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STRUCTURAL INSTABILITY OF HUMAN RIBOSOMAL RNA GENE CLUSTERS

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ABSTRACT OF DISSERTATION

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2010
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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine, Graduate Center for Toxicology at the University of Kentucky

By
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Lexington, Kentucky
2010

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ABSTRACT OF DISSERTATION

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The human ribosomal RNA genes are critically important for cell metabolism and viability. They code for the catalytic RNAs which, encased in a housing of more than 80 ribosomal proteins, link together amino acids by peptide bonds to generate all cellular proteins. Because the RNAs are not repeatedly translated, as is the case with messenger RNAs, multiple copies are required. The genes which code for the human ribosomal RNAs (rRNAs) are arranged as clusters of tandemly repeated sequences. Three of four catalytic RNAs are spliced from a single transcript. The genes are located on the short arms of the five acrocentric chromosomes (13, 14, 15, 21, and 22). The genes for the fourth rRNA are on chromosome 1q42, also arranged as a cluster of tandem repeats. The repeats are extremely similar in sequence, which makes them ideal for misalignment, non-allelic homologous recombination (NAHR), and genomic destabilization during meiosis, replication, and damage repair. In this dissertation, I have used pulse-field gel electrophoresis and in-blot Southern hybridization to explore the physical structure of the human rRNA genes and determine their stability and heritability in normal, healthy individuals. I have also compared their structure in solid tumors compared to normal, healthy tissue from the same patient to determine whether dysregulated homologous recombination is an important means of genomic destabilization in cancer progression. Finally, I used the NCI-60 panel of human cancer cell lines to compare the results from the pulsed-field analysis, now called the gene cluster instability (GCI) assay, to two other indicators of homologous-recombination-mediated genomic instability: sister chromatid exchange, and 5-hydroxymethyl-2’deoxyuridine sensitivity.

KEYWORDS: Non-allelic homologous recombination (NAHR), genomic instability, cancer, DNA repair, ribosomal RNA

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DISSEETATION

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LIST OF ABBREVIATIONS

DSB: Double strand break
NHEJ: Non-homologous end joining.
HDR: Homology-directed repair
HR: Homologous recombination
SDSA: Synthesis-dependent strand annealing
BIR: Break-induced replication
DSBR: Double strand break repair
ssDNA: Single-stranded DNA
D-loop: Displacement loop
HJ: Holliday junction
CNV: Copy number variation
SD: Segmental duplication.
NAHR: Non-allelic homologous recombination
CGH: Comparative genomic hybridization
GCI: Gene cluster instability
rRNA: Ribosomal RNA
NOR: Nucleolar organizing region
rDNA: Ribosomal DNA
CHEF: Clamped homogenous electric fields
PACE: Programmed autonomously controlled electrodess
FIGE: Field inversion gel electrophoresis
PARP: Poly(ADP-ribosose) polymerase
BER: Base excision repair
SCE: Sister chromatid exchange
SSB: Single strand break
BrdU: 5-bromo-2'-deoxyuridine
FPG:  Fluorescence plus Giemsa
UV:  Ultraviolet
DAPI:  4’,6-diamidino-2-phenylindole
EtOH:  Ethanol
KBP:  Kilobase pairs
MGP:  Megabase pairs
CHAPTER 1: BACKGROUND AND INTRODUCTION

1.1 HOMOLOGOUS RECOMBINATION

In mammals, DNA double strand breaks (DSBs) arise by a variety of mechanisms. During meiosis, they are generated by Spo11, which facilitates the alignment of parental homologues [Baudat F et al 2000; Keeney S and Neale MJ 2006; Fledel-Alon A et al 2009]. They occur in mitotic cells as a result of stalled or collapsed replication forks or replication fork barriers during DNA synthesis. Single strand breaks which are not repaired prior to synthesis also generate DSBs when the affected region is replicated. DSBs can also result from exogenous damage such as ionizing radiation or chemicals which generate inter-and intrastrand crosslinks or covalently bound adducts [reviewed in Ohnishi T et al 2009].

DSBs are repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR), depending on a variety of factors, including when the DSB occurs [Shrivastav M et al 2008]. Briefly, NHEJ involves excision of DNA to generate complementary ends which are ligated together. Although it is the most common and efficient means of repairing a DSB, NHEJ is not error-free. Some DNA is excised during this type of repair as the two ends are generated, causing a small loss of genetic information [Weterings E and Chen DJ 2008; Mao Z et al 2008; Rothkamm K et al 2003].

HDR, on the other hand, is considered to be error-free, and involves either the borrowing of genetic information by one sister from the other, or the actual physical exchange of DNA between sisters in order to effect the repair [Richardson C et al 1998]. In this chapter, I will explore eukaryotic HDR, some of what is known of the human genetics involved, and the consequences of malfunctioning or dysregulated repair, paying particular attention to mitotic recombination and the potential for genomic instability and cancer. There are numerous reviews on this subject, as evidence of somatic HDR and its importance has emerged over the last dozen years [Moynahan M and Jasin M 2010; Helleday T 2010; Reliene et al 2007].

The nomenclature associated with HDR is confusing and sometimes inconsistent in the literature. A few initial clarifications are necessary. For the purposes of the work
presented here, I consider a “double-strand break” as any two-stranded DNA molecule that is discontinuous within a chromosome, including, as previously mentioned, a replicated region of DNA which originally contained a single-break. A DSB does not have to possess frank blunt ends. Strictly speaking, the DSBs that arise by Spo11 during meiosis are physiologic, rather than pathologic, and referring to those breaks as “damage” is not accurate. They are resolved by homologous recombination (HR), however.

An exploration of meiotic versus mitotic HR and the differential regulation of each is not germane to the data presented here, and thus HR may be referred to as “repair” within the context of either mitosis or meiosis. I consider homology-directed repair (HDR) as any repair of a DSB which utilizes homologous sequence, whether from the sister, the parental homolog, or non-allelic and not necessarily homologous location. Pathways of HDR include synthesis-dependent strand annealing (SDSA), homologous recombination (HR) with and without crossing over, single-strand annealing (SSA), and break-induced replication (BIR).

In the literature, HR with and without crossing over is often referred to as “canonical DSB repair,” and the term “homologous recombination” is sometimes used to describe any form of HDR. For the sake of clarity, in this work, HR refers to the canonical DSBR pathway of HDR, specifically the repair of a DSB by means of double-Holliday junction formation and resolution which may or may not result in crossing over and the physical exchange of DNA between sisters, homologues, or non-allelic sequences.

E. coli and S. cerevisiae are convenient organisms in which to study HR; and much of the modeling and biochemistry in the literature is based on experimentation in yeast and bacteria, although regulation of HDR and its pathways is obviously quite different in more complex organisms such as humans. Although the experiments and results presented are conducted entirely in human subjects or human cancer cell lines, it is impossible to avoid using S. cerevisiae references to generate the necessary context in which to interpret this data.
The initial steps are common to the two major mammalian HDR pathway models, SDSA and HR. First, the DSB is recognized and processed to create single-stranded DNA with a 3'-hydroxyl overhang. In mammals, this is performed by the MRN complex which tethers the free ends to keep them near each other and, in the case of post-replicative repair, near the identical sister [Lavin MF 2004; Moreno-Herrero F et al 2005; Hopfner KP et al 2002]. The 3’ overhangs are coated by RPA [Wang X and Haber JE 2004]. This process is not yet fully characterized in humans or other mammals, but is known to involve CtIP, BLM, BRCA1, and EXO1, as well as other unidentified proteins [Reviewed in West SC 2003; Hartelrode AJ and Scully R 2009]. It is thought to be regulated by cyclin-dependent kinase 1 (Cdk1) which restricts the mechanism to the S and G2 phases [Ira G et al 2004]. RPA prevents the ssDNA from forming secondary structures which would prevent HDR from proceeding [Sung P et al 2003]. RPA is subsequently displaced and, in a reaction mediated in mammals by BRCA2, RAD51 is loaded onto the single-stranded DNA (ssDNA) to generate a protein-coated filament [San Filippo J et al 2006].

Next, the RAD51-coated filament invades the homologous duplex DNA and displaces the identical strand, generating a displacement loop (D-loop). Although NHEJ repairs the vast majority of DSBs during G1 and early S, RAD51 knockout is lethal in mice, giving some indication of the critical role RAD51-catalyzed HDR for genome stability and survival [Rothkamm K 2003]. Either SDSA or HR ensues thereafter, depending upon whether or not the D-loop, which migrates, captures the second free DNA end. About 50% of the time during meiosis, and 90% of the time during mitosis, the second free end is not captured by the D-loop, resulting in the SDSA pathway being preferred, yielding repair without cross-over [Bzymek M et al 2010]. These findings are not unexpected, given the fact that during meiosis, the chiasmata associated with cross-overs help to ensure appropriate segregation and are thus permitted [Hunt PA and Tassold TJ 2002]. During mitosis, however, inappropriate cross-overs may potentiate genomic instability and are therefore very infrequent in non-malignant dividing cells.
In the SDSA model, once the RAD51-coated filament has invaded the duplex DNA, a single Holliday junction (HJ) is formed and migrates as new DNA is synthesized along the homologous template (Figure 1.1). At some point following extension, the invading strand is displaced and reanneals with the end from the original broken molecule.

In the HR model, sometimes referred to as canonical DSBR, the second free end is also captured by the D-loop, resulting in the formation of two Holliday junctions (Figure 1.1). Synthesis takes place at both of the free ends, using both strands of the unbroken homologous duplex as template. The Holliday junctions can migrate along the DNA, either extending or decreasing the size of the region of heteroduplex DNA (annealed duplex DNA comprised of two strands of DNA from different origins). It is this double-Holliday junction DNA conformation which presents the possibility for the physical exchange of DNA known as crossing-over. If these HJs are “dissolved” by BLM-Topo III, no cross-over product is generated. If the HJs are “resolved”, in a process which involves cleavage of DNA, a cross-over product may result [Bzymek M et al 2010; Wu L and Hickson ID 2003; Colavto S et al 2010]. As we have noted previously, this is a very rare occurrence in mitotic cells, but a consequential one. It is this pathway, HR with cross-over, with which we are concerned. The data presented in this work explores the results of dysregulated or erroneous cross-over.

Single-strand annealing (SSA) is another HDR pathway in humans and other mammals but is distinct from HR and SDSA in that it is not catalyzed by a RAD51-coated filament-mediated strand invasion. SSA requires regions of repeated sequence flanking the DSB. Once the 5’ ends are resected, regions of homology are exposed and aligned, followed by removal of non-homologous ends, DNA synthesis, and ligation. The genetic information between the repeated regions is lost during this process [Pastink A et all 2001].

Break-induced replication (BIR), as it occurs in yeast, is a means for restarting collapsed replication forks and maintaining telomeres. In both cases, there is no second end for annealing after repair. Repeated rounds of strand invasion and synthesis must
occur before replication can be restarted and processivity restored. BIR requires large regions of homology which are not consistent with the organization of human DNA. Also, because both strands of the donor are used in order to provide the template for extending from the one-ended recipient, BIR in humans could potentially be a source of gross chromosomal rearrangements, and is thought not to be favored for this reason [Smith CE et al 2007].

HDR repair processes must be tightly regulated to maintain genomic stability, and defects associated with HDR pathways are associated with genomic instability and cancer in humans. BRCA1 and BRCA2 mutations are perhaps the most well known of these defects. BRCA1 is a component of several complexes at crucial steps of HDR, including damage response, cell cycle checkpoint progression, and actual repair of the DSB [reviewed in O’Donovan PJ and Livingston DM 2010]. The role of BRCA2 in HDR is less varied than that of BRCA1, but its activity is not well defined. What is known is that RAD51 recruitment for HDR requires BRCA2, and that it also assists with RPA displacement and RAD51 loading and stabilization of the filament [Moynahan ME et al 2001; San Fillipo J et al 2006]. Tumors which manifest defects in either BRCA1 or BRCA2 demonstrate chromosomal rearrangements and aneuploidy characteristic of loss of genomic stability [Kraackman-van der Zwet M et al 2002; Huen MS et al 2010]. BLM is another HDR-associated protein with defects associated with cancer. Bloom’s syndrome is an autosomal recessive disease in which both copies of the BLM protein are inactivated. These patients develop a variety of cancers very early in life [German J 1997]. Cells with inactivated BLM show 10-fold higher number of sister-chromatid exchanges, which are thought to arise as a result of dysregulation of cross-over HR. These cells also show structural instability in the ribosomal RNA gene clusters. BLM, a member of the RecQ helicase family, is involved in migration and resolution of Holliday junctions, and effectively functions to suppress cross-over during HR repair [Wu L and Hickson ID 2003; Killen MW et al 2009]. A great deal remains to be elucidated regarding HDR components and regulation. However, it is apparent at this point that HDR, including HR, is vital for cell viability and genome stability. HR-associated defects in cell
cycle regulation, checkpoints, or damage recognition may allow proliferation with misrepaired DSBs, leading to genomic instability. At the other end of the spectrum, a hyperactive or dysregulated recombination mechanism may also lead to genomic instability, as is the case with BLM, where cross-overs are no longer suppressed.

We have recently shown evidence of extensive cross-over-mediated structural instability in solid tumors from clinical cancer patients. Approximately 50% of patients show rearrangement of ribosomal RNA gene clusters resulting in amplification or deletion of repeat units. These results indicate that dysregulated recombination, at least with regard to the ribosomal RNA gene clusters, is a fairly common feature of solid tumors and begins early in tumor progression [Stults DM et al 2009].

1.2 NON-ALLELIC HOMOLOGOUS RECOMBINATION, SEGMENTAL DUPLICATION, AND COPY NUMBER VARIATION

It has recently become apparent that human genomic structural variation, specifically copy number variability is an important characteristic of the human genome, and that the resulting dosage differences and positional effects likely contribute as much, if not more, than single nucleotide polymorphisms to normal phenotypic variation [Freeman JL et al 2006; Feuk L et al 2006; Redon R et al 2006; Goidts V et al 2006]. A copy number variation (CNV) is defined as a copy number change in a region of greater than 1 kilobase [Feuk L et al 2006]. CNVs can be tandem duplications or involve gains or losses of homologous sequence from disparate regions of the genome [Redon R et al 2006]. Several groups have pursued identification of CNVs through various experimental approaches tailored to focus on CNVs of particular size or characteristics. We now know that CNVs are ubiquitous throughout the genome, often occurring in gene-rich regions. According to one group, two individuals could vary in total genome size by as much as 9 Mb, including as many as 1,500 genes [Wong KK et al 2007].

CNVs are often located at or flanked by segmental duplications (SDs) [Iafrate AJ et al 2004; Sharp AJ 2005; Sebat J et al 2004]. These duplications, also called low copy
repeats (LCRs), are regions of >1 kb which bear >90% sequence identity [Eichler EE et al 2004]. About 5% to 15% of the human genome is believed to be comprised of SDs [Bailey JA and Eichler EE, 2006; Sharp AJ et al 2006; Gibcus JH et al., 2007; Darai-Ramqvist E et al., 2008; Gu W et al., 2008; Mefford HC and Eichler EE 2009]. They may be scattered throughout the genome across many chromosomes, arranged in clusters of tandemly or inverted oriented repeats, or both. Their repetitive nature is inherently confounding to genome assembly strategies, making them difficult to map and subsequently grossly underrepresented by the early Human Genome Project [Sharp AJ et al 2005; Eichler EE 2006; Bailey JA et al 2002].

Such sequences are hotspots for meiotic non-allelic homologous recombination (NAHR). When homologous recombination with crossing-over occurs between homologous sequence in a non-homologous context, such as misalignment of tandemly repeated sequences, unequal crossing-over occurs and results in duplication, deletion, translocation, or inversion of sequence (Figure 1.2) [Shaw CJ and Lupski JR 2004; Lupski JR and Stankiewicz P 2005]. Several heritable genetic syndromes are known to arise by this mechanism, including 22q11.2 deletion syndrome, Charcot-Marie-Tooth syndrome, hereditary neuropathy with liability to pressure palsies, Williams-Beuren syndrome, Prader-Willi syndrome, Angelman syndrome, and Smith-Magenis syndrome [Stankiewicz P and Lupski JR Trends 2002]. NAHR is also believed to be a basis for primate speciation and variation within the human species [Bailey JA and Eichler EE 2006]. In 2004, Barbouti et al showed that i(17q), a common structural abnormality in human neoplasias, likely arises by NAHR, which indicates a role for NAHR in somatic, as well as meiotic rearrangements (Barbouti A et al 2004).

Comparative genomic hybridization (CGH) has been used to identify CNVs and to specifically uncover regions of segmental duplication. Recently, this array-based approach has yielded three previously unknown loci associated with pathogenic copy number changes in clinical cytogenetics patients [Rudd MK et al 2009]. However, sensitivity is limited to a 3-fold change in copy number, and this technique does not address the genomic architecture at a given locus that gives rise to the change [Redon R
et al 2006; Sharp AJ et al 2005. For clusters of repeated SDs, where there may be hundreds of copies of the repeat present at a single chromosomal region, CGH does not have the capacity to evaluate whether expansion or contraction by a single repeat is occurring or determine the heritability or relative stability of these regions. We have therefore developed an assay, the gene cluster instability (GCI) assay (described in chapter 2) to specifically characterize the physical genomic structure of repeat clusters and evaluate the level of NAHR-mediated instability from both the meiotic and mitotic perspective.

1.3 HUMAN RIBOSOMAL RNA GENES

This dissertation represents the use of human ribosomal RNA genes to explore human genomic instability that arises from NAHR in SDs. Each eukaryotic ribosome contains four RNA molecules which together do the catalytic work of translation and peptide bond formation. They are housed in two subunits comprised of more than 80 proteins. Because these single, individual RNAs are not repeatedly translated, multiple copies of the ribosomal RNA (rRNA) genes are required in order to generate sufficient rRNA molecules to accomplish all of the ongoing protein translation required for metabolism and viability in a eukaryotic cell. In S. cerevisiae, 60% of the total RNA is rRNA [Ide S et al 2010; Doudna JA and Rath VL 2002].

In humans, the genes that code for three of the four catalytic ribosomal RNAs are located on the short arms of the five pairs of acrocentric chromosomes (13, 14, 15, 21, and 22) [Henderson AS 1972]. The genes are contained within tandemly arranged SDs, repeated units of 43.3 kb each (OMIM 180450-180454) [Caburet S et al 2005; Schofer C et al 1998; Worton RG et al 1998]. In the nucleus, the short arms of the acrocentric chromosomes which contain these genes are arranged surrounding the nucleolus, in a region called the nucleolar organizing region (NOR). Their isolation allows them to be transcribed and regulated separately from the rest of the genome. They are transcribed by Pol I to form a single, 45S transcript, which is subsequently spliced into the 18S, 28S, and 5.8S rRNAs. [Reviewed in Bartova E et al 2010]. The size
and extreme sequence identity of the ribosomal DNA (rDNA) repeats prevents them from being properly assembled and mapped by the Human Genome Project, and as a result, the rDNA clusters are one of the largest remaining components of the human genome with no representation in the Human Genome Project. The total number of rDNA repeats per diploid human cell, based on work from 1972, is estimated to be about 400 copies [Henderson AS 1972].

Early quantitative hybridization studies indicated that the size of the clusters, though polymorphic in a population, is heritable [Guanti G and Petrinelli P 1974]. This idea was subsequently substantiated by silver staining; though the technique yields results which are a function of both DNA quantity and transcriptional activity [Miller OJ et al 1976; Markovic VD et al 1978]. Although it has been demonstrated that the rDNA clusters are subject to interchromosomal and intrachromosomal recombination, the degree of variability and stability of the clusters in the human population has not been directly determined [Arnheim N et al 1980; Worton RG et al 1988; Kuick R et al 1996; Gonzalez IL and Sylvester JE 2001].

The genes for the fourth catalytic rRNA, called the 5S rRNA, are located on chromosome 1q42. Like the 45S genes, they are arranged as clusters of tandemly repeated units. Each repeat is 2.2 kb in length [Sorensen PD et al 1990]. According to the Human Genome Project (build 36.2), the 5S rDNA cluster contains 17.2 tandemly repeated units (OMIM 180420). Although the unit repeats are smaller, enabling the chromosomal context to be determined, the size of the cluster cannot be accurately predicted by the shotgun approach utilized by the Consortium, and thus the estimation of 17.2 repeats is very likely a substantial underestimation. Prior to sequencing of the human genome, one group estimated that each array was approximately 200 kb in length and contained 90 tandem repeats of the 2.2 kb unit [Little RD and Braaten DC 1989]. Grain count analysis of seven individuals using an 125I-labeled 5S RNA probe showed differences indicating the region is likely polymorphic for copy number [Henderson AS et al 1980]. The 5S rRNA genes are located in the nucleoplasm away from the rest of the rDNA in the NOR. They are transcribed by Pol III, rather than Pol I
[Bartova A et al 2010]. I undertook structural characterization of these genes in my experiments with healthy human subjects to determine their stability and heritability, but they were not included in later experiments with clinical cancer patients or human cancer cell lines.
FIGURE 1.1: REPAIR OF DOUBLE STRAND BREAKS BY DSBR AND SDSA


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Aa) Recombination between homologous sequence in upstream and downstream repeats which flank gene A results in duplication of gene A on one chromosome and deletion of gene A on the other. b) Recombination between homologous sequence on different chromosomes results in translocation. c) Recombination between homologous sequence from repeats which are inverted with respect to one another results in inversion of the intervening sequence.

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Physical characterization of the human rRNA gene clusters was achieved by pulsed-field gel electrophoresis using a CHEF Mapper (Bio-Rad) system followed by in-blot Southern hybridization with 5S and 45S rDNA-specific 32P-radiolabeled DNA probes. The main theoretical concepts behind these techniques are described below.

Standard agarose gel electrophoresis does not resolve DNA fragments of greater than 15kb to 20kb. A single ribosomal 45S rDNA repeat is 43.3kb, and available literature predicts that the individual clusters may contain dozens of repeats in head to tail fashion. Fragments of this size are exponentially beyond the capacity of conventional electrophoretic separation. The same is true for the 5S cluster. Although a single repeat, at 2.2kb, would easily be resolved by conventional means, a single cluster contains a minimum of 35kb according to by the Human Genome Project, and is very likely much larger.

Pulsed-field gel electrophoresis technology was developed to overcome the limitations of conventional electrophoresis. Manipulating the direction of the electrical field, as well as the duration of the field, allows large fragments, up to 10 Mb, to be fractionated. Rather than moving straight through the gel toward the opposite pole, manipulating and pulsing the electrical field allows the DNA to alter speed and direction, allowing the smaller fragments to become separated from the larger ones [Schwartz DC and Cantor CR 1984].

Bio-Rad’s CHEF-Mapper XA system was used to accomplish these experiments. The system uses CHEF (clamped homogeneous electric fields) and PACE (programmed autonomously controlled electrodes) technology. It is comprised of a gel box which contains 24 electrodes arranged in a hexagon. They are controlled by a computer which allows six parameters to be manipulated: the angle of the electrical field in 5 degree increments, strength of the field, switching of time at any given field, ramping of the switching time as a linear, concave or convex hyperbolic function, and total run time.
The computer also allows for programming of blocks of completely independent sets of conditions. Field inversion (FIGE) is also an option, where the direction of the field changes by 180 degrees, and the field strength is alternated. A free-standing chiller unit is connected to a sensor in the box, such that the temperature of the buffer can be maintained during long run times. Having such a great degree of control over how the DNA migrates through the gel allows for optimal resolution of any given size range between 50 kb and 10 MB, and it is even possible to “zoom in” on a particular range, providing maximum separation in a predetermined area [Bio-Rad].

Our gene cluster instability (GCI) assay involves isolating very high molecular weight DNA (10 Mb) from human lymphocytes, solid tissue, or cultured cells. This is accomplished essentially by suspending intact cells in agarose, and using Proteinase K and detergent to digest away proteins and lipids, leaving only nucleic acid lying relatively undisturbed to minimize mechanical shearing [Jackson DA and Cook PR 1985; Cook PR 1984]. Live cells are quantified using a Partec bench-top flow cytometer and acridine orange staining, in order to ensure that there is a standard concentration of DNA per mL of agarose. DNA isolation by digestion is an overnight procedure, followed by several rinses to dilute away the Proteinase K and detergent. In final isolation step, agarose DNA suspension is equilibrated in glycerol to prevent it from freezing and splintering when stored at -20 C.

Next, the ribosomal RNA genes, either 45S or 5S, are released from genomic DNA by restriction enzyme digest. I use an enzyme which does not have a predicted restriction site within a single repeat. Since the sequence of the repeats is highly conserved, there is usually not a restriction site in any of the subsequent repeats; and thus, the entire cluster is liberated. Enzyme digestion of approximately 1 µg of DNA in agarose is accomplished overnight at 37 C. Depending on the efficiency of the enzyme, 10 to 20 units of enzyme in 200 µl of appropriate buffer is usually sufficient for complete digestion of an approximately 12 µl agarose “slice” which contains about 1 µg of high molecular weight genomic DNA.
Digested DNA undergoes separation by pulsed-field gel electrophoresis on the CHEF Mapper system described above, altering the programmed parameters in order to provide optimal resolution at the desired size range. Total run times are usually between 20 and 48 hours.

After electrophoresis, gels are soaked in ethidium bromide to stain and provide visualization of a standard DNA ladder, which indicates whether the electrophoresis was successful. Very large fragments of DNA present a technical challenge at this stage of the protocol, as well, since they do not migrate easily out of the gel and onto a customary membrane for Southern blotting. Our approach involves leaving the DNA in the agarose gel and dehydrating it [Tsao SG 1983; Purrello M and Balazs I 1983]. Glycerol is added to the ethidium bromide soak, to prevent the gel from shattering as it is dried at 65 C in a hybridizing oven. Once dehydrated, the gel is very thin and supple [Son M et al 1990; Leuders KK and Fewell JW, 1994; Ehtesham NZ and Hasnain SE 1991].

Conceptually, Southern blotting proceeds from this point much as it would under conventional circumstances. A 32P-labeled, human rDNA specific probe to a region in either the 5S or the 45S sequence, is amplified by PCR, and added to a carafe containing hybridization buffer and the rehydrated gel. Hybridization of the probe is accomplished overnight at 65 degrees, followed by several rinses of varying stringency to wash away the remaining probe and minimize background signal. The gel is exposed on a PhosphoImager cassette and visualized by PhosphoImager scanner.

In order to ascertain the rate of rDNA gene cluster instability in a given cell line, a subcloning process is undertaken prior to DNA isolation. Cultured cell suspension undergoes serial dilution plating in order to form colonies derived from a single cell. Plates are grown up from the colonies and DNA is isolated. The “parental” plates are also subjected to another round of serial dilution to grow up colonies from single cells. About a dozen of these “subclone” colonies are grown up into plates and DNA is isolated. By comparing the electrophoretic GCI karyotype of each of the subclones in comparison to the parents and to the other subclones, we can establish the rate at which the size of the rDNA gene clusters is changing (Figure 2.1).
1. Cultured cell suspension undergoes serial dilution plating in order to form colonies derived from a single cell. 2. Plates are grown up from the colonies and DNA is isolated. 3. The “parental” plates are also subjected to another round of serial dilution to grow up colonies from single cells. About a dozen of these “subclone” colonies are grown up into plates and DNA is isolated.

By Michael W. Killen, University of Kentucky College of Medicine, Department of Microbiology, Immunology, and Molecular Genetics. Used with permission.
CHAPTER 3: DIFFERENTIAL FLUORESCENCE PLUS GIEMSA STAINING OF SISTER CHROMATIDS

3.1: SYNOPSIS

A method is presented for measuring homologous-recombination mediated sister chromatid exchange. After undergoing two rounds of division in the presence of 5-bromo-2’-deoxyuridine, cells are dropped onto microscope slides to generate metaphase spreads. Fluorescence plus Giemsa staining is used to differentially stain sister chromatids. Recombination-mediated physical exchange of DNA between sisters is easily visualized and quantified by brightfield microscopy.

3.2: INTRODUCTION

Homologous recombination (HR) is a mechanism for repairing double-strand breaks (DSBs). In contrast to non-homologous end joining (NHEJ), which results in a loss of genetic material, homologous recombination is considered error-free repair because it uses the available, identical sequence from the sister chromatid to repair the DSB. HR is preferred for repairing the DSBs that arise at stalled or collapsed replication forks or from replication at single strand breaks and cross-links [Rothkamm K et al 2003]. Inhibition or deficiency of poly(ADP-ribose) polymerase (PARP), which is involved in the base excision repair (BER) pathway causes accumulation of single-strand breaks but also increases levels homologous recombination [Schultz N et al 2003; Pachkowski BF et al 2009]. PARP inhibitors can induce synthetic lethality in cells with mutations in the tumor suppressors BRCA-1 or BRCA-2, which are components of the HR pathway [Kyle S et al 2008; Ashworth A 2008]. Mitotic HR is a complex, varied, and tightly regulated process, and defects in several of the components of HR have long been associated with cancer [reviewed in Reliene R et al 2007; Helleday T 2010].

The most established means of detecting dysregulated homologous recombination, whether in cells with defective/deficient HR capacity, or in response to damage, is the sister chromatid exchange assay (SCE) which differentially stains sister
chromatids, allowing for microscopic detection of the physical exchange of DNA which occurs with cross-over HR [Wilson DM and Thompson LH 2007]. The SCE assay has been in use since the 1970s for the purpose of identifying potential “chromosomal mutagenicity” of chemical agents [Perry P and Evans HJ 1975]. Chemicals that generate interstrand crosslinks, such as mitomycin C, are potent inducers of SCE, since HR is required to repair the resultant blockage during replication [Thompson LH 2005]. Conditions and drugs which increase the number of single-strand breaks (SSBs) also increase the number of SCEs, presumably by overburdening the base-excision repair (BER) pathway such that unrepaired SSBs remain, become DSBs during replication, and elicit repair by homologous recombination [Wilson DM and Thompson LH 2007]. Mutation or knockdown of BLM, which is involved in double-strand break repair, causes increased levels of recombination as well as a 10-fold elevation in sister chromatid exchange [Killen MW et al 2009].

The protocol described below utilizes 5-bromo-2’-deoxyuridine (BrdU) incorporation and fluorescence plus Giemsa (FPG) staining to make exchanges between sister chromatids visible [Perry P and Wolff S 1974; Wolff S and Afzal V 1996]. BrdU is a nucleotide analog which resembles thymidine and is efficiently incorporated into replicating DNA. Since DNA replication is semiconservative, after BrdU has been made available to cells, it is incorporated as the nascent strand is elongated. After two rounds of replication, paired sister chromatids in the four daughter cells differ in the amount of BrdU each contains. The sister with the original template has one strand of normal DNA and one strand with BrdU. Both of the strands from the other sister contain BrdU. Subsequent binding of the ultraviolet (UV) light sensitive Hoechst 33258 dye to DNA, followed by UV light exposure, causes “bleaching” of DNA which is proportional to the amount or incorporated BrdU in the double-stranded molecule. Subsequent staining with Giemsa makes the differential bleaching apparent by light microscopy (Figure 3.1 and figure 3.2) [Wilson DM and Thompson LH 2007].

Other methods for measuring sister chromatid exchange following differential BrdU incorporation involve fluorescent imaging and the use of either acridine orange in
place of UV treatment followed by Giemsa staining [Yankiowski V et al 2001], or an anti-BrdU antibody with either propidium iodide or DAPI counterstain for bulk DNA [Wilson DM and Thompson LH 2007; Pinkel D et al 1985]. Rapid photobleaching is a potential pitfall, however, especially with the use of acridine orange, and fluorescent filters are required. We have achieved excellent results with optimized FPG staining (described in this work), and prefer the convenience of brightfield microscopy and the permanence of this staining method, including the possibility for repeated and extended viewing of the same spread.

This protocol has been tested in a wide variety of human cancer cell lines which have been immortalized to undergo unlimited rounds of replication. It has not been tested by us in primary cell lines, plant cells, or non-cultured tumor cells extracted from donors. It is not effective for terminally differentiated cells which are not actively dividing due to the requirement for differential BrdU incorporation.

3.3: MATERIALS, REAGENTS, AND STOCK SOLUTIONS
THAWING CRYOPRESERVED CELLS

1. Latex or nitrile gloves
2. 10-cm tissue culture plates (or tissue culture flasks for non-adherent cells).
3. Well characterized line adherent or non-adherent mammalian cells.
4. Tissue culture incubator
5. Laminar flow hood with standard tissue culture setup, including serological pipettes, micropipettes, and vacuum aspiration apparatus.
7. Tissue culture medium such as Dulbecco’s Modified Eagle medium or RPMI 1640
8. 95% reagent grade ethanol.
9. Fetal bovine serum
10. L-glutamine/Penicillin/Streptomycin 100X solution.
11. Plasmocin 25 mg/mL (for mycoplasma prophylaxis)
STOCK SOLUTIONS

Complete culture medium: Medium appropriate for in vitro growth of cells. We typically use MEM or RPMI supplemented with 5% to 10% fetal bovine serum, 1% Pen-Strep and L-glutamine, and 1:10,000 of Plasmocin 25 mg/mL.

70% Ethanol: 95% Reagent grade ethanol diluted to 70% with sterile distilled water.

GROWING CELLS AND ESTABLISHING DOUBLING TIME
1. Trypsin/EDTA (0.05% Trypsin, 0.53 mM EDTA).
2. 15-mL conical centrifuge tubes
3. Clinical centrifuge.

ADDING 5’-BROMO-2’-DEOXYURIDINE
1. 5-bromo-2’-deoxyuridine (BrdU) MW 307.1. Available from Fisher Bioreagents, Catalog #: BP2508250
2. Sterile distilled water
3. Aluminum foil

STOCK SOLUTIONS

10 mM BrdU stock aliquots: make 200 µl aliquots and store at -20 C. NOTE: BrdU MUST BE PROTECTED FROM LIGHT AT ALL TIMES IN ORDER TO AVOID UV-LIGHT MEDIATED FREE RADICAL DECOMPOSITION. The 10mM stock solution should be made in the dark and stored in 1.5 mL Eppendorf tubes which have been covered with aluminum foil.

ARRESTING CELLS IN METAPHASE
Colcemid (demecolcine solution 10 µg/mL in HBSS, available from Sigma, catalog # D1925)
HARVESTING CELLS FOR METAPHASE SPREADS

1. Ca\(^{2+}/\)Mg\(^{2+}\)-free phosphate-buffered saline (PBS): 200 mg/L KCl, 200 mg/L KH\(_2\)PO\(_4\), 8g/L NaCl, 2.16 g/L Na\(_2\)HPO\(_4\)-7H\(_2\)O (Available commercially from Invitrogen as D-PBS 1X liquid, catalog #: 14190-144 )
2. Potassium chloride
3. Sodium Citrate
4. Methanol
5. Glacial acetic acid

STOCK SOLUTIONS

Hypotonic Solution: 46.5 mM KCl / 8.5 mM Na\_2\cdot Citrate

3:1 methanol/acetic acid fixative: Add 1 volume glacial acetic acid to 3 volumes of reagent grade ethanol. MAKE FRESH BEFORE EACH USE!!

PREPARING AND STORING SLIDES FOR METAPHASE SPREADS

1. Microscope slides
2. Glass Coplin jars or other glass container suitable for holding a rack with at least a dozen slides.
3. Slide racks and containers for staining, such as EasyDip™ slide staining system (available from ISC Bioexpress, as well as other suppliers), which allows for easy transfer of an entire rack of slides from one solution to another.
4. Refrigeration 4 C for chilling and holding slides.

MAKING METAPHASE SPREADS

1. Small rubber suction bulbs.
2. 5” glass Pasteur pipettes
3. Slide warmer with adjustable temperature.

DIFFERENTIAL STAINING SISTER CHROMATIDS WITH GIEMSA

1. Hoechst 33258 98%, available from Acros Organics, catalog #: 229891000
2. Na$_2$HPO$_4$
3. KH$_2$PO$_4$
4. NaCl
5. UV light source: Two 20-watt blacklight bulbs (We use F20T10BLB/RS from Sanyo-Denki.
6. Shaker, hybridization oven, or warm room which can be maintained at 50 C.
7. Concentrated Giemsa stain solution (50% Giemsa in methanol and glycerin), Available from Acros Organics, catalog #: AC612051250.

STOCK SOLUTIONS

1mg/mL Hoechst 33258 in H$_2$O (protect from light), stored at 4 C. Note: Hoechst 33258 works equally well when diluted in PBS. However, it is much less soluble in phosphate buffers than in water.

Sorensen buffer: 0.1 M, pH 6.8: mix equal volumes 0.1 M Na$_2$HPO$_4$ and 0.1 M KH$_2$PO$_4$

20X SSC: 3M NaCl and 300 mM sodium citrate

10% Giemsa in Sorensen buffer: Add concentrated Giemsa stain solution to Sorensen buffer such that the concentration of Giemsa stain solution (not total concentration of Giemsa itself) comprises 10% of the total volume. It is convenient to mix this 100 mL at a time and store in a 100 mL glass bottle protected from light. The stain solution can be poured back into the bottle after staining and reused several times.

PREPARING SLIDES FOR VISUALIZATION

1. Cytoseal-60 low viscosity mounting medium
2. Coverslips for slides: 24 x 50 x 1 mm.

3.4: METHODS

THAWING CRYOSPRESERVED CELLS

Wear latex or nitrile gloves. Wipe down the working surface of the laminar flow hood with 70% EtOH. Add 8 mL of tissue culture medium to a 10-cm tissue culture dish
or flask for non-adherent cells and warm for 5 minutes in the tissue culture incubator to equilibrate temperature and pH.

Remove a vial of cryopreserved cells from liquid nitrogen freezer. In the laminar flow hood, thoroughly wipe down the outer surface of the vial and gloves to limit bacterial contamination. Unscrew the cap of the cryovial to equalize gas pressure inside the vial with normal atmospheric pressure. Screw the lid back down to seal the vial. Hold the vial in your gloved hand to rapidly warm the contents until partially thawed. Remove the tissue culture plate or flask from the incubator and place it in the tissue culture hood. When the cells are sufficiently thawed that the still frozen portion moves freely in the tube, unscrew the cap and dump the entire contents onto the tissue culture plate. Quickly replace the lid to the tissue culture plate and GENTLY agitate the plate so that the frozen pellet thaws and is evenly distributed in the pre-warmed medium. Place the plate in the humidified tissue culture incubator and leave overnight at 37 C and 5% CO₂. Non-adherent cells should be stored in the incubator with the flask cap in place but not screwed tight, to permit appropriate gas and humidity equilibration.

FOR ADHERENT CELLS: The next day, vacuum aspirate the medium to remove the cryopreservtative and dead cells. Replace with room-temperature complete culture medium.

FOR NON-ADHERENT CELLS: The next day, remove the cells and medium from the flask to a 15-mL conical tube and centrifuge at 200 x g for 5 minutes to pellet the cells. Aspirate medium and replace with fresh, pre-warmed medium. Add 1 mL and pipette up and down to disburse, then add 7 more mL. Transfer cell suspension to a new flask and put back in the incubator.

FOR ADHERENT CELLS: Visualize the plate with 10X objective inverted microscope to estimate survival. Cells should be checked under the microscope at least once a day to determine the morphology of a healthy, dividing culture.

FOR NON-ADHERENT CELLS: Flasks or plates may also be viewed with dissecting microscope. A healthy non-adherent culture will have perfectly round surface and
bright, glowing edges on phase contrast. Dead cells and debris is generally irregularly shaped and opaque. Trypan blue, and propidium iodide exclusion techniques are also commonly used to determine the viability of a non-adherent culture.

EXPANDING THE POPULATION AND ESTABLISHING DOUBLING TIME

Cells usually need at least one day to recover from the thawing and begin to divide. Depending on how many cells survived freezing, expansion may be required in concert with removal of dead cells and cyropreservative above; or, depending upon the health of the culture and the number of cells originally frozen, cells may require a day or two before they are ready for expansion.

FOR ADHERENT CELLS: A culture is ready for expansion when it is at about 80% confluence on the plate. If, the day after thawing, there are only a few cells adhering to the plate, another vial should be thawed. It should not require more than a day or two after thawing for cells to reach 80% confluence. Ideally, at least 50% of the frozen cells should survive thawing.

FOR NON-ADHERENT CELLS: The medium will begin to change color from peach to yellow as the pH becomes more acidic; the result of metabolic wastes being released into the medium. For a healthy culture, the medium will begin to turn during the first 48 hours after thawing. Upon microscopy, the population of healthy cells should have significantly increased in proportion to the debris observed upon thawing. Medium that is still peach-colored two days after thawing indicates that most or all of the culture is dead. Check for viability via propidium iodide or trypan blue staining. It may be necessary to thaw another frozen stock, or put cells into a smaller volume container (such as 6-well plate) to concentrate the suspension, as some cell lines fare better when grown in a dense culture. Expand the population by dividing the dense culture into five plates, wells, or flasks, and adding warmed complete medium to the appropriate volume.

FOR ADHERENT CELLS: Once cells have reached 80% confluence they are ready for expansion. Aspirate medium and add 2 mL of 0.05% Trypsin/EDTA to detach cells.
Trypsin works by cleaving the cadherin proteins that attach cells to the plate and one another. After about 2 minutes, the bottom of the plate will appear a little cloudy to naked-eye visual inspection, indicating that cells are beginning to “ball up” and disengage themselves from the plate. This is visible under the microscope, as cells become circularized and show brightly glowing edges on phase contrast microscopy. Detachment time may vary depending upon how tightly the cells adhere to the plate, but should not be more than 10 minutes. Once cells have begun to ball up, tap the plate gently on the side of the hood or carefully but firmly shake it to detach the cells. Tilt and swirl the plate to coax the cells off of the bottom of the plate.

Once cells are completely detached, add 5 mL complete culture medium including at least 5% FBS to “stop” the action of the trypsin/EDTA. Leaving cells for an extended period of time in Trypsin/EDTA is undesirable and decreases cellular viability. Using a 5 mL serological pipette, remove the cells/trypsin/medium to a 15 mL conical centrifuge tube and centrifuge for 5 minutes at 200 x g to pellet the cells.

During centrifugation, prepare five tissue culture plates with 7 mL each of room temperature complete culture medium.

Use vacuum apparatus to aspirate the medium overlaying the centrifuged cell pellet, taking care not to aspirate the pellet itself. Leave a small volume of medium on top of the pellet in order to minimize the risk of aspirating the pellet.

Using a P1000 variable volume pipette (e.g. Pipetman), add 1 mL of fresh complete culture medium and gently pipette up and down to break up and resuspend the pellet. Add 4 mL of medium and mix by inverting in the conical tube.

Pipette 1 mL of resuspended cells onto each of the five plates and swirl to evenly distribute. Each of the plates should contain approximately the same number of cells. While the cells are still in suspension, BEFORE they have had a chance to settle to the bottom of the plate, remove a few µl from at least three of the plates and determine the initial concentration using a hemocytometer or benchtop flow cytometer with volumetric counting to count live cells. The concentration values should not be very far apart. Average them to approximate the initial concentration in each of the plates. This
is merely to get an idea of the number of cells on each plate and verify that there is equal distribution amongst the plates.

After 24 hours, use trypsin/EDTA to harvest the cells from ONE of the plates as before as a quantification control. Count the number of cells from this plate by methods given above. Record this value as the concentration at T0 for the remaining uncounted plates. Remember that it takes the cells several hours to recover from plating. The initial concentration should not be used as the value for T0.

The remaining plates should be harvested and counted at convenient intervals less than 24 hours, such as +18 hours, +36 hours, +48 hours, and +60 hours. Record the concentrations for each of these times and plot them on a curve to determine how quickly the cells are dividing. Calculate the amount of time required for cells to reach a population of approximately twice the concentration at T0. You will use this doubling time to determine when to harvest after BrdU has been added. The process for establishing doubling time is inexact. The objective is to get a general idea, within a few hours, of how quickly the cells are dividing, in order to optimize the differential incorporation of BrdU. Most immortalized human cells double about once every 24 hours in culture and adequate results may therefore be obtained by adding BrdU 48 hours before harvest. Some may proliferate substantially more rapidly or more slowly. The effects of having added BrdU too soon or too late will not be apparent until the procedure is complete, however; and so, especially for a new or unfamiliar cell line, it is worth investing the time to establish how fast a particular cell line is actually dividing in culture under your specific laboratory conditions.

**ADDING BRDU FOR INCORPORATION**

After having established the doubling time for the cell line of interest, split cells into a fresh plate for growth at approximately 10% to 20% confluency.

When (ADHERENT) cells appear to be 80% confluent, split and expand the culture by dividing the cells evenly across five plates prepared as described above. Incubate overnight at 37 C in the tissue culture incubator to allow the cells to recover.
FOR BOTH ADHERENT AND NON-ADHERENT CELLS: The next day, thaw an aliquot of 10 mM stock of BrdU. The tube should be protected from direct light while it is thawing and throughout the procedure. Turning off the nearest room lights and the fluorescent lamp in the laminar flow hood is usually sufficient.

Add BrdU to each of the four plates at final concentration of 20 µM (1/500 dilution from 10 mM stock). Gently swirl the medium to hasten the even distribution of BrdU. Cover the plates LOOSELY with aluminum foil to block out ambient light and place in the incubator.

The fifth plate from the expansion should remain in culture until it is 80% confluent. It may be then be frozen. Alternatively, the fifth culture may be carried until the procedure is complete and satisfactory results have been obtained, in order to avoid the delay from having to thaw, grow, and split a new culture in the event of failure.

ARRESTING CELLS IN METAPHASE

Cells should be harvested after two rounds of cell division. Approximately 4 hours before harvest is scheduled, add Colcemid to 0.02 µg/mL final concentration and swirl to distribute. This should be performed using aseptic technique in a laminar flow hood, in the dark, as above. Replace the aluminum foil and return the plates to the incubator. In general, about 5% of an actively dividing culture is in metaphase at any given time. Colcemid works by inhibiting mitotic spindle formation, preventing cells from segregating chromosomes. The prescribed concentration of Colcemid in the medium is sufficient to arrest cells in metaphase and prevent them from continuing through the cell cycle. The length of time in the presence of Colcemid allows for enrichment of the percentage of the population of cells in metaphase by arresting and accumulating cells as they reach metaphase. Adding a higher concentration of Colcemid WILL NOT increase the number of cells in metaphase. The amount of time the cells are left in Colcemid will influence the number of metaphases, however, and should be adjusted depending on how quickly the cells divide. The longer the time in Colcemid, the higher the percentage of cells in metaphase will be. However, if cells are arrested in
metaphase by the Colcemid for too long, the chromosomes will condense and shrink somewhat, rendering them suboptimal for staining and scoring later in the procedure. We have found that for a cell line which doubles approximately every 24 hours, 4 hours in Colcemid is sufficient for enriching the number of metaphases with minimal numbers of shrunken chromosomes.

HARVESTING CELLS FOR METAPHASE

FOR ADHERENT CELLS: Remove cells from the incubator and aspirate medium as usual. Add 2 mL 0.05% Trypsin/EDTA, wait for cells to detach fully, and harvest by adding back complete medium followed by centrifugation to recover a cell pellet, as usual. Note: Some investigators prefer to enrich for metaphase cells from adherent cultures by using dilute trypsin/EDTA to slow the detachment process so that cells can be recovered by agitation as they begin to ball up. Theoretically, the metaphase cells will preferentially round up on the plate and become gently mechanically detachable first, enabling non-metaphase cells to be left behind. Many protocols include this step for preferential harvest of cells in metaphase. We do not recommend this method, since we find it difficult to perform reproducibly, and to generally result in a much smaller yield without significantly increasing the proportion of metaphases. By this method, the overall size of the pellet is reduced by 80% or 90%, and even though the pellet may contain a much larger percentage of cells in metaphase, the pellet itself becomes physically difficult to work with and is easily lost during repeated rinses in fixative and/or dropping onto slides. By harvesting the entire plate, the non-metaphase cells add volume to the pellet, making it easy to work with. Using four confluent plates of cells and a dense suspension eliminates the need for further metaphase enrichment by preferential harvest.

FOR NON-ADHERENT CELLS: No harvest with Trypsin/EDTA is necessary. Centrifuge cell suspension to pellet and proceed with adding hypotonic solution as described below.
Aspirate medium. Gently flick the sides of the tube with your finger to mechanically loosen the pellet and then add 1 mL hypotonic solution. Pipette up and down gently with a P1000 pipetman to completely and homogenously resuspend the pellet in the hypotonic solution.

Add 7 more mL hypotonic solution, mix by gentle inversion, and place in the incubator for 12 minutes. The hypotonic solution causes water to diffuse into cells by osmosis, making them swell. This change in morphology is visible under the microscope. The amount of time is approximate. About 12 minutes is usually sufficient. Leaving the cells in hypotonic solution for too long will cause delicate cells to lyse.

Add 2 mL freshly prepared 3:1 methanol/acetic acid fixative and invert the tube a few times to mix and fully resuspend cells. If cells have lysed due to an overlong exposure to hypotonic solution, it will be apparent at this time, since DNA released from lysed cells will appear “gooey” and will not resuspend.

Centrifuge for 5 minutes at 200 x g to pellet the swollen, partially fixed cells. Remove the hypotonic/methanol/acetic acid by aspiration, taking care not to aspirate the pellet.

Add 5 mL of 3:1 methanol/acetic acid fixative and resuspend the pellet. Cells will appear opaque white and the pellet will only require gentle inversion of the tube for complete resuspension after this step. The protocol may be stopped at this point and cells stored in the fixative solution at 4 C indefinitely.

PREPARING AND STORING SLIDES FOR METAPHASE SPREADS

We find that a very slight “roughening” and degreasing of the slide surface with dilute HCl helps cells adhere better to the slide upon dropping. This process should be completed the day before dropping to ensure that slides are completely chilled. Fill a slide rack with new, dry microscope slides.

Completely immerse the slides in a solution of 0.1 N HCl in 95% EtOH at room temperature for 20 minutes.

Remove the rack and completely immerse in a container filled with 95% EtOH.
Remove the rack, dump the EtOH, replace with fresh 95% EtOH, and immerse slides. Repeat for a total of three rinses in EtOH.

Follow with three rinses in fresh distilled H$_2$O.

Store slides completely submerged in distilled H$_2$O at 4°C. It takes several hours for the water and slides to chill to 4°C. Although the process may be hastened by chilling in a -20°C freezer, there is a risk for freezing, container breakage, and slide breakage. We find it most convenient to prepare the slides at least a day in advance of dropping. Unused slides can be stored indefinitely, submerged in distilled H$_2$O, at 4C.

MAKING METAPHASE SPREADS

Use 3:1 methanol/acetic acid fixative made fresh on the day that cells are to be dropped onto the slides. According to some protocols this procedure works best on wet days, when the room is air particularly humid due to the manner in which atmospheric conditions affect the speed of evaporation of the fixative solution on a microscope slide. We have not found normal room air humidity to be problematic. This procedure works well at normal humidity, 28% to 35%. It does seem to work better, even on sunny days, if the room temperature is slightly high, around 27°C.

Centrifuge swollen/fixed cells at 200 x g for 5 minutes, aspirate fixative, resuspend in 5 mL fresh fixative.

Repeat for a total of three aspirate/resuspend/centrifuge cycles.

Resuspend cells in the minimal amount of fixative that causes the suspension to have a slightly translucent, milky appearance similar to looking through Scotch tape.

Remove a slide from the container of chilled water. Prop one end on a disposable 10 mL serological pipette so that it angles slightly downward.

Attach a rubber suction bulb to a 1 mL glass Pasteur pipette with a 5” barrel. Insert the pipette into the cell suspension and squeeze the bulb to draw up the cells. Position the end of the pipette about 5 inches above the slide and quickly distribute 7 to 10 drops of cell suspension along the length of the still-wet slide. NOTE: These steps
should be done as rapidly as possible, to ensure that the slide is still very wet with
distilled water when the cells are dropped onto it. It may be helpful to fill the pipette
with cell suspension and rest it in the centrifuge tube. Remove the slide from the H₂O
container with one hand and rest it on the serological pipette. IMMEDIATELY drop the
cells using the other hand.

Allow most of the liquid to pool at the bottom of the slide, and quickly follow
with 7 or 8 drops of 3:1 methanol/acetic acid fixative.

Once this has also pooled at the bottom of the slide, blot the excess liquid from
the bottom edge of the slide using a paper towel. Hold the slide about an inch from your
open mouth and exhale a single breath (don’t blow!), then fan the slide once back and
forth in the air. Set the slide vertically upright at approximately 90 degrees to the
benchtop, leaning against a vertical surface (we use an empty test tube rack).

After about 30 seconds, pick up the slide and watch for the fixative to begin to recede
from around the edges of the slide as it evaporates. When this occurs, place the slide on
a slide warmer at 42 C and allow to completely dry. If the slides are too dry, the
chromosomes will be too close to each other to allow for appropriate staining and
scoring. If the slides are too wet, individual chromosomes will float away from one
another and not form obvious spreads which can be visualized in a single microscope
field.

Repeat this process to make several slides from the cell suspension. The cells will
settle relatively rapidly in the tube. Handle them carefully, inverting gently to remix.
Vigorous pipetting will cause cells to rupture while still in the tube.

Once slides are dry (about 10 minutes on the slide warmer at 42 C), metaphases can be
located using a 25X objective on the inverted microscope. The vast majority of cells will
be round and intact (predominantly non-metaphase cells) but occasionally, there should
be easily visible, tiny, x-shaped chromosomes arranged in small spreads. Screen each
slide to ensure that there are at least twenty or thirty metaphases. Allow slides to dry
overnight in the open air at room temperature on the benchtop.
DIFFERENTIAL SISTER CHROMATID STAINING WITH GIEMSA

Immerse slides with metaphases in 10 μg/ml (1/100th volume of 1 mg/mL stock) Hoechst 33258 in water for 20 minutes. The Hoechst 33258 is a UV sensitizer.

Rinse by immersing slides in Sorensen’s buffer. Note: Some protocols call for using McIlvaine’s buffer at this step, but in our hands, this solution tends to precipitate and leave debris on the slide. We get acceptable quality results with the use of Sorensen’s throughout the procedure.

Remove each slide from the rack, quickly pipette a few drops of Sorensen’s buffer along the length of the slide, and immediately add a coverslip to prevent slides from drying out. DO NOT SEAL THE COVERSIP TO THE SLIDE.

Expose slides coverslip-side-up on a 55 C slide warmer to long wavelength (approximately 365 nm) UV light (for example, at a distance of 5-10 cm from two 20 watt blacklight-blue bulbs) for 20-30 minutes. Exposure to UV preferentially nicks DNA with more highly incorporated BrdU, preventing it from accepting Giemsa stain later in the procedure.

Carefully remove and discard the coverslips and place the slides back in an empty rack. Immerse the rack in 1X SSC and incubate for 1 hour at 50 C. We use one of the EasyDip containers immobilized in a shaker oven, but a regular hybridization oven or warm room would work equally well.

Remove the slides from SSC and immerse the slides in 10% Giemsa made up in Sorensen’s buffer for 30 minutes at room temperature.

Rinse by quickly immersing the slides in distilled H₂O and allowing them to drain on a paper towel. Two or three quick rinses in water may be required to remove the Giemsa solution, although it is not necessary for all of the Giemsa stain to be completely rinsed away. The slides will retain a purplish tint at the end of this procedure. Only rinse the slides sufficently to keep droplets of relatively concentrated Giemsa solution from collecting and drying on the slide surface, which would obscure the results.

Allow the slides to dry face up without a coverslip on the benchtop overnight at room temperature.
PREPARING SLIDES FOR VISUALIZATION

Place one or two drops of Cytoseal-60 low viscosity mounting medium onto slide and add coverslip. Ideally, slides should be left face-up overnight to allow the Cytoseal-60 to dry completely. Although the medium is characterized as fast-drying, we have experienced some difficulty with coverslip displacement when the slides were not allowed to dry completely before microscopy.

VISUALIZING AND DIGITAL IMAGING OF CHROMOSOMES

Observe SCE’s using brightfield microscopy and a 63x or 100x high-quality oil-immersion objective lens. A microscope with good optics is absolutely required. At a minimum, a 63x plan apochromatic oil-immersion objective lens with a numerical aperture of 1.4 is necessary. Otherwise, the differentially stained sister chromatids will fail to resolve, and the lighter staining chromatid will appear washed out to the point of invisibility. This assay is impossible with inferior quality optics. High-quality digital imaging equipment and interface is also essential for effective and efficient data acquisition. Adjust the exposure and gain settings to capture as optimal an image as possible. Use the microscope/camera’s imaging software and manufacturer’s instructions to save captured images as TIFF or high-quality JPEG files. Images may be saved as grayscale.

After capture and saving, open the image in image processing software such as Adobe Photoshop or Macintosh Preview and adjust the magnification, contrast, and grayscale levels to maximize the differential staining of the sister chromatids.

SCORING SISTER CHROMATID EXCHANGES AND ANALYZING DATA

Well-stained metaphase spreads are straightforward to score for sister chromatid exchanges. It is convenient to print a high-resolution hardcopy of the image file and score the number of exchanges and chromosomes on the paper printout, then input the numbers into a spreadsheet program such as Microsoft EXCEL. A properly differentially stained chromatid pair with no SCEs will appear as one black stripe (the
mono-strand BrdU-substituted sister) and one lighter gray stripe (the doubly BrdU-substituted sister). A single exchange is any time the dark and light staining on the chromatids switches places. The BrdU itself induces a low level of SCEs so it is rare to find an entire spread with no exchanges whatsoever. Count the total number of exchanges in the spread and also the total number of scorable chromosomes in a spread. If the change happens at the centromere, this may be due to the chromosome being twisted and does not represent a true sister chromatid exchange. Do not include these chromosomes. If the image is ambiguous and it is not possible to determine with reasonable certainty that an exchange has taken place (i.e. partially stained chromosome) do not include that chromosome in the count. Even very small chromosomes, if they show two distinctly differentially stained chromatids, should be included in the score. For each metaphase spread, divide the number of exchanges by the total number of scorable chromosomes in the spread. This is the number of exchanges per chromosome. Scoring 20 metaphases should provide a general idea of the level of stability. If there is a great deal of variability between the scores of each spread, 50 or even 100 metaphases may be required to provide adequate statistical power when assessing SCE differences between varying experimental conditions.

3.5: NOTES, TIPS, AND TROUBLESHOOTING

For non-adherent cells, omit the trypsin/EDTA steps entirely. Use 6-well plates or tissue culture flasks for growing non-adherent cells and establishing doubling time. Add BrdU and Colcemid per protocol, then centrifuge the entire culture, aspirate medium, and replace with hypotonic solution. Continue protocol steps as described.

Depending on membrane characteristics, some cell lines are more amenable to this procedure than others. It may be useful to practice making metaphase spreads using HeLa or K562 cells first.

If only one chromatid is visible after staining or if chromosomes appear extremely dessicated, try decreasing the UV exposure time. Overexposure to UV will
essentially obliterate the sister which contains BrdU, and render it invisible, rather than very lightly stained.

If both chromatids appear lightly stained, but there is no differentiation, or if there is only partial staining of one chromatid, decrease the amount of time between adding BrdU and harvest. Allowing more than two rounds of division in the presence of BrdU will cause incorporation into both of the sisters, preventing differential staining.

If both chromatids appear darkly stained, try increasing the time between adding BrdU and harvesting. Insufficient incorporation of BrdU will cause the sisters to accept Giemsa equally well, resulting in no differential staining.
FIGURE 3.1: BRDU INCORPORATION FOR FPG STAINING OF SISTER CHROMATIDS

First round of replication

1. Fully replicated chromosome with two double stranded sisters.
2. Sisters segregate into different cells.
3. Double stranded chromatids are replicated, incorporating BrdU, resulting in double stranded sisters with one normal and one BrdU-incorporated sister.

Second round of replication

4. Fully replicated chromosome with two double stranded sisters, each with one normal and one BrdU-incorporated strand.
5. Sisters segregate into different cells.
6. Double stranded chromatids are replicated, incorporating BrdU, resulting in one sister with one normal and one BrdU strand. The other sister has incorporated BrdU into both strands.

BLUE: Unsubstituted DNA strand. PINK: BrdU-substituted DNA strand
FIGURE 3.2: DIFFERENTIALLY STAINED SISTER CHROMATIDS IN A METAPHASE SPREAD

RED CIRCLES: True sister chromatid exchanges

BLUE CIRCLE: Change at the centromere. Not definitively arisen by SCE. Possibly chromosome is flipped at centromere. Changes at the centromere should not be
CHAPTER 4: GENOMIC ARCHITECTURE AND INHERITANCE OF HUMAN RIBOSOMAL GENE CLUSTERS *

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4.1: SYNOPSIS

The finishing of the Human Genome Project largely completed the detailing of human euchromatic sequences. However, the most highly repetitive regions of the genome still could not be assembled. The twelve gene clusters which generate the structural RNA components of the ribosome are critically important for cellular viability, yet fall into this unassembled region of the Human Genome Project. To determine the extent of human variation in ribosomal RNA gene content (rDNA), and patterns of rDNA cluster inheritance, we have determined the physical lengths of the rDNA clusters in peripheral blood white cells of healthy human volunteers. The cluster lengths exhibit striking variability between and within human individuals, ranging from 50 kilobase pairs (kbp) to over 6 megabase pairs (Mbp), manifest essentially complete heterozygosity, and provide each person with their own unique rDNA electrophoretic karyotype. Analysis of these rDNA fingerprints in multi-generational human families demonstrates the rDNA clusters are subject to meiotic rearrangement at a frequency greater than 10% per cluster, per meiosis. With this high intrinsic recombinational instability, the rDNA clusters may serve as a unique paradigm of potential human genomic plasticity.

4.2: INTRODUCTION

Each human ribosome contains four RNA molecules (rRNA) that play indispensable roles in all protein translation. In order to produce sufficient rRNA for the highly abundant ribosomes, the genes encoding the rRNA are represented genomically in multiple copies. The 5S rRNA molecule (OMIM 180420) is produced from a cluster of repeated 2.2 kbp genes [Little RD and Braaten DC 1989; Sorensen PD and Frederiksen S
Similarly, the remaining three rRNA molecules, 18S, 5.8S, and 28S, are produced by post-transcriptional processing of a 45S precursor transcript expressed from clusters of repeated 43 kbp genes [Gonzalez LL and Sylvester JF 1995] on maternal and paternal 13p12, 14p12, 15p12, 21p12 and 22p12 [Henderson AS et al 1972] (OMIM 180450 – 180454). Overall, the rDNA clusters consist of repeated individual rRNA genes abutting each other in a largely head-to-tail orientation, devoid of intervening non-rDNA sequences [Caburet S et al 2005; Little RD and Braaten DC 1989; Schofer C et al 1998; Shiels C et al 1997; Sorensen PD and Frederiksen S 1991].

The repetitive, clustered nature of the rDNA was noted in early quantitative hybridization studies [Schmickel RD and Knoller M 1977], and lead to the hypothesis that, “the number of rRNA genes, although a polymorphic character, is an inheritable attribute of [a] given nucleolar organizer.” [Guanti G and Petrinelli P 1974] This prediction was supported by tracking patterns of silver-stained acrocentric chromosomes in human families [Markovic, VD et al 1978; Taylor EF and Martin-DeLeon PA 1981], although the stain was noted to be a function of both rDNA quantity and transcriptional activity [Miller DA et al 1976]. Through analysis of sequence polymorphisms within individual rDNA repeat units, it was determined that there was strong recombination activity both intra-and interchromosomally within the rDNA, leading to concerted evolution of rDNA sequences and clusters [Arnheim N et al 1980; Gonzalez LL and Sylvester JE 2001; Kuick R et al 1996; Worton, RG et al 1988]. Nevertheless, direct biochemical evidence of meiotic rDNA cluster recombination and quantitation of rDNA within individual rDNA clusters has proven elusive. We present here the determination of individual rDNA cluster lengths, the corresponding degree of person-to-person variability, and patterns of meiotic rDNA cluster inheritance and recombination.
4.3: RESULTS

Our experimental approach is shown in Figure 4.1. Very high molecular weight genomic DNA is prepared from human peripheral blood [Birren B and Lai E 1993], and intact rDNA clusters are released from the bulk genomic DNA by restriction digestion. Since the individual rRNA genes are very highly self-similar [Gonzalez IL and Sylvester JE 2001; Sorensen PD et al 1990], liberating intact rDNA gene clusters is feasible, as a restriction enzyme without a recognition site in a single rDNA gene will typically lack a recognition site throughout the entire cluster. The intact clusters are separated by size in a pulsed field gel, and detected by hybridization with either a 5S or 45S rDNA-specific radiolabeled probe.

5S rDNA clusters (Fig. 4.2A) isolated from anonymous human blood donors demonstrate tremendous allelic diversity, with most of the donors showing two distinct 5S rDNA cluster lengths that clearly indicate differentially-sized 5S rDNA clusters between maternal and paternal 1q42. Indeed, we have yet to observe a person homozygous for 5S rDNA cluster lengths. Occasionally, as seen in donor #10, we resolve three 5S rDNA bands; the presence of an extra band is enzyme specific, and we infer that a chance polymorphism in one of the 5S repeats has coincidentally created this specific restriction site within the gene cluster.

The Human Genome Project [Consortium 2004] (build 36.2) shows 17.2 5S rDNA repeats on 1q42 with an overall sequence conservation of 99.6% [Clamp M et al 2004]. The Genome Project and other sources [Little RD and Braaten DC 1989; Sorensen PD and Frederiksen S 1991] give a unit 5S rDNA repeat length of 2236 base pairs. Using our 5S rDNA cluster length data and the lengths of the unit 5S rDNA repeat and non-rDNA flanking sequences [Consortium 2004], we directly calculated the number of human 5S rDNA repeats (Table 1) in a total of 27 human donors (18 shown in Fig. 4.2A and nine additional unrelated individuals from Fig. 4.3A). Our average 5S rDNA cluster length of 98 repeats is consistent with earlier hybridization studies [Sorensen PD and Frederiksen S 1991], however, the Human Genome Project's total of 17.2 repeats is over 2.3
standard deviations outside this value, likely as a result of the difficulty the Genome Project has in correctly assembling very highly self-similar sequences [She X et al 2004].

45S rDNA cluster lengths from the same anonymous donors (Fig. 4.2B) also exhibit striking person-to-person variability. Consistent with 45S clusters on the five pairs of human acrocentric chromosomes, our pulsed-field gel analysis revealed 8 to 10 bands per person. We assume that individuals showing less than 10 bands have yet additional bands larger than 10 Mbp that are incapable of entering a pulsed-field gel [Birren R and Lai E 1993], although complete loss of rDNA sequences on specific acrocentric chromosomes is an alternative possibility. The 45S rDNA cluster size variation is consistent with our 5S rDNA cluster data: the 45S rDNA clusters display distinct and independent size variation on parentally homologous chromosomes. The 45S rDNA clusters shown here range in length from approximately 70 kbp to over 6 Mbp. For a unit 45S rDNA repeat size of 43 kbp [Gonzalez IL and Sylvester JE 1995], these data indicate cluster sizes from a single 45S rDNA unit alone up to over 140 repeats. While the lack of resolution above 6 megabases precludes an exact determination of human total diploid 45S rDNA content, our results are consistent with the upper range of earlier hybridization experiments of approximately 600 copies per diploid human cell [Schmickel RD 1973]. In contrast to the 5S rDNA clusters on 1q42, there are currently no data from the Human Genome Project concerning 45S rDNA cluster lengths.

Beyond variation in cluster length between individuals, we observed other intriguing features of 5S and 45S rDNA architecture, including evidence of significant meiotic rearrangement. In our analysis of the 5S rDNA clusters from four different human families (Fig. 4.3B), it can be seen that the paternal 5S rDNA cluster of PYFI-02B was inherited with a meiotic rearrangement. We verified the presence of this de novo 5S rDNA cluster length, relative to the 5S clusters in PYFI-02B’s parents PYFI-10 and PYFI-20, with a panel of three different restriction enzymes (Fig. 4.3B). Commercial
short tandem repeat (STR) testing verified that PYFI-20 is indeed the biological father of PYFI-02B.

The higher resolution of the 5S rDNA clusters in Fig. 3b also revealed an additional faint band in PYFI-02B of intermediate length at approximately 2% of the intensity of the two major 5S rDNA cluster bands for this individual. The intensity of the bands is directly proportional to both the cluster length and the percentage of cells in the sample harboring a particular 5S rDNA cluster in these experiments. Therefore, we interpret this fainter band to be the product of a somatic recombination event occurring in the hematopoietic stem cell population during the embryogenesis of PYFI-02B, making the peripheral blood of PYFI-02B genetically mosaic for the 5S rDNA clusters. Given that three distinct restriction enzymes yielded the same banding pattern, we can rule out the possibility of partial digestion as a source of this band.

The 45S rDNA clusters display an even larger degree of both meiotic rearrangement and somatic mosaicism (Fig. 4.4A). There are clear examples of inherited rearrangement in five of the offspring (ARDO-10, PYFI-02B, VAGR02A, VAGR-02B, and THAE-02), as indicated by the bands enclosed in dotted circles. For example, VAGR-02B possesses a cluster of 4.6 Mbp that is 1.6 Mbp larger than the largest cluster from either his mother (VAGR-10) or father (VAGR-20). A similar expansion is observed in the 6 Mbp cluster of PYFI-02B that is at least 0.5 Mbp larger than the largest rDNA cluster in either of his parents. Since clusters of repeated genes are potentially susceptible to recombinational erosion by single strand annealing [Stark JM et al 2004], the ability to extend the cluster lengths through meiotic recombination may be evolutionarily important for maintaining a steady-state level of rRNA genes.

A high-resolution gel for the 45S rDNA clusters smaller than 1 Mbp in size (Fig. 4.4B) provides a superior view of this meiotic rearrangement. VAGR-02A has clusters of 625 kbp and 715 kbp (dotted circles) that are not found in either his mother (VAGR-10) or father (VAGR-20). Likewise, the 950 kbp cluster found in THAE-02 is not found in either of his parents (THAE-10 and THAE-20). Collectively, we observe 11 clear examples of 5S or 45S rDNA cluster meiotic restructuring in 5 of the 8 parent-child trios analyzed.
Similar to the 5S rDNA cluster analysis of PYFI-02B (Fig. 4.3B), minor bands arising from 45S rDNA cluster somatic mosaicism are also apparent (Fig. 4.4B). PYFI-10 shows the strongest minor banding with a 910 kbp length cluster at approximately 25% of the intensity of her major 45S rDNA bands in that size range. The VAGR-02A, VAGR-20, THAE-10, and THAE-02 lanes all possess minor bands ranging from 5% to 10% the intensity of their respective major banding patterns. It is possible the minor band in THAE-10 at 4.3 Mbp represents a 45S rDNA mosaicism that exists in some fraction of her germline, such that her son THAE-02 inherited this cluster in Mendelian fashion from her. In total, we can detect minor-intensity mosaic banding patterns in about 1/3 of the individuals tested.

4.4: DISCUSSION

Collectively, we observed one occurrence of 5S and ten occurrences of 45S rDNA gene cluster meiotic restructuring in 5 of 8 parent-child trios analyzed, for a total of 11 clearly identifiable events. Given a potential total of 96 different rDNA clusters (8 children, each with two 5S and up to ten 45S rDNA clusters), this yields an average recombination rate of 11% per generation per gene cluster (95% confidence interval [Agresti A and Coull BA 1998]: 6.4% to 20%). This rapid meiotic restructuring likely accounts for the present-day inter-person variability in both rDNA quantity and genomic organization. We cannot currently ascertain the relative degree to which this diversity is generated by recombination between rDNA clusters on non-homologous chromosomes, parental homologs or sister chromatids, although familial analysis of flow-sorted acrocentric chromosomes may be able to answer this question.

Molecular combing experiments indicate that the rDNA clusters largely consist of repeats oriented in a head-to-tail configuration, but also with a substantial fraction of individual repeats within any given rDNA cluster in the inverted or palindromic orientation [Caburet S et al 2005]. Intermolecular recombination between such inverted rDNA repeats would generate a dicentric chromosomal translocation product linked by an rDNA cluster, reminiscent of the Robertsonian translocations between the various
acrocentric chromosomes frequently observed in the human population. Actual Robertsonian translocations, however, almost invariably have fusion breakpoints not in the rDNA, but rather in satellite III DNA proximal to the centromere, with resulting complete loss of rDNA sequences [Page SL et al 1996; Sullivan BA et al 1996], and this is also true for dup(21q) chromosomes that account for 2% of Down syndrome patients [Antonarakis SE et al 1990]. Although there are scattered examples [Boyd LJ et al 2005], why rDNA recombination mediated acrocentric chromosome fusions are generally not observed is unclear. Conceivably, the greater distance between centromeres in such an rDNA acrocentric fusion, relative to the smaller inter-centromeric distance of satellite III fused Robertsonians, would allow both centromeres to retain activity and lead to fusion chromosome loss, whereas inactivation of one of the two Robertsonian centromeres allows stable chromosomal transmission [Page SL and Shaffer LG 1998].

The human 5S and 45S rDNA gene clusters are clearly capable of very rapid copy number evolution. Although there may be considerable flexibility in rRNA expression at the transcriptional level via epigenetic control [Kawasaki K et al 1992; Maye C et al 2006], for long-term maintenance of population equilibrium rDNA levels there must be underlying evolutionary pressures constraining the total rDNA copy number between specific minimum and maximum values. It is not intuitively obvious what the phenotype of an rDNA deficiency would be. Chicken strains in which rDNA levels are reduced to 56% of wild-type levels are viable [Delany, ME et al 1995], however, a reduction to 45% of wild-type rDNA levels results in teratogenic embryonic failure [Delany ME et al 1994]. We suggest that in humans, a partial rDNA insufficiency may manifest as bone marrow failure, similar [Ellis SR and Massey AT 2006] to the way in which ribosomal protein haploinsufficiency can cause Diamond-Blackfan anemia [Draptchinskaia N et al 2006]. We anticipate that further study of the rDNA gene clusters, which are fundamentally required for cellular metabolism yet are highly variable in copy number and exhibit significant instability, will provide further insight into the role of copy number variation in human genomic architecture and stability.
4.5: METHODS

High molecular weight genomic DNA was prepared in agarose from peripheral blood via ammonium chloride erythrocyte lysis (generally according to methods described in Birren and Lai [Birren B and Lai E 1993]) with a final concentration of 1x10^7 white blood cells per ml of 0.8% low-melting temperature agarose. Agarose/DNA slices of approximately 15 µl volume were digested with 50 units of indicated restriction enzymes (New England Biolabs) overnight at 37°C and subsequently sealed with low melting temperature agarose into gels for electrophoresis. All electrophoresis was performed using a CHEF Mapper® (Bio-Rad) system. Gels were subsequently processed according to Lueders and Fewell [Lueders KK I and Fewell JW 1994] and Ehtesham and Hasnain [Ehtesham NZ and Hasnain SE 1991] with minor modifications.

Hybridization probes:

5S rDNA: Human genomic DNA was PCR amplified using primers: 5’-gggctcgaggacaaaagtagcgcgaggtc and 5’-gggtctagacagccaccgggaaacag yielding a 489 bp fragment.

45S rDNA: Human genomic DNA was PCR amplified using primers: 5’-gggctcgagatttgggacgtcagcttctg and 5’-gggtctagagtgctcccttcctgag yielding a 532 bp fragment.

PCR products were subsequently digested with XhoI and XbaI, subcloned individually into pBluescript II SK-(Stratagene) and verified by DNA sequencing. XhoI / XbaI fragments from these plasmids were 32P radiolabeled using a Prime-It II kit (Stratagene).

Pulsed field gels:

Figure 4.2A: Genomic DNA digested with EcoRV. 1% Pulse Field Certified (PFC) agarose (Bio-Rad), 0.5x TBE (44.5 mM tris, 44.5 mM borate, 1 mM EDTA pH 8.0), 14C, 6 V/cm, 120° included angle, 3-90 second field switching with linear ramp, 24 hours total electrophoresis.
Figure 4.2B and 4.4A: Genomic DNA digested with EcoRV. 1% SeaKem Gold agarose (Cambrex), 1x TAE (40 mM tris, 40 mM acetate, 1 mM EDTA pH 8.0), 14C, 2 V/cm, 106º included angle, 5-40 minute field switching with linear ramp, 92 hours total electrophoresis.

Figure 4.3A: Genomic DNA digested with HindIII. 1% PFC agarose (Bio-Rad), 0.5x TBE, 14C, 6 V/cm, 120º included angle, 10-40 second field switching with linear ramp, 20 hours total electrophoresis.

Figure 4.3B: 1% PFC agarose, 0.5x TBE, 14C, 6 V/cm, sequence of electric field vectors relative to 0º as "forward": -60º, 180º, +60º, -60º, +60º, 180º, -60º, +60º, field durations: ±60º field vectors: 10 seconds, 180º field vectors: 5 seconds, 42 hours total electrophoresis. Pulse field parameters adapted from Clark et al. [Clark SM et al 1988].

Figure 4.3b: as Figure for Figure 4.2A, except using a "ramp factor" (Bio-Rad) of 0.357.

Gel post-electrophoresis processing and hybridization:

Gels were equilibrated to a final concentration of 0.5% glycerol in water, dried at 65C and subsequently rehydrated with two exchanges of distilled water. Rehydrated gels were denatured with 0.4N NaOH / 0.8 M NaCl followed by neutralization in 0.5 M Tris pH 8.0 / 0.8 M NaCl. Gels were pre-hybridized in 2x SSC / 7% SDS / 0.5% casein at 65C and then hybridized to radiolabeled probe sequences overnight at 65C in fresh pre-hybridization solution. Gels were rinsed at 65C with 2x SSC / 0.1% SDS for 30 minutes and then again with fresh solution for 60 minutes. Gels were then rinsed at 65C twice with 0.5x SSC / 0.1% SDS for two hours per rinse. Gels were finally rinsed briefly with 2x SSC at room temperature and imaged using a PhosphorImager® system (Molecular Dynamics).

Commercial STR allele testing: Dried blood spotted on FTA Micro Cards (Whatman) was analyzed by The Genetic Testing Laboratories, Inc. (GTL), 3655 Research
Drive, Las Cruces, NM 88003. We use simplified identifying labels for the PYFl family in the main text.

Human Subjects: All human subjects protocols were reviewed and approved by the Institutional Review Board of the University of Kentucky.
FIGURE 4.1: EXPERIMENTAL STRATEGY

Release of intact rDNA clusters from bulk genomic DNA. (Open rectangles)
Individual rDNA repeat units; (double line) non-rDNA genomic DNA; (arrows)
hypothetical restriction sites.

TABLE 4.1: HUMAN 5S RIBOSOMAL REPEATS (27 DONORS)

<table>
<thead>
<tr>
<th></th>
<th>Each haploid array</th>
<th>Diploid totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average no. of repeats</td>
<td>98</td>
<td>195</td>
</tr>
<tr>
<td>Standard deviation (repeats)</td>
<td>35</td>
<td>51</td>
</tr>
<tr>
<td>Fewest repeats observed</td>
<td>35</td>
<td>93</td>
</tr>
<tr>
<td>Most repeats observed</td>
<td>175</td>
<td>299</td>
</tr>
</tbody>
</table>
Eighteen healthy anonymous human donors are shown. (A) 5S rDNA gene clusters. (B) 45S rDNA gene clusters. Cluster lengths are based upon chromosome size standards from S. pombe, H. wingei and lambda phage concatemers wingei and S. cerevisiae and lambda phage concatemers.
A) Four families, “ARDO,” “PYFI,” “VAGR,” and “THAE” are shown. Relationship codes: (01) daughter, (02) son, (10) mother, (20) father, (40) maternal grandmother, (60) paternal grandmother, (70) paternal grandfather. Siblings of the same sex and generation are additionally designated “A,” “B,” in descending birth order. (Dotted oval) A band not represented in either parent. rDNA cluster lengths are measured in kilobases and calibrated using lambda phage concatemers. (B) PYFI family 5S rDNA structure at high resolution and contrast. Restriction enzymes used to liberate 5S gene clusters from bulk genomic DNA are shown above sets of family samples. (Dotted ovals) Bands not represented in either parent. (Square brackets) Bands of nonstoichiometric signal intensity. (Left) Lambda phage concatemer size references: $\lambda_3 = 145,506$ bp, $\lambda_4 = 194,008$ bp, $\lambda_5 = 242,510$ bp.
(A) Four families, “ARDO,” “PYFI,” “VAGR,” and “THAE” are shown. Relationship codes: (01) daughter, (02) son, (10) mother, (20) father, (40) maternal grandmother, (60) paternal grandmother, (70) paternal grandfather. Siblings of the same sex and generation are additionally designated “A,” “B” in descending birth order. (Dotted ovals) Bands not represented in either parent. (Square brackets) Bands of non-stoichiometric signal intensity. Cluster lengths are calibrated on chromosomal size standards from S. pombe, H. wingei, and S. cerevisiae and lambda phage concatemers. (B) High resolution. Note resolution of two bands at 600–700 kb in VAGR-02A with respect to the unresolved doublet in this size range in panel A.
CHAPTER 5: RECOMBINATION PHENOTYPES OF THE NCI-60 COLLECTION OF HUMAN CANCER CELLS

5.1: SYNOPSIS

Double strand breaks which arise during the S or G2 phase of the cell cycle are generally repaired by homologous recombination, which occasionally results in crossing over and the physical exchange of DNA between sister chromatids. Genes arranged in clusters of repeats are subject to structural rearrangement via this mechanism, as their size and extreme sequence identity generate the potential for exchange between misaligned repeats in a process known as non-allelic homologous recombination. Dysregulated homologous recombination is believed to be one mechanism of genome destabilization by which cancer cells may acquire selective advantage. The sister chromatid exchange assay is a well known technique for visualizing large-scale chromosomal rearrangements which arise by homologous recombination but cannot measure rearrangement at the submicroscopic level, where non-allelic homologous recombination occurs. We have developed an assay to measure structural instability in gene clusters, and have shown that the ribosomal RNA gene clusters demonstrate marked instability in human solid tumors. This work represents our use of the extensively characterized NCI-60 panel of human cancer cell lines to determine whether gene cluster instability assay correlates with the sister-chromatid exchange assay to reflect genomic instability arising from dysregulated homologous recombination.

5.2: INTRODUCTION

Genomic instability is a fundamental characteristic of most solid tumors and adult leukemias. The term encompasses a broad range of defects which arise by a variety of damaging events and/or mechanistic failures of individual DNA repair pathways. Whatever its source, genomic destabilization is believed to begin early in tumor progression, creating heterogeneity within a population of cells, and conferring, in concert with other events, a selective advantage to a given cell which dominates in
proliferation [Anderson GR 2001; Stoler DL et al 1999]. Instability may or may not continue as the tumor progresses. One means of genomic destabilization is defective or dysregulated homologous recombination.

Homologous recombination (HR) is a mechanism for repairing double-strand breaks (DSBs) during S and G2 phase of the cell cycle. In contrast to non-homologous end joining (NHEJ), which results in a loss of genetic material, homologous recombination is considered error-free repair because it uses the available, identical sequence from the sister chromatid to repair the DSB. Although NHEJ is capable of repairing frank DSBs during G2/M, HR is preferred, especially for repairing the DSBs that arise at stalled replication forks, for example from forks that encounter single strand breaks or cross-links [Rothkamm K et al 2003]. Mitotic HR is a complex, varied, and tightly regulated process, and defects in several of the components of HR have long been associated with cancer (reviewed in [Helleday T 2010; Reliene R et al 2007]). One study shows overexpression of several HR-associated genes in patients with non-small cell lung cancer [Saviozzi S et al, 2009]. Approximately 5% of the human genome is comprised of large repetitive elements called low copy repeats (LCRs), also known as segmental duplications, which possess sufficiently high sequence identity to cause structural genomic instability via non-allelic homologous recombination (NAHR) between regions of identical sequence but differing genomic context, resulting in insertions, deletions, and translocations ([Consortium IHG, 2004], reviewed in [Gu et al 2008]).

The most established means of detecting dysregulated homologous recombination, whether in cells with defective/deficient HR capacity, or in response to damage, is the sister chromatid exchange assay (SCE) that differentially stains sister chromatids, allowing for microscopic detection of the physical exchange of DNA which occurs with cross-over HR [Wilson DM and Thompson LH, 2007]. The SCE assay has been in use since the 1970s for the purpose of identifying potential “chromosomal mutagenicity” of chemical agents [Perry P and Evans HJ 1975]. Chemicals that generate cross-linking of DNA are potent inducers of SCE, since HR is required to repair the
resultant blockage during replication [Thompson LH 2005]. Conditions and drugs which increase the number of single-strand breaks (SSBs) also increase the number of SCEs, presumably by overburdening the base-excision repair (BER) pathway such that unrepaired SSBs remain, become DSBs during replication, and must be repaired by homologous recombination [Wilson DM and Thompson LH 2007]. HeLa cells with downregulated XRCC1, a key component in the base excision repair pathway, show a 1.7-fold increase in SCEs, and an almost 2-fold increase when methyl methansulfonate (MMS), a DNA methylating agent, is added [Fan J et al 2007]. Likewise, the thymidine analog 5-hydroxymethyl-2'-deoxyuridine (hmdUrd) at a 1 µM dose induces sister chromatid exchanges resulting in a 6-fold increase over background in Chinese hamster ovary (CHO) cells [Kaufman ER 1989], presumably through stimulation of BER activity. Inhibition or deficiency of poly(ADP-ribose) polymerase (PARP) also increases levels of sister chromatid exchange [Schultz N et al 2003]. Essentially, the HR pathway compensates at least partially, for the defects or inadequacy of the BER response. PARP inhibitors can induce synthetic lethality in cells with mutations in BRCA-1 or BRCA-2, which are components of the HR pathway [Ashworth A 2008; Kyle S et al 2008]. Similarly, hmdUrd (or 5-chloro-2'-deoxyuridine) exposure is synthetically lethal with loss of key BER components such as XRCC1 [Horton JK et al 2008].

Despite the striking visible result upon staining, sister chromatid exchange is genetically silent. It represents a very large scale physical relocation of genetic material which is the consequence of a cross-over recombination event; but there is no gain or loss of genetic information between two identical sisters. Presumably, these cross-overs happen at the submicroscopic level as well, which is where true genomic destabilization occurs. Our lab has developed an assay which measures non-silent, molecular level changes to genomic architecture by monitoring the stability of the length of gene clusters or tandemly repeated segmental duplications which are the result of NAHR [Killen MW et al 2009]. For this gene cluster instability (GCI) assay, we use the gene clusters which encode the 45S precursor transcript to the 18S, 5.8S and 28S ribosomal
RNA molecules. These clusters of tandemly repeated genes are divided amongst the five acrocentric chromosomes, generating approximately 600 copies of the 43kb unit gene [Henderson AS et al 1972]. We characterized the lengths of these rDNA gene clusters from healthy blood donors and found complete heterozygosity on each of the five chromosomes, and between the parental pairs of homologs. We also detected abundant evidence of both human meiotic [Stults DM et al 2008] and mitotic rearrangement [Killen MW 2009]. We recently used the GCI assay to compare matched normal tissue to tumor tissue in patients with lung or colorectal cancer and found that approximately 50% of the tumors show changes in the sizes of the clusters compared to the normal tissue, as well as evidence of ongoing instability and heterogeneity within the tumor population indicating that HR has at some point become dysregulated within the tumor cells [Stults DM et al 2009]. Notably, loss or knockdown of the RecQ homolog defective in Bloom syndrome (BLM) causes a remarkable 100x increase in rDNA gene cluster instability rates along with the well-characterized 10-fold elevation in rates of sister-chromatid exchange in these cells [Chaganti RS 1974], suggesting elevated HR with crossing-over as the most likely mechanism [Wu L and Hickson ID 2003] for this destabilization. Cells with defects in either of two other disease-associated human RecQ homologs, Rothmund-Thompson syndrome protein (RTS) and Werner syndrome protein (WRN), did not show destabilization by either assay, nor did wild-type fibroblasts ([Killen MW 2009] and our unpublished results). We also demonstrated that loss of the ataxia-telangiectasia-mutated (ATM) protein causes a 10x elevation in rDNA GCI instability, even though loss of ATM in the absence of exogenous DNA damaging agents does not increase levels of sister chromatid exchange [White JS et al 2010; Bartram CR 1976; Galloway SM and Evans HJ, 1975].

We are interested in the manner by which elevated and/or dysregulated recombination may be involved in the etiology of cancer and the development of chemotherapeutic resistance. We reasoned that elevated recombination could be caused either by an increase in recombination initiating lesions as the result of BER deficiency as seen in XRCC1 mutants, or by alterations in the downstream biochemistry
of recombination causing an increase cross-over vs. non-crossover recombination as seen in BLM mutants. Accordingly, we screened the NCI-60 panel of human cancer cell lines for defective BER by sensitivity to hmdUrd, and for altered recombination outcomes by the gene cluster instability assay. Lines exhibiting either phenotype were subsequently characterized by sister chromatid exchange in order to cross-compare three potential mitotic recombination indicators.

5.3: MATERIALS AND METHODS

Cell culture

NCI-60 cell lines were acquired frozen from the National Cancer Institute’s Developmental Therapeutics Program, thawed and grown overnight in RPMI-1640 culture medium supplemented with pen/strep/L-glutamine solution and 10% fetal bovine serum. The following day, the medium was replaced with RPMI-1640 culture medium with pen/strep/L-glutamine solution and 5% fetal bovine serum. Cells were maintained in this medium, at 37° C, in 5% CO2, in a humidified incubator for the duration of these experiments.

High molecular weight DNA isolation

After harvest with 0.025% trypsin (adherent cells only), all cells were quantified using a Partec benchtop flow cytometer. Single cell suspensions were adjusted to 1x10^7 cells/mL in low melting point agarose, drawn into a 1-mL syringe, and chilled on ice until solid phase. High molecular weight DNA was prepared from the solid-phase agarose cell suspension by means of digestion with 1% sarkosyl/500 mmol/L EDTA/0.5 mg/mL proteinase K solution at 50° C for at least 16 h, after which it was treated with phenylmethylsulfonyl fluoride in 10 mmol/L Tris/1 mmol/L EDTA (TE), extensively rinsed in TE/50% glycerol, and stored at -20° C.
Gene cluster instability analysis

The rRNA gene clusters were analyzed by pulse field gel electrophoresis and Southern blotting essentially as described [Stults DM et al 2008]. Briefly, approximately 1 µg of genomic DNA in solid phase was equilibrated in 1X NEBuffer 3 (100 mM NaCl; 50 mM Tris-HCl; 10 mM MgCl₂; 1 mM Dithiothreitol pH 7.9 @ 25°C) and digested with EcoRV (New England Biolabs) overnight at 37°C to release intact gene clusters from bulk genomic DNA. Digested DNA was placed into wells of a 1% Pulse Field Certified (Bio-Rad) agarose in 0.5X TBE (44.5 mmol/L Tris / 44.5 mmol/L boric acid / 1.0 mmol/L EDTA pH 8) gel and sealed using low-melting-point agarose. Gels were run using a CHEF-MAPPER (Bio-Rad), adjusting parameters and settings according to desired size resolution. Following electrophoresis, gels were equilibrated in glycerol and dried at 65°C. Dried gels were rehydrated and the DNA was denatured using 0.4 N NaOH/ 0.8 mol/L NaCl solution, neutralized in 0.5 mol/L Tris pH 8/0.8 mol/L NaCl, then prehybridized at 65°C in hybridization buffer of 2X SSC (300 mmol/L NaCl/30 mmol/L Na-citrate) with 7% SDS and 0.5% casein. Gels were probed in fresh hybridization buffer with an rDNA-specific, 32P-labeled probe (radiolabeled PCR products amplified from a plasmid containing cloned human rDNA sequence using primers 5'-GGGCTCGAGATTTGGGACGTCAGCTTC TG and 5'-GGGTCTAGAGTGCTCCCTTCGTGAG) at 65°C overnight. Following two rinses in 2X SSC/1% SDS solution and two rinses in 0.5X SSC/1% SDS, the gels were exposed using a PhosphorImager cassette (Molecular Dynamics) with subsequent imaging in a PhosphorImager.

Sister chromatid exchange analysis

Sister chromatid exchanges were prepared and visualized essentially as described [Perry P and Wolff S 1974]. BrdU was added to growing cells at a concentration of 20 µM, and cells were grown in the dark for two rounds of division. Colcemid (0.02 µg/mL) was added to medium. Approximately 4 hours later, cells were harvested with trypsin 0.025% (adherent cells only) and centrifuged for 5 minutes at 200 X g. Medium was aspirated and cells were resuspended in 8 mL hypotonic solution (46.5
mM KCl/8.5 mM sodium citrate). Cells were incubated at 37° C for 12 minutes, after which 2 mL of 3:1 methanol:acetic acid fixative was added. Cells were centrifuged at 200 X g for 5 minutes. Hypotonic/fixative solution was aspirated and cells were resuspended in 3:1 methanol acetic acid fixative and centrifuged at 200 x g. This process was repeated for a total of three washes in 3:1 methanol:acetic acid fixative. Finally, cells were dropped onto cold, wet pretreated (0.1N HCL in 95% EtOH) microscope slides and dried completely on a 45° C heat block. For staining, slides were incubated at room temperature in 10 µg/mL Hoechst 33258 in water, rinsed in Sorensen’s buffer (0.1M, pH 6.8: equal volumes 0.1 M Na2HPO4 and 0.1 M KH2PO4) and exposed with cover slips to 365 nm UV on a 55° C heat block for 25 minutes. Cover slips were removed and slides were incubated for 1 hr in 1X SSC at 50° C. Slides were then stained in 10% Giemsa in Sorensen’s buffer for 30 minutes, rinsed in water, and dried overnight on the benchtop. Individual metaphase spreads were photographed using bright-field microscopy and a 60X oil immersion objective.

Drug sensitivity screen

The in vitro growth inhibition screen of NCI-60 screen with a single 10 µM dose of 5-hydroxymethyl-2'-deoxyuridine (hmdUrd) was performed by the National Cancer Institute’s Developmental Therapeutics Program according to the methods described (http://dtp.nci.nih.gov/branches/btb/ivclsp.html).

5.4: RESULTS AND DISCUSSION

For these experiments, we used our gene cluster instability (GCI) assay to measure dysregulated recombination by identifying changes in the lengths of ribosomal RNA gene (rDNA) clusters. High molecular weight genomic DNA was digested with EcoRV (New England Biolabs), which cuts approximately every 6600 bases in the human genome [NEB 2010], but does not have a recognition site within the single rDNA repeat. The sequence of these repeats is highly conserved, and thus, an enzyme which does not cut within a single repeat generally does not cut anywhere in the cluster of tandemly
repeated genes, and the entire cluster can be separated from rest of the genomic DNA by pulse-field gel electrophoresis, and identified by Southern blot. We have found that a pulse-field gel with a resolution limit of 1MB is the most informative for tracking changes in cluster length associated with GCI.

We used this approach to screen the entire NCI-60 panel of 59 cancer cell lines to identify those which showed evidence of rDNA cluster instability (Figure 5.1). We have previously shown that Bloom syndrome cells, which demonstrate a ladder-like banding pattern, are highly unstable on GCI analysis [Killen MW et al 2009]. It was on this basis that we identified six lines which demonstrated laddering indicative of instability as candidates for further analysis. Three of these were lung cancer lines (A549, EKVX, and NCI-H23), one was leukemia (K562), one was breast cancer (T47D), and one was renal cancer (TK10). According to our previous findings in tumor versus non-tumor tissue from the same patient, the frequency of rDNA cluster instability is about 50%, as indicated by any variation in banding pattern between the normal and malignant tissue at 1 MB resolution limit [Stults DM et al 2009]. For these experiments, we did not have access to normal tissue for comparison, and it is likely that many more than the six lines we chose are unstable.

Central nervous system (CNS) cell lines SNB19 and U251 were derived from the same patient. According to our GCI results (Figure 5.1), they demonstrate a similar pattern, with four common bands, but also deviate from one another with a total of five bands which are not shared, indicating that the rDNA has continued to undergo rearrangement in culture. NCI/ADR-RES and OVCAR-8 are also derived from the same patient, but NCI/ADR-RES has acquired adriamycin resistance. These lines share three common bands, but NCI/ADR-RES appears to have acquired an additional band in culture. The fact that these two lines demonstrated so little divergence in culture despite the adriamycin-mediated DNA damage and acquired resistance indicates that accumulated damage is not sufficient to cause gene cluster instability. A third pair, MDA-MB-435 and M14, are also derived from the same donor. However, these lines have only a single band below 1MB and it appears stable.
Nine of the NCI-60 lines (HCT-116, HCT-15, KM12, DU-145, CCRF-CEM, MOLT4, SK-MEL2, IGR-OV1, SK-OV-3) are known to demonstrate microsatellite instability indicative of mismatch repair defects [CGP 2008]. It is notable that none of the mismatch-repair deficient lines showed evidence of dysregulated recombination on the initial GCI screen. Although genomic instability is a hallmark of cancer, it is possible that a tumor cell may only need a single means of acquiring instability to confer a selective advantage; and thus mismatch repair defects and recombination defects are mutually exclusive within a given tumor cell.

For the six lines which showed laddering similar to Bloom syndrome, we undertook a subclone analysis to determine whether dysregulated recombination and subsequent instability was an ongoing process. We duplicated our approach from the previous Bloom syndrome experiments. We began by isolating and growing up colonies derived from a single cell from the populations which demonstrated laddering. In general, the parental population derived from a single cell usually demonstrates an initial, well-defined banding pattern. We called this pattern the “major banding pattern.” Colonies from single cells from the cloned parental plate were grown up and GCI analysis was performed. As each clonal population expands, if the rDNA clusters are completely stable, the initial cluster lengths found in the parental line will be faithfully transmitted to all subsequent daughter cells (Fig. 5.2, ‘No GCI’). Alternatively, recombination in the expanding population can generate sub-populations with altered gene cluster lengths. Since these sub-populations only represent a fraction of the total population, bands will be reduced in intensity accordingly. We called these reduced-intensity bands the “minor banding pattern” (Fig. 5.2, ‘Low GCI’). The amount of this minor-intensity banding found in any cell population is indicative of the degree of GCI in that population. Since recombination requires precise alignment of homologous sequences, cluster lengths can only change by integer multiples of the unit repeat length. This constraint upon allowable gene cluster lengths (Fig. 5.2, dotted lines) means that very high levels of instability will generate a ladder-like pattern of bands (Fig. 5.2, ‘High GCI’), consistent with a recombination-based mechanism. If cluster length
alterations were due to random breakage and rejoining, a smear would be observed, rather than a ladder [Killen MW 2009]. As in the case of Bloom’s syndrome, lines which demonstrated a laddering pattern in the population sometimes showed non-stoichiometric bands within the parental population, indicating a high degree of instability even within the first parental expansion.

Lung cancer cell line A549 does not appear to demonstrate ongoing rDNA cluster instability (Figure 5.3A). The major banding pattern from the clonal parental line is faithfully transmitted to each of the daughter lines with the exception of a single additional major band in subclone E (open triangle). Lung cancer line NCI-H23 shows similar results, with the disappearance of a single band in subclone D (Figure 5.3B). All nine subclones also appear to have lost two bands that are non-stoichiometric but apparent in the parental line. This is not believed to be due to dysregulated recombination. NCI-H23 does not form single-cell-derived colonies easily. Cells tend to migrate toward one another, and it may be that the parental population was not purely derived from a single cell. The third lung cancer cell line, EKVX is classified as having Low GCI. The parental line shows a single non-stoichiometric band thought to have arisen during expansion. Three of the subclonal lines appear to have inherited this band (subclones C, E, and G), while the other five (A, B, D, F, and H) did not. (Figure 5.3C). In contrast, the renal cell cancer TK10 was characterized as having high GCI. Among the nine subclones there were a total of nine new minor bands (black arrows), 24 major bands either new or inherited from minor bands in the parental population (open triangles), and 35 deletions of either major or minor bands from the parental population (brackets) (Figure 5.3D). K562 also showed high GCI, with three new minor bands (black arrows), six new major bands (open triangles), and nine major band deletions (brackets) among the 11 subclones (Figure 5.3E). Interestingly, K562 subclones also showed a stoichiometric laddering pattern of bands that differ by a length of a single unit repeat (black dots) in the lower range of the gel. We do not believe these arose by dysregulated homologous recombination, but the mechanism is unclear. Although we did not observe this phenomenon in any of the other subcloned lines, it does sometimes
develop in solid tumor and non-tumor tissue. This phenomenon may be due to a fragile site within the repeat that causes breakage of the clusters during processing. T47D also appeared unstable on subclone assay, with the appearance of eight new minor bands (black arrows), five new major bands (open triangles), and one major band deletion (bracket) across eight subcloned lines (Figure 5.2F). We also expanded a parental clone and conducted subclone analysis for MCF7, a breast cancer line that is well characterized and frequently used for molecular biology experiments. MCF7 did not demonstrate laddering on the initial screen and showed only two major bands below the 1MB resolution limit of the gel. MCF7 subclones showed two new minor bands (black arrows) and no new major bands or deletions among the eight subclones (Figure 5.3G). In summary, of the six lines we identified as definitively demonstrating rDNA GCI instability on initial screen, only three (T47D, TK10, and K562) continue to show evidence of ongoing instability at the present time.

We performed sister chromatid exchange analysis on five of our six lines which showed initial and/or ongoing instability (EKVX, NCI-H23, T47D, TK10, and K562), in order to explore whether there was any correlation between gene cluster instability and sister chromatid exchange (Figure 5.4A). There appears to be some inherent variability in the range. In general, the SCE distributions are different across cell lines (Kruskal-Wallis test p-value = 0.0001). However, none is notably unstable by SCE, as the distributions all overlap. In pair-wise comparisons of the mean values for each of the 5 cell lines, the mean value for TK10 was significantly lower than the other four cell lines tested (p<0.05) (raw, unbinned data not shown), meaning that TK10 exhibits significantly fewer exchanges per chromosome than the other cell lines. In no case was there a correlation between elevated instability on GCI and notable instability on SCE. Previous experiments with wild type fibroblasts (GM00637), which are stable on GCI, show similar values by SCE analysis (unpublished data). Single isolated metaphases from K562 (1/39) and NCI-H23 (1/20) did show between three-and four-fold elevation in the number of sister chromatid exchanges, but the significance of these findings is unclear.
In addition to its ongoing instability on GCI, K562 also appeared to demonstrate evidence of increased instability by sister chromatid exchange in two subcloned lines (Figure 5.4B). Although the original population of K562 cells did not show gross elevation above other cell lines or wild type, K562p1 and K562p3, both of which were lines expanded from a single cell of the population, did show a 1.5-fold elevation in sister chromatid exchanges in 37% and 38% of the metaphases scored, respectively. In addition, 13% of K562p1 metaphases and 25% of K562p3 showed exchanges of greater than 0.3125 per chromosome. Comprehensive analysis of metaphases from individual subclones for all of the lines tested for GCI was beyond the scope of these experiments; and it is possible that upon examination, all of five of the cell lines would have demonstrated instances of increased instability by SCE. However, it is interesting to note that K562 did show ongoing instability by GCI, as well as a trend toward increased instability on SCE, which points to a shared mechanism or partial mechanism for both phenomena. On the other hand, TK10, which is highly unstable by GCI, is remarkably stable by SCE in our hands.

XRCC1 knockdown in CHO and HeLa cells has been shown to lead to an increase in sister chromatid exchange [Fan J et al 2007; Thompson LH 1982]. Downregulation of XRCC1 also confers sensitivity to 5-hydroxymethyl-2’-deoxyuridine in HeLa cells [Fan J et al 2007]. We wished to determine whether there was any correlation between hmdUrd sensitivity and genomic instability by SCE assay, GCI assay, or both. In collaboration with the Developmental Therapeutics Program Screening Service at NCI, we screened the entire NCI-60 panel for sensitivity a 10 µM dose of hmdUrd (Figure 5.5A). Six lines were notable for sensitivity to hmdUrd at this dose. Hop62, SNB19, and Ovcar-5 were moderately sensitive (mean growth approximately -50%), U251 and NCI-Adr/Res were highly sensitive (mean growth approximately -75%), and 786-0 was extremely sensitive to hmdUrd (-100% mean growth). None of these lines were notable for pronounced genomic instability by GCI or SCE analysis (Figure 5.5B).

Interestingly, however, U251 showed higher hmdUrd sensitivity than did SNB19, which is from the same donor. They show similar ranges and mean values on SCE.
analysis, but U251, the more sensitive line, showed a higher proportion of metaphases with greater than 0.1875 exchanges per chromosome. Ovcar-8 displayed no sensitivity to hmdUrd at the 10 µM dose, but NCI-Adr/Res, the adriamycin-resistant line from the same donor, was highly sensitive to the drug. We did not detect any differences between these two lines on SCE. Renal cell line 786-0, the most sensitive line for hmdUrd, showed a normal range of stability for SCE. However, on GCI, 786-0 showed no rDNA bands below 1 Mb (Figure 5.1). We did not find this remarkable since Southern blot showed there was indeed ribosomal DNA beyond the resolution limit of the gel; and we did no further analysis. However, in light of the marked hmdUrd findings, we speculate that there may be a connection between loss of smaller rDNA gene clusters and hmdUrd sensitivity.

We were surprised to find laddering on rDNA GCI did not correspond with markedly elevated sister chromatid exchange in the NCI-60 panel. In normal cells, homologous recombination which results in crossovers and physical restructuring of DNA is suppressed due to its potential to cause damage. In searching the NCI-60 database, we did not find any consistent mutations or remarkable changes in repair-associated mRNA levels across the lines which demonstrate laddering. However, HR is an extremely complex and not fully understood process involving over 200 DNA repair and checkpoint proteins [Helleday T, 2010]. Checkpoint defects or mutations in lesser known components of HR may allow cells to proceed through the cell cycle with partially duplicated and broken rDNA. The ribosomal RNA gene contains its own origin of replication and also possesses sequences which make formation of G-quartets and fragile sites very likely, and it is possible that BLM suppresses rDNA cluster instability via a different mechanism than the one for suppressing SCEs. We were also surprised to find that the hmdUrd results for the NCI-60 panel of human cell lines did not correspond well to what has been shown in CHO cells, but perhaps this finding is not so remarkable and points to the vast differences in physiology and metabolism between humans and rodents, emphasizing the need to abstain from making direct inferences with regard to issues of human health based on findings in model organisms.
FIGURE 5.1: GCI SCREEN OF THE NCI-60 PANEL OF HUMAN CANCER CELL LINES

NCI-60 panel of cell lines grouped according to primary histology. Open star (☆) indicates resolution limit 1 Mb. Red boxes indicate lines with laddering indicative of GCI. Red text: NCI/ADR-RES and OVCAR-8 from same donor. Blue text: MDA-MB-435 and M14 from same donor. Fuscia text SNB-19 and U251 from same donor. Green text: Lines with known microsatellite instability according to the Cancer Genome Project.
Schematic of GCI patterns in clonal cell lines. A single cell shows a well-defined pattern of gene cluster lengths ('Initial Pattern'—thick bands). Allowable, but currently unrepresented gene cluster lengths are shown as dotted lines. As mitotic division expands the clonal cell population in the absence of instability, gene cluster lengths are faithfully preserved ('No GCI'). Alternatively, instability generates sub-populations within the expanding population with altered cluster lengths giving rise to lower intensity 'minor bands' ('Low GCI'—thin bands indicated by arrows). High levels of instability generate a ladder-like pattern of minor banding with individual bands on the ladder differing by integer multiples of the unit repeat length ('High GCI').
A clonal population of lung cancer cells derived from a single cell is shown in the leftmost lane. Sub-clones were derived by dilution plating to single cells with subsequent expansion. Changes to the major banding pattern are shown by triangles (new bands).
A clonal population of lung cancer cells derived from a single cell is shown in the leftmost lane. Sub-clones were derived by dilution plating to single cells with subsequent expansion. Changes to the major banding pattern shown by brackets (missing bands).
A clonal population of lung cancer cells derived from a single cell is shown in the leftmost lane. Sub-clones were derived by dilution plating to single cells with subsequent expansion. Minor bands indicated by arrows. Changes to the major banding pattern shown by triangles (new bands) and brackets (missing bands).
A clonal population of renal cancer cells derived from a single cell is shown in the leftmost lane. Sub-clones were derived by dilution plating to single cells with subsequent expansion. Minor bands indicated by arrows. Changes to the major banding pattern shown by triangles (new bands) and brackets (missing bands).
A clonal population derived from a single cell is shown in the leftmost lane. Sub-clones were derived by dilution plating to single cells with subsequent expansion. Minor bands indicated by arrows. Changes to the major banding pattern shown by triangles (new bands) and brackets (missing bands).
A clonal population of breast cancer cells derived from a single cell is shown in the leftmost lane. Sub-clones were derived by dilution plating to single cells with subsequent expansion. Minor bands indicated by arrows. Changes to the major banding pattern shown by triangles (new bands) and brackets (missing bands).
A clonal population of breast cancer cells derived from a single cell is shown in the leftmost lane. Sub-clones were derived by dilution plating to single cells with subsequent expansion. Minor bands indicated by arrows.
Sister chromatid exchange analysis of populations from cell lines that are unstable by GCI assay. X-axis: Average number of sister chromatid exchanges per chromosome. Y-axis: Proportion of metaphases scored. All five lines appear relatively stable by sister chromatid exchange assay. However, K562 and H23 do show occasional metaphase spreads which contain a markedly elevated number of exchanges.
Sister chromatid exchange analysis of metaphase spreads from two subclones expanded from K562 population. X-axis: Average number of sister chromatid exchanges per chromosome. Y-axis: Proportion of metaphases scored. In general, most of the metaphase spreads from both subclones are stable range. However, 22.5% of the K562p3 metaphases showed > 3.6 fold elevation. K562p1 subclones showed 5% of metaphases with >2 fold elevations.
NCI-60 panel was tested for sensitivity to a single 10 μM dose of 5-hydroxymethyl-2'-deoxyuridine, a thymidine analog. The lines shown in red had reduced growth in the presence of the drug.
Sister chromatid exchange analysis of metaphases from populations of cell lines that are sensitive to 5-hydroxymethyl-2’-deoxyuridine. X-axis: Average number of sister chromatid exchanges per chromosome. Y-axis: Proportion of metaphases scored. There is no correlation between hmdUrd sensitivity and increased number of sister chromatid exchanges.
CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation represents my efforts to characterize the genomic architecture of the human ribosomal RNA gene clusters and evaluate their heritability and stability in normal healthy individuals. Using the NCI-60 panel of cell lines in culture, I have also attempted to determine whether cancer-associated architectural destabilization in these clusters correlates with two other known recombination-mediated functional phenotypes: increased levels of sister chromatid exchange, and sensitivity to 5′-hydroxymethyl-2′-deoxyuridine. Key findings are summarized below.

Chapter 4 describes my initial use of a new assay, the gene cluster instability (GCI) assay, to determine the actual number of repeats in ribosomal RNA gene clusters (rDNA), since they cannot be adequately assembled by the Human Genome Project. The assay, developed by Dr. Andrew Pierce, Michael W. Killen, and me, utilizes restriction enzyme digestion of very high molecular weight DNA fragments followed by pulsed-field gel electrophoresis and in-blot Southern hybridization to monitor changes in the lengths of the clusters. Much attention has been paid recently to gene copy number variation in the human population. It is believed that copy number differences and subsequent dosage variation accounts for a great deal of the phenotypic differences between individuals [Smith S et al 2006; Redon R et al 2006; Sebat J et al 2004]. Comparative genomic hybridization (CGH) is often used as a means for tracking these changes; but sensitivity is limited to those genes that demonstrate at least a 3-fold difference in copy number. CGH is not effective for tracking changes in gene cluster lengths which vary by single integer repeats. Using our GCI approach, I found that normal, healthy humans are completely heterozygous for their repeat cluster lengths within each individual and throughout the population. In general, cluster lengths are inherited from both parents. However, meiotic rearrangement of the clusters does occur frequently, and I also saw evidence of mitotic non-allelic homologous recombination. I determined that there are about 600 copies of the 45S ribosomal genes in each human diploid cell. There are about 200 copies of the 5S ribosomal RNA genes. This work established the baseline level of stability and rate of change for ribosome RNA gene clusters in humans,
definitively demonstrating that these genes do undergo NAHR as predicted based on their structure. The results were published in 2008, in Genome Research [Stults DM et al, 2008].

Based on the results from my initial studies, I sought to determine whether cancer cells may be subject to increased levels of NAHR compared to normal tissue. I hypothesized that the characteristic genomic destabilization in cancer may in some cases be due to dysregulated homologous recombination. I used our GCI assay to compare 45S rDNA gene cluster instability in normal tissue and tumors from clinical cancer patients at the University of Kentucky Markey Cancer Center. The results, shown here in Appendix A, were part of an MS thesis project and were published in the journal, Cancer Research. From analysis of samples from 39 patients, I found that over half of the adult solid tumors show detectable rDNA rearrangements relative to either surrounding non-tumor tissue or normal peripheral blood. This instability may cease before expansion, or it may continue. About 25% of the tumors demonstrate a laddering pattern of heterogeneous cluster lengths within the tumor cell population, similar to that of BLM fibroblasts [Killen MW et al 2009] indicating ongoing instability. Pediatric leukemias did not demonstrate laddering. These findings indicate that rDNA restructuring is among the most common chromosomal alterations in adult solid tumors, and may prove to have either prognostic or predictive value [Stults DM et al 2009].

Chapter 5 of this dissertation represents my use of the NCI-60 panel of human cancer cell lines to correlate dysregulated and non-allelic homologous recombination as evidenced by the GCI assay with two other phenotypic measures of recombination: the sister chromatid exchange assay, and hmdUrd sensitivity. Based on evidence from Bloom syndrome cells which show both laddering on GCI and 10-fold elevation in sister chromatid exchange, I hypothesized that those cell lines from the NCI-60 panel which demonstrate laddering would also demonstrate evidence of recombination-mediated instability on sister chromatid analysis. I further hypothesized that hyper-recombinative cell lines may also show increased sensitivity to hmdUrd. I speculated that in those cell
lines where recombination is already up-regulated, as evidenced by GCI or SCE assay, interfering with base-excision repair by using hmdUrd may cause synthetic lethality, as hyper-recombinative cells may not be able to compensate by further up-regulating HR. I had hoped that by using the extensively characterized NCI-60 panel and the Cancer Genome Project database, I might be able to uncover a common genetic mechanism for dysregulated recombination as a means for conferring genomic instability in cancer.

Surprisingly, I did not find any correlation between rDNA cluster instability on GCI compared to SCE or hmdUrd sensitivity, although these experiments did yield interesting findings. I was able to determine rDNA cluster instability on GCI is anti-correlated with microsatellite instability, the result of a mismatch repair defect, which led me to speculate that in general, genomic destabilization arises by a single mechanism.

Sister chromatid exchange analysis of populations from twelve cell lines indicates that the level of baseline sister chromatid exchange in the presence of BrdU varies between cell lines, though none of the lines demonstrated the harlequin pattern of gross destabilization characteristic of Bloom syndrome chromosomes, and thus are all considered “stable” by SCE analysis. Sister chromatid exchange is therefore thought to be cell-line dependent and able to change in culture; as even cell lines originally derived from the same patient show varying levels of instability.

Unlike the XRCC-1 deficient CHO cells, none of the human cell lines which demonstrated sensitivity to a single 10µM dose of hmdUrd were notable for base excision repair defects, according to the Cancer Genome Project database, which indicates that HmdUrd sensitivity may be conferred via some mechanism other than inability to overcome damage by base excision repair.

At the present time, other members of the lab are pursuing some of the genetics underlying rDNA gene cluster instability, particularly with regard to Bloom syndrome and other RecQ helicases. Tandemly repeated gene clusters located elsewhere in the genome are also being evaluated to determine whether gene cluster instability is an rDNA-specific phenomenon.
In the future, I will expand on the findings from Appendix A. I will undertake a comprehensive analysis of rDNA GCI in clinical lung cancer patients to determine whether their tumor GCI status has prognostic value or can be correlated with response to chemotherapy. Current clinical staging is based on a number of factors including the size and appearance of the tumor, whether or not there are multiple loci, lymph node involvement, and metastasis to other organs. Our preliminary data indicate that patients who demonstrate molecular GCI instability are otherwise clinically identical their stable counterparts on presentation. It is reasonable to predict that although from the clinical perspective patients may be similar, whether or not they are undergoing remarkable genomic rearrangement at the molecular level may influence their prognosis and/or clinical response to treatment.

Regardless of the outcome of my studies, the profoundly different GCI results between patients of identical clinical status merits further investigation. I speculate that the patients who demonstrate the laddering pattern on GCI indicative of ongoing rDNA repeat destabilization may have a better long-term prognosis and/or response to treatment. I have proposed using late-stage patients because lung cancer survival is abysmal, less than five years; and a study of two years' duration will be sufficient to determine whether there are any differences in survival between those with and without ongoing instability. However, if I am able to extract reasonably intact DNA from frozen tissue, it may be possible to use banked samples from patients with earlier stage disease. I could use earlier stage samples, and thus interpret response to treatment in patients whose intervention has been early enough to have some effect.

The marked ongoing instability in some but not all of the solid tumors I tested is also intriguing. It has become apparent that the tumors of some non-small cell lung cancer patients demonstrate genomic alterations such as fusions and translocations, representing new targets for custom-designed therapeutics (Horn and Pao, 2009). Because of their tendency for rearrangement, the rRNA genes, in addition to playing a direct role in cell metabolism, may also drive oncogenesis via this mechanism. By determining whether there is altered sequence context for the rRNA genes, I may
uncover an exciting new therapeutic target for a clinical subset of NSCLC patients. I hypothesize that for those patients who demonstrate temporal rDNA cluster instability, translocation of the rRNA gene, promoter, and/or immediate upstream regulatory region may be influencing expression of oncogenes to promote increased metabolism, unrestrained proliferation, and/or avoidance of apoptosis. I have proposed experiments using restriction digest, cloning, PCR, and sequencing to determine whether there are alternative sequence contexts for the rRNA genes or regulatory elements that serve to drive tumorigenesis in clinical cancer patients.
Molecular Biology, Pathobiology, and Genetics

Human rRNA Gene Clusters Are Recombinational Hotspots in Cancer

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Abstract

The gene that produces the precursor RNA transcript to the three largest structural rRNA molecules (rDNA) is present in multiple copies and organized into gene clusters. The 10 human rDNA clusters represent <0.5% of the diploid human genome but are critically important for cellular viability. Individual genes within rDNA clusters possess very high levels of sequence identity with respect to each other and are present in high local concentration, making them ideal substrates for genomic rearrangement driven by dysregulated homologous recombination. We recently developed a sensitive physical assay capable of detecting recombination-mediated genomic restructuring in the rDNA by monitoring changes in lengths of the individual clusters. To prove that this dysregulated recombination is a potential driving force of genomic instability in human cancer, we assayed the rDNA for structural rearrangements in prospectively recruited adult patients with either lung or colorectal cancer, and pediatric patients with leukemia. We find that over half of the adult solid tumors show detectable rDNA rearrangements relative to either surrounding nontumor tissue or normal peripheral blood. In contrast, we find a greatly reduced frequency of rDNA alterations in pediatric leukemia. This finding makes rDNA restructuring one of the most common chromosomal alterations in adult solid tumors, illustrates the dynamic plasticity of the human genome, and may prove to have either prognostic or predictive value in disease progression. [Cancer Res 2009;69(23):9096–104]

Introduction

The human rDNA gene clusters (rDNA clusters) produce 45S precursor transcripts, subsequently processed to make three of the four structural RNA components of the ribosome, and are critically required for protein synthesis and cellular viability. The rDNA clusters comprising collectively hundreds of gene copies are located on the short arms of the five human acrocentric chromosome pairs (13p12, 14p12, 15p12, 21p12, 22p12; OMIM 180450-180454) and are generally organized with individual genes in head-to-tail tandemly repeated arrays (1). Each individual gene is each 43-kb in length (2), with near perfect sequence conservation. Extremely high sequence similarity combined with the length of the individual repeats makes both shotgun-based and directed sequencing approaches to genome sequence assembly of the rDNA impossible. As a result, the rDNA clusters are one of the largest remaining components of the human genome with no representation in the Human Genome Project.

The highly repetitive nature of these clustered genomic repeats also prevents the application of commonly used techniques to investigate genomic instability such as array-based comparative genome hybridization, single nucleotide polymorphism (SNP) analysis, and high-throughput paired end sequencing approaches. Nevertheless, the very considerations that make the rDNA clusters difficult to study by conventional means also make the rDNA biologically interesting from a genomic stability perspective. The sequence conservation, repeat length, and high relative local concentration of the repeats involving multiple chromosomal loci are, in principle, conducive to cluster restructuring by homologous recombination. We previously physically characterized human rDNA cluster lengths, finding an average of 600 repeats per diploid genome spread over the 10 rDNA clusters, subject to wide person-to-person variability driven by strong meiotic recombination; the number of gene repeats per cluster changes with a frequency of ~10% per cluster per meiosis (3). The high meiotic recombination frequency phenotype of the rDNA leads us to consider whether the rDNA clusters could serve as sentinel biomarkers for dysregulated mitotic recombination in cancer.

Dysregulated recombination has the potential to mediate genomic restructuring in cancer (reviewed in ref. 4). We recently showed that changes in rDNA cluster lengths in cultured mitotic human cells are potential indicators of recombination-mediated genomic destabilization by showing a 10- or 100-fold increase in the frequency of spontaneous rDNA cluster restructuring in cells deficient for the ataxia-telangiectasia mutated (ATM) protein or the Bloom syndrome protein, respectively (5), in line with the increased prevalence of malignancy in ATM (6) and Bloom syndrome (7) patients. To determine whether the rDNA clusters are restructured in sporadic cancers generally as well as in cancer predisposition syndromes such as ATM and Bloom syndrome, we prospectively recruited lung cancer, colorectal cancer, and pediatric leukemia patients for molecular analysis of their disease.

Lung and colorectal cancers are the leading and second leading cause of American deaths from cancer, accounting for 28% and 9% of all cancer deaths, respectively (8). Notably, both lung and colorectal cancers derive from tissues with potentially substantial exposure to environmental genotoxic agents. Many potential environmental carcinogens, including specifically those in tobacco smoke, are potent inducers of sister chromatid exchange (9–11), which is thought to occur by a recombination-based mechanism (12). Ordinarily, mammalian cells efficiently suppress recombination-mediated genomic rearrangements (13, 14), but damage or loss of this control of recombination, combined with protracted recombination induction by environmental agents, may significantly impact the etiology of tumors in these organs. Evidence suggesting potential cancer etiology relevance for dysregulated recombination, particularly in the...
case of lung cancer, includes both the overexpression of recombination
genesis (15) and ATM defects commonly found in this tissue (16).
Conversely, pediatric leukemia is thought to be driven by specific
chromosomal events, rather than the gradual accumulation of ma-
lignant features caused by many years accumulating molecular
genomic insults (17). This work reports the result of testing the
hypothesis that dysregulated recombination is a significant feature
of primarily sporadic adult lung and colorectal cancers rather than
pediatric leukemias.

Materials and Methods

Human investigations were performed after approval by the University
of Kentucky Institutional Review Board and Markey Cancer Center Protoc-
ol Review Committee, and in accordance with an assurance filed with and
approved by the Department of Health and Human Services. Informed
consent was obtained from University of Kentucky Medical Center patients
undergoing surgical resection for either lung or colorectal cancer, or from
the parents of pediatric patients being treated for leukemia. For patients
with solid tumors, peripheral blood was drawn before surgery. After sur-
gery, resected specimens were examined by the Division of Surgical Pathol-
gy, and pieces of both tumor and surrounding nontumor tissue were
removed for analysis. Leukemia patients had either peripheral blood or
bone marrow aspirates removed for analysis periodically throughout the
course of their treatment. All patient samples are referred to in this work
by study code names.

Sample processing. Solid tissues were rinsed on ice in RPMI1640 con-
taining antibiotics and 10% fetal bovine serum (RPML/FBS). Tissues were
then disaggregated to single cells mechanically either by gentle scraping
and mincing, or by mincing in RPMI/FBS and processing in a 50-μm pore
size Medicon unit in a Medimachine followed by debris removal by filtra-
tion with a 70-μm pore size Filcon (all from Becton Dickinson). For whole
blood and bone marrow aspirates, leukocytes were isolated by ammonium
chloride lysis. No digestive enzymes were used in the preparation of any
of the single-cell suspensions. Single-cell suspensions of either leukocytes or
solid tissues had DNA-containing cells quantified by vital staining with 2
μg/mL acridine orange followed by flow cytometric analysis with a 488-nm
argon-ion laser triggering acquisition on green fluorescence (DNA content)
and red fluorescence (proliferative activity). DNA was isolated from the
isolated cells with a GenElute Mammalian Genomic DNA kit (Sigma).

Sample preparation. Approximately 300 mg of tissue was minced in
RPMI/FBS and processed in a 50-μm pore size Medicon unit in a Medimac-
chine followed by debris removal by filtration with a 70-μm pore size Filcon
(all from Becton Dickinson). The final cell suspension was centrifuged,
resuspended in RPMI/FBS, and allowed to solidify. High-molecular-weight genomic DNA was prepared from this solid phase cell suspension by treatment with 1% sarkosyl/500 mmol/L EDTA, 0.5 mg/mL protease K at 50°C for at least 16 h, fol-
lowed by treatment with phenylmethylsulfonyl fluoride, extensive rinsing,
and final equilibration in 50% glycerol/10 mmol/L Tris/1 mmol/L EDTA
(pH 8.0) and long-term storage at −20°C.

Southern blot analysis. The rDNA clusters were analyzed by pulsed-
field gel electrophoresis and Southern blotting as described (3). Approx-
imately 1 μg genomic DNA in a 10-μL solid-phase agarose slice was
equilibrated with appropriate restriction digestion buffer and was digested
by EcoRV (New England Biolabs) to liberate intact rDNA clusters from bulk
genomic DNA. Agarose slices containing digested genomic DNA were sealed
into the wells of a 1% Pulse Field Certified agarose (Bio-Rad) gel in
44.5 mmol/L Tris/44.5 mmol/L boric acid/1.0 mmol/L EDTA (pH 8.0, 0.5×
Tris-borate EDTA (TBE)). The gels were run using a CHEF-MAPPER (Bio-
Rad) in 0.5× TBE for 24 h at 6 V/cm field strength, switching between 120°
separated field vectors (frequency, 3–90 s) using a "ramp factor" of 0.357
(Bio-Rad) at a constant temperature of 14°C to achieve approximately linear
size resolution of between 30 kb and 1 Mb.

Following electrophoresis, gels were equilibrated to 0.5% final concen-
tration glycerol and dried at 65°C. Dried gels were rehydrated with water, de-
natured with 0.4 N NaOH/0.8 mol/L NaCl, neutralized with 0.5 mol/L Tris
(pH 8.0)/0.8 mol/L NaCl, and prehybridized at 65°C in 2× SSC (300 mmol/L
NaCl/30 mmol/L Na-citrate) with 7% SDS and 0.5% casein. Gels were
probed overnight at 65°C in fresh prehybridization solution using the
rDNA-specific radiolabeled PCR products of primers 5′-GGGTCTAGATGCTCCC-
TTCCCTCTGAG on pBSrdNA11, a pBluescript-based (Stratagene)
plasmid containing a subcloned and sequenced segment of the human
rDNA intergenic spacer, generated by using the aforementioned primers
to amplify human genomic DNA (3). Gels were rinsed at 65°C with 2× SSC/0.1% SDS followed by 0.5 × SSC/0.1% SDS, briefly equilibrated with
2× SSC, and developed by exposing a PhosphorImager cassette with subse-
quent imaging in a PhosphorImager (Molecular Dynamics). Raw data were
obtained in TIFF format and processed using Adobe Photoshop. All images
were processed en bloc. In no case did image processing either obscure
bands from the raw data, or cause bands not apparent in the raw data
to appear.

Results

We assay for dyregulated recombination in the rDNA by exam-
ining changes in the physical length of individual rDNA clusters
(Fig. 1). Every person has a unique configuration of these rDNA
clusters (3), so the cluster lengths vary from person to person.
Where possible, we compare the rDNA cluster lengths found in
surgically excised nearby nontumor tissue to the gene cluster
lengths found in the tumor itself, and to gene cluster lengths from
the patients’ peripheral blood as an additional control (lanes N, T,
and B, respectively). Our prior experiences with both human blood
and cell lines indicated that analysis of gene cluster lengths from
50 kb to ~1 Mb is the most informative range for detecting recom-
bination-mediated alterations (3, 5), so this is the methodology we
followed in this present work. Gene clusters larger than 1 Mb all run

![Figure 1. Patterns of genomic instability in the rDNA. In each case lanes are
nontumor tissue (N), tumor tissue (T), and peripheral blood (B). The grouped
bands at the star represent unresolved gene clusters larger than 1 Mb. The lower
limit of resolution of the gel is 50 kb. •, breakage from fragile DNA not involving
actual chromosomal restructuring. ◀, new bands found in tumors and not in
nontumor tissue. ▶, new bands found in a fraction of tumor tissue but not
nontumor tissue. Arrows, bands found in nontumor tissue but underrepresented
in tumor tissue.](image-url)
together unresolved at the top of the gel in the region (indicated by the ★) under these electrophoretic conditions, otherwise all unique gene clusters shorter than 1 Mb are resolved as individual bands. If the rDNA clusters are stable under mitotic cellular expansion, the banding pattern found in tumor cells will match that found in nontumor cells (Fig. 1, Stable). Occasionally, we observe a relatively low-molecular-weight ladder of bands at the bottom of the gel in tumor and normal tissues (Fig. 1, Sample fragility), consistent with the 43 kb rDNA individual gene repeat length. These bands appear to arise from stochastic, low-frequency fragile-site breakage in individual rDNA repeats during the genomic DNA isolation procedure itself rather than from bona fide chromosomal alterations in cells, since the intensities of these bands follow a smooth Gaussian distribution and is not dependent on liberation of the gene clusters from bulk genomic DNA by restriction enzyme digestion. Although this fragility may contribute to initiating chromosomal alterations...
in gene cluster lengths, we do not consider a low-molecular-weight banding pattern on its own to be indicative of genomic instability.

On the other hand, if a tumor has lost control over recombination, the configuration of gene cluster lengths in the tumor will be different, relative to nontumor cells (5). If the rDNA cluster length restructuring occurred before clonal expansion of the prototumor cell, for example in the lungs of a heavy smoker, the altered length gene clusters will be present in the entirety of the subsequent tumor. In this case, some bands found in nontumor tissue may be lost in the tumor, and new bands become evident (Fig. 1, Instability before expansion). These new bands (Fig. 1, *) show intensities proportional to their length. Tumor samples are commonly contaminated with nontumor tissue, so fainter bands in the tumor sample occurring at the same position as bands found in normal tissue likely represent contaminating normal tissue rather than heterogeneity of cluster lengths within the tumor itself. Finally, gene cluster lengths may have changed both before clonal expansion of the prototumor cell and also during the clonal expansion of...
of the new tumor. Alterations that occurred after the tumor began clonal expansion will manifest as new bands, but with reduced intensity (Fig. 1, Ongoing instability, ▲). The degree to which the intensity of these "minor bands" is reduced relative to the intensities of the "major new bands" in the tumor is determined by the fraction of the tumor made up of cells with these alterations; the later in the clonal expansion the gene cluster alteration occurred, the fainter the minor intensity bands will be. In contrast...
to the “Sample fragility” bands, new minor bands in the tumor occur at high molecular weights, do not vary smoothly in intensity, and are dependent on liberation from bulk genomic DNA by restriction digestion before visualization on the gels.

We first analyzed lung squamous cell carcinoma (Fig. 2). Four of 11 patients, TIBBO-JU, PANIU-HA, AFILA-PA, and JIMIL-GE show no differences between observed gene cluster lengths in tumor relative to nontumor tissues indicating rDNA cluster stability. Two more patients, RESCA-BO and OFIAT-MA, show the same gene cluster profiles between tumor and nontumor cells, with the exception of an even laddering of bands in the lower part of the gel (\(\ast\)) consistent with fragility in the isolated genomic DNA. We therefore also consider these tumors to be gene cluster stable. The stability of TIBBO-JU is particularly remarkable because this tumor was a recurrence following both chemotherapy and radiation treatment. Clearly, the intense genomic damage from treatment does not necessarily cause loss of gene cluster stability, nor is loss of gene cluster stability necessarily required for poor response to therapy.

The remaining five lung squamous cell carcinomas all show evidence of structural alteration in the rDNA, XEPRY-DA, QIPPI-SH, and VEKOR-CH show bands found in tumors not present in either blood or surrounding nontumor cells (Fig. 2, \(\ast\)). In XEPRY-DA and VEKOR-CH, each novel band is \(\sim\)30% the intensity of the bands found in nontumor tissue. This is consistent with either the rearrangements occurring early in the clonal expansion of the initial prototumor cell, or in the pretumor lung tissue before the initiation of clonal expansion, in which case the tumor would make up 30% of the cells in the tumor sample, with the remaining 70% being normal tissue. The novel bands in QIPPI-SH are on the order of intensity of bands in nontumor tissue, suggesting that these genomic alterations preceded clonal tumor cell expansion.

Finally, two of the poorly differentiated lung squamous cell carcinomas, BONTA-DA and WOFUT-BR, show evidence of extensive and ongoing recombinational instability. In both cases, there are new major bands in the tumors not found in nontumor tissue (Fig. 2, \(\ast\)). Significantly, there are additionally several high-molecular-weight minor intensity bands (Fig. 2, \(\ast\)). The presence of both new major and minor intensity bands indicates that not only were the rDNA clusters altered in the pretumor tissue as might be expected for cells under genotoxic pressure from tobacco exposure, but that these gene clusters continued to structurally rearrange in the early history of the tumor after the prototumor cells had already become committed to clonal tumor cell expansion.

We see a similar level of gene cluster restructuring in lung adenocarcinomas (Fig. 3). In 6 of 14 cases, there is no evidence for rDNA cluster rearrangement. In PHAZL-SA, SEBON-CL, and IBINA-KE gene, cluster lengths in all of nontumor, tumor, and blood samples are longer than the \(\sim\)1-Mb resolution limit of the gels. This absence of resolved size differences in the gene clusters from the tumor samples compared with the nontumor controls indicates that these tumors did not undergo recombination-mediated gene cluster alterations, although we cannot preclude alterations in the unresolved gene cluster lengths larger than 1 Mb in these patients. The banding patterns in MILST-BR, PARLA-CA, and CEVAT-MA are also the same between tumor and either peripheral blood or normal lung, indicating gene cluster stability; however, UGOBI-FO, KABIG-NI, and EVIEN-RA show evidence of pretumor alteration with new major bands, but no new minor banding. The remaining five tumors, TIPOR-PA, GLOAK-EU, MEERY-BE, MUVID-DO, and FOBIK-TE, all show both new major and minor intensity bands, consistent with a period of dysregulated recombination both before and immediately following commitment to clonal tumor cell expansion.

The colorectal tumor patients show a similar distribution of rDNA cluster instability as the lung cancer patients. Of the 10 colon cancer samples (Fig. 4), 4 appear stable (SAGOF-GR, FONET-VI, ARPIC-RO, and EBETH-DA), 5 more show preclonal expansion alterations (VOBLE-JO, DAPEB-MA, COBEZ-WI, RIZON-HE, and SNARG-GE), whereas 1 shows both new major and minor bands indicative of gene cluster restructuring both before and after tumor cell commitment (PULAB-GE). The process of metastasis does not necessarily involve recombinational gene cluster restructuring, as seen in the colon tumor from EBETH-DA, which was surgically recovered from a colon metastasis to lung. Likewise, it is notable that unlike the lung cancer patients, in nearly all of whom have a smoking history, rDNA cluster alterations are also seen in colon cancer patient tumors from either nonsmokers (RIZON-HE, PULAB-GE) or individuals with a relatively modest smoking history (VOBLE-JO).

The rectal cancers follow a similar pattern (Fig. 5). Two of four patients (CLIMP-HA and DOOVA-CL) show light stochastic breakage but are otherwise gene cluster stable. Two other patient tumors show evidence of either pre-expansion (BINTA-CH) or preclonal and postclonal expansion (WIVIT-HE) gene cluster instability. Our experience was that nontumor rectal tissue was difficult to work with; so generally, we compared tumor gene cluster structure to that from peripheral blood.

Architectural rearrangements in the rDNA clusters are less frequent in the 12 pediatric leukemias we have examined (Fig. 6).
Comparing peripheral blood containing blasts before or shortly after the initiation of treatment to peripheral blood from the same patients in remission with no residual detectable blasts after recovery from induction chemotherapy shows the same pattern of bands in 10 of the 12 different pediatric patients. Evidence of pre-expansion rearrangement is detected in NIPRY-JA and RAMAH-LE, with novel band intensities proportional to the fraction of blasts found in these patients’ peripheral blood. PALT-BR is an
interesting, if tragic, case. We first encountered this 5-year-old boy in remission 3 years after a diagnosis of and treatment for pre-B-cell acute lymphocytic leukemia before his allogeneic stem cell transplant. After his transplant, his peripheral blood shows the rDNA cluster pattern of the engrafted donor marrow. Shortly after the transplant, the patient relapses and we see a re-establishment of his pretransplant rDNA cluster pattern 2 weeks before his death. Although this individual underwent years of intensive chemotherapy and full body irradiation, his disease shows no evidence of rDNA cluster instability (remission versus posttransplant leukemia rDNA cluster pattern, ignoring the bands from the donor marrow).

Discussion

Of the solid tumors analyzed for rDNA cluster instability, the overall frequency of any observed rDNA cluster alteration is 54% (21 of 39; 95% confidence interval, 39–68% by adjusted Wald method). Thirty-one percent (12 of 39) showed alterations consistent with preclonal expansion alterations only, and a further 23% (9 of 39) showed evidence of recombination-mediated rDNA cluster alterations both before the start of clonal tumor expansion as well as in the several rounds of cell division immediately following commitment to clonal expansion. No evidence of rDNA cluster instability was observed in 46% (18 of 39) of cases. Because the rDNA clusters range in size up to 6 Mb and we only analyzed clusters smaller than 1 Mb, the frequency of 54% observed gene cluster alterations is likely an underestimate.

Considering that the rDNA clusters only make up ~0.5% of the diploid human genome, this alteration frequency of over 50% in human solid tumors indicates that the rDNA clusters are extraordinarily sensitive indicators of the capacity for human cancer to use recombination to alter submicroscopic genomic structure, and that this submicroscopic genomic restructuring is one of the most frequent chromosomal aberrations found in nonhereditary solid tumors. In comparison, rDNA alteration in pediatric leukemia is only found in ~15% of cases. This frequency difference in cluster recombinational restructuring between pediatric leukemia and adult solid tumors (P = 0.04 Fisher’s two-tailed exact test) may reflect the adult tumors’ progressive accumulation of genomic insults from both time and genotoxic environmental exposure, compared with more specific transplantation-driven disease in the pediatric patients.

The rDNA clusters are critical components of cellular metabolism, so recombinational dysregulation in the rDNA may directly alter the capacity for tumor cell growth. The hypermetabolic nature of cancer cells requires expanded capacity for protein synthesis. Because rRNA production is rate limiting for the construction of new ribosomes, instability in the rDNA clusters will allow for positive selection of subpopulations of cancer cells that have expanded their rDNA complement. Upregulation of rRNA expression through epigenetic derepression is already known in lung cancer (19), making selectable gene amplification of the rRNA an additional effective mechanism for producing the large number of required ribosomes in hypermetabolic, relatively rapidly dividing tumor cells.

In addition to its direct contribution to tumor cell metabolism, instability of the rDNA may be indicative of broader recombinational-based genomic instability in repetitive genomic elements and may serve as a sentinel biomarker for genomic alteration mediated by recombination between other high-similarity low-copy repeated sequences (20). For example, dysregulated recombination causing genomic restructuring of centromeric α-satellite repeats may result in centromeric dysfunction, contributing to the aneuploidy commonly seen in adult solid tumors (21). Intriguingly, other highly self-similar clustered gene loci in the human genome (22) express protein products found with relative specificity only in cancer and in highly recombination-active tissues such as testis: the so-called “CT” genes (23). We are actively pursuing whether these loci are also recombinationally destabilized in cancer and with what potential clinical relevance.

From a predictive standpoint, because DNA double-strand breaks are potent inducers of homologous recombination in human cells (24) and many chemotherapeutic agents exert their genotoxic effects through DNA double-strand breaks, the use of these agents in tumors with dysregulated recombination may prove to be problematic. Dysregulated recombination in tumor cells may facilitate large-scale genomic restructuring in response to radiomimetic chemotherapy accelerating establishment of chemotherapeutic resistance. Similarly, exposure to genotoxic agents from tobacco in potentially numerous precancerous cells with dysregulated recombination may contribute to the synchronous dissimilar primary lung tumors sometimes found in heavy smokers.

We are continuing to follow and expand our enrolled patient population to ascertain whether assaying rDNA restructuring in tumor cells has prognostic and/or predictive value. Specifically, now that we can divide solid tumor cancer patients into two approximately evenly populated cohorts, based on whether tumors either do or do not display evidence of dysregulated recombination, we will determine whether dysregulated recombination is informative with regard to risk of tumor recurrence, with the disease-free survival interval postsurgery, and with overall survival. For patients that undergo adjuvant chemotherapy or radiation therapy postsurgery, we will also determine whether the degree of responsiveness to adjuvant therapy is influenced by the presence of dysregulated recombination in the primary tumors. The dysregulated recombination cohort can be further subdivided, based on the presence or absence of minor banding in the tumor sample, into patients for whom the tumor was still actively recombining the rDNA clusters during the initial phase of tumor clonal expansion and into those for whom dysregulated recombination appeared to be restricted to the interval before primary tumor clonal expansion, allowing further fine-tuning of the potential clinical application of the assay.

In summary, we have detected structural alterations to the rRNA gene clusters in over 50% of the adult solid tumors examined, making these genomic rearrangements one of the most common specific chromosomal alterations in adult solid tumors. The functional significance of the rDNA for the capacity of tumor cells to produce protein, as well as the potential sentinel biomarker nature of the rDNA for recombination-driven genomic alterations involving other highly conserved low-copy repetitive genomic elements, suggests assaying rDNA cluster instability in tumors may prove to have prognostic and/or predictive value.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


REFERENCES


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