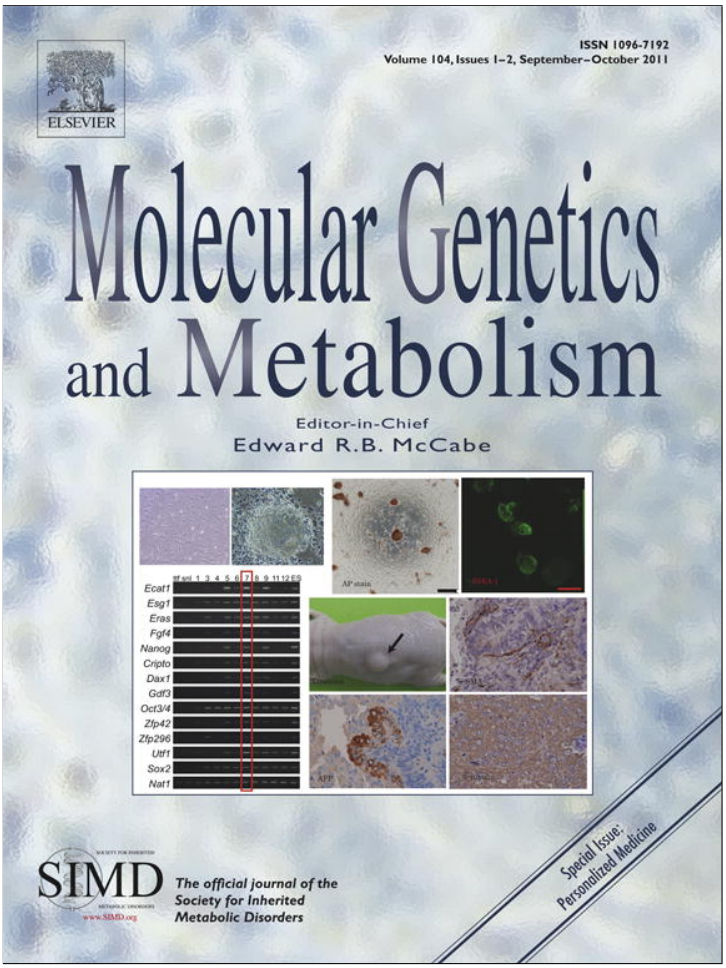

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Minireview

The evolution of the search for novel genes in mammalian sex determination: From mice to men

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ABSTRACT

Disorders of sex determination are a genetically heterogeneous group of rare disorders, presenting with sex-specific phenotypes and variable expressivity. Prior to the advent of the Human Genome Project, the identification of novel mammalian sex determination genes was hindered by the rarity of disorders of sex determination and small family sizes that made traditional linkage approaches difficult, if not impossible. This article reviews the revolutionary role of the Human Genome Project in the history of sex determination research and highlights the important role of inbred mouse models in elucidating the role of identified sex determination genes in mammalian sex determination. Next generation sequencing technologies has made it possible to sequence complete human genomes or exomes for the purpose of providing a genetic diagnosis to more patients with unexplained disorders of sex determination and identifying novel sex determination genes. However, beyond novel gene discovery, these tools have the power to inform us on more intricate and complex regulation-taking place within the heterogeneous cells that make up the testis and ovary.

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1. Introduction

Identifying the genetic origins of testis and ovarian determination is no easy task. Traditional linkage approaches for mapping disease genes required large families with multiple affected members. However, patients with disorders of sex development are sub- or infertile and therefore most families are too small to perform genetic linkage analysis. Some of the major breakthroughs relied on identification of karyotype abnormalities followed by positional cloning to identify the disrupted gene. Using this approach, SRY was identified as the gene responsible for initiating male sex determination in humans. As the human genome project evolved, it provided the tools to identify many of the important genes in sex development.

Disorders of sex development (DSD) constitute a rare set of genetic disorders in which the chromosomal, gonadal, and phenotypic sexes are incongruous. These disorders are extraordinarily stressful for both the child and parents and in the majority of cases the genetic etiology of the DSD remain unknown. To date, there exists little evidence-based data by which parents can make the difficult decisions regarding gender assignment, medical management, and surgery. The advent of next generation sequencing has identified many of the genes responsible for a variety of Mendelian traits, including those responsible for DSD. Genome sequencing will ultimately be central in the development of novel diagnostic tools and allow clinicians to personalize disease management. This review will cover the history of novel gene identification in sex determination and the future role of sequencing technology in personalized medicine for patients with DSD.

2. Mouse models of mammalian sex determination

Beyond the initial discovery of the testis-determination gene, *SRY*, in humans, the discovery of other novel sex determination genes was

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still limited by the size of the families, the rareness of these conditions, and access to fetal gonad tissue. To elucidate the complex interactions that result in the formation of a testis or an ovary, much of the work in identifying novel genes and understanding their interactions was done in inbred mouse strains. In the developing mouse there is easy access to the tissue of interest, in this case, the bipotential gonad, at the timepoint of divergence into either a testis or an ovary. Studies performed in mouse fetuses would be not only impractical but unethical in human fetuses. Furthermore, the technology exists to genetically manipulate mice, creating transgenic or knockout mice to directly assess the effect of overexpression or loss of specific genes. All of these things have catapulted mouse inbred strains as one of the primary model organisms for studying mammalian sex determination.

The modern laboratory mouse has its origins in the 20th century around the time when the laws of Mendelian genetics were "re-discovered" and integrated into classical genetics. Fancy mouse breeders realized that valued traits, such as coat color and fur length, were also likely to be genetically determined. Ms. Abbie Lathrop developed the first fancy mouse strains for laboratory use on her farm in Granby Massachusetts. In order to solidify a place for mouse models as paramount in the study of human health and disease, Clarence Little recognized the importance of generating homogenous inbred mouse strains to remove the genetic heterogeneity from experiments therefore allowing experiments conducted in different locations to be more easily compared. After moving to Cold Spring Harbor in 1918, Little began working on several strains of inbred mice, including C57BL/6, C3H, and BALB/c [1]. Little ultimately went on to co-found the Jackson Laboratory in Bar Harbor, Maine, which today remains a non-profit institution dedicated to using mouse models to better understand and treat a wide variety of human disease.

Mouse models are ideal for studying human diseases. First, mice have the same internal organs as humans, they can be housed in small cages, easily fed, and with age and diet mice develop many of the same diseases as humans, including cancer and diabetes. Despite the evolutionary distance between mice and humans, the mechanisms of sex determination and sex development are highly conserved. The trigger for placental mammals' testis-determining pathways is the sexually dimorphic expression of a single gene, *SRY*, on the Y chromosome. SRY is believed to have evolved 150 million years ago, after the divergence of placental and marsupial mammals from monotremes [2]. In lower organisms, the sex determination trigger can be either genetic, environmental, or a combination of both. While trigger mechanisms to differentiate between two sexes may be different, many of the downstream actors remain conserved.

Prior to the discovery of *SRY*, careful investigation of sex development in mouse models also identified specific strains as susceptible to XY sex reversal. A leader in the field of mouse genetics and sex development, Eva Eicher, pioneered many of the crosses and genetically mutated strains in which there is abnormal sex development [3]. Consomic crosses, in which a single chromosome is derived from one strain, while the remaining chromosomes come from a different strain, further illuminated the role of the Y-chromosome in genetic sex determination. Using this approach, the Y-chromosome from various wild derived mouse strains was backcrossed onto inbred strains to determine if there was a sex development phenotype. The Y-chromosome of POSA, a semi-inbred strain generated from the mating of a wild-derived *Mus musculus domesticus poschiavinus* male and a Naval Medical Research Institute (NMRI) Swiss female, when backcrossed onto the C57BL/6 (B6) background resulted in a large proportion of XY-female mice. When mice were examined during embryonic development at E14.5, none of the B6-Y^{POS} embryos developed fully normal testis. All of the XX mice had normal appearing ovaries, indicating that this trait resulted from an interaction between the Y^{POS} and one or more autosomal determinants on the B6 background [4]. This phenomenon is not particular to the Y^{POS} chromosome. On it's original genetic background, Y^{POS} initiates normal male sex determination. Backcrossing the Y^{POS} onto other inbred backgrounds, such as BALB/c, C58/J, and DBA/2J did not result in any XY sex reversal [5] implying that that the aberrant interaction was specific to the B6 inbred strain and the Y^{POS}. Placing other wild derived Y-chromosomes on the B6 background confirmed the propensity of the B6 strain to XY sex reversal. Y-chromosomes from *Mus musculus domesticus AKR* (Y^{AKR}), *andMus musculus domesticus tirano* (Y^{TIR}) showed varying degrees of sex reversal when placed onto a B6 background. Interestingly, Y^{AKR} merely showed delayed testis determination but by adulthood all XY mice were phenotypically male and fertile. These studies confirmed that the B6 genetic background is exquisitely sensitive to XY sex reversal.

Studies utilizing recombinant inbred strains focused on exploiting the genetic differences between the sex reversing B6 strain and the non-sex reversing DBA2 strain. B6-Y^{POS} males were crossed to DBA females and F1 offspring were intercrossed to create a genetically heterogeneous population with varying phenotypes. The genotype–phenotype correlation can be used to map genetic regions of B6 origin that contribute to XY hermaphroditism. However, these studies did not have the power or the resolution to definitively identify the genes or mutations responsible for B6 sensitivity to sex reversal [6].

Complementing the increased sensitivity to XY sex reversal, the B6 strain is also protected from XX-sex reversal. In genetic models of XX-males, when mutations are transferred to the B6 inbred strain, the XX-Sex reversal phenotype is lost. In both the *Odsex* mutant and the Tg-*Sox3* mutant, the resulting XX-male phenotype is lost when the genetic mutation is transferred to a B6 background. Together, the sensitivity of B6 background to XY sex reversal and protection from XX male sex reversal implies that at the genomic and transcriptomic level, the balance of the bipotential gonad is tipped in favor of ovarian formation.

The B6 strain's protection from XX-sex reversal and promotion of XY-sex reversal can be partially explained by the differing expression levels of "female" or "male" promoting genes within the developing gonad of B6 mice. B6 animals have a higher expression of "female-promoting" genes, compared to 129 animals. Therefore, B6 animals are already tipped in favor of ovarian sex determination. To compensate for a female-tipped balance, there is an increased level of SOX9 expression that is sufficient for male sex determination [7].

The use of mouse models of sex development has elucidated the function of genes of large effect size (i.e. *SRY, SOX9*), but also has shed light on the intricate balance of genes that take place within the differentiating gonad. The developmental choice between an ovary or a testis is not a passive process, but one that is actively battling to repress the other throughout gonadal development and even long into adulthood.

3. The role of the human genome project on disorders of sex development

The early history of novel gene identification in sex development relied heavily on karyotype and *in situ* hybridization to identify regions of interest within the genome from which a gene could be positionally cloned. Through positional cloning of Yp translocations to the X-chromosome, the testis-determining gene on the Y-chromosome was identified as SRY, a homeobox gene [8]. This initial discovery paved the way for the discovery of a second gene in the SOX gene family, *SOX9*, in patients with Campomelic Dysplasia (CD) and XY autosomal sex reversal. Translocation breakpoints identified through karyotyping were followed by *in situ* hybridization to identify the gene, which was followed up by sequencing for deleterious mutations in *SOX9* [9,10].

An alternative source of genome variation lies in duplications and deletions of larger chromosomal regions known as copy number variation (CNV) and can give rise to a number of polymorphic traits.

Karyotype and chromosome painting approaches can detect large duplications or deletions, on the order of megabases, while SNP arrays can detect copy number variations (CNV) that are as small as several kilobases. Most CNVs are benign, representing the range of genomic variation that exists within the population. However, *de novo* copy number variations are responsible for many developmental conditions. CNVs in patients with disorders of sex development (DSD) can be inherited from either the mother or the father, as the phenotype is sex-specific [11,12]. Large-scale CNV analysis in a wide range of DSD patients has identified multiple candidate genes for XY sex reversal and hypospadias [13,14]. Even more interesting are recent reports that highlight the importance of regulatory regions in the proper expression of genes within the developing gonad. Deletions and duplications in the regulatory region of *SOX3, SOX9, DAX1,* and *GATA4* can result in misregulation (either mislocalized, over- or underexpressed) of a gene within the developing gonad resulting in a sex reversal phenotype [13–16].

The road to sequencing the entire human genome began long before the idea of sequencing all three billion base pairs even seemed feasible. The Human Genome project had its roots in an ambitious project to generate a complete linkage map of the human genome based on restriction fragment length polymorphisms (RFLP) in the early 1980s. The genetic map of the human genome paved the way for linkage analysis in large families to follow specific disease phenotypes looking for a gene with a large effect size. The first linkage study in 1984 was limited to the X chromosome and identified Factor IX for the X-linked trait, Hemophilia B [17].

Identifying disease linkage peaks and/or genes in the 1980s was no small task, in which mapping a single gene comprised a graduate student's thesis. Even in the mid-1980s, the sequencing technology had not advanced to the point where sequencing model organisms was a viable option, as sequencing reactions were run on large pulse field gels and manually read. This technique was both labor-intensive and prone to human error. Upon the development of fluorescent dideoxysequencing, miniaturization, and automation the nucleotide calling the Human Genome Project (HGP) was officially born. The HGP's goal was to generate a dense physical map of the human genome and to sequence 20 Mb of model organisms in 15 years, a goal that was ultimately dwarfed by the actual accomplishments of the HGP.

The availability of a dense physical map of the human genome allowed for linkage analysis and positional cloning efforts to identify a gene in which there were large families or multiple families with a well-phenotyped disorder of sex development. In 2001, *FOXL2* was identified as the causative gene in blepharophimosis, ptosis, epicanthus inversus (BPES) syndrome after linkage analysis and positional cloning [18,19]. The conservation of sex development pathways is highlighted by the fact that this same gene is a candidate for a nearly identical condition in XX male goats known as *polled/intersex* condition. This same approach has been used to identify novel genes in sex development, including Rspondin-1(*RSPO1*) in XX-Males with palmoplantar keratoderma with squamous cell carcinoma of skin and sex reversal [20] and in a family with 46, XY gonadal dysgenesis revealed *MAP3K1*[21,22] as an important signaling pathway in early sex determination. Interestingly, this same map kinase pathway was discovered using an analogous method in the mouse models, in which ENU mutagenesis generated a spontaneous mutant, which was mapped back to chromosome 17. Positional cloning identified *Map3K4* as the mutated gene and illuminated the genetic cause of an unexplained mouse model of sex determination, T-associated sex reversal (Tas) originally described in 1983 by Eva Eicher [23–25].

The sequencing of the human genome was completed in 2001, 10 years after the official start of the HGP, by two independent groups, the public international venture led by Eric Lander [26] and a private venture, led by Craig Venter [27]. These simultaneously published drafts of the human genome shed light on the true amount of genetic variation and paved the way for the advent of next generation sequencing technologies that are, as of 2011, aiming to sequence an entire genome for $, which would make the technology feasible for use in clinical diagnostics and in large-scale research projects for novel disease gene identification. The human genome project has revolutionized our knowledge about the variation within the human genome. The immense amount of data provided by genome sequencing has shed light not only on the huge number of common variants but also on the large number of rare, personal, and deleterious variants present in each of us [28].

The traditional linkage analysis approach and disease gene identification has become even more powerful as next generation sequencing can sequence an entire linkage peak that contains hundreds of genes. Instead of using *a priori* evidence to pick a gene out of hundreds to sequence, one can take an unbiased approached to identify the disease causing gene [29]. These unbiased approaches rely heavily on bioinformatic expertise to reliably identify causative gene variants and eliminate false positive signals. However, they will identify novel pathways and genes not previously described in the sex development literature. These novel discoveries will ultimately lead to a systems biology approach to understanding the genetic regulation behind the developmental choice of the bipotential gonad to become either a testis or an ovary.

4. The future of sex determination research

Strategies to identify sex development genes have evolved alongside genomic technologies. Next generation sequencing is just the beginning of a new chapter in personalized medicine and sex development research. Sequencing the entire exome, and even the whole genome, in unexplained cases of DSD will continue to enlighten and broaden our understanding about both normal testis and ovarian development and patients who have disorders of sex development. However, the future of sex determination research will not end in the identification of disease genes through sequencing of genomic DNA. Based on the hundreds of variants identified of "undetermined significance," identification of the disease-causing variant is difficult and reliant on functional data generated in *in vitro* or *in vivo* model systems. This technology will not only revolutionize how we identify novel genes, but also how we diagnose and treat patients in the clinical setting. From the research perspective, these tools provide an unprecedented view into the various layers of gene regulation in a highly specific and quantitative way that ultimately may provide better long-term management and treatments for patients with DSD.

Currently, targeted capture approaches can sequence all known genes for a specific condition, such as breast cancer [30] and hereditary hearing loss [31]. Providing a genetic diagnosis that explains the molecular defect in every patient presenting with DSD would be an invaluable tool to patients, clinicians, and to the research community. A personalized diagnosis would allow for clinicians to create standardized guidelines for the complex medical, surgical, and psychological management of patients with DSD.

As the costs of whole genome sequencing continue to drop, the cost-savings from targeting specific exons for sequencing will ultimately disappear. In such a scenario, whole genome or exome data could be targeted at the bioinformatics level, and one would analyze only a subset of genes relevant to the disease phenotype. The major benefit to only sequencing or analyzing a subset of the genomic material is that we limit our findings to genetic variants that are known to cause disease and can be functionally validated. This targeted approach removes the ethical quandary in which whole genome or exome sequencing may identify a genetic finding completely unrelated to the reason the patient came in for testing (i.e. identification of a *BRCA1* mutation in a newborn presenting with a 46, XX DSD). Furthermore, current ethical guidelines do not support

genetic testing of children for adult onset diseases, yet there exist no guidelines on inadvertent genetic diagnoses that might arise in reporting results from the whole exome analysis. To date, there is no research on whether releasing large amounts of semi-predictive genetic data is harmful to the patient, and therefore we caution overzealous use of whole genome or whole-exome analysis on a clinical and predictive basis. In absence of studies that examine the best methods to provide this complex genetic information and the effects of receiving large-scale genetic data, we recommend a more conservative approach, to limit the genes analyzed to those specific for the disease process in question. Despite these dilemmas, there is no question that next generation sequencing will revolutionize the diagnostic approach for patients presenting with genetic disorders. For patients with DSD, this molecular phenotyping will ultimately give rise to a greater understanding about the clinical outcomes in this groups of patients and inform our understanding of the genetics of gonadal determination.

The sequencing technologies reach far beyond variant discovery and diagnostics, as these technologies can query the entire transcriptome, epigenetic markers, chromosomal conformation, to name a few. Exploring the epigenome will unravel how methylation changes throughout development can affect differentiation and function of cells and tissues. Combining genomic and transcriptomic studies can identify genes that are subject to allele-specific cis- or trans- effects [32]. In the aftermath of the Human Genome Project, we are still discovering new exons, some of which are tissue and time specific, and introduce new domains into known genes [33]. Understanding the genetic and transcriptomic regulation within the developing gonad is a lofty goal, requiring well-conceived experiments that ultimately require intensive bioinformatic analysis to identify the relationships between genome, transcriptome, epigenome, or as some would say: the "interactome." These are only a few of the ways that sequencing technology has given us tools that early geneticists could only dream of.

While most novel sex determination genes are identified in human patients with DSD, much of our understanding of how sex determination genes interact within the developing gonad comes from well-designed studies in mouse models. Our understanding of genes and pathways important in gonadal determination will be elucidated from overexpression and knockout studies in inbred mice and other model organisms. The mouse model has the added benefit that it is a controlled environment in which each cell type within the heterogeneous gonad can be dissected and analyzed for their specific gene expression profile.

Several genes that when mutated can result in 46, XY GD have roles in sex determination that remain to be explained. One of these is Chromobox-2 (*CBX2*), which was first described in a knockout mouse model M33 and more recently found in a patient with 46, XY GD [34]. Interestingly, the chromobox gene family is a known regulator of gene expression, through chromatin and histone modification, and likely plays a role in programming and regulating the environmental niche that allows for proper sex development. The interaction of *CBX2* and other epigenetic regulators are likely to play a large role in controlling timing and level of expression of sex determination genes and repressing down the expression of the genes for the opposing sex. Ultimately, understanding the genome, transcriptome, and epigenome within the developing gonad will allow us to paint a comprehensive portrait of the interactions within the developing gonad. As we can better understand the complexity that has been built into the regulatory regions of the genome, we might be able to decipher how genetic elements of small effect sizes can contribute to fertility and reproductive health.

The synergistic use of mouse and human models has contributed greatly to our understanding of early sex determination. The fruits of the decades of research, both in identifying genes for DSD and genomic technology, have made it possible to provide a genetic diagnosis for the majority of patients with DSD and personalized management for the patient and his or her family based on the gene mutation. The human genome project has provided us with the tools and technology to query the human genome in ways that were never before possible. As the costs continue to decrease and bioinformatics analysis become more standardized, next generation sequencing will become an integrated part of the clinical diagnostic process for all patients. As we move forward, there are still remarkable discoveries to be made within the field of sex development, which will help us unravel the age-old question: why are men and women different?

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