CALIFORNIA STATE UNIVERSITY SAN MARCOS

PROJECT SIGNATURE PAGE

PROJECT SUBMITTED IN PARTIAL FULLFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

PROJECT TITLE: Phenotypic Characterization of Horse and Rhinoceros Spermatogonial Stem Cells

AUTHOR: CHING-YU SU

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Phenotypic Characterization of Horse and Rhinoceros Spermatogonial Stem Cells

Institute for Conservation Research, San Diego Zoo Global

Ching-Yu Su

05/07/2018

Professional Science Masters Degree Program

California State University San Marcos

The Northern White Rhinoceros (*Ceratotherium simum cottoni*) is facing extinction, and germ cell transplantation can be a potential approach to save the species. However, the expression of Spermatogonial Stem Cell (SSC) markers in rhinoceros and horse has not been fully characterized. In the present study, The number of testis tissue collected from horses and rhinoceros was 14 and 4, respectively. After isolation, cells were stained with antibodies specific for SSC (GDNFα1, GPR125, PLZF), and pluripotency (OCT-4, SSEA-1, SSEA-4) in both horse and rhinoceros and TRA-1-60, TRA-1-81 for rhinoceros. Flow cytometry analysis showed in both species a positive expression of all markers, but not for PLZF. It also revealed that a low number of cells were positive for GPR125 and SSEA-1 in horses, and GPR125, SSEA-1, OCT-4, TRA-1-60 and TRA-1-81 in rhinoceros. For further characterization, molecular detection of germ cell-specific gene VASA, pluripotent genes OCT-4, and SOX-2, SSCs genes BMI1, UTF1 and UCHL1, and Sertoli cell-specific gene SOX9 was performed in germ cells and Sertoli cells isolated from one testis of a pubertal horse and in horse fibroblasts. RT-qPCR showed that VASA was expressed only in germ cells, while BMI1 and UTF1 were expressed in both germ cells and Sertoli cells, and UCHL1 expressed by the three cell types. SOX-2 was detected only in Sertoli cells and OCT-4 had none detectable levels within the range of the standard. To obtain a purified population of horse and rhinoceros SSC from differentiated spermatogonia and somatic testicular cells, we cultured testicular cell suspensions of horses (n=4) and rhinoceros (n=4) with a serial differential plating and analyzed the expression of markers for SSC and pluripotency. Flow cytometry after cell purification showed in horses a significant increase of cells positive for GDNFα1 and GPR125, and in OCT-4 for rhinoceros.

Phenotypic Characterization of Horse and Rhinoceros Spermatogonial Stem Cells

Ching-Yu Su

Committee Member:

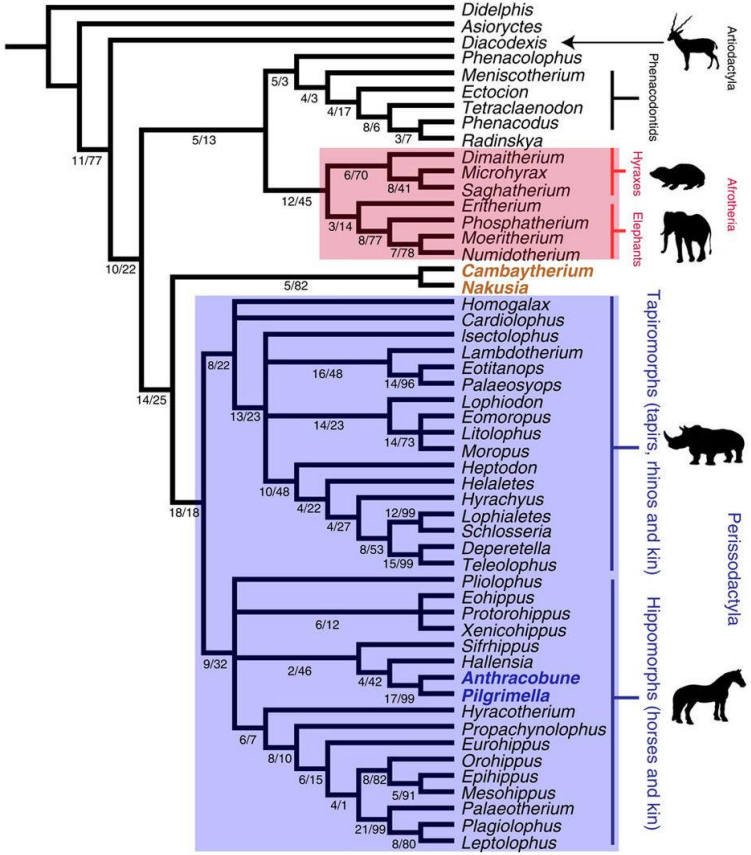
Martha Gómez, Ph.D. Daun Barr Stansfield, Ph.D. Chandrasen Soans, Ph.D. Betsy Read, Ph.D.

The last male northern white rhino, Sudan, has died

https://www.livescience.com/62068-sudan-last-northern-white-rhino-dies.html

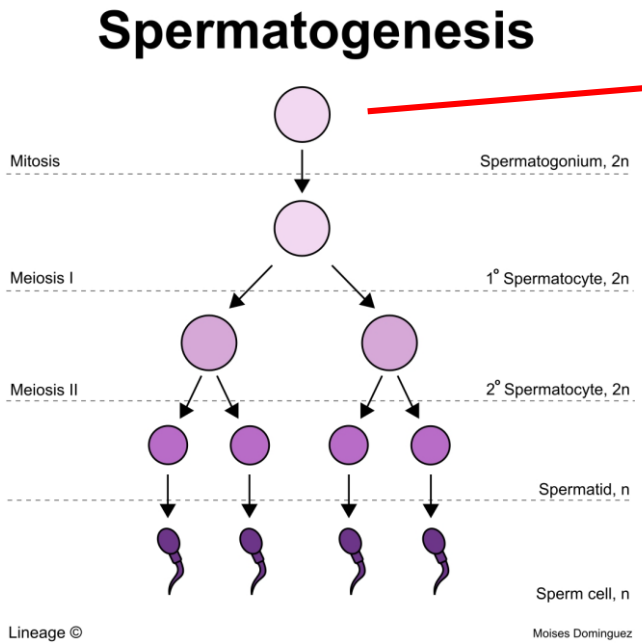
San Diego Zoo, Frozen Zoo®

Horse is the closest relative to rhinoceros



Rose et al. 2014.

Spermatogonial Stem Cell(SSC) in Horse

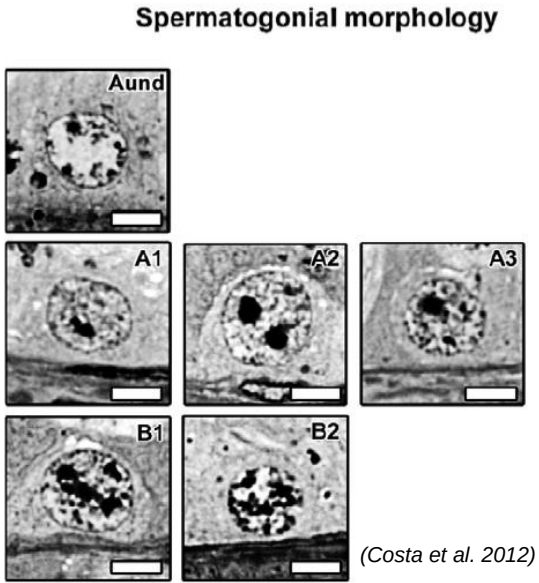


https://step1.medbullets.com/reproductive/116008/spermatogenesis

$Aund \Rightarrow A_1 \Rightarrow A_2 \Rightarrow A_3 \Rightarrow B_1 \Rightarrow B_2 \Rightarrow$ Spermatocyte

$\{ As \Rightarrow Apr \Rightarrow Aal \}$

SSC (Self-renewal and differentiation)
(Pluripotency)



(Costa et al. 2012)

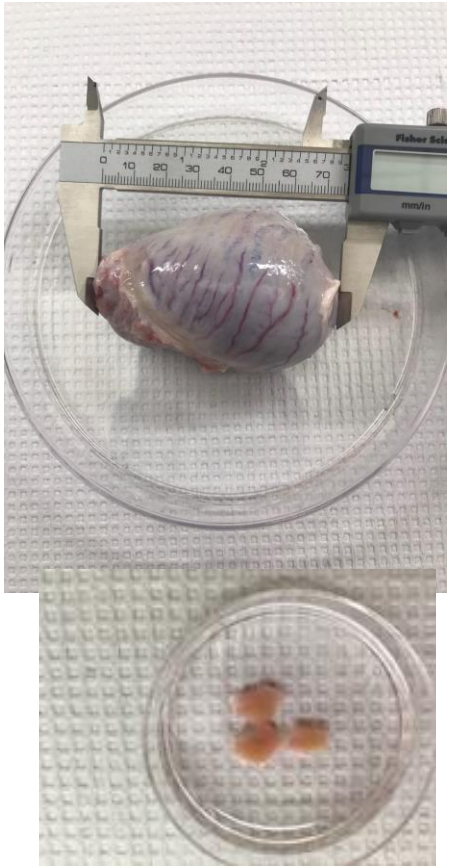
Specific Aims

1) To phenotypically characterize horse and rhinoceros SSC by the expression of common surface markers, specific for SSC in other mammalian species, and the expression of pluripotent markers.

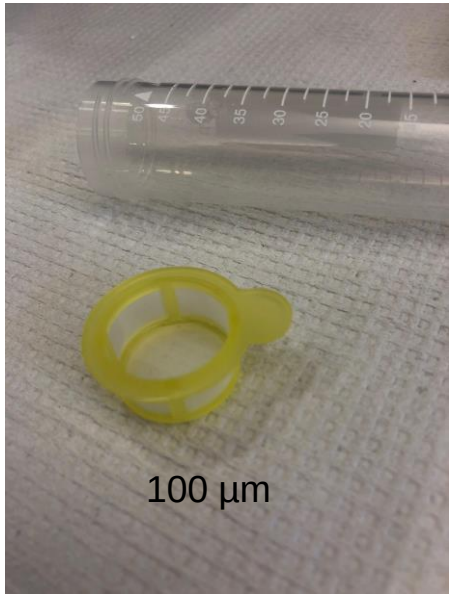
2) To evaluate if horse and rhinoceros SSC can be isolated from cryopreserved testicular tissue.

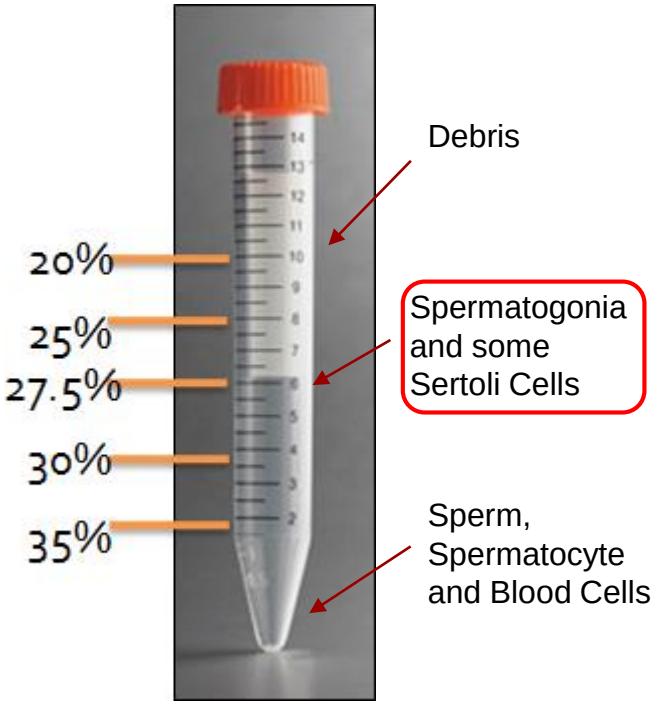
3) To evaluate if a serial differential plating can purify horse and rhinoceros SSC population.

Isolation of Spermatogonial Cells from Testicular Tissue



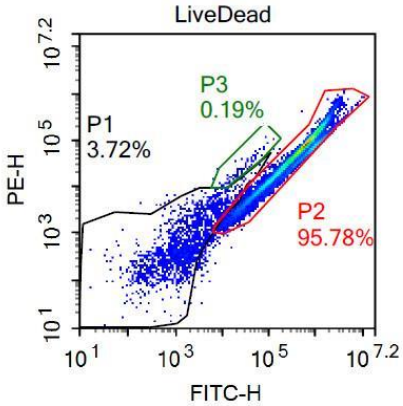
- **Collagenases:**
  break the peptide bonds in collagen
- **Hyaluronidase:**
  catalyse the degradation of hyaluronic acid (HA)
- **Trypsin:**
  protease





Experiment 1:
Phenotypic and Molecular Characterization of Horse SSC

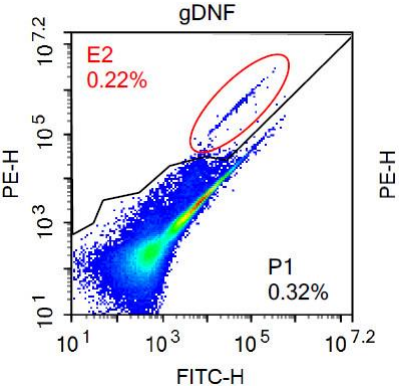
https://en.wikipedia.org/wiki/Horse

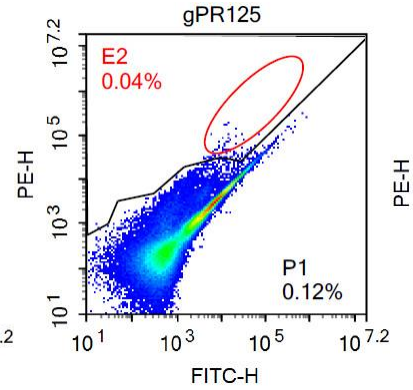


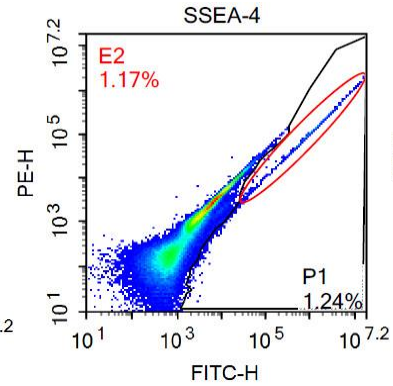
- Fresh/Refrigerated(4°C, overnight)
  Adult horses(n=5; 5 to 10 years old)
- 8.25 gram
- 5.2 ± 1.7 X 10^6 cells
- 94.17% ± 1.2% of live cells.

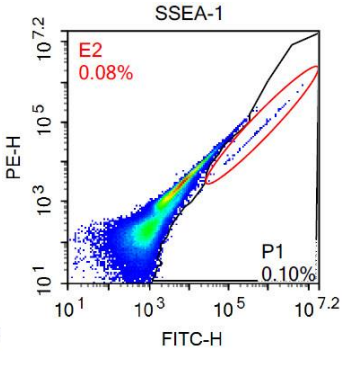
LIVE/DEAD™ Cell Imaging Kit, Invitrogen:
Green fluorescent ⇒ calcein AM ⇒ intracellular esterase activity
Red fluorescent ⇒ bind to DNA ⇒ damaged membranes

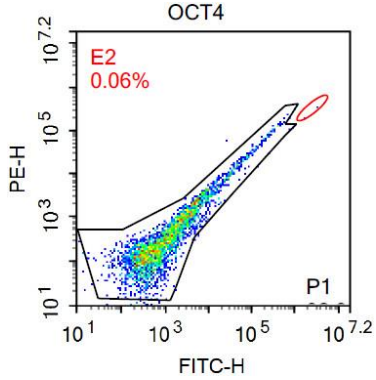
Expression of spermatogonia and pluripotency markers on horse spermatogonial cells isolated by gradient of Percoll











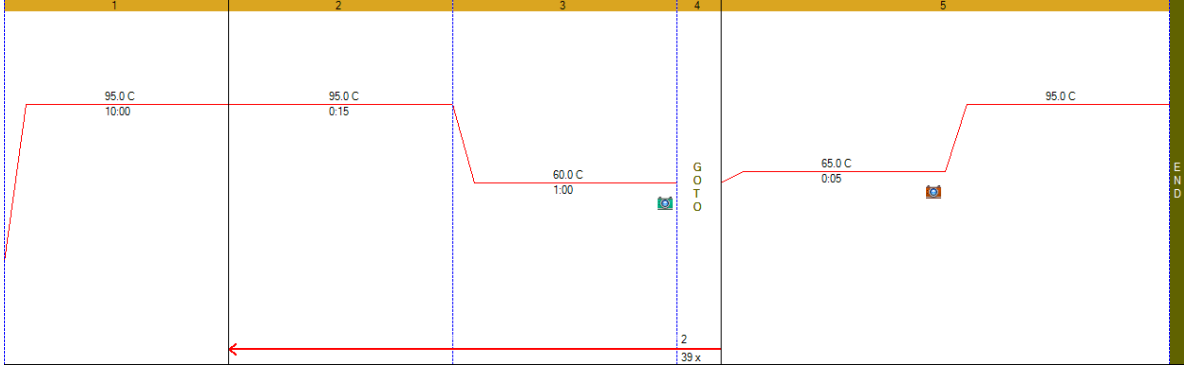
GDNFα1	GPR125	SSEA-4	SSEA-1	OCT-4
0.55% ± 0.2%	0.24% ± 0.2%	0.64% ± 0.3%	0.04% ± 0.01%	0.33% ± 0.3%
Aund in horse (Costa et al. 2012)	*SSC in mouse and human (Seandel et al. 2008; Sachs et al. 2014.)*	*Pluripotent marker (Powell et al. 2016.)*	*Pluripotent marker (Powell et al. 2016.)*	*Pluripotent marker (Shi and Jin. 2010)*

GPR125, SSEA-1 and OCT-4 can be a potential SSC marker in horse

RT-qPCR

a. Ambion® Cells-to-cDNA™ II Kit
(non-reverse transcriptase control)

b. Power SYBR™ Green PCR Master Mix
(one sample, triplicate)



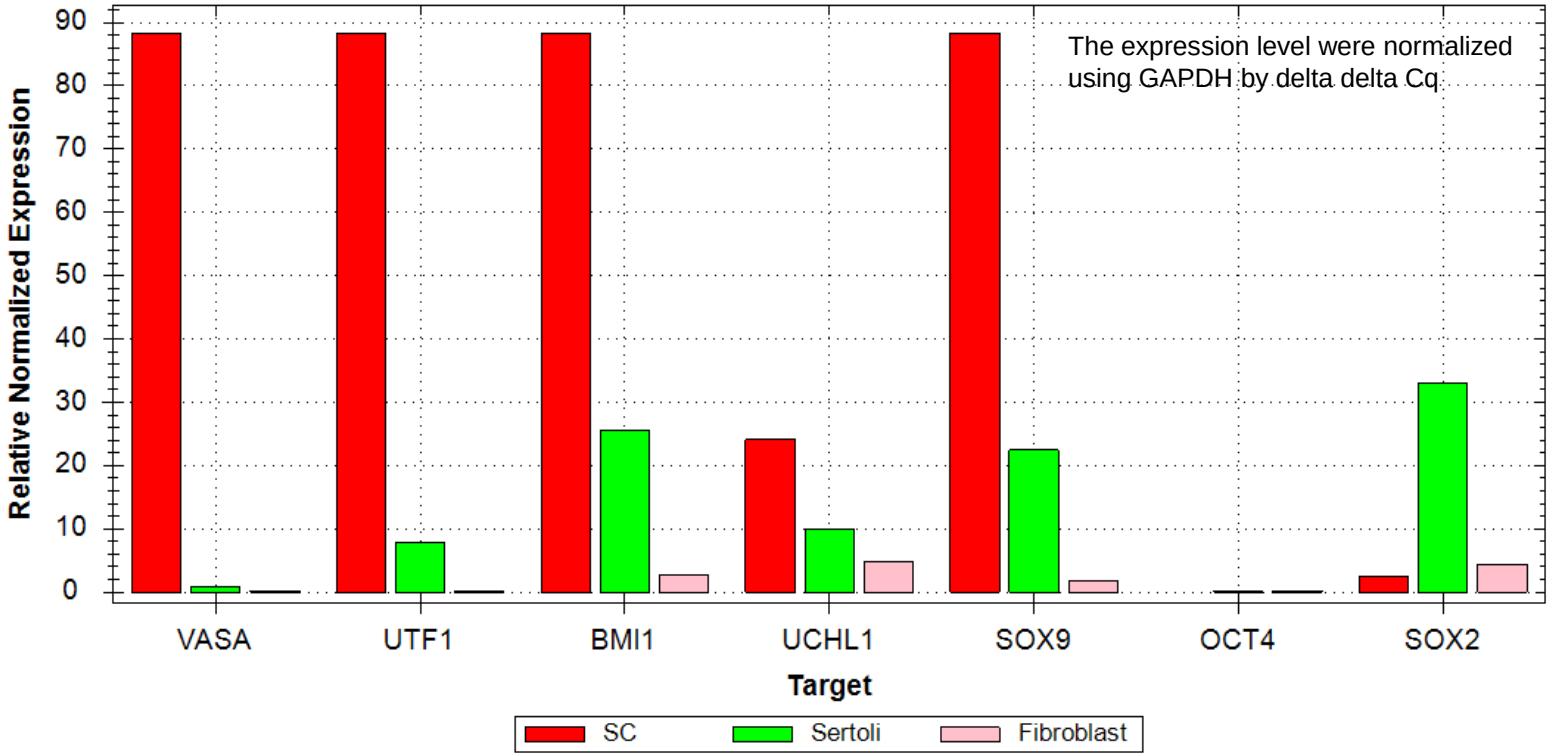
c. Sequencing (GAPDH, SOX9, UTF1, BMI1 and UCHL1)

Gene	*Forward Primer*	*Reverse Primer*	*Product(bp)*
$GAPDH_1$	CATCATCCCTGCTTCTACTGG	TCCACGACTGACACGTTAGG	117
$OCT4_2$	GGGACCTCCTAGTGGGTCA	TGGCAAATTGCTCGAGGTCT	318
$SOX2_3$	CGCTGCACATGAAAGAGCAC	CAGGCAGCGTGTACTTATCC	90
$SOX9_4$	CAGCCACTACAGCGAGCAG	CGATGGGGGTGTACATGG	230
VASA	AATTGGGCGTACTGGTCGTT	CCTGTTGAGCATCCGACAGT	116
BMI1	TCTTGTTTGCCTAGCCCCAG	TGGCAAAGGAGGATTGGTGG	121
UTF1	GACCAACTGCTGACCCTGAA	GGCAGCACTGCCTAAGATGA	99
UCHL1	GGCAGCCTAATGCCCTGTAA	TCTGCTAGTGCTTGGGTGTG	172

1. Breton et al. 2012
2. Nagy et al. 2011
3. Sharma et al. 2014
4. Bugno et al. 2008

Bio-Rad

Expression of spermatogonia and pluripotency markers at mRNA level



In horse: UTF1, BMI1, SOX9 and SOX2 expressed by Sertoli cell; UCHL1 expressed by all cell types we tested; Confirm VASA is only expressed in spermatogonia

Experiment 2:

To evaluate if horse/rhinoceros SSC can be isolated from cryopreserved testicular tissue

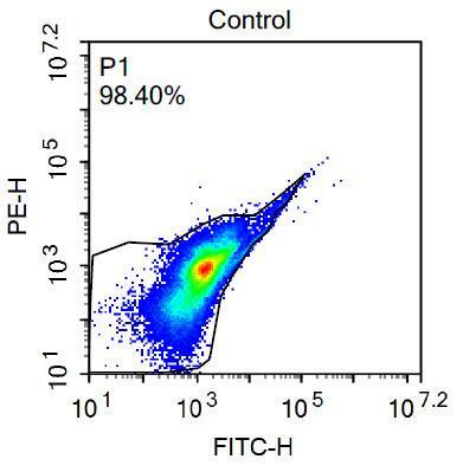
- Fresh/ref testicular tissue
  n=5; adult horse (5 to 10 YO)

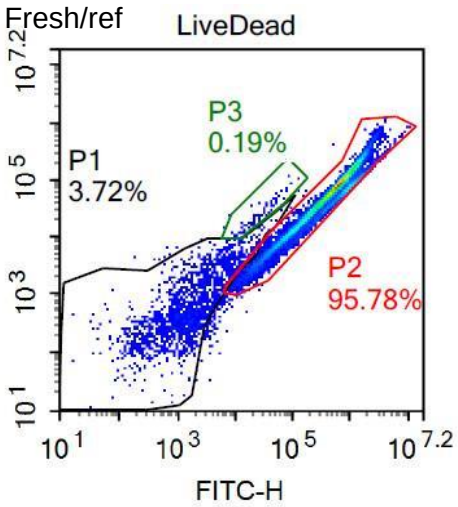
- Frozen testicular tissue
  n=9; pubertal horse (1 to 2 YO)
  1.68 gram; 1.58 ± 0.3 X 10^6 cells

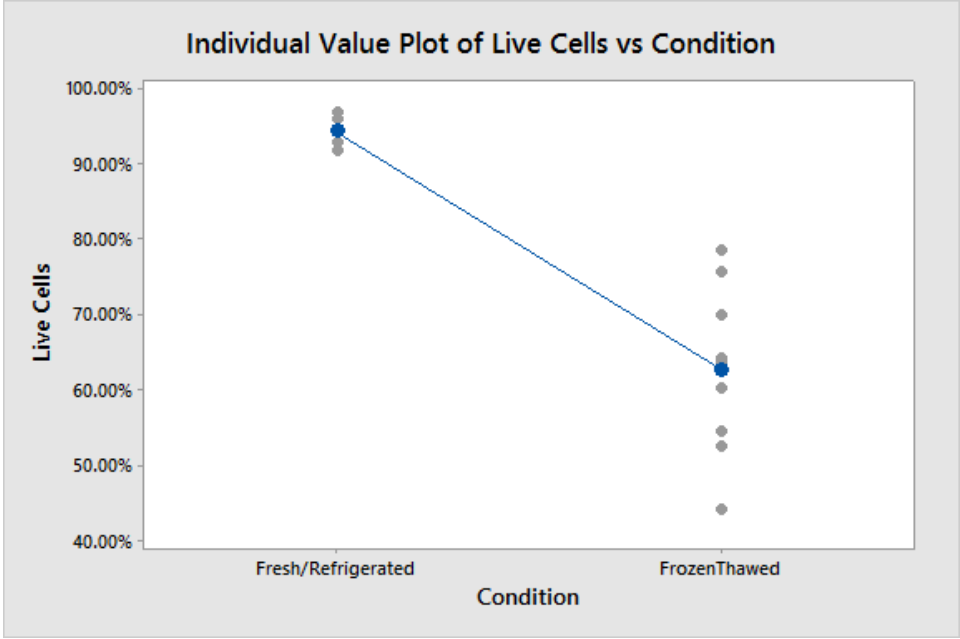
Freezing media:
⇒ PBS + 1.5 M DMSO

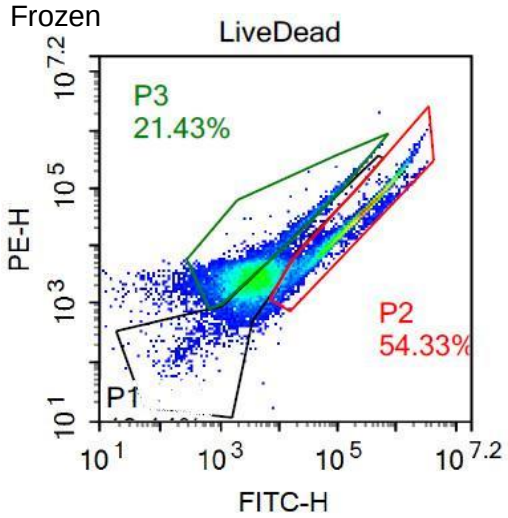
Cooling Rate:
1)From 0℃ at 2℃/min to -7℃ (hold 5 min)
2) 0.3℃/min to -40℃ - LN

San Diego Zoo, Frozen Zoo®









(P-value <0.05)

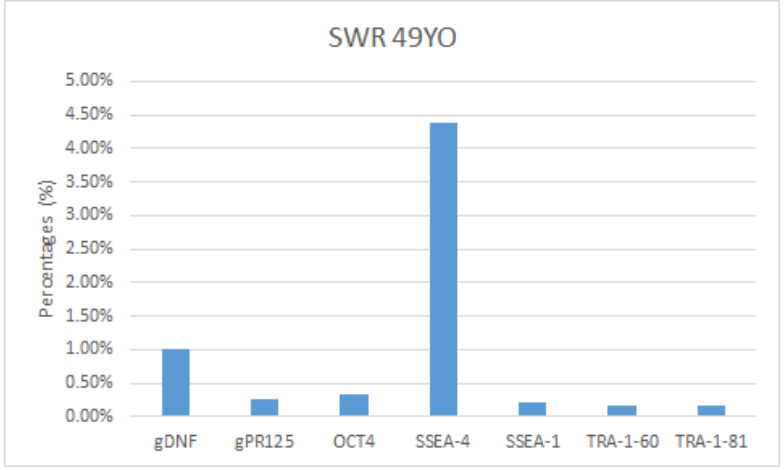
Tissue sample	Live Cell % ± SEM
fresh/refrigerated	94.17% ± 1.2%
frozen	62.52% ± 3.0%

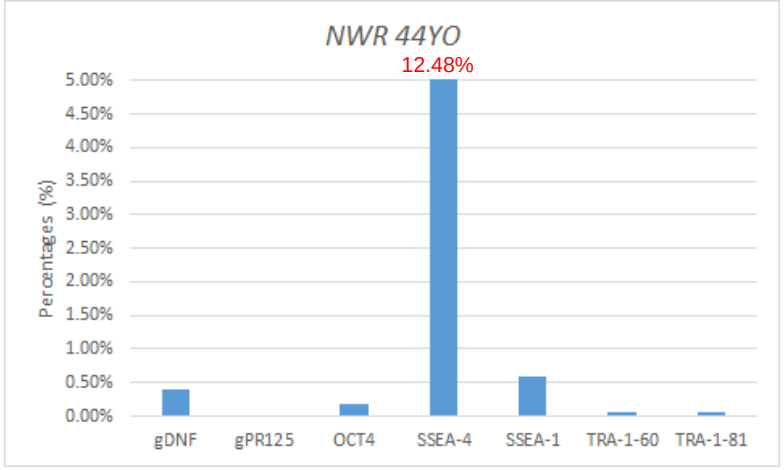
Although the live cell rate in frozen-thawed tissue is around 30% lower than fresh tissue, we are still able to isolate the cell from it

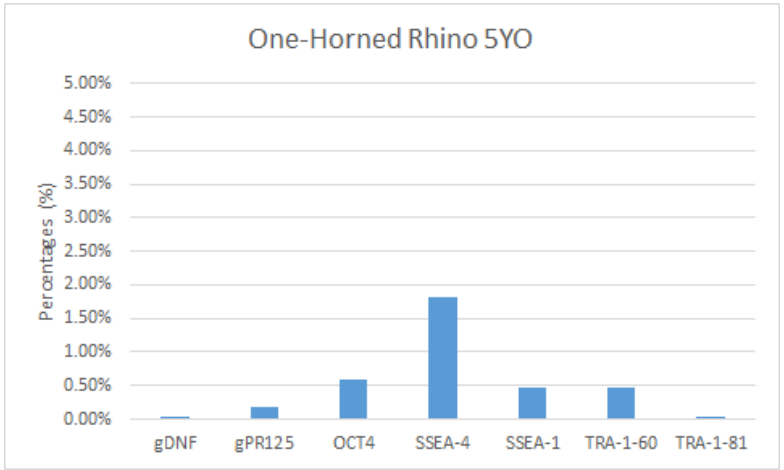
Experiment 3:
Phenotypical Characterization of Rhinoceros SSC

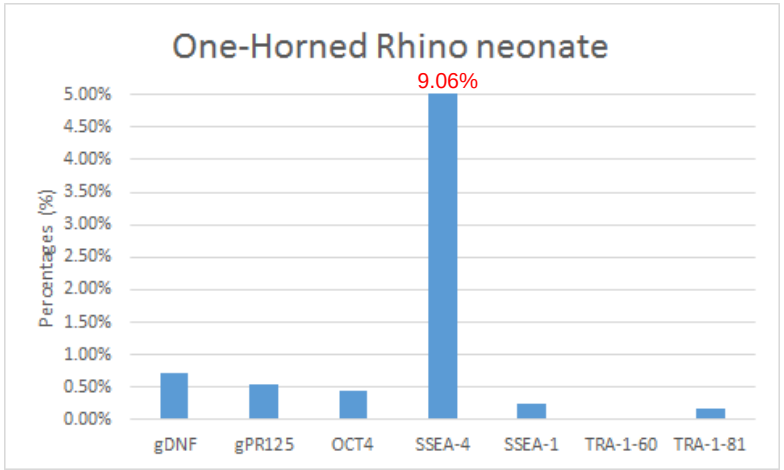
	Animal	Gram	Total Cells	Live Cell %
Fresh	Southern White Rhino(SWR) 49YO	8.0	2.6×10^6	47.15%
Fresh/ref	One-Horned Rhino 5YO	1.6	6.0×10^6(No Percoll)	85.44%
Fresh/ref 2 days	One-Horned Rhino neonate	2.0	2.0×10^6	67.00%
Frozen	Northern White Rhino(NWR) 44YO	0.7	0.2×10^6	**10.55%**

San Diego Zoo, Angalifu, 44







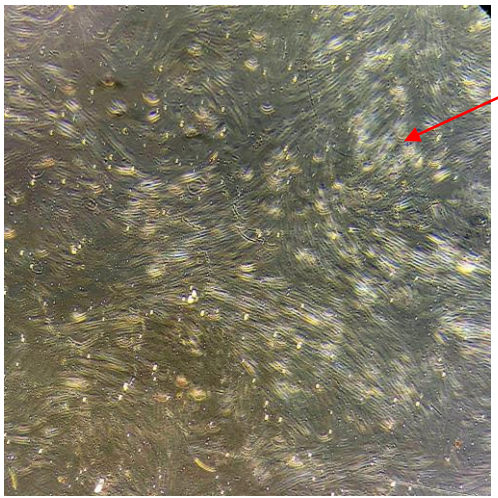


GPR125, OCT-4, SSEA-1,TRA-160 and TRA-181 can be a potential SSC marker in rhinoceros

Experiment 4:

Evaluate if a serial differential plating can purify a population of horse/rhinoceros SSC

Gelatin-Coated Dish → Collagen-Coated Dish → Laminin-Coated Dish

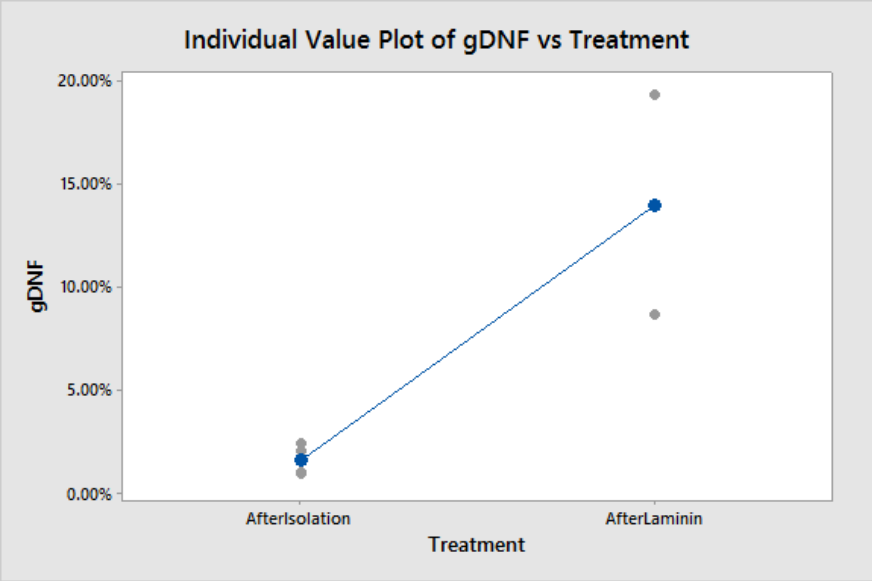


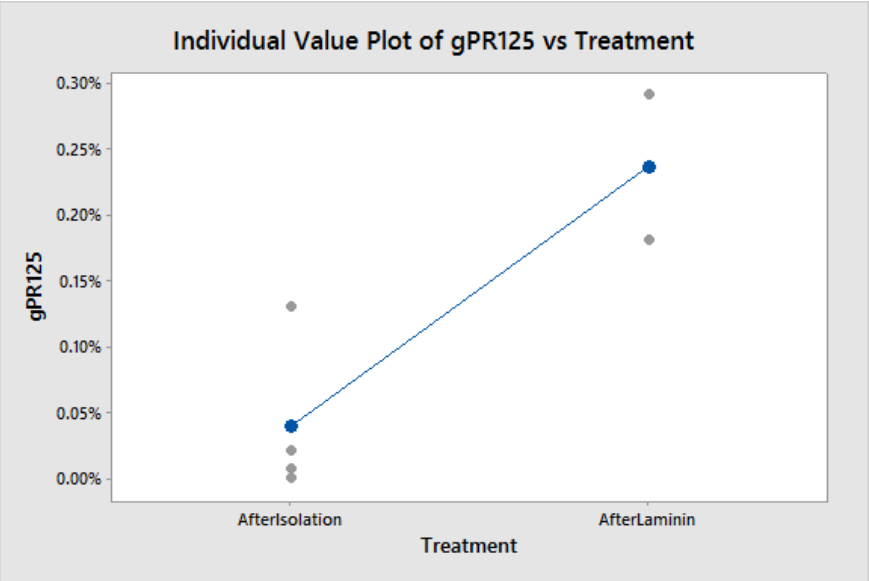
Horse: Frozen testicular tissue, n=4
Rhinoceros: Fresh/ref, n=3; Frozen, n=1

(Hamra, 2008)

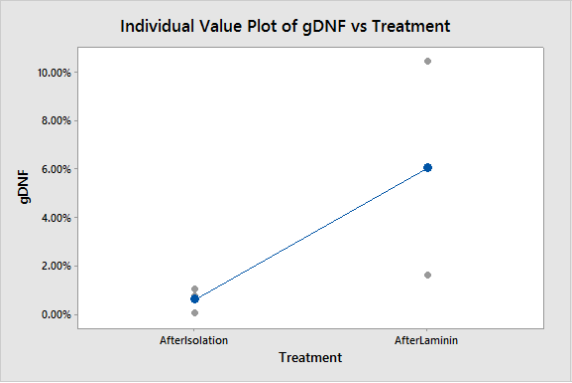
Flow after isolation (frozen; n=4)

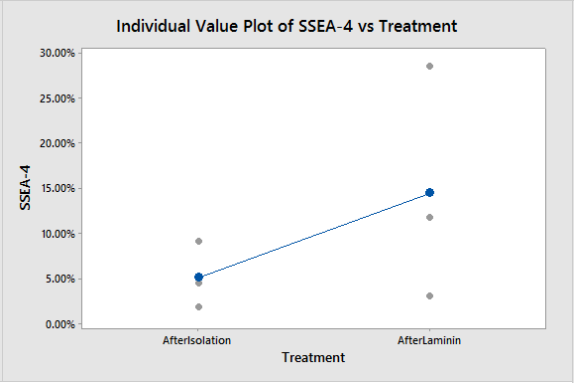
Flow after differential plating (frozen; n=2)

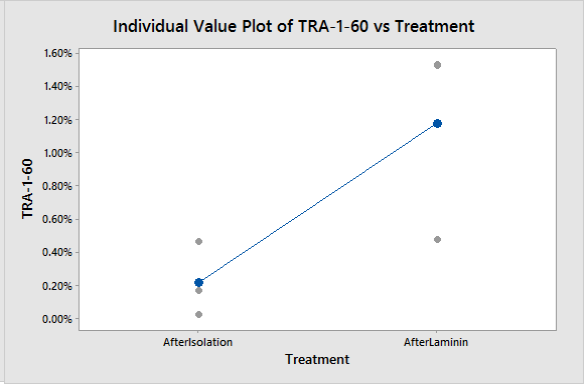




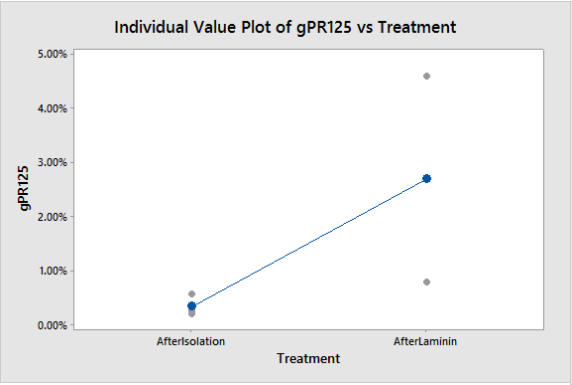
GDNFα1 and GPR125 population tend to increase in horse

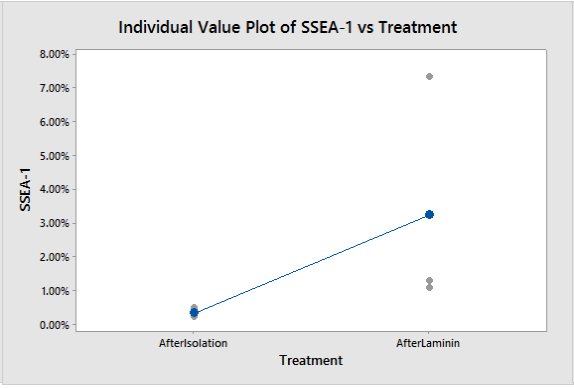


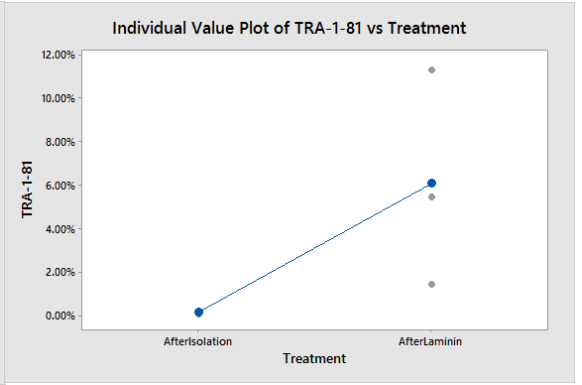




Flow after isolation (Fresh/ref; n=3)
Flow after differential plating (Fresh/ref; n=3)

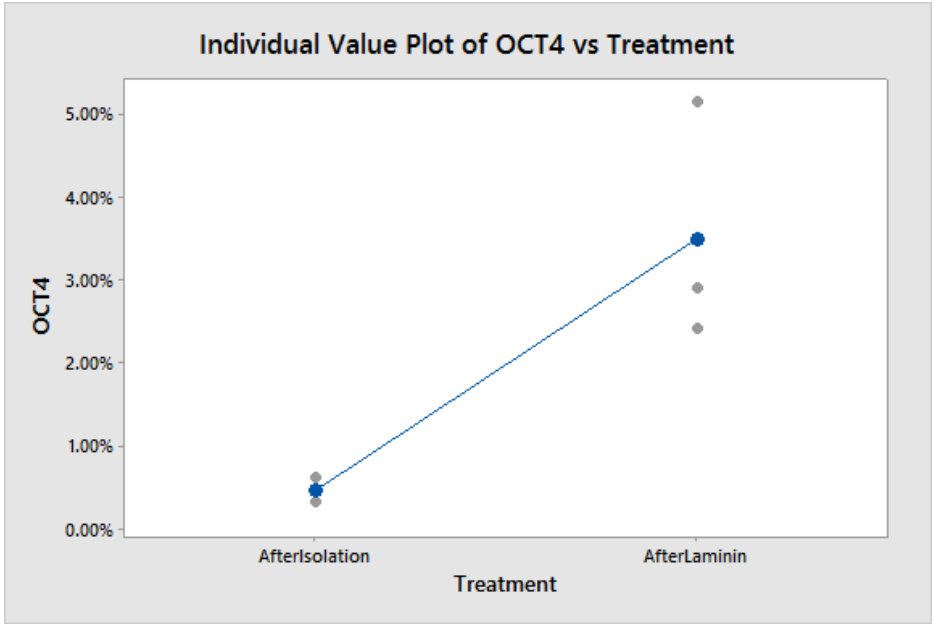






Increase percentages of GDNFα1, GPR125, SSEA-4, SSEA-1, TRA-1-60 and TRA-1-81 positive cells in rhinoceros after differential plating, but no significant difference

Flow after isolation (Fresh/ref; n=3)
Flow after differential plating (Fresh/ref; n=3)



OCT-4 population tend to increase in rhinoceros

Conclusions

1. Horse/rhinoceros SSC can be isolated from cryopreserved testicular tissue.

2. Differential plating can purify a horse/rhinoceros SSC population.

3. Markers tested in other mammalian species can be used in these two species, but differences in the expression of the markers are observed.

4. GPR125 and SSEA-1 are potential SSC marker for horse.

5. OCT-4 seems to be a specific marker for rhinoceros SSC, and GPR125, SSEA-1, TRA-1-60 and TRA1-81 are also potential SSC markers.

Future Work

1. Process more horse/rhinoceros samples, collect more data.
2. Purify horse/rhinoceros SSCs by Fluorescence-Activated Cell Sorting (FACS).
3. Evaluate UTF-1, VASA and GATA4 antibodies by flow cytometry and FACS.
4. Produce cDNA from horse/rhinoceros SSCs, and run RT-qRCR.

Long Term Goals

1. Isolate the SSCs from cryoperserved NWR testicular tissue.
2. Culture and enrich the NWR SSCs.
3. Transplant the NWR SSCs to horse testis.
4. Obtain NWR sperm from the host species.
5. Produce baby NWR.

http://www.zooborns.com/