Transgenic mouse models in the study of reproduction: insights into GATA protein function

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Abstract

For the past 2 decades, transgenic technology in mice has allowed for an unprecedented insight into the transcriptional control of reproductive development and function. The key factor among the mouse genetic tools that made this rapid advance possible is a conditional transgenic approach, a particularly versatile method of creating gene deletions and substitutions in the mouse genome. A centerpiece of this strategy is an enzyme, Cre recombinase, which is expressed from defined DNA regulatory elements that are active in the tissue of choice. The regulatory DNA element (either genetically engineered or natural) assures Cre expression only in predetermined cell types, leading to the guided deletion of genetically modified (flanked by loxP or 'floxed' by loxP) gene loci. This review summarizes and compares the studies in which genes encoding GATA family transcription factors were targeted either globally or by Cre recombinases active in the somatic cells of ovaries and testes. The conditional gene loss experiments require detailed knowledge of the spatial and temporal expression of Cre activity, and the challenges in interpreting the outcomes are highlighted. These studies also expose the complexity of GATA-dependent regulation of gonadal gene expression and suggest that gene function is highly context dependent.

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Introduction

Transgenic technologies in mice

Global (germline) targeting technology

What we observe is not nature itself, but nature exposed to our method of questioning

Werner Heisenberg

The introduction of the first transgenic mouse lines 30 years ago opened the new chapter in biomedical science (Costantini & Lacy 1981, Wagner *et al.* 1981). Soon thereafter, the isolation of mouse pluripotent embryonic stem cells (ESCs) coupled with the technique of targeted (homologous) DNA recombination formed the foundation for carrying out gene deletion or modification in mouse genome (Thomas *et al.* 1986, Thomas & Capecchi 1987). These two closely related transgenic technologies revolutionized biomedical and biotechnological enterprises and quickly established genetically engineered mice as an unrivaled model system for understanding human development and disease. Today, mice remain the most widely used model organism in health sciences due to their genetic and physiological similarities to humans, fairly low maintenance costs, a remarkably short reproductive period, and robust

fecundity. The importance of the mouse as a biomedical species of choice is reflected in the fact that its full-genome sequence became available shortly after that of a human (Lander *et al.* 2001, Waterston *et al.* 2002).

Global, or germline, gene targeting describes a process in which genetic modifications (including complete or partial promoter or gene deletions or gene mutations) are introduced into a specific locus through the use of homologous recombination in ESCs. The targeting DNA cassette is delivered to ESCs by electroporation and the recombinant clones are selected by their drug resistance. One copy of the WT gene can be replaced with the targeting construct by homologous recombination. Once the ESC clone containing the desired recombination event is identified, the modified ESCs are injected into the blastocysts that are transferred into foster mothers. The offspring born from the foster animals is comprised of cells that originated from two genetically unrelated strains (blastocyst and ESCs) and is termed chimeric founder animals. If a chimeric animal possesses germ cells derived from the ESC clone, the genetic alteration will be propagated to the offspring, generating heterozygous mutant mice. Animals that are heterozygous for the desired mutation are interbred. This method is widely used for the generation of a null allele (a knockout) or a modified allele (a knock-in).

Despite many technical advances, the ESC-based knockout technology in mice remains far from trivial and requires highly trained professionals to avoid numerous hurdles along the way. Over the years, many laboratories attempted to simplify the targeting process, make it more versatile and applicable to species other than mice. While the detailed survey of these technologies is outside of the review's scope, several new developments in genome editing are noteworthy. The TALEN (transcription-activator-like effector nuclease) approach employs a fusion protein comprising non-specific double-strand break-inducing nuclease domain of FokI restriction enzyme and a TALE, a modular sequence-specific DNA-binding protein. TALE repeat domains could be designed and assembled for recognizing any desired DNA sequence following a simple 'one-domain-to-one-nucleotide' code (reviewed in Pennisi (2012)). Recently TALEN technology has been successfully used for overcoming a long-standing problem of targeting Y chromosome in mice (Wang *et al.* 2013).

Another genome-editing strategy based on the bacterial CRISPR (clustered, regularly interspaced, short palindromic repeat)-associated protein 9 nuclease from *Streptococcus pyogenes* (Cas9) holds even more promise. Cas9 is an RNA-guided nuclease that uses conventional base-pairing rules to deliver its nuclease moiety to a target DNA sequence with a swappable genetically engineered RNA. CRISPR–Cas9 system eliminates the need for ESC step altogether, allows targeting several genes at once, and is applicable to a wide variety of species (for recent reviews, see Mali *et al.* (2013) and Sander & Joung (2014)).

Transgenic Cre/Lox technology

Similar to global gene targeting, conditional gene targeting also produces gene deletions or mutations, but these modifications are intended to be limited in a temporal and spatial manner. The so-called 'Cre/Lox' targeting technology is modeled after a bacteriophage P1 recombination system and is currently the most commonly used approach for gene modification in mice. This strategy, which also employs ESC, is initiated by incorporating two strategically positioned 34-bp DNA sequences (LoxP sites) into the targeted locus. The P1 Cre recombinase specifically recognizes a pair of LoxP sites in the whole genome and performs DNA strand exchange between them. This process results in the excision of the intervening sequence. In a targeting DNA construct, the two LoxP sites are usually inserted in the nonessential intron sequences flanking exonic DNA coding for a critical portion of the protein. This arrangement allows the gene that is flanked by LoxP sites ('floxed') to retain its WT activity until Cre recombination removes or replaces the essential coding element, leading to gene inactivation or modification respectively.

For the experiment, a mouse line derived from ESCs that contain the floxed gene of interest (Floxed strain) is crossed with another strain that expresses the Cre recombinase (Cre strain) in a specific tissue or cell type. Recombination and inactivation of the target gene are restricted to cells that activate the expression of Cre recombinase; in all other cells and tissues, the floxed gene retains both of the LoxP sequences and remains active. A critical improvement in the versatility of the Cre/Lox system came with the introduction of inducible Cre lines. Inducible Cre mouse strains express a modified, latent form of Cre recombinase that can be activated by an inducing agent (e.g. tamoxifen) administered at a desired time point in embryonic development or in adult life (see Jensen & Dymecki (2014) for a recent review).

A critical element of a typical Cre/Lox experiment is the characterization of Cre recombinase activity. This experiment is normally carried out by crossing a Cre-strain animal with a mouse of the opposite sex, harboring a transgenic cassette that includes the reporter gene controlled by the ubiquitously expressed promoter (Reporter strain). The floxed interrupter 'stop' sequence separates the reporter gene from its regulatory element and prevents the synthesis of the gene product in the Reporter strain. When these animals are crossed, the progeny that inherits both Cre gene and the reporter is used to assess Cre activity. The Cre-mediated excision of the interrupter sequence then brings the regulatory sequence in close proximity to the reporter gene and activates its expression. The promoter element used in these experiments is usually that of the ubiquitously expressed ROSA locus (Zambrowicz *et al.* 1997). Reporter genes normally encode proteins that can be detected without relying on a specific antibody, and the ones most commonly used are fluorescent proteins (enhanced green fluorescent protein (EGFP) or RFP), β -galactosidase (β -gal), or alkaline phosphatase. Importantly, not only cells expressing Cre recombinase but also their direct progeny will stain positive for the reporter protein. As activation of a reporter gene permanently labels the progenitor cell where Cre recombination occurred, that expression is retained in all of the cell's descendants even in the absence of concomitant Cre activity. This experiment is a centerpiece of the lineage tracing (fate mapping) studies.

Transgenic technology in the analysis of GATA protein in gonadal function

Expression of Gata genes in mice

Transcription factors of the GATA family possess a signature double zinc finger DNA-binding domain that recognizes the GATA motif (WGATAR) found in the regulatory regions of numerous genes (reviewed in Molkenin (2000), Cantor & Orkin (2005), Zaytouni *et al.* (2011) and Chlon & Crispino (2012)). Six GATA

proteins present in vertebrates are customarily divided into two groups of three, based on the expression pattern. GATA1–3 are most prominent in hematopoietic cell differentiation; in addition, they perform important functions in several other tissues (e.g. endothelial cells and neurons). GATA4–6 are widely expressed outside of the hematopoietic system, including the gonads, heart, liver, lung, pancreas, and gut (reviewed in Patient & McGhee (2002), LaVoie (2003) and Viger *et al.* (2008); see also Carrasco *et al.* (2012) and Xuan *et al.* (2012)). GATA proteins cooperate with other transcription factors to form a regulatory network that also includes non-DNA-binding interacting partners as well as other proteins with various enzymatic activities. It is hypothesized that GATA proteins serve to amplify (rather than specify) the expression of lineage-restricted genes, but the definitive proof of this hypothesis is still lacking.

Expression of Gata genes in reproductive organs

Gata4 appears to be the only GATA family member active in the development of bipotential gonad before sexual determination in mice. At embryonic day (E) E10.5–E11.5, *Gata4* is already expressed in the somatic cells of the developing genital ridges (Defalco *et al.* 2011, Buganim *et al.* 2012, Hu *et al.* 2013). At E13.5, *Gata4* expression becomes sexually dimorphic; in the testis, expression is notably upregulated in Sertoli cells and reduced to some extent in interstitial cells, whereas strong-to-moderate GATA4 expression is observed in the majority of ovarian somatic cells (Heikinheimo *et al.* 1997, Viger *et al.* 1998, Anttonen *et al.* 2003, Manuylov *et al.* 2008, Efimenko *et al.* 2013). A similar pattern of *Gata4* expression has been reported in rats (Lavoie *et al.* 2004). In the postnatal ovaries, GATA4 is abundantly expressed in the granulosa cells of primary, preantral, and antral follicles as well as in endocrine theca cells, but not in primordial follicles or luteal cells (Viger *et al.* 2008). The other GATA protein, GATA6, closely follows GATA4 early in the ovarian development, with continuous expression in granulosa cells and theca cells of large follicles. In contrast to GATA4, GATA6 is present in oocytes and luteal cells (Heikinheimo *et al.* 1997, Viger *et al.* 1998). *Gata2* expression in the murine embryonic ovary has also been described and is restricted to the germline from E11.5 to E15.5 (Siggers *et al.* 2002, Bhardwaj *et al.* 2008); however, the functional significance of this expression remains unknown.

In the testes, *Gata4* expression persists in the somatic cells throughout life, with both Sertoli and Leydig cells expressing the protein (Viger *et al.* 1998, Anttonen *et al.* 2003, Imai *et al.* 2004). Similar to the ovary, GATA6 is another protein present in the somatic cells of the testis, and its expression was reported to commence at E14.5 in Sertoli cells (Robert *et al.* 2002). The expression of GATA6 in fetal Leydig cells has not been analyzed in detail; the data from our laboratory show that at

E15.5 GATA6 expression in fetal Leydig cells appears higher than that in Sertoli cells, while adult Leydig and Sertoli cells express the protein at a comparable level (SG Tevosian, unpublished observations).

In addition to GATA4 and GATA6, postnatal Sertoli cells express a third GATA protein, GATA1 (Yomogida *et al.* 1994). GATA1 expression is first observed in Sertoli cells in postnatal day 7 (PND 7) mice, and GATA1 is evenly expressed in all seminiferous tubules until 3 weeks of age. Shortly after, solitary Sertoli cells lacking GATA1 start to appear in some seminiferous tubules; in the adult testis, GATA1 expression is restricted to seminiferous tubules at stages VII, VIII, and IX of the seminiferous epithelial cycle (Yomogida *et al.* 1994). This expression pattern suggests that *Gata1* expression in Sertoli cells is suppressed during embryonic development and by a signal originating from germ cells in the adult. Sertoli cell expression of GATA1 is noteworthy as this is the only place outside of the hematopoietic system where this protein is found. Despite the intriguing expression pattern, experiments aimed at determining the role of GATA1 in the testis suggest that GATA1 is not essential for Sertoli cell function (Fujiwara *et al.* 1996, Lindeboom *et al.* 2003). The expression of GATA3 or GATA5 has not been reported for mammalian gonads.

GATA protein function in the cells of the reproductive system

Before transgenic techniques became widely available in mice, the established approach for understanding gene function was overexpression studies in cultured cells. Here again, most studies focused on the role of the GATA4 protein and revealed several prospective targets of GATA4 regulation in the testis. Specifically, these studies suggested that GATA4 participates in the regulation of anti-Müllerian hormone (*Amh*; Viger *et al.* 1998, Tremblay & Viger 1999, Watanabe *et al.* 2000), steroidogenic factor 1/nuclear receptor subfamily 5, group A, member 1 (*Sf1/Nr5a1*; Tremblay & Viger 2001), aromatase (*Cyp19a1*; Tremblay & Viger 2001), inhibin alpha (*Inha*; Ketola *et al.* 1999), *Star* (Silverman *et al.* 2006, Nishida *et al.* 2008), and *Rhox5* (Bhardwaj *et al.* 2008; see Viger *et al.* (2008) and Lavoie & King (2009) for review). In granulosa cells, GATA4 was implicated to regulate a partially overlapping gene set: *Cyp11a1* (Sher *et al.* 2007), *Star* (Silverman *et al.* 1999, 2006), *Cyp19a1* (Cai *et al.* 2007, Stocco *et al.* 2007), and *Inha* (Anttonen *et al.* 2006). Similarly, *Inha* (Ketola *et al.* 1999, Feng *et al.* 2000), *Star* (Martin *et al.* 2012), and luteinizing hormone receptor (*Lhcgr*; Rahman *et al.* 2004) were identified as genes that are dependent on GATA4 in immortalized Leydig cells. While these studies illuminated a wide range of gonad-expressed genes as likely GATA4 targets, definitive conclusions regarding their temporal and spatial regulation in gonadal cells by GATA proteins could only emerge as a result of gene manipulation *in vivo* in mice.

GATA protein function revealed by global gene deletions

An analysis of the function of GATA genes in reproduction through a global knockout has not been possible due to the embryonic lethality of *Gata4*^{-/-} (~E8.0) and *Gata6*^{-/-} (~E5.5) mice several days before the gonads arise (Kuo *et al.* 1997, Molkentin *et al.* 1997, Koutsourakis *et al.* 1999). However, a knock-in mutation was quite informative in defining the role of GATA4 in gonadal development. The function of GATA proteins is modified by binding to multi-type zinc finger cofactors of the FOG (Friend of GATA) family of transcription factors (for reviews, see Cantor & Orkin (2005), Zaytouni *et al.* (2011) and Chlon & Crispino (2012)). Two of these proteins are found in mammals: FOG1 and FOG2. FOG2 is co-expressed with GATA4 in the somatic cells of the gonads between E11.0 and E13.5; after E13.5, *Fog2* gonadal expression is notably reduced and remains low during subsequent embryonic development ((Lu *et al.* 1999, Svensson *et al.* 1999, Tevosian *et al.* 1999, Anttonen *et al.* 2003, Manuylov *et al.* 2007a); SG Tevosian, unpublished observations).

The interaction between the two protein partners GATA4 and FOG2 allowed for exploring the role of GATA4 in gonadal development *in vivo*. A *Gata4* knock-in allele (*Gata4*^{ki}, a V217G amino acid substitution) generates a mutant form of the GATA4 that is incapable of interacting with FOG proteins (Crispino *et al.* 2001). Homozygous *Gata4*^{ki/ki} embryos survive till E13.0 and die from cardiac malformations similar to those described in *Fog2*^{-/-} embryos (Tevosian *et al.* 2000, Crispino *et al.* 2001). An analysis of gonadal development in these animals demonstrated that both GATA4 and FOG2 partner proteins, as well as the physical interaction between them, are essential for male sex determination and testis differentiation (Tevosian *et al.* 2002). *Fog2*^{-/-} and *Gata4*^{ki/ki} mutant XY gonads express lower levels of the master regulator *Sry* (Gubbay *et al.* 1990) compared with the controls, and the expression of genes crucial for normal Sertoli (*Sox9*, *Amh* and Desert hedgehog (*Dhh*)) and Leydig (*Cyp11a1*, *3βHsd*, and *Cyp17a1*) cell development is absent (Tevosian *et al.* 2002).

Similarly, ovarian gene expression program is profoundly disrupted upon loss of the GATA4–FOG2 interaction (Manuylov *et al.* 2008). The majority of ovarian genes essential for granulosa cell function is no longer expressed in the *Gata4*^{ki/ki} and *Fog2*^{-/-} XX gonads. Specifically, both *Foxl2* and the canonical Wnt/β-catenin pathway, two critical pieces of the ovarian developmental program, are silenced in the absence of the GATA4–FOG2 interaction. Moreover, the *Dkk1* gene, which encodes a secreted inhibitor of canonical β-catenin signaling, is strongly (approximately tenfold) activated in these mutants as early as E11.5 (Manuylov *et al.* 2008).

GATA protein function revealed by conditional gene targeting

A gene expression analysis in mice with a global loss of the GATA4–FOG2 interaction revealed that GATA4 and its transcription partner, FOG2, are indispensable for initiating embryonic development of both the testis and ovary. However, the *Gata4*^{ki} mutation is not synonymous with a *Gata4* loss of function, as the mutant GATA4 protein is impaired only in its ability to interact with the cofactor FOG proteins (Crispino *et al.* 1999). Moreover, the analysis of GATA4 gonadal function is restricted at the earliest stages of gonadogenesis. This limitation is normally attributed to the mid-gestational embryonic lethality of the GATA4/FOG2 mutants that prevents further examination. However, another reason is just as important; in the absence of the proteins, gonadal development becomes unhinged at the sex determination step. For example, deletion of a *FOG2* gene (or *Gata4*^{ki} mutation) leads to the downregulation of *Sry* expression, which alone could account for the subsequent demise of the testis gene expression program (Tevosian *et al.* 2002). Other experimental approaches were clearly needed to understand GATA gene function beyond the sex determination stage.

The first *in vivo* evidence that the GATA4–FOG2 partnership has gonadal regulatory targets other than *Sry* came from the analysis of dominant XX sex reversed transgenic mouse models. The serendipitous insertion of the tyrosinase transgene upstream of *Sox9* in Odd sex animals (ocular degeneration with sex reversal, *Ods*) leads to the elevated expression of *Sox9* in XX mice (Bishop *et al.* 1999). Similarly, in *Wt1–Sox9* mice, *Sox9* is overexpressed in gonads from the *Wt1* regulatory elements within a yeast artificial chromosome (Vidal *et al.* 2001). In both lines, ectopic *Sox9* initiates testis differentiation and a dominant, female-to-male, sex reversal is observed. This sex reversal is completely suppressed in *Fog2* heterozygous animals and XX *Fog2*^{+/-}; *Wt1–Sox9* or *Fog2*^{+/-}; *Ods* mice develop into fertile females. The suppression of sex reversal in *Fog2* heterozygous females results from an ~50% downregulation of the *Sox9* transgenic expression. Importantly, neither of two transgenes is capable of initiating *Sox9* expression in *Fog2* null or *Gata4*^{ki/ki} embryos. These results suggested that female-to-male sex reversal observed in the transgenic XX gonads required an intact GATA4–FOG2 complex and relied on gene targets other than the Y chromosome-linked *Sry* gene that is absent in genetically female (XX) animals (Manuylov *et al.* 2007a). Similar conclusions were derived through the analysis of heterozygous *Gata4* and *Fog2* animals (Bouma *et al.* 2007).

Importantly, Cre/Lox recombination technology could be used successfully to identify multiple developmental requirements for a given protein for which the later roles are concealed by its early loss in a global knockout.

To show the requirement of GATA proteins in sexual development after birth, several groups of investigators generated loss-of-function mutations in gonads using various Cre recombinase strains. To reveal the nature of GATA regulatory targets, Cre-mediated *Gata4* gene deletion was carried out in the somatic cells of the testis. This approach circumvented the early embryonic lethality of global *Gata* knockouts and permitted the analysis of gonadal development that resulted from temporally and spatially restricted GATA inactivation.

GATA proteins in testis development

Sf1 (bacterial artificial chromosome (BAC)) Cre-mediated gene deletion demonstrates *Gata4* function in testis development beyond sex determination. A Cre line of mice based on a BAC containing the *Sf1* (*Nr5a1*) locus has been described (Bingham *et al.* 2006). This *Sf1Cre* is robustly expressed in places where the *Sf1/Nr5a1* gene is active, and the expression initiates before sex determination in the somatic cells of the developing gonads. *Sf1Cre* has been used successfully by several investigators to target genes expressed in gonads and adrenal gland (Kim *et al.* 2007a, 2008, Huang & Yao 2010). In the testis, a *Sf1Cre*-mediated *Gata4*^{fllox/fllox} conditional deletion (designated as *Sf1Cre; Gata4cKO*) leads to the loss of GATA4 by E12.5 (Manuylov *et al.* 2011). These animals reveal a committed function of GATA4 in forming discrete testis cords and helped to identify genes that rely upon GATA4 regulation starting at E12.5.

Microarray analysis comparing control and mutant testis identified numerous gene expression changes upon *Sf1Cre*-mediated GATA4 loss. Interestingly, the gene that provided the most intriguing clues to GATA4 role in testis transcriptional regulation, *Dmrt1*, did not emerge through the microarray analysis, but was instead identified in a candidate gene approach. Expression and function of *Dmrt1* gene have been extensively studied in multiple species (Matsuda *et al.* 2002, Smith *et al.* 2009, Yoshimoto *et al.* 2010). Remarkably, DMRT1's penchant for sex regulation is conserved not only among vertebrates, but also in *Drosophila* and nematodes (Raymond *et al.* 1998). This is quite unusual given that sex-determining mechanisms have little in common across phyla and even between vertebrates. In mammals *Dmrt1* is highly specific to embryonic gonads where it is expressed in the developing Sertoli cells as well as in germ cells of both sexes. In the adult animals *Dmrt1* expression becomes confined to the postnatal testis. Reports of disorders of sexual development in human patients strongly suggest that DMRT1 is important for male sex determination and embryonic testis differentiation (Raymond *et al.* 1999). Surprisingly, knockout of *Dmrt1* in mice (Raymond *et al.* 2000) did not reveal the requirement for this gene during embryogenesis and prenatal testis differentiation of *Dmrt1*^{-/-} mutants is

unremarkable. Cre/Lox approach was instrumental in establishing the functions for *Dmrt1* separately in the testis somatic and germ cell compartments. Postnatal differentiation of Sertoli cells is impaired upon Sertoli cell-specific DMRT1 loss, while centrifugal migration of developing germ cells to the tubular periphery and gonocyte mitotic reactivation and viability require germ cell DMRT1 expression (Kim *et al.* 2007b). Recent research has highlighted a role for *Dmrt1* in maintaining the fidelity of testis sex determination (Matson *et al.* 2011).

Manuylov *et al.* (2011) have shown that DMRT1 protein expression is lost from the Sertoli cells of *Sf1Cre; Gata4cKO* embryonic testis (Manuylov *et al.* 2011). The expansion of Sertoli cells in these mutants to completely fill the developing testis cords (Manuylov *et al.* 2011) strongly resembles the phenotype in postnatal *Dmrt1*^{-/-} mutants (Raymond *et al.* 2000). Importantly, the testis cord defect in the *Sf1Cre; Gata4cKO* animals is manifested embryonically, considerably earlier than that in *Dmrt1*^{-/-} animals at PND 14. The most parsimonious explanation for this outcome is the deregulation of the expression of other genes in the absence of GATA4. Microarray analysis identified multiple genes that are strongly downregulated as a result of GATA4 loss. The expression of two such genes, cystatin 9 (*Cst9*) and clusterin (*Clu/ApoJ*), is easily detectable by *in situ* hybridization in the control testis but no longer observed in the E14.5 *Sf1Cre; Gata4cKO* organ. No testis abnormalities have been reported in mouse mutants deficient for either gene (Bailey *et al.* 2002, Tohonen *et al.* 2005, Hasegawa *et al.* 2006), however their combined loss could be a contributing factor in the *Sf1Cre; Gata4cKO* phenotype. Moreover, a role for *Clu/ApoJ* in augmenting cell death in the testis has been proposed (Kaisman-Elbaz *et al.* 2009), and the downregulation of *Clu/ApoJ* upon GATA4 absence may allow Sertoli cells to evade apoptosis.

In summary, the range of genes controlled by GATA factors in the somatic cells is highly dynamic (see also below), however these results provide *in vivo* evidence for GATA4 requirement in Sertoli cell *Dmrt1* expression and in organizing the development of testis cords. Importantly, the loss of *Dmrt1* expression is not merely a part of the overall failure of Sertoli cell differentiation in the absence of GATA4, as the majority of Sertoli-specific genes are expressed normally. Furthermore, the requirement for GATA4 in maintaining *Dmrt1* expression is not absolute, and other GATA proteins are likely competent to perform this task. Specifically, while GATA4 is obligatory for DMRT1 expression in embryonic Sertoli cells where it is a sole GATA factor, the DMRT1 protein reappears in the *Sf1Cre; Gata4cKO* postnatal testis where GATA6 and GATA1 remain strongly expressed. Similarly, after the acute loss of *Gata4* in the inducible *Wt1CreERT2; Gata4cKO* adult mice (see below), the somatic expression of *Dmrt1* remains unchanged (Manuylov *et al.* 2011).

The upstream regulatory DNA elements guiding Sertoli cell *Dmrt1* expression were examined in primary cultures of rat Sertoli cells (Lei & Heckert 2004, Lei *et al.* 2009). It was reported that the minimal region necessary for Sertoli cell-specific transcriptional activity is located between -3.2 and -2.8 kb upstream of the transcriptional start site and contains several regulatory elements capable of binding GATA4. Whether the same enhancer element is engaged by GATA4 in species other than rat remains to be established.

Sf1Cre; Gata4cKO male mice exhibit neither the early arrest of testis differentiation that is characteristic of the GATA4^{ki} phenotype (Tevosian *et al.* 2002) nor sex reversal. Although their testes eventually succumb to a developmental block and undergo germ cell attrition, gonadal differentiation in these mutants appears to initiate normally (Manuylov *et al.* 2011). This phenotype occurs despite the fact that *Sf1Cre; Gata4cKO* animals lose GATA4 protein expression, while GATA4^{ki} gonads express the full-length protein that retains most of its function. The likely explanation for an improved outcome is the timing of the *Gata4* gene deletion that follows rather than precedes sex determination in *Sf1Cre*-conditional animals. At E11.5, many GATA4-positive cells remained detectable in the *Sf1Cre; Gata4cKO* gonads (Manuylov *et al.* 2011). These residual GATA4-positive cells are present within a critical window of time (E11.0–E11.25) during which SRY is competent to activate the Sertoli cell pathway in a sufficient number of precursor cells ((Bullejos & Koopman 2005, Hiramatsu *et al.* 2009); see Kashimada & Koopman (2010) for review). This timing of excision hypothesis was further explored using an inducible *Wt1CreERT2* as described below.

Dynamic phenotypes of *Gata4* loss during gonadal development. To evaluate the hypothesis that the timing of *Gata4* gene deletion relative to the window of sex determination is critical, Manuylov *et al.* (2011) used tamoxifen-inducible *CreERT2* expressed from the endogenous *Wt1* locus (*Wt1CreERT2*) (Zhou *et al.* 2008). Pregnant females carrying litters containing mutant fetuses (*Wt1CreERT2; Gata4^{flox/flox}*, further *Wt1CreERT2; Gata4cKO*) were injected with tamoxifen to activate *Cre* function, and the fetuses were harvested several days later. Upon injection with tamoxifen at E10.5, *Wt1*-driven *CreERT2* was active in somatic progenitor cells, recapitulating endogenous *Wt1* gonadal expression. Unlike *Sf1Cre; Gata4cKO* embryos, E15.5 XY embryos that underwent E10.5 *Wt1CreERT2*-mediated deletion of floxed *Gata4* (*Wt1CreERT2; 10.5_Gata4cKO*) exhibited a comprehensive block of testicular differentiation and male-to-female sex reversal. Specifically, expression of male-specific genes (e.g. Sertoli marker SOX9 or Leydig 3 β HSD) in the XY *Wt1CreERT2_10.5_Gata4cKO* embryos was either strongly diminished or not observed. Instead, the gonadal cells in the mutants initiated the expression

of the early ovary-specific marker FOXL2. In summary, compared to the *Sf1Cre; Gata4cKO* mutants that develop atypical, but unambiguous testes, male pathway of differentiation in *Wt1CreERT2_10.5_Gata4cKO* gonads was profoundly disturbed by the absence of the GATA4 protein (Manuylov *et al.* 2011). In contrast, the activation of *Wt1CreERT2* just 1 day later at E11.5 (*Wt1CreERT2; 11.5_Gata4cKO*) led to a phenotype very similar to that of *Sf1Cre; Gata4cKO* animals. This result suggests that both *Sf1Cre; Gata4cKO* and *Wt1CreERT2; 11.5_Gata4cKO* males do not experience sex reversal, as sufficient numbers of GATA4-positive cells persist to support male sex determination during the window of opportunity.

These results establish the requirement for the GATA4–FOG2 complex in somatic sex specification. In developing gonads, supporting cell progenitors depend on the transcriptional regulatory network that includes GATA4 and FOG2 to successfully transition from their undifferentiated bipotential state to acquire a male or female sex-specific identity (Tevosian *et al.* 2002, Buganim *et al.* 2012). This is corroborated by the fact that gonads in $\text{Gata4}^{\text{ki/ki}}$ or *Fog2*^{−/−} mutants no longer express male-specific (*Sox9*, *Dhh*, *Amh*, or somatic *Dmrt1*; Tevosian *et al.* 2002, Manuylov *et al.* 2011) or female-specific (*Foxl2*, *Fst*, or *Sprr2d*; Manuylov *et al.* 2008) genes. In contrast, conditional gene deletion is fully complete only past the point of sex determination. After somatic cells commit sex determination, the majority of Sertoli-specific genes no longer require GATA4 for their continuous expression. Hence, the range of GATA4 duties in differentiated Sertoli cells appears to focus on regulating a subset of its former targets (e.g. *Dmrt1*). The limited set that remains under GATA4 control includes genes required for Sertoli cell maturation, testis cord organization and germ cell viability.

Recently, Hu *et al.* (2013) demonstrated an even earlier requirement for GATA4 in the developing reproductive system. A tamoxifen-inducible *Cre/Lox* system where the ubiquitously expressed synthetic chicken β -actin (*CAG*) promoter drives *CreER* expression was used to conditionally delete *Gata4* in mouse embryos after E8.75. This approach allowed conditional mutant embryos to survive to $\sim$ E11.5. Embryos deficient for *Gata4* at this developmental time point show neither coelomic epithelial thickening nor expression of the early gonadal differentiation factors LHX9 and NR5A1. This study indicates that *Gata4* is required for the formation of the genital ridge in mice prior to its role in gonadal sex determination.

Thus far, no *Cre/Lox* approach had been described that inactivated *Gata4* to produce a phenotype similar to that of a *Fog2* deletion or $\text{Gata4}^{\text{4ki}}$ mutation with a sex determination block, early demise of both sex differentiation programs, and a characteristic change in gene expression pattern. For example, the tenfold increase in *Dkk1* gene expression observed in $\text{Gata4}^{\text{4ki/ki}}$ gonads at

E11.5 (Manuylov *et al.* 2008) has not been recapitulated in any conditional setting. Most likely, this result is due to a technical difficulty of generating a suitable Cre carrier line capable of deleting genes just in time to block sex determination in a newly developed genital ridge (Hu *et al.* 2013). An attempt to delete *Gata4* using Wt1CreERT2 with induction a day earlier, at E9.5, led either to a sex reversal (as described above for the E10.5 induction) or to embryonic lethality (most likely due to the loss of *Gata4* function in the pericardium) (Zhou *et al.* 2008). It is formally possible that, under condition of GATA4 loss, GATA6 could now associate with FOG2 to form the GATA6–FOG2 complex to substitute for the missing GATA4–FOG2 partnership. If GATA4^{ki} isoform acts as a dominant negative and prevents GATA6–FOG2 complex from interacting with DNA, this complex may become active in the absence of GATA4 protein. While this will explain a milder phenotype of *Sf1Cre*; *Gata4cKO* animals compared with the *Gata4^{ki/ki}*, this possibility is remote. Before sexual differentiation, *Fog2* gene deletion or *Gata4^{ki}* mutation led to synonymous phenotype in XY or XX gonads arguing against dominant negative role for GATA4^{ki} (Tevosian *et al.* 2002, Manuylov *et al.* 2008). Moreover, the somatic cells of the developing testis exhibit no overt expression of other GATA family members (including GATA6) at least until E13.5 (unpublished observations).

The deletion of *Gata4* by an *Amhr2Cre* (see below) was also reported (Kyrölähti *et al.* 2011a). In contrast to the *Gata4* deletions described earlier, no overt defects in embryonic or early postnatal testicular development in *Amhr2Cre*; *Gata4^{flox/flox}* males were observed. Gene expression in both Sertoli and Leydig cells was also normal. The absence of a prenatal testicular phenotype probably reflects the delayed or inefficient *Amhr2Cre*-mediated recombination in the embryonic and fetal testes. Notably, the reproductive organs of aged animals developed testicular atrophy and loss of fertility. The authors report Sertoli cell vacuolization and progressive tubule destruction with concomitant germ cell loss. In this respect, Wt1CreERT2 was also activated in the sexually mature adult animals to produce a *Gata4* deletion in the testis. Wt1CreERT2-mediated deletion of *Gata4* in the Sertoli cells of the adult organ was efficient but had no immediate deleterious effect on the testis appearance or spermatogenesis (Manuylov *et al.* 2011). These data strongly suggest an overlap of the functions of GATA proteins in the adult testis where all three GATA family members (GATA1, GATA4, and GATA6) are robustly expressed.

GATA proteins in ovarian development

Conspicuous expression of the *Gata4* and *Gata6* genes in the mouse ovary was discovered a number of years ago (Heikinheimo *et al.* 1997, Viger *et al.* 1998). Given the prominent roles attributed to GATA family

members in the regulation of developmental gene expression, these proteins had long been suspected to have essential functions in the female gonad. Between the two proteins, the role for GATA4 in ovarian development and function is understood much better. As described earlier, gene expression analysis in mice carrying a *Gata4^{ki}* mutation revealed that GATA4 and its transcription partner, FOG2, are required for establishing the ovarian differentiation program. While these studies provided important clues concerning GATA4's function in ovarian cells, the embryonic lethality of the global mutants limited the analysis of ovarian differentiation to the stages before birth. Conditional deletion using the Cre/Lox recombination system was critical in uncovering the role of GATA proteins in subsequent ovarian differentiation and function.

i) *Sf1Cre*-mediated loss of *Gata4*

Efimenko *et al.* (2013) used *Sf1Cre*-mediated gene targeting to determine the requirement for GATA4 and FOG2 in ovarian development and folliculogenesis. The results from this study identified an essential role for the GATA4 (but not FOG2) in the ovarian morphogenetic/differentiation program. This result is in stark contrast to the sex determination phase that requires both proteins for its correct execution. Although *Gata4* was efficiently deleted in females shortly after sex determination, the effects of ovarian gene loss became most pronounced after birth. The conditional deletion of *Gata4* within the embryonic ovary led to a decrease in granulosa and theca cell proliferation/recruitment as well as the depletion of primordial follicles in the ovarian cortex, causing a failure in follicular development. However, folliculogenesis was not completely extinguished in *Sf1Cre*; *Gata4cKO* ovaries, and a few GATA4-deficient follicles developed to preantral stages when they became atretic. Adult *Sf1Cre*; *Gata4cKO* females developed ovarian cysts and were sterile (Efimenko *et al.* 2013).

ii) *Amhr2Cre*-mediated loss of *Gata4*

Amhr2Cre was originally described by Behringer *et al.* (Jamin *et al.* 2002) and was one of the first available Cre lines that is active in embryonic gonads. The authors indicated that the activity of Cre recombinase was detected in the Müllerian duct mesenchyme and female gonads as early as E12.5 using the *ROSA26* flox-stop-flox *lacZ* reporter (R26R) mice. For the past decade, this Cre line has been used successfully by many investigators to delete or activate their gene of interest in cells of the reproductive system.

Kyrölähti *et al.* (2011a,b) produced *Amhr2Cre*; *Gata4^{flox/flox}* (*Amhr2Cre*; *Gata4cKO*)

mice (Kyrönlahti *et al.* 2011b). While ovarian development appeared overtly normal in young animals, the authors noted impaired fertility and cystic ovarian changes in aged females. Upon gonadotropin stimulation, immature *Amhr2Cre; Gata4cKO* mice released fewer oocytes. These findings were similar to those that the authors observed in *Gata4^{+/-}* (germline heterozygous) animals. Compared with WT mice, the ovaries of gonadotropin-stimulated *Gata4^{+/-}* mice were smaller, released fewer oocytes, and produced less estrogen. In stimulated *Gata4^{+/-}* animals, the authors observed a downregulation in the expression of the putative GATA4 target genes *Star*, *Cyp11a1*, and *Cyp19a1*. In contrast, the only significantly downregulated gene in the *Amhr2Cre; Gata4cKO* animals was *Cyp19a1*.

To better interpret the results of this Cre/Lox experiment, the authors carefully examined the pattern of *Amhr2Cre*-mediated *R26R* excision within ovaries. While the authors confirmed that the *Amhr2Cre* transgene directs Cre expression to granulosa cells in secondary follicles, the expression was not uniform, with both lacZ-positive and lacZ-negative granulosa cells present. The variable Cre expression in granulosa cells likely contributed to the variable penetrance of the phenotype in *Amhr2Cre; Gata4cKO* mice. In post-pubertal mutant animals, the GATA4 protein was still present in theca cells and granulosa cells of primary, preantral, and antral follicles. The authors concluded that the extensive residual GATA4 activity presumably reflects cells in which the *Amhr2Cre* did not inactivate both *Gata4^{fllox}* alleles.

- iii) Double inactivation of the *Gata4* and *Gata6* genes
The *Cyp19Cre* transgenic mice drive Cre expression from the 304 bp *Cyp19a1* (aromatase) promoter (Fan *et al.* 2008, 2009). To monitor Cre recombination in ovaries, *Cyp19Cre* mice were crossed with the *R26R* reporter strain. Robust Cre activity was present in the granulosa cells in antral follicles and in luteal cells; Cre activity was low or undetectable in all immature follicles, theca cells, and oocytes. The activities of *Amhr2Cre* and *Cyp19Cre* were compared directly in their ability to induce recombination of KrasG12D in the ovary (Fan *et al.* 2008). The authors demonstrated that *Cyp19Cre* is expressed later in development compared with *Amhr2Cre*, with no *Cyp19Cre*-induced recombination observed before PND 10 (Fan *et al.* 2008).

The role of GATA4 within the ovary has been studied by using three conditional mutant models (*Amhr2Cre*, *Cyp19Cre*, or *Sf1Cre* where *Cyp19Cre* and *Amhr2Cre* are active postnatally, and *Sf1Cre* is active embryonically).

The severity of follicular development disruption varied in the ovaries from animals that underwent GATA4 loss through these different courses. However, all mutant mice had an abnormal response to exogenous gonadotropins and the presence of ovarian cysts was frequently observed (Kyrönlahti *et al.* 2011b, Bennett *et al.* 2012, Efimenko *et al.* 2013). In all cases, mutant females were either sub-fertile or infertile. The variations found among these phenotypes can be largely attributed to the completeness of the deletion in granulosa cells and the time when Cre recombination occurred. The earliest excision timing for *Sf1Cre* at E12.5 could be essential due to the emerging expression of GATA6 at E13.5 that partially compensates for ovarian follicular development in the absence of GATA4 (Kyrönlahti *et al.* 2011b, Bennett *et al.* 2012, Efimenko *et al.* 2013).

Recently, a mutant line harboring a deletion of *Gata4* and *Gata6* within the ovary using the *Cyp19Cre* line has been produced by Bennett *et al.* (2012). Not surprisingly, given the *Cyp19Cre* expression pattern, primary and multilayer follicles were present in ovaries from *Cyp19Cre; Gata4^{fllox/-}; Gata6^{fllox/flox}* females, but large pre-ovulatory follicles or corpora lutea were not formed (Bennett *et al.* 2012). Moreover, abnormal estrous cycle and infertility were noticed in *Cyp19Cre; Gata4^{fllox/-}; Gata6^{fllox/flox}* females. The authors also examined fertility in the single mutants and observed subfertility in *Cyp19Cre; Gata4^{fllox/-}* mice, while normal reproductive function was noted in *Cyp19Cre; Gata6^{fllox/-}* animals.

The follow-up work by Bennett *et al.* (2013) attempted to delineate the pathways regulated by GATA factors in adult granulosa cells (Bennett *et al.* 2013). Microarray analyses compared gene expression patterns in WT animals, single conditional mutants, and double conditional mutants after *in vivo* treatment with equine chorionic gonadotropin. In line with their previous observations, the authors confirmed that GATA4 is a dominant regulatory GATA factor in granulosa cells. *Cyp19Cre*-mediated *Gata4* gene deletion affected a greater number of genes than *Gata6* deletion. An even greater number of genes were affected by the deletion of both transcription factors. The authors demonstrated that GATA4 and GATA6 functionally compensate for each other in the regulation of key ovarian genes. When compared with single GATA-deficient animals, the change in the expression of numerous genes critical for ovarian function (e.g. *Fshr*, *Lhcgr*, *Inha*, *Inhbb*, versican, pregnancy-associated plasma protein A, and the regulatory unit 2b of protein kinase A) was much more

pronounced in the double conditional mutant. Rescue experiments involving the treatment of cultured granulosa cells with cAMP or insulin-like growth factor 1 (IGF1) to circumvent follicle-stimulating hormone and IGF1 signaling defects were largely inefficient, confirming that most of the affected genes are regulated by GATA factors directly (Bennett *et al.* 2013).

In a complementary approach, Padua *et al.* (2013, unpublished results) deleted both genes (*Gata4* and *Gata6*) within the ovary by using *Sf1Cre* (conditional double mutant). Embryonic loss of both GATA factors produced an early ovarian phenotype with a marked reduction in the expression of genes involved in ovarian development (e.g. *Foxl2*, *Fst*, and *Irx3*). In addition, neonatal conditional double mutant ovaries contained no follicles beyond primordial stage, suggesting a block in follicular development that is caused by an impaired somatic cell proliferation. Moreover, only rare oocytes survive in ovaries from conditional double mutant females and most of them disappear before animals reach puberty. The results from this study demonstrate the key roles of GATA4 and GATA6 in mammalian ovary development, cell proliferation, and folliculogenesis.

Conclusion

The combination of a conditional gene knockout and subsequent unbiased analysis of gene expression (e.g. microarray) has become a fundamental approach in understanding protein function. Early on, the elegant austerity of a knockout technology stirred expectations that these genetic experiments will provide absolute solutions. Now, it is becoming increasingly clear that even the most comprehensive answers require further qualifications, and what information can be gleaned from targeting genes *in vivo* is limited. Some of the important points one has to consider in interpreting the results of a gene targeting experiment are discussed below. The detailed characterization of Cre lines is an obligatory part of the gene targeting experiment, and its importance cannot be overemphasized. Comparisons of phenotypic and molecular outcomes generated by germline gene targeting and multiple Cre enzymes (as outlined for *Gata* genes in Fig. 1) will be helpful in describing the complexity of reproductive genetics.

Cre excision: location and efficiency considerations

In evaluating the results of a gene targeting experiment, the excision location and efficiency for each developmental stage need to be determined precisely. As described above, this analysis is normally performed in

advance of the actual 'gene-of-interest' experiment using a *ROSA26 lacZ* reporter (R26R) in place of the gene. A *ROSA mT/mG* reporter has been used more recently for the same purpose. In this reporter line, the excision of the stop cassette switches the fluorescent gene product from red to green in the excised cells (Muzumdar *et al.* 2007).

The use of different Reporter lines for the same Cre may provide valuable additional clues to the expected excision pattern. For example, Deutscher & Hung-Chang Yao (2007) crossed the *Amhr2Cre* line with the Cre reporter line Z/EG or B6.Cg-Tg(ACTB-Bgeo/GFP)21Lbe, which expresses EGFP upon Cre-induced recombination. The authors reported that EGFP expression began to appear only at E15.5, about 72 h later than originally described (Jamin *et al.* 2002), and was mostly restricted to the Müllerian duct mesenchyme with very few EGFP-positive cells present in the ovary. The authors proposed that the delay in the detection of *Amhr2Cre* activity could result from the time required for proper EGFP folding or the accessibility of the reporter construct to the Cre recombinase. Similarly, Jorgez *et al.* (2004) used the *Amhr2Cre* line to ablate the *Fst* gene in the granulosa cells of the ovary (Jorgez *et al.* 2004). The authors evaluated Cre activity in the postnatal ovaries by crossing the *Amhr2Cre* mice with R26R. After X-gal staining, the authors detected β -gal activity in the E17.5 ovaries of *Amhr2Cre*; R26R double heterozygous females, but the β -gal activity appeared to be fairly low. Furthermore, in the postnatal ovary, β -gal activity was abundant in the granulosa cells of all secondary and small antral follicles but was at much lower or undetectable levels in granulosa cells of primordial and primary follicles. These observations are consistent with the endogenous *Amhr2* expression pattern and introduce several possibilities one has to consider in choosing this Cre line: *Amhr2Cre* activity may become efficient fairly late (past E15.5) in ovary development, *Amhr2Cre* activity may be gene-dependent, or the timing of *Amhr2Cre* activity may depend on the animal background or some other non-traceable genetic variable.

Another important factor to consider is off-target expression. In the same study (Jorgez *et al.* 2004), low Cre activity was found in some theca cells and oocytes. Others have demonstrated that in addition to its ovarian expression, *Amhr2Cre* is expressed in additional reproductive tissues, including ovarian surface epithelial cells and the uterus (Boerboom *et al.* 2005, Fan *et al.* 2008, Hernandez Gifford *et al.* 2009). The activity of another popular Cre, *Sf1Cre*, in the pituitary and hypothalamus (Bingham *et al.* 2006) may also be of concern in evaluating adult reproductive outcomes, especially if a targeted gene is ubiquitously expressed. While early follicular development is gonadotropin-independent, gonadal pathology and abnormal folliculogenesis in the adult animals may result from the extra-gonadal gene loss. This possibility should not be automatically discounted.

Regardless of the excision pattern observed in the Cre reporter experiment, these results should only serve as an advisory preview guide in selecting an appropriate Cre, as the recombination efficiency for a gene of interest may

differ from that of the ROSA locus and should always be tested separately. This consideration is especially true for the inducible Cre lines (e.g. tamoxifen-activated), and one needs to be aware of the possibility of animal-

Figure 1 Transgenic approaches to defining GATA protein function in murine sexual development. Before sex determination stage GATA4 is required to initiate the formation of the genital ridge in the bipotential gonad. Subsequent sexual determination and activation of XY and XX sex-specific gene expression programs require GATA4-FOG2 complex. In males, GATA4-FOG2 complex function is essential for, but not limited to, *Sry* activation and this partnership remains active during subsequent sexual differentiation. GATA4 is required to establish Sertoli cell identity and *Gata4* gene ablation (represented by double strikethrough) during sex determination stage leads to a male-to-female sex reversal. Loss of GATA4 in pre-Sertoli cells shortly after sex determination is compatible with testis formation; however, Sertoli-specific genes that continuously require GATA for their expression are no longer maintained (e.g. *Dmrt1*). In females, the embryonic loss of GATA4 results in germ cell demise, postnatal block in granulosa cell proliferation, and severely curtailed follicular development. The embryonic loss of both GATA4 and GATA6 factors (double strikethrough) is markedly more detrimental, with drastically reduced numbers of pre-granulosa cells and follicular block at the primordial stage in the mutant animals. Targeting *Gata* genes in granulosa cells of the secondary follicles disrupts subsequent follicular maturation and leads to sterility. Globally targeted, transgenic, floxed, and Cre lines used for these experiments are listed below. The original publications describing the transgenic lines as well as the publications (*) describing specific applications discussed in the review are referenced. Note that three different lines carrying *Gata4*-floxed allele are available. (1) Cre/Lox, *Gata4*^{flox/flox} (Watt et al. 2004), CAG-CreER (Hayashi & McMahon 2002), WT1-CreERT2 (Zhou et al. 2008), and *Osr1*^{eGFP-CreERT2} (Mugford et al. 2008, Hu et al. 2013*). (2) *Fog2*^{-/-} (Tevosian et al. 2000), *Gata4*^{ki/ki} (Crispino et al. 2001), and MyHC-FOG2 (Tevosian et al. 2002, Manuylov et al. 2008)*. (3) *Fog2*^{-/-} (Tevosian et al. 2000), *Gata4*^{ki/ki} (Crispino et al. 2001), MyHC-FOG2 (Tevosian et al. 2002), *Wt1-Sox9* (Vidal et al. 2001), and *Ods* (Bishop et al. 1999) (Tevosian et al. 2002, Bouma et al. 2007, Manuylov et al. 2007a, 2008)*. (4) Cre/Lox, *Gata4*^{flox/flox} (Watt et al. 2004), *Gata4*^{flox/flox} (Zeisberg et al. 2005), and *Wt1ERT2Cre* (Zhou et al. 2008, Manuylov et al. 2011*). (5) Cre/Lox, *Sf1Cre* (Bingham et al. 2006), *Gata4*^{flox/flox} (Zeisberg et al. 2005), *Gata4*^{flox/flox} (Watt et al. 2004), *Gata4*^{flap/flap} (Ma et al. 2008), *Gata6*^{flox/flox} (Sodhi et al. 2006), and *Fog2*^{flox/flox} (Manuylov et al. 2007b) (Manuylov et al. 2011, Efimenko et al. 2013)*. (6) Cre/Lox, *Amhr2Cre* (Jamin et al. 2002) and *Gata4*^{flox/flox} (Watt et al. 2004, Kyrönlähti et al. 2011b*). (7) Cre/Lox, *Cyp19Cre* (Fan et al. 2008), *Gata4*^{flox/flox} (Watt et al. 2004), and *Gata6*^{flox/flox} (Sodhi et al. 2006) (Bennett et al. 2012, 2013)*.

to-animal variation due to the variability of injection efficiency or lot variations in drug potency. If gene excision in a plainly homogenous cell population remains incomplete, the decision of whether one still proceeds with the analysis of the phenotype is not an easy one. While the evaluation of a partial excision can still lead to valuable information, the interpretation of the results could be cumbersome, and a different choice of Cre line may be more prudent in these circumstances.

In the past decade, the conditional knockout technology in mice has matured into an indispensable part of the armamentarium for genetic analysis. The increased availability of mouse lines carrying 'floxed' genes and Cre recombinases from public repositories has allowed for the widespread use of this technology in numerous laboratories across the world. The analysis of reproductive outcomes (phenotypes) of Cre-mediated gene deletions in mice has been extremely helpful in expanding our understanding of reproductive development. While global gene knockouts were key in elucidating the functions of many genes in the reproductive system, these studies were limited, especially for genes required for survival during embryogenesis. The loss-of-function mutations in these genes led to early embryonic phenotypes and often lethality, thus precluding a thorough examination of their roles in organs that are non-essential for viability. The conditional gene knockouts became less of a risky undertaking than the global/germline knockouts, as the outcome of the gene modification is no longer dictated by the 'weakest link', but could be steered in a reasonably predictable manner toward differentiated adult tissues.

Theoretically, a gene loss that is limited to a defined cell type should simplify the interpretation of the outcome. However, gene regulation clearly remains highly time- and context-dependent. Once the cell has lost one or two of its major regulatory genes, a definition of the 'cell type' is becoming much more fluid and is in flux. Instead of delivering succinct diagrams, transgenic approaches have led to a more comprehensive and nuanced understanding of genetic interactions. These 'methods of questioning' have also revealed a need for new definitions of gene function that are temporally and spatially confined.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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