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Karyotype Heterogeneity and Unclassified Chromosomal Abnormalities

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Abstract
In a departure from traditional gene-centric thinking with regard to cytogenetics and cytogenomics, the recently introduced genome theory calls upon a re-focusing of our attention on karyotype analyses of disease conditions. Karyotype heterogeneity has been demonstrated to be directly involved in the somatic cell evolution process which is the basis of many common and complex diseases such as cancer. To correctly use karyotype heterogeneity and apply it to monitor system instability, we need to include many seemingly unimportant non-specific chromosomal aberrations into our analysis. Traditionally, cytogenetic analysis has been focused on identifying recurrent types of abnormalities, particularly those that have been linked to specific diseases. In this perspective, drawing on the new framework of 4D-genomics, we will briefly review the importance of studying karyotype heterogeneity. We have also listed a number of overlooked chromosomal aberrations including defective mitotic figures, chromosome fragmentation as well as genome chaos. Finally, we call for the systematic discovery/characterization and classification of karyotype abnormalities in human diseases, as karyotype heterogeneity is the common factor that is essential for somatic cell evolution.

Various large scale projects have generated a great amount of genomic data including DNA sequencing, copy number variation, and massive chromosomal changes across the genome [Feuk et al., 2006; Heng et al., 2011b; Podlaha et al., 2012]. This data has raised the question of whether traditional karyotype analysis will soon be replaced by molecular methodologies. Paradoxically, this flood of information has resulted in a great deal of confusion [Hayden, 2010]. First, these studies (especially those focused on pathological processes) have produced a large amount of heterogeneous data with few clear-cut patterns, challenging the clinical applicability of such studies. Hundreds of diverse mutations exist in each normal, healthy individual, as revealed by the personal genome project [The 1000 Genomes Project Consortium, 2010]. Second, it has been a challenge to integrate the multiple types of information and even harder to decide which information is more valuable when data conflicts. Despite the bias in favor of higher-resolution molecular methodologies, there is increasing support for karyotypic analyses, as karyotype alterations reflect genome level

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changes, and more karyotype abnormalities are significantly linked to diseases [Ye et al., 2007; Heng, 2010; Heng et al., 2011a, b].

Karyotypic analyses confer a number of benefits over molecular-based analyses, including the fact that they provide information about both individual cells and the entire population. This is important because the karyotype, rather than other sublevels of genetic organization, defines the genome system [Heng, 2009; Heng et al., 2011b]. Karyotypic analysis gives information about structural changes that is often not provided by molecular methods. Further, most molecular methods are based on a cell population average and overlook non-clonal chromosome aberrations (NCCAs). Finally, karyotypic analyses are more cost-effective than most molecular-based assays when analyses must be performed on a large number of cells from a heterogeneous population.

One of the new developments in karyotype analysis was the introduction of using NCCAs to study genome instability and related disease conditions including cancer [Heng et al., 2004, 2006a–c; Ye et al., 2007]. Traditionally, NCCAs were considered to be insignificant genetic 'noise', in favor of clonal chromosome aberrations (CCAs) [Mitelman, 2000; Heng et al., 2006a, c]. By treating the genome as a system, the importance of these seemingly random genome changes becomes apparent as each change results in a new cellular system, providing population variation. Thus, the measurement of stochastic genome alterations can be used as an index to study system instability and, in particular, genome instability [Heng et al., 2006a, c; Ye et al., 2007].

Potential links between NCCAs and many human diseases are significant [Heng, 2010], and they offer a new approach to study somatic cell evolution. Based on this, we have developed the concept of 4D-genomics which focuses on the 3-dimensional features of the genome context (genes plus genomic topology) and integrates this within an evolutionary timescale [Heng, 2013a]. Significant karyotype changes not only impact expression of hundreds if not thousands of genes, but also change the overall network structure. Therefore, alteration of a karyotype creates a new system [Heng et al., 2011a, b]. In order to facilitate incorporation of karyotype-based evolutionary principles into the current biological framework, we have proposed a 4D-genomics approach. The 4D-genomics approach places 1-dimensional genes into the 4D-genomic reality, explaining the importance of karyotype variation in an array of diseases. It is thus logical to refocus on karyotype analyses in disease studies, using the new framework of tracing stochastic genome alterations across the genome and within a cell population.

To recognize the importance of and further the study of NCCAs, we will first briefly describe the 4D-genomics concept, the importance of stochastic genome alterations, and then introduce a number of unclassified karyotype abnormalities, most of which were previously ignored. Finally, we describe the application of the 4D-genomics concept by calling for systematic analyses of karyotype heterogeneity in human diseases in the search for the missing genetic link.

The Ultimate Importance of Karyotypes in Natural and Somatic Cell Evolution – A 4D-Genomics Perspective

Genetics has traditionally focused on the gene with karyotype analysis serving as a powerful tool in an attempt to identify the genes responsible for disease. As the possibility of direct sequencing and/or detection of copy number variation becomes feasible and affordable, there is the perception that karyotype analysis will be outdated as it is lower resolution than gene-based analyses, is not 'molecular enough', and is more time-consuming.

In contrast, the recently established genome theory emphasizes the importance of analyzing the karyotype instead of profiling genes, stating that genes do not serve as independent information units as the true function of individual genes is defined by a given genome (thus the same gene can have different functions within different genome contexts) [Ye et al., 2007; Heng, 2009]. Surprisingly, it has been realized that system inheritance is achieved at the genome level (genes interacting within genomic topology) influenced by specific karyotypes. The inheritance of parts (determined by genes) has little to do with the inheritance of the entire system assembly (determined by karyotypes and maintained by sex) [Heng, 2007b; Gorelick and Heng, 2011; Heng et al., 2011b; Horne et al., 2013]. Such an important concept has been recently established in both somatic cell evolution and organismal evolution.

The concept of clonal evolution has dominated cancer research since Peter Nowell's seminal paper [Nowell, 1976]. Cancer research has been influenced by the successful characterization of CML where the *BCR-ABL* fusion gene on the Philadelphia chromosome is a strikingly clonal event. This framework has since been applied to other cancers and stepwise cancer progression has been generally accepted [Vogelstein and Kinzler, 1993; Sjob-

lom et al., 2006]. It is believed that accumulation of cancer gene mutations is the evolutionary driving force in this process. However, by watching cancer evolution in action in a spontaneous cellular immortalization model, we have unexpectedly discovered that cancer evolution occurs in 2 phases [Heng et al., 2006c]. The first phase is the punctuated, discontinuous phase (non-clonal phase) where massive karyotype changes occur and are eliminated stochastically. The second phase is the stepwise, gradual phase (clonal phase) where most cells share similar karyotypes that evolve in a traceable manner. Further synthesis links the punctuated phase and stepwise phase to macro- and microevolution, respectively [Heng et al., 2006a–c, 2011a, b]. The important message here is: cancer evolution is driven by a system replacement (karyotype replacement) process, and genome alterations leading to population heterogeneity are more important than the gene mutations that dominate the stepwise phase of somatic cell evolution [Ye et al., 2009]. In agreement with this concept, the elevated levels of NCCAs (including structural NCCAs and numerical NCCAs such as aneuploidy) have been linked to tumorigenicity and drug resistance in vitro [Heng, 2007c] as well as in clinical studies [Chandrakasan et al., 2011].

Interestingly, the karyotype is the cornerstone of not only somatic evolution, but also organismal evolution. In organismal evolution, the genome serves as a major constraint for maintaining the system. In sexually reproducing species, the function of sex is to maintain the identity of the species by filtering out all significantly altered genomes, ensuring the faithful passing of the genome, as only normal chromosomes can be properly paired during meiosis and also undergo the entire sexual reproduction process [Heng, 2007b; Ye et al., 2007]. The realization of the main function of sex has drastically altered a century-long viewpoint on meiosis as the major mechanism to increase genetic diversity [Wilkins and Holliday, 2009]. It is likely that the genetic combination of mixing genes only serves as a secondary function. Thus, the genome serves as a key evolutionary constraint both for somatic cell and organismal evolution [Heng, 2009; Gorelick and Heng, 2011]. In somatic cell evolution, when genome constraint is broken, disease evolution accelerates [Heng, 2010; Heng et al., 2010, 2011a, b]. In organismal evolution, the genome constraint maintains the species regardless of the gene dynamics. When a new genome forms and is passed on, a new species is formed [Heng, 2013a].

Based on the above syntheses, we propose a new genome-based framework of genomics, called 4D-genomics [Heng, 2013a]. 4D-genomics combines 3D-genome complexity with time and serves as the biological basis for passing genetic information and provides a platform for evolution including somatic cell evolution that drives disease progression [Heng, 2009, 2013a]. Clearly, to understand genome complexity, it is necessary to categorize karyotype heterogeneity rather than extensively focus on gene level change. Despite a decade's worth of research describing different types of karyotype abnormalities, most effort has focused on specific chromosomal aberrations. Even though there are large amounts of non-specific chromosomal alterations detectable in clinical samples, most have gone unreported and ignored. Here, we refer to these structures as unclassified chromosomal aberrations. They are extremely important as they are all considered NCCAs, which can be used as an index of karyotype heterogeneity. The application of 4D-genomics will be discussed later.

Ignored and Unclassified Chromosomal Aberrations

The discovery, characterization of various chromosome abnormalities, and linking them to diseases has been a central focus of medical cytogenetics. Identification of trisomy 21 and the development of various chromosome banding methodologies led to the recognition of a number of chromosomal aberrations associated with disease. These have since served as an important tool for clinical diagnosis. Researchers often eschew older technologies in favor of newer technologies, specifically molecular methods utilizing high resolution. We would like to point out that an important key to proper study design is to identify the correct or most influential level of study, rather than always pushing towards the molecular level [Ye et al., 2007; Heng, 2013a, b]. Indeed, even at the microscopic levels, there still are many types of chromosomal aberrations that have been largely ignored due to conceptual limitations rather than the methodologies themselves. The following are some examples.

Free Chromatin

On slides of conventional cytogenetic preparations (hypotonic treatment, fixation and air-drying), among mitotic figures and interphase nuclei, there are spindle- or rope-shaped structures (fig. 1). Originally, they were thought to be non-chromatin contamination or slide preparation artifacts. We have demonstrated that these structures are chromatin released from interphase nuclei as they contain the same amount of DNA as nuclei, but they lack nuclear envelopes [Heng and Chen, 1987].

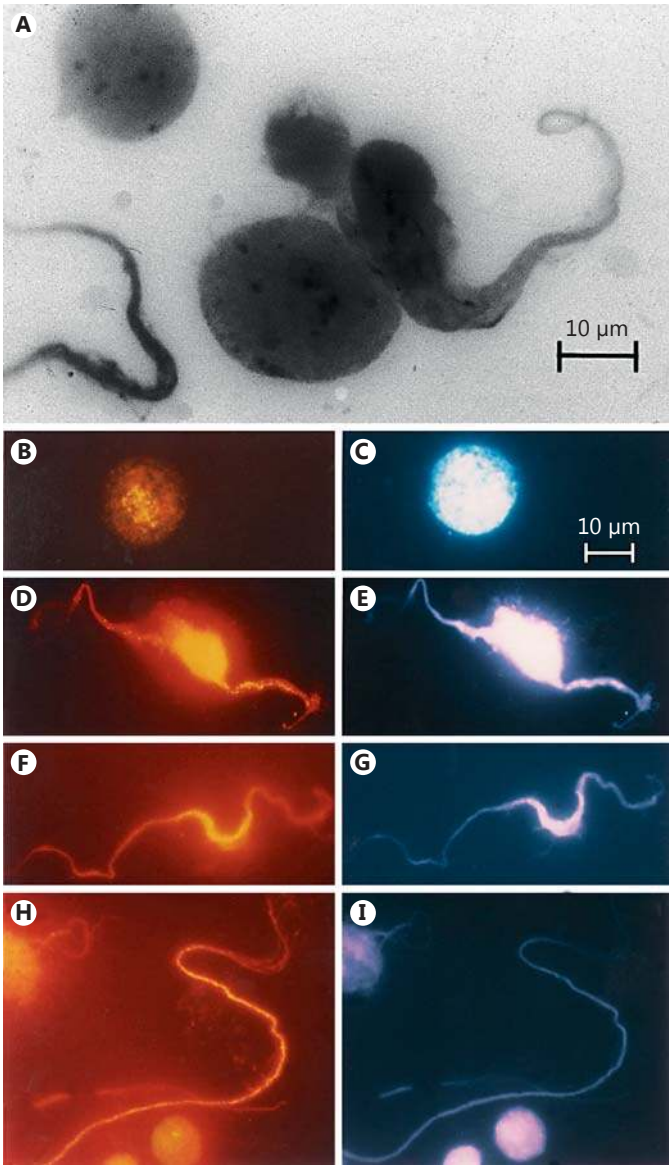

Fig. 1. Examples of free chromatin. **A** Example of the typical morphology of free chromatin (spindle and rope shapes) and 3 interphase nuclei detected from routine chromosome preparations without any treatment (reverse DAPI staining image). **B–I** FISH signal and morphological comparison between interphase nuclei and various free chromatin generated from protocols releasing free chromatin [Heng et al., 1992]. Interphase nuclei (**B**, **C**) and free chromatin (**D–I**) were prepared from a human-hamster hybrid cell line 4AF/106/KO15, which contains an altered human chromosome 7. **B**, **D**, **F**, **H** FISH detection results. The yellow signals represent a human chromosome (the FISH probe used is total human DNA). **C**, **E**, **G**, **I** Corresponding DAPI staining. From **D** to **H**, there is an increased degree of stretching.

In spite of the fact that these structures have been used to develop high-resolution fiber FISH [Heng et al., 1992; Heng and Shi, 1997], which has made an important contribution to physical mapping and has been used by the Human Genome Project, their biological significance is not clear even today, due to the lack of outside follow-up studies. These structures can be related to hypotonic conditions and cell cycle stages, but under standard slide preparation conditions, detection of higher frequencies of these structures can be linked to different cell lines, specific chemical reagents and individual differences. For example, some cell lines have higher free chromatin frequencies, and there is a clear dose-response relationship between free chromatin and many chemotherapeutics. It is possible that these frequencies are related to nuclear envelope instability and overall genome instability. It has also been suggested that free chromatin could be used to monitor toxicity [Heng et al., 1988a, b; Heng and Zhao, 1989; Heng and Shi, 1997].

Defective Mitotic Figures

During the development of high-resolution banding methods for frog chromosomes [Heng et al., 1987], one of us (H.H.Q.H.) discovered structures called defective mitotic figures or DMFs (fig. 2). DMFs were initially described as 'uncompleted-packing-mitotic figures' [Heng and Chen, 1985; Heng et al., 1988b]. This nomenclature was proposed based on the co-existence of condensed chromosomes and undercondensed chromatin fibers within one mitotic figure.

DMFs represent an ideal tool to illustrate and determine the high-order model of chromosomal packaging due to the transitional structures that connect the condensed chromosomal regions and undercondensed regions. Unfortunately, since our initial report nearly 30 years ago, there has been only limited interest in these structures. An undesirable aspect of DMFs is that their existence makes it difficult to reconcile the generally accepted idea that there is a scaffold within metaphase chromosomes, and it was very difficult to imagine how DMFs form if there is a scaffold within chromosomes [Laemmli, pers. commun.]. Another challenge was inducing DMFs in high frequencies. Induced frequencies are often low and individual samples can vary widely. It has been difficult to pinpoint the mechanism of DMF formation; however, we have demonstrated that the direct molecular mechanism of DMFs is a combination of a condensation defect and a G2-M checkpoint defect. Interestingly, DMFs are commonly detected in various cancer cell lines and patient samples, suggesting that the chromosomal

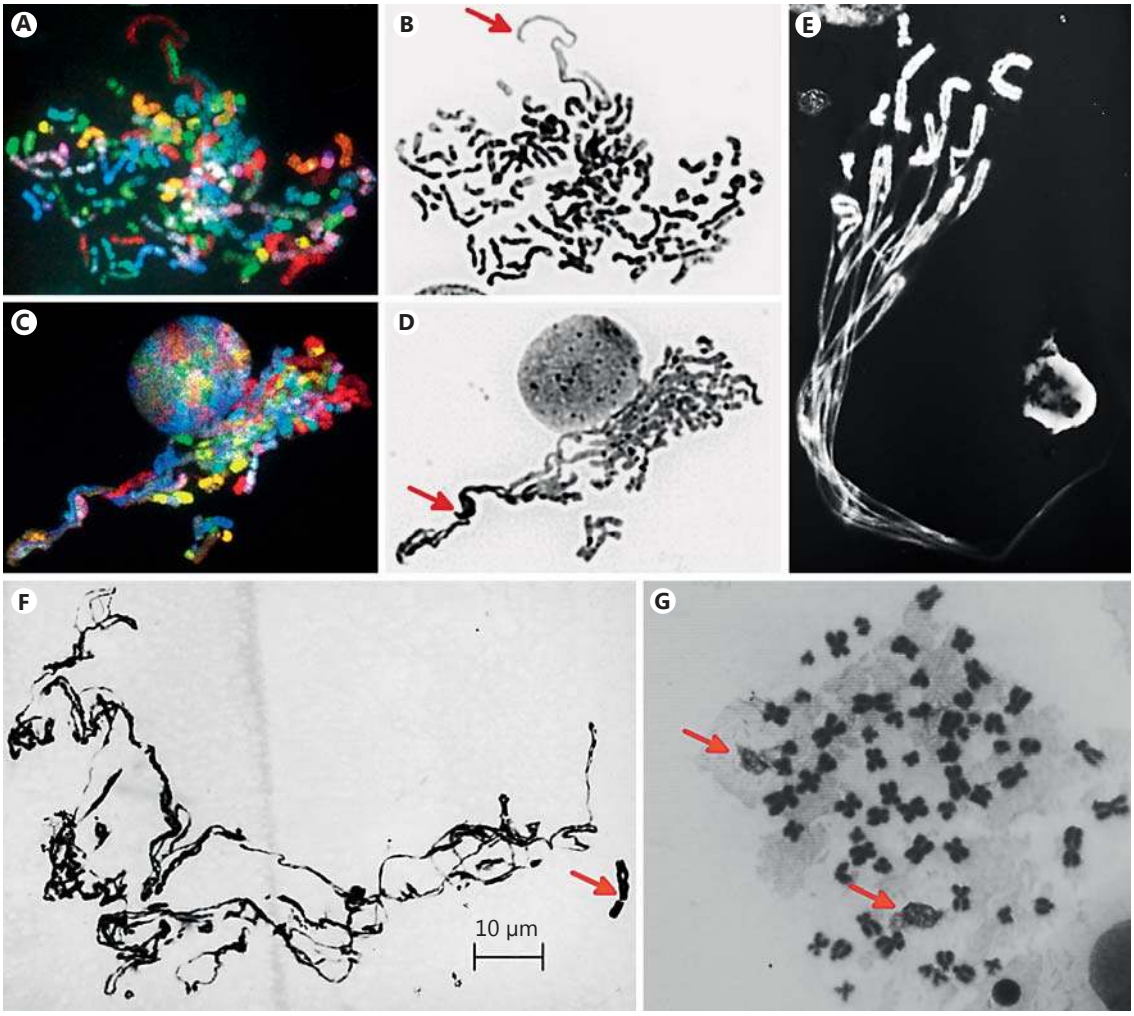

Fig. 2. Examples of various types of DMFs. **A–E** Typical DMFs detected from treated blood cultures of human (**A–D**) and frog (**E**), respectively. **A**, **C** SKY images. **B**, **D** Corresponding reverse DAPI images. The arrows indicate the uncondensed chromatin regions. **E** DAPI image of a frog DMF, where both the condensed chromosomes and uncondensed chromatin fiber are clearly illustrated. In these DMFs, the condensed mitotic chromosomes are distributed at one end, which is the main form of DMF. **F** DMF with an atypical pattern of distribution, but the differential condensation among different chromosomes is evident. One normal, condensed chromosome is indicated by an arrow. **G** New type of DMF displaying diffused chromosomes detected in a patient with chronic fatigue syndrome. As indicated by the arrows, some chromosomes seem to be decondensed. For all types of DMFs, the common key feature is the differential condensation among chromosomes.

condensation process is an important factor in cancer [unpubl. data].

It should be pointed out that the issue of condensation defects has been addressed by a few other groups. It was found that treatment of cells with 5-Aza-dC can induce heterochromatin undercondensation. An example of induced heterochromatin undercondensation in the giant X chromosomes of *Microtus agrestis* is very striking [Haaf and Schmid, 1989]. A similar phenomenon to DMFs has also been reported under the heading of replication delay and condensation delay [Smith et al., 2001]. DMFs can be generated by many factors, but a replication error is not the main reason for the formation of DMFs [Heng, 2013a]. The appearance of DMFs raises an interesting question; is there a condensation order among chromosomes? Using specific cancer cell lines, it seems that some chromosomes have higher than expected frequencies of DMFs, indicating that these chromosomes may condense later than others. For example, in a prostate cancer cell line, the one copy of chromosome 1 is often found amid

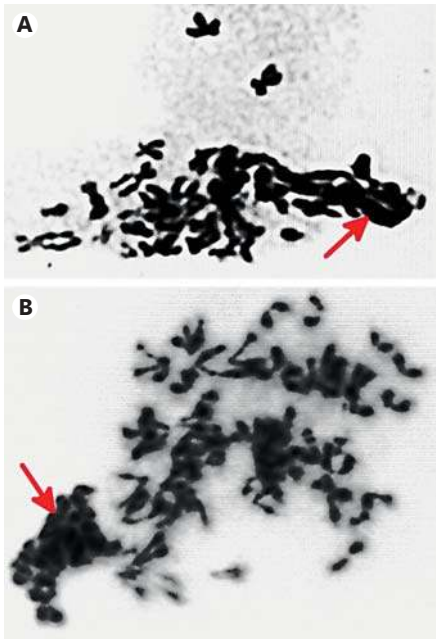

Fig. 3. A, **B** Example of sticky chromosomes. Chromosomes stick to each other and are tangled by chromatin fibers (arrows). If a cluster of tangled chromosomes is observed within well-spread chromosomes, it might indicate sticky chromosomes. When chromosomes are sticky, chromatin fibers are often visible among chromosomes. These sticky chromosomes also display a fuzzy morphology.

a few uncondensed chromosomes (which are among the last to condense). This observation has also been confirmed by FISH painting using a chromosome 1-specific probe. Interestingly, we recently detected high rates of DMFs and other related abnormalities in chronic fatigue and immune dysfunction syndrome patients.

Sticky Chromosomes

Sticky chromosomes can be observed following treatments that interfere with DNA replication, chromosomal condensation and methylation. It is known that the sticky chromosomes can be observed in high frequencies after drug treatment such as ethidium bromide (EB). Some cancer samples that display high frequencies of DMFs also have increased sticky chromosome frequency, possibly due to abnormal replication and condensation (fig. 3). There seems to be a correlation between sticky chromosomes and difficulties in preparing suitable mitotic figure spreads. In some cases, 'fuzzy' chromosomes are closely associated by sticking to each other which is not caused by improper slide preparation but is likely due to condensation status. It has been suggested that alteration of DNA methylation can prevent the synchronization of chromatin compaction leading to improper condensation [Flagiello et al., 2002]. Clearly, when high frequencies of sticky chromosomes appear in analyses, additional attention is needed such as checking the methylation and condensation status, rather than disregarding them as slide preparation artifacts. Interestingly, pulling a single nucleolus or chromosome out of interphase or mitotic cells using a microsurgical technique under isotonic conditions leads to the sequential removal of the remaining nucleoli and chromosomes that are interconnected by a continuous elastic thread [Maniotis et al., 1997]. This suggests that there is a structure connecting all chromosomes within the nucleus. The general stickiness of chromosomes should be considered as a possible mechanism for this. An approach to this would be to use spectral karyotyping (SKY) to identify the order of chromosome chains. Consistency among the order of chromosomes would provide essential information.

Unit Fibers

Using metaphase chromosome isolation methods, Bak et al. [1979] described 'unit fibers' as substructures of metaphase chromosomes. These 'unit fibers' have a constant diameter of about 0.4 μm, which is approximately 5-fold less than the final condensed chromatids in metaphase chromosomes. Interestingly, such structures can also be induced by drug treatment (such as topoisomerase II inhibition) in short-term lymphocyte cultures from various species. Frog chromosomes in particular produce unit fibers with clear morphology (fig. 4). Chemically induced unit fibers differ from Bak's unit fibers isolated from metaphase chromosomes in that the former produces 2 parallel fibers called sister unit fibers whereas the latter results in a single unit fiber. It is likely that the isolation procedure separates the sister chromatids of the metaphase chromosomes prior to decondensation into unit fibers. The existence of the unit fibers strongly suggests that the metaphase chromosomes are packaged by multiple levels of coiling organization and the unit fiber is the substructure of the last level of packaging.

Chromosome Segregation Errors

Vig [1983] has extensively investigated the order of chromosome segregation. It was observed that at metaanaphase, the centromeres of chromosomes in a given genome do not randomly separate into 2 sister units. Such controlled and species-specific sequence is independent of the length of the chromosome or the position of the centromere, but apparently dependent upon the quantity and quality of pericentric heterochromatin [Vig and

Willcourt, 1998]. Alteration in the physical state of heterochromatin, such as decondensation, is associated with aberrations in the pattern of centromere separation. Interestingly, centromere separation errors are implicated in human disease. This line of investigation should be followed using available methods such as SKY to trace the segregation order changes in cancer and other diseases.

Chromosome Fragmentation

Chromosome fragmentation (C-Frag) is a form of mitotic cell death where condensed chromosomes are progressively degraded [Stevens et al., 2007, 2011]. C-Frag has been observed for several decades, but its mechanism was previously unknown. Initially, C-Frag was confused with chromosome pulverization (also known as premature chromosome condensation) [Stevens et al., 2010]. Recently, C-Frag has been characterized as a main form of mitotic cell death. It differs from apoptosis as well as mitotic catastrophe (fig. 5) [Stevens et al., 2007]. Importantly, C-Frag has been linked to diverse types of cellular stress including gene mutations, ER stress, infection, drug treatment, and centrosome dysfunction. C-Frag thus represents a general response to system stress rather than a specific type of stress [Stevens et al., 2011]. This explains why so many individual molecular mechanisms can be linked to this phenomenon [Stevens et al., 2013]. C-Frag can lead to both aneuploidy (when fragmentation occurs involving only one or a few chromosomes) or genome chaos (see next session), and thus it can contribute to karyotype abnormalities [Stevens et al., 2007, 2013].

Genome Chaos

Genome chaos or karyotype/chromosome chaos has been used to describe the massive, rapidly acquired genome changes that occur during the punctuated phase of cancer evolution [Heng et al., 2006c, Heng, 2007c, Duesberg, 2007]. Karyotype chaos can be divided into structural chaos, numerical chaos, or a combination of both types (fig. 6). Structural chaos is determined by either the percentage of cells that display a chaotic phenotype or by the presence of massive rejoining (more than 5 translocation events on one chromosome, for example) within each cell. The types of chromosomal aberrations of a chaotic phenotype are diverse and include chromatid breakage, translocations, rings, individual chromatids, C-Frag, large-scale chromosome fusion, rings formed by chromatids, combinations of structural and numerical changes, and other striking phenotypes. Genome chaos has been observed in various cancer progression and drug resistant models as well as in clinical samples [Wahab et al., 2008].

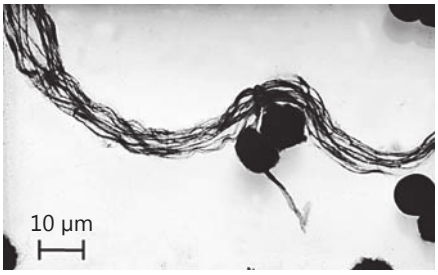

Fig. 4. Example of unit fibers (duplicated from Heng et al., 1988a). These Giemsa-stained unit fibers were prepared from a frog chromosome culture treated with BrdU (2 h) prior to slide preparation. There are a few interphase nuclei. The bundle of all unit fibers comes from one cell. Note that sister unit fibers exist in parallel. The diameter of these unit fibers is approximately 0.2 μm.

Recently, the cancer genome sequencing project revealed massive reorganization localized within a single chromosome in patient samples. This subtype of genome chaos was named chromothripsis [Stephens et al., 2011]. Though the discovery of the unique rearrangement of chromothripsis by deep sequencing is exciting, we would like to point out that massive genome reorganization is well documented in the cytogenetic community, genome chaos was discovered by karyotype analysis years ago, and finally, that molecular cytogenetic methods are crucial to understanding genome chaos. We recently introduced a model explaining the mechanism of genome chaos in which stress-induced C-Frag serves as material for genome reorganization [Heng et al., 2011a, b]. While the majority of chaotic genomes will eventually be eliminated, the diversity of genomes created during this important process increases the probability that an evolutionary winner will be selected [Liu et al., unpubl. observation]. Clearly, both the process of genome chaos and its clinical implications particularly for cancer treatment need to be systematically analyzed.

Micronuclei

Micro- and multiple nuclei can arise from a number of events and are normal in some cell types such as megakaryocytes. Micronuclei (MN) contain whole chromosomes, fragments of chromosomes or combinations thereof [Huang et al., 2011]. MN are produced by at least 7 different mechanisms including chromosomes displaced during metaphase, slow separation of chromosomes in bipolar and multipolar anaphase to telophase transition, fragments of broken chromosome bridges, inheritance from mother cells, nuclear fragments that move at the same speed as chromosomes but are slightly

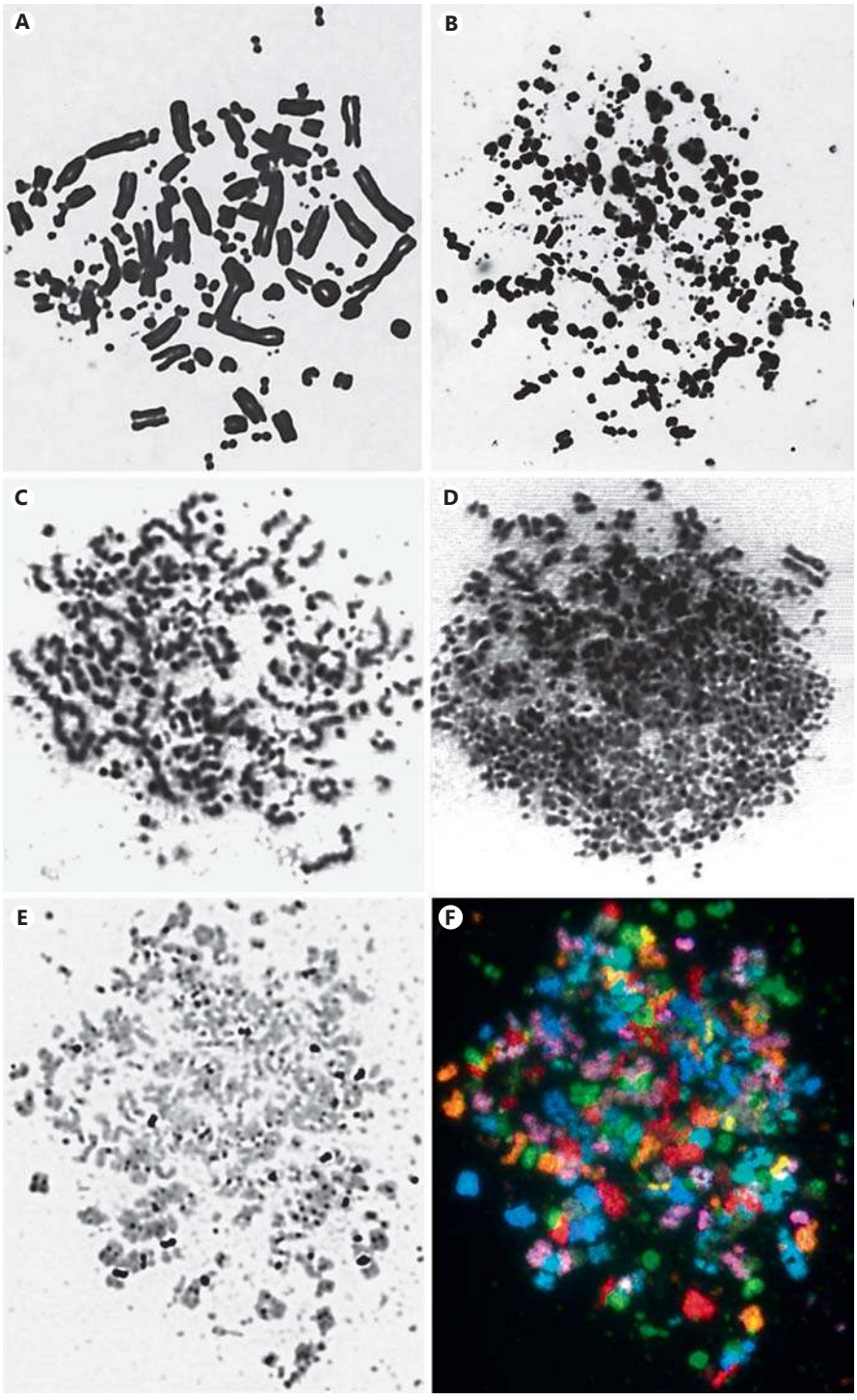

Fig. 5. Examples of C-Frag. **A** In early-stage C-Frag, many individual chromosomes are normally condensed. **B** In late-stage C-Frag, most of the chromosomes are fragmented. **C–E** In addition, C-Frag can occur in both early (**C**) and late mitotic figures (**D**, **E**). **F** SKY image of C-Frag in **E**. **D** Intact chromosomes and completely fragmented pieces can be detected from the same mitotic figure.

separated during anaphase, extrusion of a chromosome to a mini cell which then fuses to a daughter cell, and nuclear buds during interphase [Huang et al., 2011]. An example of budding interphase nuclei as well as MN from mitotic chromosomes is shown in figure 7. MN formation has been linked to dysfunction of gene networks involving DNA damage responses that are common to cancer, and some genetic polymorphisms that increase MN frequency have been identified [Iarmarcovai et al., 2008; van Leeuwen et al., 2011], but the exact mecha-

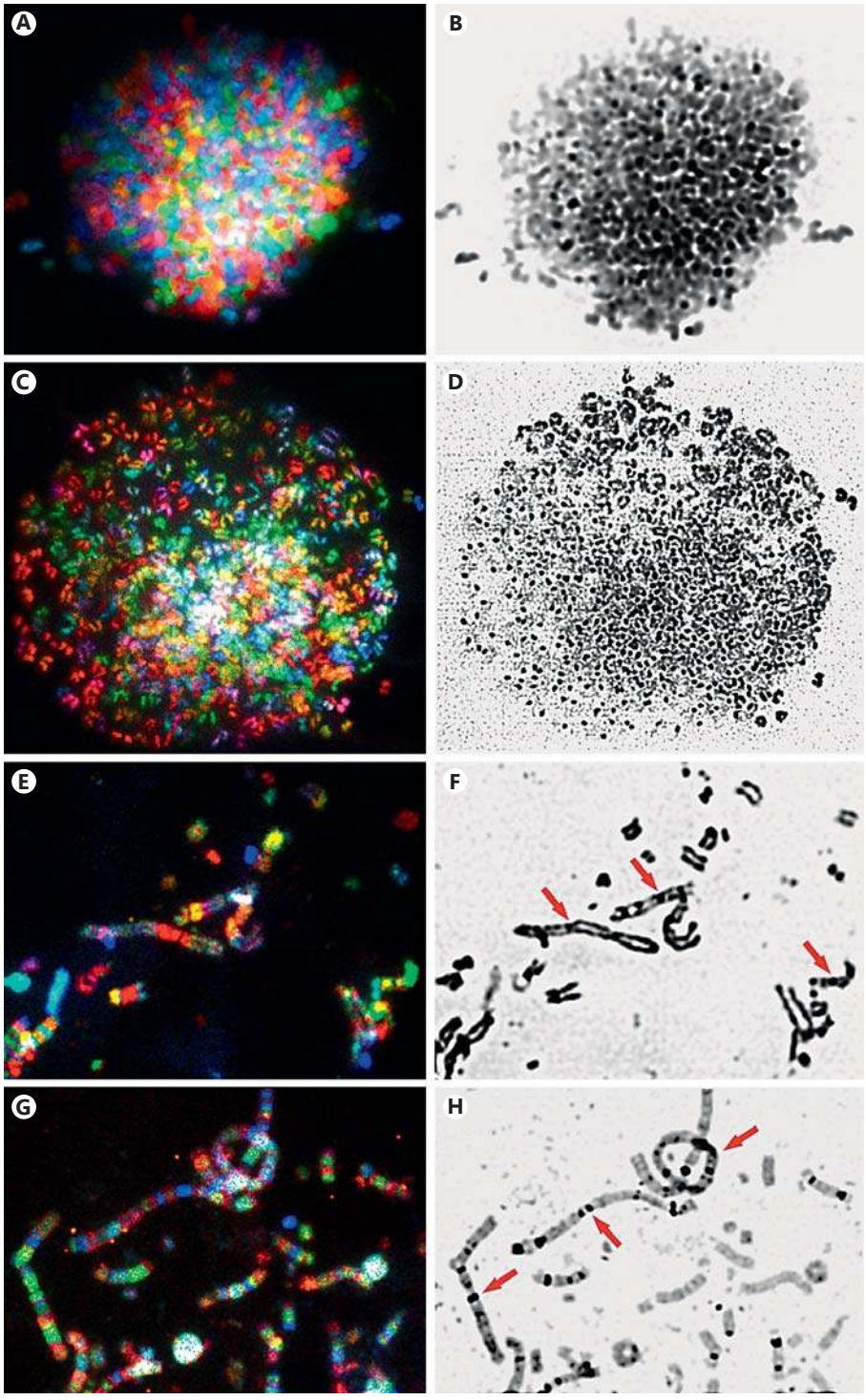

Fig. 6. Examples of genome chaos. **A–D** SKY and DAPI images of numerical chaos. In human (**A**, **B**) and mouse (**C**, **D**) cancer cell lines, the chromosome number can be hundreds (**D** with >550 mouse chromosomes). Note that these chromosomes are of normal size and are not fragments. **E–H** Images of structural chaos where a large number of chromosomal abnormalities including massive translocations, long fused chromosomes indicated by arrows, single sister chromatids, ring chromosomes and C-Frag are visible. **E**, **F** Genome chaos induced from a human cancer cell line. **G**, **H** Induced from mouse cancer line. Arrows indicate the newly formed long chromosomes containing multiple parts of other chromosomes. For all images, the left panels are SKY images where the mixed color indicates multiple translocations, the right panels are reverse DAPI images.

nisms of MN formation are not yet clear. In addition, it is known that MN can be associated with various types of cell death, in particular. It is therefore necessary to systematically analyze MN and their relationship with cell death.

For 15 years, the HUMN (International Human Micronucleus) project has coordinated the study of MN formation associated with DNA damage. MN frequency in peripheral lymphocytes has been shown to increase with age especially after 30 years of age, is higher in males than

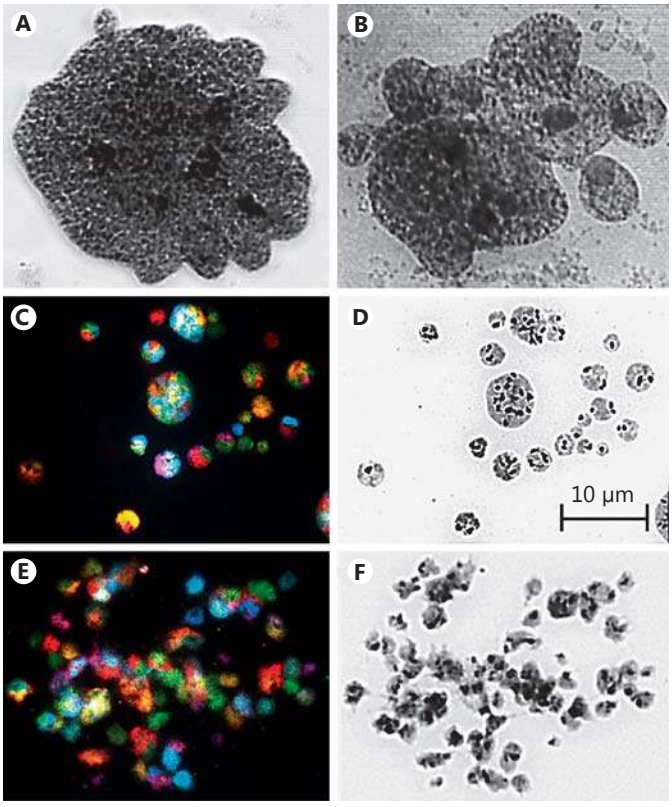

Fig. 7. Examples of micronuclei. **A**, **B** Illustrations of the various MN including multilobulated nuclei (**A**). **C**, **D** SKY and reverse DAPI images of MN. The smallest-size MN often display one color, indicating formation of material from one chromosome. According to the reverse DAPI image (**D**), the 1-color MN are from one chromosome as judged by the number of centromere signals within each MN. The bigger MN is linked to a number of chromosomes. **E**, **F** Some MN can form directly from chromosomes. As determined by morphological features (**F**), there are a mixture of chromosomes and MN, indicating that these chromosomes were decondensed and then formed into MN.

females, and is elevated upon exposure to harmful chemicals and radiation [Bonassi et al., 2011; Fenech et al., 2011]. Interestingly, MN frequency is depressed in smokers, but elevated in smokers that go on to acquire lung cancer [Fenech et al., 2011]. In fact, increased MN frequency is associated with cancer in general, especially urogenital and gastrointestinal cancers. MN formation has also been applied to the identification of dietary deficiencies [Fenech, 2002]. Although the exact results of MN formation are not known, MN formation is indicative of overall genomic instability. The presence of MN indicates genome change, but the lack thereof has not been shown to indicate the opposite. Further work is therefore required to determine the exact relationship between MN and karyotypic change. In the meantime, however, MN should be treated as NCCAs as they alter the genome topology. Future studies should seek to identify the quantitative contribution of MN to overall NCCAs.

Other Unclassified Structures

There are many unclassified types of chromosomal or nucleus aberrations detectable from conventional chromosome slides, particularly from cancer samples. Many of these aberrations have unknown mechanisms but appear to be associated with various treatments. In order to encourage more attention to these structures, we will share some observations on structures that have not previously been extensively studied or considered. The following are examples:

Nuclei with a Small Hole

Nuclei with small holes, or donut shapes were observed during chromosome preparation of bone marrow from irradiated mice. This phenomenon also occurs following drug treatment, such as pingyanymycin (antitumor-antibiotic complex which belongs to the bleomycin family) or BrdU (fig. 8A). There appears to be a dose-response link to the frequency of this type of nuclei. Treated samples show a frequency increase of 20–25-fold compared to untreated animals.

Abnormal Nuclear Morphology

Chemotherapeutics such as doxorubicin can induce interphase nuclei with strange morphologies (fig. 8C–E, G, H). The pattern of chromatin condensation is rather different depending on the dosage and treatment duration.

Giant Nuclei

In normal cytogenetic slides, we can observe big nuclei that are a few times larger than average. In particular, drug treatment (such as doxorubicin) increases giant nuclei frequency. As demonstrated in figure 8G, the size of a giant nucleus is much bigger than a regular interphase nucleus.

Further understanding of the above described structures opens the door to a new area of cytogenetic research interest. It is very unsettling not to know what the structures are despite frequent observations of these structures. It is possible that some of these structures are only generated from specific drug treatment, but it may still be important to consider using them to monitor drug treatment effects and consequences. Due to the importance of the identification of DMFs and C-Frag, we are confident that the systematic characterization of types of aberration that contribute to karyotype heterogeneity is very valuable.

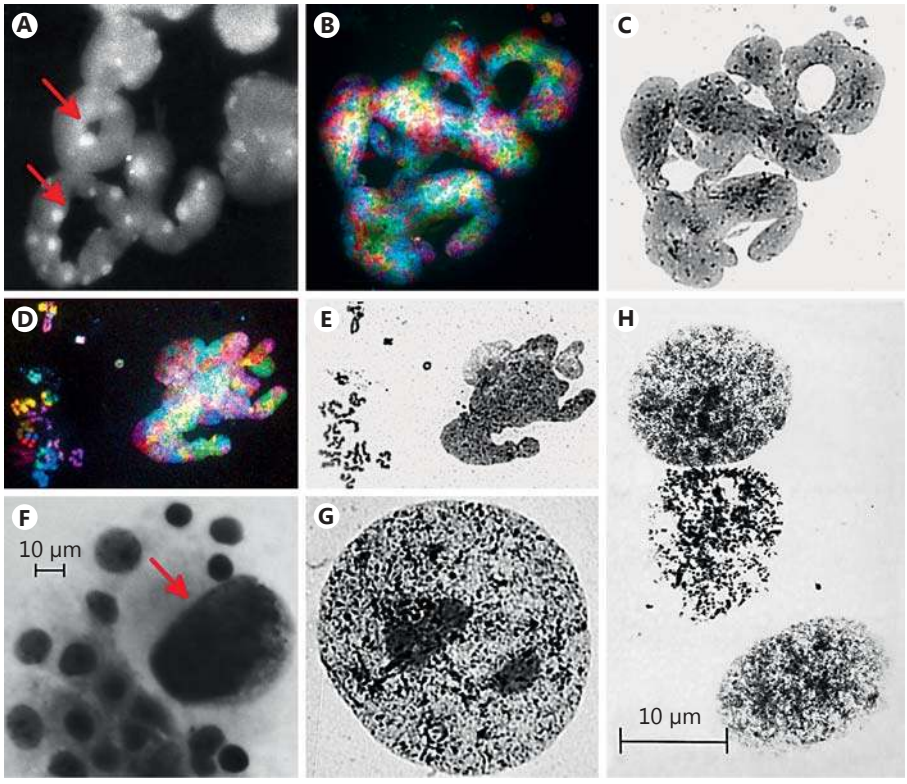

Fig. 8. Unclassified chromosomal or nuclear abnormalities/variations. **A** DAPI image of a mouse nucleus with 'holes' following BrdU treatment. The holes are indicated by the 2 arrows. The slide was made by routine cytogenetic preparation of mouse bone marrow following BrdU treatment. The bright spots are heterochromatin. **B–E** Irregular shapes of nuclei observed from cancer lines (both SKY and reverse DAPI images). **F** Huge interphase nuclei in a human blood cell culture following doxorubicin or EB treatment. The large nuclei (indicated by an arrow) are clearly bigger than average size nuclei (the rest of the nuclei). **G**, **H** Special morphology of nuclei following doxorubicin treatment. Some nuclei show morphology similar to late-stage C-Frag.

The Significance of Using NCCAs to Monitor Karyotype Heterogeneity

Many of these ignored types of chromosome aberration are NCCAs, as they often involve different chromosomes and alter genome context. Using cancer research as an example, we have advocated the importance of studying the relationship between NCCAs and CCAs, and have applied NCCAs to measure genetic instability [Heng et al., 2004, 2006a–c]. Increasingly, reports have linked overall genome changes (karyotype abnormality) to many common diseases and specific physiological or pathological conditions [Ye et al., 2007; Iourov et al., 2008; Astolfi et al., 2010; Duncan et al., 2010]. For the convenience of our readers, we will briefly summarize the main points of our reasoning.

Using Stochastic Genetic Alterations to Monitor System Dynamics

Cells are biosystems which function primarily in complex manners. No matter how dedicated we are in studying them, this complexity cannot be distilled to discrete linear relationships and specific genetic changes cannot predict most common and complex diseases. There has been success in the past in determining causative factors of single gene diseases, but it is a major challenge to pinpoint a specific causative relationship in common diseases like cancer. In cancer, there is no fixed specific molecular mechanism shared within a patient population due to the diverse factors involved in progression and the contribution of the environment-time interaction. To address this reality, seemingly stochastic changes of the system must be used to study the patterns of system dynamics. According to the system control principle, monitoring nonspecific genomic changes should be the most effective way to study general system dynamics. A recent report using random gene expression to predict cancer represents just such an example [Venet et al., 2011]. Interestingly, genome level stochasticity should be a better predictor than any gene level change, as the genome is the highest level of genetic organization and the information control level of the biosystem. We have illustrated this point by linking NCCAs to overall tumorigenicity and acquisition of drug resistance.

The Evolutionary Meaning of Karyotype Heterogeneity

The next phase of genomics, 4D-genomics, applies evolutionary principles to genome research [Heng et al., 2011a; Stevens et al., 2013]. As most diseases, including cancer, involve an evolutionary process at the somatic cell level, it is necessary to understand the significance of somatic cell evolution and karyotype heterogeneity. For example, the 3 key evolutionary components of cancer evolution are: (a) there must be variation in the cell population from which the tumor arises; (b) the variation must be heritable, and (c) the variation must affect survival or reproduction. Karyotype heterogeneity is an essential aspect of these key components.

When discussing variation, one must ask what types of genetic variation are more dominant; and the answer surely is the genome. When discussing inheritance, the question is what defines genetic inheritance, the gene or genome? And the answer is that system inheritance is ensured by the genome context and simply can be thought of as the integrity of the karyotype (as unaltered karyotypes reflect the unchanged genome context). Finally, when discussing achieving fitness, the question of whether it is a rapid punctuated or slow stepwise process, must be posed, and the answer to this is both. There are 2 phases of evolution, and the more crucial punctuated phase is driven by a high level of karyotype heterogeneity.

We recently described the evolutionary mechanism of cancer [Ye et al., 2009; Heng et al., 2011b; Heng, 2013a] which can be condensed to 3 key components including stress-induced system instability, population variation, and genome-mediated macroevolution. Again, the direct relation of all 3 components to karyotype heterogeneity suggests its primary importance in cancer evolution. Evolutionary mechanisms are explained by widely varying and different types of independent molecular mechanisms, which also suggest the presence of widely diverse genome level alterations. The degree of karyotype heterogeneity can thus be considered to be an indication of the evolutionary potential of a population.

It is crucial to realize that genetic heterogeneity and particularly karyotype heterogeneity is the driving force behind many diseases. Evolution only occurs when there is genetic variation and competition, and the potential for rapid macroevolution is dependent on high levels of karyotype heterogeneity. In general, evolution is not dependent on a specific pathway (not only because of the plethora of pathways in the first place, but also because so many factors can alter pathway function during evolution, rendering it an unpredictable process), but rather depends on the presence of genetic heterogeneity, which is the rationale for focusing on heterogeneity rather than on specific pathways for most common and complex diseases. This approach is strongly supported by the results of the current cancer genome sequencing project which demonstrates that there is overwhelming genetic heterogeneity at the gene mutation level, copy number variation level and gross karyotype level in all the major types of cancer. In cancer, heterogeneity is the rule, and high penetration of any single specific genetic alteration is the exception. We predicted these results when the cancer genome project first began [Heng, 2007a]. This prediction was based on an appreciation of the multiple levels of the genetic/epigenetic heterogeneity that exists in most cancers [Heng, 2007a]. It is now time for the research community to take action and reevaluate the overall concepts and strategies applied to cancer and other common diseases.

Returning Karyotype Analysis to the Driver's Seat with 4D-Genomics

We have consistently stated that the karyotype is an important feature of a biological system and is not reducible. Therefore, karyotype analysis requires a dominant position in genomics and cannot be replaced by higher-resolution molecular methods focusing on levels below the genome. Somatic cell evolution cannot be understood if the explanation is solely based on the parts of the genome. If we do not appreciate the governing role of the karyotype as we develop a holistic understanding of somatic cell evolution, we will never understand complex diseases. Traditional efforts focusing on the gene make sense under the gene-centric paradigm, but in contrast, the importance of karyotypic heterogeneity is enlightened by the 4D-genomics concept. Of course, we welcome the combinational approaches of molecular cytogenomics, but the karyotype must remain the core factor when studying diseases like cancer where karyotype alterations are the driving force. Therefore, the genetics field as a whole needs to seriously consider the importance of karyotype heterogeneity and strategies to use NCCAs for this purpose.

Knowing the existence of high levels of karyotype heterogeneity in vivo is a big shock to many, as biology has taught us that there is a high level of fidelity in biological systems including DNA repair, multiple check levels, and the mechanism of cell death to eliminate any altered genomes. One frequently asked question is: Why are there high levels of genome variation in the first place, and how do biological systems tolerate this heterogeneity? The answer is related to the fact that there are large numbers of

cell division events and a seemingly unlimited source of bio-stress. After all, it is not a perfect world where all outside influences can be avoided or corrected. The existence of many diseases illustrates this point. However, in normal situations, system homeostasis can tolerate certain levels of heterogeneity, and the key might be quantitative levels of heterogeneity relative to the increased probability of hitting some important pathways. It is interesting to point out that one function or by-product of karyotype heterogeneity at the somatic cell level might be to provide increased adaptability (system dynamics), which might also lead to disease conditions when it surpasses a certain threshold [Heng, 2013a].

Following the reading of this report, we hope that readers will go back to their microscopes or photo files and (re) discover more interesting karyotype abnormalities, characterize, classify, and publish them. There are some immediate tasks as well which include: (1) confirm some of the above findings and discover/classify additional aberrations. In order to more efficiently apply this new concept of using karyotype heterogeneity to monitor the evolution of the system, effort is needed to discover remaining, unknown NCCAs and complete the whole picture. This can be done by re-examining archival slides. (2) From these samples we should establish the baseline for NCCA frequencies in normal populations of different ages. Although this is a large task, it can be done, as evident by a similar goal of the HUMN project. (3) Characterize the 4D-genomic landscape of major types of disease, comparatively analyze the contributions of different levels of genetic alterations (gene, epigene, genome), and evaluate the clinical prediction power of analyses at the various levels.

Recently we have begun to apply the 4D-genomics concept to other common diseases including Gulf War illness and chronic fatigue and immune dysfunction syndrome. The goal of these studies is to focus on the evolutionary process of these diseases and link them to overall genome instability. Using these idiopathic diseases as an example, we have begun to show that karyotype heterogeneity and resulting system instability is the missing genetic link to many diseases [Heng, 2010, 2013a, b].

Conclusion

By briefly summarizing the various forms of karyotype heterogeneity, this article does not intend to provide a comprehensive list of chromosomal/nuclear aberrations, but instead seeks to provide increased awareness of these ignored abnormalities and to stimulate further discussion on improving studies of cytogenetic aberrations. Genome level alterations are an important subject, and stochastic genome changes previously held to be unimportant are in fact a universal finding overwhelmingly present in experimental and clinical samples; these clearly are not an artifact and should no longer be disregarded. Now is the time for a discussion regarding the future path of cytogenomic analysis, particularly in light of the fact that there is a call to move away from traditional karyotype analysis towards more molecular profiling. Systematic analysis of karyotype heterogeneity clearly represents an important new direction for the field of cytogenetics and genomics.

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