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Perspectives

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The Fifties and the Renaissance in Human and Mammalian Cytogenetics

Orlando J. Miller

Center for Molecular Medicine and Genetics and Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, Michigan 48201

THE period from 1956 to 1962 was seminal for human and mammalian cytogenetics. The human chromosome number and normal human karyotype were established, along with those of many other mammals. The high incidence and severe effects of human aneuploidy were discovered, along with the critical importance of the *Y* chromosome in mammalian sex determination, the nature of the sex-chromatin (Barr) body, the mechanism of dosage compensation for genes on the *X* chromosome, and the late replication of constitutive and facultative heterochromatin. The involvement of chromosome changes in malignancy began to be clarified, setting the stage for understanding their role in activating cellular oncogenes and the discovery of tumor suppressor genes. The single active *X* hypothesis (LYON 1961) remains the most powerful theoretical statement in mammalian cytogenetics.

The watershed publication by JOE HIN TJIO and ALBERT LEVAN (1956) established the correct human chromosome number as $2n = 46$, not 48 as stated in all the textbooks at that time. This discovery was made possible by advances in cell culture technique and by the use of colchicine as a spindle poison and hypotonic treatment prior to fixation as a way to improve the spreading of metaphase chromosomes (the serendipitous discovery of T. C. HSU in 1952). It also took courage to deny a universally accepted "fact." The renaissance of mammalian cytogenetics was marked by several bold rejections of accepted observations or hypotheses, and this was the first. TJIO and LEVAN pointed out that their finding might not be completely general, because it was based on the study of somatic cells in culture. It was, therefore, important that CHARLES FORD and JOHN HAMERTON (1956) found $2n = 46$ in human spermatogonia and $n = 23$ in testicular first meiotic divisions, thus ruling out the presence of germline-limited chromosomes and confirming $2n = 46$. Their chiasma counts (mean of 56 per cell) provided a still-useful minimum estimate of 28 morgans as the genetic length of human chromosomes in older males.

The human chromosome number, $2n = 46$, was confirmed in at least 74 individuals by 1958. Mitotic chromosomes showed clear morphologic features, such as length and arm ratio, that enabled workers to distinguish three to five chromosome pairs individually and to place all the chromosomes into seven groups: *1–3*, *4–5*, *6–12* + *X*, *13–15*, *16–18*, *19–20*, and *21–22* + *Y*. A standard nomenclature for the karyotype was proposed in Denver by the seven groups who had published papers on the normal karyotype by early 1960. This was almost universally accepted and used with minimal modification for ten years. A number of methodological improvements, such as the air-drying technique for flattening chromosomes that K. H. ROTHFELS and L. SIMINOVICH introduced in 1958, made chromosome studies easier. Most important was the discovery by MOORHEAD *et al.* (1960) that phytohemagglutinin is a potent mitogen for human peripheral blood lymphocytes; this made it possible to do a chromosome study on virtually anyone, using only a few drops of blood instead of a tissue or bone marrow biopsy. The demonstration by STEELE and BREG (1966) that amniotic fluid cells could be grown in culture and karyotyped opened the floodgates still wider, permitting prenatal screening of pregnancies at high risk for chromosomally unbalanced complements.

There was great excitement when JEROME LEJEUNE and associates announced in late 1958 that individuals with Down syndrome, then called mongolism, have 47 chromosomes, as first suggested by WAARDENBURG in 1932, and are trisomic for a G-group chromosome, which they called number *21*. They confirmed this in a total of nine patients with Down syndrome and published the results in January, 1959. The race was on to find other disease states due to a chromosome imbalance, and some journals pushed the pace by publishing particularly timely reports in as little as two weeks from receipt of the manuscript. That's how long my first chromosome paper (FORD *et al.* 1959a) took, in April of that year.

The presence of multiple malformations involving almost every organ system in *21*-trisomic individuals led to the idea that trisomy for other chromosomes might cause malformation syndromes as distinctive as Down syndrome. Sure enough, two such syndromes were reported in 1960 in back-to-back papers, the one by EDWARDS *et al.* dealing with trisomy for an E-group chromosome and the one by PATAU *et al.* with trisomy for a D-group chromosome. Despite vigorous efforts, no further autosomal trisomies (nor any monosomies) were found in people until 1966, when THORBURN and JOHNSON reported a case of G-group monosomy. Because there was no reason to expect nondisjunction to be limited to only three of the 22 autosomes, an alternative explanation for the failure to observe most trisomies or monosomies gained favor: that most of these severe chromosome imbalances have lethal effects during embryonic or fetal development. Indeed, PENROSE and DELHANTY (1961) had found a macerated fetus to be triploid. DAVID CARR (1963 and later) carried out an intensive study of aborted embryos and fetuses and found that about 40% of these were chromosomally abnormal, with trisomy being most common, and involving chromosomes of every group. Because 15% of recognized pregnancies are spontaneously aborted, these results indicated that about 3% of pregnancies are trisomic, 1% triploid, and 1% *XO*, almost all being embryonic lethals. The meiotic process was error-prone!

LEJEUNE's original observations on Down syndrome were quickly confirmed by other groups, who then turned their attention to the exceptional cases: those born to young mothers (the incidence rising exponentially with increasing maternal age), and familial cases. In 1960, three groups reported Down syndrome in patients with 46 chromosomes, including what they interpreted as a D/G or a G/G Robertsonian-type translocation. The report by PENROSE *et al.* (1960) included examples of both types, and one parent had not only the same G/G translocation as the affected child, but also a tiny fragment thought to represent the reciprocal translocation product, an extremely rare finding. The slightly earlier report by MARCO FRACCARO, K. KAIJSER and JAN LINDSTEN illustrates some of the limitations of nonbanded karyotype analysis. The affected child had 46 chromosomes, but the father had 47: both had an extra F-group (*19–20*) chromosome, probably a G/G translocation, but that would have meant the father had two extra G-group (*21–22* + *Y*) chromosomes. Was he also *XYYY*? The authors did not suggest that, but concluded he might be *19*-trisomic, even though that left his normal phenotype and the translocation trisomic child unexplained. Most of us experienced similar difficulties in interpretation because of the limited ability to identify extra or rearranged chromosomes. Fortunately, interphase sex chromatin bodies provided an independent means to evaluate the *X* chromosome complement.

Sex chromosome abnormalities were quickly found to be quite common in humans and responsible for relatively mild phenotypic abnormalities. PATRICIA JACOBS and J. A. STRONG's report of an *XXY* complement in a chromatin-positive male with Klinefelter syndrome appeared in January 1959, and three months later CHARLES FORD *et al.* (1959a,b) reported an *XXY*, *21*-trisomic complement in a man with both Klinefelter and Down syndromes, and an *XO* complement in a female with Turner syndrome. The choice of these patients for karyotype analysis was based on the earlier observations that females with Turner syndrome, like normal males, lack a sex chromatin body (are chromatin negative), and males with Klinefelter syndrome, like normal females, are chromatin positive. Each was considered a type of sex reversal by some investigators, although in 1956 PAUL POLANI and associates suggested that chromatin-negative Turner females were *XO*, and E. R. PLUNKET and M. L. BARR suggested that chromatin-positive Klinefelter males were *XXY*. In 1957, MATILDA DANON and LEO SACHS observed patches of skin that were chromatin-positive mixed with patches that were chromatin-negative in two females with Turner syndrome and suggested that these patients were *XO*/*XX* or *XY*/*XX* mosaics. Chromosome studies in 1959 led CHARLES FORD and associates to the direct demonstration of *XO* and *XO*/*XX* mosaic karyotypes in Turner females.

My involvement in human cytogenetics began in 1958 when, after an obstetrics and gynecology residency at Yale, I went to the Galton Laboratory in London to work with LIONEL PENROSE to delineate genetic causes of infertility and sexual abnormality. The slight degree of mental subnormality of some men with Klinefelter syndrome led us, and others, to screen institutions for the mentally retarded, PENROSE's favorite place for research. In this way, we identified a large number of males with Klinefelter syndrome and variants and were thus well positioned to apply the new chromosome techniques in collaborative studies with CHARLES FORD and DAVID HARNDEN. This led to the identification of the first *XXY*, *21*-trisomic male (FORD *et al.* 1959a) and the first *XXYY* male (ELLIS *et al.* 1961). (I also screened a prison, with comparable results, probably reflecting a comparable concentration of mildly retarded individuals in both types of institution.) I continued this approach after moving to Columbia University and, with ROY BREG (Yale), analyzed other sex chromosome abnormalities. In 1961, we reported a chromatin three-positive *XXXXY* male who was phenotypically similar to the one MARCO FRACCARO and JAN LINDSTEN had first reported in 1960 as an *XXY*, *8*-trisomic, *11*-trisomic Klinefelter male but later reinterpreted after finding three Barr bodies in some cells. This case serves to re-

emphasize the limitations of the techniques and the lack of information on the phenotypic effects of most autosomal trisomies in 1960.

The discovery of individuals with unusual sex chromosome complements provided the key to understanding mammalian sex determination. *XO* individuals were female and *XXY* individuals male, indicating that the *Y* chromosome is male-determining. This is quite different from the situation in Drosophila, where sex is determined by the balance between the number of *X* chromosomes and the number of autosome sets. Thus, in Drosophila, diploid *XO* flies are male and *XXY* flies female, just the reverse of the human situation. Even the presence of three or four *X* chromosomes in the human complement did not overcome the male-determining effect of a single *Y* chromosome. However, intersexual development could occur when only a fraction of the cells had a *Y* chromosome, as in an *XO*/*XY* mosaic (KURT HIRSCHHORN *et al.* 1960) and an *XX*/*XY* chimera produced by double fertilization (STAN GARTLER *et al.* 1962).

Individuals with three or four *X* chromosomes in their diploid complement provided a critical insight into the nature of the sex chromatin (Barr) body discovered by MURRAY BARR and M. A. BERTRAM in 1949. This was present as a nucleolus-associated chromatin mass in the neurons of female, but not male, cats and other mammals, and as a nuclear membrane-associated chromatin mass in epithelial cells of female mammals. Barr suggested that this frequently bipartite body arose from paired heterochromatic segments of the two *X* chromosomes. This hypothesis became so well established it initially led SUSUMU OHNO and his associates in 1958 to interpret the single heteropycnotic *X* chromosome of mouse mitotic prophase cells in the following way: "At prophase the two X chromosomes, in positively heteropycnotic state, were found, without exception, to be in end-to-end association." Hypothesis influences (and sometimes misguides) observation! However, a year later, OHNO *et al.* (1959) offered a different interpretation of identical findings in the rat, reporting that these showed a single heteropycnotic prophase chromosome, and proposing that the sex chromatin body arises from a single *X* chromosome. What led to this critical reinterpretation? The authors never said. However, at about the same time, JACOBS *et al.* (1959) reported an *XXX* female who had two sex chromatin bodies in many cells, and in the same year Murray Barr's group reported the presence of two sex chromatin bodies in three males with Klinefelter syndrome (later shown to be *XXXY*). Seven more *XXX* females were reported in 1960; they, as well as the two *XXXY* males reported by FERGUSON-SMITH *et al.* (1960), had two chromatin masses, while the *XXXXY* males referred to above had three. OHNO's hypothesis offered a simple explanation of these results and was an important precursor of the LYON hypothesis.

The most profound theoretical insight to come out of the renaissance in mammalian cytogenetics was the single-active-*X* hypothesis formulated by MARY LYON (1961). This short paper in *Nature* is a model of terse, critical argument: (1) *XO* mice have a normal female phenotype (reported by WILLIAM WELSHONS and LIANE B. RUSSELL in 1959); (2) all sex-linked mutants affecting coat color in the mouse have a mottled or dappled phenotype, with patches of normal color and patches of mutant color; (3) a similar phenotype, described as "variegated," is seen in female mice heterozygous for coat color mutants translocated on to the *X* chromosome (reported by RUSSELL and BANGHAM in 1959 and 1960).

MARY LYON's hypothesis followed: "It is here suggested that this mosaic phenotype is due to the inactivation of one or the other X-chromosome early in embryonic development. If this is true, pigment cells descended from cells in which the chromosome carrying the mutant gene was inactivated will give rise to a normal-coloured patch and those in which the chromosome carrying the normal gene was inactivated will give rise to a mutant-coloured patch." The utter simplicity of this formulation allowed no misinterpretation. Furthermore, the two final arguments she presented indicate her awareness that the single-active-*X* hypothesis applied to all mammals: (4) in embryos of the cat, monkey, and human, sex chromatin is first found in nuclei of the late blastocyst stage (with reference to two 1957 papers); (5) the sex chromatin is thought to be formed from one *X* chromosome in the rat and opossum (referring to 1959 and 1960 papers of OHNO *et al.*).

In 1962, LYON gave a fuller discussion of the various components of her powerful hypothesis, with particular reference to human disease phenotypes. In this paper, she tried to share some credit, pointing out that, simultaneously with the original publication of her own hypothesis, L. B. RUSSELL (1961) put forward a very similar but more limited one concerning variegation due to sex-linked translocations in the mouse. Russell considered that the variegation was "presumably a heterochromatic effect" and, from the fact that two *X* chromosomes were essential for its expression, together with cytological evidence, postulated that "in animals, genic balance requires the action of one X in a manner which precludes realization of its heterochromatic potentialities, so that only additional X's present assume the properties characteristic of heterochromatin." In this paper and another published the same year in GENETICS, RUSSELL called this phenomenon "some kind of V-type position effect," a well known but poorly understood phenomenon in Drosophila. In fact, transcriptional inactivation of the variegating gene was first demonstrated by STEVEN HENIKOFF in Drosophila only in 1979.

Although her formulations lacked the clarity and generality that has made the LYON hypothesis so useful, L. B. RUSSELL was closer, in 1961, to understanding *X* inactivation than anyone else. More limited attempts had been made to account for the sexual dimorphism in sex chromatin. In a short letter to *Lancet* in 1960, J. S. S. STEWART stated, "There is a very simple explanation for the presence of the sex chromatin body: In the intermitotic metabolic nucleus the heterochromatin of one X chromosome is apparently necessary for and engaged in the metabolic business of the cell and therefore not stainable. The heterochromatin of any other X chromosome is, however, superfluous to metabolic requirements, functionally inert at this time, and therefore stainable." Little attention was paid to this hypothesis because no supporting evidence was presented, and heterochromatin was generally regarded as having no metabolic functions; "facultative heterochromatin" was not yet an established concept in mammals.

MARY LYON's 1961 paper in *Nature* was like a bolt out of the blue, providing the insight that allowed the rest of us to make sense out of a diverse array of findings. For example, J. HERBERT TAYLOR (1960) had observed asynchronous replication of one arm of the two *X* chromosomes of the Chinese hamster, which GEORGE YERGANIAN said bolstered his own hypothesis, based on morphologic differences, of an X_1X_2/X_1Y sex-determining mechanism in this species. The LYON hypothesis favored a different explanation: that the arm of the *X* chromosome which replicates early in *XY* cells but late in *XX* cells is active and euchromatic in *XY* cells but inactive and heterochromatic in *XX* cells. This fit well with the finding by LIMA-DE-FARIA (1959) that heterochromatin is late-replicating in the insect Melanoplus and the plant Secale, and was supported by later studies, such as that of GRUMBACH in 1963, showing that the number of late-replicating *X* chromosomes in humans is the same as the number of Barr bodies and one less than the number of *X* chromosomes.

Tests of the LYON hypothesis were not long in coming. MEL GRUMBACH and associates showed in 1962 that the level of *X*-linked G6PD enzyme activity was the same in individuals with one, two, three, or four *X* chromosomes. ERNEST BEUTLER and his associates demonstrated in the same year that two populations of red blood cells are present in G6PD heterozygotes, and the following year RON DAVIDSON and associates showed the clonal nature of G6PD-A and G6PD-B fibroblasts in such heterozygotes, using a method that has been used many times since then to determine whether an *X*-linked gene shows "Lyonization" or escapes *X* inactivation. BARID MUKHERJEE and ANIL SINHA showed in 1964 that *X* inactivation was random in *XX* cells, taking advantage of the dimorphic *X* chromosomes in a horse–ass hybrid, the mule. Exceptions to one or another aspect of the LYON hypothesis have been discovered, such as non-random inactivation in *X*-autosome translocation heterozygotes and reactivation of the second *X* in oocytes, but despite such exceptions this hypothesis continues to spark novel experiments and lead to new insights. One of these was the clonal origin of many neoplasms, such as chronic myeloid leukemia, and the common origin of erythroid and granulocyte lineages (FIALKOW *et al.* 1967). One of the most interesting was OHNO's recognition that the presence of a single active *X* in mammalian somatic cells would greatly restrict the transfer of genes between *X* and autosomes because of dosage effects, and his resultant hypothesis that the *X* chromosome of all placental mammals should carry the same genes and have the same amount of euchromatin. Measurements in a diverse series of mammals supported this hypothesis (OHNO *et al.* 1964), as have more recent mapping studies.

Throughout the 1950s, SAJIRO MAKINO, ALBERT LEVAN, GEORGE KLEIN, and others had demonstrated that ascites and some other cancer cell lines tend to be mitotically unstable and show highly variable chromosome numbers. Aneuploid cells with a specific number were usually most common within a line and tended to persist, leading to a "stem cell" concept, the precursor of today's much better established "clonal" origin of most cancer cell lineages. The first definitive evidence of an association between a specific chromosome change and a particular malignancy was the discovery of a partially deleted G-group chromosome in human chronic myeloid leukemia (CML) cells by PETER NOWELL and DAVID HUNGERFORD (1960). They initially interpreted this as a deletion involving the *Y* (both patients being male), but soon discovered the same Ph1 (Philadelphia) chromosome in CML in females. The occcurrence of a constitutional deletion involving a D-group chromosome in one of six patients with a retinoblastoma was described by LELE *et al.* (1963), who pointed out that the deletion might be causal and indicate the location of the retinoblastoma gene. In fact, although only a small number of such deletions have been studied, their cytogenetic analysis guided the mapping of the autosomal dominant retinoblastoma gene to the 13q14 region, its positional cloning, and its recognition as a tumor suppressor gene.

Despite much effort, additional insights into chromosomal causes of cancer were slow in coming in the prebanding era. Increased chromosome breakage and rearrangement was observed in 1964 in two autosomal recessive disorders associated with an increased risk of cancer: Fanconi anemia by TRAUTE SCHROEDER and Bloom syndrome by JAMES GERMAN. Perhaps the first evidence for tumor suppressor genes was derived by chromosome segregation analysis in somatic cell hybrids between malignant and nonmalignant murine cells (HARRIS *et al.* 1969). HENRY HARRIS showed that these hybrids were initially nonmalignant but tended

to regain their ability to grow as tumors when injected into histocompatible mice. While on sabbatical leave with HARRIS, I showed that the return of the malignant phenotype was associated with loss of chromosomes from the nonmalignant parent, suggesting that loss of a specific tumor suppressor gene on one chromosome was responsible. Unfortunately, the methods then available did not permit identification of individual mouse chromosomes.

Improvement in methods for chromosome identification was very limited throughout the sixties. Symbolic of this were the minimal modifications adopted at the Conference on Standardization in Human Cytogenetics in 1966 at the International Congress of Human Genetics in Chicago: (1) "chromosome short arms are designated p and long arms q" (p for petite, at JEROME LEJEUNE's suggestion, and q because all geneticists know that p + q = 1!); and (2) "autoradiographic DNA replication patterns may help identify chromosomes *4, 5, 13, 14, 15, 17* and *18*." Thus, GERMAN *et al.* (1964) showed that the deleted (*5*p) chromosome in the cri du chat syndrome discovered the year before by JEROME LEJEUNE had a characteristic replication pattern, and WOLF *et al.* (1965) emphasized that the deleted (*4*p) chromosome in their patient with a clinically different syndrome had the other replication pattern found in the B group. We and others identified abnormal chromosomes in B, D, and E groups in this way, but were unwilling to accept unusual conclusions by another group on the basis of this rather limited technique! The excitement of the early years was gradually replaced by increasing frustration at the severe limitations imposed by the inability to identify individual chromosomes or chromosome segments in the mammals of most interest, the human and the mouse. Clearly, most inversions and translocations were being missed, and those detected were often difficult to interpret. The location of most deleted segments could not be determined, nor could the identity of extra or missing chromosomes in highly aneuploid cancer cells. Thus, by the mid to late sixties, it seemed that little more could be learned by cytogenetic analysis with the existing methods.

This rather gloomy state of mind was quickly abolished by TORBORN CASPERSSON's discovery of chromosome banding, which permitted accurate identification of every normal human chromosome and of an impressive array of structural abnormalities. His original findings, in 1968, were made in plants and enabled a distinction to be made only between euchromatin and heterochromatin. Application of his quinacrine mustard fluorescent staining technique to human chromosomes (CASPERSSON *et al.* 1970) revealed the power of the method to delineate a hitherto unknown level of diploid mitotic and meiotic chromosome organization, the band. Each band contains 1 to 50 or more megabase pairs of DNA, roughly 10 to 100 or more genes, and is thus totally different from a band in polytene chromosomes. JOHN EVANS, MARINA SEABRIGHT, JEROME LEJEUNE, and others quickly discovered methods to produce a very similar banding pattern (G banding) or the reverse pattern (R banding) using Giemsa stain, and my group showed that almost every banding pattern could be produced by selective denaturation of chromosomal DNA and binding of labeled single-strand-specific antinucleoside antibodies. Most exciting was the discovery by SAM LATT and BERNARD DUTRILLAUX of a nonradioactive method for analyzing replication timing. This produced either a G-band or an R-band pattern, depending on whether BrdU is incorporated early or late in the S phase, and demonstrated that G bands replicate late and R bands replicate early.

The introduction of chromosome banding made individual identification of every chromosome routine and led to an explosive growth in knowledge. Trisomies of every chromosome were identified in abortuses. Translocations, deletions and inversions were identified in great abundance in malformation syndromes or cancer. The specific chromosome change in chronic myelogenous leukemia was shown by JANET ROWLEY to be a specific translocation. The role of this translocation in activating the *c-abl* proto-oncogene by placing it 3′ to the strong promoter of the *bcr* gene was demonstrated in the present molecular era. Dozens of additional translocations have since been shown to be specifically associated with other cancers, some activating other proto-oncogenes. Banding analysis made it possible to identify any human chromosome remaining in mouse-human hybrid cells and thus to map a specific gene quickly (MILLER *et al.* 1971b). This technique has been widely used to maps hundreds of genetic markers to specific chromosomes in the human and a few other mammals, and it set the stage for the Human Genome Initiative. We showed that even the 20 pairs of similarly sized telocentric chromosomes of the mouse could be individually identified by their banding patterns, and we were able to assign mouse linkage groups to specific chromosomes by identifying the chromosomes involved in a series of translocations involving known linkage groups (MILLER *et al.* 1971a). JONASSON, HARRIS, KLEIN and their colleagues showed in 1974 that specific loss of mouse chromosome *4* contributed by the nonmalignant parent of hybrid cells led to malignancy, *i.e.*, mouse chromosome *4* carries a tumor suppressor gene.

Chromosome banding made possible a second renaissance in human and mammalian cytogenetics, but in retrospect we can only marvel at how much was accomplished with the simple tools available in the pre-banding days of the fifties and early sixties, a time that truly deserves to be called the first renaissance in this field.

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