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***CBFB* and *MYH11* in inv(16)(p13q22) of Acute Myeloid Leukemia Display Close Spatial Proximity in Interphase Nuclei of Human Hematopoietic Stem Cells**

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Abstract

To gain a better understanding of the mechanism of chromosomal translocations in cancer, we investigated the spatial proximity between *CBFB* and *MYH11* genes involved in inv(16)(p13q22) found in acute myeloid leukemia patients. Previous studies have demonstrated a role for spatial genome organization in the formation of tumorigenic abnormalities. The non-random localization of chromosomes and, more specifically, of genes appears to play a role in the mechanism of chromosomal translocations. Here, two-color fluorescence in situ hybridization and confocal microscopy were used to measure the interphase distance between *CBFB* and *MYH11* in hematopoietic stem cells, where inv(16)(p13q22) is believed to occur, leading to leukemia development. The measured distances in hematopoietic stem cells were compared to mesenchymal stem cells, peripheral blood lymphocytes and fibroblasts, as spatial genome organization is determined to be cell-type specific. Results indicate that *CBFB* and *MYH11* are significantly closer in hematopoietic stem cells compared to all other cell types examined. Furthermore, the *CBFB-MYH11* distance is significantly reduced compared to *CBFB* and a control locus in hematopoietic stem cells, although separation between *CBFB* and the control is ~70% of that between *CBFB* and *MYH11* on metaphase chromosomes. Hematopoietic stem cells were also treated with fragile site-inducing chemicals since both genes contain translocation breakpoints within these regions. However, treatment with fragile site-inducing chemicals did not significantly affect the interphase distance. Consistent with previous studies, our results suggest that gene proximity may play a role in the formation of cancer-causing rearrangements, providing insight into the mechanism of chromosomal abnormalities in human tumors.

INTRODUCTION

Chromosomal translocations often result in cancer development through disruption of genes involved in important cellular processes such as cell proliferation and survival. Despite the

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high prevalence of chromosomal translocations in tumor cells, the mechanism by which this process occurs is still unclear. The formation of translocations is a multi-step process that is initiated by DNA strand breakage (Meaburn et al., 2007). DNA double-strand breaks (DSBs), which often occur through exposure to ionizing radiation or genotoxic chemicals (Weterings and Chen, 2008) must be present in at least two places in the genome. There are several major pathways that contribute to DSB repair: homologous recombination (Kanaar et al., 1998; Elliott and Jasin, 2002), non-homologous end joining (Shrivastav et al., 2008), and microhomology-mediated end joining (Klugbauer et al., 2001). In order for a translocation to occur, these pathways must fail to repair the breaks, and the broken chromosome ends must physically meet and become joined together (Kanaar et al., 1998; Elliott and Jasin, 2002). The molecular basis underlying why certain genes undergo specific chromosomal translocations remains elusive. However, their recurrence in human tumors suggests a role for spatial genome organization in the formation of cancer-causing rearrangements.

In an increasing number of studies that have examined potential mechanisms, non-random spatial genome organization has emerged as an important factor in the generation of chromosomal abnormalities. Interphase nuclei are compartmentalized into discrete, three-dimensional regions, known as chromosome territories, according to gene density, whereby gene-rich chromosomes tend to cluster toward the nuclear center while chromosomes that are gene-poor preferentially locate toward the periphery (Meaburn et al., 2007). The position of genes is non-random with respect to each other, which is functionally important for understanding cancerous transformation. In addition to human cells, higher-order genome organization is also observed in mice and other vertebrates, which supports an important role for the spatial arrangement of chromosomes and genes in interphase nuclei.

The concept that the spatial proximity of chromosomes may lead to an enhanced susceptibility to translocations was recently demonstrated. The nuclear architecture of bone marrow cells was examined and revealed that chromosomes 8 and 21 tended to co-localize with one another in the nucleus rather than be separated, thus promoting the t(8;21) observed in acute myeloid leukemia (AML) of the M2 subtype (Manvelyan et al., 2009). In 2003, Roix et al. examined the spatial proximity between the *MYC* oncogene and three of its translocation partner genes, *IGH*, *IGL* and *IGK*, taking advantage of the fact that in Burkitt's lymphoma, *MYC* translocates with each partner at different frequencies. The distances between each gene set were measured and compared to their clinically observed frequencies, with results indicating a strong correlation between the spatial proximity of genes during interphase and their translocation frequencies (Roix et al., 2003). In addition, a number of studies have looked at the spatial proximity of genes involved in translocations observed in thyroid cancer, since there is a well-established association between DNA damage caused by radiation exposure and the occurrence of thyroid tumors. Examination of the spatial proximity between *RET*, a gene commonly rearranged in papillary thyroid carcinoma (PTC), and two of its partner genes, *CCDC6* (Nikiforova et al., 2000) and *NCOA4* (Gandhi et al., 2006), revealed close proximity in both gene pairs that was cell-type specific. Furthermore, Roccato et al. determined that the interphase distance between *TPR* and *NTRK1* genes, separated by ~30 Mb and also rearranged in PTC, was significantly closer in human thyrocytes than in lymphocytes, potentially favoring a rearrangement (Roccato et al., 2005).

More recent reports have elegantly demonstrated the ability of genes located in close spatial proximity to give rise to a number of cancer-specific translocations following DNA damage, providing compelling evidence of a role for spatial genome organization in the formation of chromosomal translocations. Gandhi et al. showed that the *RET/PTC1* rearrangement, which involves closely-positioned genes *RET* and *CCDC6* (Nikiforova et al., 2000), could be generated in normal human thyroid cells upon fragile site breakage (Gandhi et al., 2010).

NPM1 and *ALK*, genes participating in t(2;5)(p23;q35) associated with anaplastic large cell lymphoma (ALCL), are located in close spatial proximity within the nuclear space of t(2;5)-negative ALCL cells, and translocate with each other upon irradiation (Mathas et al., 2009). Similar results have been shown for translocations involving *TMPRSS2* with two of its partner genes, *ERG* (Lin et al., 2009; Mani et al., 2009) and *ETV1* (Lin et al., 2009), whereby irradiation of LNCaP cells induced both fusion transcripts, and represented the authentic fusion junctions commonly observed in prostate cancer patients.

According to the Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer (Mitelman et al., 2010), there are over 700 chromosomal translocations reported in various types of human tumors. To date, most studies of spatial gene proximity have focused on solid tumors, including PTC (Nikiforova et al., 2000; Roccato et al., 2005; Gandhi et al., 2006), lymphoma (Roix et al., 2003; Mathas et al., 2009) and prostate cancer (Lin et al., 2009; Mani et al., 2009). Fewer studies have investigated the spatial proximity of genes involved in translocations observed in leukemia, with the exception of the Philadelphia chromosome translocation commonly found in patients with chronic myelogenous leukemia. The results support the concept that participating genes *ABL* and *BCR* are closely positioned during interphase, thus promoting their rearrangement (Kozubek et al., 1997; Lukasova et al., 1997; Neves et al., 1999). To our knowledge, there has been no such study examining the interphase distance of genes participating in an intrachromosomal translocation associated with leukemia. Therefore, the objective of this study was to determine whether short interphase distances between translocation-participating loci involved in leukemia-specific intrachromosomal rearrangements could further support the role of spatial genome organization in the formation of cancer-causing rearrangements.

The intrachromosomal rearrangement inv(16)(p13q22) is one of the most common abnormalities observed in AML, occurring in ~12% of adult patients (Kuo et al., 2006) and 100% of patients of the M4 subtype with accompanying eosinophilia (M4Eo) (Liu et al., 1993). This rearrangement involves the *CBFB* and *MYH11* genes, both of which are located on chromosome 16 at ~50 Mb apart, which is further apart than any gene pair formerly investigated for spatial proximity of intrachromosomal rearrangements. CBF β is part of the core binding factor (CBF) complex (Kuo et al., 2006), a heterodimeric transcription factor comprised of CBF β and core binding factor alpha (CBF α) subunits that regulate a variety of genes involved in hematopoiesis (Otto et al., 2003). The fusion product of inv(16)(p13q22) blocks hematopoietic differentiation in a dominant-negative manner by inhibiting the normal function of the CBF transcription complex (Castilla et al., 1996; Yergeau et al., 1997). In addition, breakpoints within both *CBFB* and *MYH11* coincide with human chromosomal fragile sites FRA16B/FRA16C and FRA16A, respectively (Burrow et al., 2009). Fragile sites are specific regions of the genome that are highly susceptible to DNA breakage following conditions of partial replication stress (Richards, 2001). These regions are often targets of environmental and chemical agents, including ethanol, cigarette smoke, caffeine and pesticides (Richards, 2001), and play a direct role in the generation of oncogenic *RET/PTC* rearrangements (Gandhi et al., 2010).

To determine whether spatial gene proximity is involved in the formation of this rearrangement, the distance between *CBFB* and *MYH11* loci was examined in interphase nuclei of normal human hematopoietic stem cells (HSCs). Two-color fluorescence in situ hybridization (FISH) and confocal microscopy were used to map the locations of *CBFB* and *MYH11*. The distances were compared to cell types of different lineages and differentiation stages, since previous studies have shown that spatial gene proximity may be cell-lineage and cell differentiation stage-specific (Neves et al., 1999). We found significant differences for *CBFB-MYH11* measured distances in HSCs compared with human mesenchymal stem cells (MSCs), peripheral blood lymphocytes (PBLs) and fibroblasts. Furthermore, the

distance between *CBFB* and *MYH11*, which are ~50 Mb apart on chromosome 16 was determined to be significantly reduced during interphase in HSCs compared to *CBFB* and the control 16p11.2 locus, which is separated from *CBFB* by ~37 Mb. We also examined the spatial proximity between *CBFB* and *MYH11* following treatment with fragile site-inducing chemicals to determine any potential changes in their interphase distance. Treatment of HSCs did not cause any significant differences compared to untreated HSCs, indicating that mild replication stress and/or fragile site breakage had little effect on *CBFB-MYH11* spatial proximity. Here, we present evidence of a role for interphase gene proximity in the formation of inv(16)(p13q22) whereby *CBFB* and *MYH11* are located at a significantly reduced distance in normal human HSCs compared to other cell types. These results further support a critical role for non-random spatial genome organization in the generation of cancer-specific chromosomal rearrangements.

MATERIALS AND METHODS

Cell Culture

Fresh primary human CD34⁺ HSCs were purchased from Lonza (Walkersville, MD) and maintained in HPGM hematopoietic progenitor growth medium (Lonza) containing a StemSpan CC100 cytokine cocktail of recombinant human (rh) Flt-3 ligand, rh stem cell factor, rh IL-3 and rh IL-6 (STEMCELL Technologies, Vancouver, British Columbia). Human MSCs were obtained from Texas A&M University (College Station, TX) and grown in Dulbecco's Modified Eagle Media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA) and 1% penicillin-streptomycin (Invitrogen). PBLs from a healthy donor were established according to routine procedures (Department of Medical Cytogenetics, WFUBMC). The normal human fibroblast cell line GM08402 was obtained from Coriell Institute for Medical Research (Camden, NJ) and maintained in Minimum Essential Media (Invitrogen) with 10% fetal bovine serum (ThermoScientific) and 1% penicillin-streptomycin (Invitrogen). All cells were grown in a humidified incubator at 37°C with 5% CO₂. For the induction of fragile sites, cells were treated with 0.4 μM APH (Sigma-Aldrich, St. Louis, MO), 2 mM 2-AP (Sigma-Aldrich) and 150 mg/L berenil (Sigma-Aldrich) for 24 h.

FISH

Cells were harvested according to a standard cytogenetic procedure. Colcemid was directly added to cells for 30 min. After centrifugation, 0.075 M KCl hypotonic solution was added and cells were suspended in 3:1 methanol/acetic acid, dropped onto slides and pretreated with 0.001% pepsin (Sigma, St. Louis MO) in 0.01M HCl for 10 min at 37°C followed by a rinse in PBS (Sigma) and a 5 min fixation in 1% formaldehyde. Slides were dehydrated through an ethanol series and dried. Bacterial artificial chromosome (BAC) clones spanning the *CBFB* (RP11-5A19) and *MYH11* (RP11-585P8) chromosomal loci to be used as probes were purchased from Children's Hospital Oakland Research Institute (CHORI, Oakland, CA). The *CBFB* probe was labeled with SpectrumOrange - dUTP (Abbott Molecular, Abbott Park, IL) and *MYH11* probe was labeled with SpectrumGreen - dUTP (Abbott Molecular) according to instructions in the Nick Translation Kit (Abbott Molecular). As a control, a probe spanning the 16p11.2 locus (RP11-74E23) was purchased pre-labeled with SpectrumGreen from BlueGnome (BlueGnome Ltd., Great Shelford, Cambridge, UK). A hybridization mix of each probe (300 ng) was prepared by adding 7 μL of Abbott Molecular LSI Hybridization mix and 3 μL of distilled water to the BAC pellets. The hybridization mixture was applied to the slides seated in a Hybrite heating block set to 37°C, sealed with a coverslip and denatured at 73°C for 3 min. After overnight incubation at 37°C, slides were washed in 0.4X Sodium Citrate Saline (SSC)/0.3% NP-40 at 73°C for 1.5 min, followed by a brief rinse through 2X SSC/0.1% NP-40 and then dried.

Slides were counterstained with DAPI II (Abbott Molecular) and cover-slipped, and fluorescence was viewed using a Zeiss Axioplan 2 microscope with a triple filter to simultaneously show the probes and counterstain. In order to illustrate the presence of signals, fluorescent images of probe hybridization were made using the Cytovision capture system equipped with a CCD camera (Photometrics). The fluors were captured in sequential order as follows: SpectrumOrange (excitation 500–600 nm/emission 550–650 nm), SpectrumGreen (excitation 400–550 nm/emission 500–600 nm) and then DAPI II (excitation/emission 400 nm). The images were captured in gray scale, merged and pseudo-colored by the software to resemble the color of the original signals.

For the analysis of *CBFB* and the control locus, slides containing HSCs were stripped and re-probed with *CBFB* and 16p11.2 BACs according to the same procedure.

Confocal Microscopy

A total of 105 interphase nuclei from each cell type were examined using a Zeiss Axiovert 100M confocal microscope. Each nucleus displays two distinct hybridization signals per probe (Fig. 1), and therefore, a total of 210 pairs of *CBFB* and *MYH11* signals were then analyzed for each cell type. Since previous studies have determined that two pairs of heterologous signals are located in two separate areas of the nucleus with shorter distances than any other possible distance between the signals (Nikiforova et al., 2000), it was assumed that the two shorter distances were between loci on the same chromosome. Distances between hybridization signals were measured using Zeiss LSM Image Browser Software's overlay feature.

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics v19 software (SPSS Inc., Chicago, IL). Data are presented as the mean $\pm$ standard deviation (SD) or as a percentage. The distribution of measured distances between *CBFB* and *MYH11* in HSCs and other cell types were compared using student's t-test. Student's t-test was also utilized to determine significant differences between *CBFB-MYH11* and *CBFB-16p11.2* distances. A chi-square test was used to compare the percentage of gene pairs in which *CBFB* and *MYH11* were located 0–0.99 μ m, 1.00–1.99 μ m, 2.00–2.99 μ m, 3.00–3.99 μ m or 4 μ m apart in HSCs and other cell types. A p value < 0.05 was considered statistically significant and was adjusted by a Bonferroni correction for multiple comparisons.

RESULTS

Interphase Distance of *CBFB* and *MYH11* in Human Hematopoietic Stem Cells Compared with Human Mesenchymal Stem Cells

The inv(16)(p13q22) is believed to occur very early in differentiation at the stem/progenitor level in HSCs (Kundu et al., 2002). Therefore, to investigate whether *CBFB* and *MYH11* display close spatial proximity in HSCs, interphase distances between *CBFB* and *MYH11* loci were measured in HSCs and compared to those in MSCs. MSCs were examined since they are also found in the bone marrow and have the potential for multi-lineage differentiation and self-renewal but give rise to different cell types. The distribution plots of measured *CBFB-MYH11* distances in HSCs and MSCs are shown in Figure 2. The shorter distance (mean $\pm$ SD) for HSCs ($1.90 \pm 1.14 \mu$ m) as compared to MSCs ($3.78 \pm 2.67 \mu$ m) was highly significant ($p = 2.24 \times 10^{-18}$) (Table 1). To estimate the range of distance that may be related to translocation events, the measured distances between *CBFB* and *MYH11* were categorized into 0–0.99 μ m, 1.00–1.99 μ m, 2.00–2.99 μ m, 3.00–3.99 μ m and 4 μ m groups, respectively (Fig. 3). The overall distribution of interphase distance between these two stem cell types was significantly different ($p = 7.27 \times 10^{-16}$). Interestingly, HSCs had a

significantly higher percentage of short interphase distances ($<2 \mu\text{m}$) (20.50% vs. 7.60% for 0–0.99 μm and 40.50% vs. 18.10% for 1.00–1.99 μm), and similar or lower percentages of long interphase distance ($>2 \mu\text{m}$) compared with MSCs (0–1.99 μm vs. $>2 \mu\text{m}$, $p = 7 \times 10^{-13}$). Comparison of the frequency of *CBFB-MYH11* co-localization (distance = 0.00 μm) between the two cell types also revealed differences. HSCs had a higher percentage (3.81%) of gene pairs that co-localized compared to MSCs, which did not have any co-localized pairs (Table 2). These results show that *CBFB* and *MYH11* are indeed located in close spatial proximity in HSCs compared with MSCs, providing a potential mechanistic explanation of why breakage at *CBFB* and *MYH11* promotes the formation of inv(16)(p13q22) in HSCs but not in MSCs.

Spatial Gene Proximity of *CBFB* and *MYH11* in Specialized Cell Types

The measured distances between *CBFB* and *MYH11* were examined in specialized cell types and compared to the distances observed in HSCs. PBLs and fibroblasts were chosen because they are fully-differentiated cell types derived from HSCs and MSCs, respectively. Histograms of measured distances are shown in Figure 2. The distribution of *CBFB-MYH11* distances was statistically significant between HSCs and PBLs ($p = 1.09 \times 10^{-11}$), as well as between HSCs and fibroblasts ($p = 1.09 \times 10^{-11}$) (Table 1). The percentage of chromosomes with gene loci 0–0.99 μm , 1.00–1.99 μm or 2.00–2.99 μm apart was also determined. In PBLs, 7.10% and 20.50% of gene pairs were 0–0.99 μm and 1.00–1.99 μm , respectively. The examination of fibroblasts proved similar, with 12.90% and 25.70% of *CBFB* and *MYH11* loci separated by 0–0.99 μm and 1.00–1.99 μm , respectively (Fig. 3). Chi square tests showed significant differences between HSCs and PBLs ($p = 2.08 \times 10^{-13}$) and fibroblasts ($p = 8.34 \times 10^{-7}$). Furthermore, there were observed differences among the frequency of co-localized gene pairs between HSCs (3.81%), PBLs (2.86%), and fibroblasts (0.95%) (Table 2). Therefore, it was concluded that the distance between *CBFB* and *MYH11* is significantly greater in PBLs and fibroblasts than in HSCs, supporting the idea that spatial proximity is cell-type and differentiation stage-specific.

While *CBFB-MYH11* distances are significantly reduced in HSCs compared to MSCs, PBLs and fibroblasts, due to potential differences in cell size, we next examined the distance between *CBFB* and a control locus (16p11.2) in HSCs to determine whether spatial gene proximity is a contributing factor in the formation of inv(16)(p13q22). 16p11.2 is separated from *CBFB* by ~37 Mb, and is located in-between *CBFB* and *MYH11* on metaphase chromosomes. This region is not known to participate in a rearrangement with the *CBFB* gene, nor does it co-localize with a fragile site. The mean distance between *CBFB* and 16p11.2 ($2.78 \pm 1.68 \mu\text{m}$, Supplementary Fig. 1) was significantly higher than the distance between *CBFB* and *MYH11* in HSCs ($p = 4.11 \times 10^{-10}$). Furthermore, there was no co-localization between *CBFB* and 16p11.2, which is in contrast to 3.81% of co-localizing pairs between *CBFB* and *MYH11* in HSCs. These data further support that *CBFB* and *MYH11* are physically in close proximity during interphase in HSCs.

Proximity of *CBFB* and *MYH11* Following Treatment with Fragile Site-Inducing Chemicals in HSCs

To determine whether treatment with fragile site-inducing chemicals affects spatial gene proximity of *CBFB* and *MYH11*, HSCs were treated with 0.4 μM aphidicolin (APH), 2 mM 2-aminopurine (2-AP) and 150 mg/L berenil for 24 h. APH, an inhibitor of DNA polymerases α , δ and ϵ (Cheng and Kuchta, 1993; Glover, 2006), induces the majority of fragile sites (Sutherland, 1991) including FRA16B/FRA16C (Zlotorynski et al., 2003), which co-localize with breakpoints within *CBFB*. 2-AP is a non-specific inhibitor of ataxia telangiectasia and Rad3-related (ATR) kinase (Sarkaria et al., 1999; Dimitrova and Gilbert, 2000), a protein required for fragile site maintenance (Casper et al., 2002), and significantly

increases fragile site breakage up to 20-fold in combination with APH (Casper et al., 2002). Berenil was also used because it is the most common inducer of fragile site FRA16B (Sutherland, 1991). The concentrations and incubation times were chosen because they are optimal for the detection of fragile site breakage. Induction of fragile sites was confirmed by cytogenetic analysis following treatment (Supplementary Fig. 2). Distribution plots of measured distances revealed no obvious differences between untreated and APH-treated HSCs (Fig. 2). Statistical analysis did not show any significant differences in mean distances for treated HSCs (Table 1) or in the proportion of chromosomes with *CBFB* and *MYH11* at different distance intervals compared to untreated cells (Fig. 3). Furthermore, there was no difference in the frequency of co-localization between untreated and treated HSCs (Table 2). These results show that treatment with fragile site-inducing chemicals does not significantly affect the distance between *CBFB* and *MYH11* in interphase nuclei of HSCs.

DISCUSSION

The high occurrence of chromosomal aberrations in cancer has prompted a closer investigation into the molecular basis of structural rearrangements in tumor cells. Here, we found statistically significant differences between *CBFB* and *MYH11* interphase distances in HSCs compared to human MSCs, PBLs and fibroblasts. The data also demonstrate significant differences in the percentage of chromosomes at different distance levels. In HSCs, *CBFB* and *MYH11* are separated by 1.99 μm or less in 61.43% of chromosomes, whereas in MSCs, PBLs and fibroblasts, *CBFB* and *MYH11* are separated by 1.99 μm or less in 26.67%, 28.10% and 39.05% of chromosomes, respectively (Fig. 3). In contrast, distances greater than 2 μm were similar or less frequent in HSCs compared to other cell types, indicating that at lengths $>2 \mu\text{m}$, the spatial proximity of *CBFB* and *MYH11* is dominated by random influences. These results suggest that spatial genome organization is cell-type specific and support a role for reduced interphase distances between translocation-participating loci in the formation of chromosomal rearrangements (Kozubek et al., 1999; Neves et al., 1999; Nikiforova et al., 2000; Roix et al., 2003; Roccato et al., 2005).

As an additional control, the distance between *CBFB* and 16p11.2, a non-translocation participating locus was measured to determine whether a close physical relationship exists between *CBFB* and *MYH11* during interphase in HSCs. Although 16p11.2 is located ~ 37 Mb away from *CBFB* during metaphase ($\sim 70\%$ of the distance between *CBFB* and *MYH11*), the mean interphase distance was significantly higher than that between the *CBFB* and *MYH11* genes, which are separated by ~ 50 Mb. These results suggest that chromosome 16 may form a loop structure during interphase, bringing some loci in close proximity while separating others. Therefore, translocation-participating loci are in close physical proximity during interphase which may promote the formation of translocations, and supports the hypothesis that spatial genome organization is a contributing factor in the generation of chromosomal rearrangements.

Since differences in cell size could potentially have an effect on spatial gene proximity, separation distances between *CBFB* and *MYH11* were also determined as a fraction of the nuclear diameter. We found that the mean *CBFB-MYH11* distance in HSCs ($16.03 \pm 9.85\%$) was not significantly different compared to other cell types (Supplementary Fig. 3). However, HSCs have the smallest average diameter of the cells examined (Supplementary Fig. 3). These results suggest that nuclear size may be a factor in cell-type specific spatial genome organization, and overall support the notion that spatial gene proximity plays a role in the formation of chromosomal translocations and is likely the reason why certain chromosomal aberrations are associated with specific tissues in human tumors (Meaburn et al., 2007). It is also important to note that the methodology utilized herein involves hypotonic swelling of cells followed by fixation, which is known to exaggerate interphase

distances. Therefore, the distances reported here may not reflect the true interphase separation of these genes in vivo. However, during harvest, attached cells were first trypsinized and then processed in the same manner as cells grown in suspension, allowing for an accurate comparison between cell types.

Normal HSCs were treated with fragile site-inducing chemicals to evaluate the role of chromosome breakage caused by fragile site expression on the spatial proximity of *CBFB* and *MYH11*. Previous studies have shown that DNA damage caused by γ -radiation can significantly reduce the proximity of genes in interphase (Kozubek et al., 1997), most likely due to chromatin remodeling, and rearrange the positioning of chromosome territories (Folle, 2008). Based on these results, we hypothesized that treatment with fragile site-inducers would reduce the distance between *CBFB* and *MYH11* in interphase nuclei. Interestingly, there was no significant difference between the average distances in untreated HSCs versus treated HSCs, or in the percent distribution of chromosomes at different distance intervals. Furthermore, the frequency of co-localization between *CBFB* and *MYH11* did not change upon treatment (Table 2). These data suggest that treatment with agents causing mild replication stress does not have as great an effect on chromosome mobility as irradiation. One explanation for this difference is that ionizing radiation produces DNA double-strand breaks whereas chemicals used for the induction of fragile sites likely only cause single-strand breaks or gaps (Casper et al., 2002). Double-strand breaks may be more mobile, making it possible for other ends to join.

The conserved nature of spatial genome organization across species and the specificity of whole chromosome and gene locations within the nucleus point to a significant role in the mechanism of chromosomal rearrangements. As the molecular basis of these events is being elucidated, two models have been proposed to explain how chromosomal translocations occur. According to the breakage-first theory, DNA damage occurs at two separate locations. Broken DNA ends then “scan” the nuclear space for potential translocation partners that are subsequently joined together (Savage, 2000; Aten et al., 2004). This model suggests that chromosome ends are able to move over large distances within the nucleus, searching for other DNA breaks. The contact-first model predicts that structural rearrangements take place after DNA damage occurs at a site where genes co-localize within the nucleus (Savage, 1993; Savage, 2000). In this model, broken chromosome ends are expected to have limited ability to move within the nuclear space. Evidence for both models exists, although more recent data favor the contact-first model as the underlying mechanism in the formation of chromosomal translocations (Neves et al., 1999; Nikiforova et al., 2000; Roix et al., 2003; Meaburn et al., 2007). The results presented in this study support the contact-first model of translocations since *CBFB* and *MYH11* were significantly closer with respect to one another in HSCs compared with other cell types, and this distance did not change following treatment with fragile site-inducing chemicals. However, additional studies will be necessary to make a more definitive determination of which model is most relevant in AML.

We initiated these studies in an effort to better understand the molecular basis of chromosomal translocations and to define a potential mechanism for the formation of *inv(16)(p13q22)* in AML. The interphase distance between participating genes *CBFB* and *MYH11* was examined based on a growing body of evidence that supports a role for spatial gene proximity in the generation of chromosomal rearrangements found in cancer. Our results are consistent with studies of other gene rearrangements, demonstrating that *CBFB* and *MYH11* are located within close proximity in HSCs, and support the concept that spatial proximity of translocation-prone loci may favor gene rearrangements. Furthermore, the highly significant difference between *CBFB-MYH11* distances in HSCs compared with MSCs, PBLs and fibroblasts is in agreement with the fact that spatial genome organization is

tissue-specific. Mild replication stress did not affect *CBFB-MYH11* distances, perhaps because the damage was not significant enough to alter chromatin structure or produce ends able to search for a potential partner. Overall, the data presented in this study provide further evidence of a role for spatial gene proximity in the formation of cancer-specific chromosomal translocations and offer new insight into the mechanism of *inv(16)(p13q22)* in AML.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Two-color FISH of normal human HSCs with the *CBFB* probe (RP11-5A19, orange) and *MYH11* probe (RP11-585P8, green). Nuclei showing two distinct hybridization signals per probe were selected for interphase distance analysis, assuming that the two shorter distances were between loci on the same chromosome. Scale bar = 4 μ m.

Figure 2. Distribution of measured interphase distances between *CBFB* and *MYH11* loci in HSCs (untreated), MSCs, PBLs, fibroblasts, and treated HSCs, $n = 210$.

Figure 3.

Percentage of chromosomes with *CBFB-MYH11* located at 0–0.99 μm, 1.00–1.99 μm, 2.00–2.99 μm, 3.00–3.99 μm and 4 μm apart. Distances were measured in HSCs (untreated), MSCs, PBLs, fibroblasts, and treated HSCs. The percentage of chromosomes was determined from each range of distances (0–0.99 μm, light gray; 1.00–1.99 μm, black; 2.00–2.99 μm, white; 3.00–3.99 μm, medium gray; 4 μm, dark gray), and results were compared using the chi-square test to determine statistical significance.

TABLE 1Analysis of Measured *CBFB-MYH11* Interphase Distances

Cell type	Mean $\pm$ SD (in μm)	p value ^a
HSCs	1.90 $\pm$ 1.14	N/A
MSCs	3.78 $\pm$ 2.67	2.24 $\times 10^{-18}$
PBLs	3.50 $\pm$ 2.34	1.09 $\times 10^{-11}$
Fibroblasts	3.19 $\pm$ 2.39	1.09 $\times 10^{-11}$
Treated HSCs	2.07 $\pm$ 1.35	0.146

^aSignificance compared to HSCs. n=210

TABLE 2Frequency of *CBFB* and *MYH11* Co-localization

Cell type	number of co-localized gene pairs	% co-localization ^a
HSCs	8	3.81
MSCs	0	0
PBLs	6	2.86
Fibroblasts	2	0.95
Treated HSCs	8	3.81

^aPercentage of chromosomes in which the measured distance between *CBFB* and *MYH11* = 0.00 μm . n= 210