Genetic Modification, *In Vitro* Culture and Cryopreservation of Bovine Spermatogonial Stem Cells

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Characteristics & Practical Application Of Spermatogonial Stem Cells

- Introduction
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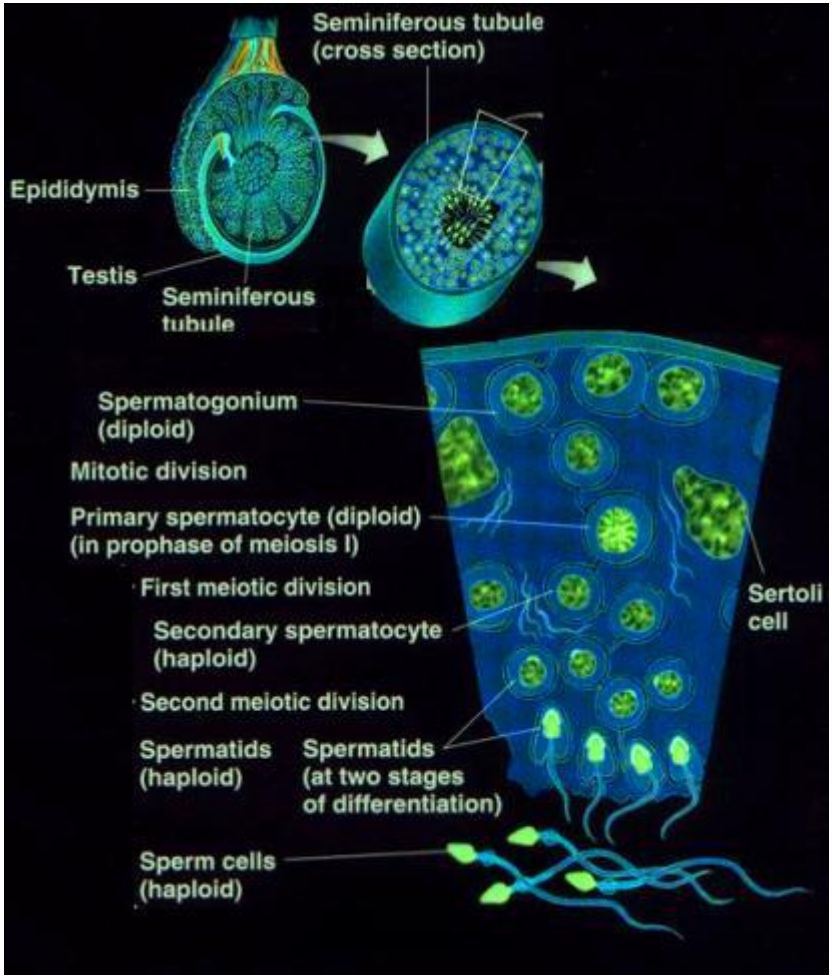
- Conclusion

INTRODUCTION

INTRODUCTION

- Spermatogonial stem cells (SSCs)
  - Self-renewal
  - Differentiation
  - ✓ Foundation of spermatogenesis
  - ✓ Continuation of the species
  - ✓ Genetic modification is the germ-line modification

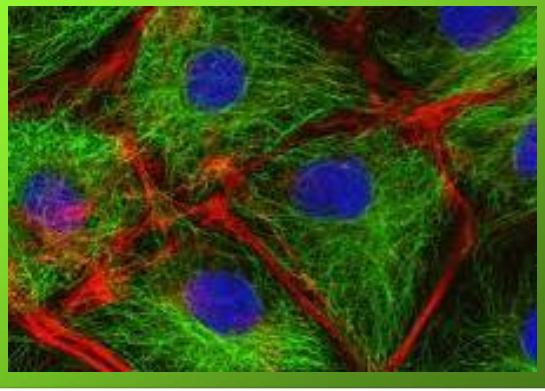


Applications of Spermatogonial stem cells

Produce Transgenic animals

Regenerative medicine – Male infertility

Stem cell biology, Molecular cell biology



Zoological, agricultural animals & Endangered species

INTRODUCTION

- **Enrichment**
  - ✓ **The identification and isolation of bovine germ cells has been difficult.**
    - **– The numbers of SSCs within testes are extremely rare.**
    - **– The lack of specific markers.**
  - ✓ **Enrichment and purification of bovine SSCs will enhance the ability to characterize bovine germ cells.**

- **Genetic Modification**
  - ✓ **In rat, a single SSC can produce 4096 mature spermatozoa through spermatogenesis.**
  - ✓ **The transgenesis of a single SSC can potentially produce thousands of modified spermatozia.**

INTRODUCTION

❖ ***In Vitro* Culture**

- ✓ **Within testes, only 0.03% and 0.2% of adult mouse and rat testicular cells are SSCs.**
- ✓ **The limited number of SSCs in the testis hampers studies of their biological characteristics.**
- ➢ **To acquire large quantities of bovine germ cells, *in vitro* culture methods must be developed.**
- ➢ ***In vitro* culture can serve as a model for comprehensively understanding the biology of SSCs and the factors that regulate male fertility.**
- ➢ **No establishment except mouse, rat, and rabbit.**

INTRODUCTION

❖ **Cryopreservation**

- ✓ **Cryopreservation is a process where cells or whole tissues are preserved by freezing.**
- ✓ **Any biological activity is effectively stopped**
  - **– To avoid the effects of aging**
  - **– To reduce contamination**
  - **– To diminish transformation in finite lines**
  - **– To minimize genetic change in continuous lines**

➢ **The best method for long-term preservation of SSCs.**

Enrichment & Genetic Modification

JOURNAL OF ANIMAL SCIENCE
The Premier Journal and Leading Source of New Knowledge and Perspective in Animal Science

Lentiviral modification of enriched populations of bovine male gonocytes

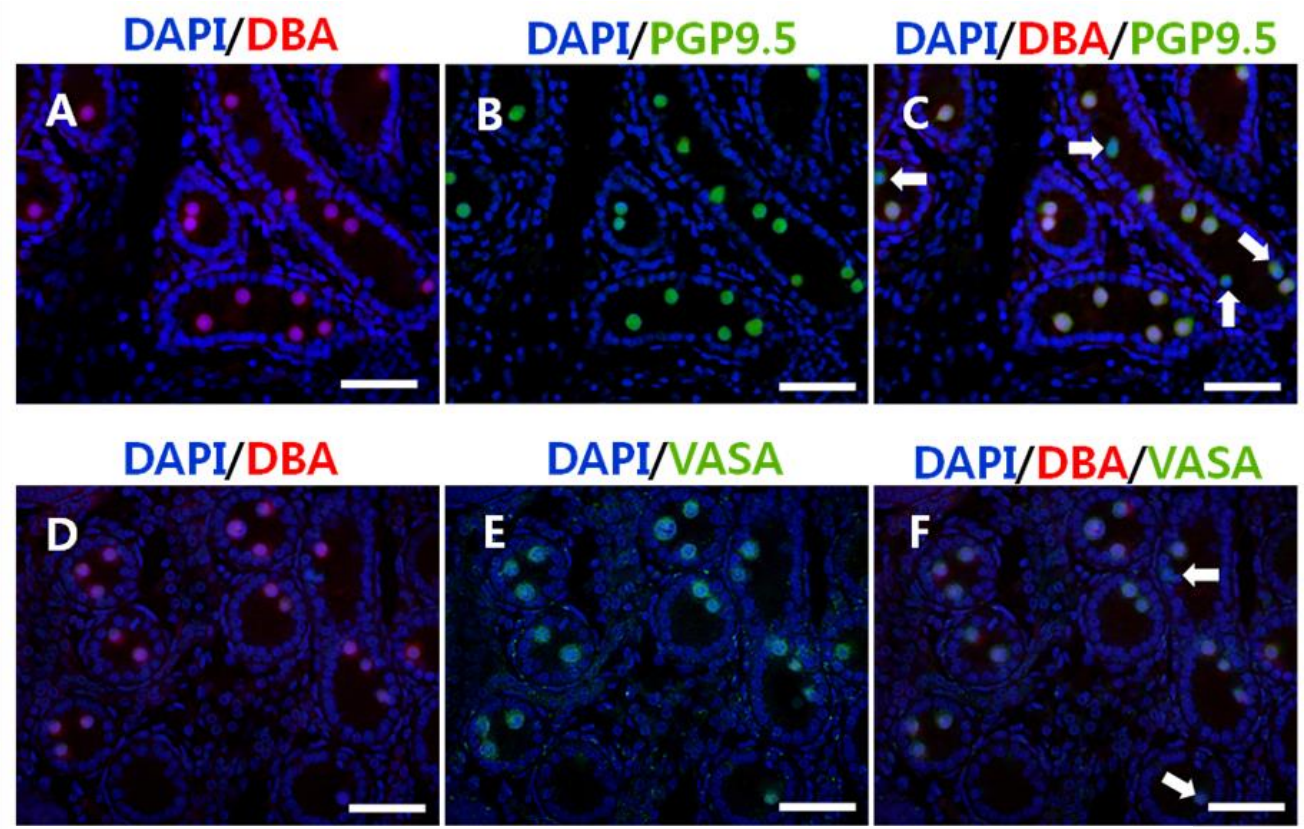
K.-J. Kim, C. M. Cho, B.-G. Kim, Y.-A. Lee, B.-J. Kim, Y.-H. Kim, C. G. Kim, J. A. Schmidt and B.-Y. Ryu

MATERIALS AND METHODS

- ✓ **Donor Animals**
  - **– 10- to 14-wk-old pre-pubertal Holstein**
- ✓ **Enzymatic digestion**
  - **– Collagenage type Ⅳ, Hyaluronidase, 0.25% trypsin-EDTA, Dnase Ⅰ**
- ✓ **Percoll density gradient**
  - **– 20% and 40%**
- ✓ **Extracellular Matrix (ECM) molecules**
  - **– Laminin, fibronectin, collagen and gelatin**
- ✓ **Immunocytochemstry**
  - **– DBA, PGP 9.5, VASA**
- ✓ **Lentivirus**
  - **– Subclass of retroviruses**
  - **– Deliver target genes to the genome of non-dividing cells**

RESULTS

Immunohistochemistry of donor testis



Germ cell marker

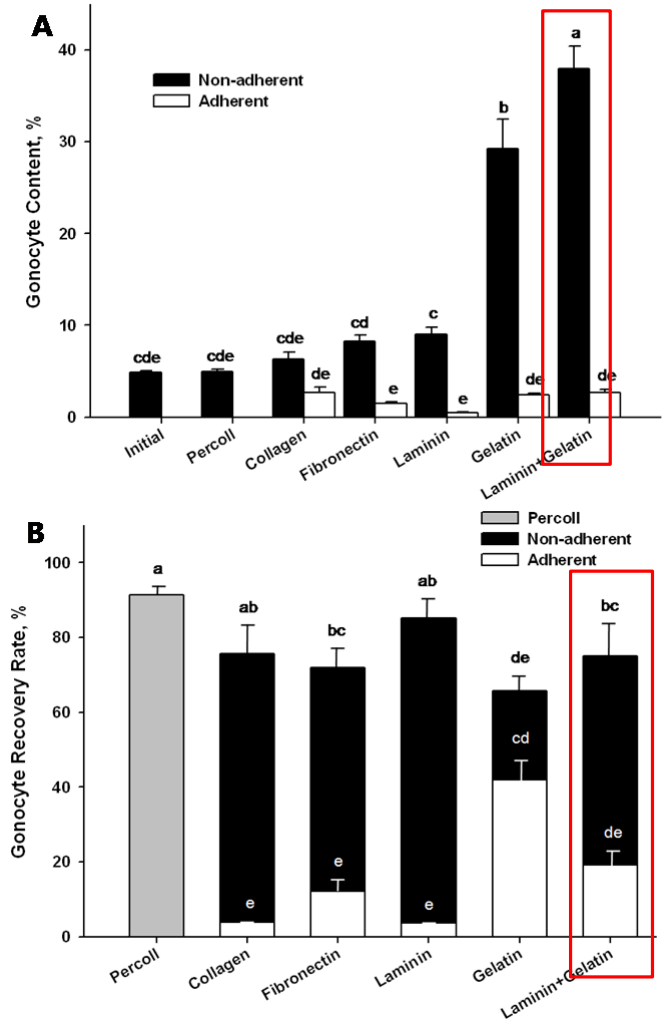
***Dolichos biflorus agglutinin* (DBA)**

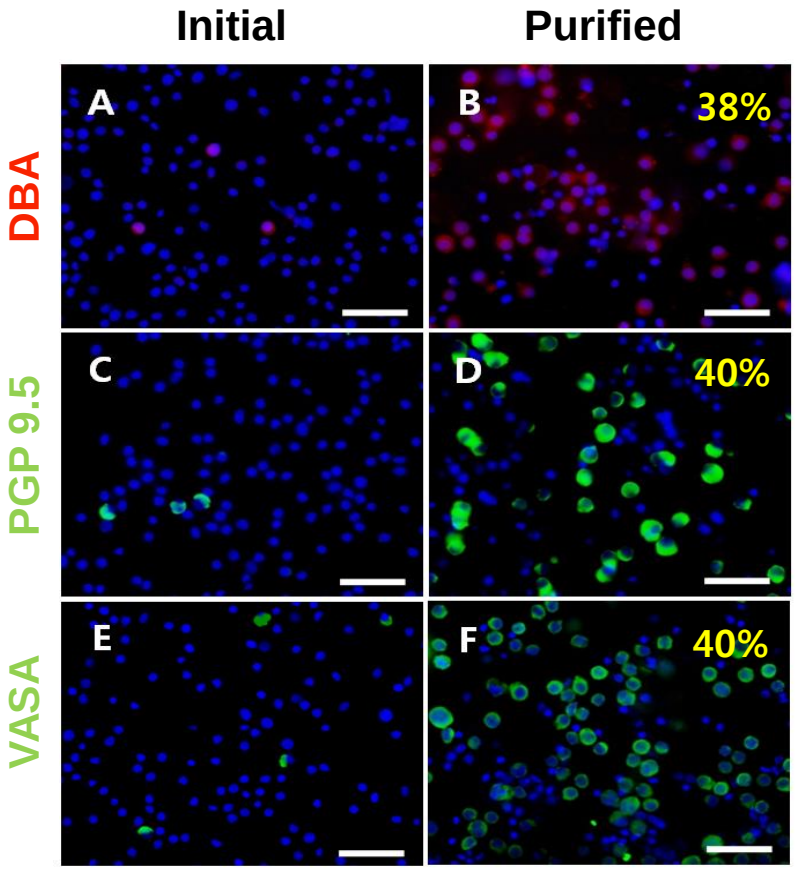
Protein gene product 9.5 (PGP 9.5)

VASA homolog (VASA)

RESULTS

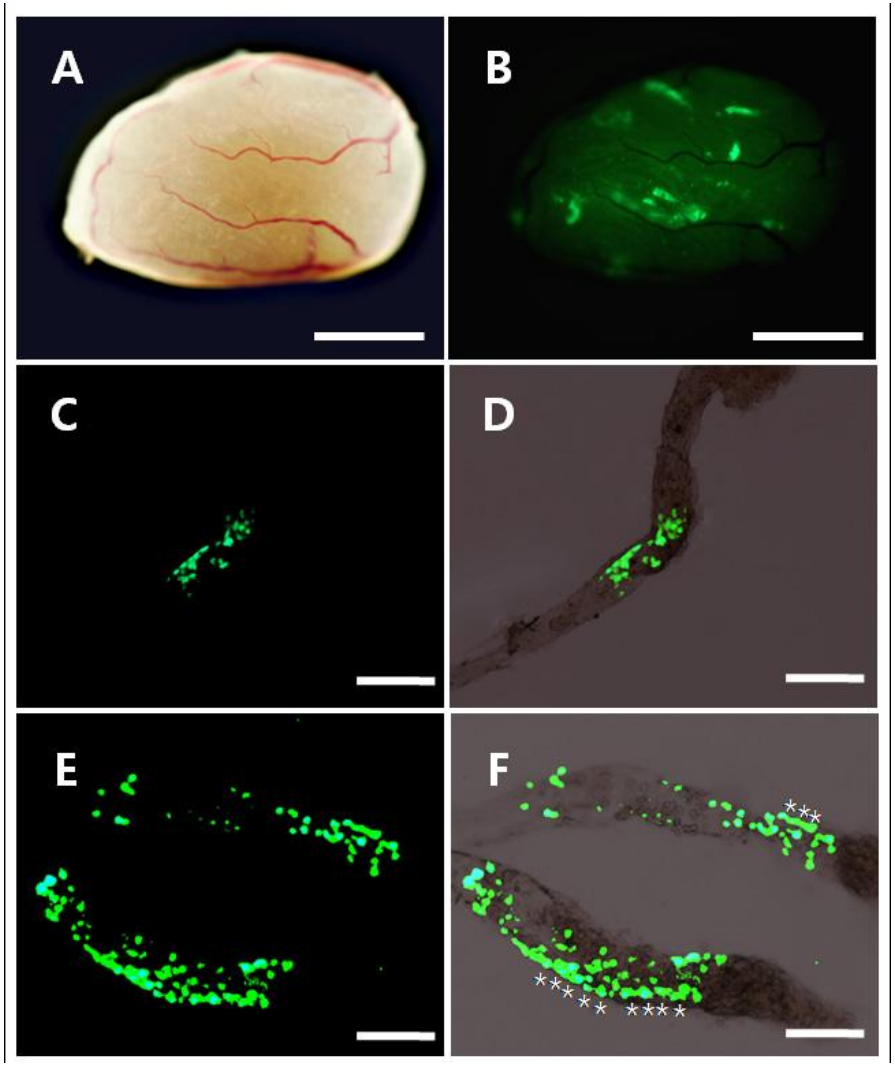
Purity and recovery rate of germ cells using ECM molecules





RESULTS

Detection of transgenic eGFP-expressing germ cell colonies



SUMMARY AND DISCUSSION

- The combinatorial isolation method with laminin and gelatin yielded 8-fold increase in terms of germ cell purity.

- The transplanted bovine SSCs proliferated and colonized the recipient testes.

- We successfully transduced bovine germ cells using a lentiviral vector.

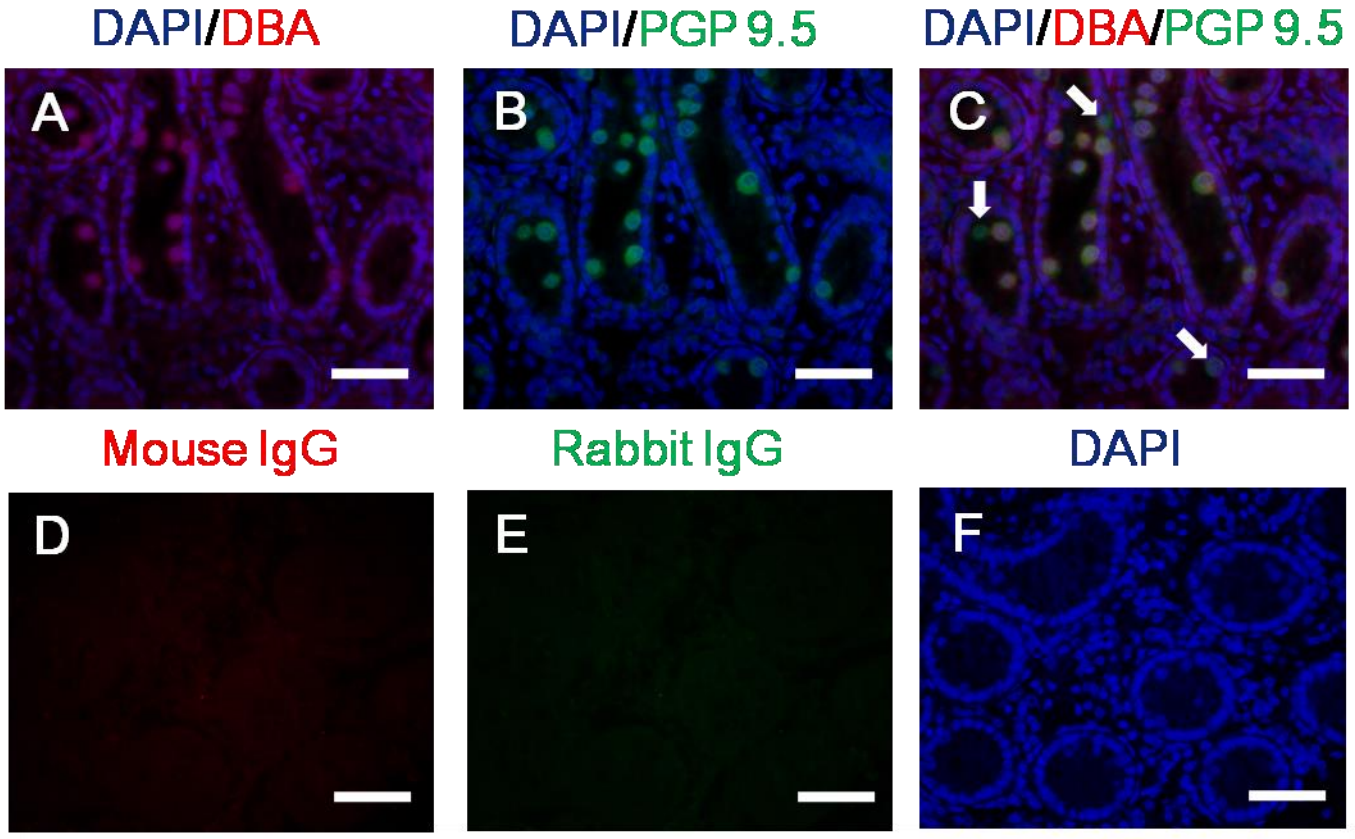
In Vitro Culture

MATERIALS AND METHODS

- ✓ **Cell Culture**
  - **– Seeding cells: combinational selection with laminin and gelatin**
  - **– Basic growth factor condition: GDNF, GFRα1, and bFGF**
  - **– Feeder-free conditions**
  - **– Subculture: picked by scraping**
  - **– The medium was changed every 2–3 days.**
  - **– Medium: 1× rSFM, 2× rSFM. and StemPro**
  - **– Serum: FBS 0%, 0.1%, and 1.0%**
  - **– Additive Growth factors: EGF, LIF, CSF-1, and IGF-1**

RESULTS

Immunohistochemistry of donor testis



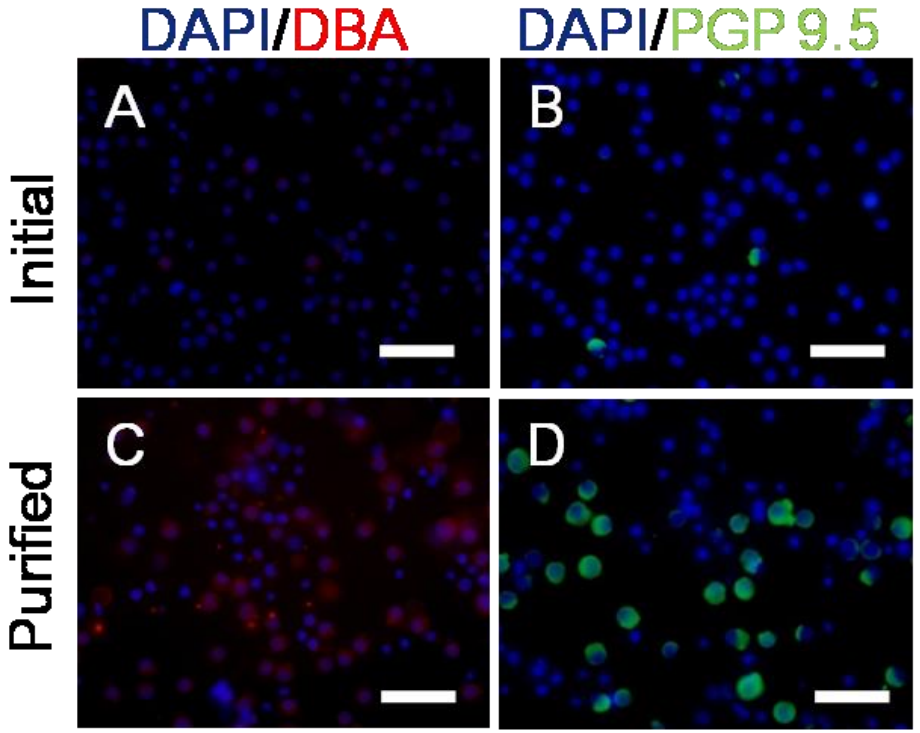
Undifferentiated spermatogonia marker

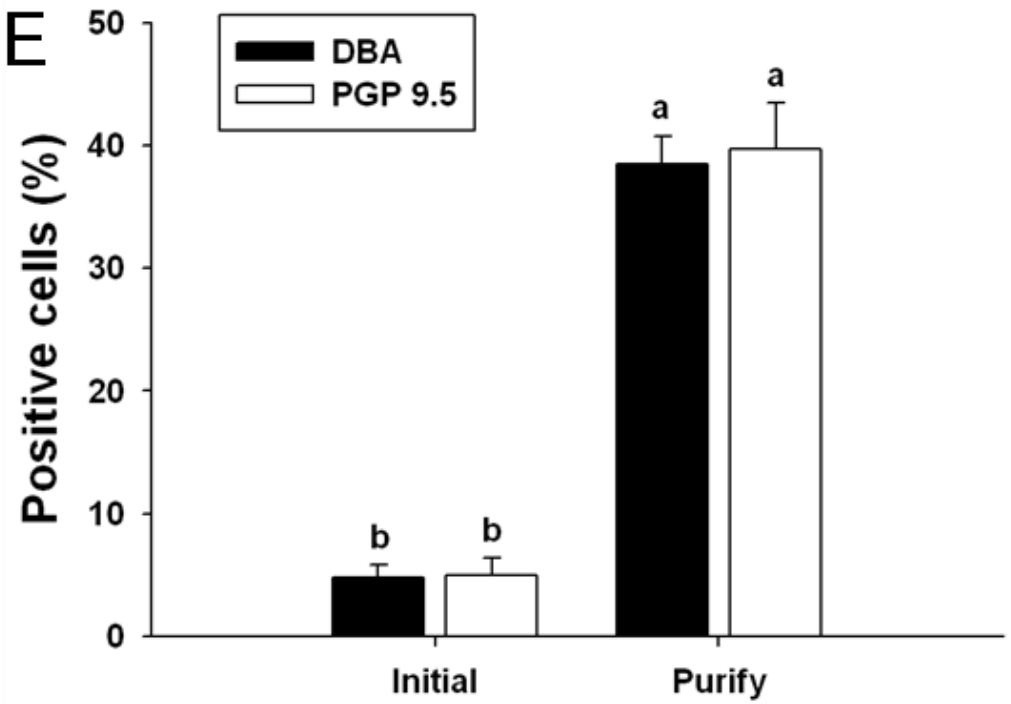
Dolichos biflorus agglutinin (DBA)

Protein gene product 9.5 (PGP 9.5)

RESULTS

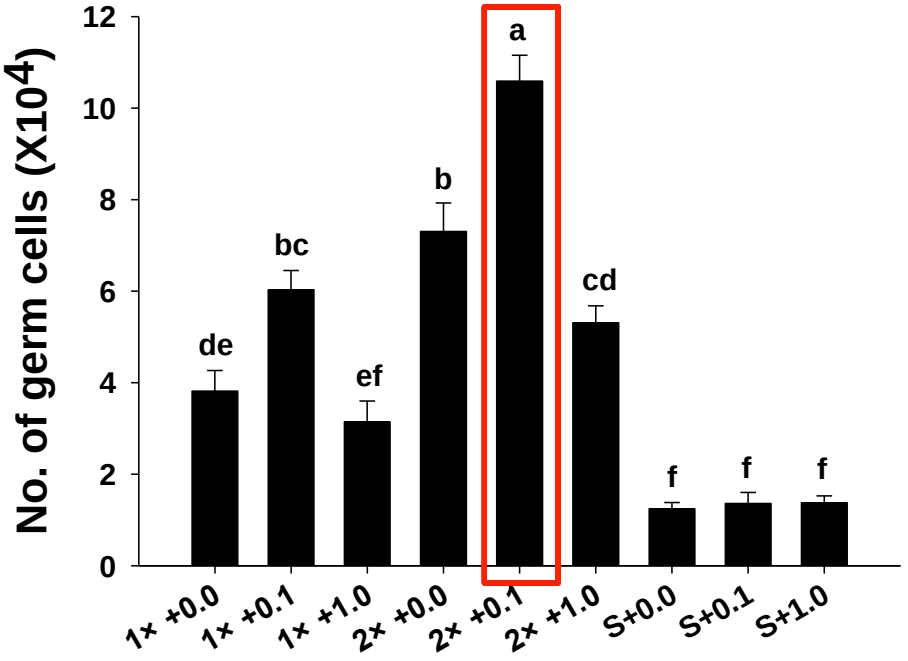
Enrichment of bovine SSCs





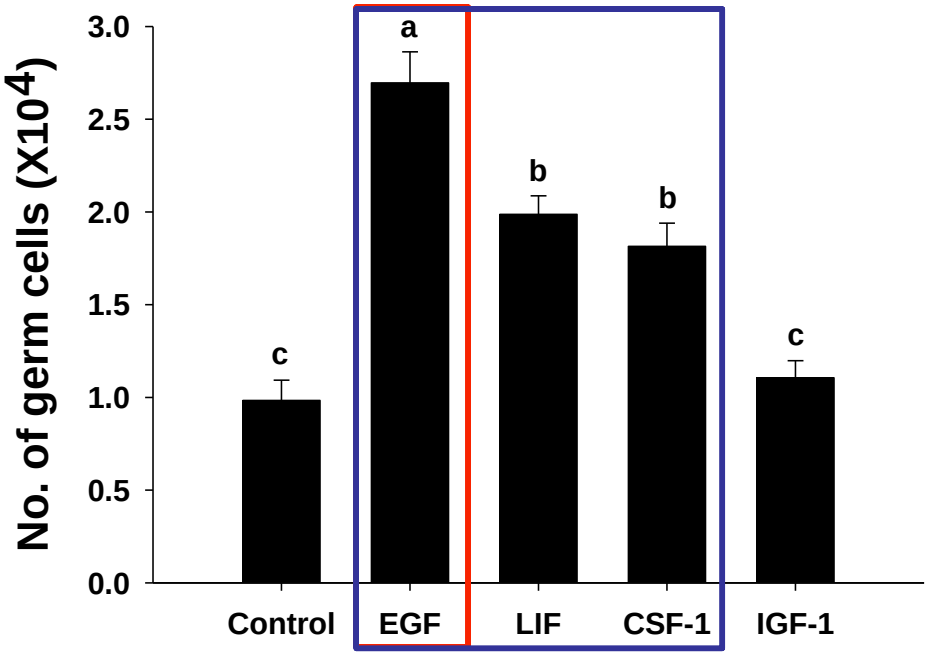
RESULTS

Effects of medium, serum, and growth factors on cell proliferation



1×: 1× rSFM
2×: 2× rSFM
S: StemPro

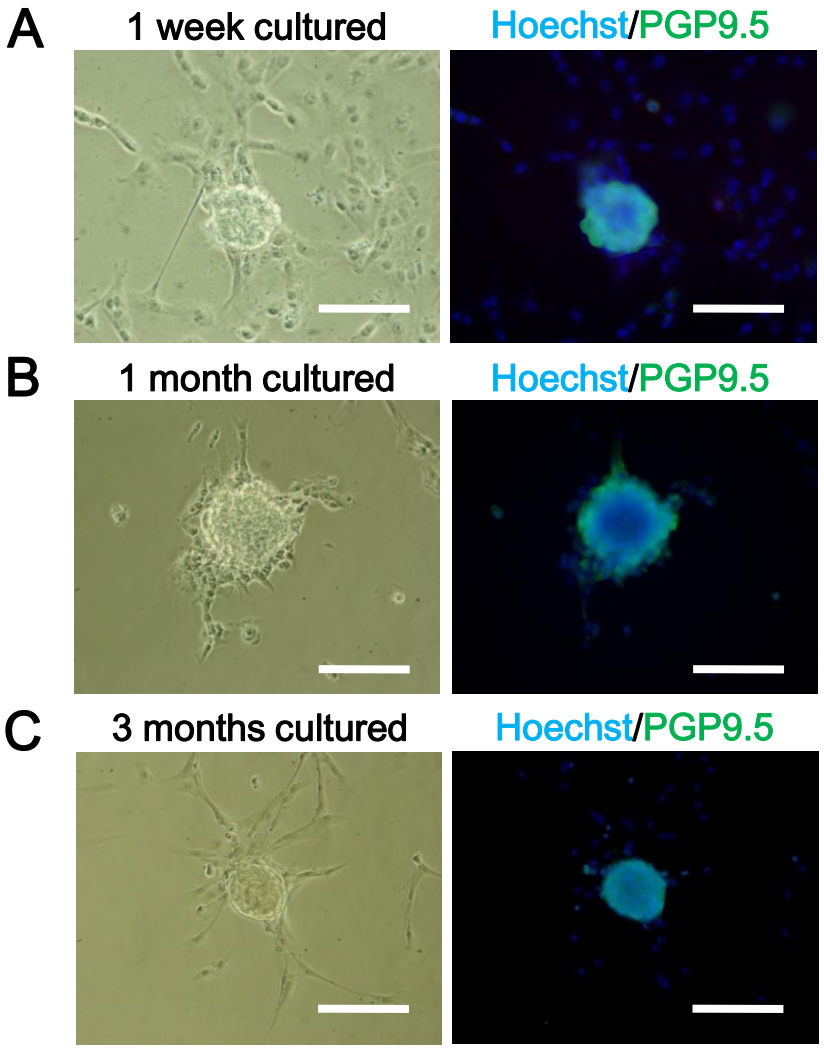
0.0: 0.0% FBS
0.1: 0.1% FBS
1.0: 1.0% FBS



Control: GDNF + GFRα1 + bFGF
EGF: Control + EGF
LIF: Control + LIF
CSF-1: Control + CSF-1
IGF-1: Control + IGF-1

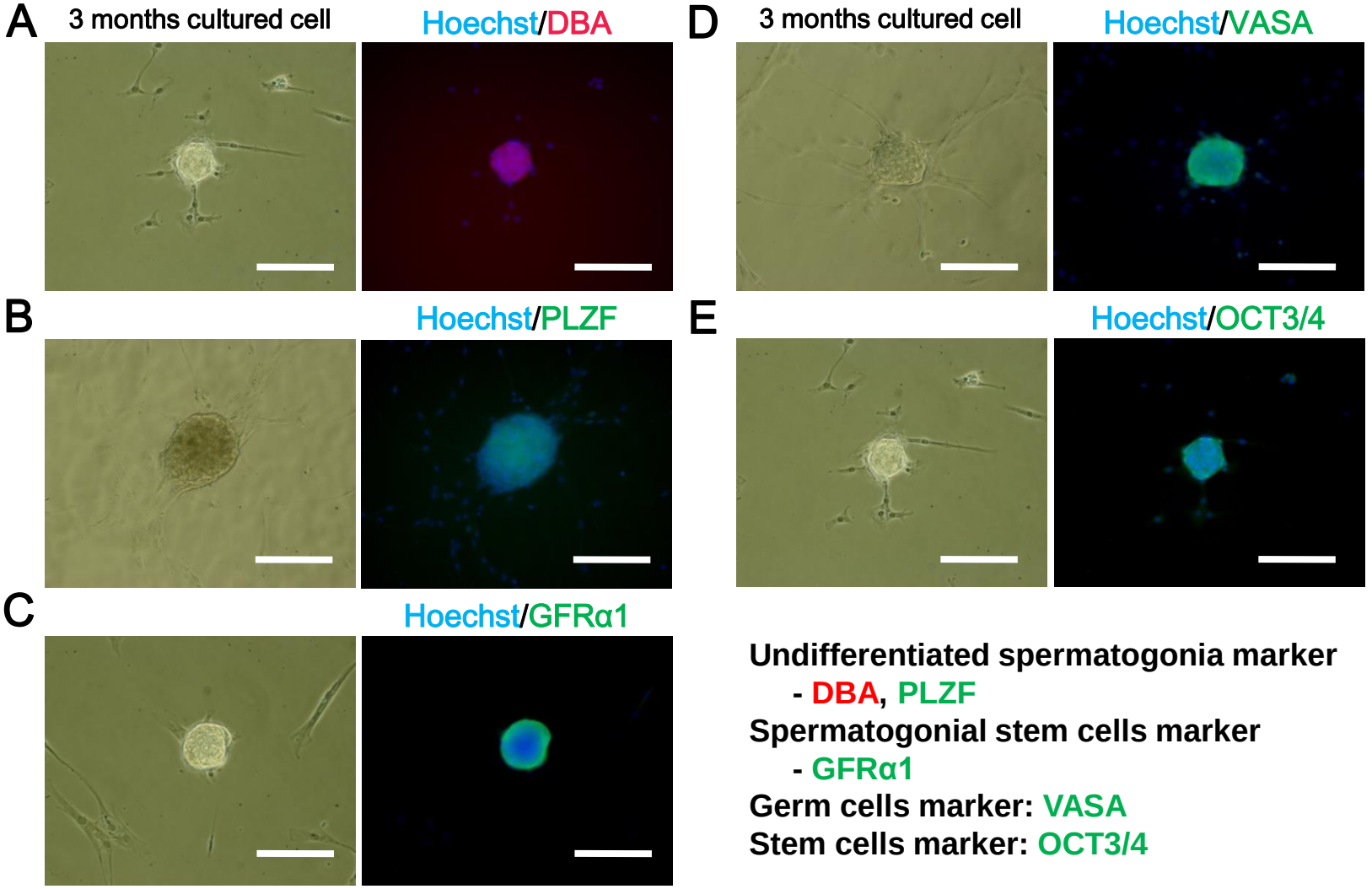
RESULTS

Morphology and characteristics of cultured germ cells



RESULTS

Morphology and characteristics of long-term cultured cells



Undifferentiated spermatogonia marker
- **DBA**, **PLZF**

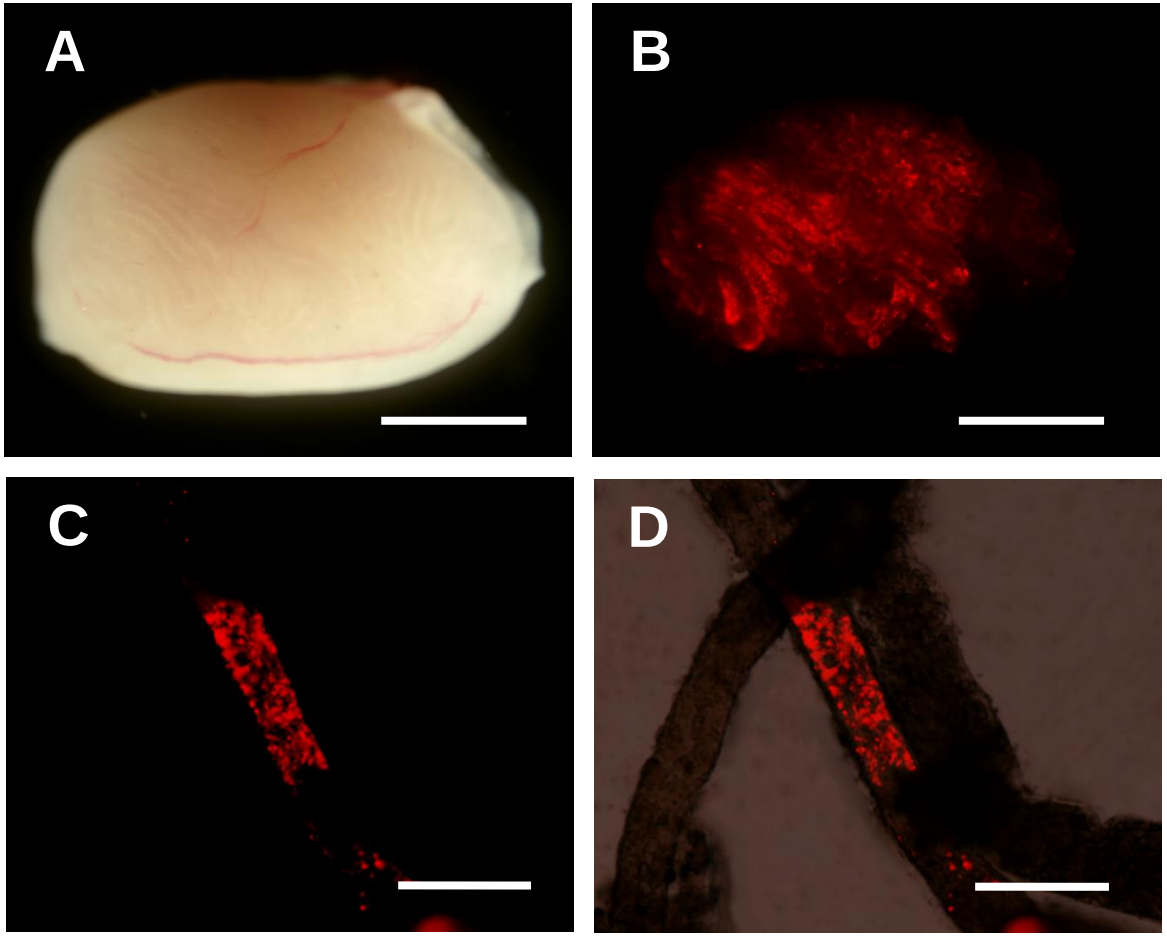
Spermatogonial stem cells marker
- **GFRα1**

Germ cells marker: VASA

Stem cells marker: OCT3/4

RESULTS

Detection of colonies derived from cultured bovine germ cells



Transplanted cultured cell for 3 months

Culture condition

- Medium: 2× rSFM
- Serum: 0.1% FBS
- Growth factors:

  GDNF + GFRα1 + bFGF

  + EGF + LIF + CSF-1

SUMMARY AND DISCUSSION

- We have identified a specialized medium that can promote the proliferation of bovine germ cells.

- We developed the optimal culture conditions for maintaining bovine SSCs over long periods under feeder-free conditions.

- Cultured cells could be maintained for long-term periods without any alteration of stem cell characteristics and functions.

Cryopreservation

Cryobiology 70 (2015) 175–183

Contents lists available at ScienceDirect

Cryobiology

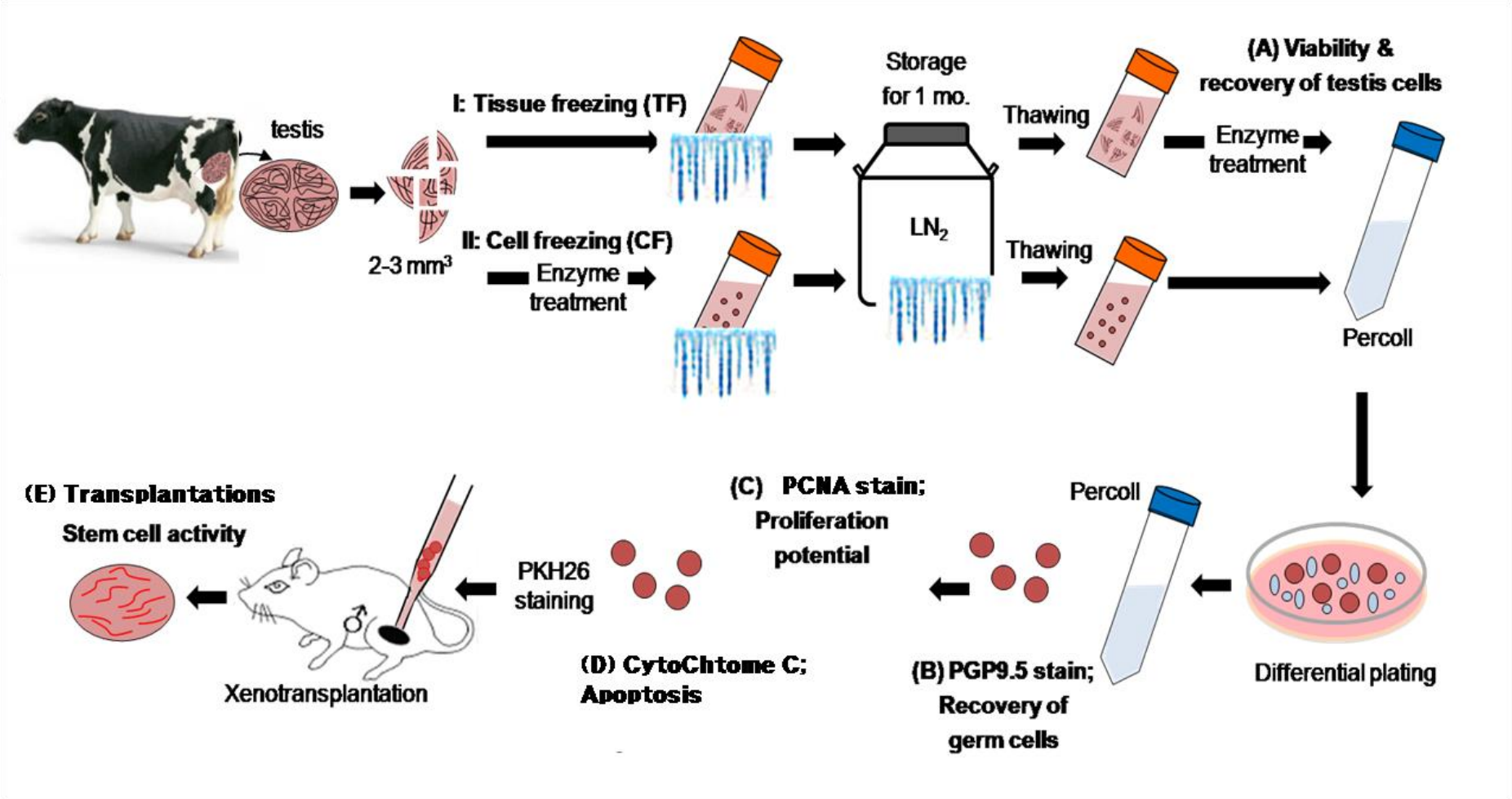
journal homepage: www.elsevier.com/locate/ycryo

Cryopreservation of putative pre-pubertal bovine spermatogonial stem cells by slow freezing ☆

Ki-Jung Kim [a], Yong-An Lee [a,1], Bang-Jin Kim [a], Yong-Hee Kim [a], Byung-Gak Kim [b], Hyun-Gu Kang [a], Sang-Eun Jung [a], Sun-Ho Choi [c], Jonathan A. Schmidt [d], Buom-Yong Ryu [a,*]

MATERIALS AND METHODS

Process of experiment

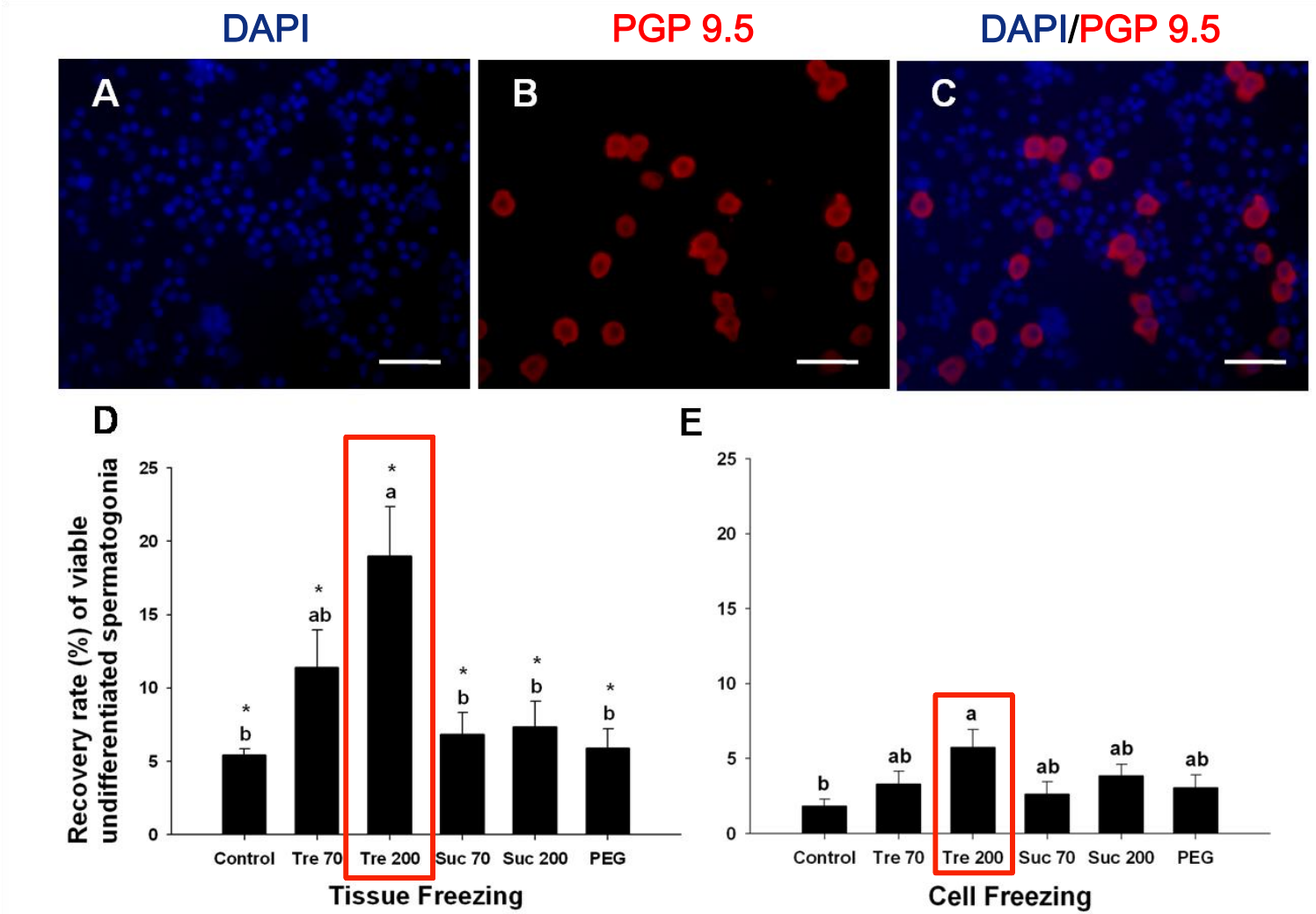


MATERIALS AND METHODS

- ✓ **Cryopreservation**
  - **– Freezing media: 10% FBS, 10% DMSO, 80% Medium**
  - **– Freezing period: 1 month**
  - **– Freezing-thawing methods: slow freezing and rapid thawing**
  - **– Enrichment: differential plating with gelatin**
  - **– Cryopreservation methods: tissue freezing-, cell freezing-methods**
  - **– Cryoprotective agents: trehalose, sucrose, and polyethylene glycol (PEG)**

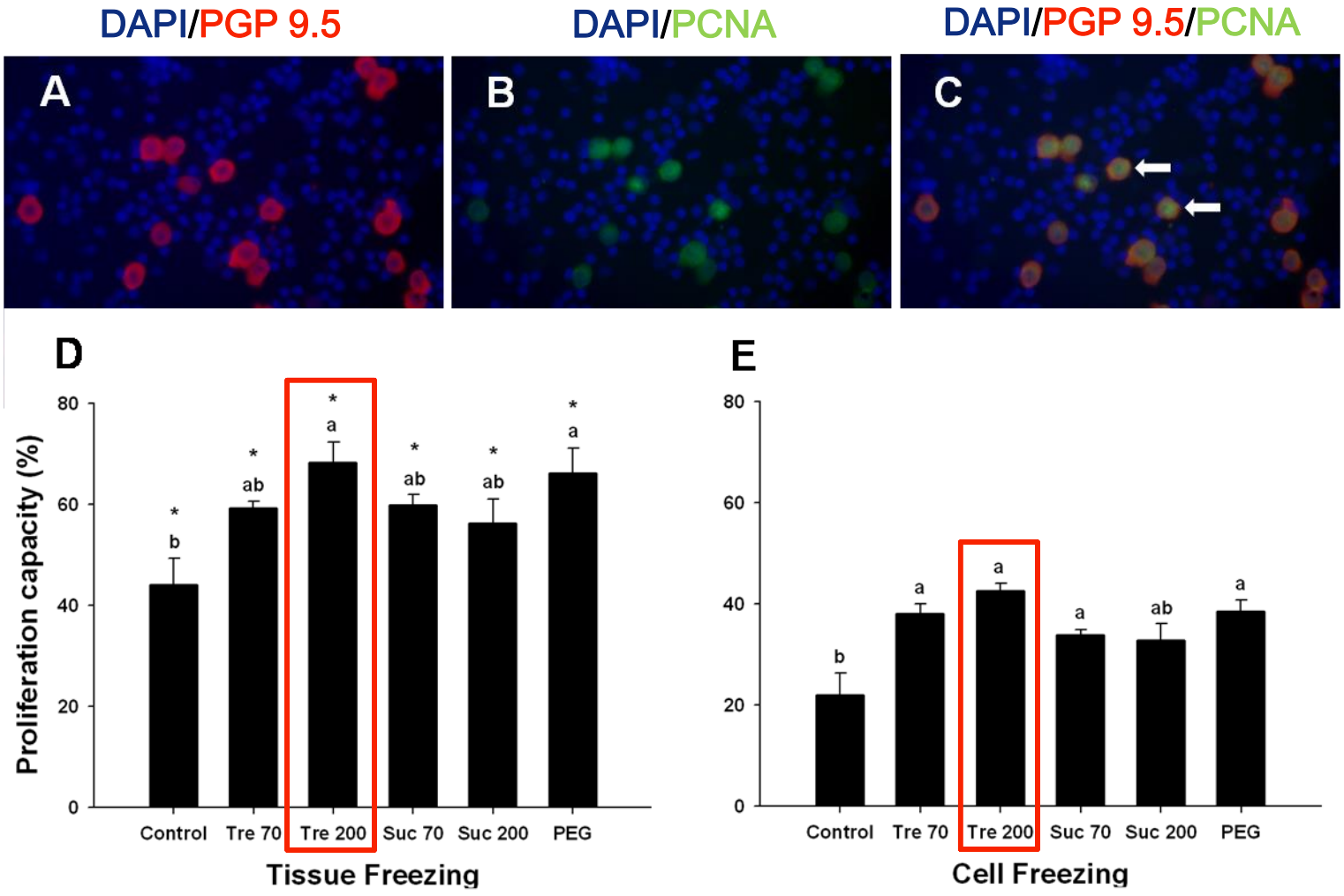
RESULTS

Recovery of viable germ cells after differential plating



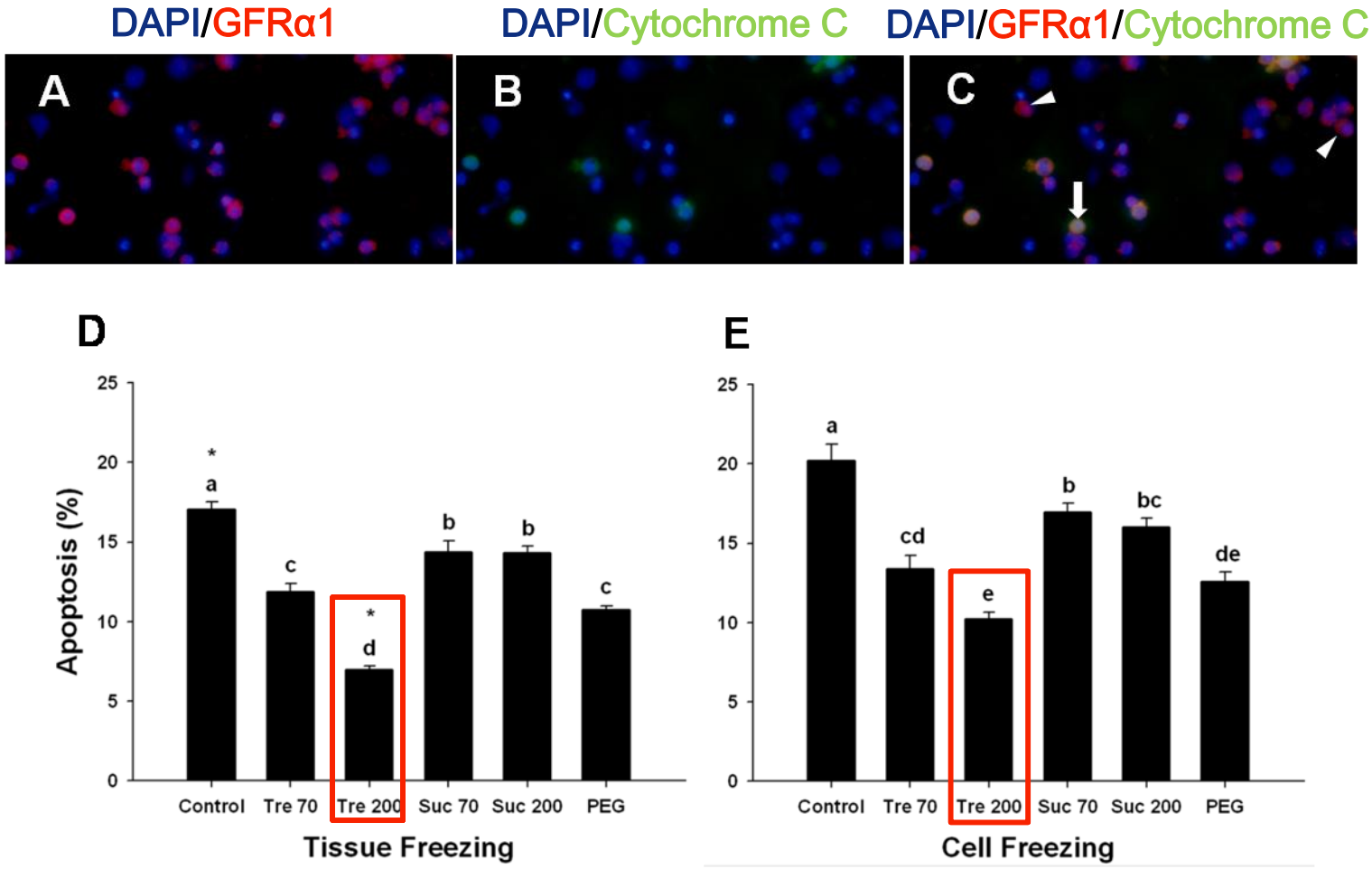
RESULTS

Proliferation capacity of undifferentiated spermatogonia



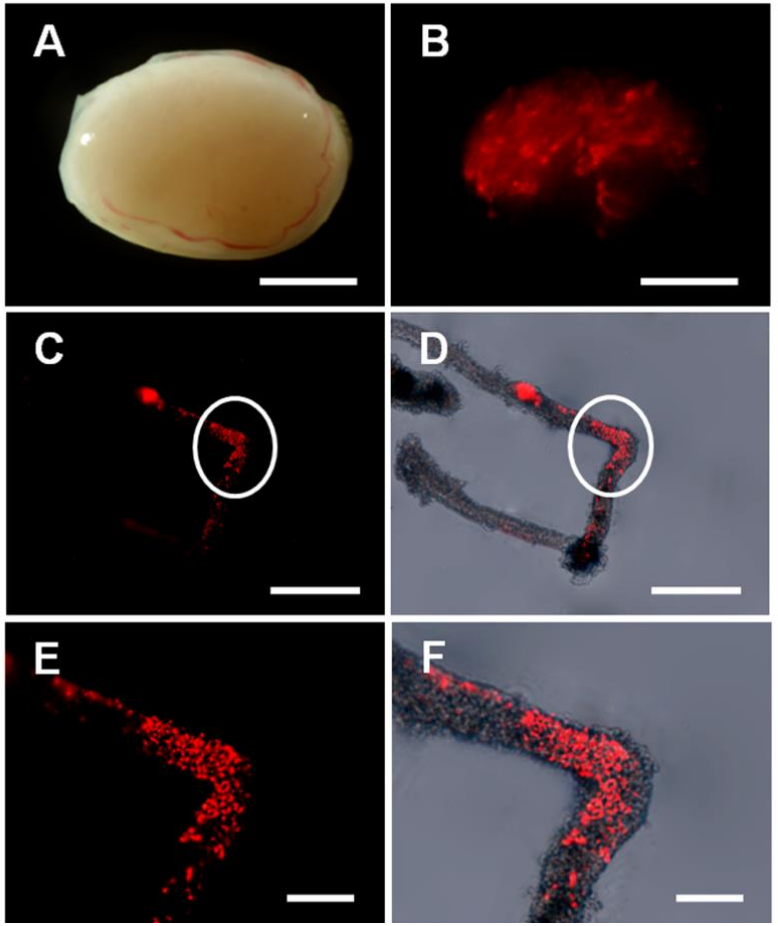
RESULTS

Undifferentiated spermatogonia undergoing apoptosis



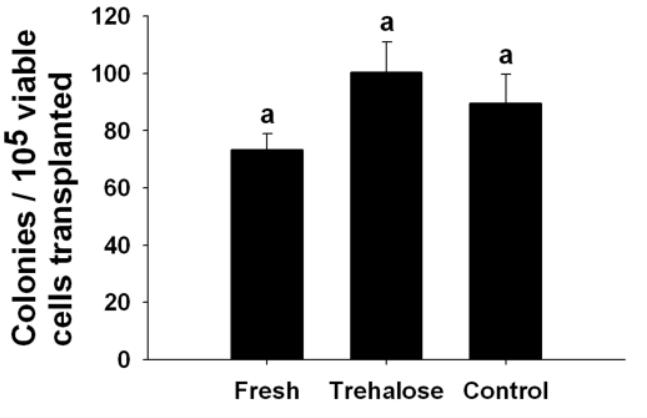
RESULTS

Detection of putative bovine spermatogonial stem cells



Frozen-thawed cells using tissue freezing methods with 200 mM trehalose

G



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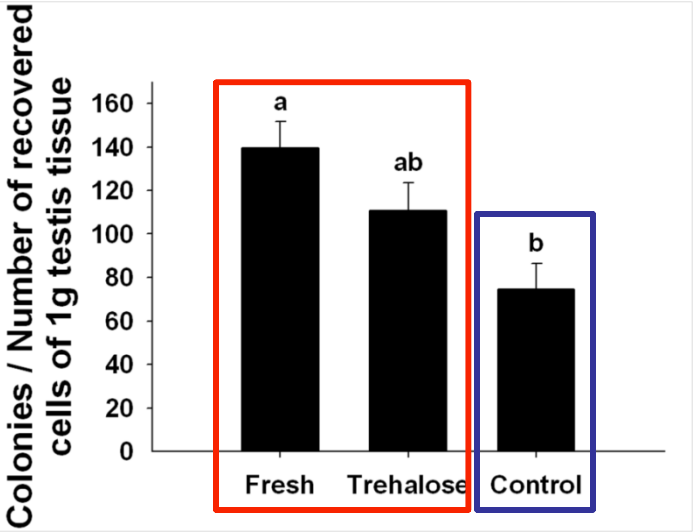

SUMMARY AND DISCUSSION

- **Tissue-freezing method results in greater recovery, proliferation capacity, and less apoptosis compared to cell-freezing method.**

- **The addition of 200 mM trehalose to the cryopreservation media provides the most effective method for cryopreservation of bovine SSCs.**

- **Tissue freezing with 200 mM trehalose in the cryopreservation media by slow freezing is an effective method for the cryopreservation of bovine SSCs.**

CONCLUSION

CONCLUSION

- ✓ **We were to develop an effective selection method for bovine germ cells and to genetically modify enriched populations of bovine germ cells using lentiviral transduction.**

- ✓ **Bovine SSCs were successfully maintained and proliferated for culturing long-term periods *in vitro*.**

- ✓ **We developed an effective cryopreservation protocol for bovine SSCs using slow freezing methods.**

- ➢ **These studies can serve as a model for comprehensively understanding the biology of SSCs and will contribute to the development of new therapeutic techniques for male infertility.**


Thanks