Alteration of chromosome positioning during adipocyte differentiation

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Summary

Chromosomes are highly restricted to specific chromosome territories within the interphase nucleus. The arrangement of chromosome territories is non-random, exhibiting a defined radial distribution as well as a preferential association with specific nuclear compartments, which indicates a functional role for chromosome-territory organization in the regulation of gene expression. In this report, we focus on changes in adipocyte differentiation that are related to a specific chromosomal translocation associated with liposarcoma tumorigenesis, t(12;16). We have examined the relative and radial positioning of the chromosome territories of human chromosomes 12 and 16 during adipocyte differentiation, and detected a close association between the territories of chromosomes 12 and 16 in differentiated adipocytes, an association not observed in preadipocytes. Although further studies are required to elucidate the underlying reasons for the adipocyte-specific translocation of chromosomes 12 and 16, our observations indicate that alteration of relative chromosome positioning might play a key role in the tumorigenesis of human liposarcomas. In addition, these results demonstrate the potential impact of higher order chromatin organization on the epigenetic mechanisms that control gene expression and gene silencing during cell differentiation.

Key words: Chromosome territory, Adipocyte differentiation, TLS-CHOP

Introduction

Chromosomes are not randomly arranged within the interphase nuclei of plant and animal cells; instead, each chromosome occupies its own distinct region, known as a ‘territory’ (Cremer and Cremer, 2001). The radial organization of chromosome territories has been well-characterized. Typically, gene-poor chromosomes are located in a zone close to the nuclear perimeter, whereas gene-rich chromosomes are found at the center of the nucleus (Boyle et al., 2001; Bridger et al., 2000; Cremer et al., 2003; Cremer and Cremer, 2001; Habermann et al., 2001; Sun et al., 2000; Tanabe et al., 2002a; Tanabe et al., 2002b). We have previously shown that such a gene-density-correlated radial arrangement of chromosome territories is evolutionarily conserved in the genomes of higher primates (Tanabe et al., 2002b). Furthermore, several studies have shown that non-random radial chromosome arrangements are maintained in many different cell types, with the exception of some tumor cells (Boyle et al., 2001). However, because technical limitations render the spatial analysis of chromosome position difficult, it remains unclear whether radial positioning is conserved in all normal cell types.

Specific chromosomal translocations have consistently been found in particular cancers and might promote tumorigenesis through the activation of specific oncogenes or the creation of fusion proteins (Rabbitts, 1994). Human myxoid liposarcomas are associated with the chromosomal translocation t(12;16)(q13.3;p11.2), which creates a chimeric oncogene comprising part of the TLS/FUS gene found at 16p11.2 and part of the CHOP gene found at 12q13.3 (Aman et al., 1992; Crozet et al., 1993; Eneroth et al., 1990; Rabbitts et al., 1993). The resultant fusion protein is crucial to the transforming activity of the translocation, through its promotion of the unscheduled expression of the adipocyte differentiation gene DOL54 (Kuroda et al., 1997; Kuroda et al., 1999). The reasons underlying the occurrence of this specific translocation in liposarcomas, however, have remained elusive.

Recent studies have reported that the close juxtaposition of interphase chromosomes plays an important role in such basic cellular processes as gene expression. Moreover, the induction of chromosomal translocations is influenced by proximity of chromosomes, with chromosomes in close proximity to one other presumably more likely to undergo translocations than those that are further apart (Bickmore and Teague, 2002; Parada and Misteli, 2002; Sachs et al., 1997). Interestingly,
such proximity effects have been described in analyses of cancer-causing translocations involved in both leukemia and Ewing sarcoma (Kozubek et al., 1999; Taslerova et al., 2003).

In this study, we examined the relative and radial positioning of human chromosomes 12 and 16 in both preadipocytes and adipocytes to address the question of whether or not chromosome-territory (CT) repositioning occurs during adipocyte differentiation. We observed an alteration in the positioning of these CTs, suggesting that the translocation t(12;16), which might play a key role in liposarcoma tumorigenesis, is induced by the alteration in CT location. In addition, these data indicate that chromatin and nuclear compartments are dynamic during cell differentiation, and that these changes might play a role in the regulation of transcriptional activity in chromatin.

**Materials and Methods**

**Cell culture and adipocyte differentiation**

Human preadipocytes were obtained by Zen-Bio from a group of approximately six healthy, non-diabetic, non-obese (body mass index of 25) women (aged 35-38 years) undergoing elective cosmetic liposuction procedures. For adipocyte differentiation, preadipocytes cells were first cultured to confluence in preadipocyte medium (#PM-1; Zen-Bio). Adipocyte confluence was then induced by replacing the preadipocyte medium with differentiation medium (#DM-2; Zen-Bio). The differentiated adipocyte cells were maintained in adipocyte medium (#AM-1; Zen Bio).

**Probe preparation, three-dimensional fluorescence in-situ hybridization and fluorescence detection**

In order to obtain three-dimensionally preserved cell nuclei, cells were cultured on coverslip slides and fixed in 4% paraformaldehyde in 0.3× PBS. Permeabilization was performed as previously described (Solovei et al., 2002), by treating the cells with 0.5% Triton X-100 in PBS and then 20% glycerol in PBS, followed by repeated freeze-thaw cycles in liquid nitrogen before a final incubation in 0.1 N HCl.

For the delineation of the human chromosome-12 territory (CT12) and chromosome-16 territory (CT16), we used whole-chromosome painting probes provided by T. Cremer (Ludwig-Maximilians University of Munich, Munich, Germany). Probe labeling was performed by DOP- (degenerated oligonucleotide primer) PCR (Telenius et al., 1992) in the presence of biotin-16-dUTP for human chromosome 12 or digoxigenin-11-dUTP (DIG-11-dUTP) for human chromosome 16 (both from Roche). Three-dimensional fluorescence in-situ hybridization (3D-FISH) and the detection of labeled probes were performed according to protocols described elsewhere (Cremer et al., 2001; Solovei et al., 2002). Briefly, biotinylated human chromosome 12 was detected by avidin-conjugated fluorescein isothiocyanate (FITC) and biotinylated goat-anti-avidin antibody (both from Vector), followed by another round of avidin-FITC binding. Simultaneously, DIG-labeled human chromosome 16 was detected in a similar fashion using a rabbit-anti-DIG antibody (Sigma) and Cy3-labeled goat-anti-rabbit antibody (Amersham Pharmacia Biotech). DNA counterstaining was carried out using TOPRO-3 (Cy5-like fluorescence peak; Molecular Probes), and slides were mounted in Vectashield medium (Vector).

**Confocal image**

Serial nuclear images were acquired with an axial separation of 200 nm using a confocal laser-scanning microscope (LSM 410; Carl Zeiss) equipped with a 63x/1.4 Plan-Apochromat objective. For each optical section, sequential images were recorded for all three fluorochromes (FITC, Cy3 and Cy5). Stacks of 8-bit grayscale two-dimensional (2D) images were obtained with a pixel size of 66 nm and with 512x512 pixels in each channel. The image stacks were processed with Adobe Photoshop 7 and the distances between fluorescence peak centers (FPCs) were measured after converting image stacks into 256×256 pixels. Three-dimensional (3D) reconstructions of hybridized nuclear image stacks were created using Amira 3.0 TGS (http://www.amiravis.com/) software. Amira software was used only for visualization, not for data analysis.

**Segmentation**

A probability density of intensity was modeled by a finite normal mixture with m components (m=3 or 4):

\[ p(x_j) = \sum_{i=1}^{m} w_i f(x_j | \mu_i, \sigma_i) . \]

where \( x_j \) is an intensity value at pixel \( j \), \( f(x_j | \mu_i, \sigma_i) \) is the normal density of component \( i \) with mean \( \mu_i \) and standard deviation \( \sigma_i \), and \( w_i \) is a mixture ratio. The parameters (i.e. mixture ratio, intensity mean and standard deviation) were estimated so as to maximize the likelihood using the expectation-maximization (EM) algorithm (Dempster et al., 1977).

Each pixel in 3D space was classified based on a posterior probability \( \pi(i,j) \) calculated as:

\[ \pi(i,j) = \frac{w_i f(x_j | \mu_i, \sigma_i)}{\sum_{k=1}^{m} w_k f(x_j | \mu_k, \sigma_k)} . \]

That is, if \( \pi(k,j) \) was the largest out of \( \pi(i,j) \) (i=1,..,m) then pixel \( j \) was classified as a member of component \( k \). Pixels belonging to the component with the largest mean intensity were segmented as nucleus or CT. A threshold was then determined based on the minimum intensity of pixels classified into the largest mean intensity component. To obtain a smooth boundary, an Epanechnikov filter (bandwidth 0.65 μm) was applied to the boundary pixels and their neighboring pixels. The boundary was then resegmented using the threshold determined by the procedure explained above.

**Distance measurement**

FPCs of the CTs were detected in 3D space. To avoid the effects of local fluctuation, an Epanechnikov filter (bandwidth 0.65 μm) was applied. We defined the pair of chromosomes 12 and 16 with the minimum FPC distance as the proximal pair and the other pair as the distal pair. The distance between CT12 and CT16 of the proximal pair was normalized using the standardized radius of the nucleus. We simulated the distribution of the normalized proximal-pair distances using Monte Carlo simulation (Kozubek et al., 2002) and compared the simulation results with experimental results. The distribution of normalized proximal-pair distances was simulated as follows. First, we generated the radial positions of four FPCs (two for CT12 and two for CT16) in a unit 2D disc using a random-number generator modulated by the experimental radial distributions. Second, the positions were determined assuming a uniform angular distribution (Kozubek et al., 2002). Third, the CT12-CT16 pair with the minimum distance of the two possible combinations was selected and the proximal distance was determined. After 100,000 repetitions, the distributions of the proximal-pair distances were determined.

**Normalization of the nucleus size and shape**

To remove the effects of size and shape change during cell differentiation, we standardized the nucleus size and shape as follows.
Chromosome territories in adipogenesis

(1) The nucleus area and the FPCs of CT12 and CT16 were projected onto the x-y plane.

(2) The radius of the standard nucleus disc ($r_0$) was calculated so that the disc area was equal to the projected nucleus area.

(3) The boundary of the original nucleus was extended (or receded) in the 2D plane if the distance from the center of gravity of the nucleus ($P_0$) to the boundary ($R$) was shorter (or longer) than the radius of the standard nucleus.

(4) The same transformation was performed on each pixel in a territory region ($x$) to obtain a deformed position ($x'$). Radial positions and mutual distances between CTs were evaluated using the values relative to the standardized nucleus radius.

Statistical analysis

We used 38 preadipocyte cells and 41 adipocyte cells for the distance and radial distribution analysis. Welch’s $t$ test was used to determine the significance of differences in the proximal-pair distance, and the Kolmogorov-Smirnov (KS) test was applied to the radial distribution differences. The statistical analysis software R version 1.8.1 (http://www.r-project.org/) was used.

Results

Induction of adipocyte differentiation in primary human preadipocyte cells

We cultured human preadipocyte cells until they reached confluence and then induced them to differentiate into adipocyte cells (Fig. 1A,B). The 2D-FISH karyotype of the preadipocyte cells during metaphase appeared normal: 2n=46, XX, with one pair each of chromosomes 12 and 16 (Fig. 1C).

Relative 3D positioning of the human CT12 and CT16 in preadipocytes and adipocytes

We performed 3D-FISH in 3D-preserved fixed nuclei using painting probes for human chromosomes 12 and 16. Both preadipocyte and adipocyte cells were arrested in the G0/G1 phase in response to cell confluence. Fluorescent signals from both human CT12 and human CT16 were successfully visualized using a two-color analysis, with green and red representing chromosomes 12 and 16, respectively.

We observed the nuclei under a microscope to determine whether or not there was a close association between CT12 and CT16. By visual inspection, we could clearly detect a close association between one CT12 and one CT16 in 81% (33/41) of adipocyte nuclei, whereas this association was observed only in 45% (17/38) of preadipocyte nuclei. Next, we imaged nuclei with a confocal laser-scanning microscope for a more precise quantitative evaluation of the distances between the CTs. A series of optical sections was recorded for each cell, with successive sections separated by an axial distance of 200 nm. All three fluorochromes were recorded sequentially for each section using a box size of 512x512 pixels. A representative z-axis series and 3D reconstruction of both preadipocyte and adipocyte nuclei are displayed in Fig. 2.

For a quantitative evaluation of relative CT positioning, we first measured CT and nucleus size in both preadipocytes and adipocytes (Table 1). We found that the nucleus size was reduced by approximately 10% during adipocyte differentiation. In addition, the CT16 size increased by 50%, whereas the CT12 size showed no significant changes. Furthermore, the nuclei of preadipocyte and adipocyte cells exhibited a range of shapes. Therefore, to eliminate the effects of size and shape change of CTs during cell differentiation, we standardized the nucleus size (Fig. 3). We used the minimum...
distance between the FPCs of all four possible pairs of CT12 and CT16 in each nucleus as a measure of the level of CT association (Fig. 4A,B). The mean value for the minimum FPC distance relative to the standardized 2D nucleus radius in adipocyte nuclei was significantly smaller than that in preadipocyte nuclei ($P=0.016$) (Fig. 4C). These results suggest that repositioning of one pair of CTs occurs during adipocyte differentiation. This proximity effect (Fig. 4), as well as the magnification of CT16 (Table 1), substantially increases the probability of an interaction between CT12 and CT16, and is likely to lead to a t(12;16) translocation during adipocyte differentiation. Although the parental origin of this pair of chromosomes is unknown, this raises the intriguing possibility that an association between a specific combination of chromosomes of paternal or maternal origin is established by as yet unknown epigenetic mechanisms.

Table 1. Sizes of nuclei and chromosome territories (CTs), showing the means ± s.e.

|        | Nuclear volume ($\mu m^3$) | CT12 volume ($\mu m^3$) | CT16 volume ($\mu m^3$) |
|--------|---------------------------|------------------------|------------------------|
| Preadipocyte ($n=38$) | 1147.1±51.8              | 29.7±2.1               | 15.3±1.4               |
| Adipocyte ($n=41$)    | 1028.9±71.0              | 29.9±2.4               | 22.6±1.4               |

Radial distribution of the CT12 and CT16 does not affect their relative positioning during adipocyte differentiation

Previous studies have showed that the radial rearrangement of genetic structures changes during myogenesis (Chaly and Munro, 1996) and granulopoiesis (Bartova et al., 2002; Bartova et al., 2001; Bartova et al., 2000b). Therefore, we next examined the radial distributions of CT12 and CT16 in preadipocyte and adipocyte cells. We investigated total radial distributions over the standardized 2D nucleus and compared them for the cell types studied using the KS test. Although there were no significant differences between CT12 and CT16 during adipocyte differentiation, the means of the total radial distribution appeared to shift towards the center for CT16 (from $<r>=59.0±22.0$ to $56.8±21.5$, $P>0.49$, where $<r>$ stands for the mean radial position) and the mean of the total radial distribution for CT12 shifted toward the periphery (from $<r>=57.7±21.4$ to $59.8±21.4$, $P>0.7$) (Fig. 5). This shift of the CT might account for the reduction in relative distances between CT12 and CT16.

Thus, to determine whether the difference of minimum FPC distances described above was affected by the fluctuation of the radial distribution of CT12 and CT16 during cell differentiation, we conducted a Monte Carlo simulation (Kozubek et al., 2002). The minimum distances obtained through the simulation were not significantly different (0.45±0.25 for adipocyte and 0.46±0.26 for preadipocyte). Thus, the change in distance between CT12 and CT16 during adipocyte differentiation was not caused by changes in radial distributions.

Discussion

Insight into the epigenetic function of CT repositioning is important for understanding how nuclear architecture is organized in different cell types, and how it might be involved in cell differentiation and tumorigenesis (Cremer and Cremer, 2001; Jenuwein and Allis, 2001; Strahl and Allis, 2000). There are two aspects to consider with respect to CT positioning: the absolute radial location within the nucleus and the position of CTs relative to one another (Parada and Misteli, 2002). The radial location of a CT is tightly correlated with its size and gene density. As a general rule, gene-dense CTs are found near the center of the nucleus, whereas gene-poor CTs localize to the periphery of the nucleus (Boyle et al., 2001; Cremer et al., 2001; Croft et al., 1999). This rule is applicable in cells with spherical nuclei, such as lymphocytes and lymphoblastoid cell lines, and its evolutionary conservation has been clearly demonstrated (Tanabe et al., 2002b). By contrast, the extent to which the rule applies to cell types with non-spherical nuclei, such as fibroblasts, epithelial cells and tumor cell lines, remains controversial (Boyle et al., 2001; Bridger et al., 2000; Cremer et al., 2003; Cremer et al., 2001; Croft et al., 1999; Kozubek et al., 2002; Tanabe et al., 2002b).

In this study, we evaluate the arrangement of CT12 and CT16 in preadipocyte and adipocyte cells. Interestingly, we...
observed that the minimum FPC distance of CT12 and CT16 changed during adipogenesis. Furthermore, we examined whether or not the total radial distribution of CT12 and CT16 changed during adipogenesis. We could not detect a significant radial shift between the two cell types using the KS test (Fig. 5). Thus, we conclude that the relative positioning of CT12 and CT16 was altered during adipogenesis. The reasons for the difference in behavior between chromosomes 12 and 16 are at present unknown. However, it is possible that the difference is due to variations in local gene expression from each chromosome.

The process of cellular differentiation represents a remarkably coordinated program of gene regulation that directs multipotent stem-cell precursors down various lineages into fully mature and functionally distinct cell types. During adipocyte differentiation, many genes have been shown to be regulated in a differentiation-dependent manner. It is has been clearly shown that adipogenic transcription factors such as C/EBP and PPAR play an important role in the regulation of gene expression through conventional genetic mechanisms. However, recent reports indicate that the spatial arrangement of chromatin in the nucleus is correlated with cell differentiation (Bartova et al., 2002; Bartova et al., 2001; Bartova et al., 2000a; Manuelidis, 1990). Moreover, gene positioning and heterochromatin-mediated gene silencing appear to play an important role in cell differentiation (Bartova et al., 2002). Our results and other studies of CTs (Mahy et al., 2002a; Mahy et al., 2002b) suggest that chromosome distribution might also control epigenetic mechanisms that affect regulation of genes. Thus, changes in CT location might act as an epigenetic factor that functions on a different level than the genetic code.

**Fig. 4.** Relative positions of human chromosome-12 and chromosome-16 territories (CT12 and CT16, respectively). (A) The definition of a representative peak point for a CT. Green dots indicate the fluorescence peak center (FPC). The location of the CT is represented by the position of its FPC. (B) Schema of the minimum FPC distance. We measured the pixel-to-pixel distance between the FPCs of CT12 and CT16, and then recorded the shortest of the four distances obtained from the four possible pairings as the minimum FPC distance. (C) Quantitative evaluation of the minimum FPC distance between CT12 and CT16. The distances are normalized using the radius of the standardized 2D nucleus. The significance of the difference between minimum FPC distances in preadipocytes and adipocytes was evaluated using the Welch’s $t$ test ($P=0.017$).

**Fig. 5.** Radial positions of human chromosome-12 and chromosome-16 territories (CT12 and CT16, respectively). Total radial distributions of CT12 and CT16 over the standardized 2D nucleus ($n=76\pm38\times2$ for preadipocyte, and $n=82\pm41\times2$ for adipocyte). The mean values ($<r>$) and standard errors of all distributions are also shown.
The relative positioning of CTs with respect to one another is known to influence the translocation frequencies between two chromosomes. For example, human myoid and round cell liposarcomas are associated with specific chromosomal translocations (Sreekantaiah et al., 1992). The TLS-CHOP fusion gene derived from t(12;16) is present in 95-98% of myoid and round cell liposarcomas but, in rare cases, a variant t(12;22) translocation is observed that results in the EWS-CHOP fusion gene (Hosaka et al., 2002). The TLS-CHOP chimeric protein has transforming activity (Kuroda et al., 1997) and induces the expression of the DOL54 gene, which is normally associated with adipocyte differentiation (Kuroda et al., 1999). In addition, histological diagnosis of myoid liposarcoma reveals the presence of immature adipose cells called lipoblasts, suggesting that myoid liposarcomas come from an immature mesenchymal or adipocytic cell lineage. However, it is still unknown why the chromosomal translocation t(12;16)(q13;p11.2) and the creation of the TLS-CHOP fusion gene occur specifically in liposarcomas. Our observations demonstrate that the minimum FPC distance between CT12 and CT16 is reduced, and the size of CT16 is magnified during adipocyte differentiation. These phenomena might lead to the t(12;16) translocation event specific to liposarcomas.

In conclusion, we have demonstrated that the particular CTs involved in a specific translocation event implicated in cancer development can physically associate with one another following differentiation in human cell lines. These results also suggest that the t(12;16) translocation, which has been implicated in liposarcoma tumorigenesis, occurs because of an alteration in chromosome location.

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