Identification of reproductive genes by gene targeting strategies

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Reproduction is a process delicately choreographed to ensure the survival of species. In terms of precision and beauty, reproductive physiology is just as amazing as the flamboyant display of reproductive behaviors. Any misstep during the scripted development of functional gonads and gametes may result in reduced fertility or infertility. Fertility disorders in human are a major health problem and also distressing experience for couples expecting children in their life. For diagnostic purposes, some genetic analyses for known defects and other tests are conducted in fertility clinics. However, almost a quarter of infertility cases are idiopathic, depicting our lack of knowledge in the underlying mechanisms of reproduction. Although assisted reproduction is widely available in the clinics to help these couples, quite a few infertile women still fail to become pregnant. Even when the procedures are successful, there are also substantial risks associated with the passing of defective genes to their children.

To expand our understanding of fertility disorders in humans, animal models have been providing invaluable insight into reproductive physiology. Mouse models have advantages including the relatively short life cycle, the economy of mouse husbandry, and the available techniques to manipulate its genome (reviewed in [1]). The breakthrough in utilizing homologous recombination to generate targeted mutations in the mouse genome [2] and subsequent application of targeted mutagenesis in ES cells [3-5] heralded the precise engineering of genetic material in mice. Due to the small footprint their target sites, the studies of site-specific recombination mediated by Cre/loxP [6] and Flp/FRT systems [7] in mouse cells further extended the available strategies for targeted gene manipulation [8]. Combined with rapidly evolving techniques, gene targeting techniques have become the tool of choice to genetically dissect the functions of genes.

To engineer desired modifications in mouse genome, targeting vectors are designed to contain engineered sequences and selectable markers flanked by homologous sequences (Fig. 1A). Correct gene targeting requires double reciprocal recombination in homologous arms across the modified core, and it is verified by PCR or Southern blot analysis. Targeted ES cells are injected into blastocysts to generate chimeras (Fig. 1B). The offspring are screened for germ-line transmission of the modified allele, and the F1 heterozygous mice are then intercrossed to homozygosity for further characterization of phenotypes.

Targeted disruption of genes, or gene “knockout”, is an effective way to study loss-of-function mutations. More than 200 knockout models with reproductive defects can be

found in the supplementary table from the review of mammalian fertility pathways by Matzuk and Lamb [9]. To search more knockout mice, two useful portals are the Transgenic/Targeted Mutation Database (TBASE) at the Jackson Laboratory (http://tbase.jax.org/) and the Mouse Knockout & Mutation Database at BioMedNet (http://research.bmn.com/mkmd).

With these 'designer mice' available, many aspects of reproduction can be recapitulated and studied by specific ablation of genes, even substituting them with another gene if necessary. An excellent example utilizing the combination of knockout and knockin was a study that generated mouse eggs with humanized zona pellucida (ZP) proteins [10]; these studies have given us a different insight into the structure-function relationship of ZP proteins and species-specific gamete interactions. For complex pathways, a knockin study that substituted the wild-type Kit receptor with a mutant receptor Kit-Y719F, which specifically disrupted its interaction with phosphatidylinositol 3'-kinase, demonstrated a separate pathway involved specifically in spermatogenesis and oogenesis [11]. As for gene family members showing spatiotemporal and phenotypical differences, studies such as the partial compensation of the *Inhba*-null phenotype by an *Inhbb* knockin (Fig. 2) helped our laboratory to elucidate the qualitative differences between these two alleles [12].

For conditional knockout mice to work, corresponding Cre transgenic mouse lines are required (see Table 1; more Cre mouse lines can be found at http://www.mshri.on.ca/nagy/cre.htm). For better control of the conditional gene disruption, more efficient Cre and Flp recombinases are being developed (e.g. iCre with optimized mammalian codons [13]), and the temporal control of recombinase activity has been achieved by fusion with ligand-binding domains from either progesterone or estrogen receptors [14]. Generating more tissue-specific Cre mouse lines with the improved recombinases will also have positive impact on the conditional knockout studies in reproductive functions. Besides deletion or inversion of a short stretch of DNA, much larger deletions have been produced by Cre/*loxP*-mediated chromosome engineering; for example, deletion of the vomeronasal receptor *V1r* gene cluster has been engineered for the study of chemosensory signals in reproductive behavior [15].

Genes involved in reproduction can be identified by 'forward' genetic approaches, like ENU mutagenesis screens (reviewed in [16]) directed specifically to infertile phenotypes [17]. However, deleterious phenotypes may hinder their effectiveness, demanding revised strategies for studying uncharacterized genes. Advances in genome projects have provided the raw materials for the efficient utilization of 'reverse' genetic approaches such as site-directed mutagenesis, site-selected mutagenesis, and chromosome engineering in mice. They have also facilitated the studies of molecular details in reproduction by comparative genomics. On the one hand, reproductive development involves evolutionarily conserved processes that ensure the survival of species. On the other hand, divergences in these processes, such as meiotic progression during oogenesis [18], were evident as reproductive success in every species was selected to fit its specific life cycle. Furthermore, the divergences were sexually selected to create reproductive

barriers, the major contributor of speciation. Taking both ends into consideration will surely help us to elucidate the underlying mechanisms of reproduction.

Known genes involved in reproduction can be readily used to create knockout mice for loss-of-function studies. However, at this moment, many more genes are classified as having an "unknown" function than a "known" function. With ambitious genome projects covering a wide variety of species, the identification of novel genes involved in reproduction by comparative genomics will likely catch up soon. Conserved gene orthologs among species showing similar expression patterns suggest that the functions of the proteins are also conserved. For example, our laboratory has identified *Gasz* [19], *Npm2* [20], and *Zar1* [21, 22] genes in multiple vertebrate species. Combining "wet lab" techniques, like suppression subtractive hybridization and *in situ* hybridization, with *in silico* subtraction of the microarray data or the expressed sequence tags (ESTs) in model species, we may identify uncharacterized genes showing conserved gonad-specific expression patterns as potential knockout targets to expand our understanding in reproductive physiology. Some *in silico* subtraction tools can be found on the public web interface at UniGene Digital Differential Display (http://www.ncbi.nlm.nih.gov/UniGene/info_ddd.shtml) and CGAP cDNA Digital Gene Expression Displayer (http://cgap.nci.nih.gov/Tissues/GXS).

Due to the nature of genes involved in reproduction, most of them can only be studied *in vivo* at the organism level, limiting the choices of methods for *in vivo* analysis. Although RNA interference may be qualified as an alternative to knockout techniques, more optimization is still required for it to be useful at this moment [23]. In the mean time, we will surely see fruitful achievements in understanding reproduction by using powerful, yet versatile, gene targeting techniques.

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Figure 1 Generating knockout mice. (A) Targeted disruption of the exon containing the start codon; (B) ES cells are selected and checked for correct targeting before injecting into blastocysts to generate chimeric mice. After the targeted allele is transmitted to the progeny, the heterozygous (+/-) knockout mice are crossed to homozygosity (-/-) for further analysis.

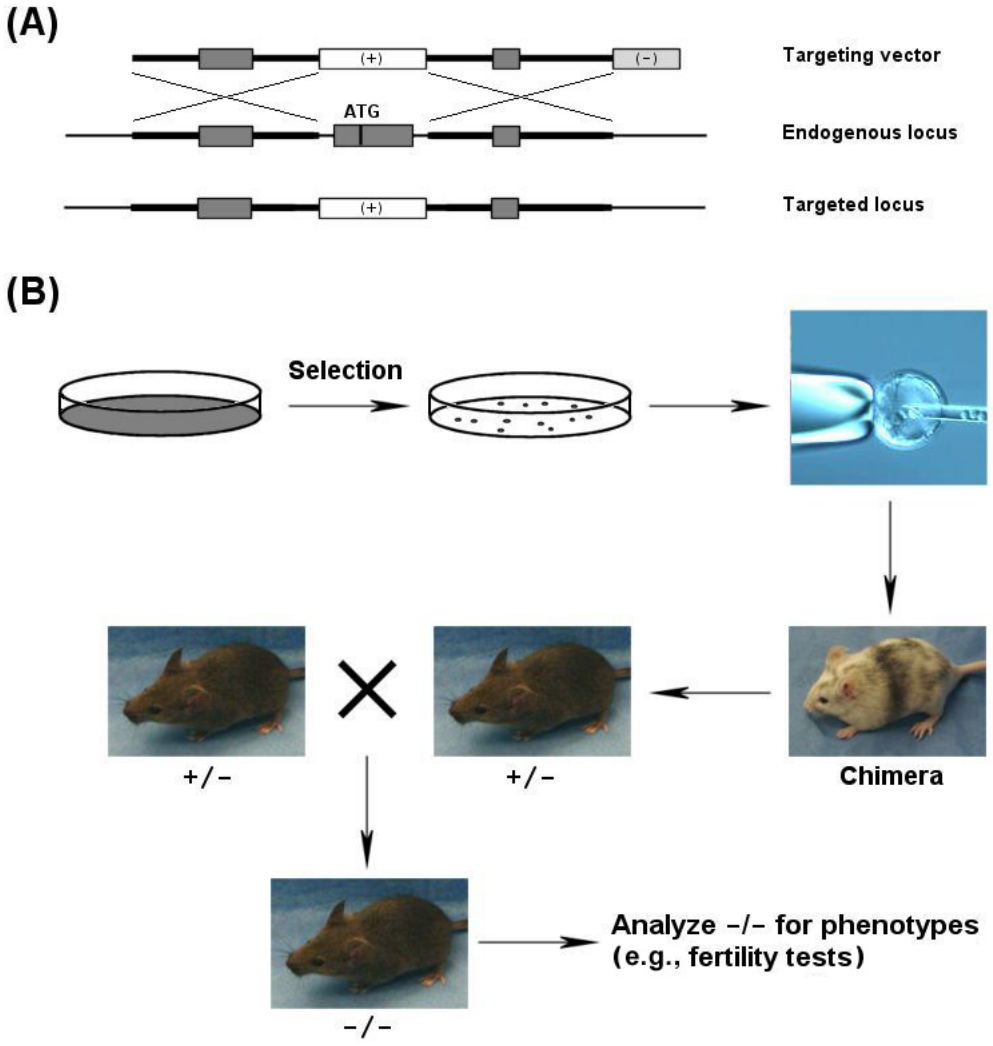

Figure 2 Example of complex gene targeting. (A) The activin βA (*Inhba*) exon 2 (E2), which contains the entire mature domain, was first replaced by the *HPRT* selection marker to generate the knock-out allele (*Inhba*$^{-}$). A second recombination then placed the mature domain of activin βB (*Inhbb*) in frame under the control of the *Inhba* promoter to generate the knock-in allele (*Inhba*^{BK}). (B) Targeted alleles shown by Southern-blot analysis using the 5' probe. (reproduced from Ref. 12 with permission)

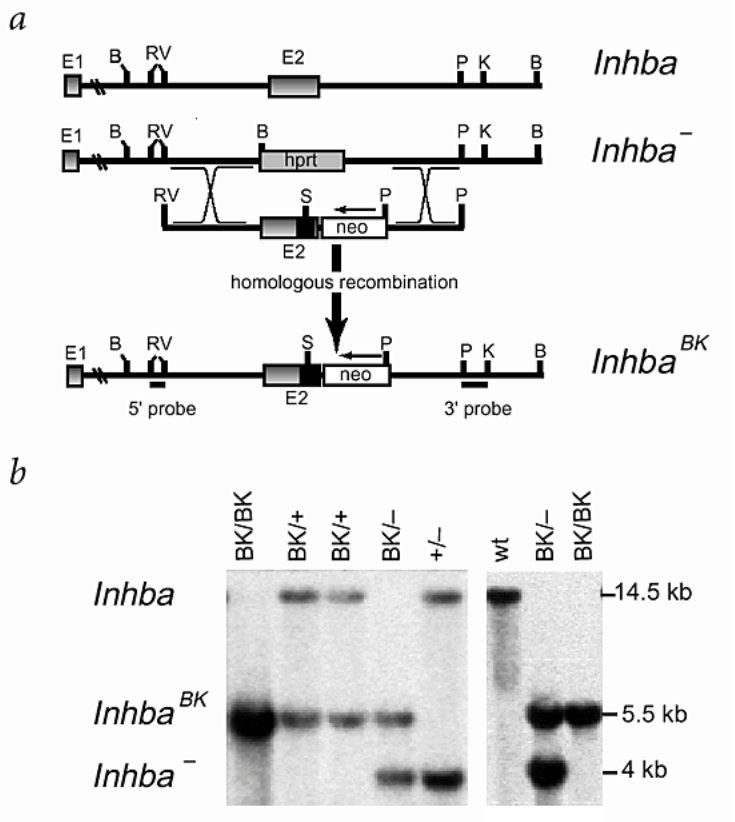

Figure 3 Visualization of *Amhr2-Cre* expression in the ovary by X-gal staining after crossing with ROSA26 mice. (A) 6-week-old whole mount ovary; (B) E17.5 ovary: Cre expressed in almost all somatic cells; (C, D) 6-week-old ovary: Cre expressed mainly in granulosa cells of preantral and small antral follicles, and Cre activity was also found in some thecal cells. The apparent β-gal activity in some oocytes may be a staining artifact. (reproduced from Ref. 31 with permission)

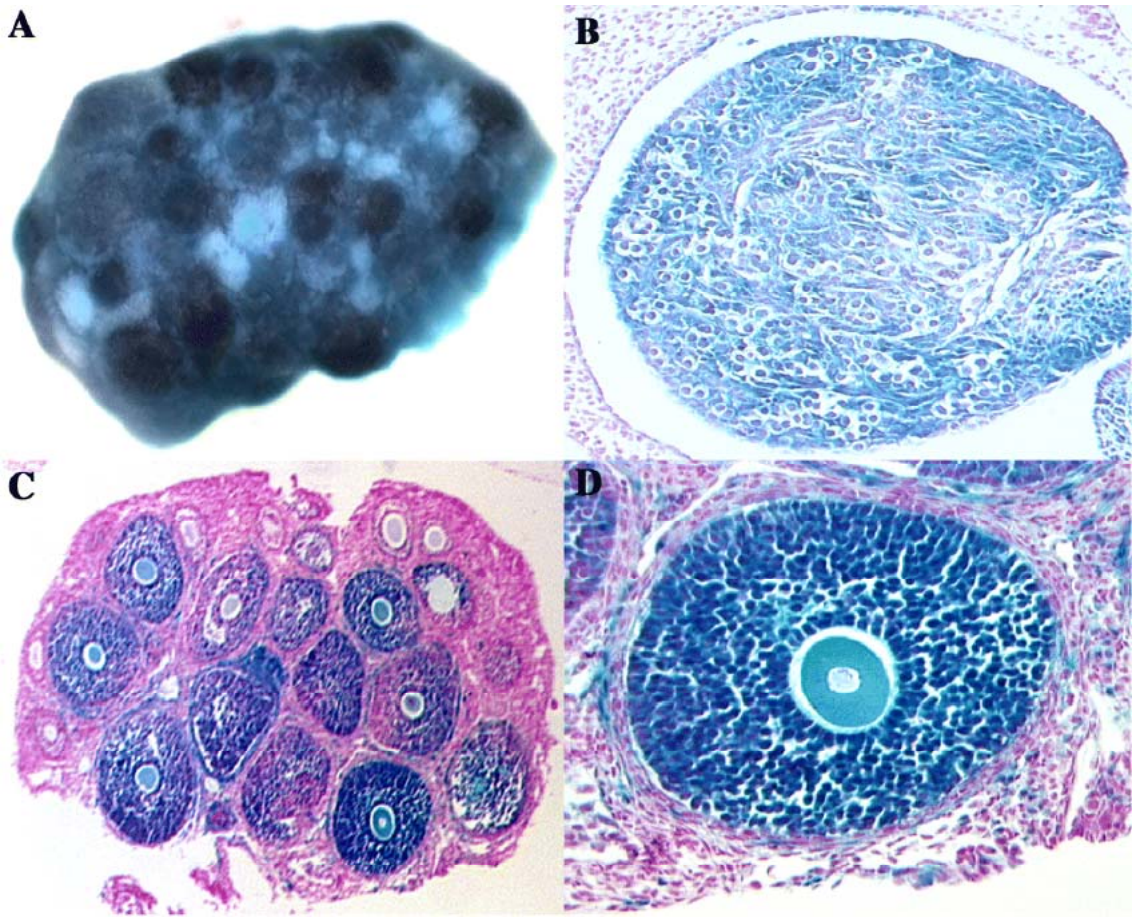

Table. 1 Mouse lines expressing Cre recombinase in reproductive tissues

Gonadotropin-releasing hormone (GnRH)-iCre	GnRH neurons	[13]
Alpha subunit of glycoprotein hormones (alpha GSU)-Cre	Gonadotropes and thyrotropes	[24]
Pgk-2-Cre	Spermatocytes	[25]
Protamine-1 (Prm1)-Cre	Spermatogenic cells	[26]
Prion protein (PrP)-Cre-ER^T	Spermatogonia and spermatocytes	[27]
Anti-Mullerian hormone (AMH)-Cre	Sertoli cells (male), granulosa cells (female-not evenly active)	[28]
Zp3-Cre(+)	Oocyte-specific	[29, 30]
Anti-Mullerian hormone type 2 receptor (Amhr2)-Cre (Fig. 3)	Granulosa cells (female), Leydig cells and Sertoli cells (male-weak expression)	[31]

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