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Origins and Directions of Human Cytogenetics

Cytogenetics is the study of the structure, function, and evolution of chromosomes, the vehicles of inheritance that reside in the cell nucleus. Cytogenetics deals with chromosome behavior during the divisions of the somatic and early germline cells that produce identical daughter cells with two sets of chromosomes (*mitosis*) or the final two germline cell divisions that produce germ cells with a single set of chromosomes (*meiosis*). Human cytogenetics is particularly concerned with how these processes may go wrong and how structural changes arise, because changes in the number or structure of chromosomes are major causes of mental retardation, multiple malformations, cancer, infertility, and spontaneous abortions.

O. J. Miller et al., *Human Chromosomes*

Origins: Cytology, Genetics, and DNA Chemistry

Human cytogenetics had its beginning in the nineteenth century, aided by the development of the compound microscope, fixatives for preserving cell structure, and chemical dyes that preferentially stain nuclei and chromosomes. These early studies in cytology (now called cell biology) led to the elaboration of the cell theory, that all living cells come from pre-existing cells. The first study of human chromosomes, by Fleming in 1889, provided limited information. However, along with the work of van Beneden, Strasburger, Waldeyer, and others, it led to a clear understanding that the behavior of chromosomes in mitosis and meiosis is consistent and that their key features are virtually identical in animals and plants. The evidence was so impressive that Weissmann, in 1892, claimed that chromosomes were the physical basis of heredity. A method of genetic analysis was needed to test this hypothesis. This was provided by the rediscovery of the principles of Mendelian inheritance in 1901. Within a year, Sutton and Boveri independently reported that the segregation of each pair of homologous chromosomes and the independent assortment of nonhomologous chromosomes in meiosis could account for the corresponding behavior of Mendelian unit factors (genes), if these were carried by the chromosomes. This was the first major theoretical contribution of cytogenetics to genetics, and it led to the realization that each chromosome must carry many different genes. Intensive efforts over the next 90 years showed that in each organism the genes were arranged in linear arrays along the length of each chromosome, with each gene having a unique location on a particular chromosome. But what was the chemical nature of the gene? The pioneering work of Avery, McLeod and MacCarty, published in 1944, established that genes are composed of deoxyribonucleic acid, or DNA, not protein.

The presence of DNA as a major component of cell nuclei had been known since the work of Miescher, late in the nineteenth century. In 1924, Feulgen developed a method for staining nuclei and chromosomes that was based on a specific chemical modification of DNA. This Feulgen reaction, or stain, allowed quantitative measurements of the DNA content of tissues and even of individual nuclei after cytophotometric methods were developed. The cells of each organism had a characteristic amount of DNA, with the amount of DNA in nondividing diploid somatic cells (2C) twice that in haploid germ cells (C). The 3.65 picograms of DNA in haploid sperm provided an estimate of the size of

the haploid *genome*, or complete set of human chromosomes: approximately 3.4 billion nucleotide base pairs of DNA.

The existence of nucleotide base pairing in DNA was unknown until 1953, when Watson and Crick, aided by the X-ray crystallographic data of Wilkins and Franklin, proposed that DNA is composed of two long, spiraling strands of nucleotides that are held together by hydrogen bonds between pairs of bases. Two hydrogen bonds linked each adenine (A) with a thymine (T), and three hydrogen bonds linked each guanine (G) with a cytosine (C). These were the only types of base pairs permitted by the proposed structure of DNA. As a consequence, the sequence of bases in one strand determines the sequence of bases in the complementary strand. Furthermore, the genetic information carried by each chromosome could be specified by the sequence of bases in its long DNA molecule. Crick showed that the information is, indeed, encoded in successive triplets of bases in DNA and in the messenger RNA (*mRNA*) that is transcribed from one strand of the DNA. Nirenberg, Mathai, and Ochoa worked out the complete genetic code—the particular nucleotide triplets (codons) that specify each amino acid in a growing polypeptide chain or protein and the stop codons that terminate chain growth. This completed the logic of genetics and explained the earlier fundamental discovery of Garrod, Beadle, and Tatum that each enzyme protein is generally the product of one gene: the "one gene-one enzyme" hypothesis.

The Midwives of Human Cytogenetics

The early cytological history of human cytogenetics has been reviewed comprehensively by Makino (1975). Few studies on human chromosomes were published before 1952. That of Painter in 1923 was responsible for the notion that the human chromosome number is 48, a mistake that went uncorrected for the next 33 years. His report was worded quite cautiously; after all, it was based almost entirely on the analysis of a few cells from one institutionalized individual! That number may have been correct for that individual, as rare institutionalized individuals with 48 chromosomes are now well known. However, it may simply have been the result of the inadequate methods available to the early investigators, who had to examine serial sections of testes because the badly overlapping chromosomes were not even in one focal plane. Accurate studies of human chromosomes became possible only after several technical developments. Improved cell culture methods provided a ready source of individual dividing

cells that could be squashed on a slide. Blakeslee and Eigsti showed in 1936 that colchicine destroys the mitotic spindle and blocks cells in metaphase, facilitating their accumulation and study. Hsu discovered in 1952 that treatment of cells with hypotonic salt solution before fixation gave a marked improvement in chromosome spreading, especially when combined with the use of single-cell suspensions and the addition of colchicine to the cells before hypotonic treatment.

Taking advantage of these new methods, Tjio and Levan (1956) established that the correct human diploid chromosome number is 46, based on their study of cultured embryonic lung cells from several individuals. The same year, Ford and Hamerton confirmed this in spermatogonia and showed that cells in meiosis have 23 paired chromosomes, or *bivalents*. Methods continued to improve. Air-drying cell suspensions directly on microscope slides gave better spreading and flattened the entire metaphase spread into a thin focal plane. An important innovation in cell culture technique came with the discovery in 1960 by Moorhead and his associates that peripheral blood lymphocytes can be induced to divide after a few days in culture in the presence of phytohemagglutinin, a bean extract. Because blood samples are so readily available, chromosome studies could be carried out quickly and easily on virtually anyone. Such cultures are still one of the most widely used sources of human chromosomes. An important additional source is amniotic fluid. In 1966, Steele and Breg reported that cells cultured from amniotic fluid could be used to determine the chromosome constitution of the fetus. This is the technique that is still most widely used for prenatal chromosome studies, although rapidly growing numbers of studies are now carried out on cells cultured from biopsies of chorionic villi taken from the placenta during the first trimester of pregnancy.

The Birth of Clinical Cytogenetics

The new techniques were soon applied to individuals who were mentally retarded or had multiple malformations. Miller (1995) gives a short review of this early phase, with extensive references. Lejeune et al. (1959) found that Down syndrome in several subjects was caused by the presence of three copies (*trisomy*), instead of the normal two, of number 21, one of the smallest human nonsex chromosomes, or *autosomes*. The same year, Jacobs and Strong found a male with Klinefelter syndrome who had an XXY complement, while Ford and his collaborators reported females with Turner syndrome who had a single X (XO or *monosomy* X) or were XO/XX *mosaics*, with both XO and XX cells. They

also reported the first case of *double aneuploidy*: an extra sex chromosome and an extra chromosome 21 (XXY-trisomy 21) in a man with 48 chromosomes who had both Klinefelter and Down syndromes. These observations indicated that *sex determination* in humans depends upon the presence or absence of a Y chromosome and not on the ratio of X chromosomes to autosome sets, as it does in *Drosophila*. The Y chromosome is male determining even in the presence of as many as three or four X chromosomes, since XXXY and XXXXY individuals are male. Individuals who have a Y chromosome in only a fraction of their cells, such as XO/XY mosaics or XX/XY *chimeras*, which arise from fertilization by two sperm, often show mixed, or intersexual, development (Chapters 19 and 21).

The presence of multiple malformations in 21 trisomic patients led to the search for trisomy of other autosomes among patients with multiple malformations. Trisomy 13 and trisomy 18 were discovered in 1960 by groups headed by Patau and Edwards, respectively (Chapter 12). No additional trisomies were found in liveborns, so attention turned to a search for chromosome abnormalities in spontaneously aborted embryos or fetuses, based on the assumption that trisomy for these autosomes might act as embryonic lethals. Carr, and later Boue's group, carried out extensive studies of spontaneous abortuses and found that autosomal trisomies represented about 3% of all recognized pregnancies. In addition, XO and triploid (3n) abortuses were also extremely common: each made up about 1% of recognized pregnancies. Clearly, these chromosome constitutions were quite lethal, and human meiosis quite error-prone (Chapter 11).

Only structural aberrations that produced large changes in the length or arm ratio of a chromosome could be detected with the methods available before 1970. These included Robertsonian translocations, which involve the long arms of two acrocentric chromosomes, such as numbers 14 and 21. Their discovery by Penrose, Fraccaro, and others was the result of studies of exceptional cases of Down syndrome in which the mother was young or there was an affected relative (Chapter 13). A number of deletions were also detected. Nowell and Hungerford noted in 1960 the consistent presence of a deleted G group (Philadelphia) chromosome in chronic myelogenous leukemia cells. Later, using banded chromosomes, Rowley showed that the aberration was really a specific translocation (Chapter 27). The first example of a deletion of a D group chromosome was found in 1963 in a patient with retinoblastoma by Penrose's group, who pointed out that if the deletion was responsible for the disease, a gene for retinoblastoma must be in the deleted segment. Deletions were shown to cause some characteristic and previously unrecognized clinical syndromes: Lejeune's cri du chat (cat cry) syndrome by a deletion of the short arm of chromosome 5

and the Wolf-Hirschhorn syndrome by a deletion of chromosome 4 (Chapter 15). Characteristic phenotypes were also noted by de Grouchy and others in patients with deletions of either the long arm or the short arm of chromosome 18. In some families, the presence of a chromosome rearrangement in one parent was responsible for the deletion or other chromosome imbalance in one or more children (Chapter 16).

The Lyon Hypothesis

In 1949, Barr and Bertram described what is called the sex chromatin, or *Barr body*, which is visible in some cell nuclei in all mammalian females. Its frequently bipartite form led to the hypothesis that it arose from pairing of highly condensed (*heterochromatic*) segments of the two X chromosomes. The finding of three X chromosomes in individuals with two Barr bodies in some of their cells (for example, Jacobs et al., 1959) provided a key piece of information and made an alternative hypothesis more attractive: that a Barr body is a single heterochromatic X chromosome. This interpretation, first formulated by Ohno, played a role in the development of the single-active-X, or Lyon, hypothesis (Lyon, 1961). The hypothesis states that in mammalian females one X chromosome is inactivated in all the cells at an early embryonic stage. The original choice of which X is inactivated is random, but the same X remains inactive in all the descendants of that cell. If a cell has more than two X chromosomes, all but one are inactivated, and each inactive X chromosome may form a Barr body (Chapter 18). The Lyon hypothesis remains one of the most important theoretical concepts in human, and all mammalian, cytogenetics. It has directed research and led to many key insights in developmental and cancer biology and to even more in other areas of genetics, as described throughout later chapters.

Adolescence: The Chromosome Banding Era

Until 1970, chromosome identification, and particularly the identification of structural changes, was severely limited. Normal chromosomes could be sorted into seven groups on the basis of length and arm ratio, but only a few chromosomes can be individually recognized. Autoradiographic DNA replication pat-

terns helped identify a few more, but this was time-consuming and still of very limited value. The introduction of chromosome banding techniques revolutionized human cytogenetics. In 1970, Caspersson et al. discovered that quinacrine mustard produces consistent fluorescent banding patterns along each human chromosome that are so distinctive that every chromosome can be individually identified. This discovery was followed by a flood of additional banding techniques, whose use greatly simplified chromosome studies and made possible the identification of an enormous range of chromosome abnormalities, especially structural aberrations, such as translocations, inversions, deletions, and duplications, that were previously undetectable. A fundamental discovery was that the DNA in each chromosome band replicated during a specific part of the DNA synthetic (S) phase of the cell cycle (Latt et al., 1973; see also Chapter 3). A standard system of chromosome nomenclature was developed through a series of conferences and publication of their recommendations. A standing committee now publishes comprehensive booklets incorporating the accepted nomenclature and modifications necessitated by new developments in the field. The most recent is ISCN (1995), an international system for human cytogenetic nomenclature.

Somatic Cell Genetics and Chromosome Mapping

Harris (1995) has published an excellent historical review of somatic cell genetics. The initial goals of this field were the development of methods for analyzing the segregation of mutant alleles and the recombination of linked genes using cultured somatic cells. Segregation of homologous chromosomes and the alleles they carry occurred in human-rodent somatic cell hybrids that had lost some of their human chromosomes. Thousands of genes were mapped to chromosomes using panels of these hybrids (Chapter 29). The linear order and physical distance separating these gene locations (*loci*) on a chromosome could be determined using hybrids carrying different segments of a particular chromosome. The most precise mapping was achieved using a panel of *radiation reduction hybrids* that contained different mixtures of the human chromosome complement (genome) or of any particular chromosome, because the human chromosomes were fragmented by massive irradiation of the cells before being hybridized with rodent cells (Chapter 23). These approaches were so successful that they continue to dominate gene mapping.

Maturity: The Molecular Era

The molecular era was ushered in by the development of methods for manipulating DNA. Marmur, Doty, Spiegelman, and Gillespie showed that the two strands of DNA fragments could be easily separated (denatured, or dissociated) and that complementary strands could be reannealed (renatured, or reassociated), even in the presence of large amounts of noncomplementary DNA. Such *molecular hybridization* became of fundamental importance in molecular biology, and *in situ hybridization* of labeled DNA probes to the DNA in cytological preparations of chromosomes and nuclei became a powerful tool in human cytogenetics. Several thousand loci have been mapped by *fluorescent in situ hybridization (FISH)*, a faster, more reliable, and more precise method for mapping genes than the autoradiographic detection of radioactively labeled DNA fragments (Chapter 8). Autoradiographic in situ hybridization led to the fundamental discovery that cytoplasmic mRNA molecules are much shorter than the genes that encode them, because of the presence of intervening sequences (*introns*) that break up the protein-coding portion of most genes into short segments called *exons*. The invention of methods to determine the sequence of nucleotide bases in DNA (*DNA sequencing*) by Gilbert and Sanger made it possible to characterize precisely the genes and other parts of the genome. Another powerful tool was the *polymerase chain reaction* (PCR), invented by Mullis. It permitted rapid amplification of any short fragment of DNA, yielding up to a million-fold increase in the number of copies, and this revolutionized many aspects of cytogenetics.

An important advance was the development of methods for cloning DNA fragments, including genes. This was based on the discovery that bacterial viruses are still infectious when they contain an inserted fragment of human DNA. Plasmids would accept inserts up to about 5 kilobase pairs (kb) in length, bacteriophages up to 15 kb, and cosmids up to 50 kb. A bacterial cell infected with a single recombinant virus could be grown into a clone of millions of cells each containing the same unique fragment of human DNA. Alternative techniques were developed to clone larger DNA fragments. Yeast artificial chromosomes (YACs), with a yeast centromere and telomeres, could accept human fragments of several hundred kb or rarely 1–2 megabase pairs (Mb) and functioned as fairly stable chromosomes in yeast. Bacterial artificial chromosomes (BACs), containing 160–235 kb of human DNA, were even more useful, being stable in bacteria and easier to purify. Any of these cloned fragments could be

labeled to produce radioactive or fluorescent DNA probes. These could be used in molecular hybridizations to detect complementary DNA that has been size-separated by gel electrophoresis and transferred to nitrocellulose filters by a simple method called *Southern blotting*. The hybridized probe could also be detected in fixed cells or chromosome spreads by in situ hybridization (Chapter 8). For further information about molecular genetics, see Strachan and Read (1996) and Lewin (1997).

Molecular hybridization was widely used to construct genetic linkage maps (Chapter 29) and to determine the parent of origin of a deleted chromosome or of the extra chromosome in a trisomic individual (Chapters 11 and 15). It was also used to show that some individuals with a normal chromosome number received both their copies of a particular chromosome from the same parent (*uniparental disomy*). This led to the discovery of a previously unrecognized cause of disease and a novel mechanism of gene regulation, called *genomic imprinting*: the normal inactivation of either the maternal or the paternal copy of a gene (Chapter 21). Molecular methods were instrumental in the identification of many of the genes that regulate the cell cycle (Chapter 2) and clarification of the mechanisms by which chromosome breakage can lead to cancer (Chapters 24–28). Molecular methods led to the discovery of abundant interspersed sequence elements repeated many times in the genome. Many of these are still capable of acting as *transposable elements*, that is, moving to new locations in the genome, sometimes disrupting a gene or breaking a chromosome. Barbara McClintock described this behavior nearly 50 years ago in maize, and it appears to be universal in eukaryotes. For an interesting account of some of the fundamental discoveries made by this great cytogeneticist, see Federoff and Botstein (1992).

The genetic basis of sex determination was advanced by the molecular characterization of a gene on the Y chromosome, *SRY*, that is required for male sexual development. A number of genes on the autosomes are also involved in the complex process of male sex differentiation (Chapter 17). The molecular basis of X inactivation was advanced by the discovery of the *XIST* gene and the demonstration that it is expressed strongly only from the copy on the X chromosome that is inactivated (Chapter 18). Surprisingly, the *XIST* gene does not code for a protein; instead, its RNA product, or *transcript*, coats the X chromosome carrying it and mediates inactivation of this chromosome, in an unknown manner (Brown et al., 1992).

Ohno (1967) suggested that the gene content of the X chromosome has remained virtually unchanged throughout mammalian evolution—some 125

million years—because the dosage compensation mechanism associated with X inactivation would interfere with transfer of genes between the X chromosome and an autosome. However, the X chromosome of marsupials and monotremes, which split from placental mammals roughly 140–165 million years ago, is only about 60% as large as that of placental mammals. Molecular cytogenetic studies suggested that most of the short arm of the human X chromosome consists of genes that are autosomal in marsupials and monotremes but have managed to make the transition to the X chromosome and a dosage-compensated system (Graves et al., 1998).

Molecular cytogenetic methods led to spectacular successes in understanding cancer (Chapters 24–28). Major causes of genome destabilization were found, and chromosome instability was shown to be a major factor in carcinogenesis and tumor progression (Chapters 24 and 26). Molecular cytogenetic studies of chromosome aberrations specifically associated with particular types of cancer led to the discovery of many tumor suppressor genes (Chapter 28) and proto-oncogenes (Chapter 27), providing key insights into the origin of cancer.

Despite the impressive growth of knowledge about human chromosomes in the last 40 years, important questions remain unanswered. The enormous databases generated by the Human Genome Project, and powerful new technologies to generate and analyze data, can be used to address these (Chapter 31). Human cytogenetics promises to remain an exciting field, both for its scientific challenges and for its rapidly growing applications in the diagnosis, treatment, and prevention of human disease.

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