MONASH University

Genetics of Disorders of Sexual Development

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MONASH SCIENCE INDUSTRY
STUDENT PLACEMENT PROGRAM

PRINCE HENRY'S INSTITUTE

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Acknowledgements

The author express his gratitude to Professor Vincent Harley and Dr. Rowena Lavery for their time and assistance as supervisors, and their keen interest in guiding the research.

The author also wishes to thank Dr Pascal Bernard, Dr Stefan Bagheri-Fam, Dr Kate York, Dr Makoto Ono, Janelle Ryan, Dimithu Alankarage and Rajini Sreenivasan for their assistance both directly and in-directly during the program.

Special thanks to Dr Theodoros Papakonstantinou and Dr Gerry Rayner for this amazing placement experience at Prince Henry's Institute.

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Gonadal Development

Gonadal differentiation is the first step of mammalian sex determination (1, 2). In mammals, male sex determination is governed by SRY-dependent activation of SOX9, whereas female development involves RSPO1, an activator of the WNT/beta-catenin signaling pathway (Figure 1) (3). Hormones such as androgens and anti-Mullerian hormone also affects sexual differentiation and is important for sex determination (1). This review will focus on the male pathway.

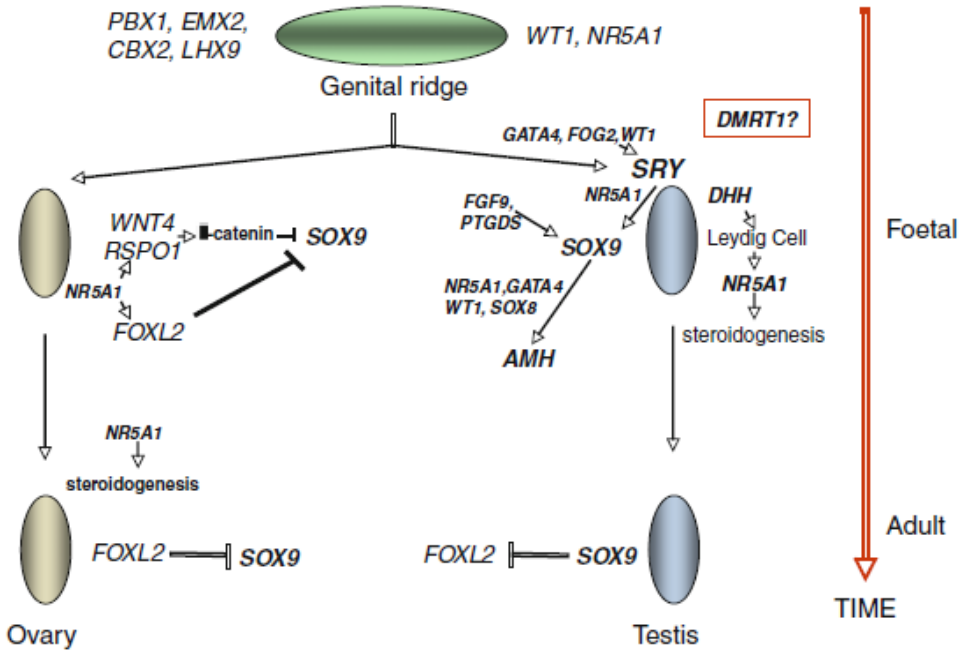

Figure 1: Molecular genetics of sex determination (3).

SRY gene

Researchers have found that SRY gene, a sex-determining region on the Y chromosome which controls testis formation, play a role in the male and female gonadal development (4). SRY is located on Yp11.3 and it encodes a DNA-binding protein with an evolutionary conserved motif termed the HMG-box (Figure 2). SRY is also a member of the SOX family of proteins, therefore showing a potential of SOX downstream genes to be implicated in disorders of sexual development (DSD) (5). The presence of SRY promotes the formation of male sexual characteristic while its absence promotes ovary formation (1).

SOX9

SOX9 is a transcription factor which contains a DNA-binding HMG domain and a transcription activating proline- and glutamine-rich domain (Figure 2) (6, 7). SOX9 is highly upregulated in the Sertoli cells (1). Since SOX9 is the downstream gene of SRY, there is a strong interaction between SRY and SO9 in particularly on gonadal development. The role of SOX9 on sexual disorders was confirmed when mutation on SOX9 gene was conducted for the understanding of sexual development (7, 8).

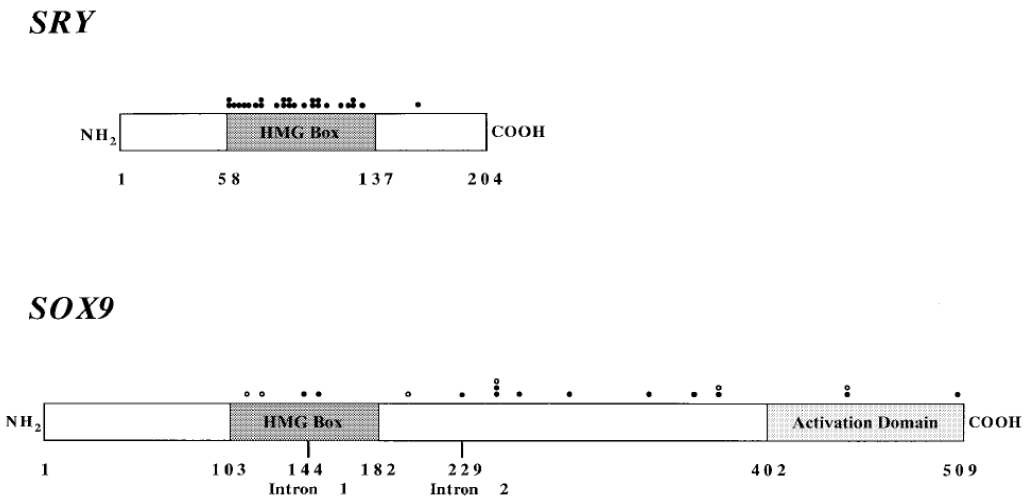

Figure 2: Schematic diagram of SRY and SOX9 gene (Modified from (6)).

From the Primordial germ cell of the bipotential gonad to sexual development

In mice Primordial germ cells (PGCs) begin to leave the dorsal gut at E9.5 and migrate to the urogenital ridges E9.5 (Figure 3A). By E10.5 mouse PGCs have left the dorsal body and cluster at the urogenital ridges (Figure 3B). At this stage the gonad of both XX and XY is bipotential. However in the XY gonad it is then the expression of the Y chromosomal testis

determining factor SRY that induces up-regulation of the transcription factor SOX9, which in turn, promotes the expression of a cascade of genes essential for testis differentiation.

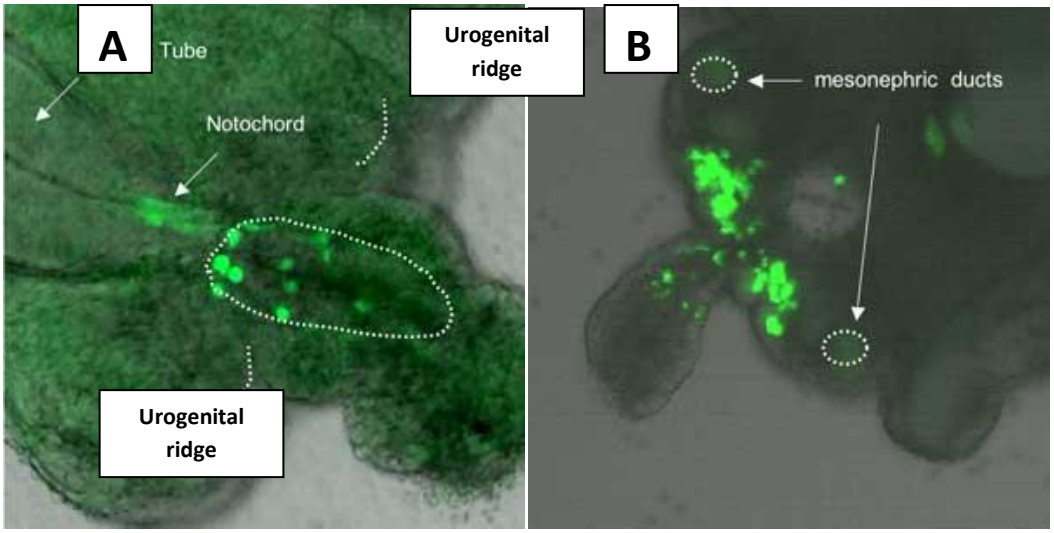

Figure 3: (A) Primordial germ cells-GFP begin to leave the dorsal gut and migrate to the urogenital ridges E9.5. (B) By E10.5 mouse PGCs-GFP have left the dorsal body and cluster at the urogenital ridges.

Why is it important to know which genes are involved in sexual development?

While the intricacies of the gonadal developmental gene cascade are still to be fully understood, it is however known that disruption of this pathway can lead to disorders of sexual development (DSD). Moreover, mouse models have shown that conditional ablation of *Sox9,* specifically in the Sertoli-precursor cells, during early stages of gonad development results in a complex mRNA signature that indicates wide-spread transcriptional de-regulation and male to female sex-reversal (9).

Identification of genes involved in sexual development is important for the elucidation of mechanisms involved in male gonadal development and, thus, has the potential to aid our understanding of the aetiology of DSD in humans (10). In fact, to date the aetiology of 80% of DSD cases remains unknown while a mere 20% are known to be due to *SRY* or SOX9 factors.

While the advances in gene expression analysis using Affymetrix™ technology present an exciting future in terms of the identification of novel genes involved in the aetiology of disease and in our case DSD , the application of molecular techniques also has the ability to move us from an era where sex reversed/ DSD newborn babies was defined by examination of external genitalia, later on in a child's development, to one where several identifiable

genetic or pathological mechanisms aid in their diagnosis (11). With this change in paradigm comes new hope of understanding idiopathic disorders of sexual development and the generation of specific and effective diagnostic measures which in the long term will aid in the early sex rearing of the individual child.

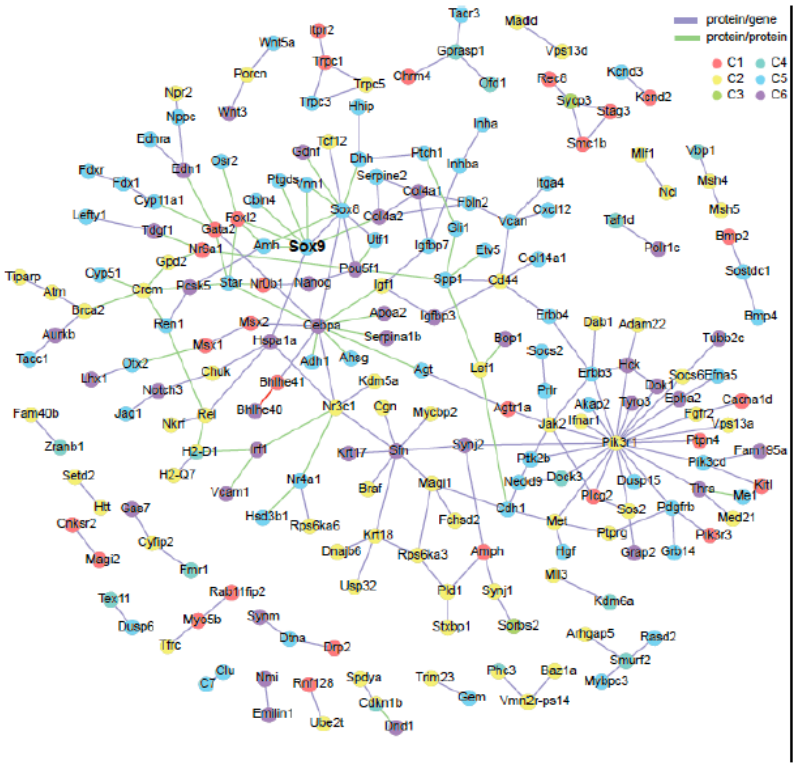

Figure 4: Sox9 predicted gene regulatory network generated by AMEN program from previously published Microarray data.

Disorders of sexual development

Disorders of sexual development (DSD) occurs when gonadal, chromosomal or anatomical sex is abnormal (12-14). DSD include both 46, XX and 46, XY disorders (15). 46, XX DSD is caused by presence of a 46, XX karyotype, resulting in reduced testes size and testosterone deficiency. 46, XY DSD on the other hand is identified by a 46, XY karyotype, no sperm production and underdeveloped gonads.

There are many DSD. This includes congenital adrenal hyperplasia which involves inheritance of adrenal gland disorders (16). The abnormal production of hormones causes unusual formation of sexual organs.

SRY and Sex Reversal

SRY has also been implicated in Sex reversal. Bullejos and Koopman (2004) has identified SRY role at causing B6-Y^{DOM} sex reversal. It was shown that a delayed ending of SRY

expression promotes sex reversal. Studies has shown that SRY expression levels below a certain threshold level causes XY sex reversal but this is not the only cause. This has also indicated the importance of SRY expression levels for proper imitation of male sex determination in mammals (9).

WNT4 mutation also contributes towards sex reversal. It was known that lack of WNT4 activity promotes masculinisation in female while excess WNT4 activity promotes feminization of male (17).

Mutation of SRY outside the 5' end of the open reading frame has also been implicated in sex reversal (18). However, mutation of SRY only accounts for less than 20% of DSD patients. This means the that majority of DSD patients are mutant for unknown genes which prompted the significance of this project.

Due to the exact molecular basis behind the events downstream of SOX9 is not fully elucidated, using the latest technology in the market, potential SOX9 gene implicated in DSD can be identified. Gene expression profiling technology such as Microarray analysis specifically in murine SOX9 mutants' gonadal RNA has allowed us to identify putative direct targets of SOX9 (19). An example of a Sox9 gene regulatory network, depicting a predictive gene cascade of events, was generated from previously published Microarray data (19).

Hence, this project focused on the analysis of previously published Affymetrix^{TM} data and of SOX9 putative target gene candidates using molecular biology techniques. Riboprobes will be made in order to characterise these genes in mouse gonadal embryonic tissues.

Table 1: Novel Genes expressed at E11.5 in gonad (GUDMAP gonadal microarray data), down-regulated in XY SOX9 mutant gonads and expressed in a gonadal somatic cell microarray **(*with exceptions)**

Gpm6b	**No papers regarding sex determination** Sertoli cell expression genepaint-Designed primer
Bex2 *	Designed primer **No papers regarding sex determination** Sertoli cell expression gudmap -designed
Rik5730 *	Designed primer

	No papers regarding sex determination Sertoli cell expression gudmap and genepaint-designed
Ace2	Designed primer Angiotensin converting enzyme2 **Publications regarding human fertility,** But no literature regarding mice 'From rat to human: regulation of Renin-Angiotensin system genes by sry.' Prokop JW et al 2012 **Ace2 may be regulated by Sry**
Gstm1	Infertility- humans **Not published in mouse**
Heyl	Designed **No papers regarding sex determination**
Dtna (dystrobrevin alpha)	Designed primers **One publication** in mice: Menke DB, et al., Gene Expr Patterns. 2002 Dec;2(3-4):359-67 Identified Dtna as a new candidate of sex determination  Homozygous targeted mutants exhibit skeletal and cardiac myopathies. Neuromuscular junctions appear to form normally, but their postnatal maturation is compromised. Dtna mutations do not increase the severity of Dmd or Utrn mutants whose products are

	also part of the dystrophin-glycoprotein complex.

Technical skills acquired

Primer sets of Gpm6b, Bex2, Rik5730, Heyl, Ace2, Dtna and Gstm1 were successful used for the generation of RT-PCR amplicons from mouse Brain cDNA in order to make riboprobes. Riboprobes are RNA probes that can by created by in vitro transcription of cloned DNA inserted in the plasmid downstream of a viral promoter. Characterisation of these genes using in situ was outside the scope of this placement. The following techniques were carried out over the industry placement period.

1. Lysis of tissue for DNA isolation
2. PCR- Genotyping
3. Embryo Dissection (observed)
4. Fixation of embryos (observed)
5. RNA extraction
6. DNase treatment of RNA
7. Quantitative and qualitative analysis of RNA
8. cDNA synthesis
9. RT-PCR
10. Aseptic technique
11. Cloning - Ligation
    -Transformation
12. Preparation of media and broth for bacterial culturing
13. Blue/white colony selection via vector containing Lac-Z fragment and X-gal screening
14. Plasmid preparation
15. Plasmid digestion for linearisation (control digest to test directionality of cloned fragments): checkpoint analysis-gel/nanodrop
16. Purification using Qiagen Quick- checkpoint analysis-gel/nanodrop
17. In vitro transcription of linearised DNA- checkpoint analysis-gel/nanodrop
18. Precipitation of Riboprobe-checkpoint analysis-gel/nanodrop

Conclusion

It is hoped that this study will make a step towards identifying bonafid SOX9 target genes and thus aid in our understanding the genetic basis of male gonadal development and disorders therein.

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