Published in final edited form as:

Trends Endocrinol Metab. 2009 December ; 20(10): 471–476. doi:10.1016/j.tem.2009.06.007.

Systems biology asks new questions about sex differences

Arthur P. Arnold¹, Atila van Nas², and Aldons J. Lusis^{2,3}

¹Department of Physiological Science, University of California, Los Angeles

²Department of Human Genetics, University of California, Los Angeles

³Department of Microbiology, Immunology & Molecular Genetics; and Medicine, University of California, Los Angeles

Abstract

Females and males differ in physiology and in the incidence and progression of diseases. The sex-biased proximate factors causing sex differences in phenotype include direct effects of gonadal hormones and of genes represented unequally in the genome because of their X- or Y-linkage. Novel systems approaches have begun to assess the magnitude and character of sex differences in organization of gene networks on a genome-wide scale. These studies identify functionally related modules of genes that are co-expressed differently in males and females, and sites in the genome that regulate gene networks in a sex-specific manner. The measurement of the aggregate behavior of genes uncovers novel sex differences that can be related more effectively to susceptibility to disease.

Sex differences in the function of biological systems

Each of us has a sex, which has a profound influence on our daily lives, including our physiology and susceptibility to disease. Yet, biologists often assume that basic physiological processes are similar in females and males, as evidenced by the huge predominance of biomedical studies in which only one sex is studied, or in which the sex of the subject is not identified [1]. The sex of the experimental subject may often be considered a variable that only complicates the job of the researcher. However, unless the effect of sex is studied, we are at risk for misunderstanding the physiology of half of the population. Because most diseases show sex differences in incidence or progression, it is important to determine the factors that cause sex differences in phenotype and the magnitude and character of their impact [2].

To date, most studies of sex differences in physiology are “single gene” studies, in which a small number of biological factors or genes are manipulated to determine their effects on phenotype. Recently however, the revolution in systems biology has generated numerous analytical tools that allow, for the first time, an appreciation of the aggregate behavior of the

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Correspondence to: Arthur P. Arnold.

Contact information

Arthur P. Arnold, Department of Physiological Science, UCLA, 621 Charles Young Drive South, Los Angeles CA 90098-1606, 310-825-2169, fax 310-825-8081, Corresponding author: arnold@ucla.edu

Atila van Nas, Department of Human Genetics, David Geffen School of Medicine at UCLA, PO Box 957088, Los Angeles CA 90095, atila@ucla.edu

Aldons J. Lusis, UCLA Med-Cardio/Microbio, BOX 951679, 3730 MRL, Los Angeles, CA 90095-1679, 310-825-1359, fax 310-825-2957, jlusis@mednet.ucla.edu

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genome. Until now, systems biology has not impacted the study of sex differences, largely because single-gene investigators have been unaware of the methods and attitudes of systems biology. Systems biologists are also often unaware of the literature on sex differences, which has identified biological factors that cause sex differences in phenotype. This situation is changing with the publication of several recent papers that begin to ask how global patterns of gene expression differ in males and females, and how these differences might explain differential susceptibility to disease.

Traditional physiological and genetic approaches have led to a few simple conclusions about the origins of sex differences in mammals. First, all sex differences in phenotype are caused at the genetic level by the sexual imbalance in the number of X and Y chromosomes, which is the only consistent sex difference in zygotes (Figure 1) [3]. In mammals, the Y gene *Sry* is dominant because when expressed in the undifferentiated gonad, it induces testis formation. Thus, the male-specific *Sry* gene is responsible for setting up life-long sex differences in the type and pattern of secretion of gonadal steroid hormones, especially androgens, estrogens, and progestins. These hormones have permanent sex-specific differentiating effects on numerous tissues such as genitalia and the brain during development (called “organizational” hormonal effects), and cause many sex differences acutely in adulthood (called “activational” hormonal effects) (Figure 1) [4]. In addition to these hormonal effects, XX and XY cells differ in phenotype because of the differential action of X and Y genes within the cells themselves, called “direct sex chromosome effects.” [5]. These conclusions come from studies in which sex-specific hormonal or genetic factors have been manipulated, one or two at a time, to determine which tissue phenotypes are affected in a sex-specific manner.

The field of systems biology has seen significant advancements because of methods that allow the simultaneous measurement of expression of almost all of the genes in the genome. The analytical tools of systems biology empower the biologist to determine which gene networks are responsible for specific traits, which regions of the genome regulate specific networks, and how the genome is organized and has evolved in a sex-biased manner [6–9]. Thus, systems biology leads to questions about sex differences that traditional biologists never asked or could not answer.

How many genes are expressed at a different level in males and females?

How large are sex differences in gene expression?

The earliest microarray studies on non-gonadal tissues reported that only tens to hundreds of genes are expressed consistently at different levels in males and females [10–17]. Those studies usually involved only a few individuals and therefore lacked statistical power, especially when significance thresholds were corrected for measurement of many variables. When larger numbers of mice are studied (365 in one study [18]), the majority (50–75%) of genes are sex-biased (i.e., expressed at a different level in the two sexes) in tissues such as liver, fat, and muscle. Large numbers of mice are needed to detect sex differences because the differences are usually modest, with a mean difference of 8–9% among sex-biased genes in most tissues. Genes are rarely expressed more than two-fold higher in one sex. Physiologists might consider an 8% difference in gene expression almost trivial, but that opinion reflects the single-gene attitude. A sex difference of that magnitude in the majority of genes seems much more impressive, and might reflect fundamental sex differences in physiology. Sex appears to control more variance across the genome than other known variables, at least for tissues such as the liver, so that, for example, one can reliably discern the sex of the animal from the global pattern of gene expression [18].

Where do sex-biased genes reside in the genome?

The sex chromosomes are subject to sex-specific selection pressures, which has made these chromosomes unusual in the types of genes that they encode, and the degree of sex bias (sex difference) in gene expression [9,19,20]. Obviously Y genes are expressed only in males and are under pressure to increase male fitness, but are freed from the need to be compatible with female physiology. The X genes spend twice as much time during evolution in females than in males (because fathers pass their X chromosomes to daughters but not to sons), and thus must function well in females. However, the hemizygous exposure of individual X alleles in males (the effect of any X allele is revealed in males because there is no other X allele present) means that X alleles are eliminated if they seriously decrease male fitness. For these and other reasons, the X and Y chromosomes are not just a random assortment of genes [19]. Among the specializations of the X chromosome is that genes with sex-biased expression in liver, muscle and brain occur at higher frequency on the X chromosome than elsewhere in the genome [18, 21]. Sex-biased expression may evolve because the effect of an allele on fitness may be high in one sex but low in the other, a situation that then could lead to evolution of mechanisms to down-regulate expression in the latter sex only to reduce the negative effects on fitness. However, some autosomes also have regions with a disproportionate number of sexually dimorphic genes [18]. Although all sex differences stem ultimately from a sexual imbalance of X and Y genes, sex hormones and other important sex-biased proximate factors cause sexual bias in gene expression by acting directly on genes throughout the genome.

Are the same genes sex-biased in different tissues?

Each tissue expresses different sets of genes, although some genes are expressed in all tissues. In one study examining gene expression in skeletal muscle, liver, and adipose tissue, about 24% of actively expressed genes were expressed in all three tissues. However, only 8% of sexually dimorphic genes (those expressed significantly higher in one sex in at least one tissue) were dimorphic in all three tissues [18]. This comparison indicates that sex-biased genes do not overlap across tissues as much as sexually unbiased genes. In other words, genes common to all cells are generally less sexually biased than other genes. That makes sense if the selection pressures on the common genes are similar in males and females. Moreover, the transcription factors and regions of the genome regulating sex-biased genes differ among tissues, suggesting different pathways regulating sexual dimorphism in various tissues [18].

Are dynamic interactions among genes (gene networks) operating similarly in males and females, or does one sex possess sets of gene interactions lacking in the other?

When thousands of genes are measured in hundreds of mice, it is possible to correlate the expression of genes pairwise, and cluster genes that show similar patterns of co-expression across individuals [22]. These clusters, or modules in the gene co-expression networks, represent groups of genes that are either driving the expression of each other, or are responding similarly to regulatory factors [23]. Because this analysis often involves measuring individual genes averaged over all cell types in a tissue, the genes that comprise a module need not be in the same cells (and hence may not directly interact), but nevertheless represent a group with important common functional properties. The identification of modules also represents a scheme that reduces the complexity of gene expression data based on biological relationships among genes that emerge from the expression data themselves rather than from the predispositions of the investigator [24]. It is therefore possible to study the coherent behavior of relatively small numbers of modules rather than tracking the noisy behavior of tens of thousands of individual genes. Reducing the number of variables also increases the statistical

power of the analysis and enhances the ability to relate patterns of gene co-expression to variability in traits or in the genome itself.

Do male and female tissues have the same co-expression modules, suggesting that the general organization of tissues is similar in the two sexes? In a study of four mouse tissues (brain, liver, skeletal muscle, and adipose tissue), the answer is “yes” [25]. When gene co-expression network modules were constructed separately for male and female tissues, most modules in one sex were similar in gene composition to one or two modules in the other sex. Because male and female tissue share 95% of their genomes (i.e., they differ in copy number of X and Y genes only), gene expression networks are quite similar. Another indication of overall similarity comes from the comparison of the connectivity of genes within modules. The network connectivity of a gene is a measure of the sum of connection strengths of the gene, which are the pairwise correlations between the level of expression of a gene and expression of each other gene across animals. The connectivity of a gene in male networks generally correlates well with its connectivity in female networks [25]. Thus, it is possible to find evidence that genes in specific functional modules are roughly similar in males and females, and that these genes have similar details of interactions with other genes as revealed by measures such as the degree of connectivity. It would be surprising if functional gene networks were not grossly similar in the two sexes within any one species.

Despite this generalized sexual similarity, measures of global patterns of gene expression have identified groups of genes that are the exception to the rule [25]. For example, one gene module in liver (“cyan module”) showed unusual sexual inequality in (1) the level of expression (higher in males) and (2) in connectivity (more connected in males), and was also (3) enriched in genes that are regulated by the level of androgens in adult mice (Figure 2 and Figure 3). Thus, viewing the group behavior of genes from three orthogonal perspectives uncovered a heretofore unknown but functionally related group of sexually biased liver genes regulated by androgens (Figure 2 and Figure 3). In future studies, this male-biased gene module can be explored further to determine its behavior (e.g., changes in pattern of expression) in different physiological or disease states and its sex-biased regulation by the genome and/or environment.

Once gene network modules are identified, their behavior (eigenvalues of gene expression [24]) can be correlated with specific traits related to physiology or disease. For example, the expression of genes in the “green” module in adipose tissue (Figure 2) was strongly correlated with the amount of body fat in female mice but uncorrelated with that in males [25]. In liver, the “blue” module was well correlated with plasma glucose and free fatty acid levels in females but not in males. These results indicate that systems variables can be used to discern group behavior of gene modules that are sex-biased or sex-specific in a manner that would not be possible using more traditional single-gene studies.

An important goal is to identify the genetic and environmental factors that influence the behavior of such a sex-biased module and account for the sex specificity in its relation to phenotype. One approach is to find quantitative trait loci (QTL) (Box 1) at which genetic variation is correlated with variation in expression of genes in sexually biased modules [25, 26]. Interestingly, QTL analysis shows that some regions of the genome perturb networks in a sex-biased manner. For example, changes in expression of cyan module genes in mouse liver (Figure 2) is caused by genetic variation at a locus on the X chromosome in males, but not in females. For the blue module in adipose tissue, expression QTL hotspots were found on chromosomes 1 and 7 in females but on chromosomes 8 and 11 in males. We envision further studies to identify genes at these hotspots that have sex-biased effects on networks, to explain how the sex-specificity is achieved, and how those sex differences impact disease.

Are most sex differences caused by hormones or by the direct effects of sex chromosome genes?

The first answer to this question came from a study comparing sexual dimorphism in global liver gene expression under different hormonal conditions, and from comparisons of mice that are XX or XY independent of their gonadal status [25]. One dramatic finding was that almost all sex differences in liver gene expression were abolished by gonadectomy of adult mice. This finding indicates that quantitatively, the most important factors causing sex differences are the “activational” or acute differences in effects of ovarian vs. testicular hormones. Importantly, some sex differences remain, which can be attributed to long-lasting (“organizational”) effects of gonadal secretions that acted prior to gonadectomy, or to constitutive tissue-autonomous differences in effects of XX vs. XY genomes [5]. However, the direct effects of XX vs. XY genomes (“sex chromosome effects”) (Figure 1) also appear to be modest, because at least in mice gonadectomized as adults, only a few genes were consistently different in XX vs. XY mice that had the same type of gonad [25]. When gonadectomized mice were treated with estradiol or with the androgen dihydrotestosterone (DHT), the effects of DHT were much more dramatic in terms of numbers of genes affected. Thus, a tentative conclusion is that androgens (or testicular secretions) might be more important globally for causing sex differences in liver function than estrogens.

How do classic endocrine manipulations (e.g., gonadectomy and hormone replacement), which eliminate sex differences, impact the organization of gene networks?

No study has yet measured perturbations in gene networks by controlled manipulations of the proximate factors that cause sex differences, i.e. the activational and organizational effects of gonadal hormones and the direct effects of X and Y genes. One reason is cost; gene co-expression network studies require large numbers of mice in each group (>50). Therefore to date, the known effects of hormones on the gene network comes from comparing sets of genes whose expression is influenced by hormones with the identified gene modules found in males and females [25]. The salient example is the cyan module in the mouse liver (Figure 2 and Figure 3), which shows male bias in expression and connectivity, and is also enriched for genes that respond to DHT. Although the identification of the cyan module is a step forward in understanding the effects of androgens on liver function, much more information is needed to determine how the cyan module behaves in males and females, and how it is influenced by organizational and activation effects of androgens, or by other factors.

Are genes and gene networks that are sexually dimorphic the same across species?

The first extensive comparison across species of global sex differences in gene expression comes from recent microarray analysis of whole body mRNA in seven species of *Drosophila* [27,28]. These species diverged from each other at different times in evolution, so that the change in sexual dimorphism can be studied as a function of degree of relationship between species. Interestingly, male-biased genes are more prevalent in most *Drosophila* species than female-biased genes. Genes with sexual bias tend to change more rapidly in the course of evolution than those that lack bias [9]. Sexual bias of genes also shows some conservation across *Drosophila* species [28] (also in the primate brain [17]), but during the course of evolution the set of sexually biased genes progressively changes [28]. Rarely, however, do genes flip their bias (e.g., male-biased becoming female-biased) even over long evolutionary distance among *Drosophila*. A major conclusion then is that there does not appear

to be a set of genes consistently prone to sexual bias because they can tolerate such bias more than other genes. In *Drosophila*, male-biased genes tend to be more species-specific than female biased genes, suggesting that male biased genes have a higher effective rate of birth and extinction.

What will systems approaches tell us about sex bias in the future?

The primary attitude of systems biology is that complex biological systems show emergent properties that cannot be predicted from the analysis of the component parts of the system. Patterns of behavior of the components emerge from analysis of coordinated changes in the system [7,23,29–34]. In practice, systems biologists build up a series of orthogonal analyses that yield different perspectives on the function of the system, and the combination of these perspectives yields new insights.

To understand sex differences at any systems level (cell, tissue, individual), it is important to begin to assemble the orthogonal systems analyses. The first steps have been taken and have yielded exciting results. These baby steps forward, however, must now be augmented by taking the findings at the systems level back to the single gene level to determine which manipulations of individual sex-biased factors are responsible for sex differences in physiology. Ultimately, it will be a combination of single-factor manipulations and systems-level analysis that will yield better understanding of sex differences in physiological networks affecting disease.

Box 1. QTL mapping

A quantitative trait, such as the amount of body fat, varies continuously rather than discretely. Such traits are often polygenic in that they are influenced by the combined action of many genes. Variations at specific loci in the genome can be measured by a variety of methods, such as microarray assessment of single nucleotide polymorphisms (SNPs). Other methods include microsatellite mapping, which is a PCR-based method to measure the length of polymorphic repeat sequences at specific regions in the genome. These signatures of the genotype at specific loci are called genetic “markers”. It is possible to map regions of the genome that cause variations in traits in a group of animals or humans by measuring both the marker genotype (SNP or microsatellite) at many loci across the genome, and the trait itself. The genotype of a small number of genomic loci (called quantitative trait loci, or QTLs) may be found to correlate with the trait, indicating that some variation in the genome near those markers has a causal influence on the trait. Expression QTLs (eQTLs) control the expression of specific genes, and eQTL hotspots are regions of the genome with an unusual concentration of eQTLs for genes in a specific gene co-expression module. The power of QTL mapping to narrow in on a critical genomic region increases with the number of individuals studied, and the number of markers mapped.

Acknowledgments

Supported by NIH grants HL28481, DIC 071673, NS045966, NS043196, DK72206 and HD07228.

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Figure 1.

Genetic and proximate factors causing sex differences in phenotype. All sex differences in phenotype arise at the genetic level because of the sexual imbalance of genes encoded on the sex chromosomes. The expression of *Sry* in the undifferentiated male gonad causes differentiation of the testes, which sets up lifelong sex differences in the secretion of gonadal sex steroid hormones. These hormones have two types of effects, permanent differentiating ("organizational") effects on genitalia, brain, and perhaps other tissues, and differences in the acute ("activational") effects of ovarian and testicular hormones. In addition, *Sry* and other X and Y genes have sex-biased differential effects ("sex chromosome effects") within non-gonadal cells that differ between the two sexes [4,5].

Figure 2.

Two orthogonal views of modules of co-expressed genes leads to identification of a new class of sex-biased genes. Genome-wide microarray analysis of liver gene co-expression leads to the identification of several groups (modules) of genes that show correlated expression across mice [25]. Individual dots represent genes in two modules, colored cyan and green, graphed according to two of their attributes. The difference in expression of each gene in males vs. females is indicated on the Y axis as the t-test statistic comparing male and female, with positive t-scores indicating higher expression in males. Thresholds for significantly different expression in males and females are shown by the horizontal lines, so that sectors 1–3 show higher expression in males, and sectors 5–7 show higher expression in females. The difference in connectivity of the genes within gene networks is shown on the X axis as the difference (in females vs. males) in the connectivity statistic k , which is a function of the summed pairwise correlations of the expression level of that gene with expression of each other gene across

animals [22]. Thresholds for significance are shown by the vertical lines. Greater connectivity in males (sectors 1, 7, and 8) results in negative k difference scores, and greater connectivity in females gives positive k difference scores (sectors 3–5). This two-dimensional analysis reveals a previously unknown subset of cyan module genes in sector 1 that are expressed higher in males and are better connected in males. The cyan module is unusually enriched in genes whose expression is sensitive to androgen levels in adults [25]. The expression of the green module is strongly correlated with the amount of body fat in female mice but uncorrelated with that in males. This combined use of several orthogonal analyses therefore leads to the identification of groups of genes with similar response to sex-specific factors.

Figure 3.

Gene co-expression analysis shows sex differences in gene networks in adult mouse liver. The figure is a simplified visual summary of sex differences in the organization of the cyan module of gene expression in liver of (a) females and (b) males. Genes (nodes) in the network are represented as dots, and the correlation between expression of genes is represented as lines (network edges). Large dots indicate genes with more than 40 connections in the module, and smaller dots genes with fewer than 40 connections. Modules are defined in females and compared to males. Most other modules defined based on co-expression in females are also found in males. In the liver cyan module, however, a significant number of genes were more connected in males (more large dots) and showed higher expression in males (Figure 2). Other data indicates that expression of a disproportionate number of cyan module genes was sensitive to the level of androgen [25]. Copyright 2009, The Endocrine Society.