



Session 2

Biological effects on early-stage spermatogenesis in cryptorchidism

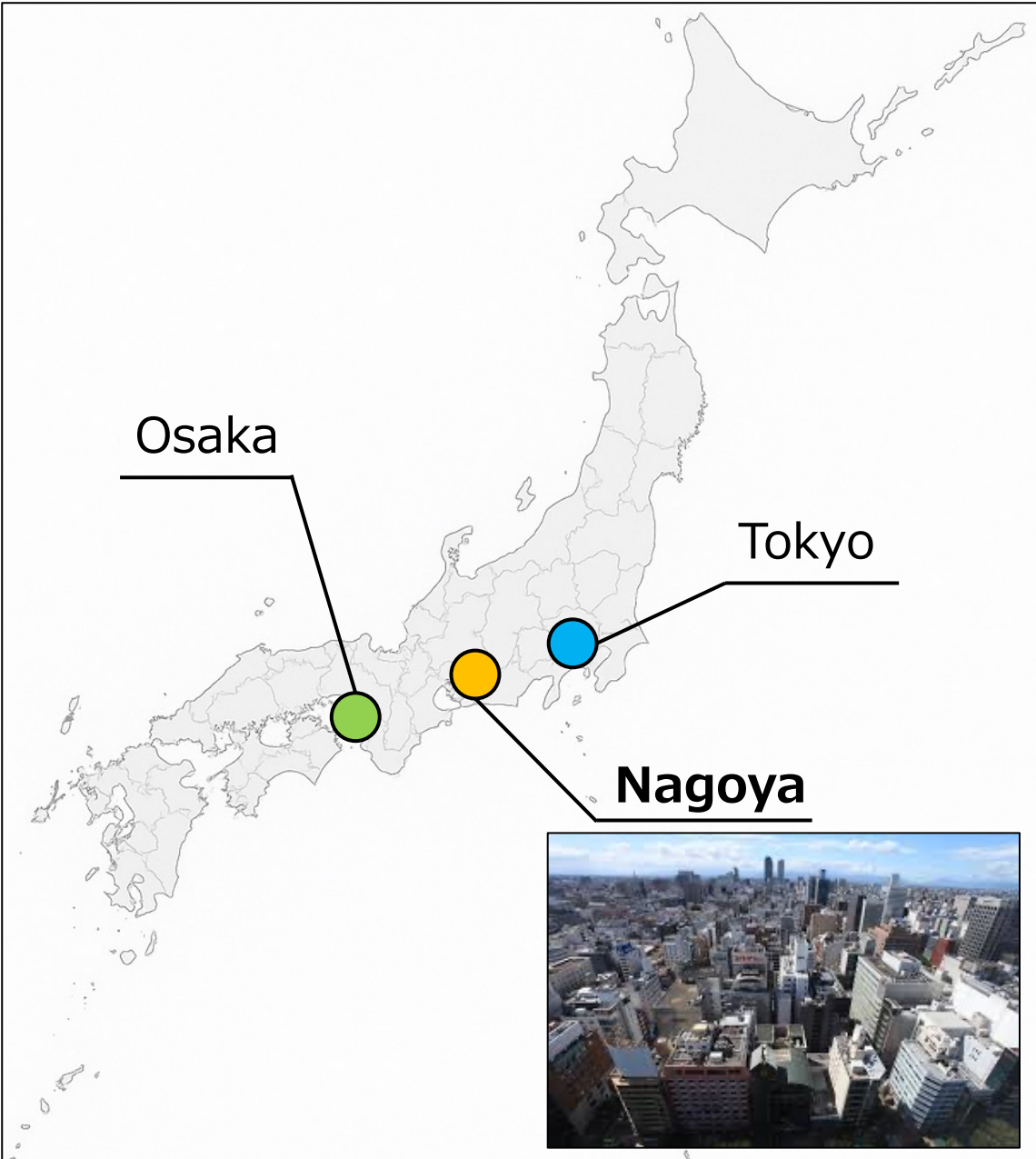
Kentaro Mizuno¹, Hidenori Nishio¹, Yutaro Hayashi^{1, 2}

1. Department of Pediatric Urology, Nagoya City University Graduate School of Medical Sciences, Nagoya, JAPAN
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The 5th International Andrology Symposium COI disclosure information

We have no conflicts of interest related to this presentation.

Introduction



Nagoya City University Hospital

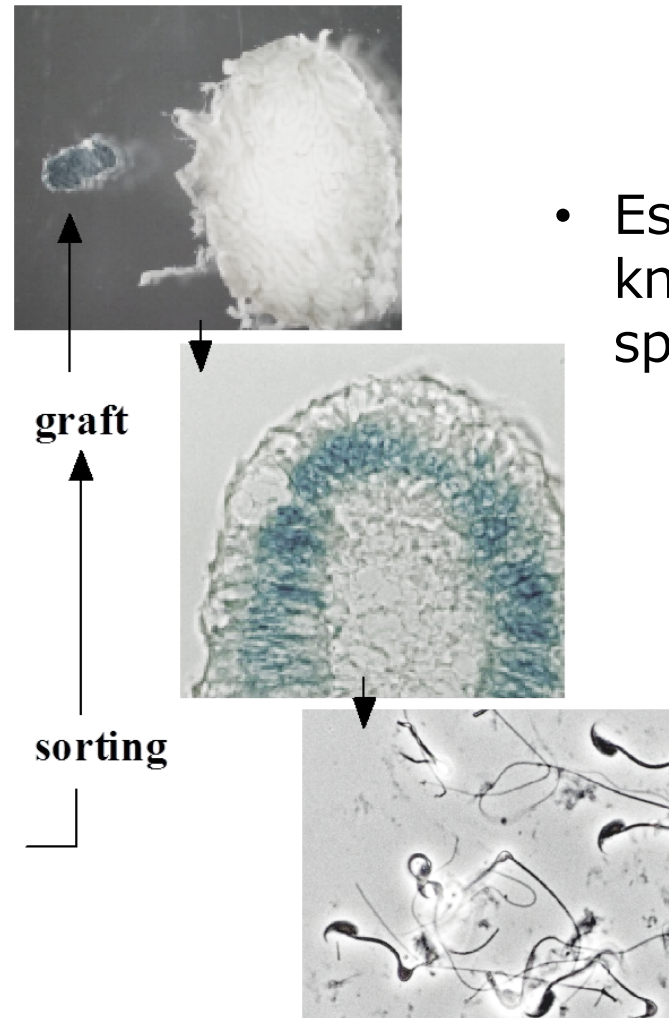
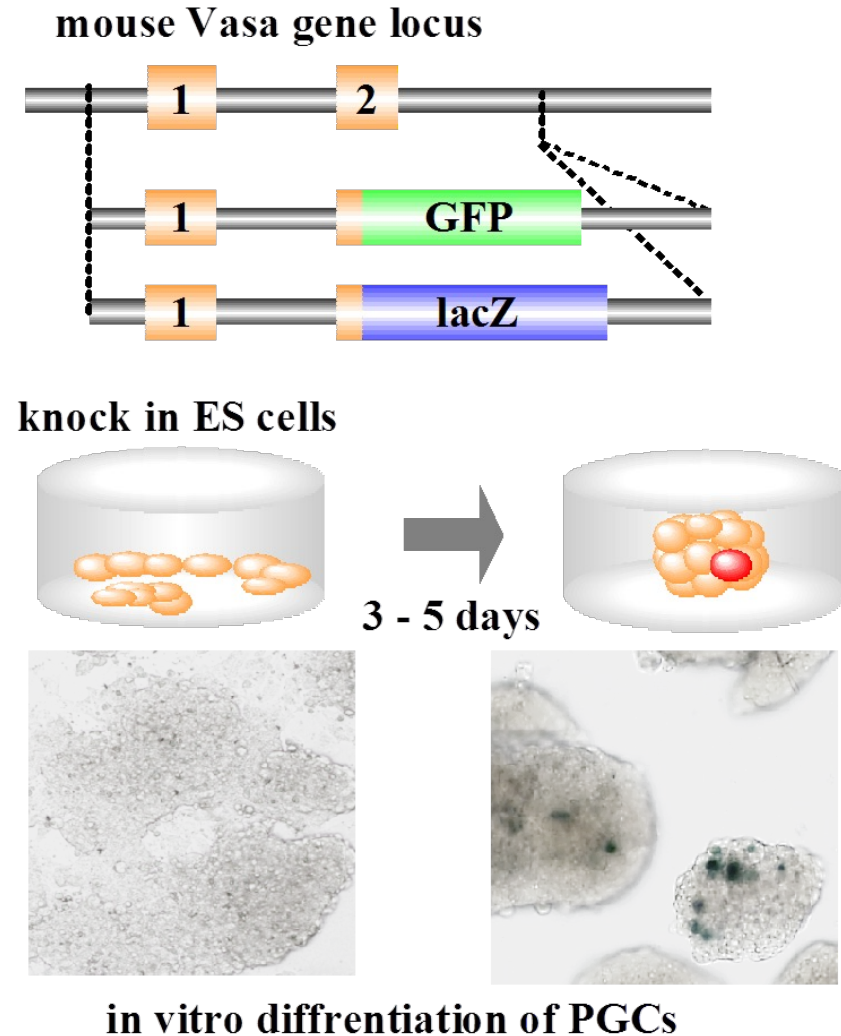


Dpt. Pediatric
Urology

Cryptorchidism
Hypospadias
Robotic surgery
...

Embryonic stem cells can form germ cells *in vitro*

Yayoi Toyooka, Naoki Tsunekawa, Ryuko Akasu, and Toshiaki Noce*

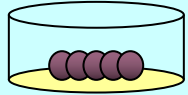


- Established ES cells with GFP or lacZ knock-in at the *Mvh* gene locus, specifically expressed in germ cells

Formed embryoid bodies
↓
sorted GFP-positive cells
↓
transplanted them into testes
↓
successfully induced donor-derived spermatogenesis

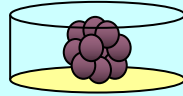
(Toyooka Y, Noce T, et al., *PNAS*, 2003)

Vasa-GFP/LacZ
knock-in ES cell



LIF(-)

EB formation



Low glucose (5.6mM)



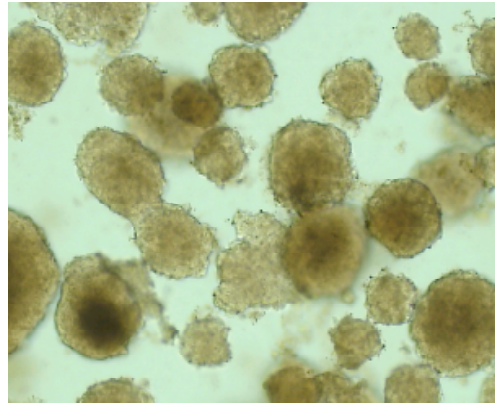
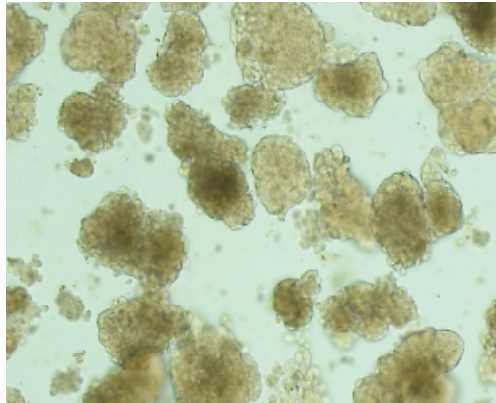
High glucose (25mM)

PGC differentiation varies
with **glucose levels**
in culture medium

ES (vasa-lacZ) alone **ES + BMP4-M15**

EB culture day 3

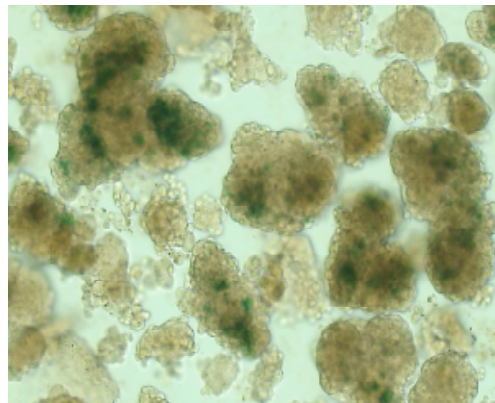
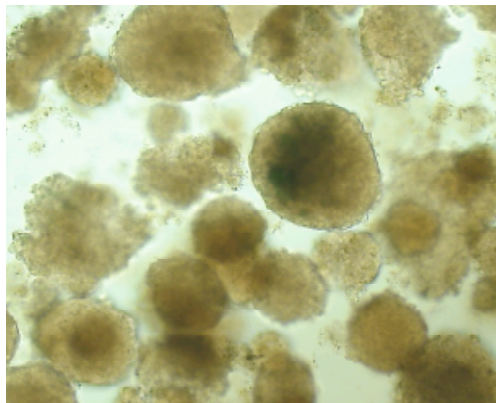
Low
glucose



PGC
differentiation

No

High
glucose



Yes

Identified candidate
genes involved in germ
cell development
using subtraction method

Genes Associated With the Formation of Germ Cells From Embryonic Stem Cells in Cultures Containing Different Glucose Concentrations

KENTARO MIZUNO,^{1,2} AKO TOKUMASU,¹ AYA NAKAMURA,¹ YUTARO HAYASHI,² YOSHIYUKI KOJIMA,² KENJIRO KOHRI,² AND TOSHIAKI NOCE^{1*}

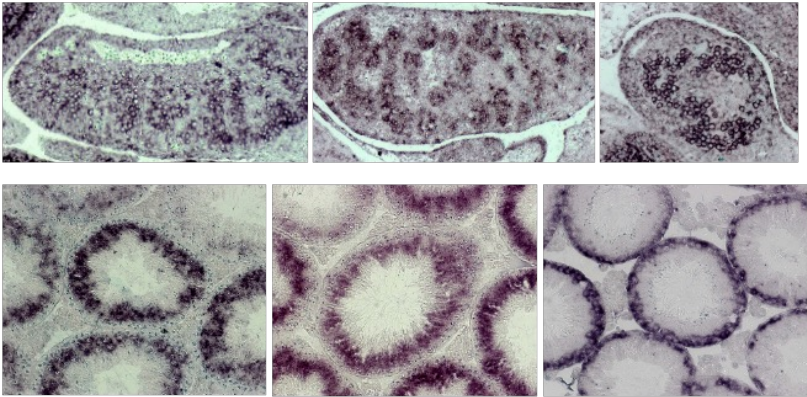
¹Mitsubishi Kagaku Institute of Life Sciences, Minami-Ooya, Machida, Tokyo, Japan
²Department of Nephro-urology, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, Japan

Identities of the subtracted clones of the high-glucose group

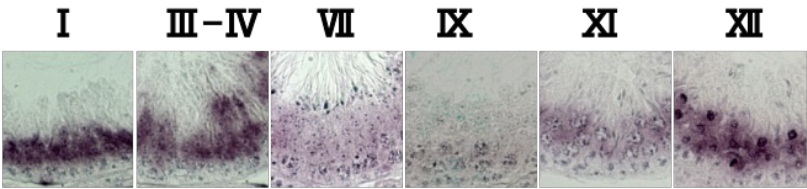
Clones	Gene symbol	Gene name
8	Txnip	Thioredoxin interacting protein
5	Pttg	Pituitary tumor-transforming 1
4	Sdc4	Syndecan 4
2	Ftl1	Ferritin light chain 1
2	Mdm4	Transformed mouse 3T3 cell double minute 4
2	Gnas	GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus
2	Sep15-pending	Selenoprotein
1	Hspa5	Heat shock 70kD protein 5
1	RuvBl2	RuvB-like protein 2
1	Dda3-pending	Differential display and activated by p53
1	Rps18	Ribosomal protein S18
1	Rpl5	Ribosomal protein L5
1	3110013H01Rik	RIKEN cDNA 3110013H01 gene
1	Paics	Phosphoribosylaminoimidazole carboxylase

In situ hybridization

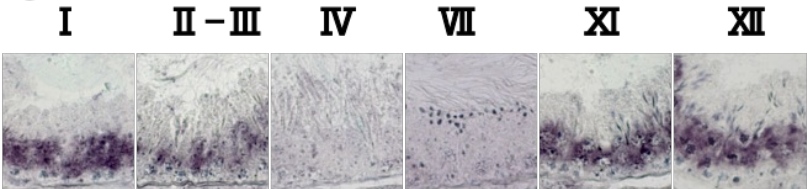
Pttg RuvBl2 Txnip



RuvBl2



Pttg



(Mizuno K, Noce T, et al., *Mol Reprod Dev*, 2006)

46,XX testicular DSD (disorders of sex development)

- Gonads are testes, with male internal/external genitalia and secondary sexual characteristics, despite 46,XX karyotype (de la Chapelle et al, 1964)
- Incidence: 1 / 20,000–25,000 births
- 80%: Translocation of SRY gene to X chromosome
- 10%: Associated with hypospadias



To elucidate the mechanism of testis development,

- 1. Elucidation of genes specifically expressed in XX male testes**
- 2. Genome-wide analysis of common mutations in XX males**

Gene Expression Profile During Testicular Development in Patients With *SRY*-negative 46,XX Testicular Disorder of Sex Development

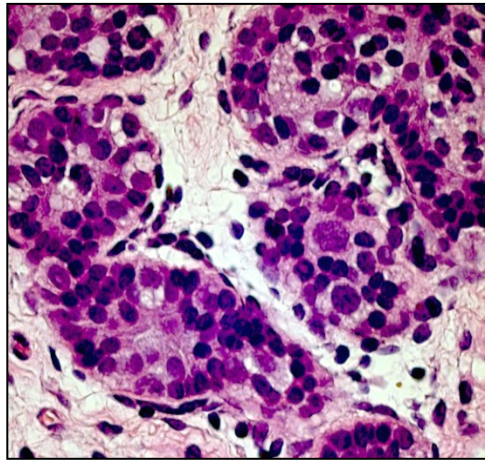
Kentaro Mizuno, Yoshiyuki Kojima, Hideyuki Kamisawa, Yoshinobu Moritoki, Hidenori Nishio, Kenjiro Kohri, and Yutaro Hayashi

In the XX *SRY*⁻ testis

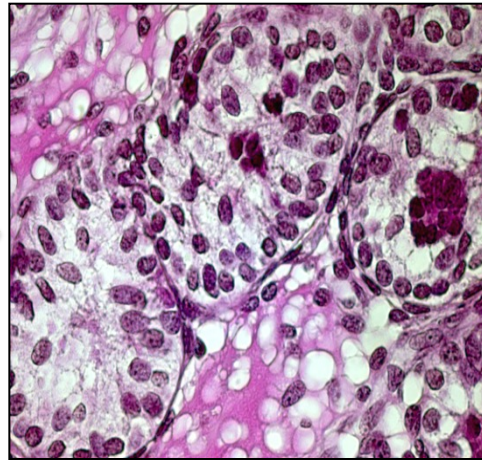
Up-regulated : 14 genes

Down-regulated : 7 genes

XY testis (2y5m)



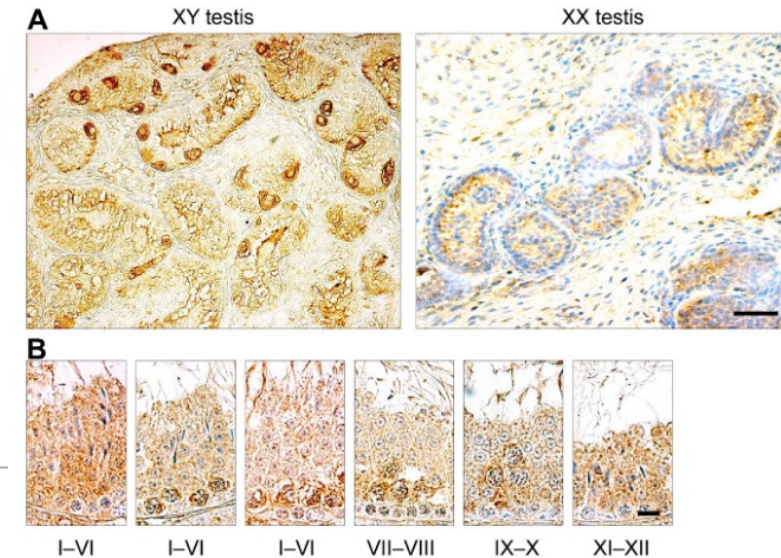
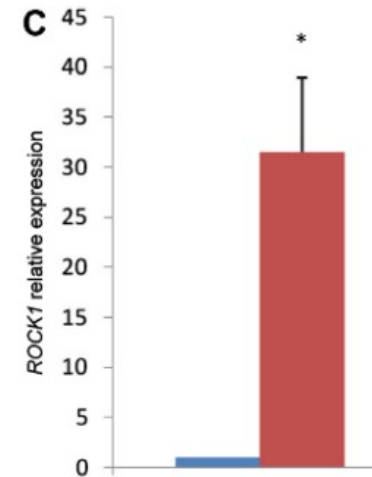
XX testis (2y7m), *SRY* negative



(x 1000, H-E stain)

- Testicular tissue obtained by intraoperative biopsy
- Subtraction method

ROCK1 gene



expressed in germ and Sertoli cells

(Mizuno K, Kojima Y, Hayashi Y, et al., *Urology*, 2013)

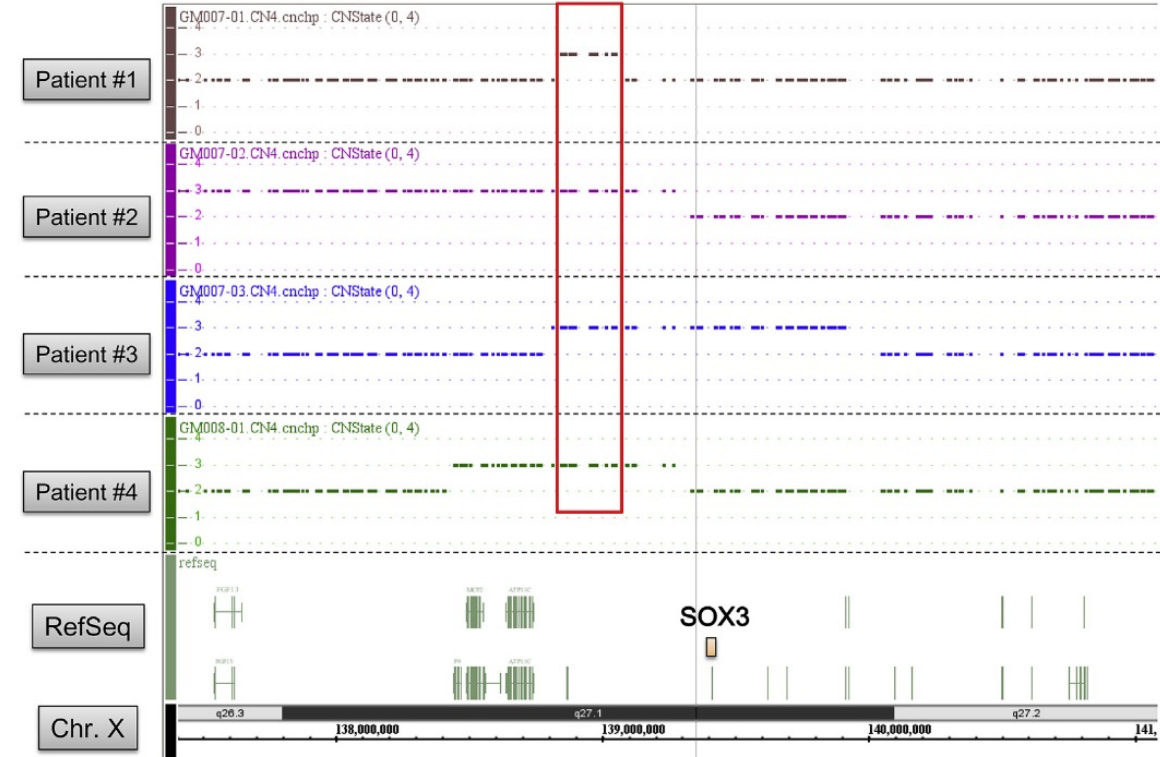
Elucidation of Distinctive Genomic DNA Structures in Patients with 46,XX Testicular Disorders of Sex Development Using Genome Wide Analyses

Kentaro Mizuno, Yoshiyuki Kojima, Hideyuki Kamisawa, Yoshinobu Moritoki, Hidenori Nishio, Akihiro Nakane, Satoshi Kurokawa, Kenjiro Kohri and Yutaro Hayashi*

- Four patients with 46,XX testicular DSD (SRY-negative)
 - Array CGH using genomic DNA extracted from peripheral blood
 - Normal female (46,XX) reference data used as control
- Identified a common copy number gain near the SOX3 gene in 4 cases
 - SOX3 pathway, independent of SRY, involved in testis development

Table 3. Common regions presenting with copy number gain

Chromosome	Genome Area	Size (kb)	Genes
2	2p11.2	45	None
14	14q32.33	150	None
19	19q11	203	None
X	Xp11.3	133	ZNF673
	Xq13.2	273	PABPC1L2A, NAP1L6, NAP2L2
	Xq27.1	206	CXorf66, upstream of SOX3

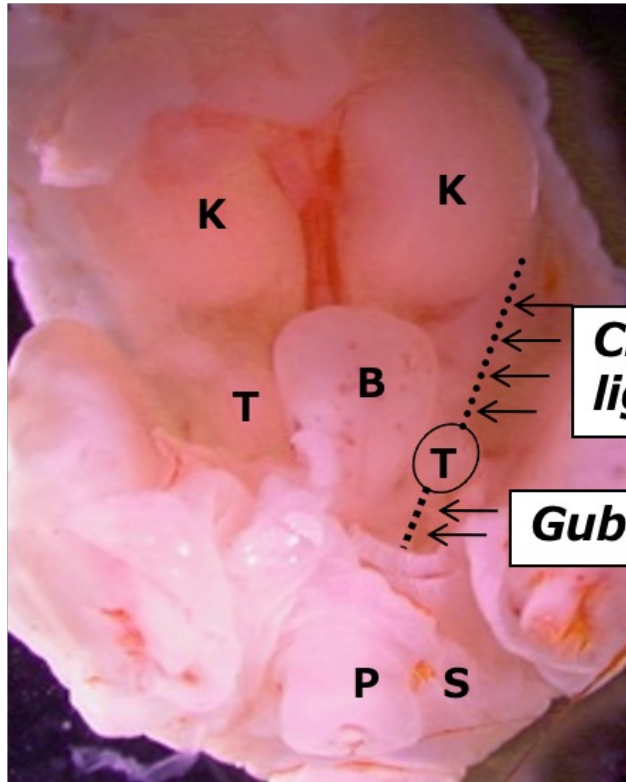


(Mizuno K, Kojima Y, Hayashi Y, et al. *J Urol*, 2014)

Nagoya castle



Biphasic descent of the testis



Cranial suspensory ligament (CSL)

Gubernaculum

Transabdominal phase

androgen independent

Inguinoscrotal phase

androgen dependent

K: kidney, B: bladder, T: testis

P: penis, S: scrotum

Basic research on cryptorchidism

Influence for testicular development and histological peculiarity in the testes of flutamide-induced cryptorchid rat model

Kentaro Mizuno, Yutaro Hayashi, Yoshiyuki Kojima, Satoshi Kurokawa, Shoichi Sasaki and Kenjiro Kohri
Department of Nephro-urology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Incidence rate (7.5mg/body):
unilateral 74.2%, bilateral 19.4%

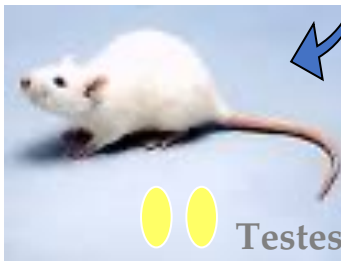
Pregnant S-D rat



2.5 - 15mg/body
Flutamide (d14-20)

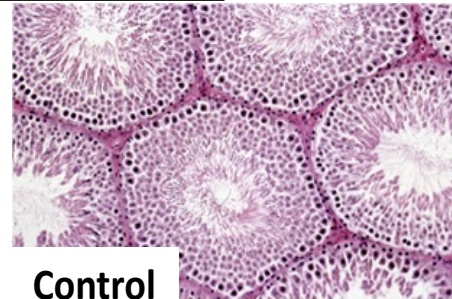
Birth

Model Rat

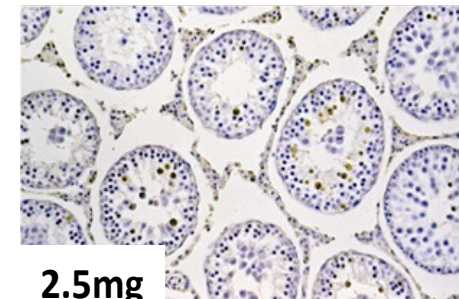


Testes

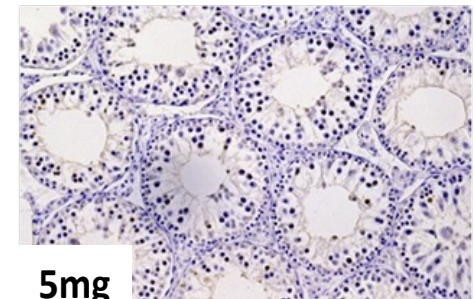
→ Evaluation



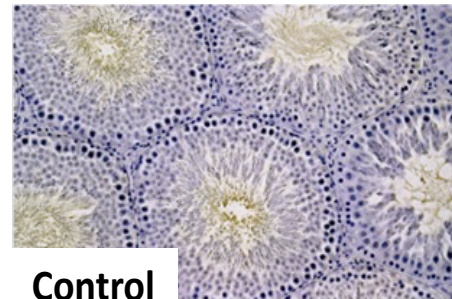
Control



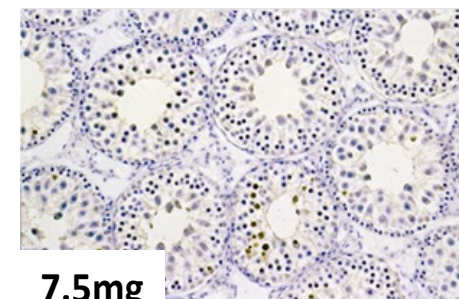
2.5mg



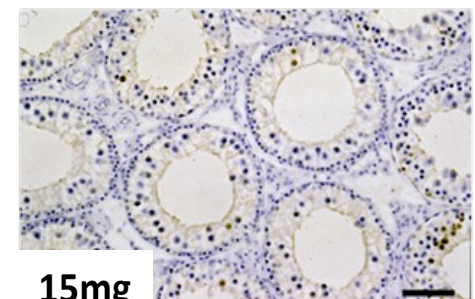
5mg



Control



7.5mg

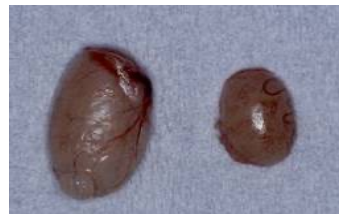


15mg



Control

Model



Control

Undescended

- Established an animal model of cryptorchidism by anti-androgen exposure during fetal period
- High incidence of undescended testes with enhanced germ cell apoptosis and reduced seminiferous tubule diameter

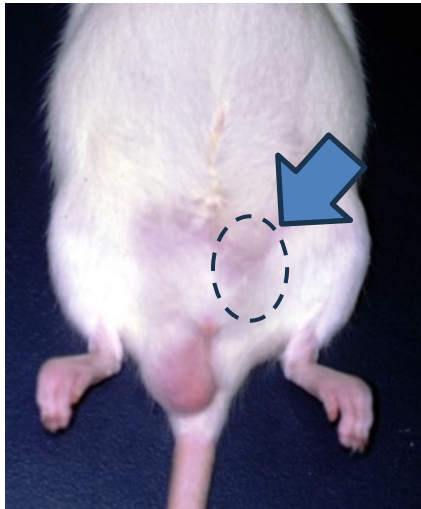
(Mizuno K, Hayashi Y, Kojima Y et al. *Int J Urol*, 2007)

Basic research on cryptorchidism

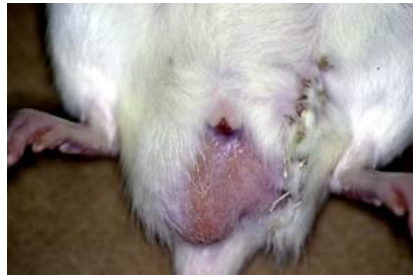
Early Orchiopexy Improves Subsequent Testicular Development and Spermatogenesis in the Experimental Cryptorchid Rat Model

Kentaro Mizuno,* Yutaro Hayashi, Yoshiyuki Kojima, Satoshi Kurokawa, Shoichi Sasaki and Kenjiro Kohri

From the Department of Nephro-urology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

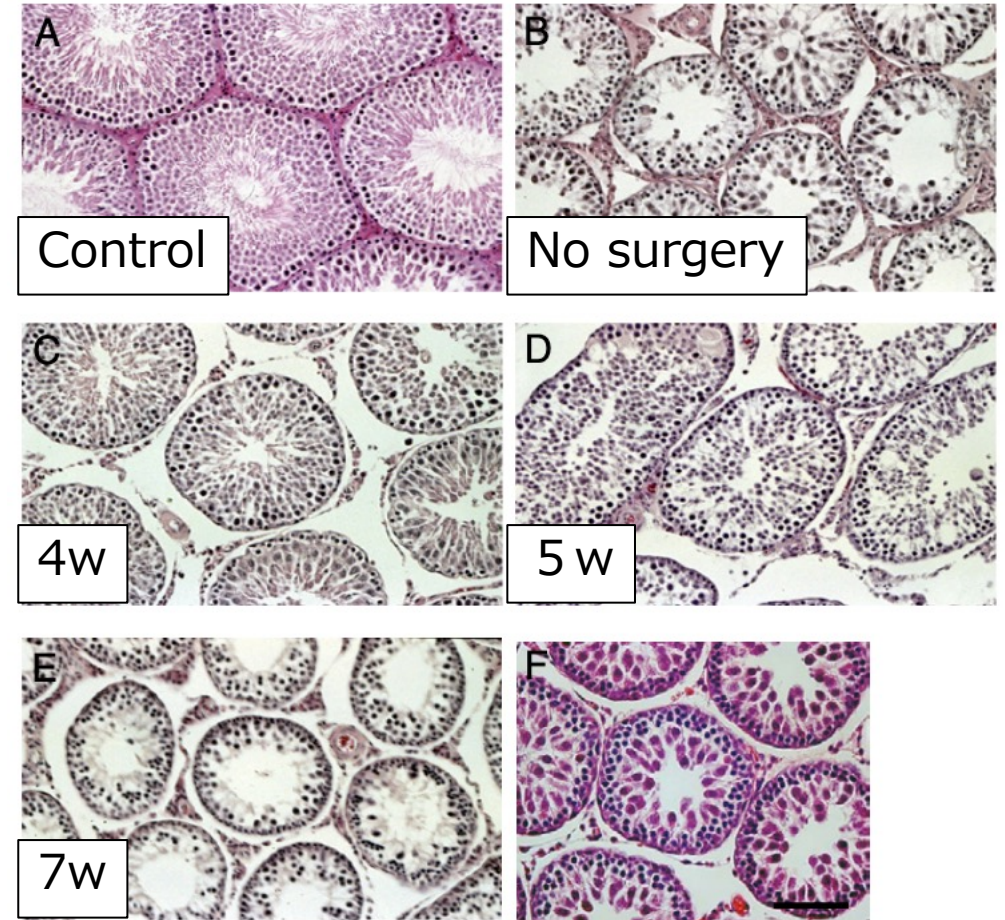


Orchiopexy for model rats at 4-7 weeks old



Evaluation of testicular tissues at 10 weeks old

- Orchiopexy reduces testicular tissue damage
- Earlier surgery further minimizes tissue damage
- Early orchiopexy brings beneficial effects to testicular tissue



(Mizuno K, Hayashi Y, Kojima Y et al. *J Urol*, 2008)

Basic research on cryptorchidism

Attenuation of Spermatogonial Stem Cell Activity in Cryptorchid Testes

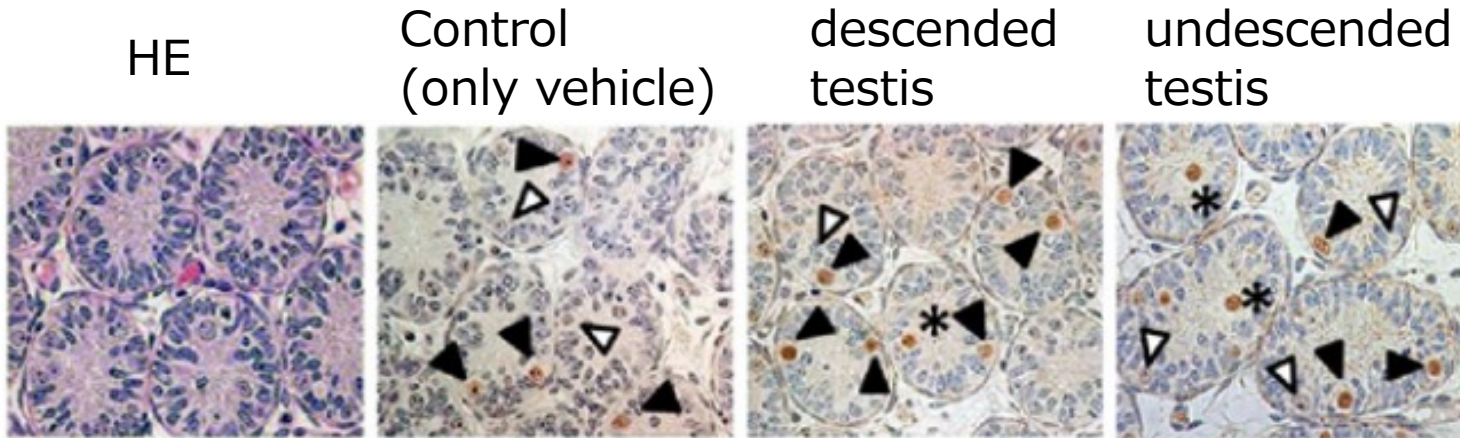
Hideyuki Kamisawa, Yoshiyuki Kojima,* Kentaro Mizuno, Makoto Imura, Yutaro Hayashi and Kenjiro Kohri

From the Department of Nephro-Urology, Nagoya City University, Graduate School of Medical Sciences, Nagoya, Japan



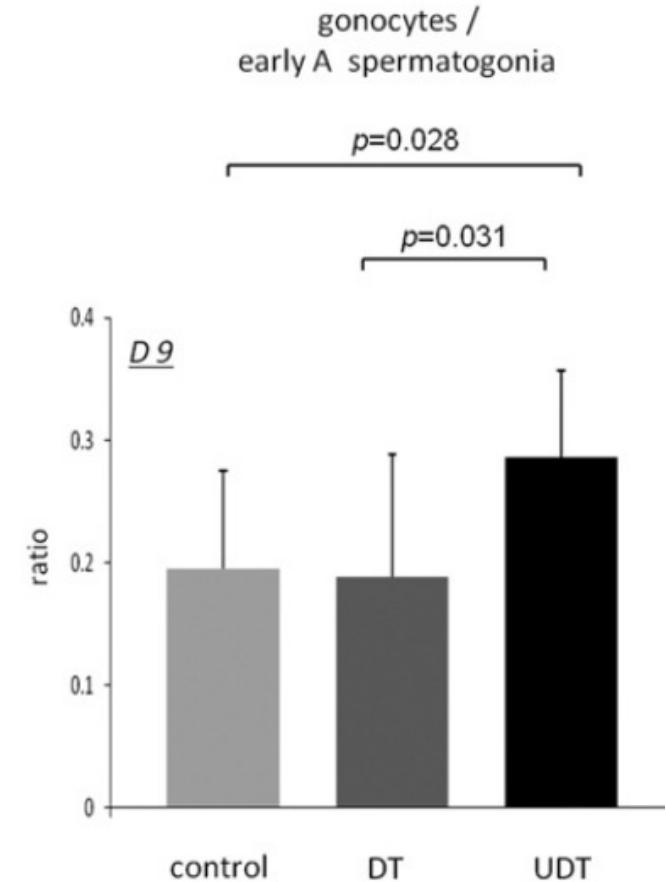
Time-course analysis of testicular tissue in a cryptorchidism model using UTF1 as a germ cell marker

【UTF1 immunohistochemistry】



(* : gonocytes, ▲ : UTF1(+) spermatogonia,
△ : UTF1(-) spermatogonia)

(day 9)



- Delayed differentiation from gonocytes to type A spermatogonia in the cryptorchidism model

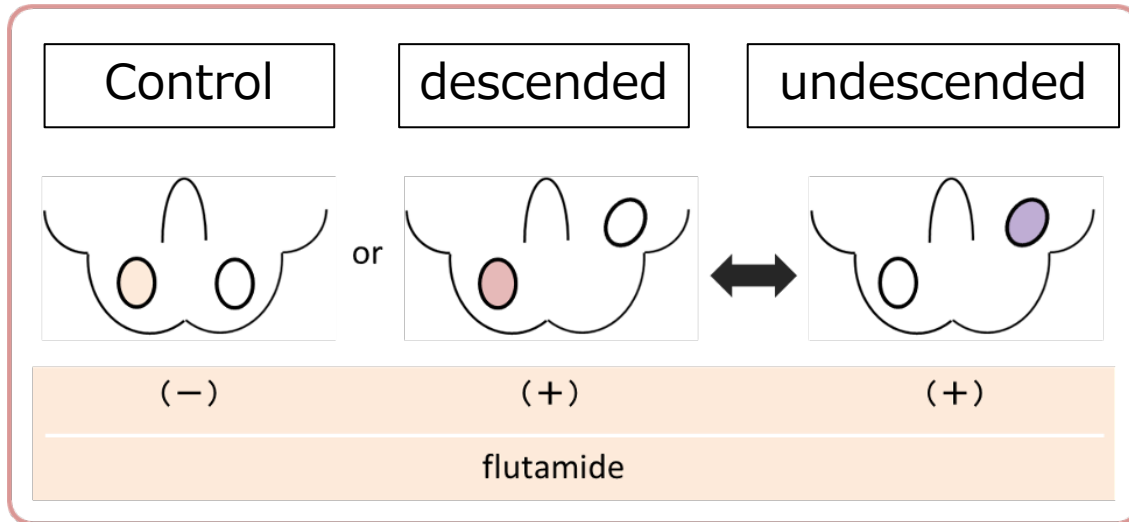
(Kamisawa H, Kojima Y, Mizuno K et al. *J Urol*, 2012)

Basic research on cryptorchidism

Distinctive Changes in Histone H3K4 Modification Mediated via Kdm5a Expression in Spermatogonial Stem Cells of Cryptorchid Testes

Hidenori Nishio, Yutaro Hayashi,* Yoshinobu Moritoki, Hideyuki Kamisawa, Kentaro Mizuno, Yoshiyuki Kojima and Kenjiro Kohri

From the Department of Nephro-Urology, Nagoya City University Graduate School of Medical Sciences, Nagoya and Department of Urology, Fukushima Medical University School of Medicine (YK), Fukushima, Japan



- Upregulated Kdm5a expression in cryptorchid testes
- Kdm5a also expressed in germ cells
- H3K4 histone modification may affect SSC differentiation

Microarray analysis

A. Twenty-four genes up-regulated in descended testes

Gene symbol Gene name
**up-regulated :
24 genes**

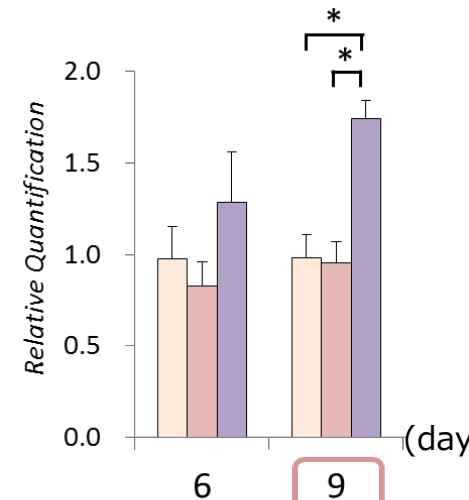
Yeast4 Yeast4 domain containing 4
Star Star
Ctcf1 Ctcf1
Kdm5a Kdm5a
Rab20 RAB20, member RAS oncogene family
Znf398 Zinc finger protein 398
Crop Cereals resistance-associated overexpressed protein
Tm9sf3 Transmembrane domain family member 3
RGD1563318 Similar to dimeric factor of hepatocyte nuclear factor 1 (HNF1)
Pdc4 Pdc4
LOC680485 LOC680485
RGD1311783 Similar to RIKEN cDNA 10012005
Atp5d ATP5D-like
Adam10 ADAM metalloproteinase domain 10
Pdyn Pdyn
Crim1 Crim1
Sed Sed
Alox3 Alox3
Polr2h Polr2h

Kdm5a

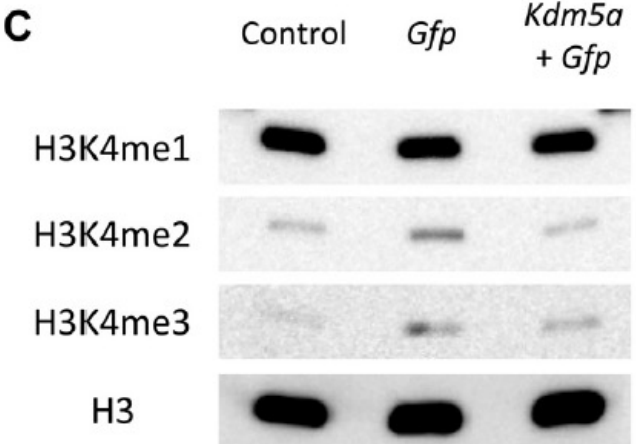


Kdm5a
expression

control
descended
undescended



C



(Nishio H, Hayashi Y, Mizuno K et al. *J Urol*, 2014)

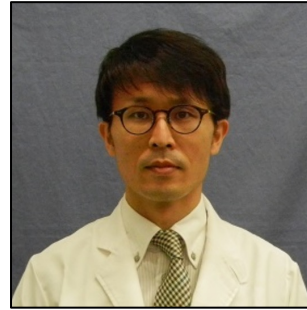
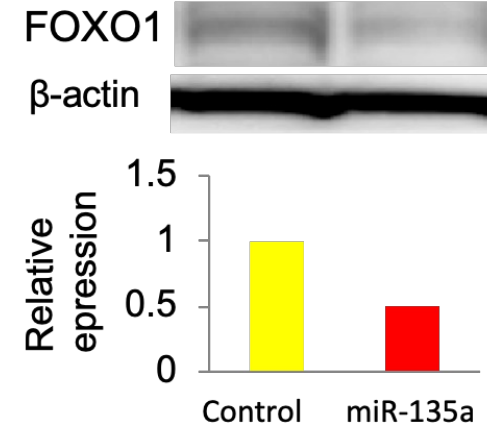
Basic research on cryptorchidism

Expression Profiling of microRNA in Cryptorchid Testes: miR-135a Contributes to the Maintenance of Spermatogonial Stem Cells by Regulating FoxO1

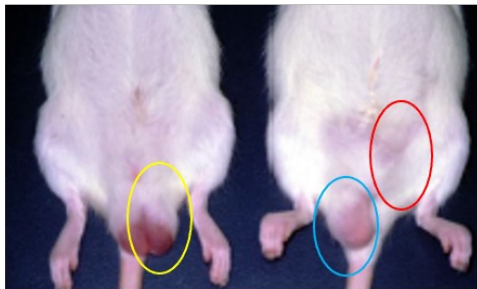
Yoshinobu Moritoki, Yutaro Hayashi,* Kentaro Mizuno, Hideyuki Kamisawa, Hidenori Nishio, Satoshi Kurokawa, Shinya Ugawa, Yoshiyuki Kojima and Kenjiro Kohri

From the Departments of Nephro-Urology (YM, YH, KM, HK, HN, SK, KK) and Neurobiology and Anatomy (SU), Graduate School of Medical Sciences, Nagoya City University, Nagoya and Department of Urology, Fukushima Medical University (YK), Fukushima, Japan

Western Blot

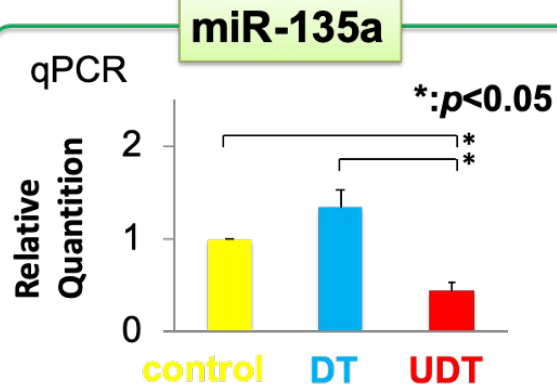


"Day 9"

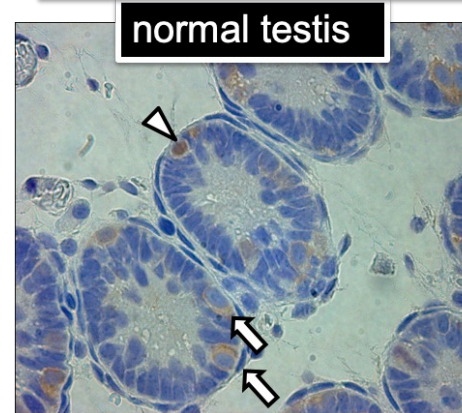


control DT UDT

- Reduced expression of miR-135a in cryptorchid testes
- miR-135a suppresses FOXO1 expression
- FOXO1-positive SSCs significantly decreased in cryptorchid testes

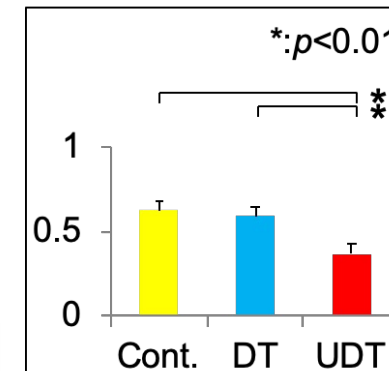


FOXO1 localize in SSCs

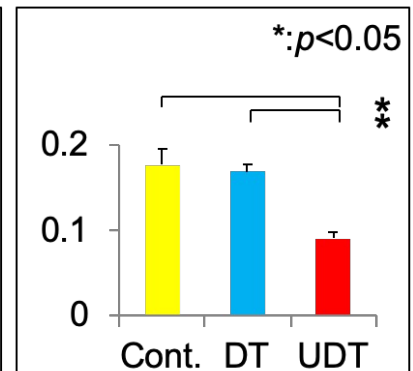


localization patterns
 $\blacktriangle$: nuclear positive
 $\blacktriangleup$: cytoplasm positive

FOXO1 nuclear posi. /tubule



FOXO1 nuclear posi. /cytoplasm posi.

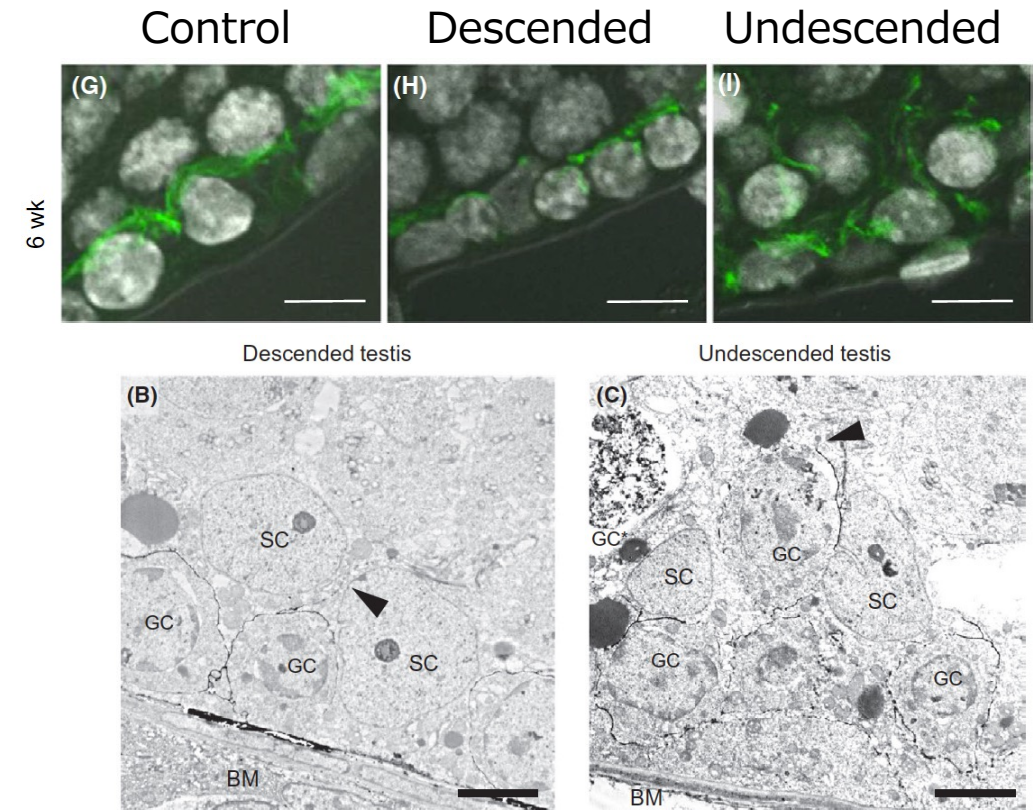
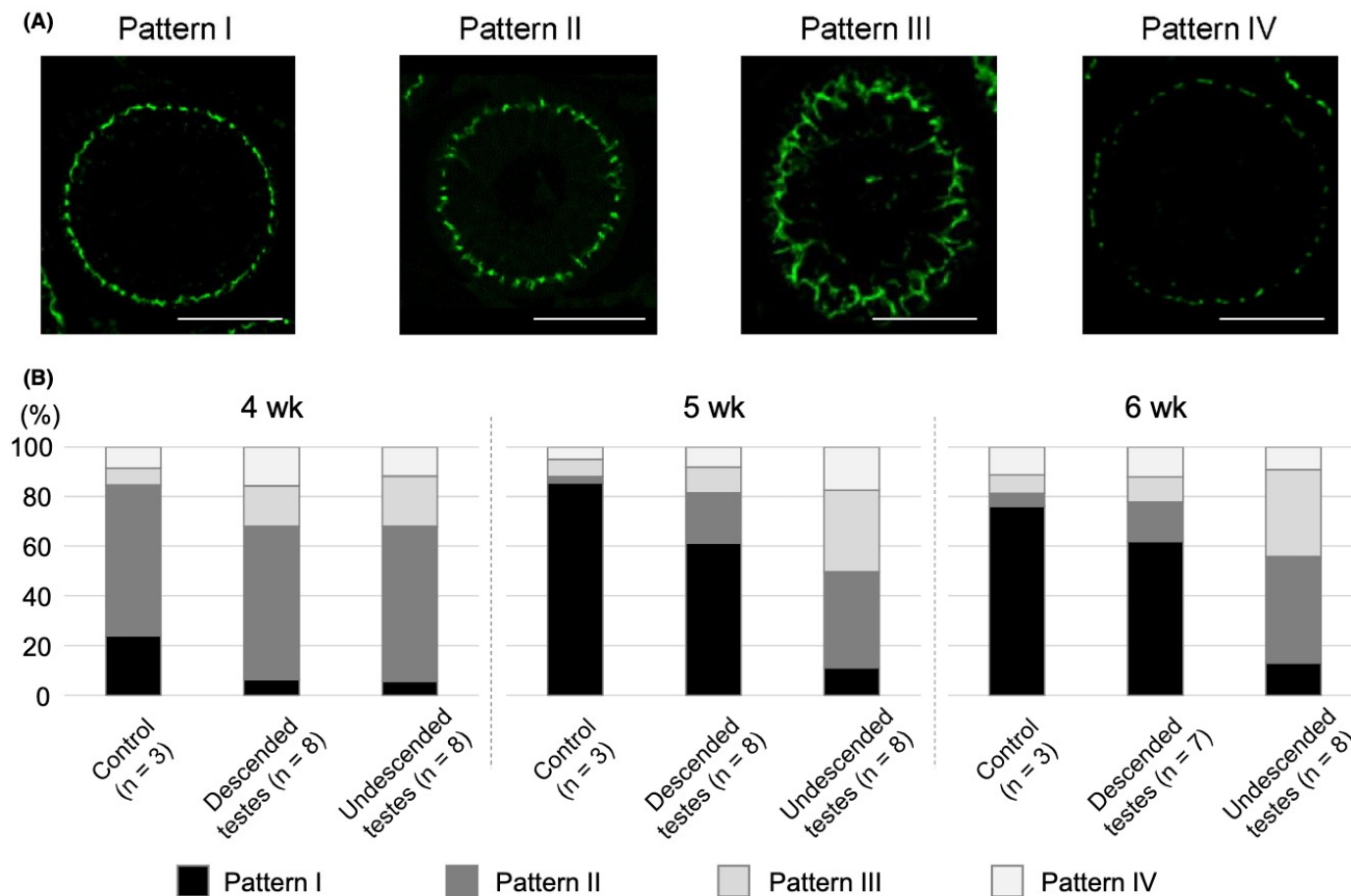


(Moritoki Y, Hayashi Y, Mizuno K et al. *J Urol*, 2014)

Basic research on cryptorchidism

Disorganization of claudin-11 and dysfunction of the blood-testis barrier during puberty in a cryptorchid rat model

Taiki Kato¹ | Kentaro Mizuno²  | Hidenori Nishio² | Yoshinobu Moritoki¹ | Hideyuki Kamisawa¹ | Satoshi Kurokawa¹ | Akihiro Nakane¹ | Tetsuji Maruyama¹ | Ryosuke Ando¹ | Yutaro Hayashi² | Takahiro Yasui¹



- Disorganization of CLDN-11 in BTB (blood testis barrier) of cryptorchid testes
- Failure of BTB formation at puberty
- Induction of germ cell apoptosis

(Kato T, Mizuno K, Nishio H et al. *Andrology*, 2020)

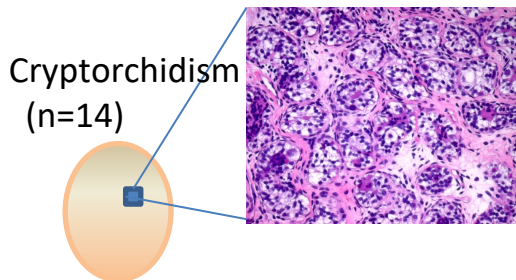
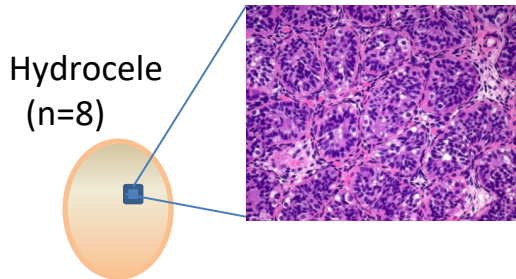
Basic research on cryptorchidism

Identification of Differentially Expressed Genes in Human Cryptorchid Testes Using Suppression Subtractive Hybridization

Kentaro Mizuno, Yoshiyuki Kojima, Satoshi Kurokawa, Tetsuji Maruyama, Shoichi Sasaki, Kenjiro Kohri and Yutaro Hayashi*

From the Department of Nephro-Urology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Aichi, Japan

- Testicular tissue obtained by intraoperative biopsy
- Subtraction method

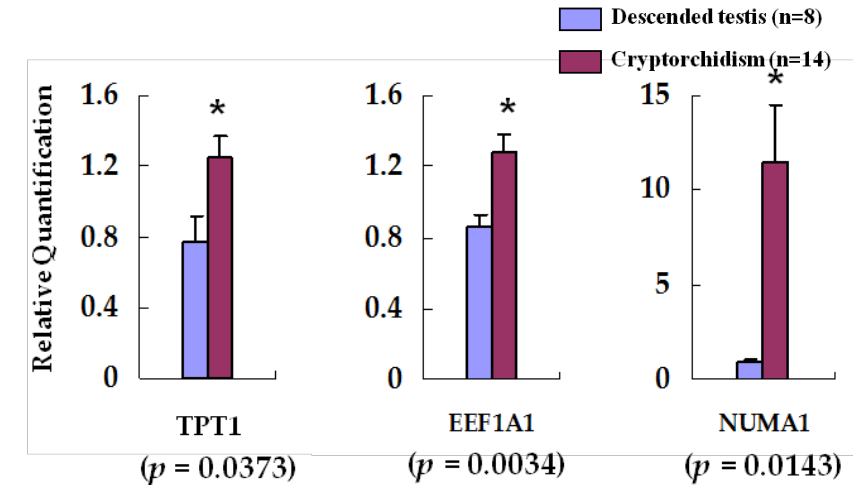


Gene symbol	Gene name	Function	Accession No.
TPT1	Tumor protein, translationally controlled 1	Anti-apoptosis	NM_003295
FTH1	Ferritin, heavy polypeptide 1	Cell proliferation	NM_002032
LOC441581	FSDH region gene 2 protein	Unknown	NM_001080998
ZSCAN2	Zinc finger and SCAN domain containing 2, transcript 1	Cell differentiation	NM_181877
EEF1A1	Eukaryotic elongation factor 1A1	Translation	NM_001013
NUMA1	Nucleoside diphosphate kinase 1	Translation	NM_001013
NBPF1	Neuroblastoma breakpoint family, member 14	Unknown	NM_015383
UBC	Ubiquitin C	Unknown	NM_021009
NBPF14	Neuroblastoma breakpoint family, member 14	Unknown	NM_015383
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1	Unknown	NR_002819
TTTY6	Testis-specific transcript, Y-linked 6	Unknown	AF332237
RPL3	Ribosomal protein L3, transcript variant 1	Translation	BC_107711
RPL7A	Ribosomal protein L7a	Translation	NM_000972
RPL32	Ribosomal protein L32	Translation	NM_000994
RPS9	Ribosomal protein S9	Translation	NM_001013
RPS14	Ribosomal protein S14	Translation	NM_001025071
RPS20	Ribosomal protein S20	Translation	NM_001023
RPS25	Ribosomal protein S25	Translation	NM_001028

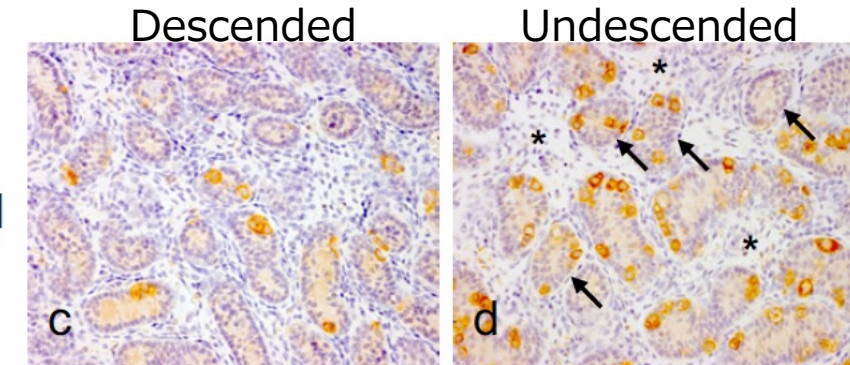
18 up-regulated genes

Gene symbol	Gene name	Function	Accession No.
HNRPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1	RNA splicing	NM_002137.2
MYL6	Myosin, light chain 6, alkali, smooth muscle and non-muscle	Muscle development	NM_021019.3
IFITM3	Interferon-induced with tetratricopeptide motifs 3	Unknown	NM_021034.1
IFITM2	Interferon-induced with tetratricopeptide motifs 2	Unknown	NM_021034.1
ZC3H7C	Zinc finger CCHC domain containing 7C	Unknown	NM_001013
SOD1	Superoxide dismutase 1	Unknown	NM_001013
PTGDS	Prostaglandin G/H synthase 2	Unknown	NM_000954.5
CLU	Clusterin, transcript variant 2	Anti-apoptosis	NM_203339.1
RPL7A	Ribosomal protein L7a	Translation	NM_000972.2
RPL17	Ribosomal protein L17	Translation	NM_001035006.1
RPL24	Ribosomal protein L24	Translation	NM_000986.3
RPL28	Ribosomal protein L28	Translation	NM_000991.3
RPS2	Ribosomal protein S2	Translation	NM_002952.3
RPS18	Ribosomal protein S18	Translation	NM_022551.2
RPS25	Ribosomal protein S25	Translation	NM_001028.2

16 down-regulated genes



EEF1A1



- Identified TPT1, EEF1A1, and NuMA1 genes upregulated in human cryptorchid testes
- All were expressed in germ cells during early spermatogenesis

(Mizuno K, Hayashi Y et al. *J Urol*, 2009)

Atsuta Shrine



Clinical research on cryptorchidism

Low Serum Inhibin B/Follicle-Stimulating Hormones and Anti-Müllerian Hormone/Follicle-Stimulating Hormones Ratios as Markers of Decreased Germ Cells in Infants with Bilateral Cryptorchidism

Taiki Kato,¹ Kentaro Mizuno ,^{2,*} Daisuke Matsumoto,³ Hidenori Nishio,² Akihiro Nakane,⁴ Satoshi Kurokawa,³ Hideyuki Kamisawa,³ Tetsuji Maruyama,¹ Shoichiro Iwatsuki,³ Yukihiro Umemoto,⁵ Takahiro Yasui³ and Yutaro Hayashi²

- To assess for associations between serum hormone level and testicular histopathology

DDX4 immunohistochemistry

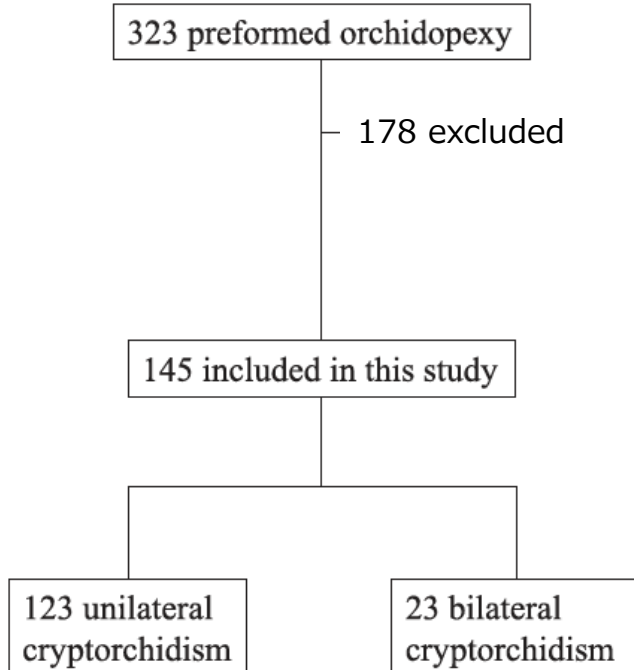
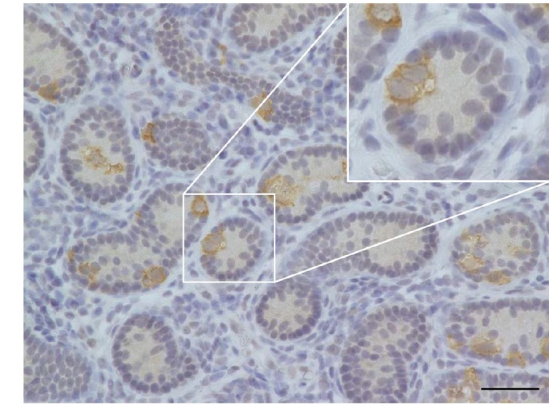


Table 3. Patient characteristics, serum hormone levels and G/T between unilateral and bilateral cryptorchid boys aged ≤ 24 months at orchidopexy

	Unilat		Bilat		p Value*
No. pts	82		12		
Median mos age at orchidopexy (range)	17 (7–24)		18.5 (11–24)		0.130
Median cm ³ individual testicular vol (range)	0.34 (0.18–1.10)		0.45 (0.23–1.12)		0.073
Median pg/ml inhibin B (range)	140 (52–354)		111 (71–197)		0.098
Median pmol/L AMH (range)	1,100 (381–2,942)		831 (408–2,913)		0.149
Median mIU/ml FSH (range)	1.0 (0.3–3.2)		1.3 (0.6–4.6)		0.131
Media mIU/ml LH (range)	0.1 (0.1–1.0)		0.1 (0.1–1.6)		0.393
Median ng/ml T (range)	0.03 (0.03–0.03)		0.03 (0.03–0.03)		1.000
Median inhibin B/FSH (range)	140 (26–885)		76 (20–237)		0.036
Median AMH/FSH (range)	1.078 (140–8,092)		649 (147–2,202)		0.063
G/T (range)	0.66 (0–4.55)		0.24 (0–0.95)		< 0.001

Statistically significant p values are bolded.

* Measured using the Mann-Whitney U test.

(Kato T, Mizuno K, Hayashi Y et al. *J Urol*, 2022)

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Inhibin-B/FSH
ratio

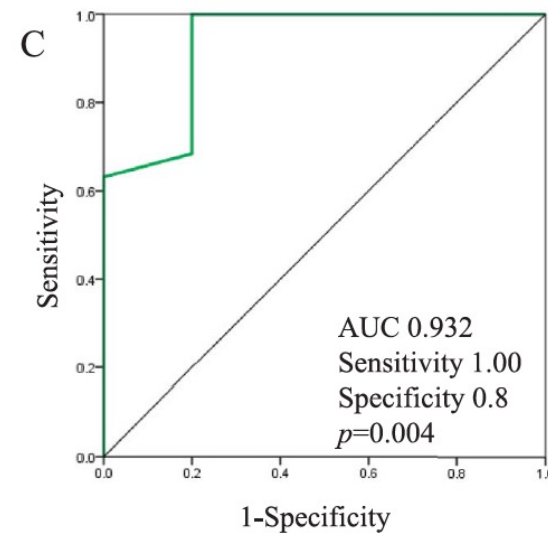
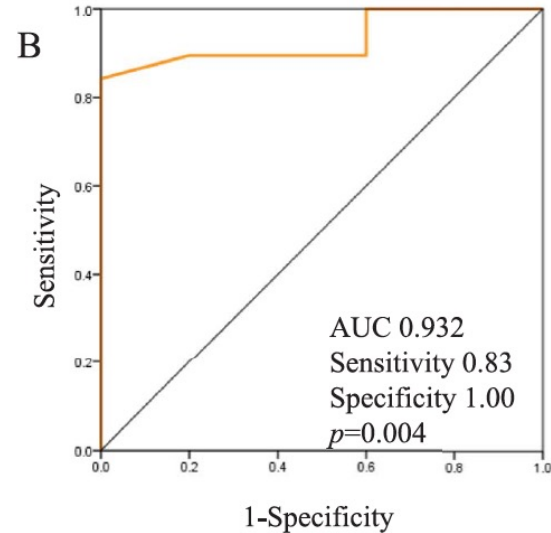
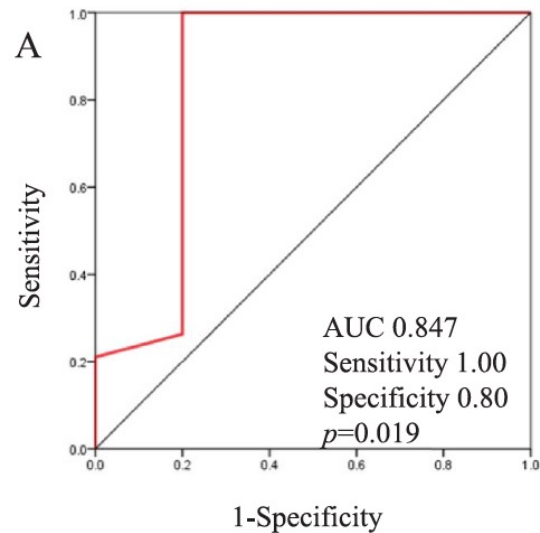
AMH/FSH
ratio

FSH value

Cut-off <134 pg/mIU

Cut-off <766 ng/mIU

Cut-off > 0.9 mIU/ml



- Bilateral cases have a higher risk of impaired spermatogenesis
- No correlation between testicular position and G/T
- In bilateral cases ≤ 24 months, G/T ratio can be predicted by combining serum hormone levels (Inhibin B, AMH, FSH)

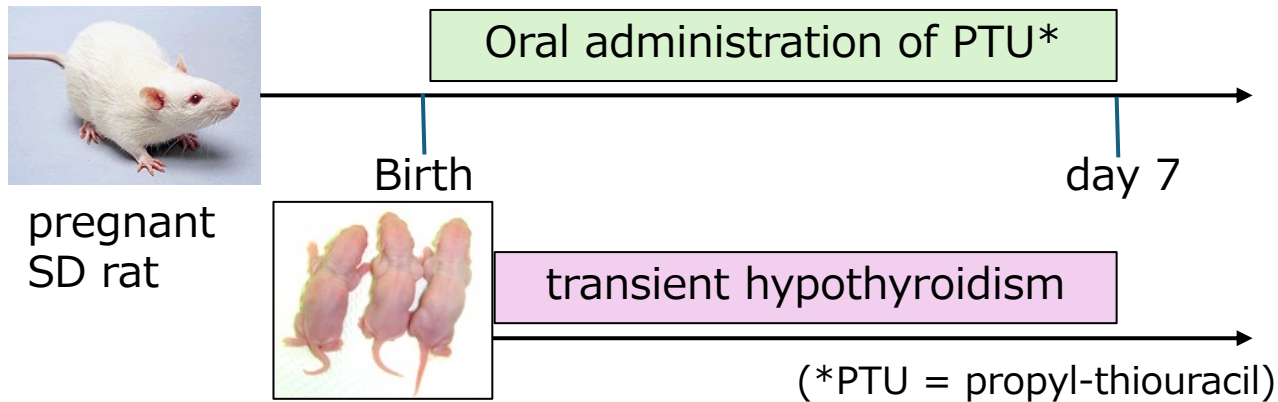
(Kato T, Mizuno K, Hayashi Y et al. *J Urol*, 2022)

New animal model for early spermatogenesis

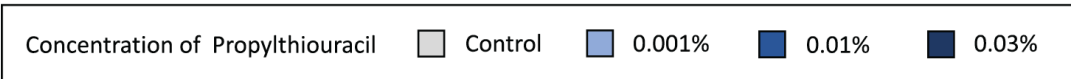
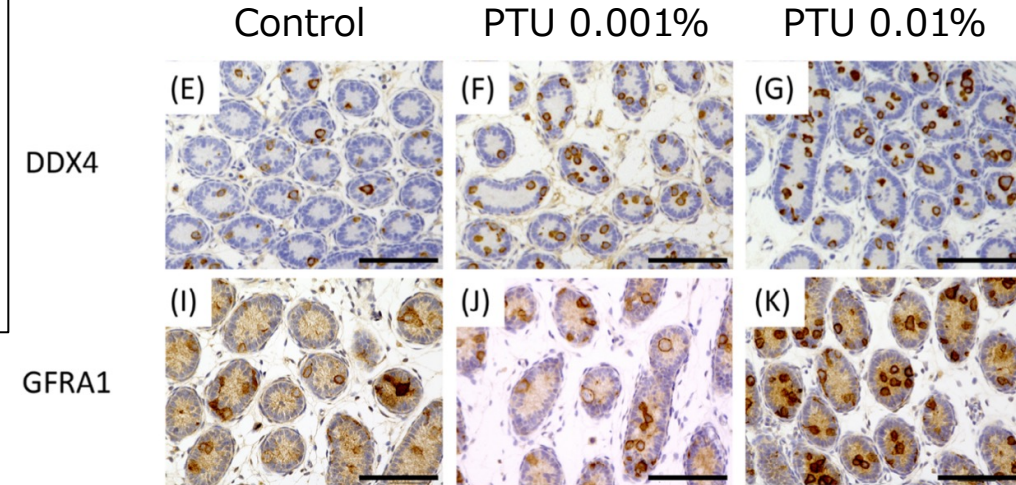
Effects of neonatal hypothyroidism on testicular development and undifferentiated spermatogonia in prepubertal rats

Daisuke Matsumoto¹ | Kentaro Mizuno² | Hidenori Nishio² | Hideyuki Kamisawa^{1,3} |
Takuya Sakata¹ | Taiki Kato^{1,4} | Akihiro Nakane^{1,5} | Satoshi Kurokawa^{1,3} |
Tetsuji Maruyama⁶ | Yutaro Hayashi² | Takahiro Yasui¹

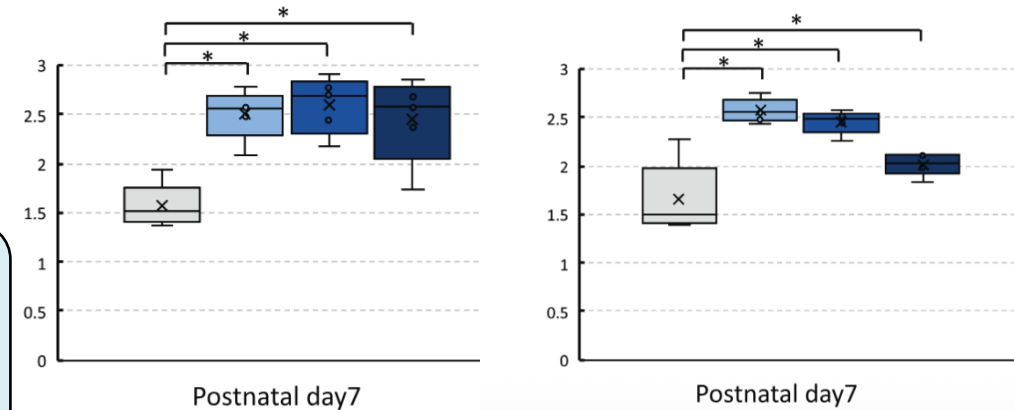
- To investigate the effects of hypothyroidism on early spermatogenesis, we developed novel rat model.



- Established a neonatal transient hypothyroidism animal model
- This model showed an increased number of undifferentiated spermatogonia
- Thyroid function may be involved in early spermatogenesis



(A) DDX4-positive cells per tubules (C) GFRA1-positive cells per tubules



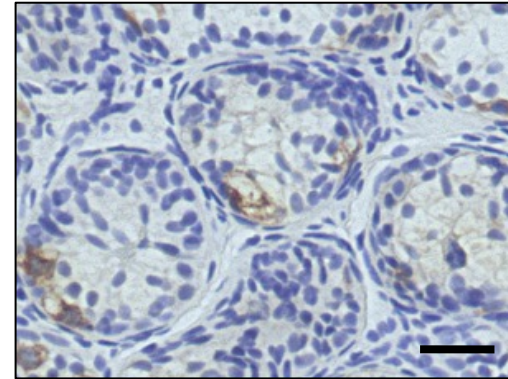
(Matsumoto D, Mizuno K, Hayashi Y et al. *Andrology*, 2025)

Translational research

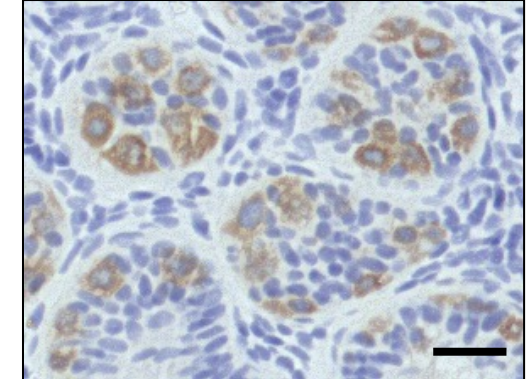
- Aimed to elucidate the effect of hypothyroidism on early spermatogenesis in cryptorchidism
- We retrospectively reviewed patients who underwent orchiopexy between January 2008 and February 2021.
- Divide group by past history of congenital hypothyroidism (CH)

DDX4 immunohistochemistry

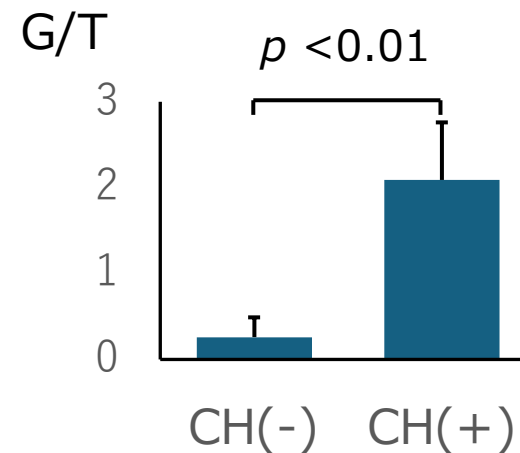
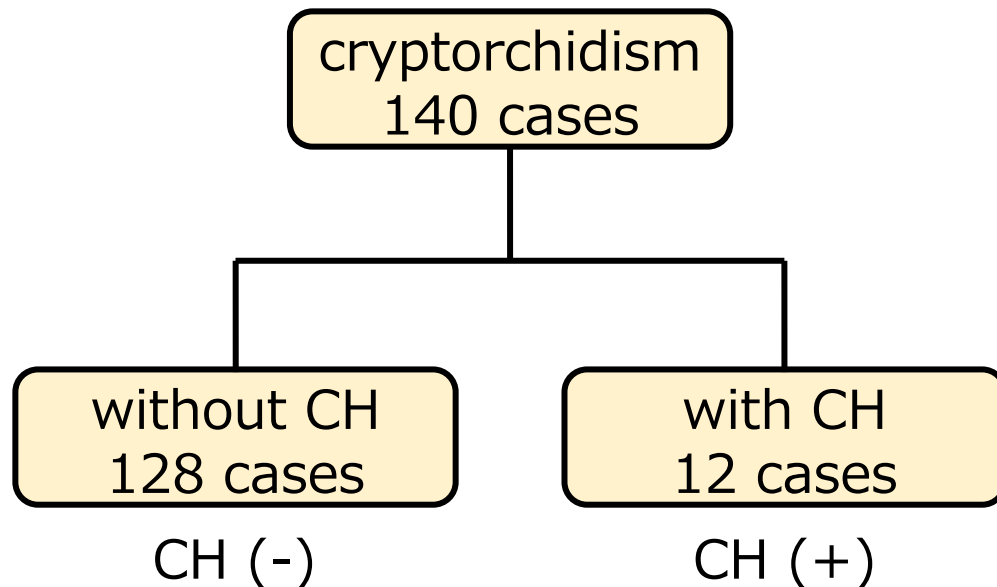
CH (-)



CH (+)



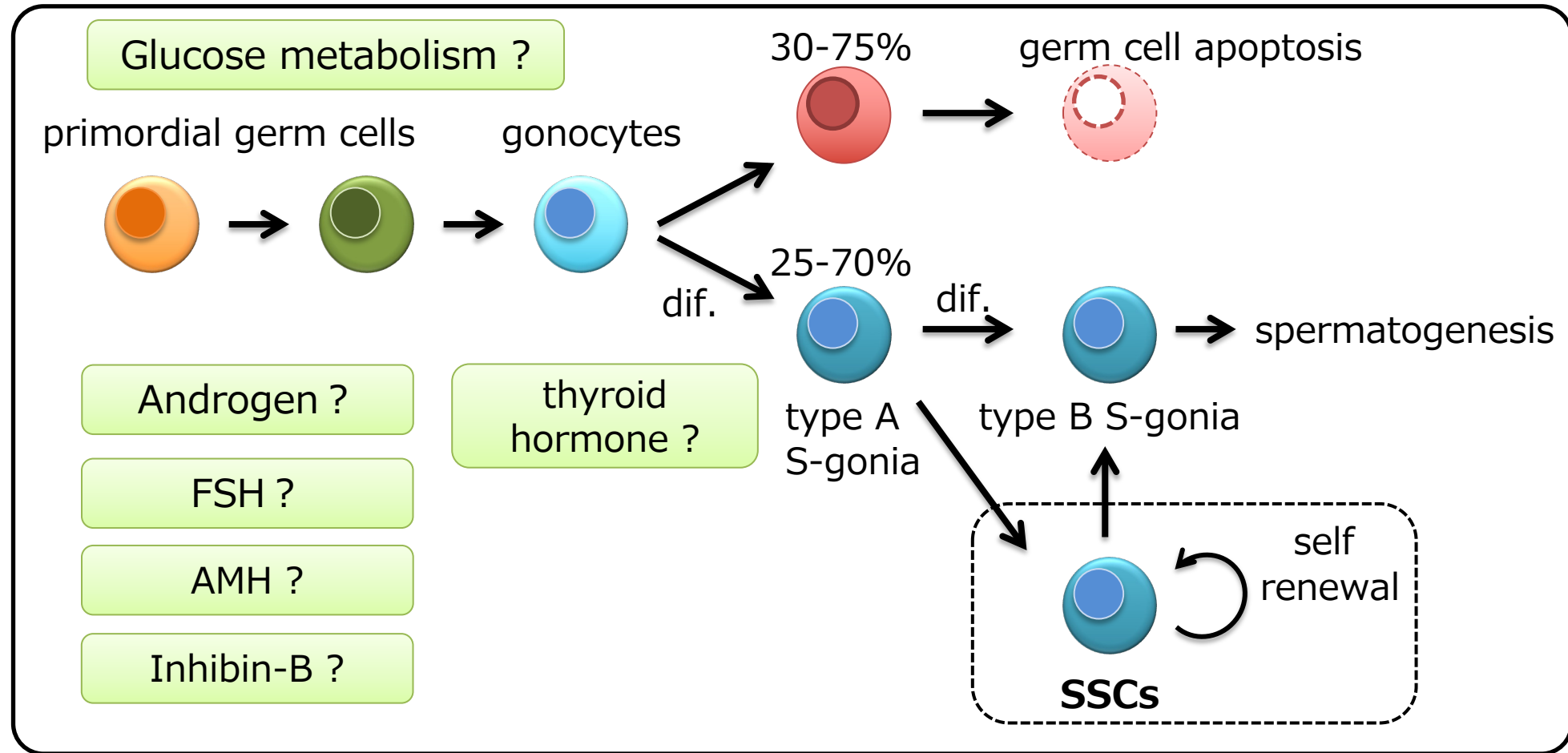
(scar bar = 25μm)



- As in the animal model, the CH(+) group showed an increased number of germ cells
- Early-life thyroid dysfunction may affect early spermatogenesis

(unpublished data)

Biological effects on early-stage spermatogenesis in cryptorchidism



Acknowledgement

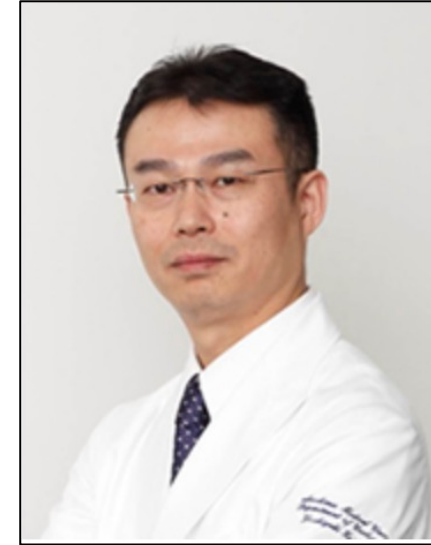


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Thank you for your attention