Good planning and serendipity: exploiting the *Cre/Lox* system in the testis

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Abstract

Over the past 20 years, genetic manipulation has revolutionised our understanding of male reproductive development and function. The advent of transgenic mouse lines has permitted elegant dissection of previously intractable issues. The development of the *Cre/Lox* system, which has permitted spatial and temporal localisation of genetic manipulation, has expanded upon this, and now makes up one of the primary approaches underpinning our increasing understanding of testis development and function. The success of conditional gene targeting is largely reliant upon the choice of Cre recombinase expressing mouse line, which is required to specifically target the correct cell type at the correct time. Presupposition that Cre lines will behave as expected has been one of the main oversights in the design of *Cre/Lox* experiments, as in practice, many Cre lines are prone to ectopic expression (both temporal and spatial), transgene silencing or genetic background effects. Empirical validation of the spatiotemporal profile of Cre expression prior to undertaking conditional gene targeting studies is essential and can be achieved through a combination of molecular and immunohistochemical approaches, along with *in vivo* examination of reporter gene expression in targeted tissues. This paper details the key considerations associated with exploitation of the *Cre/Lox* system and highlights a variety of validated Cre lines that have utility for conditional gene targeting within the testis.

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Introduction

Over the past 20 years, genetic manipulation in the mouse has revolutionised our understanding of male reproductive development and function (Jorgez *et al.* 2005, Roy & Matzuk 2006). Knockouts, knockins, gene-trapped, ENU-derived and other transgenic mouse models have permitted elegant dissection of previously intractable issues (for reviews, see Cooke & Saunders (2002), O'Bryan & de Kretser (2006), Borg *et al.* (2010), Jamsai & O'Bryan (2010) and Yatsenko *et al.* (2010)). The development of the *Cre/Lox* system of conditional gene targeting, which has permitted spatial and temporal localisation of genetic manipulation, has expanded and refined the toolbox of genetic manipulation available to researchers (for reviews, see Hadjantonakis *et al.* (1999) and Nagy (2000)), and there are now > 500 independent Cre recombinase expressing mouse lines, many of which can be accessed via the publicly available CreXmice database (Nagy *et al.* 2009). Exploitation of the *Cre/Lox* system has become a key mechanism for dissection of novel problems within the male reproductive system, and is certain to remain an important tool for many years to come.

The cyclisation recombination (Cre) enzyme of bacteriophage P1, a 38-kDa site-specific recombinase,

was first developed as an *in vivo* molecular tool over two decades ago (Sauer & Henderson 1988, 1989). While other recombinase systems, such as *Flp/FRT* and ϕ C31/*att*, are being employed with increasing frequency (Branda & Dymecki 2004), Cre has come to prominence as the pre-eminent recombinase of choice for molecular genetic analysis primarily because it recombines DNA at high efficiency, and does not require accessory proteins or specific DNA topology to mediate efficient recombination in target cells (Sauer 1987). Since its initial use, several improved versions of Cre have been developed, which utilise eukaryotic codon preference to improve the expression of the Cre, such as iCre (Shimshek *et al.* 2002) or add a eukaryotic nuclear localisation signal to increase recombination efficiency (Kalderon *et al.* 1984).

The general tenet behind the *Cre/Lox* system relies upon the ability of Cre recombinase to identify bind and recombine DNA between two *LoxP* (locus of crossing (X-ing) over in P1) sites, each of these 34 bp target DNA sequences consists of two 13 bp inverted repeat sequences, flanking a central, 8 bp, directional core (Fig. 1). Application of this technology to the mammalian system is possible because the mammalian genome lacks high affinity *LoxP* sites (cryptic *LoxP* sites are found naturally in the genome and can be targeted by Cre, at least at low efficiency and *in vitro* (Thyagarajan *et al.* 2000,

Figure 1 Cre mediated DNA recombination. (a) Cre expression is driven by an exogenous promoter of choice, enters the cell nucleus and binds to *LoxP* sites flanking a target gene. (b) The DNA surrounding the target gene undergoes a conformational change to bring the two *LoxP* sites into close proximity where a synaptonemal complex is formed. DNA strand exchange occurs at the sites of homology leading to the excision of the target gene (c) and the presence of a single *LoxP* site (generated from two half-sites) at the original genomic location.

Semprini *et al.* 2007)), and thus artificially engineering these specific sequences into DNA regions flanking a target locus of interest 'primes' that site for recombination by Cre recombinase (Kwan 2002). Consequently, target genes/DNA sequences flanked by *LoxP* sites are said to be 'floxed' (Lewandoski 2001, Gossen & Bujard 2002, Branda & Dymecki 2004). While the *Cre/Lox* system has been used to induce DNA translocation and DNA inversion (Branda & Dymecki 2004), the predominant application in the mouse has been its use in conditional gene targeting, where it has been exploited to knock out, insert, replace, activate or modify the expression of a gene of interest.

For recombination to take place *in vivo*, Cre recombinase itself must be expressed within the target cells. To achieve this, Cre recombinase, as an exogenous gene, must be inserted downstream of a promoter to drive expression, and it is the choice of promoter that primarily defines specificity of Cre expression. Thus, the limitation of Cre recombinase as a molecular genetic tool is only bound by the necessity of identifying a promoter with the correct specificity and strength to drive Cre at a sufficient level to promote recombination. The most widely used approach has been to characterise a cell-specific promoter of interest, place Cre downstream of this promoter and generate transgenics through pronuclear injection (Wang 2009). This approach has the advantage of being technically relatively simple, with production of new transgenic lines extremely rapid. The disadvantage is that, in most cases, these constructs use fragments of promoter and are thus prone to ectopic expression. Furthermore, the random nature of genomic insertion can lead to transgene silencing, and the

propensity to insert and express multiple copies from a single insertion site can impact upon cellular viability. Presupposition that Cre lines will behave as expected has been one of the main oversights in the design of *Cre/Lox* experiments.

To deal with these issues, an alternative but more technically challenging approach has been to 'knock in' Cre to a specific genomic locus through gene targeting in embryonic stem cells, such that Cre is driven from the endogenous promoter (and thus should faithfully follow the expression of the gene in question; Wang 2009). This approach simultaneously provides a null allele of the gene in question (and KO when homozygous) and a useful Cre model when heterozygous (Nagy *et al.* 2009). The caveat to this approach is that mice inheriting the Cre transgene have also lost one allele of the endogenous gene, leading to the potential of a sensitised genetic background that can impact upon later experimental analysis. In some instances, where this is not acceptable, researchers have chosen to insert Cre downstream of an internal ribosome entry site (IRES) in the 3'UTR of the target gene. These 'gene trap' lines provide reliable promoter-driven expression without ablation of the endogenous gene (Nagy *et al.* 2009).

Validation of Cre expression and function

As touched on above, for the vast majority of Cre lines, expression of Cre does not follow the documented expression pattern expected for the gene from which their promoter has been taken. Many review articles begin their description of the Cre line by describing the sites of expression of the original gene from which

the promoter for Cre has been derived. While this can be helpful in guiding individuals in choosing which Cre line to use, this can also be misleading as it encourages a level of expectation of the line that may, in practice, be flawed. While the Cre line may have originally been generated to target a specific cell type of interest, where it *actually* functions is much more important to know. Thus, Cre lines should always be considered in terms of their utility rather than in terms of their original purpose. To establish this, empirical validation is essential.

Establishing the spatial and temporal expression of Cre recombinase in a mouse line can be addressed in several ways; however, each has its limitations and a combination of several approaches should be considered essential. Many Cre lines now exist as fusions or bicistronic transcripts with fluorescent marker proteins (Nagy *et al.* 2009). Fluorescent microscopy or immunohistochemistry using an antibody against either the Cre protein or the fluorescent marker protein can be used to establish the expression of Cre. However, this approach can only ever establish where Cre is *currently* expressed, not where it has been expressed and functioned in the past. A Cre-mediated recombination event at an earlier time point will persist in all daughter cells, regardless of whether these continue to express Cre, and thus it is possible to be deceived when defining the sites of expression using a Cre antibody at a single, later, time point. To address this, a wide variety of mice carrying one of a series of Cre-inducible reporter genes have been generated, whose expression relies upon Cre-mediated recombination at *LoxP* sites flanking an upstream stop signal. Thus were Cre ever active in a cell, the reporter protein is switched on, and continues to be expressed in all daughter cells, providing a lineage-tracing profile of where Cre *has* been active (Fig. 2). The first reporter lines developed expressed genes such as alkaline phosphatase and/or lacZ, which can be exploited via colourimetric reactions to aid histological localisation of expression (Tsien *et al.* 1996, O'Gorman *et al.* 1997, Sakai & Miyazaki 1997, Lobe *et al.* 1999, Mao *et al.* 1999, Soriano 1999, Yamauchi *et al.* 1999). These were followed by reporter lines that express various fluorescent reporter proteins (Kawamoto *et al.* 2000, Novak *et al.* 2000, Mao *et al.* 2001, Srinivas *et al.* 2001, Weber *et al.* 2003, Vintersten *et al.* 2004, Luche *et al.* 2007, Muzumdar *et al.* 2007, De Gasperi *et al.* 2008), and more recently bioluminescent proteins (Lyons *et al.* 2003, Safran *et al.* 2003, Ishikawa & Herschman 2011), both of which have the utility for *in vivo* analyses in addition to *ex vivo* analyses. However, these reporter lines are not without fault. One note of caution is the seductive appeal that increased reporter expression in one cell type over another suggests a 'better' targeting in that cell type, while this may simply reflect the cell-specific expression of the reporter within that cell type or the cellular turnover of the reporter protein. This can result in focussing downstream analysis on one

Figure 2 Validation of Cre recombinase expression using a reporter line. (a) Expression of a fluorescent reporter gene driven from the *Rosa26* promoter is blocked by a *LoxP*-flanked upstream stop signal. Expression of Cre within the same cell elicits a recombination event that removes the stop signal and activates expression of the reporter gene, thus identifying in which cell types Cre has functioned. (b) Examples of YFP reporter expression in several Cre lines (left panels, whole testis; right panels, testis sections; bar = 100 μ m). (i) Spermatid (AQP2-Cre), (ii) Sertoli (Amh-Cre) and (iii) endothelial (Tie2-Cre).

cell type over another, when two or more cell types have in fact been targeted.

Further to this, in some instances, spatiotemporal expression of Cre can be very different depending on genetic background (Hebert & McConnell 2000), and thus switching strains to examine Cre expression in a reporter line should be avoided if at all possible. In addition, the reporter gene and the floxed target gene, which is to be exploited for later experimental analysis, can be subjected to differences in recombination efficiency due to their divergent genomic location (Wang 2009). Thus, the reporter may not provide an accurate representation of sites of recombination for other floxed target genes. To address this, the use of

reporter lines should always be supported by immunohistochemistry against the target protein derived from the floxed gene in experimental animals to demonstrate that the cells highlighted by the reporter gene do indeed show evidence of recombination.

Using reporter gene expression as a *de facto* measure of gene-targeting success also has temporal as well as spatial implications. If early targeting of a specific floxed gene results in a detrimental phenotype in a developing tissue, targeted cells could be outcompeted or no longer contribute to the final tissue. In this instance, immunohistochemistry at later ages may show minimal evidence of recombination, suggesting that the Cre line has not functioned, contradicting the reporter gene data on where Cre should be expressed. Thus, the onset of Cre expression must also be ascertained (usually by RT-PCR), and tissues were examined throughout development to determine the impact of Cre-mediated recombination.

Cre recombinase can itself produce a phenotype

Once the spatiotemporal pattern of Cre expression has been confirmed, the first control in any study is to determine whether the Cre recombinase line itself induces a phenotype in the mouse. The significance of this has been succinctly highlighted in a paper analysing the RIP-Cre line, which has been used widely to target β -cells in the pancreas. This Cre line was used in 21 studies to target different floxed genes; in the majority of cases, the main observation was glucose intolerance or diabetes. However, in a seminal paper, Lee *et al.* (2006) demonstrated that on certain genetic backgrounds, the

RIP-Cre line itself, in the absence of genes targeted by *LoxP* sites, exhibits glucose intolerance. Fifteen of the previously published studies had not included phenotypic analysis of the RIP-Cre line as a control in their study.

Targeting the testis

The testis provides specific and unique challenges when it comes to Cre recombinase choice and use. With over 500 lines to choose from, one line targeting a specific cell type can seem as good as another. However, all Cre lines have their own idiosyncrasies depending on the site of expression, timing of expression, copy number, strain background and input from targeting of other body systems. This review cannot possibly cover every Cre line that exhibits expression in the testis (for an expanded list, see Nagy *et al.* (2009)), but will highlight the most commonly used Cre lines (Fig. 3) and the caveats inherent with their use.

The choice of promoter for driving Cre expression falls broadly into three groups, namely global, cell/tissue-specific and inducible. The use of a global promoter ensures targeting of the floxed gene of interest in every, or almost every, cell type. These are used to determine the global impact of targeting of the gene of interest, or where the target gene is only expressed in a specific cell type of interest and thus targeting elsewhere is unlikely to have a phenotypic impact. Global targeting is usually achieved through driving Cre expression in gametes, such that recombination results in offspring with targeting of the floxed allele in every cell type, and thus global Cre lines and germ cell (GC)-specific Cre lines are often one and

Figure 3 Cre lines in the testis. A schematic section through the testis highlighting Cre lines that target of each key cell type. SC, Sertoli cell; LC, Leydig cell; PTM, peritubular myoid cell; VSM, vascular smooth muscle; E, endothelial cells; Sg, spermatogonia; Spc, spermatocyte; Sd, spermatid.

the same thing; the difference being whether analysis is focussed upon the gonad of the parent, or analysis is focussed upon the whole body of the offspring (for review, see [Hammond & Matin \(2009\)](#)). One real advantage of targeting specifically in gametes rather than simply driving Cre ubiquitously is that the floxed target gene and the Cre transgene can segregate independently, and thus it is possible to obtain recombined mice that have not inherited the Cre transgene, an important control, especially where cellular toxicity arising from systemic Cre expression is a concern ([Higashi *et al.* 2009](#), [Jae Huh *et al.* 2010](#)). Many Cre lines have been developed that target GCs, albeit unwittingly. In many cases, this is due to promiscuous expression of Cre in lines not originally designed to recombine in the germline ([Weng *et al.* 2008](#)). This ectopic GC expression can result in inheritance of a recombined allele of a floxed target gene in all tissues, which, if not controlled for, has the potential to complicate downstream analysis of any gene that is sensitive to haploinsufficiency. Thus, as a general point, validation of any Cre line should include an examination of reporter gene expression in the gametes of both sexes.

Spermatids

Cre lines have been developed that target recombination to GCs at all stages of development; the researcher's aim is therefore to identify the most appropriate Cre line to target their floxed allele of choice. If the target gene of interest is expressed at several stages of GC development but the experiment requires targeting in haploid cells only, a range of Cre lines with expression restricted to spermatids are available. One Cre line with testis expression limited to spermatids (although also expressed in the brain) is the Tspy-Cre, which is driven by 2.4 kb of the testis-specific protein Y-encoded (TSPY) promoter ([Kido & Lau 2005](#)). When bred with WT females, males containing both the Tspy-Cre transgene and the Z/EG (LacZ/EGFP) reporter ([Novak *et al.* 2000](#)) produced recombined pups, demonstrating that Tspy-Cre males are fertile, with Cre able to function in spermatids in addition to acting as a global deleter.

A second line expressed in spermatids is the AQP2-cre, which is driven by 14 kb of the human aquaporin 2 promoter ([Nelson *et al.* 1998](#)). Owing to technical reasons, in the original publication, Cre could not be localised within the testis; however, breeding to a reporter line ([Srinivas *et al.* 2001](#)) demonstrates that expression of the Cre mirrors AQP2 with expression within the testis limited to spermatids ([Fig. 2](#)). Therefore, the AQP2-Cre represents a further spermatid-specific Cre line and a useful global deleter.

Targeting of floxed genes in most meiotic GCs can also be achieved through the use of the protamine-1 (Prm1)-Cre ([O'Gorman *et al.* 1997](#)), which is highly expressed in haploid spermatids (and at 100 times lower levels in

most other tissues). Interestingly, while the Cre transgene segregates during meiosis, which means in an animal heterozygous for the transgene, only 50% of the spermatids will contain the transgene and thus expected recombination should be limited to 50% of spermatids, in practice, 92% of target alleles undergo recombination, which was attributed to sharing of gene products by haploid spermatids via cytoplasmic bridges.

Development of a second Prm1-Cre during an independent study neatly highlights the fickle nature inherent in Cre line design. Utilising a larger fragment of the Prm1 promoter (4.1 kb rather than 652 bp) and a different UTR produced incredibly different outcomes ([Schmidt *et al.* 2000](#)). In this model, 100% of males were infertile due to Cre-mediated chromosomal rearrangements, which led to abortive pregnancies in matings to WT females, possibly due to increased expression or nuclear accumulation of Cre, or subtle changes in timing of expression during meiosis.

Spermatocytes

The observation of unwanted chromosomal rearrangements through activation of Cre in GCs has been recorded in other Cre lines, each of which expresses Cre during meiosis. The synaptonemal complex protein 1 promoter has been used to generate a Sycp1-Cre line ([Vidal *et al.* 1998](#)). Initial analyses demonstrated expression of Cre in leptotene/zygotene spermatocytes, with Cre-mediated recombination occurring in pachytene spermatocytes ([Chung *et al.* 2004](#)). Intriguingly, a further study using the Sycp1-Cre line demonstrated that, while Sycp1-Cre functions as an excellent deleter in the first generation, expression of this Sycp1-Cre during meiosis leads to epigenetic methylation of *LoxP* sites (observed independently in several target genes) and a heritable silencing of *LoxP* sites, which prevented Cre-mediated recombination in all tissues of the next generation ([Rassoulzadegan *et al.* 2002](#)). These observations were supported by a further independent study ([Rasoulpour & Boekelheide 2006](#)). While these issues can be circumvented by maintaining the Sycp1-Cre alongside floxed alleles through the female germline (as Sycp1-Cre is not expressed there), this brings into question the utility of the Sycp1-Cre as a good choice for a GC-specific deleter. However, while targeting of floxed alleles was problematic, the induced silencing, which was observed to spread to alleles that had never been exposed to Cre recombinase, meant that the Sycp1-Cre line has utility as a serendipitous model of transvection in the mammalian genome ([Rassoulzadegan *et al.* 2002](#)).

Further Cre recombinase models targeting the spermatocytes have been developed. A 1.4 kb promoter fragment from the phosphoglycerate kinase 2 (*Pgk2*) gene was used to generate a *Pgk2*-Cre line ([Ando *et al.* 2000](#)). Molecular analysis and reporter gene expression

demonstrated GC-specific expression within the testis, which could first be detected in pachytene spermatocytes. However, effective Cre-mediated recombination was limited to 70% of spermatocytes. A second study demonstrated ectopic Cre expression during embryogenesis, which potentially limits the utility of the line (Bhullar *et al.* 2001). However, an independent Pkg2-Cre line, generated by Kido *et al.* (2005) (albeit using the same promoter fragment), has been used successfully to overexpress PER15 in spermatids.

Recently, a further line targeting spermatocytes was generated using a 640 bp promoter fragment of the heat shock protein 2 gene (*Hspa2*), with the specific aim of overcoming the limitations of both the Sycp1-Cre and the Pkg2-Cre (Inselman *et al.* 2010). The *Hspa2*-Cre transgene is expressed from day 20 in spermatocytes from the leptotene/zygotene stage onwards and additionally in spermatids by day 30, and has a functional efficiency of 100% when GFP reporter expression was examined. However, again, the *Hspa2*-Cre transgene is also expressed during embryogenesis, which would need to be controlled for were the study aimed at targeting genes important both for embryo development and spermatogenesis.

The synapsin1-Cre (SynCre) was originally generated to target neurons; however, Rempe *et al.* (2006) have demonstrated that SynCre also functions in male GCs. Exact spatiotemporal expression is inferred based upon inheritance of a recombined floxed allele in offspring, which suggests spermatocytes as the target cell type. The SynCre has utility as a global deleter.

The cKit-Cre transgene, driven by 300 bp of the *Kit* oncogene promoter, is expressed in secondary spermatocytes and spermatids (Bergqvist *et al.* 1998). However, while recombination in GCs is stably inherited, ectopic expression during embryogenesis leads to widespread mosaicism (ranging between individuals from 20 to 100% of cells) in offspring, limiting the utility of the cKit-Cre line for GC-specific analyses.

PGC/gonocytes/spermatogonia

The problem of chromosomal rearrangement and epigenetic silencing associated with Cre-mediated recombination during meiosis suggests that, where at all possible, choice of Cre lines for targeting GCs should focus upon those that function in pre-meiotic diploid GCs, either PGCs, gonocytes or spermatogonia. Many such lines are available, but even these differ in their utility.

The Nanos3-Cre was generated through targeted insertion into the *Nanos3* gene (Suzuki & Saga 2008, Suzuki *et al.* 2008). Cre expression is first detected by reporter gene expression in migrating primordial GCs at e7.75 in both sexes. While specific for GCs, the reported recombination is limited to 25% of GCs, potentially limiting the utility of the line for analysis of GC transcripts. However, in practice, this limited

recombination has been seen as an advantage, and the line exploited extremely successfully for lineage tracing and dissecting the process of spermatogonial stem cell division and renewal (Sada *et al.* 2009).

The TNAP-Cre was generated through targeted insertion into the tissue non-specific alkaline phosphatase (TNAP) gene (Lomeli *et al.* 2000). While the Cre is expressed in PGCs, targeting is limited to 60%, with further widespread expression during foetal life that limits the utility of the line for specific targeting of postnatal GC products.

The Ngn3-Cre is driven by a 23 kb promoter fragment initially isolated from a BAC clone containing the neurogenin 3 gene and regulatory region (Yoshida *et al.* 2004). Ngn3-Cre is expressed from day 7, and within the gonad, it is limited to pre-spermatogonia and spermatogonia. Ngn3-Cre recombines with high efficiency, and in adulthood reporter, gene expression can be detected in the vast majority of spermatocytes and spermatids, making this an excellent Cre line of choice for targeting GCs.

The Vasa-Cre (also called DEAD box polypeptide 4, *Ddx4*; Gallardo *et al.* 2007), which is GC-specific, expressed from embryonic day 15, and induces recombination in >97% of GCs in both sexes, also represents a good choice to circumvent issues associated with targeting during meiosis. Interestingly, because the Vasa-Cre is expressed in GCs of both sexes parent of origin, effects of the transgene make a significant difference to the outcome of progeny. When the female parent carries the Vasa-Cre transgene, persistence of Cre protein in the fertilised oocyte induces recombination in the zygote and results in global Cre-mediated recombination of the inherited floxed gene in all offspring. Conversely, because Cre does not persist in spermatozoa, inheritance of the Vasa-Cre from the male parent results in GC-specific expression of Cre in the resulting offspring.

Of all of the GC Cre lines, the Stra8-Cre could perhaps be considered the ideal for targeting GCs specifically in the adult testis (Sadate-Ngatchou *et al.* 2008). A 1.4 kb promoter fragment from the stimulated by retinoic acid gene 8 (*Stra8*) gene drives Cre expression from early postnatal life. Expression is male-specific and limited to spermatogonia and preleptotene spermatocytes throughout life, negating the problems associated with spermatocyte-Cre lines discussed above. Identification of GFP expression in Z/EG-positive pups from Stra8-Cre:Z/EG matings to wild-type females demonstrates that recombination efficiency in GCs is >95%.

Somatic cell Cre lines

The majority of Cre recombinase lines have been designed such that Cre is driven from a somatic cell type- or tissue-specific promoter. These now number in the hundreds (Nagy *et al.* 2009), and several have been

specifically designed or serendipitously demonstrated to target somatic cells of the testis. Several Cre lines express the transgene in more than one cell type within the testis. While in some instances, this can be problematical, making interpretation of results difficult, in some instances, for example when the floxed gene is only expressed in one of the two cell types, or the researcher wishes to target both cell types simultaneously, then this can be an advantage.

The SF-1-Cre of Bingham *et al.* (2006) utilises a BAC containing 111 kb of the steroidogenic factor-1 (nuclear receptor subfamily 5, group A, member 1, *Nr5a1*) locus to drive Cre recombinase through insertion of Cre into the ATG initiator methionine. Reporter gene expression was observed in the adrenal cortex, testes and ovaries. Within the testis, β -galactosidase expression was detected in Leydig cells (LCs) with expression also likely within other interstitial cells and peritubular myoid cells (PTM). Sertoli cell (SC) expression was also observed consistent with the normal expression pattern of SF-1. No reporter expression was detected in GCs. The early onset of the Cre (reporter expression detectable at E10.5) has led to exploitation of this line for studies of gonadogenesis (Kim *et al.* 2007, Maatouk *et al.* 2008, Barsoum *et al.* 2009, Krentz *et al.* 2009, Huang & Yao 2010).

A further Cre line targeting testicular somatic cells was generated through a targeted knockin to the anti-Müllerian hormone receptor 2 (*Amhr2*) locus (Jamin *et al.* 2002). Cre activity was observable at 11.5 dpc in the genital ridge, and at 12.5 dpc, it has been found exclusively in the mesenchyme surrounding the Müllerian duct epithelium and in somatic cells of the male and female gonad. In the testis, expression was identified in SCs and in LCs. The Amhr2-Cre line has been used to target genes of interest in the somatic cells of the testis (Jeyasuria *et al.* 2004, Tanwar *et al.* 2010), but the majority of studies utilising this mouse line have focussed upon targeting within female reproductive tissues.

The influence of site of transgene insertion is elegantly shown in three mouse lines that all arose through targeting of the same construct, namely the Inhibin- α iCre (Jorgez *et al.* 2006). Utilising the identical 6 kb of promoter, lines derived from three founders expressed Cre either predominantly in ovaries – line 1, predominantly in the anterior pituitary and adrenal medulla – line 2, or predominantly in LCs and SCs (with some expression in GCs) – line 3. Thus, the choice of line to use follows the empirical data, not the expectation based upon original promoter choice.

The DMRT1-Cre is driven from 2.6 kb of the pig doublesex and mab-3-related transcription factor 1 (*Dmrt1*) 5' region (Boyer *et al.* 2002). Mating to an YFP reporter (Srinivas *et al.* 2001) revealed detectable expression in genital ridges of both sexes from e12.0. In the developing testis, expression was restricted to the testis cords. Breeding from male mice transgenic for both DMRT1-Cre and YFP resulted in the production of

embryos that expressed YFP ubiquitously confirming that, in addition to SCs, DMRT1-Cre is expressed in GCs. Outside of the testis, expression could be detected in somites and the brain from e11.5.

A Dhh-Cre was generated and utilised to knock out *Gata1* selectively from SCs (Lindeboom *et al.* 2003). The Dhh-Cre utilises a 19 kb fragment of the desert hedgehog (*Dhh*) gene which drives Cre expression in SCs and endothelial cells of the blood vessels, which is detectable using a LacZ reporter gene at e15. Outside of the testis, the Dhh-Cre is expressed in Schwann cells of the peripheral nervous system (Darbas *et al.* 2004).

Sertoli cells

Several Cre lines have been developed that specifically target the SCs. In Abp-Cre mice, 1.5 kb of the rat androgen-binding protein alpha (*Abpa*) promoter was used to drive Cre. Expression was detectable from e17 and restricted to SC (Lim *et al.* 2009). Two independent groups have used the anti-Müllerian hormone (*Amh*, or *MIS*) promoter (Lecureuil *et al.* 2002, Holdcraft & Braun 2004) to generate Amh-Cre mice. Both Amh-Cre lines display cell-specific expression from foetal life and have been used widely due to their highly restricted expression pattern and robust efficiency. That there are not more SC-specific Cre lines is probably a reflection that the ones already generated not only tick all of the boxes, but also have been widely distributed.

Leydig cells

LC-specific targeting has been a relatively recent addition to the toolkit of the researcher. Two published lines aimed at targeting steroidogenic cell lineages exhibit expression restricted to LCs within the testis (while also targeting steroidogenic organs outside of the testis such as adrenals). Use of 4.4 kb of the human *Cyp11a1* promoter (which makes P₄₅₀-SCC) has been exploited to make a SCC-iCre (Wu *et al.* 2007). In addition to LC expression, the SCC-iCre is expressed in adrenals and the brain. The *Cyp17*-iCre (Bridges *et al.* 2008), which uses 3.34 kb of the mouse P₄₅₀ 17 α -hydroxylase/17,20-lyase promoter, also displays expression within the testis limited to LCs (when examined at E16.5). Outside of the testis, expression was observed in adrenals, the intermediate lobe of the pituitary. Minor reporter gene expression was also detected in other tissues, and widespread expression was detected in liver.

Endothelial cells

While to date, targeting of endothelial cells within the testis has not been a major focus for researchers, several papers describing endothelial cell-specific Cre lines do

show images of testicular expression, demonstrating this is a possibility (Alva *et al.* 2006, Monvoisin *et al.* 2006, Kawashima *et al.* 2009). Characterisation of the testicular expression of the endothelial-specific receptor tyrosine kinase Tie2-Cre, first reported by Kisanuki *et al.* (2001), confirms that Tie2-Cre mice are fertile and express Cre in all endothelial cells of the testis making this an excellent choice for targeting of floxed genes in endothelial cells (Fig. 2).

Smooth muscle (PTM cells and vascular smooth muscle)

Targeting of PTM cells has remained impossible for many years due to the absence of a usable and specific Cre line. This was recently overcome through utilising a combination of two Cre recombinase lines to control for extra sites of expression (Welsh *et al.* 2009). To target androgen receptor (AR) in PTM cells, the smooth muscle myosin heavy chain (*smMHC*-Cre) line (Xin *et al.* 2002), which targets both PTM cells and vascular smooth muscle (VSM; with occasional expression noted in GCs, which do not express AR), was used, along with a second line (transgelin, SM22-Cre; Boucher *et al.* 2003), which is VSM only to ensure any effects observed reflected targeting of the PTM cells directly (Welsh *et al.* 2009). Further to this, the same *smMHC*-Cre line was exploited to determine the role of AR in seminal vesicle smooth muscle (Welsh *et al.* 2010a), and the SM22-Cre line was exploited to determine the role of AR in testicular arterioles (Welsh *et al.* 2010b).

Inducible promoters

Inducible Cre lines are increasing in popularity due to the ability for elegant temporal as well as spatial targeting of genes (Kuhn *et al.* 1995, Sauer 1998). However, the systems currently available appear to have been specifically designed to make targeting of the postnatal testis difficult.

The most widely used inducible Cre lines utilise a Cre fused to mutant ligand-binding domain of oestrogen receptor (Feil *et al.* 1996). This exploits the system of signalling of the steroid hormone receptors, which remain inactive in the absence of ligand. Delivery of the selective oestrogen receptor modulator tamoxifen leads to Cre entry into the nucleus and recombination. Thus, Cre can be expressed, but does not function until ligand is added. The first lines to use this utilised CreER; however, this was found to be quite leaky, with recombination occurring in the absence of ligand, and was quickly superseded by the CreER^{T2} to provide higher specificity (Feil *et al.* 2009). However, tamoxifen treatment can impact upon the architecture and function of the rodent testis (Fisher *et al.* 1999, Motrich *et al.* 2007). Unless adequately controlled for, such effects may severely

curtail the ability to elucidate meaningful results from a study. Despite these caveats, with appropriate controls, tamoxifen-inducible Cre lines can and have been used successfully to target genes in reproductive tissues (Weber *et al.* 2003, John *et al.* 2008, Qin *et al.* 2008).

Another approach has been to utilise a Cre driven by a heat shock promoter (Dietrich *et al.* 2000). In this case, mild heating of the mouse leads to activation of the Cre and recombination. While this has proven extremely useful for experiments focussing on early testis development (DiNapoli *et al.* 2007, Kim *et al.* 2007), the 'mild' increase in temperature required to induce Cre also results in spermatocytes death and a stress response that leads to a major disruption to testicular architecture (Paul *et al.* 2008, 2009), making this unsuitable for studies aimed at targeting genes in the testes of adolescent or adult mice. The widely used Tet-On system of inducible gene activation (Sprengel & Hasan 2007) has been modified to encompass the use of Cre recombinase (Belteki *et al.* 2005). However, as yet, this methodology does not appear to have been widely applied to the testis. For more focussed analyses, other options available are use of a cell-permeable Cre, which has been used to target spermatogonial stem cells *ex vivo*, prior to testicular transplantation (Grisanti *et al.* 2009). Further to this is the development and application of Cre adenoviral constructs (Takehashi *et al.* 2007), which have the advantage of not integrating into the genome, yet are prone to induction of an immune response *in vivo* (Hooley *et al.* 2009). This could be avoided by moving towards the less immunogenic lentiviral system, which has been validated *in vivo* where it selectively transduces SCs (Ikawa *et al.* 2002).

Future perspectives

The widespread and ever-increasing availability of large numbers of Cre recombinase-expressing lines suggests that future use of the Cre/Lox system for analysis of the testis is assured. The development of conditional alleles of 20 000 protein-coding genes that is currently underway (<http://www.knockoutmouse.org/>, EuComm: www.eucomm.org, NorComm: www.norcomm.org, KOMP: www.komp.org) will make it possible to target any gene in any cell type at any time of choosing. Such powerful technologies will allow ever more complex experiments and more detailed dissection of the key events underpinning testis development and function, information that will be essential for development of novel therapies for reproductive or steroidogenic conditions in humans. The testis it seems is about to give up its secrets.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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