Genomic Imprinting and Environmental Disease Susceptibility

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Genomic imprinting is one of the most intriguing subtleties of modern genetics. The term "imprinting" refers to parent-of-origin-dependent gene expression. The presence of imprinted genes can cause cells with a full parental complement of functional autosomal genes to specifically express one allele but not the other, resulting in monoallelic expression of the imprinted loci. Genomic imprinting plays a critical role in fetal growth and behavioral development, and it is regulated by DNA methylation and chromatin structure. This paper summarizes the Genomic Imprinting and Environmental Disease Susceptibility Conference held 8–10 October 1998 at Duke University, Durham, North Carolina. The conference focused on the importance of genomic imprinting in determining susceptibility to environmentally induced diseases. Conference topics included rationales for imprinting: parental antagonism and speciation; methods for imprinted gene identification: allelic message display and monochromosomal mouse/human hybrids; properties of the imprinted gene cluster human 11p15.5 and mouse distal 7; the epigenetics of X-chromosome inactivation; variability in imprinting: imprint erasure, non-Mendelian inheritance ratios, and polymorphic imprinting; imprinting and behavior: genetics of bipolar disorder, imprinting in Turner syndrome, and imprinting in brain development and social behavior; and aberrant methylation: methylation and chromatin structure, methylation and estrogen exposure, methylation of tumor-suppressor genes, and cancer susceptibility. Environmental factors are capable of causing epigenetic changes in DNA that can potentially alter imprint gene expression and that can result in genetic diseases including cancer and behavioral disorders. Understanding the contribution of imprinting to the regulation of gene expression will be an important step in evaluating environmental influences on human health and disease. *Key words*: **environment, epigenetics, genetics, genomic imprinting, methylation.** *Environ Health Perspect* **108:271–278 (2000). [Online 7 February 2000]**
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The Genomic Imprinting and Environmental Disease Susceptibility Conference held 8–10 October 1998 on the campus of Duke University in Durham, North Carolina, convened to discuss one of the most exciting and promising areas of ongoing research related to medical genetics. The National Institute of Environmental Health Sciences (Research Triangle Park, NC) and Duke University Medical Center's Department of Radiation Oncology and Integrated Toxicology Program joined forces to organize and promote this conference. The conference created the opportunity for information exchange between researchers working in the field of genomic imprinting, and brought together an interdisciplinary group of scientists to explore the relationship between genomic imprinting and environmentally induced disease.

In the near future, developments in medical genetics are expected to impact greatly on therapeutic biotechnology, the practice of healthcare and medicine, and the understanding of human evolution and behavior. As our knowledge in these areas continues to expand, it will be critical to explore the links between genetics and the environment in influencing human health and disease. Thus, the topic of this conference is both timely and of crucial value.

The conference objective was to discuss one of the most intriguing subtleties of modern genetics: namely, the fact that although cells have a full parental complement of autosomal genes, not all of those genes are biallelically expressed. This parent-of-origin-dependent gene expression, termed genomic imprinting, plays a critical role in fetal growth and behavioral development. Genomic imprinting is regulated by DNA methylation and chromatin structure. Thus, environmental factors capable of causing epigenetic changes in DNA can potentially alter imprint gene expression and result in genetic diseases that include cancer and behavioral disorders. This conference focused on the importance of genomic imprinting in determining susceptibility to environmentally induced diseases.

This paper is a summary of the Genomic Imprinting and Environmental Disease Susceptibility Conference. It is not meant to be an inclusive record of all of the material discussed or presented at the conference. Rather, it is intended to communicate a sense of the great current interest in genomic imprinting and the immense promise that this field holds for enhancing our understanding of how environmental factors affect disease susceptibility.

Genomic Imprinting: Why Bother?

The evolution of genomic imprinting. Genomic imprinting can be defined as the influence of the past environment of a gene on its expression. This influence can extend beyond the gene's parent of origin to actual experiences of an individual gene carrier, such as famine, disease, or chemical exposure. Molecular biologists and geneticists address the mechanism of genomic imprinting, but evolutionary biologists attempt to ask questions from within a theoretical framework about the value of imprinting: that is to say, "Why bother?"

A basic tenet of evolutionary biology is that genetic actions in one individual can affect other individuals. Some genes only affect an individual, but some genes affect their relatives as well. Most relatives are asymmetrically related on either the maternal side or the paternal side. Only individuals and their direct descendants belong equally to the individual's matriline and patriline. Therefore, conflicts can occur between effects of the genome on either the maternal or the paternal side (*1*).

Imprinting is favored when genes have parentally antagonistic effects. Examples of situations that cause such antagonism include postzygotic maternal care with multiple paternity, or sex-biased dispersal (usually male biased). In these circumstances, a change in reproductive partners creates paternal demands and antagonistic effects on the fetus and results in a bias in the social group. In patterns of male-bias dispersal, the coefficient of paternal relatedness within a pedigree decreases relative to the more constant coefficient of maternal relatedness. Thus, the social group is composed of individuals who are more related to each other on the maternal side than on the paternal side. As a result, the fitness effects of the maternal genome can be

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equivalent for the self and the mother but not for the self and the father. In this scenario, it can be argued, based on evolutionary models, that selection on the maternal genome will account for social behavior, and the maternal genome will favor genes for social behavior more than genes for selfish behavior. A similar selective process will not act on the paternal genome. Thus, change in sexual partners selects for a smaller effect of the male genome on social behavior.

Genes on a paternal X chromosome are an exception to this rule. All autosomes have a 50% chance of transmission to daughters and sons. In contrast, the paternal X is transmitted only to daughters, and sons carry only a maternal X. Because all females and no males carry a paternal X, there is a bias in favor of the presence of social behavior genes on the paternal X.

These evolutionary models explain certain effects *in utero* and also predict that genes affecting adult behavior should show imprinting. In addition, it is possible to hypothesize a role for imprinting in response to environmental experience or signals. A study of pregnancy-induced hypertension provides support for this line of thinking (*2*). In this study, the level of pregnancy-induced hypertension was determined and correlated with the duration of the relationship between the mother and father. When conception occurred during the first 4 months of a relationship, the incidence of pregnancy-induced hypertension was 30%. As the duration of the relationship between mother and father before conception increased to 12 months, the incidence of pregnancy-induced hypertension decreased 10-fold. If a subsequent partner change occurred, the incidence returned to a high level during the first 4 months of the new relationship before decreasing again. Environmentally responsive imprinting of the paternal genome is a potential mechanism to explain these results.

Imprinting and speciation. Imprinting has not been observed in yeast, *Drosophila melanogaster*, or *Caenorhabditis elegans*. It is possible that imprinting is important for mammals and that the study of imprinting in mammals is also important. If yeast, flies, and worms exist without imprinting, then it is important to ask, "What is the function of imprinting?"

Imprinted genes regulate and control growth during development (*3*). In mice, defects in genomic imprinting alter the adult size or the birth size of mice. When imprinting patterns are changed, growth patterns change. According to evolutionary biology theory, imprinting may arise when there is a parent/offspring conflict. Several predictions can be developed from this concept, including the prediction that imprinting might be absent in a monogamous species.

To explore imprinting in a monogamous mammal, two closely related rodent species were identified, one of which is monogamous and one of which is polygamous. Hybrids of *Peromyscus polionotus* (monogamous species) and *Peromyscus maniculatus* (polygamous species) demonstrate dramatic parent-of-origin-dependent growth effects. The mean body weight of adult animals of each species is approximately 18 g at day 45. The F_1 progeny of a cross between *P. polionotus* and *P. maniculatus* are either abnormally large or abnormally small, depending on whether the mother or the father is *P. polionotus*. When the mother is *P. maniculatus*, the F_1 progeny are only approximately 10 g, but they are viable. When the mother is *P. polionotus*, the F_1 progeny are extremely large and usually die. The placental size of the hybrid animals varies to an even larger extent than the animals' size, with differences of up to 5-fold.

Several hypotheses are possible to explain the characteristics of these F_1 hybrids. One hypothesis is that the polygamous rodent species carries imprinted genes that promote and retard growth, but the monogamous species does not imprint the analogous genes. The hybrids would then become biallelic for growth promoters or retarders in a parent-of-origin-dependent manner. This hypothesis was not substantiated, because both the monogamous and polygamous species of *Peromyscus* imprint growth regulatory genes such as mannose 6-phosphate/insulin-like growth factor 2 receptor (*M6p/Igf2r*), insulin-like growth factor 2 (*Igf2*), and *H19*. Another hypothesis is that the hybrids do not imprint properly. Imprinting was examined in the parental species and hybrid animals for several genes including *Igf2, H19,* small nuclear ribonucleoprotein-associated polypeptide n (*Snrpn*), *M6p/Igf2r,* paternally expressed gene 1/mesoderm-specific transcript (*Peg1/Mest*), paternally expressed gene 3 (*Peg3*), growth factor receptor-bound protein 10 (*Grb10*), and mouse achaete-scute homolog 2 (*Mash2*). Although *Igf2* is imprinted properly in the hybrid, the other genes show disrupted imprinting in the hybrid. For example, *Peg3*, *Snrpn,* and *Mest* are monoallelically expressed in the small hybrid but biallelically expressed in the large hybrid, leading to overexpression of paternal genes. *H19*, which is maternally expressed, is also monoallelically expressed in the small hybrid and biallelically expressed in the large hybrid. In contrast, maternally expressed *M6p/Igf2r* is biallelically expressed in the small hybrid and monoallelically expressed in the large hybrid. Thus, the hypothesis that the hybrids would show disruption of imprinting was substantiated, suggesting that imprinting signals may not be reciprocally recognized from one species to the other.

To confirm that improper imprinting might be directly involved in the growth defects of the hybrid animals, either adult body weight or placental size was scored and correlated with polymorphisms near the imprinted genes. The results suggest that at least one gene that maps in the vicinity of the imprinted gene *Peg3*, and another gene that maps on the X chromosome near the zinc finger protein X-linked (*Zfx*) gene, may be directly involved in the oversized phenotype in the hybrids.

These results raise the possibility that genomic imprinting may play a role in speciation. Speciation is often described as developing out of allelic incompatibilities that are promoted by geographic isolation of closely related populations. Because allelic incompatibility is diminished through heterozygosity in each of two diverging populations, imprinting can accelerate speciation by counteracting this effect. In particular, incompatibility between paternally expressed imprinted genes and X-linked genes (always monoallelically expressed) may be observed. This is a novel and potentially important function for genomic imprinting in mammals.

Imprinted Gene Identification

The study of imprinted genes has accelerated rapidly in recent years, in part because of the increasing number of methods available to identify imprinted genes.

Screening for imprinted genes by allelic message display. Allelic message display (AMD) is a reverse transcriptase-polymerase chain reaction (RT-PCR)-based method derived from the well-established method called differential display PCR (DDPCR). Although DDPCR is used primarily to compare the expression of many gene sequences in different stages or different physiologic states, AMD simultaneously compares the expression status of multiple polymorphic transcripts (i.e., transcribed alleles) in two parental mouse strains, reciprocal F1 hybrids and pooled backcross progeny. Paternally and maternally expressed transcripts can be unequivocally identified from the displayed pattern. The screening can be accelerated using a fluorescence-based message display system as well as a set of mice that share identical mitochondrial DNA generated by nuclear transplantation.

AMD was applied in a search for imprinted mouse genes using two parental mouse strains, B6 and JF1. More than 1,000 polymorphic transcripts were compared, and three potential imprinted genes were identified. One such gene is imprinted ancient (*Impact*). There are homologues of *Impact* in the human, yeast, and bacterial genomes; it is an ancient imprinted gene. In the mouse, *Impact* maps to chromosome 18A2-B2,

thereby providing the first imprinted gene on this chromosome.

In contrast to previous screening methods requiring positional cloning or androgenetic and parthenogenetic embryos, AMD is much less labor intensive and can be applied to any tissue or developmental stage.

A novel system for the detection of human imprinted genes. Another approach to identify imprinted genes uses monochromosomal mouse/human hybrid cells. A study demonstrating the potential of this approach was carried out using A9 mouse cells and a human fibroblast cell line carrying a selectable marker (*4*). In this approach, micronuclei are formed from the human cells, fused with A9 cells, selected for the marker, and then analyzed for chromosome content. The parent of origin of the human chromosome in the hybrid is then identified by PCR analysis using known repeat polymorphisms. Paired cell lines carrying either maternal or paternal specific chromosomes can be generated and maintained.

Using known imprinted genes, it was established that a human imprinting signal is functional and can be maintained in the A9 cell while it is passaged in culture. The methylation status of human DNA is also maintained in the mouse A9 cell. In contrast, the imprinting status of human DNA is not maintained when a human chromosome is transferred into an undifferentiated mouse embryonic stem (ES) cell. The expression status of the human chromosome can be further altered if the ES cell is induced to differentiate. This result demonstrates that imprinting signals function in a tissue-specific context, and that the imprinting mark can be remembered through many cell generations, and even through cycles in which it is alternately repressed or derepressed.

To demonstrate the usefulness of the monochromosomal hybrid lines, expressed sequence tag (EST) expression analysis was carried out using hybrid cell lines with maternal or paternal human chromosomes 11 and 15. Of 190 ESTs screened, 6 new imprinted genes were identified.

Human 11p15.5 and Mouse Distal 7: Analysis of an Imprinted Gene Cluster

Control of fetal growth by genomic imprinting. A large and relatively well-characterized region of human chromosome 11 at 11p15.5 carries a cluster of 9–10 imprinted genes that are interspersed with several nonimprinted genes. Distal mouse chromosome 7 has a related cluster of imprinted genes. Many characteristics of the region are conserved between the human and mouse genomes. This region plays a crucial role in growth regulation, particularly during fetal development *in utero*. Coordinate regulation of these genes has been demonstrated, and functional relationships among them are likely (*5*).

IGF2 and *H19* are adjacent genes in this cluster that are coordinately but reciprocally imprinted. *IGF2* is expressed from the paternal chromosome, and *H19* is expressed from the maternal chromosome. The expression of *H19* and *IGF2* are regulated by several endoderm-specific enhancers that lie downstream of the *H19* coding region. Sequences that regulate mesodermal expression are expected to be present. The promoter of *H19* is methylated on the silent paternal allele and unmethylated on the active maternal allele. There are signals required to maintain *H19* in a methylated state, including a differentially methylated region element in the 5′ promoter region of the gene. The mechanism that causes this element to direct methylation only to the paternal chromosome is not yet clear.

Studies of the expression and function of *Igf2* were undertaken using mice that carry mutations in or near the *Igf2* gene. One allele, called minute (*Mnt*), with a mutation made by radiation that is tightly linked to *Igf2*, causes a small growth phenotype when it is paternally transmitted. In an *Mnt* mouse receiving a paternal copy of the deleted chromosome, endodermal expression of *Igf2* is maintained, but mesodermal expression is absent. In contrast, with maternal transmission of the mutated chromosome, *H19* expression is absent from both endoderm and mesoderm. Thus the mutated chromosome is deficient in the regulation of mesodermal expression and is also deficient in the imprinting of the *H19* locus. The mutation may include a mesoderm-specific *Igf2* enhancer sequence and a second sequence that participates in methylation protection of the maternal *H19* allele. There is currently no evidence to distinguish whether these effects are mediated by two separate elements or by the same element.

Another *Igf2* mutant allele was generated to study the role of *Igf2* expression in the placenta. A single promoter region was identified that is active in placental tissue, and a deletion in this region was generated by homologous recombination. The function of this region in placental expression and growth was confirmed by the fact that mice receiving this allele can achieve normal size despite small size at birth. This effect requires postnatal *Igf2* function.

Genomic imprinting: epigenetic variation, epimutation, environment, and cancer. Wilms tumor pathogenesis is frequently associated with chromosome aberration events in the human 11p15.5 imprinted domain (*WT2*). Frequent loss of heterozygosity (LOH) is observed in Wilms tumor patients, involving either loss of the maternal allele or duplication of the paternal allele. Deletions of the maternal chromosome cover a large area that often encompasses the entire imprinted domain; no subregional deletion events are found. This result raises the question of whether Wilms tumor *WT2* is a multigene locus, or whether there are one or a few genes within the region that can be specifically implicated in the Wilms tumor phenotype.

In Wilms tumor patients, *IGF2* is activated and *H19* is inactivated. More than one mechanism can explain this expression pattern, including deletion, mitotic recombination leading to allele loss, and aberrant methylation (*6*). Aberrant methylation is a likely mechanism in tumors with no apparent lesion in the 11p15.5 region. In some of these cases, gene-specific hypermethylation of *H19* has been observed.

The 11p15.5 region includes the following imprinted genes, all of which can be considered as having a potential role in Wilms tumor development: imprinted in placenta and liver (*IPL*), imprinted multimembrane-spanning polyspecific transporter-like gene-1 (*IMPT1*), p57 kinase inhibitor protein 2 ($p57^{KIP2}$), potassium channel involved in Long QT syndrome (*KvLQT1*), target of an antiproliferative antibody (*TAPA1*), Achaete-Scute homolog 2 (*ASCH2*), *IGF2*, and *H19*. Feinberg's group also reports *TSSC3, TSSC5,* and *LIT1* as genes in the 11p15.5 imprinted domain. Throughout the domain encompassing these genes, variable imprinting effects are observed in both normal and Wilms tumor patients. The methylation patterns of *IPL* and *IMPT1* show no systematic change when *H19* hypermethylation is observed. Outside the domain, *ZNF195* remains unmethylated when *H19* becomes hypermethylated. *TAPA1*, which is imprinted weakly in the same direction as *H19*, is not silenced when *H19* is silenced by hypermethylation. Expression of $p57^{KIP2}$ is somewhat down modulated in Wilms tumor cells. Whereas $p5^{KIP27}$ mutation and methylation events are associated with Beckwith-Wiedemann syndrome (BWS), similar events are not found in Wilms tumor cells.

Although *H19* epimutation appears to be an early permissive event in Wilms tumor progression, it is not likely to be sufficient for tumor development. The epigenetic lesion may be restricted to *H19*, but the Wilms tumor phenotype affects the whole imprinted domain. A two-step model for Wilms tumor development is suggested. The first step causes the epigenetic alteration in the imprinted domain, and the second event, which is likely to be rate limiting, leads to the Wilms tumor phenotype.

BWS is a hereditary overgrowth syndrome associated with altered expression of genes in the imprinted cluster on human 11p15.5. This syndrome is often associated with predisposition to cancer and Wilms tumor. The BWS disease phenotype is correlated with loss of imprinting (loss of repression or gain of expression) in the 11p15.5 imprinted domain affecting $p57^{KIP2}$, *IGF2*, *H19*, and *KvLQT1*.

A hypothesis has also been advanced by Feinberg concerning the organization of the 11p15.5 imprinted domain into subdomains, in which genes are regulated in *cis* over a distance in a chromatin-structure-dependent manner. Six pieces of evidence are offered in support of this concept.

- A common genetic change occurs in cancer cells in which loss of imprinting affects several of the genes in the imprinted domain.
- The hereditary disorder BWS, which predisposes humans to cancer and causes prenatal overgrowth, involves frequent epigenetic alterations within this domain.
- Imprinting within this domain appears to be developmentally regulated.
- Both genetic alterations (mutations) and epigenetic alterations (DNA methylation) are found in cancer cells.
- A partial reversal of abnormal imprinting of both *IGF2* and *H19* can be achieved using 5-aza-2′-deoxycytidine, an inhibitor of DNA methylation.
- The chromosome 11 domain is itself organized into subdomains (the BWS/$p57^{KIP2}$ is distinct from the cancer/*IGF2*/*H19* domain and is separated by at least two newly isolated nonimprinted genes, *TSSC4* and *TSSC6*).

Together these observations have led to a proposed model of genomic imprinting as a dynamic developmental process involving a chromosomal domain. According to this model, cancer involves both genetic and epigenetic mechanisms affecting this imprinted domain and the genes within it (*7*).

A boundary region centromeric to the 11p15.5 imprinted domain has been identified. The boundary region separates it from adjacent nonimprinted genes. Within the boundary region, a repeat element was found that possesses a telomere-like silencing function in yeast. Similar regions or factors may mediate epigenetic silencing in diverse species.

X Chromosome Inactivation: A Paradigm for Understanding Epigenetic Controls

Xist *promoter switch mediates RNA stability at the initiation of random and imprinted X chromosome inactivation.* In mammals, the X chromosome is subject to a parent-of-origin independent epigenetic process known as X inactivation (*8*). By this process, the cell achieves dosage equivalence for the X chromosome in males and females. In females, one of the two X chromosomes is silenced. X inactivation is normally random and is stably maintained. X inactivation is a very early, developmentally regulated event that is under the control of a single master switch locus. The master switch locus is called X inactivation center (Xic), and is required in *cis* for X inactivation to proceed. A 15–17kb transcript called X inactive specific transcript (*Xist*), associated with the inactivated X chromosome, maps to the Xic region. *Xist* expression correlates with the onset of X inactivation. The transcript stays in the nucleus, has no known protein coding potential, and binds to the inactive X chromosome.

Xist expression was studied in mouse ES cells. When these cells are cultured without being stimulated to differentiate, both X chromosomes are active. When ES cells are induced to differentiate, X inactivation proceeds, providing a convenient system to study the process. A comparison of *Xist* transcript stability in ES and somatic cells demonstrates a large difference in the two cell types. Whereas the half-life of *Xist* is < 0.5 hr in ES cells, it is indefinitely stable in somatic cells. The stable and unstable forms of *Xist* initiate at different promoter sites. Stable transcript is produced from two promoter regions: the P1 promoter reported previously, and a second promoter, P2, located 1.5 kb downstream of P1. In contrast, a novel promoter, P0, located 6.5 kb upstream, was involved in the synthesis of the unstable *Xist* transcript. Thus, the switch from P0 transcription to P1/P2 transcription correlates with a change in transcript stability and the initiation of the inactivation process.

In mouse embryos *Xist* is specifically expressed from the paternal X from the 2–4 cell stage onward, whereas *Xist* begins to be produced from the maternal chromosome in the mid–late blastocyst stage. In early stages of the mouse embryogenesis, the maternal allele does not synthesize *Xist*, and the paternal allele, which is imprinted, functions from P1/P2 but not from the P0 promoter. At later embryonic stages, before a commitment occurs to inactivate either parental chromosome, P0 is active on both alleles. Commitment to chromosome inactivation correlates with the transcription switch from P0 to P1/P2 on one chromosome or the other. Preliminary data suggests that CpG island methylation in these promoter regions may contribute to their regulation during development.

Variability in Imprinting

Imprint erasure and non-Mendelian inheritance ratios. In addition to studying the mechanisms and consequences of imprinting, it is important to examine the consequences of the failure to imprint. As in other research areas, the exception, the mutation, or the process gone awry are often highly informative about the normal, wild-type, and fully functional process or component. It is often assumed that imprinted genes are invariably imprinted in all individuals in the human population. Although this can be observed in small groups of individuals, it may not be true in large populations.

Mendel's second law states that units of heredity segregate independently in each generation, resulting in an allelic inheritance ratio of 1:1. It is unlikely that the presence of epigenetic marks on chromosomes can affect the allelic inheritance ratio. In contrast, it is expected that the failure to properly establish epigenetic marks will affect allelic inheritance ratios. Consistent with this prediction, an *Arabidopsis* mutant that cannot properly methylate its DNA demonstrates a maternal-effect aberration. This mutant displays genome-wide hypomethylation and lacks any phenotypes, but a high level of segregation distortion is observed in its F1 offspring. If these mutant plants imprint aberrantly, as would be expected, then segregation distortion may occur in response to the failure to properly imprint its chromosomes.

The X chromosome in humans is subject to imprinting in certain regions (parent-of-origin-dependent epigenetic marking), and is also marked for X inactivation (parent-of-origin-independent epigenetic marking). The hypothesis that the failure to properly mark an X chromosome will cause segregation distortion leads to the following predictions: males who fail to properly mark their X chromosome will not have daughters, and females who fail to properly mark their X chromosome will not transmit their marked allele to sons.

To test these ideas, a large group of 47 families was studied for indications of segregation distortion for alleles on the X chromosome (*9*). Strong evidence of deviation from the expected Mendelian inheritance ratio of 1:1 was found at three X chromosome loci in this study (the androgen receptor, DXS1068, and DXS101). For example, in the region Xp11.4-p21.1, a bias in favor of transmission of the grandpaternal allele from mothers to sons was observed. Among the male offspring, an allelic inheritance ratio was observed as great as 1.6:1 in favor of the grandpaternal allele. Selective analysis of recombinant chromosomes confirmed that the allelic transmission bias maps to the specific loci identified.

This study suggests that variability in imprinting at the population level could be a statistically significant phenomenon. The interaction of genetic and environmental

factors in affecting the amount of this variability is an interesting area for exploration.

Genetic control of parental imprinting. Genomic imprinting modulates gene expression by providing the means to turn specific alleles of a gene on or off in a parent-of-origin-dependent manner. It is by design an inherently unstable mechanism of gene regulation. Both genetic and environmental factors can affect the imprinting process and alter the level of expression of imprinted genes. In addition to developmental and tissue-specific variation, imprinting can also be polymorphic among individuals.

One example of polymorphic imprinting involves the *M6P/IGF2R*. This membrane-associated receptor targets *IGF2* to the lysosome for degradation, binds and activates TGF-β (transforming growth factor-β), and is a key player in mediating growth regulation by *IGF2* and TGF-β. In the mouse, *M6p/Igf2r* is maternally expressed and is imprinted in all tissues except the brain. A more variable level of expression of the paternal allele is observed in human tissues. Many human fetal tissues show biallelic expression involving partial imprinting of the paternal chromosome, such that expression from the paternal allele is approximately one-half the level of expression from the maternal allele. Complete imprinting is observed only in approximately 10% of the fetal samples examined. In general, imprinting is absent by the completion of development: postnatal tissues and term placenta both show uniformly biallelic expression. Wilms tumor patients often show persistent imprinting of *M6P/IGF2R* both in the tumor and in the surrounding normal kidney tissue.

The *INS/IGF2/H19* region of chromosome 11 also shows variable patterns of imprinting in somatic tissue. In both mice and humans, *IGF2* is paternally expressed and maternally repressed during development. In contrast, freshly isolated human lymphocytes do not express *IGF2*. Stimulated lymphocytes show variable amounts of monoallelic or biallelic expression of *IGF2*; however, monoallelic expression is observed in < 10% of lymphocyte populations. The adjacent *H19* gene, whose imprinting is coregulated with *IGF2*, shows a corresponding change in expression: cells with up regulation of maternal *IGF2* lack *H19* transcripts. The pattern of *IGF2/H19* expression was consistent with lymphocytes from a given individual and showed familial clustering. However, attempts to identify genetically linked polymorphisms that correlate with a given *IGF2* expression pattern were not successful, suggesting the possibility that it is determined by a *trans*-acting regulatory mechanism.

Variable number of tandem repeats (*VNTR*) is a repeated sequence region composed of a 14-bp sequence adjacent to the insulin gene. Class I alleles of *VNTR* have a smaller number of repeats (up to 60), and class III alleles have a larger repeat number (from 120 up to several hundred). Class III alleles are correlated with expression of insulin (*INS*) in the thymus, which is protective against insulin-dependent diabetes mellitus (IDDM). In contrast, class I alleles predispose to IDDM. When the parental transmission of these alleles was examined, a bias toward maternal transmission of class I alleles was found, and it appears that the protective effect of class III alleles may require paternal transmission. Whereas class III *VNTR* alleles usually are associated with high expression of insulin in the thymus, this pattern may also have a parent-of-origin-dependent component. Although *INS* is not imprinted and is biallelically expressed in the pancreas, monoallelic expression of *INS* is seen at variable levels in human thymus samples (*2*). Thus, there may be a pattern of allele-dependent imprinting of *INS* in thymus tissue.

These examples demonstrate that imprinting is variable in human tissues and that it does not act as an all-or-none mechanism. The genetic variability within the human population, which is much higher than the genetic variability of the laboratory mouse, may be responsible for a portion of the observed variability in imprinting. However, environmental factors may be equally important in influencing the penetrance of imprinting both within an individual and within the human population.

Genomic Imprinting and Behavioral Development

Genetic studies of bipolar disorder: is there a parent-of-origin effect? Recent work has suggested that one or more regions of human chromosome 18 are involved in bipolar disorder and related depressive conditions (*10*). Patients with these syndromes have an episodic disorder characterized by manic and depressive episodes in addition to intervals without these episodes. Depressed or manic patients have symptoms that primarily affect their mood, vitality, and self-attitude. The severe form of the disease, bipolar I, occurs in approximately 1% of the population in the United States. A milder form of the illness, bipolar II, with less intense, briefer manic periods has recently been discovered. Its prevalence in the population is unknown but it was diagnosed more frequently than other phenotypes (bipolar I or recurrent major depression) in the families ascertained at Johns Hopkins University (Baltimore, MD) for one of the two large genome-wide studies. These genome scans of 70 and 96 families, respectively, have not established robust linkage to any loci, suggesting that the strong familial clustering of the illness relies in large part on multigenic mechanisms.

Recent clinical and linkage studies conducted independently in a National Institute of Mental Health (Bethesda, MD) collaboration and at Johns Hopkins focused on chromosome 18. Both studies suggest a parent-of-origin effect in multiplex bipolar families (with excess maternal transmission). In addition, linkage to chromosome 18 is also indicated in a subset of the families with paternal transmission of the phenotype. Several other multiplex samples show the same excess of maternal transmission. However, at least one consecutive series sample suggests that excess maternal transmission is seen in the multiplex families. It is not apparent in families studied without regard to the number of affected individuals in the family. Similarly, other studies demonstrate mixed results concerning linkage to chromosome 18 and whether it occurs primarily in families with paternal transmission. Further studies are needed to resolve the genetics of bipolar disorder and the possibility of a parent-of-origin effect.

Imprinting, the X chromosome, and the male brain. Turner syndrome is a rare disorder of females that causes social adjustment problems and short stature but normal intelligence in most individuals. X chromosome deletion or loss is frequent in Turner syndrome females. Thus, haploinsufficiency of genes that are not subject to X inactivation is thought to be one possible mechanism to account for many features of the Turner phenotype. Some findings suggest that imprinted loci may exist on both the paternal and maternal X chromosomes, and that these loci serve different functions in brain development (*11*).

Turner syndrome was studied in a group of monosomic females, approximately two-thirds of which carried the maternal X (45,X^m) and one-third of which carried the paternal X (45,X^p). All of these females showed slightly reduced nonverbal intelligence skills and normal verbal skills. Comparison of social adjustment skills indicated that the 45,X^m females showed twice as great an impairment. Specific neuropsychologic tests were conducted with samples of 46,XY males; 46,XX females; 45,X^m females; and 45,X^p females. Normal females performed better than normal males on a test of the ability to recall certain sorts of verbal material from memory, but 45,X^m females were significantly impaired relative to 45,X^p females. This observation is consistent with the hypothesis that the disorder is associated with an imprinted locus on the X chromosome at which the paternal allele is expressed and the maternal allele is silenced. Sex differences between normal males and

females may reflect the fact that in normal 46,XX females the imprinted locus on the paternal X chromosome would be expressed. In 46,XY males, whose single X is always maternal in origin, the locus would be silenced. On measures of social adjustment in which 45,X^m females perform less well than 45,X^p females, males performed less well than normal females, suggesting that the putative imprinted locus may predispose to sexual dimorphism in certain behavioral traits. The neuropsychologic correlates of these traits are currently under investigation. It is possible that sexual dimorphism in social communication abilities could be associated with greater male vulnerability to developmental disorders of language and social cognition such as autism, which is considerably more common among males than females.

Evidence also suggests that recall of a complex pattern was performed considerably better by 45,X^m females than by 45,X^p females. This visuospatial memory task was performed equally well by normal males and females. This suggests the possibility of an imprinted gene expressed only from the maternal X chromosome that is shared by normal males and females, and which corresponds to some aspect of visuospatial or motor memory. Both the verbal and the visuospatial memory functions mapped to two distinct areas of the brain in which structural deficits could be detected that may correspond to the functional deficiencies observed in the 45,X^m and the 45,X^p patients respectively.

Turner syndrome patients show alterations in cognition behavior that have a genetic parent-of-origin-dependent component. The altered behavior patterns in these patients may also relate to behavior differences that exist between normal females and males.

Genomic imprinting and brain evolution. Genomic imprinting has been implicated in diseases that affect behavior such as Angelman syndrome, Prader-Willi syndrome, bipolar disorder, and schizophrenia (*12*). Based on this observation, it seems possible that genomic imprinting plays a role in brain development. One means of investigating imprinting in the brain is to generate and study chimeric mouse embryos that include a fraction of androgenetic (Ag) or parthenogenetic (Pg) cells. These Ag and Pg chimeras have a much greater survival rate and are more useful than homogeneous Ag or Pg embryos. The Ag or Pg component of the chimera can be readily traced using a visible marker such as the *lacZ* gene, which allows for *in situ* chromogenic staining.

Ag and Pg cells were localized in the brain region of mouse chimeras by *lacZ* staining. Ag cells are enriched in the mediobasal forebrain, and in particular in the hypothalamus. By parturition, they are virtually absent from the telencephalic structures. In contrast, Pg cells are excluded from the hypothalamus, are not abundant in the brain stem, and accumulate in the regions from which Ag cells are excluded, especially the neocortex and striatum. These patterns are observed consistently, regardless of the relative proportion of normal/androgenetic or normal/parthenogenetic cells in the developing brain.

In brains with a significant Ag component, a relative decrease in size of the forebrain is observed, whereas in brains from Pg chimeras, the frontal area is larger than both normal brains and brains from Ag chimeras. Overall brain size is correlated to body weight, and it increases linearly within a single class of animals. The relative size of specific areas of the brain is variable in different organisms. The relative size of the frontal area of the brain (where Pg cells accumulate) increases from the insectivores to the prosimians and to the simians. The enlargement of this brain area, which is composed primarily of Pg cells, correlates with the development of social organization. The asymmetric distribution of the paternally and maternally derived genetic component in the brain suggests that the maternal genetic component may make a differentially greater contribution to social behavior than the paternal genetic component.

Methylation, Epigenetics, and the Environment

Chromatin condensation and DNA methylation during nickel carcinogenesis. The role of methylation in imprinting has been described extensively. Changes in methylation also occur in somatic cells in a nonparent-of-origin-dependent manner. In some studies, changes in DNA methylation have been induced by environmental agents and correlated with changes in chromatin structure, which may also contribute to the regulation of gene expression.

The relationship between methylation and chromatin structure was examined in studies of Chinese hamster ovary cells exposed to the carcinogen nickel (*13*). The insoluble crystalline forms of nickel metals are actively phagocytized, which can lead to an intracellular nickel concentration as high as 5 M. Transgenic cell lines carrying an insert of the guanine phosphoribosyl transferase (*gpt*) gene were exposed to nickel and tested for increased resistance to 6-thioguanine (loss of *gpt* function). Nickel exposure was mutagenic in a cell-line-specific manner, causing loss of gene function in cell line G12, but no similar effect in cell line G10. The G12 transgene is near a region of heterochromatin on chromosome 1, and the loss of function of the transgene was associated with increased methylation and increased heterochromatization of the G12 transgene, but not of the G10 transgene. Nevertheless, a decrease in global methylation is observed after nickel treatment, indicating the possibility that nickel exposure can induce site-specific *de novo* methylation in regions of heterochromatin.

The effect of nickel on chromatin structure was confirmed by studies in the yeast *Saccharomyces cerevisiae*. In yeast, gene silencing is achieved by mechanisms involving chromatin condensation but not cytosine methylation. Yeast exposed to nickel demonstrate site-specific effects on gene expression that depend on proximity to a heterochromatic region (i.e., the telomere-silencing element). This result suggests that nickel can directly affect chromatin structure under conditions where cytosine methylation does not also contribute to the extent of chromatin condensation.

CpG demethylation of the uterine* lactoferrin *gene in adult mice treated neonatally with diethylstilbestrol. Studies of the estrogen-regulated *lactoferrin* gene in the mouse uterus provide another example of an environmental influence on DNA methylation. Prenatal or neonatal exposure to the synthetic estrogen, diethylstilbestrol, can alter *lactoferrin* expression and lead to uterine tumors (*14*). This effect is mediated by diethylstilbestrol-induced changes in the methylation state of a *lactoferrin* promoter CpG site. However, it also requires that estrogen be present, because it does not occur in ovariectomized animals. Diethylstilbestrol exposure may have additional affects on CpG methylation in the mouse genome.

Liver microsomal *cytochrome P450* genes may also affect the physiologic and pathologic responses to xenochemical exposure. *Cytochrome P450* genes play a major role in metabolic detoxification/activation of xenochemicals and exhibit sexually dimorphic expression due to developmentally regulated promoter methylation.

Genomic Imprinting and Cancer

Aberrant methylation of tumor-suppressor genes: influence of environmental exposure. Allele-specific methylation is a well-established mechanism for genomic imprinting. In many cases, it has been definitively shown that methylation is required for allele-specific gene silencing. It is assumed that imprinting-associated methylation is a tightly regulated process; however, methylation has also been found in somatic tissue without an imprinting function. In particular, aberrantly methylated promoter-region CpG islands are

often found in cancer cells. Approximately 50% of all human genes have promoter-associated CpG islands that are usually maintained in an unmethylated state. Methylation of these CpG islands is a major mechanism for gene activation in cancer cells, including loci such as *HIC1* (hypermethylated in cancer 1), *p16*, *p15*, *VHL* (Von Hippel-Lindau), *IGF2*, *E-cadherin*, *ER* (estrogen receptor), O^6*MGMT* (O^6-methylguanine methyltransferase) and *MMR* (mismatch repair) genes.

The involvement of CpG island promoter methylation in the progression of lung cancer was explored by examining the methylation and expression status of tumor-suppressor genes in cells and tissues. Methylation and expression status were followed in cancers induced by exposures to various chemical and physical agents. Studies were conducted using NNK (4-methylnitrosamino-1-(3-pyridyl)-1-butanone)-induced and spontaneous mouse tumors; human tumors in smokers, nonsmokers, and radon-exposed individuals; and rat tumors induced by NNK, X rays, plutonium, beryllium, carbon black, or diesel exhaust.

The methylation status of the promoter of *p16*, a tumor-suppressor gene involved in cell-cycle regulation, was analyzed in the tissue of 18 primary rodent tumors and in cell lines derived from the tumors (*15*). Thirteen of 18 tumor tissues and 8 of 18 derived cell lines demonstrated aberrant methylation of the *p16* gene. By staging the cancer phenotype of different cells, it was established that *p16* methylation is an early event in carcinogenesis, and that an increase in the amount of aberrant *p16* methylation is associated with cancer progression.

From these data, it can be argued that the probability a patient has cancerous or precancerous lesions in the lung can be estimated by determining the methylation status of *p16* in lung tissue. This idea was tested by a blind analysis of human tissues. This study demonstrated that it was possible to predict the presence of cancerous or precancerous tissue by the extent of methylation of the *p16* promoter region (*15*).

For rodent tumors, various exposures can induce lung cancer by a mechanism involving *p16* promoter methylation. Tumors induced in rats by NNK, X rays, plutonium, beryllium, carbon black, or diesel exhaust all showed evidence of *p16* involvement. In contrast, when the methylation status of two other genes, *ER* and O^6*MGMT*, were examined in tumors induced by various agents, the correlation between methylation status and tumor incidence was less strong.

Promoter region hypermethylation and gene silencing in cancer. Cancer cells often demonstrate hypermethylation of promoter CpG islands and hypomethylation of structural genes and bulk chromatin (*16*). However, the functional significance of these methylation changes is not always clearly established, leaving open the possibility that the methylation or demethylation events are secondary to other changes. One test of the functional significance of methylation is to treat cells with 5-azacytidine. In a cell line with one unmethylated mutant and one methylated *WT p16* gene, 5-azacytidine induced demethylation of the *WT* gene and restored *p16* transcription and function to the cells. Thus, in this case, methylation functionally corresponds to transcriptional silencing.

Studies of the *E-cadherin* gene confirm that changes in its methylation status have functional significance and in some cases correlate with the gain of the transformed phenotype. *E-cadherin* is a tumor-suppressor gene that expresses a cell surface adhesion protein. The *E-cadherin* upstream promoter region has a CpG island and two methylated Alu sites. The regulatory regions also include an enhancer and 3 GC-rich regions near exon 1 that are protected from methylation. Cells that have progressed toward a cancer phenotype show changes in methylation in this region of the *E-cadherin* gene. Methylation analysis of several cell lines suggests a correlation among increased methylation, decreased expression, and cellular transformation. In addition, cells within a tumor mass generally demonstrate both heterogeneous expression of *E-cadherin* and heterogeneity in their metastatic potential, and these two parameters are correlated with one another. Because both metastasized and primary tumors are heterogeneous in these properties, expression of *E-cadherin* must be modulated during the metastatic process. It is possible that variability in *E-cadherin* expression is linked to metastatic progression. The observation that mutants of *E-cadherin* have altered metastatic potential also indicates an important role for *E-cadherin* in metastasis.

Genomic imprinting of* M6P/IGF2R *and cancer susceptibility. *M6P/IGF2R* is located near the tip of chromosome 6 at 6q26, and cancer-associated LOH often includes this region. LOH at the *M6P/IGF2R* locus is seen in approximately 65% of human HCCs (hepatocellular carcinomas), and gene-inactivating mutations are often present in the remaining allele. This is equally true for HCCs that develop in patients with or without chronic liver cirrhosis. *M6P/IGF2R* is also frequently inactivated in breast cancer and Wilms tumor, and because it contains a poly-G region, it is also an oncogenic target in gastric tumors, colon tumors, and endometrial tumors with mismatch repair deficiency. Thus, significant mutational evidence now supports the hypothesis that the *M6P/IGF2R* functions as a tumor-suppressor gene (*17*).

Dysplastic hepatic lesions, the earliest detectable liver tumor stage, show a 63% LOH, which is similar to that observed in HCCs. Furthermore, phenotypically normal hepatocytes adjacent to HCCs frequently possess a mutated *M6P/IGF2R* allele. Thus, *M6P/IGF2R* mutation is an early step in liver carcinogenesis. A model is suggested whereby hepatocytes with only a single functional *M6P/IGF2R* allele or haploinsufficiency preferentially regenerate in patients with chronic hepatic cirrhosis. The majority of HCCs then ultimately develop from these *M6P/IGF2R*-mutated premalignant cells. The tumor-suppressive effect of the *M6P/IGF2R* can now be directly tested because *Cre-LoxP*-conditional or tissue-specific *M6p/Igf2r* knockout mice have been produced, thus eliminating the embryonic lethality that occurs in *M6p/Igf2r* null mice.

Although mice are imprinted at the *M6p/Igf2r* locus, imprinting at this locus is a polymorphic trait in humans. Thus, mice may be at a higher risk for tumorigenesis than humans because of this species difference in imprinting of the *M6p/Igf2r* tumor suppressor. This possibility raises questions about the suitability of mice for human carcinogen risk assessment. It further suggests that transgenic mice with biallelic expression of the *M6p/Igf2r* gene may be more appropriate human surrogates than those presently employed in toxicologic testing.

Conclusion

It has been a long road from the first conceptualization of the gene as a hereditary unit to its modern molecular concept. As demonstrated by the discussion at the

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conference, one of the important subtleties of modern genetics is the process of imprinting. Imprinting, which allows for transient changes in expression of a gene without mutation in the gene, affects many genes that can have significant impact on development, behavior, and cancer susceptibility. Environmental effects on imprinting add one more layer of complexity to patterns of gene expression in humans and other mammals. Understanding gene expression at the level of the individual in all of its complexity will be important for the successful evaluation of the environmental influences on human health and disease.

References and Notes

1. Haig D. Parental antagonism, relatedness asymmetries, and genomic imprinting. Proc R Soc Lond B Biol Sci 264:1657–1662 (1997).
2. Vafiadis P, Bennett ST, Colle E, Grabs R, Goodyer CG, Polychronakos C. Imprinted and genotype-specific expression of genes at the IDDM2 locus in pancreas and leucocytes. J Autoimmun 9:397–403 (1996).
3. Tilghman SM. The sins of the fathers and mothers: genomic imprinting in mammalian development. Cell 96:185–193 (1999).
4. Mitsuya K, Meguro M, Sui H, Schulz TC, Kugoh H, Hamada H, Oshimura M. Epigenetic reprogramming of the human H19 gene in mouse embryonic cells does not erase the primary parental imprint. Genes Cells 3:245–255 (1998).
5. Reik W, Walter J. Imprinting mechanisms in mammals. Curr Opin Genet Dev 8:154–164 (1998).
6. Tycko B, Trasler J, Bestor T. Genomic imprinting: gametic mechanisms and somatic consequences. J Androl 18:480–486 (1997).
7. Cui H, Horon IL, Ohlsson R, Hamilton SR, Feinberg AP. Loss of imprinting in normal tissue of colorectal cancer patients with microsatellite instability. Nat Med 4:1276–1280 (1998).
8. Brockdorff N. The role of Xist in X-inactivation. Curr Opin Genet Dev 8:328–333 (1998).
9. Naumova AK, Leppert M, Barker DF, Morgan K, Sapienza C. Parental origin-dependent, male offspring-specific transmission-ratio distortion at loci on the human X chromosome. Am J Hum Genet 62:1493–1499 (1998).
10. MacKinnon DF, Jamison KR, DePaulo JR. Genetics of manic depressive illness. Annu Rev Neurosci 20:355–373 (1997).
11. Reilly SM, Skuse DH, Wolke D, Stevenson J. Oral-motor dysfunction in children who fail to thrive: organic or non-organic? Dev Med Child Neurol 41:115–122 (1999).
12. Keverne EB. Genomic imprinting in the brain. Curr Opin Neurobiol 7:463–468 (1997).
13. Lee YW, Broday L, Costa M. Effects of nickel on DNA methyltransferase activity and genomic DNA methylation levels. Mutat Res 415:213–218 (1998).
14. Li S, Washburn KA, Moore R, Uno T, Teng C, Newbold RR, McLachlan JA, Negishi M. Developmental exposure to diethylstilbestrol elicits demethylation of estrogen-responsive lactoferrin gene in mouse uterus. Cancer Res 57:4356–4359 (1997).
15. Swafford DS, Middleton SK, Palmisano WA, Nikula KJ, Tesfaigzi J, Baylin SB, Herman JG, Belinsky SA. Frequent aberrant methylation of p16INK4a in primary rat lung tumors. Mol Cell Biol 17:1366–1374 (1997).
16. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 72:141–196 (1998).
17. Jirtle RL. Genomic imprinting and cancer. Exp Cell Res 248:18–24 (1999).