Putting Medical Genetics into Practice

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Abstract

This article describes a fragment of history on the growing impact of
genetics on the practice of medicine over 50 years, as experienced by
a medical geneticist who helped to provide services to patients at risk
of genetic disorders. It is a personal account influenced by a fascination
with chromosomes that has drawn him into many studies, including
sex determination, gene discovery, fetal diagnosis, phylogenomics, and
karyotype evolution.

INTRODUCTION

In the first article of the first volume of the *Annual Review of Genomics and Human Genetics* (15), Barton Childs and David Valle comment to the effect that although the contribution of medicine to genetics has continued since the beginning of the twentieth century, until the 1960s genetics seems to have contributed very little to the practice of medicine. This struck a chord with me, as it was in 1960 that my own career was directed from internal medicine into medical genetics with the idea of exploiting the exciting new developments in human genetics for application to clinical medicine. I owe this change in direction to the guidance of Victor McKusick (see 90) when I was a fellow in medicine at the Moore Clinic at Johns Hopkins Hospital from 1959 to 1961. My decision was greeted by Glasgow colleagues with surprise: "How will you live? There are no genetics jobs in the National Health Service." This article is not just a story of how my family managed to survive this career decision, but also an account of how it has taken me on a rewarding journey along different avenues of medicine and biology in which a driving force has been my interest in human chromosome research.

BACKGROUND

I grew up in Glasgow, where my father was a dermatologist in charge of wards at Glasgow Royal Infirmary. He ran his consulting practice from home, which was where Joseph Lister had lived from 1860 to 1869 during the time that Lister developed antiseptic surgery. Many physicians and surgeons lived nearby, and so my upbringing was influenced greatly by a medical environment. It is perhaps not surprising that my three siblings and I chose careers in medicine and that all of us studied medicine at Glasgow University. My undergraduate progress was unexceptional, and I graduated with an MBChB degree in 1955. This is the only degree that I obtained by examination; my other professional qualifications arose from published work.

My aim after graduation and internships in medicine and surgery was a career in internal medicine. With this in mind, I applied for a junior post in pathology at Glasgow Western Infirmary in 1956. I believed that training in pathology was a prerequisite for specializing in medicine. The professor indicated at the interview that only those contemplating a career in pathology were considered, so I was very surprised to get a letter the next day offering me the job.

NUCLEAR SEXING AND KLINEFELTER SYNDROME

During my first months in the Pathology Department, a senior pathologist, Dr. Bernard Lennox, asked me whether I would consider assisting him in some research on patients with anomalous nuclear sex. He explained that the somatic nuclei of normal females had a sex chromatin body that was absent in the nuclei of males. His work with Paul Polani in 1954 (109) had discovered that women with Turner syndrome had "male" nuclear sex, and others had found recently that males with Klinefelter syndrome had "female" nuclear sex. He was interested to identify these sex-reversed females and learn more about them, so he suggested that I look for cases with sex chromatin among boys with cryptorchidism using cells scraped from the buccal mucosa. No Klinefelter patient was found among 115 cryptorchid boys. However, as the Urology Clinic also ran male infertility sessions, I asked permission to screen patients found to have azoospermia or severe oligozoospermia, and the ninth patient I tested had Klinefelter syndrome. Altogether, 10 of the 91 patients (11%) tested had chromatin-positive nuclear sex (47). As several of these patients had attended special schools because of learning difficulties, our buccal smear survey was extended to a local hospital for the handicapped, where I found that 3 of 283 male patients were chromatin positive (28). I identified other Klinefelter patients from a review of testis biopsy material held in the Pathology Department. The histopathology of chromatin-positive testes was

characteristic, with an apparent overgrowth of Leydig cells in among which were a few seminiferous tubules lined solely by Sertoli cells and without germ cells. Most tubules were atrophic and represented by hyalinized "ghosts" without epithelium. Absence of elastic fibers in the ghosts indicated that the degeneration had occurred before puberty. In one exceptional case, a single tubule with complete spermatogenesis was found in the biopsy (49). The presence of sperm, and spermatocytes containing an obvious XY bivalent (sex vesicle), seemed to refute the concept that these males were sex-reversed females, and indicated an urgent need for someone to look at their chromosomes and identify the Y.

Although human chromosome analysis had been developed from cell cultures about a year earlier, when Tjio and Levan discovered that humans have 46 chromosomes rather than 48, our Genetics Department was not interested to take this on. However, I was put in touch with Charles Ford at Harwell, who explained that he and his colleagues were developing a method using short-term incubation of bone marrow cells that was suitable for clinical studies (see below). He suggested that I try the method myself and offered to test our patients when the method was perfected. He had just published the results of meiotic analysis that confirmed Tjio's count of 46 human chromosomes, and suggested that we could use this method whenever our patients had testicular biopsy. Several of our subsequent patients were tested by both methods, but none had spermatogenesis, and my bone marrow preparations were poor because of my inexperience and the paucity of mitoses.

Meanwhile, others were prompted to undertake buccal smear surveys, and by 1964, 22 groups had studied over 15,000 males with learning difficulties and had detected 128 with Klinefelter syndrome (0.85%) (32). Keith Moore, who had introduced the buccal smear test (93), surveyed 1,911 newborn males in 1959 and found 5 chromatin-positive males (0.26%). By 1964 a total of 34 cases had been found in 16,463 male newborns in five separate surveys

(0.21%). Klinefelter syndrome was surprisingly common (32).

As indicated above, the paradoxical findings of nuclear sex at variance with anatomical sex were first noted by Polani in Turner syndrome in 1954, two years before the observations in Klinefelter syndrome. Polani's interest was prompted by his observation of coarctation of the aorta in several patients, a condition that was rare in females but more common in males. In an attempt to obtain additional evidence of sex reversal, Polani checked the color vision of 20 Turner patients and found 4 with color blindness, a frequency 25 times greater than expected in normal females. He considered two alternative explanations: either those affected were sex-reversed XY males, or they had only one X chromosome. He subsequently gathered together 55 chromatin-positive Klinefelter patients from Glasgow, London, and Zurich whose color vision had been tested and found no case of color blindness, suggesting that this was consistent with the female frequency and that they most likely had two X chromosomes (107). Some of these early studies were reported first at a symposium on nuclear sex held at King's College Hospital Medical School, which I attended in September 1957. Those present included Murray Barr, who discovered sex chromatin (6); Bernard Lennox; Paul Polani; Lionel Penrose; Charles Ford; Harold Klinger; Alfred Jost; Ruth Sanger; and others who were later involved in the early developments of human cytogenetics.

EARLY CHROMOSOME STUDIES AND SEX DETERMINATION

From the 1920s until 1956, at least eight studies had confirmed that the human chromosome number was 48. This was corrected to 46 by Tjio and Levan with cell cultures and better techniques, and confirmed by Ford and Hamerton in human meiosis the same year. Ford, Patricia Jacobs, and László Lajtha then developed a method that could be used in patients involving short incubation of bone marrow cells with colchicine. In their 1958

Nature paper describing their technique (57), they included results from a Klinefelter patient with a normal female karyotype, apparently confirming that he was a sex-reversed female. Reappraisal of his karyotype later suggested that this patient might have been an XX/XXY mosaic, or possibly a very rare XX male. Some months later, when Jacobs moved to work with Michael Court Brown in Edinburgh, she studied another Klinefelter patient that proved to have a 47,XXY karyotype (77). I was pleased to learn that they had found the Y chromosome that I had predicted, and that Klinefelter patients were not sex-reversed females. Meanwhile, Polani and Ford (58) investigated the chromosomes of a Turner patient and found a 45,X karyotype.

During 1957–58, Marthe Gautier, working single-handed at the Hôpital Trousseau in Paris, was studying chromosomes in human fibroblast cultures to investigate a theory held by Raymond Turpin and others that Down syndrome might be due to a chromosome abnormality. Her first successful result in an affected patient in May 1958 showed a small extra chromosome. This finding in three patients was reported briefly with Jerome Lejeune and Turpin in January 1959 (82). Precedence thus goes to France for the discovery of the first human chromosome aberration. Other papers on Down syndrome from the groups of Jacobs, Böök, Polani, and Harnden followed in 1959, as did papers on human sex chromosome aberrations, including 47,XXX (76) and XX/XXY mosaic (59). In the following year we reported two patients with 48,XXXY (46), and trisomies 13 and 18 and translocation Down syndrome were also added to the list of discoveries (11, 26, 102, 103, 108).

The early papers on human sex chromosome abnormalities failed to make the comparison with the sex chromosome aneuploidies described by Calvin Bridges (40) in *Drosophila* over 25 years previously. He showed that XXY, XO, and XXX individuals with normal autosomal complements were female, male, and "superfemale," respectively (9). Sex in flies was therefore determined by the relative dose of Xs to autosomes, and the presence of the Y was immaterial. It was thought that this might apply to all organisms. The implication from the human results was that male sex was determined by the Y chromosome, which was supported by the discovery in 1959 that the XO mouse was also female and, two years later, that the XXY mouse was male. It was likely that the Y chromosome carried the male determinant in all mammals. The drive was on to find the determinant.

The year 1959 also saw the resolution of the nature of the sex chromatin body. Susumu Ohno (101) showed that in prophase chromosomes one of the two Xs was precociously condensed, and it alone formed sex chromatin at interphase. His observation was crucial for Mary Lyon's hypothesis of random X-inactivation of one of the two Xs in female mammals, which explained dosage compensation of X-linked genes and accounted for the mosaic coat pattern of female mice heterozygous for X-linked coat color genes (86). The identification of patients with three or more Xs, and with multiple sex chromatin bodies numbering one fewer than the number of Xs, indicated that all additional Xs were subject to inactivation (46). X chromosome isochromosomes and deletions were preferentially inactivated and associated with large and small sex chromatin bodies respectively (29).

Sex chromatin was soon established in clinical medicine as a simple test for deciding the sex of babies with ambiguous genitalia. In 1968 it was used at the Olympic Games in Mexico to verify the sex of female athletes, and in 1970, when the Commonwealth Games came to Edinburgh, I was asked to undertake gender verification. I declined on the grounds that it was discriminatory and was likely to detect women with androgen insensitivity more frequently than males masquerading as females. My inquiries later showed that approximately 1 in 421 female athletes attending the Olympics had "male" nuclear sex, and most had androgen insensitivity (37, 44). Many were barred from competing and suffered personal distress from innuendo and misunderstanding. My colleagues agreed with me, and we lobbied

the Medical Commission of the International Olympic Committee and other sporting bodies on several occasions to stop the practice. As a result of these efforts, testing was finally abandoned at the Sydney Olympics in 2000.

My own chromosome studies on Klinefelter syndrome were put on hold until I reached Johns Hopkins Hospital in February 1959, when I was greeted with the news that an extra chromosome had been found in Down syndrome. Like others, I decided to see whether I could confirm this and set about obtaining the necessary equipment and access to patients undergoing diagnostic bone marrow biopsies. Following an invitation to give a talk in Houston early in May, I took the opportunity to spend three days with Albert Levan, then working with T.C. Hsu at MD Anderson Hospital. Both were very helpful with technical advice, and Levan taught me to draw chromosomes with the camera lucida. With this help, and time at last to concentrate on the bone marrow method, I managed almost immediately to obtain chromosome results in normal and Down syndrome material comparable to those published. Levan came to Baltimore some weeks later, and we looked together at my Down syndrome preparations using the camera lucida. It was clear to us both that the extra chromosome was the smallest acrocentric—i.e., the 22nd, and not the 21st as Lejeune had claimed. As many others were reporting on Down syndrome, we did not write up our results, and missed the opportunity to correct a simple error perpetuated in the literature (which now accepts that these two acrocentrics are not numbered according to size). My colleagues and I used the bone marrow method sometime later to confirm that chimpanzees, unlike humans, have 48 chromosomes (141).

My interest was then directed to Turner syndrome by Lawson Wilkins, a pioneer in pediatric endocrinology at Hopkins, who had independently discovered "male" nuclear sex in the condition in 1954 (132). He had amassed a large series of patients, chromatin negative and chromatin positive, and was glad to let me check their chromosomes. With the help of my wife, Marie; Alan Johnson; and several students, we established at the Moore Clinic what turned out to be the first diagnostic cytogenetics laboratory in the United States, for we were receiving referrals from around the country. Many of Wilkins's patients were 45,X, but others also had XX or XY cell lines, and some had structural aberrations of the X or Y. Based on this clinical material, added to data then in the literature, we could draw conclusions about the nature of the genetic factors that might be responsible for the differences in stature, genital, and other malformations. It became clear that the phenotype of short stature, amenorrhoea, ovarian agenesis, webbed neck, and other malformations typical of 45,X Turner syndrome were modified by more complex karyotypes. Those with mosaicism for XY cells tended to be taller, could be virilized, and had fewer typical malformations. Most important, those with deletions of the X short arm (or with isochromosomes of the long arm) tended to have the complete syndrome with short stature, whereas those with long-arm deletions had amenorrhoea but were taller and lacked the associated malformations. I interpreted these findings to mean that there were genes on the short arm of the X that escaped X-inactivation and had active homologues on the Y. When present in double dose, as in normal males and females, these genes prevented the Turner phenotype. In other words, haploinsufficiency of these genes caused Turner syndrome. It was expected that these genes would be located in or close to the pairing (pseudoautosomal) segments of the X and Y chromosomes. Our patients with 47,XXY and 48,XXXY (46) would have duplications of the same critical regions, explaining their increasing disabilities on the additional sex chromosomes. This hypothesis was controversial, as Lyon had shown that XO mice were phenotypically normal and that all X-linked genes were inactivated in the female. I thus had difficulty getting my views published until 1965 (31), but it subsequently became a citation classic in 1991.

The studies on color vision in Turner and Klinefelter syndrome that were so interesting

earlier now proved valuable in determining the parental origin and site of nondisjunction in sex chromosome aneuploidies. Another, more informative X-linked marker, the *XG* blood group locus, became available to us for this purpose in 1962 thanks to Rob Race and Ruth Sanger. It was soon found that the X chromosome in 45,X Turner syndrome was usually maternal (80%), indicating an abnormal event in paternal meiosis and consistent with the absence of a maternal age effect (8). In Klinefelter syndrome the extra X could be either maternal or paternal, but increased maternal age was apparent only in the former (48). Race and Sanger typed many cases of Klinefelter syndrome, and included among these were several with an apparent nonmosaic 46,XX karyotype. In two such cases the XX male had failed to inherit an *Xg(a)* allele from his Xg+ve father, suggesting that both Xs were maternal and that he was an undetected XX/XXY mosaic. However, another explanation occurred to me in a eureka moment, namely, that an illegitimate X-Y interchange in meiosis in the father could have resulted in the exchange of the male determinant on the Y for the *XG* locus on the X (33). This would place the location of both in the differential segments just outside the boundaries with the pairing segments. This hypothesis has been proved correct, and the mammalian male determinant, SRY, was isolated at the predicted site from an XX male by molecular genetic methods 24 years later by Peter Goodfellow and his colleagues (124).

CHROMOSOME IDENTIFICATION AND MAPPING

On my return from Baltimore to Glasgow in November 1961 I became a lecturer in medical genetics in the Genetics Department under Professor Guido Pontecorvo. In addition to the Klinefelter studies outlined in the previous section, I followed up my studies on satellited chromosomes that I had started in Baltimore (45) by looking at their behavior in meiosis. I had shown previously for the first time that all five pairs of human acrocentric

chromosomes had satellite bodies distal to nucleolus organizers (NORs) located on the satellite stalks and that there was a tendency for these regions to become attached to one another in mitosis, a phenomenon I termed satellite association. My meiotic studies showed that satellite association also occurred at pachytene, with all acrocentric bivalents (identified by their distinctive chromomere patterns), sometimes seen attached together to a common nucleolus (30). The significance of this was related to the occurrence of translocations, which in humans most commonly involved acrocentric chromosomes. I postulated that these translocations were the result of illegitimate recombination at meiosis between homologous repeats at NORs on nonhomologous chromosomes. This phenomenon is now referred to as nonallelic homologous recombination, and is regarded as a common mechanism in chromosome rearrangement. My interests in meiosis and the origin of human chromosome aberrations led to the first studies of human-centric and reciprocal translocations and inversions at pachytene and meiosis I in carrier patients (34, 51, 133).

The short-term blood culture method introduced by Moorhead in 1960 (94) led to higher-resolution analysis of karyotypes and easier distinction between chromosome aberrations and polymorphisms. The consensus on chromosome nomenclature, achieved at the Denver Conference in that year, arranged the human karyotype into seven groups based on length and centromere position. Only chromosomes 1, 2, 3, 16, 17, and 18 and the Y were easily identified. From the large number of metaphase photographs we had accumulated from referrals, it was possible to also identify chromosomes 6, 9, and 11 based on "secondary constrictions" (43). These proved to be the precursors of the Giemsa banding patterns described by Marina Seabright and others in 1970 (121). Further characterization of the human karyotype in 1962 was achieved by James German (60), who studied patterns of DNA replication using pulse labeling with tritiated thymidine and autoradiography. The inactive X was the last to complete replication, and other specific

patterns allowed, for example, the identification of chromosomes 13, 14 and 15, and 19 and 20.

With greater confidence in chromosome identification, cytogeneticists began to consider ways of mapping genes and genetic linkage groups to their location on chromosomes. A number of genes had been located on the X by sex linkage in pedigrees, but it was not until 1968 that a gene was assigned to an autosomal region, and this was when Roger Donahue (25) linked the Duffy blood group locus to a centric chromosome polymorphism on chromosome 1. The previous year Weiss and Green (130) had showed that hybrid cells made by fusing mouse and human cells in a selective culture medium progressively lost human chromosomes, and that this loss could be correlated with the loss of specific human biochemical markers. The thymidine kinase gene was mapped to chromosome 17 in this way, and the same technique mapped many other genes in the next few years. As my group was identifying many unbalanced chromosome aberrations in our clinical work, we chose to try to map red cell enzyme and serum polymorphisms in patients with deletions and duplications. The first success of this approach came in 1973, when we mapped the red cell acid phosphatase locus to the end of the short arm of chromosome 2 by loss of a parental allele in the deleted region, confirmed by enzyme dosage in the affected patient and his parents (50). We made other assignments by deletion mapping in our patients, including the loci for *AK1*, *GOT*, *NP*, *HPT*, *ADA*, *GALT*, and *XG*. The localization of *AK1* to chromosome 9q34 also assigned the linked *ABO*:nail-patella linkage group (42) and the locus for tuberous sclerosis type 1 (17). A useful by-product of the method was the exclusion of loci from a deleted region when both alleles were present in the patient (2).

In situ hybridization of cloned DNA is the simplest and most productive strategy for gene mapping. It was first used in humans by Angela Henderson (74) to map the ribosomal genes to the satellite stalks (NORs) of the five acrocentrics in 1970. It succeeded because of the large number of copies of the gene at those sites.

Attempts to locate single-copy genes failed until recombinant technology and DNA cloning were developed in the mid-1970s. At that time Sue Malcolm joined our group, and we proved the feasibility of the method in 1981 by mapping the alpha and beta globin genes to the short arms of chromosomes 16 and 11 (87). Both had been assigned to these chromosomes in somatic cell hybrids, but the regional location was previously unknown. We next mapped the kappa immunoglobulin light chain gene to the proximal short arm of chromosome 2 (88). The method required radioisotopic labeling and autoradiography and depended on long exposures and counts of silver grains scattered over the hybridization site. The development later of nonisotopic methods, including fluorescence in situ hybridization (FISH), was a most welcome advance, and FISH remains the standard technique to this day.

The international effort to map human chromosomes can be traced through the results presented at the eleven Human Gene Mapping Workshops, to all of which I contributed, that were held between 1973 and 1991. By the eleventh workshop, in London, 2,104 autosomal and 221 X-linked gene loci had been mapped, plus a further 8,000 anonymous DNA markers. Thereafter these workshops were replaced by Single Chromosome Workshops, organized by Bronwen Loder and myself under the auspices of the Human Genome Organisation (HUGO) in London, and continued until 1996. The chromosome maps provided a sure foundation for the Human Genome Project, which announced the draft human DNA sequence in 2001 and the finished sequence in 2004 (75).

Because of my interest in human sex determination and differentiation, I made a big effort during the late 1980s and early 1990s to construct a physical map of the Y chromosome with Nabeel Affara, and with the collaboration of Ellen Magenis from Portland. As the Y did not undergo recombination, except for small pairing regions at the distal ends of both arms, genetic linkage was not an option. We relied on deletion mapping in patients found to

have Y deletions and isochromosomes, and in XX males with variable X-Y interchange break points (41). DNA markers from our sorted Y library were mapped by Southern blotting to the Y aberrations, and a deletion map was obtained for both arms of the Y. This enabled the mapping of Y loci of the genes for testis determination (1), HY tissue antigen (123), and amelogenin (4).

The main interest of gene mapping was in its potential for diagnosis and carrier detection, and for positional cloning of the genes that caused serious genetic disease. The first of these genes to be isolated were Duchenne muscular dystrophy (DMD) and chronic granulomatous disease from Xp21 as well as retinoblastoma from 13q14 in 1986, followed by cystic fibrosis from 7q31 three years later, and then many more. I was fortunate to get a Medical Research Council program grant on the molecular pathology of disease when I came to Cambridge. With other grants to Eamonn Maher, we successfully cloned the gene for von Hippel-Lindau disease (81) and isolated and characterized the genes for glycerol kinase deficiency (119) and hereditary persistence of alpha-fetoprotein (AFP) (91). Much of our effort was in developing diagnostic techniques for use in the clinic for genes cloned by others, such as cystic fibrosis and Huntington disease. The positional cloning of some disease genes was complex and slow, as illustrated by our experience in finding the multiple self-healing squamous epithelioma (MSSE) gene.

MULTIPLE SELF-HEALING SQUAMOUS EPITHELIOMA

In 1934 my father described a patient with multiple cutaneous tumors that resembled squamous carcinomas except that they resolved after several months, leaving deep disfiguring scars (27). The tumor cells spread locally along lymphatics and occasionally into lymph nodes. In this and other respects they differed from keratoacanthomas. The original patient was adopted, and the condition was not known to be familial until his daughter became affected.

Other patients were recognized, mostly from Scotland, and the condition became known as Ferguson-Smith disease. In 1966 I examined 62 affected individuals from 11 families (54), and James Renwick tested them and their relatives for 23 blood group and serum polymorphisms in the hope of mapping the gene. Four of the families belonged to a large Scottish pedigree connected to a couple who married in 1810, and three apparently unrelated families could be traced back to their origin in 1745. The condition was inherited as a dominant trait, and there were equal numbers of affected males and females. No linkage was noted at the time, although when the results were revisited 20 years later, a weak positive LOD score was found with the *ABO* locus on chromosome 9q.

In the late 1980s many polymorphic DNA markers became available for mapping, and in 1987 David Goudie and I made another concerted effort to map MSSE. Our linkage analysis of 150 patients and their relatives revealed strong linkage to a DNA marker at 9q31 with a common haploid group extending from 9q22 to 9q31 (64). Two possible candidate genes in the region, xeroderma pigmentosum and *PATCHED* (mutated in familial basal cell carcinoma), were checked and excluded for mutations by Frances Richards in our group (115). Richards went on to exclude other candidates and to demonstrate loss of heterozygosity within the critical region in tumors, thus providing the first evidence that the MSSE gene was a tumor suppressor gene (7). Families with the condition were now being referred to us from Italy, Japan, Denmark, France, and the United States with linkage to 9q31 but with different haplotypes (19). Although there was clearly a founder effect in the large Scottish pedigrees, the disease mutations must have arisen independently many times and were not as rare as we had thought.

Meanwhile, sequencing additional candidate genes within the 4 Mb critical region failed to identify mutations. Accordingly, Birgit Lane and Goudie arranged high-throughput sequencing of a much larger 242 Mb interval in four unrelated families in 2009. This led to the

discovery of three different mutations in the gene for transforming growth factor beta receptor 1 (*TGFBR1*) in these families (63). *TGFBR1* exons were then sequenced in other families, and 11 different mutations were found in 18 of 22 families. The nature of the self-healing aspect of MSSE still remains elusive, but carrier detection is now available for the families.

GENETIC SERVICES AND PRENATAL DIAGNOSIS

Our small cytogenetics research group in the Genetics Department in Glasgow was frequently asked to provide karyotype analysis in patients, particularly for those at the Royal Hospital for Sick Children. By 1964 the demand had increased beyond our resources, and James Hutchison, the professor of child health, found space for me in the neonatal laboratory at the Queen Mother's Maternity Hospital. My wife, Marie; Elizabeth Boyd; and Irene Greig were recruited to provide a chromosome diagnostic service funded by the National Health Service, and I received a new appointment held jointly in the Genetics Department and Child Health Department. I started a weekly genetics counseling clinic at Queen Mother's that continued until 1972, when we all moved to the new Children's Hospital. Research continued at the Genetics Department, fueled by the clinical material from the hospital.

Following the report of successful fetal chromosome analysis from the United States in 1966, Marie was able to develop this method from hysterotomy samples and from amniotic fluid sampled in pregnancies with Rhesus incompatibility. Our publication in 1971 was the first in the United Kingdom to report on a prospective study of prenatal diagnosis (56). The clinical indications included previous Down syndrome, advanced maternal age, and chromosomal translocation. Although only 1 affected fetus was found in the 30 referrals, the procedure provided reassurance to the remaining couples, many of whom would not have contemplated pregnancy without prenatal diagnosis. Increased maternal age became

the most frequent indication, and by 1981, 2,700 women age 35 and over had been tested in our laboratory and 23 Down syndrome fetuses detected. John Yates and I collected amniocentesis data from European centers and calculated maternal age-specific rates for chromosome aberrations in nearly 53,000 couples in the second trimester (55). An exponential increase in risk with advancing maternal age for trisomies 13, 18, and 21 and for XXY and XXX sex chromosome abnormalities implied a similar mechanism in each. A declining frequency with advancing maternal age was evident in XO Turner syndrome and for trisomy 21 in mothers in their late 40s (a novel finding confirmed in recent years). These effects could be explained by the tendency of such mothers to miscarry before the time of amniocentesis. This view is supported by the well-known inviability of 45,X conceptions, 80% of which are lost in the first trimester. In XYY pregnancies there is no effect of maternal age, as nondisjunction is paternal. Our survey found no separate effect of paternal age in 13,000 Down syndrome pregnancies. The maternal age data were very useful in providing risk estimates in counseling.

We were fortunate in having Ian Donald, professor of midwifery and pioneer of ultrasound in medicine, at our hospital, as our patients had the benefit of ultrasound guidance for inserting the amniocentesis needle when taking samples for prenatal diagnosis. With ultrasound, gestation could be confirmed, twins identified, and the placenta located so that anterior placentas could be circumvented. Other regions were slower to introduce prenatal diagnosis until ultrasound became more widely available, and I believe that the demand for safe amniocentesis was an important factor in the development of sonar in obstetrics.

In 1972 David Brock and Roger Sutcliffe reported that neural tube defects (NTDs) could be identified by raised amniotic AFP. Anencephaly and spina bifida occurred with a frequency of over 5 per 1,000 in the West of Scotland at that time, and we received many requests to test for recurrence of NTD in subsequent pregnancies. We thus made the first

prenatal diagnosis of spina bifida from amniotic AFP in 1972 (3), and when Brock found that maternal serum AFP was also raised in affected pregnancies, we instituted a screening program in 1975 that was offered to all mothers. By 1982 we had screened over 140,000 pregnancies and detected 310 anencephalic and 195 spina bifida fetuses. In the population of three million in our region, the rate of NTD live and stillbirths declined from 4.3 per 1,000 in 1976 to 1.7 per 1,000 in 1981 (35). Although some of the decline might have been attributable to improved diet, it was estimated that at least 80% was due to prenatal screening and termination of affected pregnancies. By 1991 approximately 60% of pregnancies in Europe had maternal serum AFP screening for NTD, but since then, ultrasound detection has been used increasingly for both screening and diagnosis.

In 1984 Merkatz (92) observed that maternal serum AFP levels were lower in Down syndrome than in normal pregnancies, and we confirmed this on stored samples and in a few prospective cases. Soon other biochemical and ultrasound markers (such as nuchal translucency) were combined with maternal age in protocols to determine the risk of the condition at both the first and second trimesters (18). In 2009 it was possible to detect over 90% of Down syndrome pregnancies using this strategy, for a false +ve rate of 2%. The effect of screening has been to reduce the number of amniocenteses (and chorionic villus biopsies) required, and consequently the risk of miscarriage from invasive tests. Although in recent years Down syndrome birth frequency has been reduced by only a few percent, because of women postponing pregnancy until their age risk increases, it is estimated that without prenatal screening and diagnosis the birth frequency now would be 45% higher.

Small numbers of fetal cells have been isolated from maternal blood since 1969 (128), and many studies have exploited this for noninvasive prenatal diagnosis, with variable success. In our studies we had some success in enriching the numbers of fetal nucleated red cells in maternal blood samples by negative magnetic sorting (142). The cells were recognized as fetal by antifetal hemoglobin antibodies. However, too few were suitable for aneuploidy detection by FISH, and when we attempted to culture fetal red cell progenitors, only colonies of maternal stem cells were identified (14). Nonetheless, our probes for fetal trisomies and fetal sex determination were put to good use in speeding prenatal diagnosis in uncultured amniotic fluid cells (23). Dennis Lo's current research on determining sex (84) and detecting trisomies from fetal nucleic acids in maternal blood (85) has proved much more successful, and the fetal cell approach has been largely abandoned.

Because we were able to offer prenatal diagnosis, the West of Scotland Regional Genetics Service was one of the first to be established in the United Kingdom. Before the advent of prenatal diagnosis, the reproductive options for couples at risk were limited to avoiding pregnancy, choosing adoption, or, in cases at risk of gene defects, choosing artificial insemination with donor semen. Genetic counseling could help only with risk estimation and tests for carrier status. All this changed when it became possible to test the fetus and terminate the pregnancy if it was severely affected. At this point the need for genetic clinics grew rapidly, first in academic centers involved in genetic research and then more widely. Eventually a network of National Health Service regional centers became established in the United Kingdom, providing genetic services in the 1980s to an increasing proportion of the public. On becoming head of the Pathology Department at Cambridge University, I was also appointed director of the East Anglia Regional Genetics Service and continued to enjoy regular contact with patients at Addenbrooke's Hospital. The clinical load was heavy, but this was encouraging as it was becoming clear that genetics was having an increasing impact on medical practice everywhere.

In view of the growing activity in prenatal diagnosis, I was approached by Wiley (the publishers) in 1979 to edit a specialist journal in that field. I was reluctant to take this on, as I had an ambition to start a new journal on gene

mapping. However, I was persuaded that the time was not ripe for gene maps and so came to edit the journal *Prenatal Diagnosis* for the next 26 years. I believe the journal fulfilled a useful role, particularly in developing genetics within obstetrics and improving services to patients. Happily, gene mapping got its journal, *Genomics*, seven years after *Prenatal Diagnosis* began publication.

CHROMOSOME SORTING AND PAINTING

The development of recombinant DNA technology in the 1970s was key to many advances in gene mapping and medical genetics. The technology was responsible for the introduction of chromosomes-specific libraries cloned in bacteria and used to make markers for positional cloning. In 1975 Joe Gray and others in the United States were among the first to use the sorting of chromosomes in fluid suspension in the preparation of these libraries (66), and Bryan Young in Glasgow used the sorting method to make a human X chromosome library (20) that helped to map the DMD gene, among others. I was asked by Young in 1980 whether I could help in interpreting the flow histograms produced by a single-laser flow cytometer with sorted chromosomes from a transformed cell line. The peaks in the histogram could be correlated with chromosome size and quantity, so that the X peak with male cells was half the size found with female cells, but there was also much unexplained variation. We decided to sort chromosomes from short-term blood cultures from individuals from our diagnostic service known to have chromosomal aberrations and polymorphisms, so that the flow peaks could be correlated with known variants (140). This worked well and prompted research that has continued for 30 years.

After our first study Peter Harris joined the group and took flow karyotyping for his PhD project. With the judicious use of heteromorphisms we were able to sort pure samples of almost every human chromosome except 10, 11, and 12 (69). The DNA content of each could be measured, and our results correlated closely with those obtained previously by cytophotometry (70). Homologous chromosomes could be distinguished in the flow karyotype by differences as small as 1 part in 2,000 of the genome, and in one study we detected a 6 Mb deletion in two cousins with DMD and the carrier state in three of their relatives. De novo structural aberrations in patients could be distinguished from heteromorphisms by analysis of parental flow karyotypes (71). In most XX males with X-Y interchange, the size difference between the two Xs was sufficient to distinguish them (36).

When this research was continued after our move to Cambridge in 1987, we acquired a dual-laser flow cytometer with even greater resolution. Nigel Carter joined us, and one of the first projects was to confirm that the two X chromosomes in XX males differed in size because of X-Y interchange (13). In one XX male the size difference enabled each to be sorted separately onto nitrocellulose filters, which were then probed with radioactive X and Y clones, the latter hybridized only to the spot containing the larger X (13). A similar strategy was used later to make a detailed map of chromosome 9 in the region containing the tuberous sclerosis type I gene (17) by sorting both derivatives of reciprocal translocations onto filters (72).

In 1992, Haakon Telenius's (125) development in our department of random-primed polymerase chain reaction (PCR)—termed degenerate oligonucleotide-primed PCR (DOP-PCR)—for amplifying any DNA sequence was a major advance, with widespread application in molecular genetics. Its first application was to amplify and label DNA fragments from sorted chromosomes to make chromosome-specific probes from each chromosome for use in diagnostic cytogenetics. The probes were initially labeled with biotin or digoxigenin and detected by fluorochromes, such as fluorescein isothiocyanate or rhodamine, coupled to avidin or antidigoxigenin antibodies, so that they could be visualized by fluorescence microscopy. The probes hybridized along the length of each chromosome with greater specificity and

intensity than was possible with chromosome libraries. Chromosome painting became a most valuable tool in identifying chromosome rearrangements. Reverse painting, in which aberrant chromosomes were sorted and labeled and then painted onto normal metaphases, was particularly valuable in interpreting complex rearrangements (12). Those without access to flow cytometry could use chromosome microdissection and DOP-PCR for the same purpose.

As additional fluorochromes became available, our human and mouse chromosome-specific DNA was used to make multicolor FISH, or M-FISH (83, 120). With five fluorochromes, each chromosome probe could be labeled with a different combination of colors, so that all chromosomes could be identified separately in one metaphase at one hybridization event. Digital fluorescence microscopes, with image processing and software capable of classifying each chromosome in a karyotype, were introduced so that all major interchromosomal rearrangements were recognizable. Only intrachromosomal abnormalities, such as inversions, escaped detection. Color banding, which uses gibbon instead of human chromosome-specific probes, had higher resolution as it produces color bands along human chromosomes (**Figure 1**), and this helped with the detection of intrachromosomal abnormalities (73, 96). M-FISH continues to have practical application in analyzing constitutional aberrations and the multiple rearrangements found in cancer cells (16, 95).

Figure 1

Human metaphase to which gibbon chromosome-specific DNA, labeled with combinations of five fluorochromes, has been hybridized. The gibbon paint probes reveal homologous segments. Gibbon chromosomes are highly rearranged compared with human chromosomes, and the result is a useful technique for the analysis of human chromosome aberrations. Figure taken from Reference 53.

CROSS-SPECIES PAINTING AND KARYOTYPE EVOLUTION

Johannes Wienberg (131) used human chromosome libraries successfully in 1990 to identify homologues of human chromosomes in apes and Old World monkeys. When Fengtang Yang joined our group in 1993 to study chromosome homologies in muntjacs, we made chromosome paints from sorted Indian muntjac chromosomes to look for similar homologies in closely related species of deer. The female Indian muntjac was a good choice as it had only three pairs of chromosomes and, with only two fluorochromes available at the time, red and green dyes could be used for labeling two of the chromosomes and a mixture of the two dyes for the other. The hybridizations unexpectedly revealed for the first time a new feature—namely, that interphase nuclei were organized into six distinct chromosome territories (**Figure 2**). This result prompted others to look at the functional significance of chromosome domains at interphase. However, our interest at the time was to make homology maps by cross-species painting, and Yang found that almost all the Chinese muntjac chromosomes were painted by only one of the three Indian muntjac chromosomes, and that only single regions of the Indian muntjac chromosomes were

painted by 19 of the 23 pairs of Chinese muntjac chromosomes (135, 139) with the exception of three Chinese muntjac chromosomes that were rearranged in the other species. Therefore, the Indian muntjac karyotype consisted mainly of single Chinese muntjac chromosomes fused together along the chromosome. Moreover, a probe prepared from the muntjac centromeric sequence hybridized to most of the interstitial fusion sites. Painting both species with 35 chromosome-specific paints from the brown-brocket deer, *Mazama gouazoubira* (MGO), confirmed the centromere-telomere fusions and showed that at least five single MGO chromosomes had been conserved in both the Indian and Chinese muntjacs, indicating that they had originated in a common ancestor (139).

The interesting findings of the muntjac study prompted an extensive program of research into the use of cross-species painting in determining phylogenetic relationships across mammalian families and orders. We used the Indian muntjac idiogram to align chromosome homologies between humans, cattle, sheep, and muntjacs to show regional patterns of hybridization that were shared by species differing widely in chromosome number and karyotype (10). Collaborative studies quickly followed on carnivores (65, 100), laboratory rodents (137), and bears (98) with the help of colleagues with expertise in these species.

By 1996 our collection of animal cell cultures was expanding and flow sorting was much in demand, and Patricia O'Brien, who had joined us in 1994, undertook the responsibility for both. One particularly difficult task was to generate paint probes from the 78 dog chromosomes. Except for the X chromosome all were acrocentric, and characterizing the 17 smallest pairs by banding had not been possible. Yang (138) was able to combine painting and banding to produce a complete, definitive banded karyotype, which he used to prepare homology maps between dogs, red foxes, and humans. David Sargan and Yang (118, 136) used these results to assign 83 canine genetic linkage and radiation hybrid groups to dog chromosomes, using PCR mapping of cloned DNA sequences and FISH

Figure 2

The three pairs of chromosomes of the female Indian muntjac in metaphase and interphase nuclei show the arrangement of chromosomal territories at interphase. Chromosomes 1, 2, and X+3 are shown in light blue, green, and red, respectively. Figure prepared by Dr. Fengtang Yang.

mapping of DNA markers. The closely related red fox was very helpful in this, as it had only 34 chromosomes, which were all easily identifiable, so that dog DNA markers located on fox chromosomes could be assigned to the correct dog chromosome from the dog-fox homology map. In fact, our FISH mapping results were made with ease from mixtures of dog and fox metaphases on the same microscope slide. Our dog homology maps proved helpful for studies on the phylogeny of carnivores. Cross-species painting with human probes revealed 73 blocks of conserved synteny, indicating that among mammals, the dog had one of the most rearranged karyotypes.

Almost all phylogenetic studies on placental mammals using painting are based on identification of syntenic blocks of conserved DNA revealed by human paints. We have used this method to study representative species of all eutherian orders (i.e., excluding marsupials and monotremes) to postulate the composition of

the ancestral eutherian karyotype. It seems that the ancestor had 46 chromosomes with conservation of the X and most of 10 human autosomes (53).

Afrotheria is regarded as the most basal of all eutherian orders, and is represented by the elephant, aardvark, manatee, golden mole, tenrec, hyrax, and elephant shrew. The DNA sequence of several genes indicates that they share a common progenitor despite their apparent anatomical differences. Cross-species painting confirms this conclusion, showing that the seven species share a unique chromosomal signature—namely, syntenic associations between human chromosomes 5 and 21 as well as 1 and 19 (117, 134). The Afrotheria results are the product of our collaborations with Terence Robinson from Stellenbosch and Alexander Graphodatsky from Novosibirsk, with whom we have also collaborated on Perissodactyla (horses, zebras, rhinoceroses) (126); Cetartiodactyla (cattle, sheep, pigs, deer, camels, giraffes, dolphins, hippopotamuses) (5, 80); and Xenarthra (armadillos, sloths, anteaters) (24). We also studied South American species of Chiroptera in collaboration with Julio Pieczerka from Belem (105), and Akodon rodents with Yatiyo Yonenaga-Yassuda from São Paulo (127). In 2007 Vladimir Trifonov and I (53) reviewed painting studies and karyotype evolution in placental mammals and were able to provide links between all mammalian orders and many families by their unique chromosome signatures. These signatures seemed to us to be the ideal traits that Charles Darwin was looking for to illustrate the descent of species from a common progenitor.

One of the best illustrations of the potential of chromosome painting for investigating problems in genetics comes from our study that resolved the complex sex chromosome systems in the platypus and echidna. This arose from work on marsupials and monotremes done in collaboration with Jenny Graves and her group in Canberra. Willem Rens in our group first used painting to work out the chromosome homologies between several marsupials from Australia (113) and then went on to show the close relationship between marsupials of Australia and South America (114). It was interesting to find that although human paints did not hybridize to marsupial chromosomes and vice versa, the tammar wallaby X was an exception in that it hybridized to the long arm and proximal short arm of the human X, indicating greater conservation of the X during the evolution of the two lineages (62). This finding also showed that the eutherian X received its short arm after their divergence 148 million years ago.

Cross-species painting between human and monotreme chromosomes was also unsuccessful, but sorting and painting the platypus chromosomes resolved the long-standing questions about the presence of unpaired chromosomes in the male. We showed that there were 10 unpaired sex chromosomes in the male, and that 5 of these were Xs as they were paired in the female (111). In male meiosis the 10 sex chromosomes formed a chain of alternating X and Y chromosomes linked by pairing (pseudoautosomal) regions (68). Mapping X and Y markers in sperm showed that male and female gametes were produced by alternate segregation in the chain. We went on to show that the male shortbeaked echidna had nine sex chromosomes, the first five of which were the same as the platypus, but the order of the others was different, and in each species a different autosome replaced one of the five X chromosomes (112). The meiotic chain most likely arose by reciprocal translocation between sex chromosomes and autosomes, with further divergence of the ancestral karyotype in both lineages. With the help of the platypus draft genome sequence (129), gene mapping by both the Cambridge and Canberra groups demonstrated that the sex chromosomes of monotremes are unique and do not share homology with the X and Y chromosomes of placental mammals or marsupials. Mapping orthologues of chicken Z genes onto platypus chromosomes shows that a number (including *DMRT1*) are located on three sex chromosomes and four autosomes (112). Although there is a link between the avian Z and monotreme sex chromosomes, it is by no means clear that this implies the same sex determinant

in each. It seems likely that the monotreme male determinant may be found in one of the Y chromosomes shared by the platypus and echidna.

Most birds have complex karyotypes with 78–80 chromosomes, of which 8 or 9 are identifiable macrochromosomes and the remainder are indistinguishable microchromosomes. The macrochromosomes of the chicken can be sorted and labeled easily, but the microchromosomes sort into five groups from which single chromosomes can be obtained and amplified to make chromosome-specific paints. Darren Griffin, who was with us in 1997, tackled the difficulty of obtaining microchromosome paints by microdissection. The first results were reported in 1999 (67), and an almost complete set of chicken chromosome paints was achieved five years later (89). In the intervening period, it was found that most macrochromosomes were highly conserved among birds, including the most primitive ratites (122) and the California condor (110). Some exceptions were found, including some raptors and a few others. In many species (including the primitive ratites) chicken chromosome 4 painted one macro- and one microchromosome, indicating fusion between two ancestral chromosomes. The partridge's chromosome 4 has the same fusion as the chicken but is acrocentric rather than metacentric. Fumio Kasai showed that the order of genetic markers along acrocentric partridge 4 was the same as that along metacentric chicken 4, so that the centromere shift was due to repositioning and not inversion as had been assumed (78). This was the first evidence of a neocentromere occurring in bird evolution. Our other ventures into avian karyotype evolution included a contribution to owl phylogeny (21), characterization of the 42 chromosomes of the stone curlew (99), and the discovery of the highly rearranged karyotype in the white hawk (22).

The phylogenetic relationship between birds and reptiles is of great interest. As crocodiles and some turtles have no sex chromosomes, and sex is determined by egg incubation temperature, we looked for conservation between the chicken Z and the chromosomes of representatives of these two reptiles. Kasai (79) showed that the Z paint hybridized along the entire length of chromosome 6 in both species and that chromosome 6 in each could be painted to one another and, reciprocally, to the chicken Z. The study has been extended to 28 squamate reptiles that diverged from birds over 275 million years ago (106). In each species, the chicken Z hybridized to a single region, either to a whole chromosome or to one arm of a metacentric chromosome. It is not known whether other reptilian chromosomes are conserved to such a great extent. However, in a collaborative study with Massimo Giovannotti and Ettore Olmo from Ancona (61), cross-species painting between skinks revealed a number of rearrangements.

In our painting studies we have had least success with amphibians and fish, largely because of the high proportion of repetitive DNA in their genomes (especially in amphibians) and the similar AT:GC ratios in each chromosome in the complement, which leads to poor separation in flow sorting. In the case of the electric knifefish (*Gymnotus carapo*) the 21 pairs of chromosomes could be separated into four groups, which were subdivided into subgroups containing fewer chromosome pairs that were then amplified and labeled. Paints from various subgroups, each of which contained different combinations of pairs, were able to provide coverage of the whole complement. It was thus possible for Cleusa Nagamachi from Belem (97) to identify rearrangements with these paints that allowed fish with otherwise apparently identical phenotypes and karyotypes to be identified as different species.

Our experiments with vertebrates from many disparate taxa attest to the value of chromosome painting in various aspects of comparative genetics, sex determination, taxonomy, and evolution. Much of the work was supported by grants from the Wellcome Trust, which established our Cambridge Resource Centre for Comparative Genomics and enabled us to provide chromosome-specific DNA from over 150 different species to our colleagues on every continent without charge. This generated many of the collaborations described in this article.

BOVINE SPONGIFORM ENCEPHALOPATHY AND THE BSE INQUIRY

During the last year of my time as head of the Pathology Department at Cambridge I was asked, along with Nicholas Phillips (a judge) and June Bridgeman (a civil servant), to serve on the government inquiry into the emergence of bovine spongiform encephalopathy (BSE) and the variant Creutzfeldt-Jacob disease (CJD) (104). My role, as the scientist on the committee, was to review the relevant research.

BSE is an infectious disease of cattle caused by abnormal posttranslational modification of prion protein that renders it resistant to enzyme degradation, so that it forms aggregates that accumulate in and around cells and cause degeneration of neurons, dementia, and death. In humans, prion disease causes CJD, and in sheep it causes scrapie. The abnormal prion protein is infectious in food or by grafting from infected donors, and in humans, 15% of cases are familial and caused by prion gene mutations. The BSE epidemic was identified in southwest England in 1986, and its spread by infected meat and bone meal (MBM) rendered from animal carcasses was recognized shortly afterward. It was not until 1988 that control measures were introduced, including the banning of MBM in cattle feed. By 2000 over 180,000 cattle were affected and 5 million had to be destroyed to control the epidemic and prevent the entry of infected meat into the human food chain.

The scientific advice at the time was that the epidemic was due to the inclusion of the scrapie agent in MBM, and that this did not pose a risk to humans, as the public had been exposed to scrapie for over 200 years without harm. The controls were introduced because of the remote risk of transmission and were considered by some to be ultraprecautionary, so that enforcement tended to be lax. However, scrapie had also been exposed to cattle for at least 70 years without harming them, and the inquiry found other evidence that the agent responsible for BSE was not scrapie. The biochemistry of BSE prions was different from scrapie prions, BSE had a different host range, cats were susceptible to BSE but not to scrapie, BSE was exclusively a British disease, and the start of the epidemic was consistent with a single rather than a multiple origin (38). This information was not passed on at the time to the public, who continued to be reassured that it was safe to eat beef. Early in 1996 it came as a complete shock that BSE had infected humans, some of whom were developing variant CJD. The public was angry and felt betrayed. Over 200 have since died from variant CJD.

The inquiry report suggested that the scrapie origin was no longer plausible and that the most likely cause was a novel bovine mutation of the prion gene leading to spread of the abnormal prion protein in contaminated MBM (39). That prion gene mutation was the only known cause of prion disease strongly influenced my reasoning (as a medical geneticist). The government veterinarians at the Ministry of Agriculture strongly opposed the inquiry's rejection of the scrapie origin hypothesis that they favored. The ministry set up a review committee to consider our conclusions but could only suggest that these were speculative and that, although a mutation in cattle or sheep could not be excluded, a modified form of scrapie could not be excluded either. There matters stood until the report by Jürgen Richt and Mark Hall in 2008 (116) of an atypical case of BSE from Alabama that had a prion gene mutation that had been transmitted to her calf. The mutation (E211K) was the same as that found in the commonest form of familial CJD in humans. It is conceivable that this abnormal prion protein might cross the bovine-human species barrier should the occasion present itself. I believe that the finding of a BSE mutation in the Alabama cow strengthens the feasibility of our hypothesis about the origin of the BSE epidemic (52), and indicates the importance of continued surveillance for future outbreaks.

CONCLUSION

It seems remarkable that in the short space of 50 years, human genetics has moved from the

discovery of the correct chromosome number to a point where it is possible to sequence individual human genomes and to define chromosomal disorders by DNA sequence rather than by Giemsa bands. From contributing virtually nothing to the practice of medicine, genetics is now the basic medical science and has taken a key role in the diagnosis and understanding of both rare and common disease, from cystic fibrosis to breast cancer. It has been exciting to be in medical genetics during this time of exceptional discovery and opportunity. Genetics and medicine seem set to continue to contribute to one another and could even merge closer together in the future for the greater benefit of our patients.

DISCLOSURE STATEMENT

ACKNOWLEDGMENTS

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