. The primers for *MYL1* gene was checked on cDNA from muscle. RNA samples were taken on day 0, day 7, and day 14 of the in vitro cultures. The intensity measurements of the amplified cDNA for each gene at each time point are in Table S 4

positioning during adipocyte differentiation in human cells (Kuroda et al. 2004), the authors found changes in chromosome positions. They analyzed two chromosomes HSA12 and HSA16 which are involved in chromosome translocation in liposarcoma tumorigenesis and detected close association between chromosome territories in adipocytes when compared with preadipocytes. Additionally, they detected that during human adipogenesis HSA16 territory size increased by 50%. In our study, we did not look at relative positioning of the studied chromosomes. However, we observed very condensed chromosome territories in MSC and more decondensed chromosome territories with extended loops at day 14 of differentiation. It has been suggested that changes in the architecture of chromosome territories are produced by transcription (Croft et al. 1999; Chubb and Bickmore 2003). It is also possible that a more decondensed chromosome territory could slightly affect the

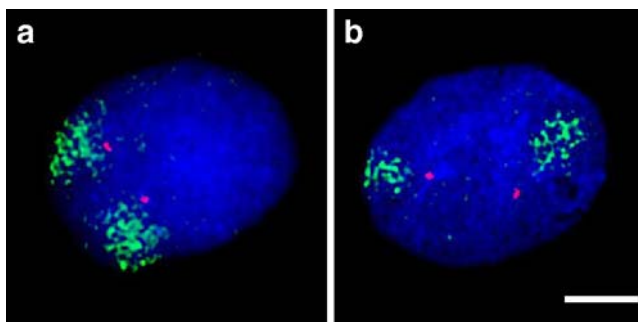


Fig. 6 **a, b** Nuclear distribution of gene signals and chromosome territories in cells from day 14 of differentiation. AD14 nuclei that have been subjected to 2D FISH for whole SSC13 delineation were subjected to RE-FISH with probes to delineate *PPARG* gene loci. Gene loci signals (*red*) were found frequently located outside of the chromosome territory (*green*). DNA was counterstained with DAPI (*blue*). Similar spatial relationships were found for *SREBP1* and *SSC12* and *CREB* and *SSC15* (not shown). Scale bar, 10 μ m

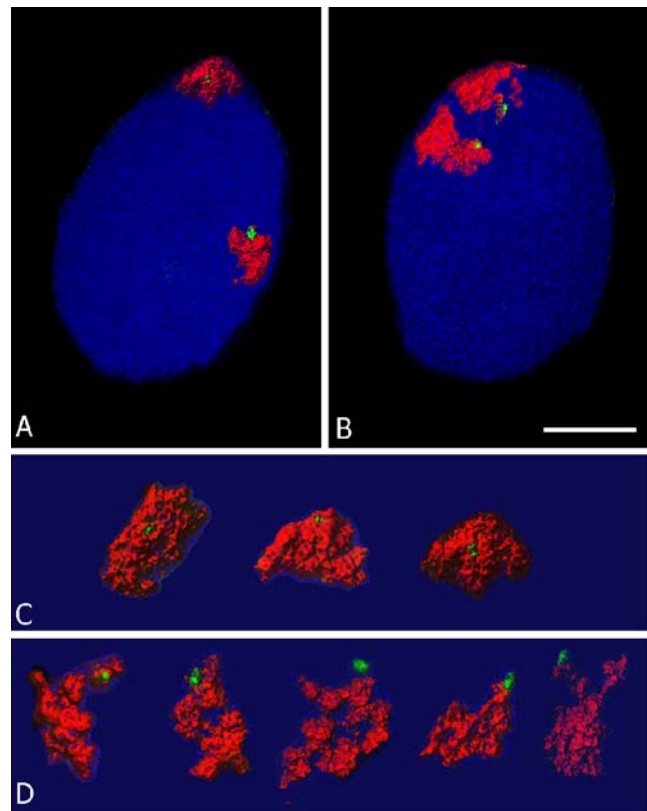
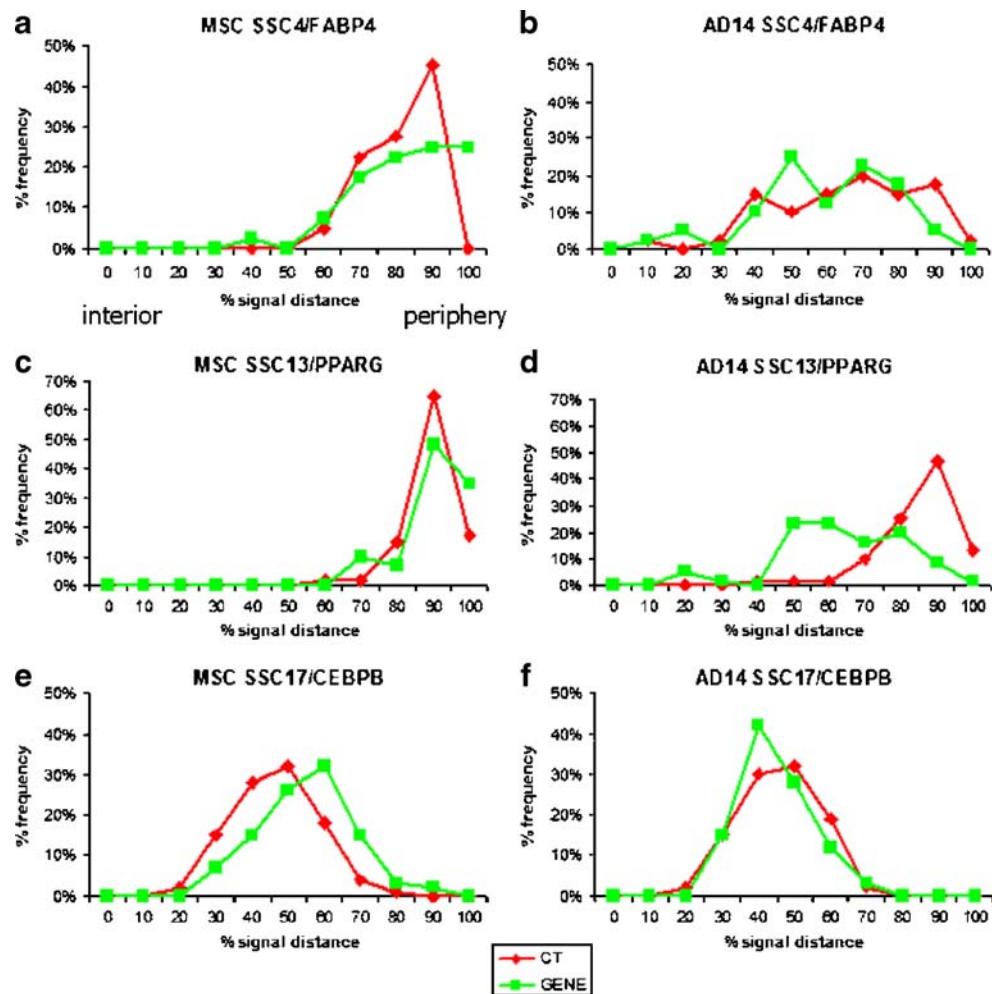


Fig. 7 Nuclear distribution of *PPARG* gene and *SSC13* chromosome territories in cells from day 14 of differentiation subjected to 3D FISH. MSC, AD7, and AD14 cells were fixed and permeabilized to preserve the 3D structure of the cells. Dual color FISH experiments were performed with whole chromosome painting probes and BAC probes for specific gene loci. Cells were optically sectioned at 0.2- μ m intervals using a Nikon 3-color laser scanning microscope. 3D reconstructions and measurements of the optical sections were performed in Imaris software. **a** 3D reconstruction of a nucleus from a MSC. **b** 3D reconstruction of a nucleus from an AD14 cell for the *PPARG* (*green*) and *SSC13* chromosome territory (*red*). **c, d** 3D reconstructions of representative chromosome territories with gene signals of MSC and AD14 cells, respectively. Scale bar, 10 μ m

number of shells a territory was found within in the 2D analyses.

When we analyzed the nuclear positions of the genes themselves, we found that substantial repositioning had occurred, with a number of genes moving from a peripheral location within nuclei to a more central position. These were genes that we knew were up-regulated in adipogenesis from studies in other organisms, i.e., mouse and human. However, when gene expression was determined using RT-PCR, we found that the movement of genes to a more central nuclear location did indeed correlate with transcriptional up-regulation. The most interesting gene was *GATA2*, whose expression is up-regulated in preadipocytes and then down-regulated again in adipocytes. The *GATA2* gene loci were located in a peripheral position in MSC nuclei and then became interior at day 7, but by day 14 of sampling,

Fig. 8 Frequency distribution plots of the nuclear positioning of chromosomes and genes in MSC and AD14 cells. **a, c, d** Frequency distributions of the radial distance measurement profiles of *FABP4* and *SSC4* (**a**), *PPARG* gene and *SSC13* (**c**), *CEBPB* and *SSC17* in MSCs (**e**). **b, d, f** The same gene/chromosome combinations are represented after 14 days in differentiation medium (*AD14*). Gene positioning is displayed with *green lines* and chromosome positioning with *red lines*; 0% of the *x*-axis represents the nuclear interior, while 100% represents the nuclear periphery



they were relocated at the nuclear periphery. This correlates with the gene expression data. Other genes that were located more towards the nuclear interior became significantly more interior when adipogenesis was induced and their expression up-regulated. Thus, this is an important model system in which to analyze the role of gene translocation within the interphase nuclei and provides a number of examples whereby genes appear to change their location with expression. Our data confirm our hypothesis that correlation between gene positioning and expression activity can be observed during differentiation when gene status has changed from silenced form to active form (Takizawa et al. 2008b). However, there are also reports where a lack of correlation between radial position of genes and their transcription activity has been described. For example, in a large study where radial positioning of 11 genes during early tumorigenesis of breast cancer was investigated, no correlation with gene expression was found (Meaburn and Misteli 2008).

There are a number of recent studies proposing that groups of related genes or genes within a certain pathway favor specific transcription factories or interchromatin

granules and are found together even though their chromosome territories may be at a distance (Osborne et al. 2004, 2007; Hu et al. 2008). However, we found that four of the adipogenesis genes we positioned by FISH within the same

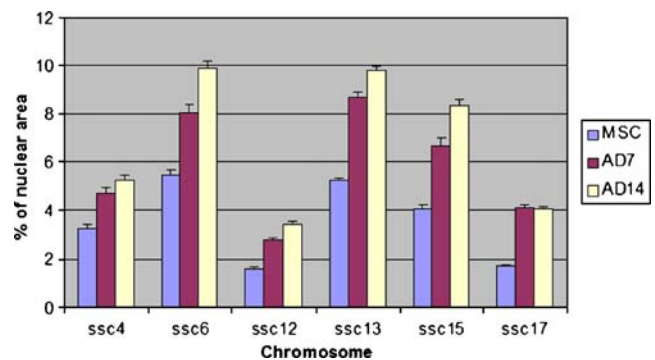


Fig. 9 Changes in chromosome territory condensation during porcine adipogenesis. Areas of chromosome territories were measured in methanol/acetic acid-fixed nuclei. Chromosome territories from 30 nuclei were analyzed in Adobe photoshop and their area is expressed as a percentage of the total nuclear area and are displayed as *histograms* with *purple bars* representing MSCs, the *burgundy bars* the AD7 cells, and the *lemon bars* the AD14 cells. *Error bars* are SEM

sample were not co-localised but the numbers of genes associated with nucleoli were increased (Fig. S 2). Furthermore, large RNA-FISH and 4C studies would be required to determine if the porcine adipogenesis genes do indeed move to a common nuclear area and/or transcription factories. Interesting data were generated in the study of gene association during erythropoiesis (Brown et al. 2008). The authors found that a tendency of genes to associate depends on the local chromatin environment and not sharing transcription factories. They demonstrated that active genes cluster around common nuclear speckles.

How genes become relocated is not yet clear, but nuclear motors containing nuclear myosin and actins may be involved (Hofmann et al. 2006; Mehta et al. 2008). Given the importance of these genes in adipogenesis, knowing how they behave in situ will help to control their expression by developing interventionist mechanisms to prevent expression. Thus, these types of analysis will help in not only understanding adipogenesis and diseases like obesity but also working towards the control of co-regulated expression of this network of genes, which will be of benefit to the health economy and agriculture.

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