UNIVERSITIY OF CALIFORNIA SAN DIEGO

Effects of Homeodomain Transcription Factor *Six3* in Reproduction and Circadian Rhythms

A thesis submitted in partial satisfaction of the requirements

for the degree of Master of Science

in

Biology

by

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THESIS APPROVAL PAGE

The Thesis of Nay Chi (Tiffany) P. Naing is approved, and is acceptable in quality and Form of on microfilm and electronically.

University of California San Diego

2022

DEDICATION

I would like to dedicate my thesis to my family and friends for all the love and support.

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LIST OF ABBREVIATIONS

IHH	Idiopathic hypogonadotropic hypogonadism
HPG	Hypothalamic-pituitary-gonadal
GnRH	Gonadotropin-releasing hormone
AVPV	Anteroventral periventricular nucleus
ARC	Arcuate nucleus
LH	Luteinizing hormone
FSH	Follicle-stimulating hormone
SCN	Suprachiasmatic nucleus
NMS	Neuromedin S
AVP	Arginine vasopressin
VIP	Vasoactive intestinal peptide
SIX3	Sine oculis-related homeobox 3
DMEM	Dulbecco's modified eagles media
FBS	Fetal bovine serum
DPBS	Dulbecco's phosphate buffered saline
hKiss-Luc	−1313/+26 human *Kiss1*-Luciferase-pGL2 plasmid
mKiss-Luc	−4058/+455 mouse *Kiss1*-Luciferase-pGL2 plasmid

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ABSTRACT OF THE THESIS

EFFECT OF HOMEODOMAIN TRANSCRIPTION FACTOR *SIX3* IN REPRODUCTION AND CIRCADIAN RHYTHM

by

Nay Chi (Tiffany) P. Naing

Master of Science in Biology

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Professor Pamela L. Mellon, Chair

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The homeodomain transcription factor sine oculis-related homeobox 3 (SIX3), is an important regulator of reproduction and circadian rhythms. SIX3 has the potential to be a novel candidate gene for infertility disorders like Idiopathic Hypogonadotropic Hypogonadism (IHH). To extend our knowledge of the importance of *Six3*, my research investigated the neural location of SIX3 regulation in the hypothalamus that influences fertility and circadian clock. Kisspeptin

neurons, in the hypothalamus, are an important regulator of endocrine pathways and circadian-regulated ovulation. To determine whether SIX3 can regulate the two immortalized kisspeptin neuron populations *in vitro*, I conducted a transient transfection and luciferase assay. I found that SIX3 is capable of regulating human *Kiss1* transcription using the human-Kiss1-Luciferase promoter. Another aim of my project was to examine if deletion of *Six3* in the neuromedin S (NMS) neurons would affect neuropeptide expression in the suprachiasmatic nucleus (SCN) and/or reproductive competency of male and female mice ($SIX3^{NMSCre}$). NMS is located in the SCN region of the hypothalamus and is an essential pacemaker for internal clock. I prepared samples for *in situ* hybridization by RNAScope to detect four different types of neuropeptides critical for circadian rhythm, and the data are pending. I also examined male fertility by measuring copulation and sperm in $SIX3^{NMSCre}$ mice. I found that these male mice had normal plugging behavior and total sperm count, however, the percentage of motile sperm is significantly lower in mutant males. To study fertility in $SIX3^{NMSCre}$ female mice, I conducted a fertility assessment which is still underway. Current preliminary results have shown that $SIX3^{NMSCre}$ female mice have normal fertility as measured by time to first litter and number of pups per litter. In conclusion, we found that *Six3* expression in kisspeptin neurons is important for female fertility and *Six3* expression in the NMS neurons is important for fertility and circadian rhythm in males.

INTRODUCTION

Reproduction is an essential process for the survival of all species. Mammals utilize sexual reproduction to ensure genetic diversity and a stable ecosystem. Around the globe, 72 million people struggle with infertility [1]. Infertility can be caused by many factors such as delayed puberty, hormone imbalance, ovulation disorder in females, and abnormal sperm function in males. Reproductive disorders such as Idiopathic Hypogonadotropic Hypogonadism (IHH) and Kallmann syndrome (KS) are rare genetic conditions with phenotypes of delayed or absent puberty, micropenis, amenorrhea, decreased sex steroids, and partial or complete loss of fertility [2]. Unfortunately, only approximately 50% of IHH cases are accounted for by known genetic defects and mutations [3]. Therefore, studying novel genes implicated in infertility, such as homeodomain-binding transcription factors, can further our understanding of genetic reproductive disorders.

The hypothalamus is the master regulator of the endocrine system and is essential for reproduction. The hypothalamic-pituitary-gonadal (HPG) axis is an example of a crucial pathway that the hypothalamus regulates [4]. As shown in Figure 1, the pathway starts at the hypothalamus with kisspeptin neurons releasing kisspeptin to stimulate gonadotropin-releasing hormone (GnRH) neurons. There are two populations of kisspeptin neurons: [1] anteroventral periventricular nucleus (AVPV), and [2] arcuate nucleus (ARC). 1) The AVPV kisspepin neuronal population is found mostly in females. It undergoes positive feedback from estrogen and releases more kisspeptin, triggering ovulation. 2) The ARC neuronal population is present in both males and females and regulates the downstream GnRH neurons to release GnRH in pulsatile fashion every 30 – 60 minutes [5-7]. When GnRH neurons are stimulated by kisspeptin, they release GnRH, which stimulates the gonadotrope cells in the anterior pituitary to release luteinizing hormone (LH) and

follicle-stimulating hormone (FSH). For successful fertility, LH and FSH act on the gonads to induce ovulation, folliculogenesis, spermatogenesis, and the synthesis of sex hormones, such as estrogen, testosterone, and progesterone [8]. In females, rising levels of estrogen produced by the developing follicle(s) stimulate the AVPV kisspeptin neurons. In turn, these AVPV kisspeptin neurons stimulate a surge of GnRH and then LH release. The bolus of LH released in response to rising estrogen is called LH surge which is circadian regulated and crucial for ovulation [9].

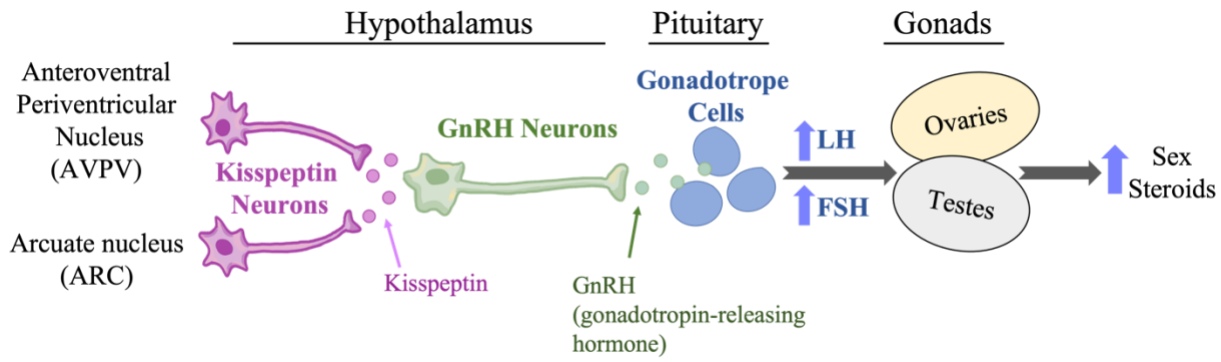

Figure 1. Hypothalamic-Pituitary-Gonadal Axis.

Modified from Sen & Hoffmann, 2020 [10].

A functional reproductive system requires normal circadian rhythms. In humans and rodents, the LH surge is temporally gated to the start of the active period [11, 12]. Circadian rhythms are controlled by a molecular clock that exists in nearly every cell in the body and oscillates with a ~24 hour period [13]. This clock drives and synchronizes our daily activity schedule of sleeping, eating, body temperature, and other fundamental homeostatic functions [13]. In the hypothalamus, the region called the suprachiasmatic nucleus (SCN) is a master clock that maintains an approximately 24-hour rhythm by utilizing transcription-translation feedback loop [10]. The SCN receives light input from the retinohypothalamic tract which synchronizes

physiological function with the environment [10]. Lesion of the SCN eliminates the LH surge and estrous cyclicity, therefore, the SCN is essential for normal reproductive functions in female [14-16].

In the SCN, there are neurons that make neurotransmitters that are responsible for generating circadian signals throughout the brain and body. Figure 2 demonstrates three neurotransmitters in the SCN that will be covered in this project: 1) neuromedin S (NMS), 2) arginine vasopressin (AVP), and 3) vasoactive intestinal peptide (VIP). 1) NMS is almost exclusively expressed in the SCN: it is an essential pacemaker and may also function in relaying photoperiod information to the master clock. NMS is present in 40% of the cells in the SCN, including most AVP and VIP neurons [17-19]. 2) AVP is mostly found in the dorsomedial shell, and it plays an important role in interneuronal communication in the SCN. AVP may influence fertility by stimulating AVPV kisspeptin neurons [10, 18]. 3) VIP is found in the ventrolateral core of the SCN. It receives direct light input and is critical for maintaining coupling between cells in the SCN. VIP neurons send projections directly to GnRH neurons to impact the HPG axis [10, 18]. To highlight, the neurotransmitters in the SCN may directly affect fertility by stimulating kisspeptin and/or GnRH neurons at the precise time of the day to induce the LH surge and ovulation in female mice [20, 21].

There are studies that have shown that alteration in clock genes can affect reproduction in mice model. Core clock genes such as brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1) and cryptochrome (CRY1 and CRY2) are responsible for the transcription-translation feedback loop that regulates circadian rhythm. Our lab has shown that deletion of *Bmal1* gene resulted in infertility and abnormal growth hormone regulation for both male and female mice. Furthermore, *Bmal1* knocked-out (KO) male mice show mating behavioral

defects and *Bmal* KO female mice have irregular LH levels [22-24]. Bur et al. (2009), has shown that *Cry* double-KO (*Cry1*$^{-/-}$/*Cry2*$^{-/-}$) mice have irregular regulation of male and female sex-specific metabolic genes and growth hormone [25]. *Cry* double-KO mice also exhibited sex-dependent liver dimorphism. Deletion of clock genes like *bmal1* and *cry* demonstrated that circadian timing and fertility are interconnected.

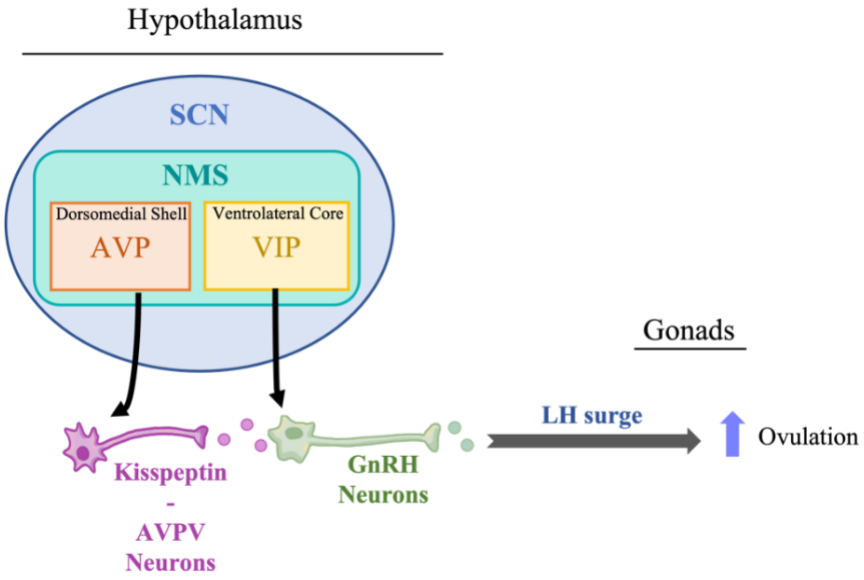

Figure 2. Neurotransmitters within the circadian clock.

Modified from Sen & Hoffmann, 2020 [10].

Recent studies have shown that homeodomain transcription factors such as sine oculis-related homeobox 3 (SIX3) have the potential to underly fertility and circadian rhythms [5, 26-28]. Homeodomain-binding transcription factors encode proteins that are essential for morphogenesis. SIX3 is critical for SCN development and the endocrine system [9, 29]. SIX3 specifically plays a role in eyes, nose, and forebrain development as well as expressed in the adult

hypothalamus [30-32]. Knockout of *Six3* in mice is lethal, which indicates that *Six3* is an essential transcription factor for survival.

Pandolfi, et al. (2018), studied whole-body *Six3* heterozygous mice and found that they exhibited phenotypes of subfertility in both male and female such as reduced GnRH neurons in the hypothalamus, longer time to first litter in both sexes, smaller litter size, and longer time to achieve vaginal plugging [26, 27]. This study revealed the importance of *Six3* in reproduction. SIX3 is also highly expressed in the GnRH neurons. However, when *Six3* was conditionally deleted only in GnRH neurons, there was an increase in the number of GnRH neurons and no subfertility as was observed in the SIX3 heterozygous mice [26]. Therefore, it raises the question of where else *Six3* could be acting in the hypothalamus to affect fertility. One candidate is kisspeptin neurons because they function upstream of GnRH neurons [28]. In this project, we examined if *Six3* in kisspeptin neurons affects fertility. Since kisspeptin neurons contribute to the secretion of GnRH, they have essential roles in the initiation of puberty, regulation of sex steroid-mediated feedback, and the control of adult fertility [28, 29, 33]. To study the role of SIX3 regulation in the kisspeptin neurons *in vitro,* we transfected the *Six3* gene into two immortalized kisspeptin cell lines that are equivalent to female AVPV and ARC neurons [34].

When Hoffmann, et al. (2020), and Meadows, et al. (2021), conditionally knocked-out *Six3* in post-developmental neurons using Synapsin^{cre}, a promoter that targets mature neurons, it resulted in disrupted circadian rhythms in constant darkness in males and females [21, 35]. While male fertility was not affected, female mice exhibited impaired fertility such as irregular estrus cycle and diminished LH surge amplitude [21, 35]. Since the LH surge is also circadian regulated, whether *Six3* is acting through the SCN or other synapsin-containing neurons is unknown [9]. Furthermore, Hoffmann, et al. (2020), investigated the effect of *Six3* in VIP and AVP

neurotransmitters, *in vitro*, and found that *Six3* does not regulate VIP expression, but enhances AVP expression. Yet, the role of *Six3* in the SCN specifically, not in all mature neurons, is yet to be explored. To study the role of *Six3* in SCN, our lab used Cre/Lox technology to create a mouse using NMS-Cre aiming to delete *Six3* in NMS neurons ($SIX3^{NMSCre}$). The NMS-Cre mouse targets 98% of NMS neurons in the SCN, including 95% of AVP and VIP neurons as well [36].

Previously, Dr. Lauren Chun from the Mellon Laboratory conducted the wheel-running experiment to determine the effect of *Six3* in NMS neurons on locomotor activity, which is influenced by circadian clock (Fig. 3). Both male and female of $SIX3^{NMSCre}$ and $SIX3^{flox/flox}$ ($SIX3^{f/f}$ nonmutant), circadian activities were monitored. As shown in Figure 3A, $SIX3^{NMSCre}$ male mice had significantly reduced free-running period in constant darkness. Therefore, *Six3* in NMS neurons maybe important for normal circadian function. In addition to the circadian rhythm of $SIX3^{NMSCre}$ mice, we examined if fertility or SCN peptide expression is altered in female and male mice.

Information about *Six3* gene regulation as a homeodomain transcription factor is limited, yet important to further our understanding of reproduction as well as circadian rhythm. SIX3 has the potential to be a novel candidate gene for infertility disorders such as IHH. Our research investigated the neural location of SIX3 regulation in the hypothalamus that influences fertility and circadian clock. I focused on 1) the regulation of SIX3 in the two immortalized kisspeptin neuron populations *in vitro*, 2) the $SIX3^{NMSCre}$ mice neuropeptide expression in the SCN, and 3) the reproductive competency of $SIX3^{NMSCre}$ mice.

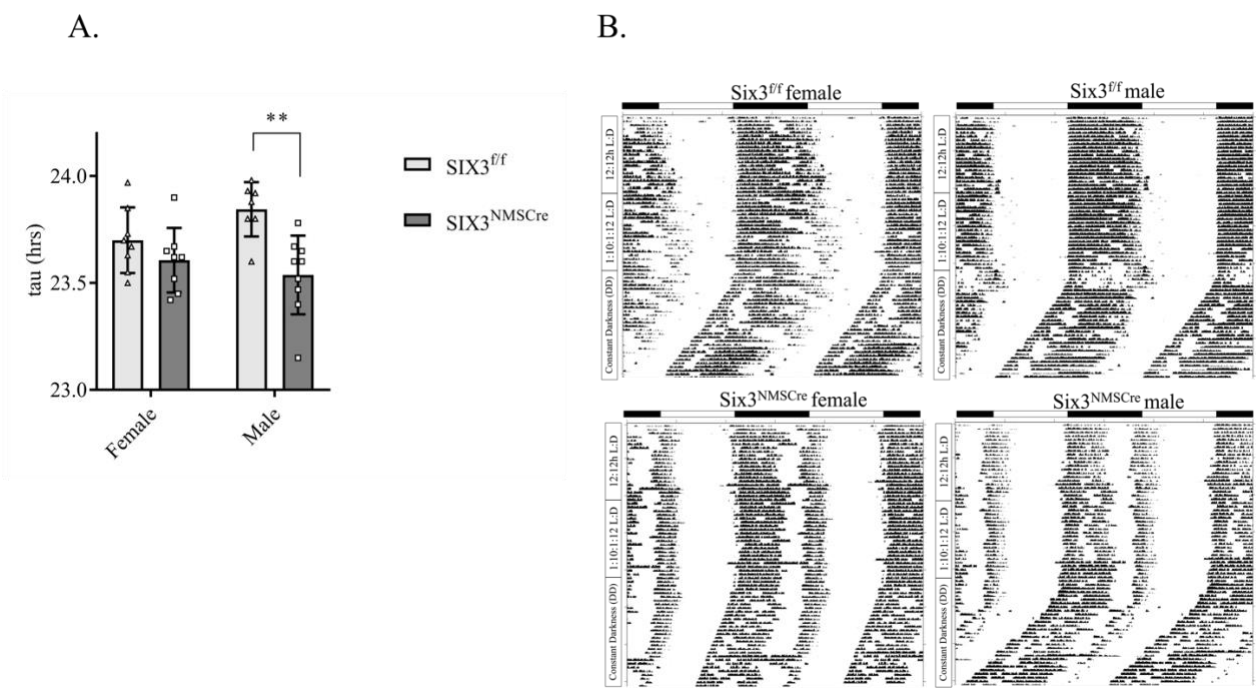

Figure 3. SIX3^{NMSCre} male mice exhibit irregular circadian activity.

Male and female SIX3$^{f/f}$ and SIX3^{NMSCre} mice were individually housed in cages with a running wheel. They were exposed to 12h:12h light:dark, then a skeleton photoperiod (1:11:1:11), then 15 days on free-running in constant darkness. A) Tau of free-running period in hours. Bars represent mean ± SEM, N=30 to 32, and data points represent each mouse. B) Wheel running activity patterns. Data were analyzed with ClockLab for Chi square analysis, Two-way ANOVA, and Tukey's multiple comparison post-hoc test to determine the period. * $p < 0.01$.

RESULTS

1. SIX3 regulates Kiss1 expression, in vitro.

To study the first hypothesis, whether SIX3 can regulate the two kisspeptin neuron populations, I designed an experiment using transient transfection and luciferase assay. I tested if overexpression of SIX3 can induce *Kiss1* transcription by transfecting the immortalized kisspeptin cell lines derived from AVPV (KTaV-3) and ARC (KTaR-1) of the female mouse hypothalamus [34]. Along with the SIX3 expression vector or pGL2-empty vector (EV), I co-transfected with either a human *KISS1* luciferase (hKiss-Luc) or mouse *KISS1* luciferase (mKiss-Luc) promoter plasmid. It was found that overexpression of SIX3 significantly repressed hKiss-Luc transcription in both KTaV-3 and KTaR-1 (Fig. 4A and 4C). In contrast, overexpression of SIX3 did not significantly affect the mKiss-Luc transcription in either KTaV-3 or KTaR-1 cell lines (Fig. 4B and 4D). SIX3 regulates human and mouse kisspeptin promoters differently: on hKiss-Luc, SIX3 acts as a repressor while on mKiss-Luc, it has no effect. These data have been published in Lavalle, et al., 2022 [28].

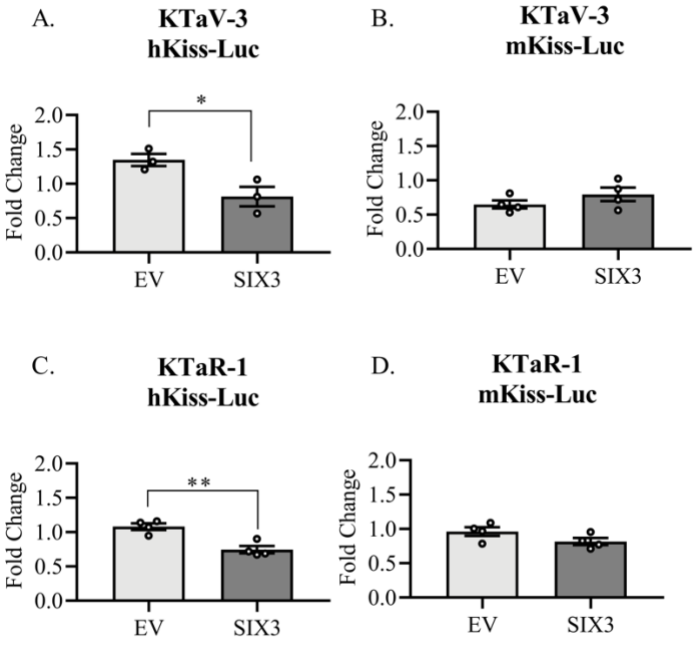

Figure 4. SIX3 represses Kiss1 transcription in both AVPV and ARC neurons using a hKiss-Luc reporter.

The hKiss-Luc plasmid was co-transfected with 20 ng of SIX3 or pGL2-empty vector (EV) in A) KTaV-3 (P=0.0327) and B) KTaR- 1 cells (P=0.2530). mKiss-Luc was co-transfected with 20 ng SIX3 or EV in(C) KTaV-3 (P=0.0032) and D) KTaR-1 (P=0.1265) cells. The luciferase expression was normalized to TK-βgal, triplicates were averaged and re-normalized to the pGL2 backbone. The data are represented as fold change relative to SIX3/EV and analyzed using an unpaired Student's t-test to compare between EV and SIX3. *P<0.05, **P<0.005. For all of the experiments, bars represent mean ± SEM, N=3 to 4 and data points represent each replicate. These data are published in Lavalle, et al., 2022.

2. In Situ Hybridization - RNAScope

To understand if *Six3* gene has an effect on neuropeptides such as *Nms, Vip,* and *Avp* in the SCN, we measured the mRNA expression of those neuropeptide genes using fluorescent *in situ* hybridization (RNAScope). After sectioning the fresh frozen brains of $SIX3^{f/f}$ and $SIX3^{NMSCre}$ mice at 20 μm thick using a cryostat, the frozen slides were shipped to our collaborator Dr. Hanne Hoffmann at Michigan State University. They will be conducting an automated RNAscope analysis for *Nms*, *Vip*, *Avp*, and the circadian clock gene *Bmal1*. The data are pending.

3. Reproductive competency of $SIX3^{NMSCre}$ male and female mice.

To investigate if conditional deletion of *Six3* allele in NMS neurons has an effect on fertility, I conducted the reproductive competence assessments for both male and female mice. For male fertility assessment, I performed a vaginal plugging assay by pairing $SIX3^{f/f}$ or $SIX3^{NMSCre}$ male with wildtype (WT) females and recorded the number of days it took for the male to leave a copulatory plug in the female (Fig. 5A). There was no significant difference between $SIX3^{f/f}$ (2.38±0.60 days) and $SIX3^{NMSCre}$ (2.88±0.44 days) time to plug. To further test the fertility of male mice, I collected their sperm and measured the percentage of motile sperm and total sperm count (Fig. 5B and 5C). Significant reduction in sperm motility in the $SIX3^{NMSCre}$ (18.98±2.67%) compared to the $SIX3^{f/f}$ (30.26±3.83%) male mice was found. The total sperm count of $SIX3^{f/f}$ (44.09±5.18 million/mL) and $SIX3^{NMSCre}$ (41.11±3.26 million/mL) were not significantly different.

Furthermore, to test the fecundity of female mice, I conducted a fertility assay by pairing $SIX3^{f/f}$ or $SIX3^{NMSCre}$ females with WT males (Fig. 6). The experiment is still underway; however, there were no significant differences in the time to deliver the first litter (Fig. 6A) and average

number of pups per litter (Fig. 6B) between $SIX3^{f/f}$ and $SIX3^{NMSCre}$ female mice. Current preliminary conclusion is that $SIX3^{NMSCre}$ female mice do not have changes in overall fertility.

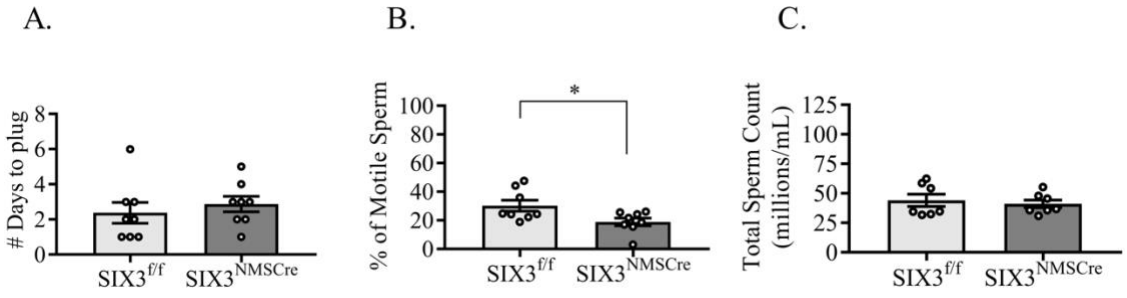

Figure 5. SIX3^{NMSCre} male mice exhibited reduced percentage of motile sperm.

A) Number of days to plug WT female mice (*P*=0.5108). B) Percentage of motile sperm (*P*=0.0297). C) total sperm count in millions/mL (*P*=0.2898). Data were analyzed using unpaired Student's t-test. For all of the experiments, bars represent mean ± SEM, N = 14 to 16, and data points represent each male mouse. **P*<0.05.

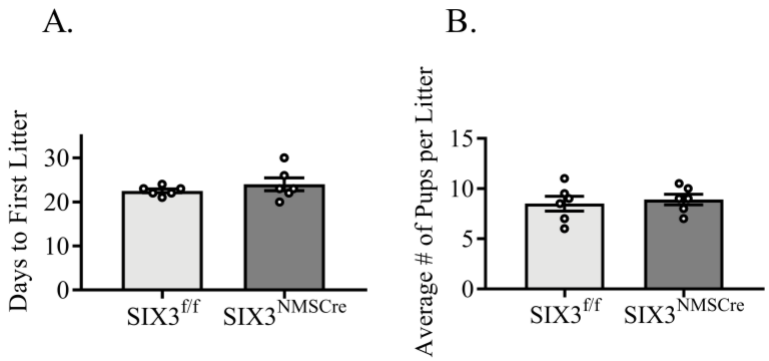

Figure 6. No difference in female fertility in SIX3$^{f/f}$ versus SIX3^{NMSCre}.

A) Number of days to deliver the first litter (P=0.3409). B) Average number of pups per litter (P=0.6527). Data were analyzed using unpaired Student's t-test. For all of the experiments, bars represent mean ± SEM, N = 12, and data points represent each female mouse.

DISCUSSION

1. Six3 is capable of regulating the kisspeptin transcription using a human-Kiss1-promoter, in vitro.

Overall, we found that SIX3 can regulate the human *Kiss1* promoter in immortalized kisspeptin neurons derived from the anteroventral periventricular nucleus (AVPV) or the arcuate nucleus (ARC) of a female mouse *in vitro*. In Figure 4, 20 ng of SIX3 overexpression repressed hKiss-Luc transcription, while we saw no effect on mKiss-Luc transcription in both AVPV and ARC cell lines. Since SIX3 has the potential to affect Kiss1 transcription in humans, we can confirm that it may be essential for regulating the HPG-axis pathways and ultimately fertility.

Lavalle, et al. (2022), suggested that the differences between human and mouse Kiss1 transcription are due to the differences in the sequence of the two promoters [28]. The SIX3 amino acid sequences of different vertebrates (human, mouse, chicken and zebrafish) are identical [37], while the human and mouse *Kiss1* promoters contain many of the ATTA and ATTA-like motifs that SIX3 binds to for transcription. However, lack of conservation in the specific positions of ATTA-like motifs between the human and mouse kisspeptin promoters may underlie the differences in transcription [28, 38-40]. In addition, human and mouse Kiss1 promoter vectors have different lengths of base pairs, therefore, selectivity and binding mechanisms can be different.

When *Six3* was deleted from *Kiss1*-expressing cells using the Kiss-Cre mouse, female mice exhibited subfertility and irregular estrous cycles, while no effect was observed in males [28]. However, the loss of SIX3 in female mice did not change the increase in AVPV *Kiss1* at the time of LH surge nor the ability to mount an LH surge [28]. Therefore, deletion of *Six3* from the kisspeptin neurons does not completely explain the reproductive phenotype of the *Six3* heterozygous mice.

In this study, *in vitro* experimental design with immortalized kisspeptin cell lines does not reflect on overall fertility in humans or mice. *In vivo*, SIX3 expression in kisspeptin neurons can be influenced by differences in sex, hormone feedback, and time of the day [28]. To further understand the effect of SIX3 between female and male mice *in vitro*, future studies could introduce different types of sex steroids such as synthetic androgen (R1881) and estrogen receptor alpha (ERα) into the transient transfection [29].

2. Six3 RNAscope, In Situ Hybridization

Data pending.

In Hoffmann et al. (2021), they conducted *in vitro* experiments to determine if SIX3 could regulate *Vip* and *Avp* transcription [21]. Similar to our project with Kiss1-luc, they utilized transient transfection and luciferase assay: transfected embryonic fibroblast cell line (NIH3T3) with *Vip* or *Avp* luciferase expression promotors. They found that SIX3 strongly promotes Avp-luciferase expression, while Vip-luciferase expression was not regulated [21]. But *Nms* luciferase expression is yet to be explored. To further test the SIX3 regulation of these peptides in the SCN *in vitro*, we will analyze the RNAScope data to determine if these mRNAs are altered with deletion of Six3 from Kisspeptin neurons. We will also compare males and females to determine if differential changes in *Avp* and *Vip* expression may underlie the different locomotor phenotype.

3. *SIX3^{NMScre} male mice have reduced sperm motility while female mice have normal reproductive competency*

Overall, SIX3^{NMSCre} mice had normal reproductive capacity except for sperm motility. The plugging behavior and total sperm count of SIX3^{NMSCre} male mice are similar to SIX3$^{f/f}$. For female fertility, since this the experiment is still continuing, we cannot make a definitive conclusion yet. But so far, there are no differences in female fertility.

Interestingly, Dr. Lauren Chun's circadian activity data (Fig. 3) showed that SIX3^{NMSCre} male mice had abnormal internal clock. In general, we have observed impaired fertility and circadian activity in male mice, and not in female mice. One of the characteristics that can cause reduction in sperm motility is obesity, and ablation of SCN can cause abnormal metabolism and weight gain [41, 42]. While conducting the sperm count, I noticed that the SIX3^{NMSCre} mice appeared more obese compared to SIX3$^{f/f}$ male mice. Therefore, to confirm the if deletion of *Six3* in the NMS-expressing cells causes weight gain that might underlie the change in sperm motility, we will be measuring the weight progression of SIX3^{NMSCre} and SIX3$^{f/f}$ mice in the future. However, in contrast, deletion of Six3 in mature neurons (SIX3^{SynCre} mice) exhibited significant reduction in growth starting at 4 weeks old due to decreases in fat mass and increases in lean mass compared to SIX3$^{f/f}$ in male mice [35]. But SIX3^{SynCre} female mice did not exhibit difference in weight compared to SIX3$^{f/f}$.

SIX3^{KissCre} mice also have reduced motile sperm 52.2% versus 72.5%; p=0.012; n=7-9) compared to SIX3$^{f/f}$ [43], but was not altered in the whole-body heterozygous mouse [27]. Kiss1^{CRE} is expressed in the testis [44], so we cannot rule out that *Six3* action in the testis underlies this phenotype. Future studies will examine whether NMS is also in the testis, as well as measuring

baseline LH and FSH levels from SIX3^{NMSCre} mice. We will also validate the knockdown of SIX3 using immunohistochemistry (IHC) in the SCN.

The manual analysis of motile sperm and total sperm count can be subjective from an observer to another. To quantify the motile sperm behavior and activity pattern further, we could use the computer-assisted sperm analysis (CASA) model [45]. This automatic technology can categorize sperm motility into distinguish and quantitate five patterns: progressive, intermediate, hyperactived, slow, and weakly motile. CASA model can allow us to examine in depth how deletion of *Six3* in *Nms*-expressing cells effect sperm behavior.

4. Six3 expression in kisspeptin and NMS neurons is important for reproduction and circadian rhythms.

In this project, we have found that SIX3 is capable of regulating transcription of the Kiss1 gene in both AVPV and ARC neurons using the human-Kiss1-luciferase promoter, indicating that it may be crucial in regulating the HPG-axis pathway. However, loss of *Six3* in *Kiss1*-expressing cells only showed subfertility in female and not male [28]. Furthermore, my project has demonstrated that the loss of *Six3* in *NMS*-expressing cells revealed mild subfertility phenotype of low sperm motility and impaired circadian activity of reduced free-running period in constant darkness. Because these effects were not as profound as the heterozygous whole-body knockout mouse, the neural location of SIX3 regulation that influences fertility and circadian clock is still not fully understood.

Six3 is essential for SCN formation during development [9]. In human, heterozygous loss of Six3 exhibits is severe condition called holoprosencephaly, which is a condition that results in abnormal development of the brain during first two or three weeks of pregnancy [46, 47]. Six3

heterozygous mice also presented phenotypes of severe infertility in both male and female [27]. However, the necessity of *Six3* expression post-development such as puberty and adulthood in the different neuronal population is yet to be explored.

MATERIALS AND METHODS

1. Cell Culture

Dr. Patrick Chappell (Oregon State University) kindly provided KTaV-3 and KTaR-1 immortalized mouse kisspeptin cell lines derived from the AVPV or ARC [34], respectively, of an adult female mouse hypothalamus. The cell lines were incubated in 10 cm plates in an incubator at 37°C with 5% CO_2 and maintained in complete media containing Dulbecco's Modified Eagles Media (DMEM), 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. When the cells reached 85-90% confluency, they were passaged by washing with Dulbecco's Phosphate Buffered Saline (DPBS), resuspending in trypsin and diluting in complete media.

2. Plasmids

Dr. Alejandro Lomniczi and Dr. Sergio Ojeda (Oregon Health and Science University) kindly provided the −1313/+26 human *KISS1*-pGL2-luciferase plasmid (hKiss-Luc) [48]. Dr. Steven Kliewer (UT Southwestern) kindly provided the −4058/+455 mouse *Kiss1*-pGL4-luciferase plasmid [49]. To create the −4058/+455 mouse Kiss1-Luciferase-pGL2 plasmid (mKiss-Luc), we subcloned this sequence from pGL4 into pGL2 backbone by using *Kpn*I-HF, *Xho*I, and *Sal*I-HF restriction enzymes in 10X Cut Smart Buffer and religated using Quick Ligase Kit.

3. Transient Transfections

Kisspeptin cells were counted using a hemocytometer and plated in 12-well plates. Each well was plated with 30,000 cells/ml of complete media. The plated cells were incubated overnight to allow cells to adhere to the plate. Plasmid DNA of TK-β-gal, either pGL2 backbone and mKissLuc or hKissLuc reporters, and pGL2-empty vector (EV) or SIX3 expression vectors were combined with the transfection reagent, Polyjet, and DMEM. The plasmid and Polyjet/DMEM

mixtures were incubated at room temperature for 15-30 mins and then 50 μL of the mixture was slowly injected into each well. The plates were returned into the incubator overnight. To stop the transfection, the media in each well was aspirated and replaced with complete media. The plates were returned to the incubator overnight.

4. Luciferase assay

Each individual well was about 90-100% confluent before harvesting. The media in each well was aspirated, then wells were washed with room temperature DPBS, then DPBS was aspirated. Lysis buffer was added to each well and shaken for 5 minutes at room temperature. Then 25 μL of lysed cells were transferred into two 96-well plates for luciferase and β-gal assays. For the β-gal assay, Galacton reagent (Galacton Diluent and Galacton+) was added to lysed cells in β-gal plate and incubated at room temperature for 15 to 60 minutes. A luminometer was used to inject room temperature accelerator into wells and record raw β-gal values. For the luciferase assay, luciferase assay buffer (ddH_2O, 0.25 M Tris pH 7.8, 1 M $MgSO_4$, 0.25 M ATP, and Luciferin) was prepared and injected by luminometer into wells and raw luciferase values were recorded.

5. Animals

All animal procedures were performed in accordance with the University of California, San Diego Institutional Animal Care and Use Committee regulations. To delete *Six3* from the NMS-expressing neurons, we crossed $SIX3^{flox/flox}$ ($SIX3^{f/f}$) mice [50] with Nms-iCre mice [36] to get $SIX3^{NMSCre}$ mice. Then we used Ai14 Rosa-tdTomato mice [51] to create a NMS reporter line by crossing them to NMS^{cre} mice to generate NMS^{cre}:tdTomato mice. All mice were on a mixed NMRI and C57/Bl6 background. Mice were group housed and maintained on a 12-h light, 12-h

dark cycle with *ad libitum* access to chow and water. All the mice used were around 3 months old unless specified.

The mice were genotyped using PCR with DNA from tail tips. *Six3* wildtype (WT), *Six3* flox alleles and germline recombination were detected using
Six3-For1: 5'-TGCCCCCTGCTAAAGAGCCAGT-3',
Six3-For2: 5'-TAGGGACAGGCACGGAGGGTTG-3',
and Six3-Rev: 5'-ATGCCCACATTGTCGGCCCATG-3' primers.
NMS was detected using iCre-F: 5'-AGATGCCAGGACATCAGGAACCTG-3',
iCre-R: 5'-ATCAGCCACACCAGACACAGAGATC-3',
iCre-CtrlF: 5'-CTAGGCCACAGAATTGAAAGATCT-3',
and iCre-CtrlR: 5'- GTAGGTGGAAATTCTAGCATCATCC-3' primers.
tdTomato was detected using Rosa-tdtF: 5'-GGC ATTAAAGCAGCGTATCC-3', Rosa-tdtR1: 5'-CTGTTCCTGTACGGCATGG-3',
Rosa-tdtF2: 5'-CCGAAAATCTGTGGGAAGTC-3',
and Rosa-tdtR2: 5'-AAGGGAGCTGCAGTGGAGTA-3'. Mice that were positive for *Six3* germline recombination in tail samples were omitted from the study.

6. RNAScope

Using a cryostat, fresh frozen brains were sectioned at 20 μm and collected the spanning length of the SCN. Sections were mounted on super plus slides and stored at -80°C. The slides were shipped to our collaborator Dr. Hanne Hoffmann (Michigan State University) to conduct a RNAScope assay using an automated system. RNA of *Nms, Vip, Avp,* and *Bmal1* will be measured and analyzed. The data from this experiment are pending.

7. Plugging and Fertility Assay

To assess male plugging behavior, $SIX3^{f/f}$ or $SIX3^{NMSCre}$ male mice were paired with virgin WT female for 10 days. During the assay period, each female vaginal opening and the cages are searched for a white mucus plug between 8 am to 9 am every day. To assess female fertility, $SIX3^{f/f}$ or $SIX3^{NMSCre}$ female mice were paired with virgin WT males for 76 days. The number of offspring, latency to first litter, and total number of litters were recorded.

8. Manual Sperm Motility and Total Sperm Count

After the male mice had been housed alone for at least a week, epididymides were harvested and placed in M2 media at room temperature. To determine the percentage of sperm motility, one of the epididymis was poked with surgical needle, then used surgical scissors and forceps to cut in half and expel sperm into 500 μL M2 media. After being undisturbed for 15 mins, sperm in M2 media were transferred by avoiding large clumps into another eppendorf tube, allowed to sit for 3 mins then diluted with M2 media. Motile sperm were counted using a hemocytometer and then placed on a 55°C heat block for 3 mins to immobilize all sperm. The intact sperm were counted. Four corners of the hemocytometer were counted and averaged. Percentage of motility was calculated by motile sperm divided by intact sperm. To determine the total sperm count, another one of the epididymis was minced in 1 mL M2 media and set in room temperature for an hour. Then it was filtered through 70 μM cell strainer and diluted with water. All the sperm heads were counted using a hemocytometer. The total sperm count is calculated by average number of heads in all four corners x dilution x 10,000 (magnification and hemocytometer).

9. Statistical Analysis

Excel was used to compile the raw luciferase and β-gal data and calculations were performed to normalize the data. GraphPad – PRISM 9 was used to analyze and graph the data. Unpaired Student's t-tests was used to determine statistical significance. Outliers were identified using Grubbs test (alpha = 0.05) and omitted from the study. *P<0.05, **P<0.005, and ***P<0.0005.

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