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SPOTLIGHT

Of sex and determination: marking 25 years of Randy, the sex-reversed mouse

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ABSTRACT

On Thursday 9 May 1991, the world awoke to front-page news of a breakthrough in biological research. From Washington to Wollongong, newspapers, radio and TV were abuzz with the story of a transgenic mouse in London called Randy. Why was this mouse so special? The mouse in question was a chromosomal female (XX) made male by the presence of a transgene containing the Y chromosome gene *Sry*. This sex-reversal provided clear experimental proof that *Sry* was the elusive mammalian sex-determining gene. Twenty-five years on, we reflect on what this discovery meant for our understanding of how males and females arise and what remains to be understood.

KEY WORDS: *Sry*, Sex determination, Transgenic mouse, Testis

Introduction

One of the most fundamental processes in biology is the development of two sexes. Many great minds, from Aristotle to Darwin, struggled with the question of how the two sexes arise. However, by 1959 it was known that in eutherian mammals a gene or genes on the Y chromosome initiates testis development in the embryo and that the testes then produce all the hormones needed to make a male. In the absence of the Y chromosome, the embryonic gonads develop instead as ovaries. It was hoped that finding the relevant gene(s), termed testis-determining factor (*TDF* in humans; *Tdy* in mice), would eventually lead to a complete understanding of the processes needed to generate a testis, providing a paradigm for elucidating the morphogenesis of other organ systems. In subsequent decades, *TDF*/*Tdy* was narrowed down to a small region on the short arm of both the human and mouse Y by analysing XX males carrying a small Y fragment. The overlap between this fragment and the Y chromosome interval deleted in XY female patients delimited *TDF* (Pritchard et al., 1987; Page et al., 1987), enabling the search for the gene or genes defining that locus to begin.

The discovery of *SRY*/*Sry*

The *TDF* gene was predicted to have five key properties. First, it should be conserved amongst mammals. Second, it should be located on the Y chromosome in all mammals and within the smallest known sex-determining fragment of the Y chromosome in mice and humans. Third, its product should most likely act cell-autonomously and control the activity of other genes (Burgoyne et al., 1988). Fourth, it should be expressed in the somatic component of the developing testis at the time of sex determination (Burgoyne et al., 1988). Finally, gene mutations should lead to failure of testis development and to XY females, whether human patients or mice.

Two research teams were strong contenders in the race to identify *TDF*. In London, UK, Peter Goodfellow's human molecular genetics group teamed up with Robin Lovell-Badge's mouse developmental biology group to bring complementary expertise to the problem. In Boston, David Page's group led the race within the USA. In December 1987, the Boston group published a strong candidate for *TDF*, the gene encoding the zinc-finger protein ZFY (Page et al., 1987). *ZFY* mapped to the smallest region of the human Y chromosome found so far in XX males, was missing in XY females and encoded a transcription factor that could act cell-autonomously; but over the following two years, evidence increasingly indicated that it was, in fact, the wrong gene. Its marsupial homologue was found not to map to the Y chromosome (Sinclair et al., 1988). *ZFY* was expressed ubiquitously in humans, which did not fit well with the biology of *TDF*, while its mouse homologues were neither expressed in somatic cells of the fetal testis (Koopman et al., 1989) nor mutated in XY female mice known to carry a heritable mutation mapping to the Y chromosome (Tdy^{m1}; Lovell-Badge and Robertson, 1990; Gubbay et al., 1990). Crucially, four variably masculinised XX patients with testicular tissue were found to possess Y-derived markers but lack *ZFY* (Palmer et al., 1989).

Fortunately, however, these same four patients held the key to identifying a new candidate, as they had in common a mere 35 kb of Y chromosome DNA. The Goodfellow group cut this region into small fragments and tested each on Southern blots containing male and female DNA from human, mice and cattle. Only one human Y-fragment was identified that showed male-specific bands in human and several other mammalian genomes (Sinclair et al., 1990), as well as in XY and XX male mice [where the latter carried a small region of the Y chromosome short arm termed Sxr(b) (Laval et al., 1995)], but not XX or XY^{Tdym1} female mice (Gubbay et al., 1990). As unique sequences conserved between the Y chromosomes of different mammalian species are very rare, these results implied that this human Y chromosome fragment probably contained *TDF*/*Tdy*. Sequencing revealed an open reading frame coding for a single exon gene, cautiously named sex-determining region on the Y chromosome (*SRY* in humans, *Sry* in mice: Sinclair et al., 1990; Gubbay et al., 1990).

With this strong candidate in hand, it was relatively straightforward to test its properties against the predictions made for *TDF*. The first two were satisfied by Southern blotting, as described above. In those experiments, additional bands were observed that were not sex-specific – an early indication of the existence of an *Sry*-related gene family later named *Sox*. Although much of the *SRY*/*Sry* open reading frame was not well conserved, both human and mouse sequences included a region encoding a

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79 amino acid domain with homology to a conserved DNA-binding motif present in the nuclear high-mobility group proteins HMG1 and HMG2, and in the eukaryotic transcription factor UBF (Jantzen et al., 1990). Shortly afterwards, a related 'HMG domain' was found in the transcription factors TCF1 and LEF1, further suggesting that SRY was itself a transcription factor (van de Wetering et al., 1991; Travis et al., 1991). RT-PCR experiments showed that *Sry* is expressed in XY mouse fetal gonads at 11.5 days post coitum – the time point immediately preceding the first morphological signs of testis differentiation (Gubbay et al., 1990). Indeed, *Sry* is expressed for only a brief period prior to sex determination in mouse gonads and, like sex determination itself, does not depend on the presence of germ cells (Koopman et al., 1990). Finally, *de novo* point mutations affecting the SRY HMG domain were detected in DNA from two human XY females, providing strong genetic evidence that the function of SRY is required for testis development and male sex determination (Berta et al., 1990). All the evidence pointed to *SRY*/*Sry* being the right gene. But how could the putative role of *SRY*/*Sry* be proved?

Making a male mouse

It was reasoned that the gene(s) representing *Tdy* should cause XX sex reversal if introduced as a transgene via pronuclear injection into mouse zygotes. A 14 kilobase (kb) genomic clone containing the mouse *Sry* open reading frame and about 8 kb of 5′ and 5 kb of 3′ flanking DNA was identified and mapped. This had been sequenced and characterised sufficiently to be confident that there was no additional ORF in the region, but there was no assurance that it contained all the necessary sequences required for correct quantitative, temporal and cell type-specific expression, or mRNA stability. Without adding a tissue-specific promoter or insulator sequences, the possibility that this small and naked genomic fragment might function to drive male development seemed remote, but it was worth a try.

Injected embryos were analysed at 14 days post coitum, some 36 h after the formation of testis cords, which, together with the formation of a male pattern of gonad vasculature, make it possible to visibly distinguish testes from ovaries. Chromosomal sex and the presence or absence of the transgene were verified by PCR and/or Southern blotting. Early experiments were disappointing. Then, in one experiment, two out of four XX transgenic embryos had testes indistinguishable from those of control XY embryos (Koopman et al., 1991). Another two transgenic XX fetuses were female and another four XX fetuses had low transgene copy numbers: six XX females, in which mosaicism and/or unfavourable integration had presumably precluded transgene function. The two sex-reversed embryos were cause for relief and an ambrosial sense of triumph, but there was little time to celebrate: there was much more work to do.

In subsequent experiments, mice were allowed to develop through to birth after pronuclear injection. Five of these were transgenic, including three XX. One of the latter looked to be an entirely normal male externally (Fig. 1), was very willing and able to mate with females, and so was given the nickname Randy. He had a normal male reproductive tract, but his testes were small and devoid of spermatogenesis, entirely as predicted for an XX male: two X chromosomes are incompatible with the proliferation of spermatogonia after birth and other Y chromosome genes are required to make sperm. Because the sex reversal in Randy was complete, this was the final proof that *Sry* was not only necessary, but also sufficient on its own to initiate male somatic development. Thus, *SRY*/*Sry* was functionally and genetically equivalent to *TDF*/*Tdy*. The paper was prepared with urgency and excitement (Fig. 2),

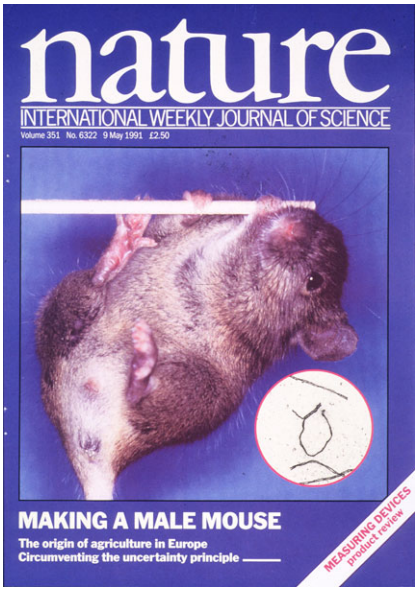

Fig. 1. Complete sex reversal of an *Sry* transgenic mouse. The external phenotype of XX transgenic mouse m33.13, also known as Randy (shown here on the cover of the 9 May 1991 issue of *Nature*), was identical to that of a wild-type XY male mouse. Image reproduced with permission from Nature Publishing Group.

reviewed positively, accepted on 5 April and published within five weeks as a feature article in *Nature* (Fig. 1).

By 1991, several transgenic mice had already hit the headlines. These included big mice that overexpressed rat growth hormone (Palmiter et al., 1982) and mice that developed specific tumours (Brinster et al., 1984). However, many hundreds of others flew below the radar and it was assumed that this one would not create much of a stir in the media, especially because Randy looked like a normal male mouse and there had already been media attention around the discovery of *SRY*/*Sry* the previous year. We could not have been more wrong!

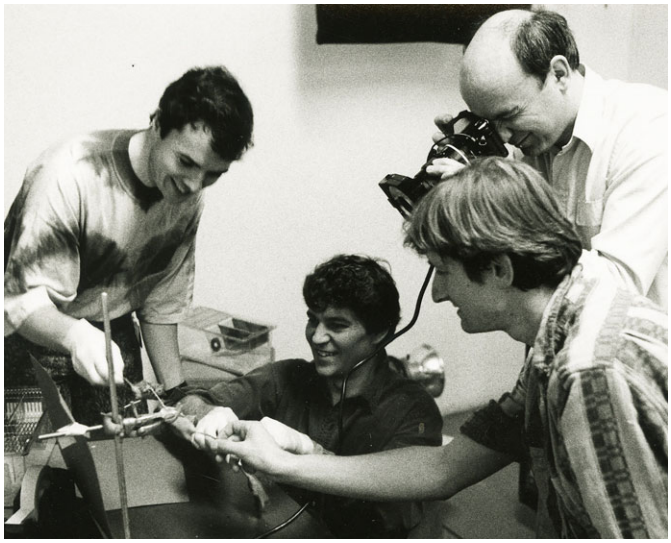

Fig. 2. Making a *Nature* cover. Never work with children or animals, the adage goes. Certainly it can take more scientists than animals when it comes to persuading mice to hang contentedly from a rod. Left-right: Nigel Vivian, Robin Lovell-Badge, Peter Koopman and Neal Cramphorn. Image courtesy of Jérôme Collignon.

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The story made front-page news in the UK and spread from there around the world (Fig. 3). Reporters, photographers, TV and radio crews invaded the lab for weeks. The technical accomplishment, the achievement of a significant milestone in genetics and possibly some cute photos of mice, resonated with many. Some stories trumped up a relevance to cancer, others questioned the ethics or talked of a slippery slope, and yet others found the concept of 'a sex-change for Mickey' mildly amusing.

The broader significance of the work undoubtedly lies in having finally provided an answer to one of our most fundamental and long-standing questions – why am I male or female? That a single gene makes all the difference was a surprise to many. Scientifically, the discovery of *SRY/Sry* helped to open the field of sex determination. It provided an entry point into the genetics and embryology underlying testis development, explained cases of sex reversal, and provided an important new diagnostic. It also spurred research into ovary development – the other half of the story of gonadal sex determination.

Progress and current challenges

Proving the testis-determining role of *SRY*/*Sry* was expected to open the gate to discovering the next gene in the pathway, and the one after, such that a comprehensive picture of how sex determination works at the molecular genetic level would be 'quickly' gained. Things did not work out quite that way. *SRY*/*Sry* proved to be extremely difficult to work with in almost every way imaginable. The gene is small, intronless and, being from the Y chromosome, embedded in a poorly conserved and highly repetitive DNA sequence (Gubbay et al., 1992). It is expressed in a small number of cells in an inaccessible fetal tissue for (in mice) only a brief period (Albrecht and Eicher, 2001; Sekido et al., 2004; Wilhelm et al., 2005). No specific antibody for SRY was produced for over a decade (Bradford et al., 2007). Even initial efforts to define its mRNA transcript literally had us going around in circles (Capel et al., 1993). Immortal cell lines expressing *SRY*/*Sry* bore questionable resemblance to fetal gonadal cells and primary gonadal cells placed into culture quickly lost *SRY*/*Sry* expression (Capel et al., 1996; Beverdam et al., 2003). The SRY binding site is small and degenerate (Harley et al., 1992), hence putative target sites are present throughout the genome. In short, standard techniques for discovering upstream or downstream genes were essentially unworkable for *SRY*/*Sry* (Larney et al., 2015).

Fig. 3. Media frenzy. Newspaper clippings reflecting the varied popular interpretations of the significance of creating a sex-reversed mouse.

Despite this, we now have a basic understanding of the SRY protein's cellular and molecular functions. SRY binds specifically to DNA in one or more enhancers of the target gene *Sox9*, in order to upregulate *Sox9* transcription (Sekido and Lovell-Badge, 2008). This occurs in just one cell lineage – the bipotential supporting cell precursors that give rise to granulosa cells in the ovary or Sertoli cells in the testis. SOX9, in turn, activates the expression of other genes required for Sertoli cell differentiation and function, including factors that instruct the other cell lineages within or adjacent to the gonad to follow a male differentiation pathway (reviewed by Svingen and Koopman, 2013). Importantly, SOX9 also promotes its own expression, so that continued *Sry* expression is not required to sustain testis differentiation or maintain testis phenotype. At the same time, SOX9 and/or its direct or indirect targets repress genes associated with ovarian development. In summary, *Sry* acts as a trigger and *Sox9* an effector in driving testis development (reviewed by Canning and Lovell-Badge, 2002; Kashimada and Koopman, 2010). Not surprisingly, transgenic expression of *Sox9* during XX mouse fetal gonad development is as effective as *Sry* in inducing testis development and maleness (Vidal et al., 2001).

As with all transcription factors, a threshold level of *SRY*/*Sry* expression is required to activate *Sox9*. It is also clear that *SRY*/*Sry* must act within a specified time window, otherwise it 'misses the boat'; transgenic mice in which *Sry* is expressed later than normal via an inducible promoter do not switch on *Sox9* and do not make testes (Hiramatsu et al., 2009). Because the timing and levels of SRY expression are integral to its sex-determining function, considerable effort has focused on understanding the regulation of *SRY*/*Sry* transcription. In mice, *Sry* expression is thought to be activated by some combination of the core transcription factors steroidogenic factor 1 (SF1), Wilms tumor 1 (WT1) and GATA binding protein 4 (GATA4), although whether and how these factors act specifically on *Sry* regulatory sequences are matters that remain to be fully explained experimentally (reviewed by Larney et al., 2014). A signal transduction pathway involving GADD45γ, MAP3K4 and p38 MAPK (MAPK14) is also crucial (Warr et al., 2012), although the extracellular ligands and their source have yet to be identified. *SRY*/*Sry* is a rapidly evolving gene, so the specific details may not be the same in all mammals.

Somewhat surprisingly, gonadal phenotype has emerged as relatively labile. The deletion of *Foxl2* from adult granulosa cells allows *Sox9* to be expressed, which then triggers transdifferentiation to Sertoli cells. This leads to gonadal sex reversal, with the ovaries exhibiting a structure and cell types resembling that of the testis (Uhlenhaut et al., 2009). Conversely, loss of *Dmrt1* in the adult testis leads to upregulation of *Foxl2* and repression of *Sox9*, and a transformation from testicular to ovarian cell types (Minkina et al., 2014). All of this suggests that there is no crucial role for chromatin modifiers to lock down the system. Could this flexibility and the

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need for continuous repression of the opposite pathway reflect an evolutionary hangover from early vertebrate ancestors that could change sex, as seen in some species of fish (Kobayashi et al., 2013)?

The discovery of *SRY*/*Sry* also encouraged a search for essential sex-determining genes in non-mammalian vertebrates, where it is absent, and consideration of how sex chromosomes and sex-determining genes arose during evolution. Mapping of *Sox3*, one of the original group of *Sox* genes co-discovered with *Sry*, and its human homologue *SOX3*, to the mouse and human X chromosomes, first suggested the prevailing model that *Sry* evolved from *Sox3* (Stevanović et al., 1993). SOX3 does not seem to have any role in sex determination, but has conserved functions during gastrulation, in the development of the central nervous system, and in a few other tissues. It seems most likely therefore that *Sox3* was originally autosomal, as it still is in prototherian mammals, and that a regulatory mutation led to expression of one copy in the early gonad, where its protein product promoted transcription of *Sox9*, which does appear to play a role in Sertoli cell differentiation in all vertebrates. This variant *Sox3* became *Sry*, the chromosome it was on became the Y chromosome, and the chromosome retaining a normal *Sox3* became the X chromosome. In support of this model, situations where SOX3 is mis-expressed in the early gonad can lead to XX male sex reversal in mice and probably in humans, perhaps mimicking events in evolution (Sutton et al., 2011).

Despite much progress from many labs, we still do not have a comprehensive picture of the regulatory networks leading to the differentiation, maturation and maintenance of either the testis or the ovary in mammals. Most causes of XY and XX gonadal dysgenesis, other than those involving *SRY*, remain unknown. As was the case with the original identification of *SRY*/*Sry*, co-ordinated efforts involving multiple species will continue to drive progress in filling these gaps. For example, transcriptomic studies in mice can identify candidate disorders of sex development (DSD) genes that can be tested for mutations in the DNA of DSD patients. Conversely, DSD patient screening can identify new candidate genes that can be characterised and functionally studied in mice. In this way, and through the application of increasingly sophisticated biochemical and genetic approaches, the field will continue to advance.

Concluding remarks

These days, it is hard to imagine the stir created by a single transgenic mouse experiment in 1991. Given the lack of current tools such as whole genome sequencing, internet-based bioinformatic tools and advanced gene-editing tools such as CRISPR/Cas9, it remains something of a miracle that within the space of a couple of years, a plausible *TDF/Tdy* candidate was identified and its function so definitively validated by the creation of Randy the mouse. While Randy did not leave any direct descendants, other transgenic mice made with *Sry* have been part of his legacy. For example, the *Tdym1* mutation, where *Sry* is deleted from the Y chromosome, can be complemented by an *Sry* transgene on an autosome to give fertile XY males. The four types of offspring these would have produced when mated with XX females would make it possible to distinguish direct effects of X- and Y-linked genes from the effects of gonadal hormones on differences between the sexes (De Vries et al., 2002). This ‘four core genotype’ model has now been used to explore the basis of many sex-specific differences, from neuroanatomy to food preferences. Moreover, it has led to a more general appreciation of how commonly such differences occur and their clinical importance (Arnold, 2014). *Sry* could itself have roles outside the gonad. Indeed, it is expressed in the brain in mice and other mammals (Loke et al., 2015). This could place male-specific traits under selection, perhaps explaining aspects of rapid *Sry* evolution. Finally, the discovery of *Sry* also, importantly, led to the identification of other members of the *Sox* gene family, of which there are 20 in man and mouse (Schepers et al., 2002). These *Sox* genes, unlike *Sry*, are highly conserved in evolution and have proved essential for many cell fate decisions during embryogenesis. They are also clinically relevant for both congenital disease and cancer. Quite an impressive legacy from a sterile, albeit frisky, XX male mouse.

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Competing interests

The authors declare no competing or financial interests.

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