Xenografting of human fetal testis tissue: a new approach to study fetal testis development and germ cell differentiation

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Introduction

- Indifferent gonad → SRY gene → Sertoli cells (Wilhelm et al., 2007)
- Fetal germ cells (gonocytes) →form seminiferous cords (start at GA 7~9wks) (Wartenberg,1981; Tilmann and Capel, 1999; Hanley et al., 2000; Gaskell et al., 2004; Ostrer et alk., 2007)
- Fetal leydig cells differentiation → secret T → masculinization (Scott et al., 2009)

Introduction

- Disorders of sex development (DSDs)
  - Failure of normal gonad development or subnormal androgen production / action . (Hughes, 2008)
  - Testicular germ cell tumor (TGCT)
    - Predispose from CIS (Skakkebaek, 1972; Rajpert-De Meyts, 2006)
    - Failure of differentiation of germ cell (expresses OCT4 and NANOG) into pre-spermatogonia (expresses VASA and MAGE-A4) . (Rajpert-De Meyts, 2006; Gaskell et al., 2004; Anderson et alk., 2007; Mitchell et al., 2008)
    - The most serious manifestation of a testicular dysgenesis syndrome (TDS).

Introduction

- Models for studying testicular developments
  - Only rodent models, development was different from primates (Wilhelm et al., 2007; Gassei et al., 2008; Ehmcke et al., 2006)
  - Human models : retrospective studies, with DSDs or TDS disorder
  - Testicular xenograft (Wistuba and Schlatt 2002; Dobrinski. 2008; Ehmcke and Schlatt, 2008; Schlatt et al., 2010)

Introduction

- Testicular xenograft
  - Immature testis tissue of several species → into a nude mouse host → full spermatogenesis of the grafted tissue (Honaramooz et al., 2004; Rodriguez-Sosa and Dobrinski, 2009)
  - Human testis xenografts → limited survival

    (Geens et al., 2006; Schlatt et al., 2009)

Aim of the study

- To investigate the suitability of human fetal testis xenografting to recapitulate normal fetal testis development.

Materials and Methods

Human fetal testes

- 1st trimester (9 wks, n=4)
- 2nd trimester (14~18wks , n=6)
- 3 groups
  - Pre- graft group
  - Xenograft
  - Age-matched control

Procedures

- 3~6 testis grafts from a single fetus were inserted subcutaneously.
- OP ~ post-OP day 5 →Analgesia + antibiotics
- Day 8 : 2nd trimester xenografts + hCG
- Retrieved and weighed grafts after 6 wks → immunostaining
- Killed host mice → check T

Table I **Antibodies and conditions used for immunohistochemistry in xenograft study using human fetal testis tissue in nude mice.**

Antigen	Source	Species	Dilution	Retrieval
AMH	Santa Cruz[a]	Goat	1:500	N
AR	Santa Cruz[a]	Rabbit	1:200	Y
MAGE-A4	Gift[c]	Mouse	1:20	N
OCT 4	Santa Cruz[a]	Goat	1:50	Y
SOX9	Chemicon[e]	Rabbit	1:80	Y
SMA	Sigma[f]	Mouse	1:5000	Y
3β-HSD	Gift[d]	Rabbit	1:1000	N
VASA	Abcam[b]	Rabbit	1:500	Y

All antibodies were raised against human peptide sequences. AMH, anti-Mullerian hormone; AR, androgen receptor; SMA, smooth muscle actin; 3β-HSD, 3β-hydroxysteroid dehydrogenase.

[a]Santa Cruz Biotechnology, CA, USA.

[b]Abcam, Cambridge, UK.

[c]Dr Guilio Spagnoli, University Hospital, Basel, Switzerland.

[d]Prof. Ian Mason, The Queen's Medical Research Institute, Edinburgh, UK.

[e]Chemicon/Upstate/Linco.

[f]Sigma, Poole, UK.

Quantitative markers of germ cell subpopulations

Table II Antibodies and conditions for triple immunofluorescence in xenograft study using human fetal testis tissue in nude mice.

Antigen	Dilution	Secondary antibody	Visualization
OCT4	1:150	[a]CAG-p	[c]Tyr-Cy3 (10 min)
MAGE-A4	1:100	[b]CAM-p	[d]Tyr-Cy5 (10 min)
Ki67	1:200	[b]CAM-p	[e]Tyr Fluor (10 min)

[a]CAG-p, Chicken anti-goat peroxidase (Sigma, Poole, UK).
[b]CAM-p, Chicken anti-mouse peroxidase (Sigma, Poole, UK).
[c]Tyr Cy3, Tyramide Cy3 (Perkin Elmer, MA, USA).
[d]Tyr Cy5, Tyramide Cy5 (Perkin Elmer).
[e]Tyr Fluor, Tyramide Fluorescien (Perkin Elmer).

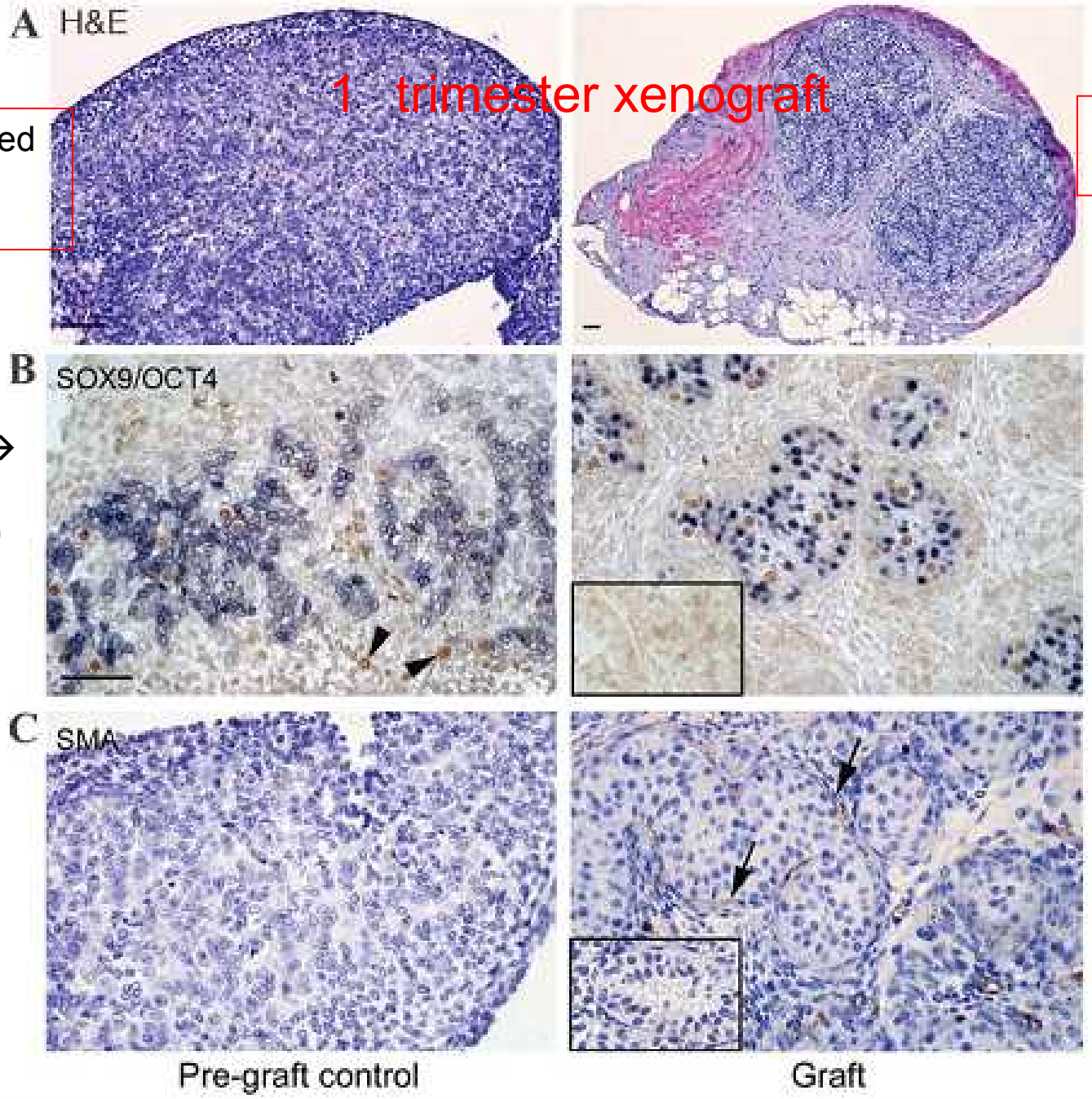
Statistical analysis

- One-way analysis of variance
- Paired t-test
- $P < 0.05$

Results

- Retrieval rates → similar
  - 1st trimester : 9/12 (75%)
  - 2nd trimester : 50/65 (77%)
- Graft size : 1-3 mm^3 in diameter
- Graft weight: 0.1~5.2 mg (mean 2.1mg)

st



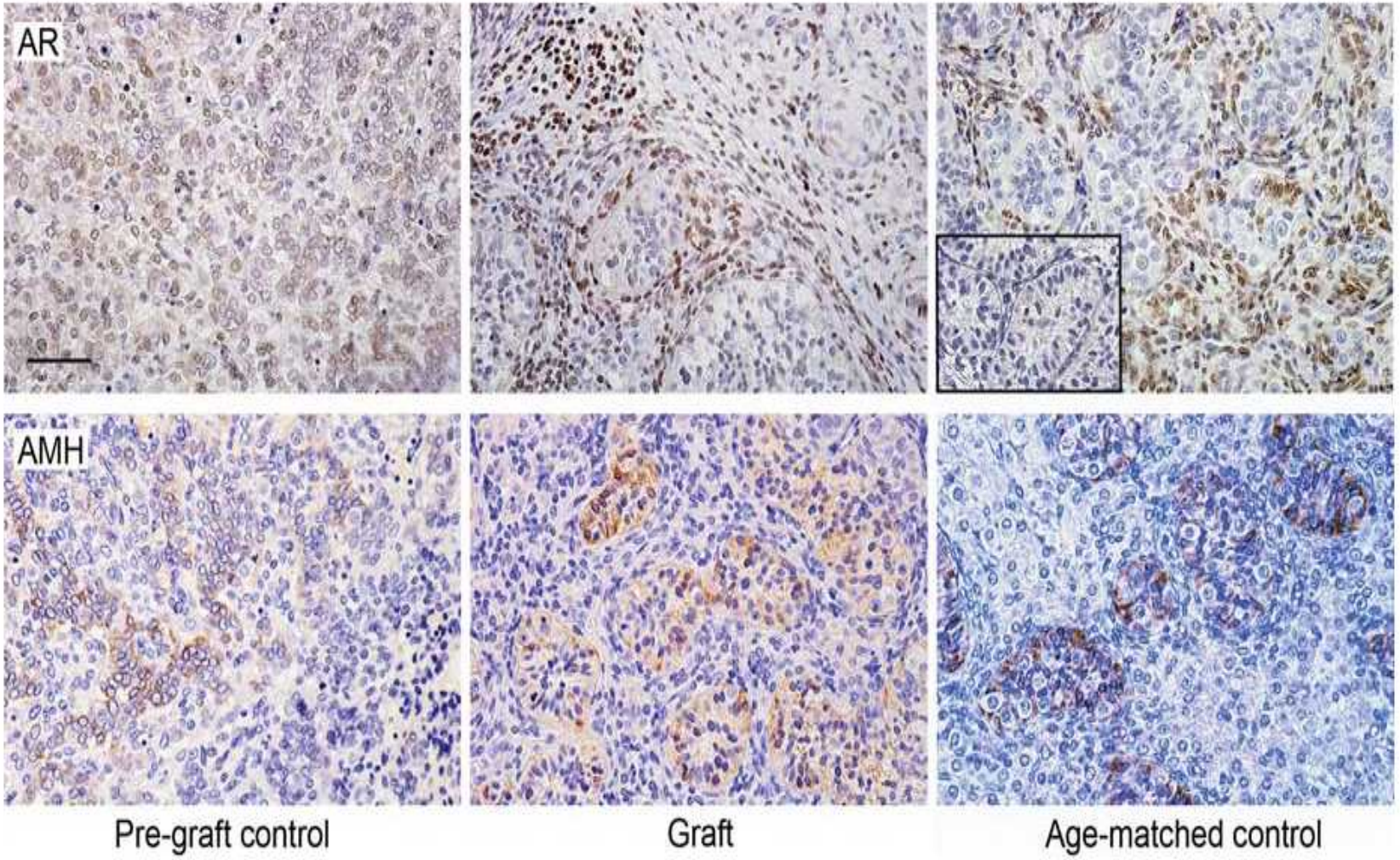
Lack completed Seminiferous cord

Seminiferous cord formed

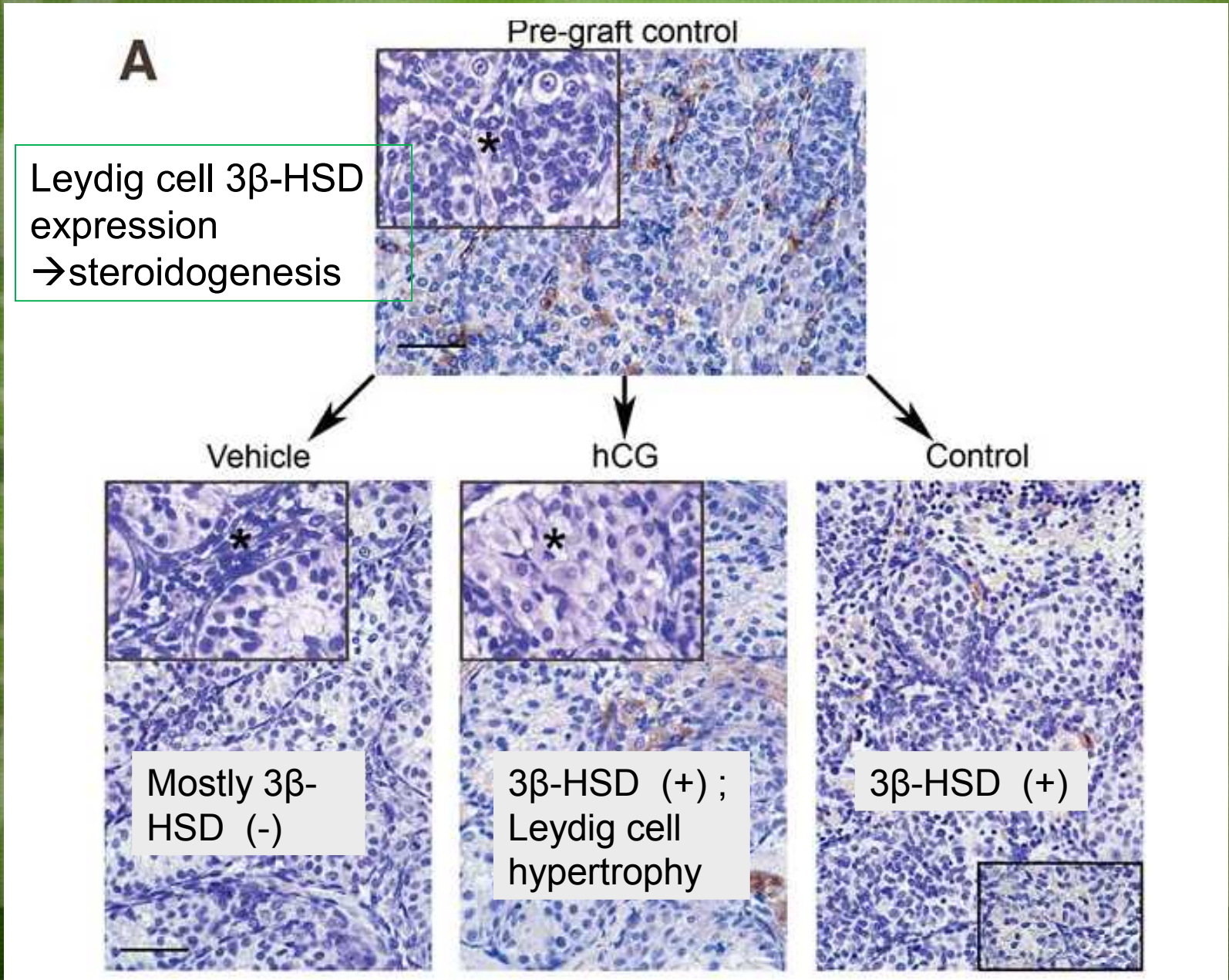
SOX9(blue) → Seroti cell
OCT4(brown) → germ cell

SMA→peri-tubal myoid cells

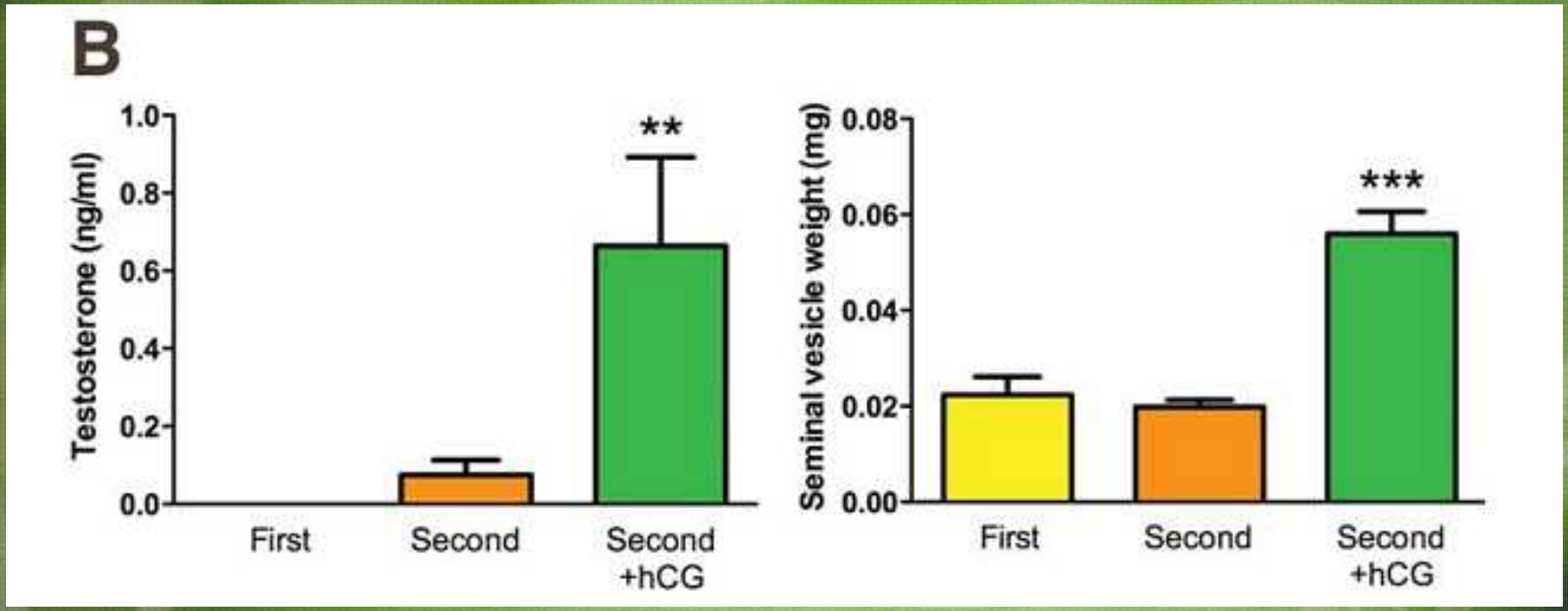
First trimester graft
Androgen receptor and AMH



Second trimester graft _ Leydig cell 3β-HSD



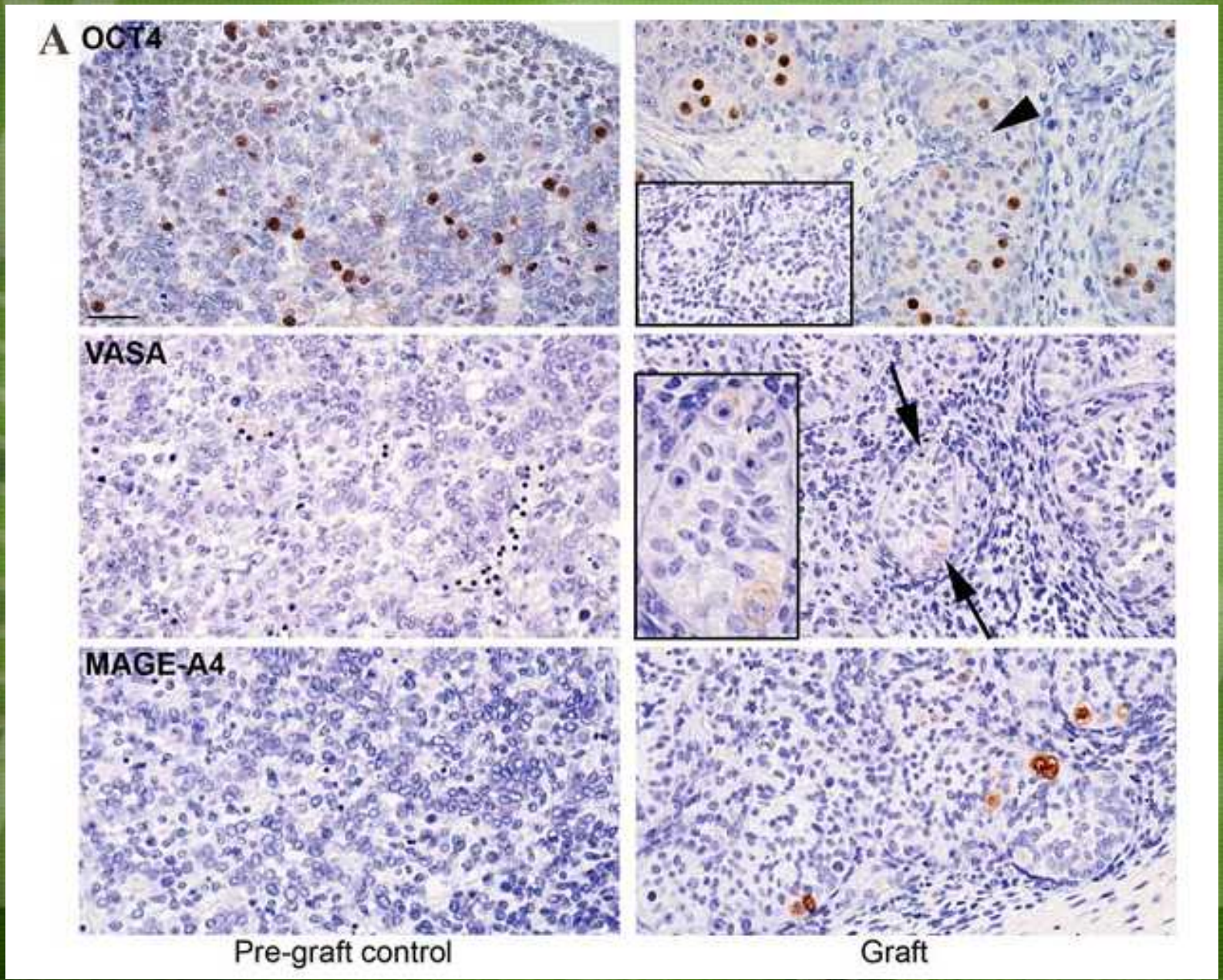
Testosterone and seminal vesicle weight



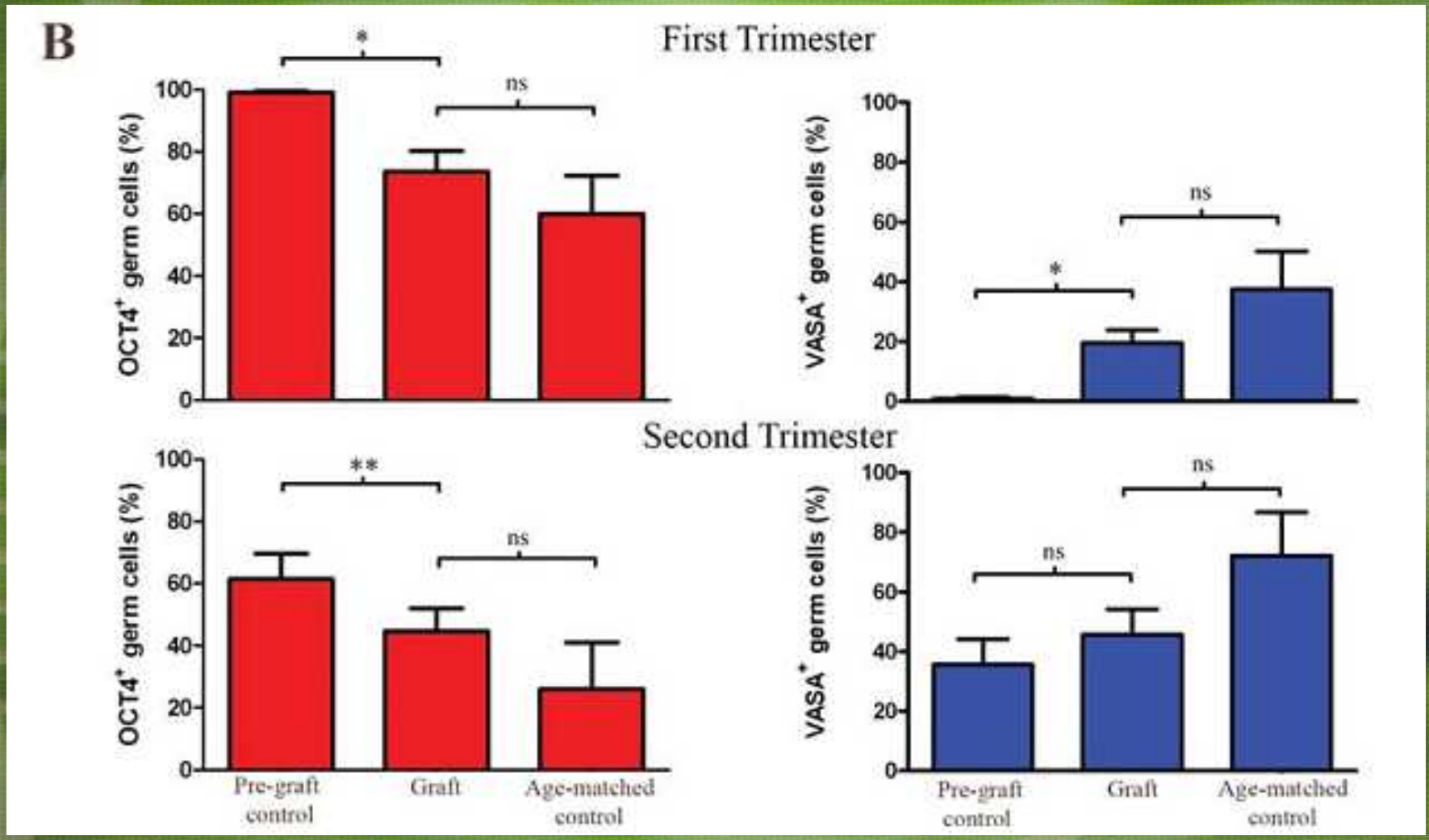
10 fold increase

3 fold increase

Differentiation of germ cell into pre-spermatogonia

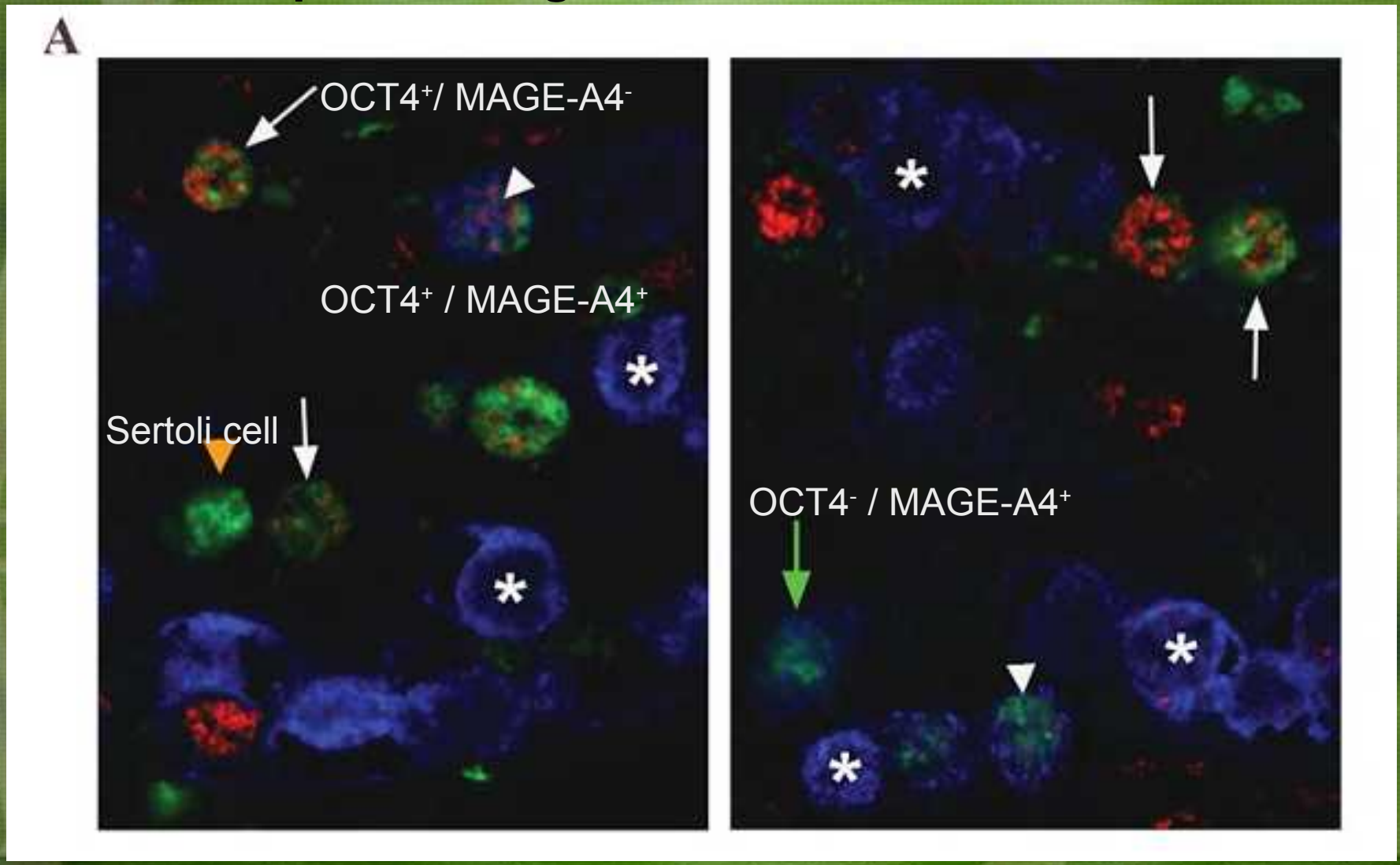


Quantification of OCT4 and VASA



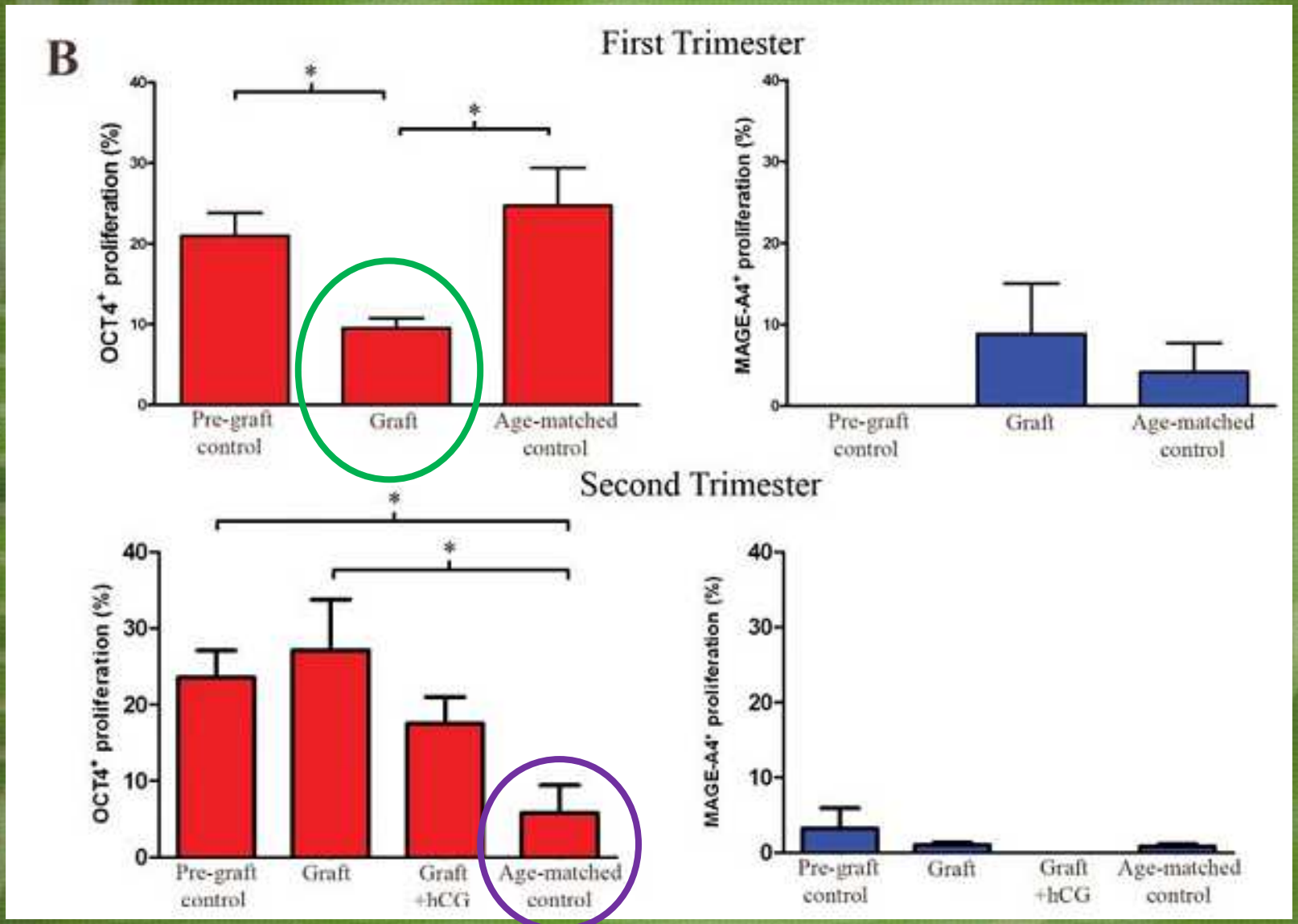
Stage-specific germ cell proliferation

Triple staining : Ki67 OCT4 MAGE-A4



Second trimester graft

Proliferation rate of OCT4 and MAGE-A4



- Higher proliferation rate of OCT4+ germ cell

Discussion

What did we find in this study?

- Human fetal testis xenografts could survive.
- Graft growth and cellular proliferation/differentiation progress normally → normal testis structure with seminiferous cords
- Excellent survival rate : > 75%
- The first demonstration of ex situ seminiferous cord formation in the human testis .

Expression of functional markers

- Expression of Sertoli (AMH), Leydig(3β-HSD) and peritubular myoid cell (SMA) are comparable with those of equivalent age-matched controls .
- AR and 3β-HSD were expressed after xenografting → steroidogenesis began and became androgen responsive (SMA : androgen- dependent)
- AMH expression became stronger within the Sertoli cells after xenografting.

Expression of steroidogenic function

- Testosterone and seminal vesicle weight
  - 2nd trimester grafts are capable to produce T and significantly increased by hCG.
  - 1st trimester grafts did not produce T.
    - Size ? No. of grafts?
    - Age-related differences in the responsiveness to host mouse LH?

Differentiation of germ cells

- Germ cells are present in 2nd trimester xenografts , but the differentiation was not demonstrated . (Povlsen et al., 1974; Skakkebaek et al., 1974; Yu et al., 2006)

- In this study
  - Gonocyte (OCT4 / MAGE-A4 / VASA) to pre-spermatogonium (OCT4 / MAGE-A4 / VASA) → normally occurred in xenografts , comparable with age-matched controls .

Differentiation of germ cells

- Exception
  - OCT4+ germ cells in the 1st trimester grafts had a significantly lower proliferation index.
- Overall result
  - Germ cell differentiation occurs in the xenografts
  - This is broadly comparable with the normal situation in vivo.

Future applications of xenograft model

- May be especially useful for investigating the origins of DSDs and TDS in humans.
- DSD often result from genetic abnormalities. (Hughes et al., 2006)
- Xenografting technique may be modified to introduce genes that either promote or disrupt normal cord formation and testis development → provide an in vivo model.

Future applications of xenograft model

- DSDs may result from impaired androgen production or action.
- Chemical effect in Rat testis models (Scott et al., 2009)
  - Phthalate esters → inhibit T → TDS-like phenotype in male offspring
  - Similar effect in the human ?
    - No effect in these studies . (Hallmark et al., 2007; Lambrot et al., 2009)
    - May have effect in this study. (Swan et al., 2005)
- Xenografting would be a relevant approach .

Conclusion

- The first demonstration that human fetal testis xenografts are a comparable *in vivo ex situ* model of normal seminiferous cord formation, germ cell development and testosterone production .

- Germ cells within xenografts differentiate from gonocytes into pre-spermatogonia and proliferate in a manner similar to that in normal age-matched control testes.

Conclusion

- Grafts are capable of producing testosterone , and and increase from basal levels can be induced by hCG treatment of the host animal.
- This system can be used to dissect the cellular mechanisms of normal human fetal testis development and the male reproductive disorders.
- Use this approach to study genetic disruption of testis development or T production → may be useful for studies related to DSDs.

Thanks for your attention!