Male factor still accounts for at least half of all cases of human infertility, suggesting that something like one in 20 of all men might be affected, and andrological analysis still indicates that greater than 50% of such cases are idiopathic (1). In about 15% of male infertility patients, genetic abnormalities may be detected, including chromosome rearrangements and single gene mutations. There is mounting evidence that infertility is a complex trait affected by many genes and the environment and may also involve polymorphisms as risk factors. At a time when assisted reproductive technology (ART) has now been used for 30 yr with over 1 million births, it may seem less important to understand completely the molecular basis of infertility, particularly where intracytoplasmic sperm injection (ICSI) is used to resolve the male factor. On the contrary, now that we know that ICSI can pass on a genetic defect from father to son (2), a better understanding of the genetic and epigenetic aspects of male infertility is urgently needed for both a better diagnosis as well as for counseling in regard to the passing on of genetic defects or infertility predisposition to the next generation. In humans, the identification of genes and mutations leading to male infertility is haphazard, and the mouse model has been our most powerful tool to systematically identify genes with a direct role in spermatogenesis with over 200 mouse mutants generated with an infertility phenotype (3). The description and thorough analysis of an Insl6 knockout mouse reported by Burnicka-Turek and colleagues (4) in this issue adds a new gene to the list of factors required for male spermatogenesis. More importantly, it is a gene coding for a member of the well-known family of relaxin-like peptides that include genes that have been conserved over hundreds of millions of years as well as genes that emerged only recently on the genomic landscape of placental mammals. The evolutionary history, enigmatic signal transduction, and a phenotype that depends on genetic background additionally illuminate a number of important aspects that go beyond the simple description of a new gene required for male fertility.

The development and maturation of sperm is a complicated and unique process of differentiation, which generates haploid and functional spermatozoa from diploid stem cells (spermatogonia) in the testes. This process continues from puberty throughout life, taking (in humans) roughly 72 d for the full differentiation of mature sperm to be completed. At the heart of sexual reproduction is the meiotic cell division, which involves chromosome pairing and recombination followed by segregation into the haploid spermatocytes. Meiosis and packaging of the genome into sperm also involves dramatic changes in terms of genome organization, epigenetic make-up, and chromatin composition, with specific gene transcription both before and after meiosis (5). This culminates in the wholesale re-packaging of almost the entire genome from histone-dominated chromatin to a protamine-compacted pseudocrystalline structure, with the chromosomes organized in a specific nonrandom fashion inside the sperm head (6, 7). All of this is required to achieve the highly specialized and evolutionarily variable morphology of the spermatozoon, with its unique ability to mature and function while in the reproductive tract of another individual. Every individual sperm must retain the capacity to decondense and reorganize its chromatin in a recipient oocyte (8) and provide a full and intact single-copy genome with which to create a new individual. This is a much more complicated process than that undergone by oocytes, although these may be

Abbreviations: ART, Assisted reproductive technology; GPCR, G protein-coupled receptor; ICSI, intracytoplasmic sperm injection; INSL3, insulin-like factor 3; LGR, leucine-rich repeat containing G protein-coupled receptor; RLN2, relaxin-2.
individually much older. However, whereas oocytes and their genetic material are protected in the safest possible place deep within the abdomen, this is not true for sperm. These are generated in an organ that in most mammals is vulnerably and paradoxically hung outside the body and exposed to all kinds of physical danger (9).

How should we understand this apparent paradox? Part of the reason lies in the notion that the higher mutation rate in the male gamete supplies evolution with more genetic variability than other cell types, including oocytes. The role of the male gamete as the principal source of genetic evolution, through recombination, high mutation rates caused by DNA damage, retroposon activity, or natural sperm selection is nevertheless still a matter of debate. The surprising dynamic of this fundamental process is also reflected by the evolutionary variability of genes associated with postmeiotic germ cell functionality. For example, there are several genes involved in sperm maturation in the testis or epididymis that are considered essential in other mammals but that in humans have been mutated or lost (10). Similarly, there are postmeiotic genes expressed in rodents that are not found elsewhere (11).

**INSL6** is a postmeiotically expressed gene belonging to the relaxin-insulin family of peptide hormones. Several of the members of this family are so-called neohormones (12), in other words molecules that have evolved important roles in specifically mammalian traits such as placentation, lactation, viviparity, a scrotal testis, or postreproductive survival. Typical examples of neohormones are relaxin-2 (RLN2) and insulin-like factor 3 (INSL3), both evolutionarily closely related to INSL6 (Fig. 1). INSL3 is a product of Leydig cells and is primarily responsible for the early phase of testicular descent during gestation, whereas RLN2 is an ovarian hormone involved in lactation or preparation of the birth canal. A recent analysis of synonymous and nonsynonymous nucleotide substitution rates (13) shows that, unlike the ancestral neuropeptide in this group, RLN3, which is barely changing, particularly INSL6, RLN1, and INSL4 are all evolving rapidly, but not much was known about the precise expression pattern and function of INSL6. The expression analysis by Burnicka-Turek and colleagues (4) provides a comprehensive analysis that shows *Insl6* expression in male and female mouse embryos before and after sex determination, with testis specificity observed in adult tissues. To narrow down the expression in the testis, the authors tested expression of *Insl6* in a variety of mutant mice elegantly showing that *Insl6* expression occurs at the pachyten stage of meiotic prophase I and postmeiotic germ cells, consistent with earlier work reported in this journal (14). To further elucidate the function of *Insl6*, Burnicka-Turek and colleagues (4) generated *Insl6*-deficient mice, which develop normally until the onset of spermatogenesis in males, where the group has discovered a phenotype uniquely associated with male gametes; this phenotype has only partial penetrance, with some *Insl6*-null males being fully fertile, whereas others show varying levels of male infertility (4). In other words, there is a degree of redundancy involved in *Insl6* function, supporting the notion that this gene has only recently arisen by DNA duplication in placental mammals and maybe has retained some overlapping functionality with other genes.

Unlike the insulin-IGF subgroup within this superfamily of peptides, which are assumed to be the ancestors of all family members and make use of single membrane-spanning tyrosine kinase receptors, the relaxin-INSL group of peptides acquired new receptors in their evolution. The older peptides RLN3 and INSL5 both signal through related class A G protein-coupled receptors (GPCR135 and GPCR142, respectively; now called RXFP3 and RXFP4), whereas INSL3 and RLN1 and -2 switched receptors again and signal through a quite different kind of GPCR (class C) with large extracellular domains (LGR8 and LGR7, respectively; now called RXFP2 and RXFP1) (15). Comprehensive database searching has now identified all structurally related receptors in various mammalian genomes; none of these appears to be a receptor for INSL6 or INSL4 (16), nor do the known receptors for other members of the relaxin-insulin family respond in any way to these peptides in *vitro* (16). Thus, the functionality evidenced by the *Insl6* knockout mouse (4) would suggest that yet another quite different kind of receptor has been acquired. Evolution continues apace.

The neohormone hypothesis (12) implies the evolution and acquisition of functions that are uniquely mammalian, but it does not preclude the retention of some ancestral functions. Relaxin-like molecules have been identified in frogs (17), sharks (18), and most recently starfish (19), all of which appear to relate to the ancestral molecule, RLN3. Although in mammals, this molecule is almost exclusively a neuropeptide (20), in lower vertebrates, it appears to be expressed at high levels also in both male and female gonads (17, 18) and in starfish is also linked to

*FIG. 1.* Consensus evolutionary tree for the relaxin family of insulin-like peptides (based on the bioinformatics analysis of Ref. 13). Insulin was used as an outgroup.
gonadal function (19). Whereas the Leydig cell hormone INSL3 in mammals appears to be mainly involved in the transabdominal migration of the fetal testis, it also appears to have a role in supporting spermatogenesis by interacting in the adult with specific receptors (LGR8) in late germ cell stages (21, 22). New evidence suggests that the specificity of INSL3 toward LGR8, and its role in testicular descent, have evolved recently after the divergence of egg-laying mammals, whose testes do not descend into a scrotum (23). There is also good evidence that INSL3 has a similar function in supporting follicle selection within the ovary (24, 25). These could represent ancestral, rather than neohormone, aspects of INSL3 physiology. In a similar way, INSL6 could also be maintaining an ancestral function, although possibly involving interaction with other receptors.

Another interesting evolutionary aspect is associated with the physical location of the INSL6 gene. It is well established that the X and Y chromosomes of marsupials and placental mammals are enriched for genes involved in reproduction and in particular for those relating to spermatogenesis. Moreover, deletions within the azoospermia factor (AZF) region on the human Y chromosome account for 8–12% of nonobstructive azoospermia (26). Nevertheless, like many other genes involved in infertility, the INSL6 gene is autosomal. However, it maps to a region that has been proposed to represent part of the ancestral mammalian sex chromosome. As Burnicka-Turek and colleagues (4) have noted, INSL6 is located in a well-known region on the short arm of human chromosome 9, which is affected in human sex reversal and is in proximity to the evolutionarily highly conserved double sex-mab-3-related transcription factor 1 gene (DMRT1). DMRT1 is now recognized as an extraordinarily conserved sex-determining gene for which orthologs have been identified in organisms ranging from worms to humans, with up-regulation of DMRT1 in the developing male gonad being observed in a variety of vertebrates engaging in different types of sex determination (27). Human chromosome 9 shares extensive homology with the chicken Z chromosomes (28) and the platypus X5 chromosome (29, 30). This region therefore represents at least part of an ancestral sex chromosome system that was established in the reptilian ancestor of birds and mammals. Today this system is maintained only in birds as a female heterogametic ZW/ZZ sex chromosome system and in the basal mammalian lineage of egg-laying mammals as part of a bizarre multiple XY sex chromosome system (29). Neither vertebrate group has evolved INSL6, which appears only after the divergence of marsupials from placental mammals about 148 million years ago (13, 23). The only related genes found on both the chicken Z chromosome and human 9p is the RLN2 gene (RLN2) on human 9p24 and on the chicken Z (Fig. 2). The exact evolutionary relationship of these related gene families is still a subject of debate, but the localization in this region does further enhance its reputation as a cluster of genes with important roles in sex determination and reproduction.

Another important feature of the study by Burnicka-Turek and colleagues (4) is the effect observed in different genetic backgrounds in Ins6-deficient males. The Ins6 homozygous knockout phenotype was observed only in hybrids of different Mus musculus strains (C57BL/6J × 129/Sv), but there is no obvious male phenotype when the mutation is carried within the inbred 129sv strain of mice. Only when the −/− mutation is introduced into an outbred strain with assumed genetic variability does the male phenotype, albeit still with partial penetrance, become apparent. This is not a new phenomenon but has been noted previously, as indicated in the article (4), as well as in earlier work by the same Goettingen group (31). In the latter study, they showed that a male phenotype induced by a similar postmeiotically expressed gene was evident only when fertilizing an oocyte with a thicker zona pellucida, as occurs in some strains of mice (31). This is not surprising given our knowledge about quantitative trait loci and male fertility from animal breeding.

FIG. 2. Position and diversification of RLN2 in birds and mammals. RLN2 and DMRT1 are syntenic (on the same chromosome) on the chicken Z sex chromosome and human chromosome 9. This conservation of syteny between placental mammals and birds that diverged 315 million years ago suggests that synteny is also maintained on platypus X5 and opossum chromosome 6 where the physical location of RLN2 is not yet clear. Chicken Z, platypus X5, opossum 6, and human 9 evolved from the ancestral sex chromosome system that birds and mammals inherited from the amniote ancestor. RLN2 has undergone recent diversification with INSL6 in placental mammals, INSL4 in primates, and RL17 in great apes after the divergence of the marsupial lineage (data from UCSC and ENSEMBL genome browsers and Refs. 16 and 23).
studies and emphasized in more recent genetic analysis of M. musculus × Mus spretus hybrids. This work also highlights a phenotype-genotype association for sperm-head shape-related traits on a large segment of chromosome 19, where in mice Ins14 and Ins16 are colocalated (32). One of the most dramatic cases of an effect of genetic background on sex determination is the classic example of C57BL/6J mice carrying Y chromosomes of Mus domesticus poschevinius (YPOS), which show sex reversal and develop as females. This has led to the idea that there are several autosomal loci (termed testis-determining autosomal, tda) affecting the regulation presumably of the Sry gene on the Y chromosome (33) or preventing the sex reversal to occur in the DBA/2J or 129S1/SvImJ (129) background (34). Interestingly, variable phenotypic effects on male fertility of Y chromosome deletions have been observed in different human populations (35). These results illustrate the complexity of factors contributing to male infertility and the limitations of our current methodological paradigms and models for dealing with such complex traits and genes with redundant function. The mouse is proving to be an excellent model system that has already provided us with many genes and loci involved in infertility. The different penetrance of phenotypes dependent on the mouse genetic background, as observed by Burnicka-Turek and colleagues (4) and others, may seem a nuisance for establishing a phenotype, but it also provides us with new tools with which to identify complex traits or modifier loci and hence unravel new genes and regulatory mechanisms that are involved in human fertility or sexual development in general.

This is important to understand in the context of human male infertility. In some countries, babies born as a result of ART make up over 4% of births. It has to be a concern that the diagnosis of infertility is often based on very superficial tests (36, 37). Consequently, we are likely to see a net shift in the prevalence of some of the genetic traits negatively affecting male fertility, without being able to identify them, and there is already growing evidence that children conceived with ICSI have a higher risk of congenital defects (38). Thus, the idea of male infertility being idiopathic and that it can be treated by ART, as advertised by some reproductive clinics, should not occlude careful consideration of a genetic and heritable reason for the male factor and the possible consequences for future generations.

Acknowledgments

Address all correspondence and requests for reprints to: Richard Ivell, School of Molecular and Biomedical Science, University of Adelaide, Adelaide, South Australia 5005, Australia. E-mail: richard.ivell@adelaide.edu.au.

Disclosure Summary: R.I. and F.G. have nothing to declare.

References

12. Ivell R, Bathgate RA 2006 Hypothesis: neohormone systems as exciting targets for drug development. Trends Endocrinol Metab 17:123
22. Amory JK, Page ST, Anawalt BD, Covello AD, Matsumoto AM, Bremner WJ 2007 Elevated end-of-treatment serum INSL3 is asso-


27. Ferguson-Smith M 2007 The evolution of sex chromosomes and sex determination in vertebrates and the key role of DMRT1. Sex Dev 1:2–11


35. Nyboe AA, Erb K 2006 Register data on assisted reproductive technology (ART) in Europe including a detailed description of ART in Denmark. Int J Androl 29:12–16

