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Renal subcapsular transplantation of 2'-deoxyguanosine treated murine embryonic thymus in nude mice

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Corresponding Author:	Shiqi Jia Jinan University Guangzhou, CHINA
Corresponding Author's Institution:	Jinan University
Corresponding Author E-Mail:	jshiqi@aliyun.com
Order of Authors:	Jianxue Wang
	Guolin Chen
	Qianwen Cui
	Erfei Song
	Cunchuan Wang
	Shiqi Jia
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TITLE:

Renal Subcapsular Transplantation of 2'-Deoxyguanosine-Treated Murine Embryonic Thymus in Nude Mice

AUTHORS & AFFILIATIONS:

Jianxue Wang[1,3,4], Guolin Chen[1,3,4], Qianwen Cui[2,3,4], Erfei Song[1,3,4], Cunchuan Wang[1,3,4], Shiqi Jia[1,3,4]

[1]The First Affiliated Hospital, Jinan University, Guangzhou, China
[2]Department of Biochemistry and Molecular Biology, School of Basic Medical Science, Jinan University, Guangzhou, China
[3]The Institute of Clinical Medicine, Jinan University, Guangzhou, China
[4]Joint institute of Metabolic Medicine Between Jinan University and University of Hong Kong

Corresponding author:

Shiqi Jia (jshiqi@aliyun.com)
Cunchuan Wang (twcc@jnu.edu.cn)

Email Addresses of Co-Authors:

Jianxue Wang (xue7524@qq.com)
Guolin Chen (chenguolin0808@foxmail.com)
Qianwen Cui (1311049048@qq.com)
Erfei Song (amossong@connect.hku.hk)

SUMMARY:

We provide a simple and efficient method to transplant 2'-deoxyguanosine treated E18.5 thymus into the renal capsule of a nude mouse. This method should aide in the study of both thymic epithelial cells function and T cells maturation.

ABSTRACT:

The thymus is an important central immune organ, which plays an essential role in the development and differentiation of T cells. Thymus transplantation is an important method for investigating thymic epithelial cell function and T cells maturation in vivo. Here we will describe the experimental methods used within our laboratory to transplant 2'-deoxyguanosine (to deplete donor's lymphocytes) treated embryonic thymus into the renal capsule of an athymic nude mouse. This method is both simple and efficient and does not require special skills or devices. The results obtained via this simple method showed that transplanted thymus can effectively support the recipient's T cells production. Additionally, several key points with regards to the protocol will be further elucidated.

INTRODUCTION:

The thymus is the central immune organ, within the thymus thymocytes undergo positive and negative selection, and become mature T cells[1,2]. Abnormal positive or negative selection results

in immunodeficiency or autoimmune pathologies respectively[3,4]. Therefore, thymus organ transplantation is an important approach to study the process of T cells selection in the donor's thymus. This method is particularly crucial when analyzing thymic epithelial function mediated by gene mutations which cause embryonic lethal phenotype when mutated[5].

In order to study the maturation of a recipient's T cells in the transplanted thymus, depletion of donor's lymphocytes within the thymus is necessary. For this purpose, embryonic 14-, 15- or 16-day (E14, E15, E16) thymus is usually selected[6,7]. Thymus from more mature stages can also be successfully depleted of the donor's lymphocytes by treating with 2'-deoxyguanosine. However, a detailed protocol for depleting lymphocytes and use of older thymus culture has not previously been described[8,9]. While transplantation protocols have been introduced by several studies[10,11], further modification and improvement of these protocols is necessary.

Our protocol is separated into two parts: (i) Depletion of T lymphocytes from late developmental stage E18.5 thymus by culture in 2'-deoxyguanosine-containing media. (ii) Transplantation of the cultured thymus into recipients. In this procedure, we developed a simple way to deliver the large tissue (E18.5 thymus) into the renal capsule with reduced chance of kidney injury. While focussing on later stage thymus, our protocol can also be used directly or with modifications for transplantation of thymus at various developmental stages or other similar sized tissues.

PROTOCOL:

The presented protocol adheres to the guidelines of the ethics committee of Jinan University regarding animal care.

NOTE: Materials used are listed in the **Table of Materials.**

1. Isolation of embryonic thymus

1.1. Autoclave all surgical instruments before the experiment, and sterilize the bench/hood with 70% ethanol.

1.2. Using carbon dioxide, anesthetize and euthanize the pregnant female mouse (18.5 days post successful mating). Then wipe the abdominal region with 70% ethanol.

NOTE: Here we mated *Insm1*$^{+/lacZ}$ females and *Insm1*$^{+/lacZ}$ males.

1.3. Using scissors, make a "V" shaped cut on the abdomen starting from the bladder and running until each horn of the uterus.

1.4. Using the scissors, cut the mesometrium and cervix/vagina, and collect the uterus. Place the uterus in a Petri dish containing cold phosphate-buffered saline (PBS) on ice. Next, expose the embryos which are in the enveloped decidua, by cutting the anterior uterine wall from one uterine horn to the other. Using fine tweezers, peel back the enveloped decidua tissues and cut

the umbilical cord to release the embryos. Place all the embryos in a new Petri dish on ice until the isolation of the thymus.

1.5. Wipe an embryo with 70% alcohol and place it in a new Petri dish. From this step on, ensure that sterile conditions are maintained.

1.6. By cutting close to the lower jaw with scissors, remove the head of the embryo and drain the blood with a paper towel. Next, fix the embryo on the same Petri dish in supine position.

NOTE: We cut a piece of the tail of each embryo for *Insm1* and *lacZ* genotyping.

1.7. Using scissors, cut the lateral chest wall horizontally along the axillary front, and then cut the diaphragm to open the chest. The thymus should now be visible as two white lobes located in front of the trachea and adjacent to the heart.

1.8. Place bent-tip forceps behind the thymus and then pull off the thymus gently. Be sure to check the integrity of the thymus to confirm that it contains two jointed lobes.

1.9. Wash the thymus with 1x PBS and trim off the connective tissues and blood vessels under a stereomicroscope.

2. Culture of the isolated embryonic thymus

2.1. Add 500 µL of culture medium (RPMI1640 + 15% fetal bovine serum (FBS) + 100 U/mL penicillin and 100 µg/mL streptomycin) to each well of a 24-well plate. Transfer the clean thymi into the wells with one thymus per well. **Figure 1** displays the isolated thymus in culture media.

2.2. To each thymus-containing well add 2'-deoxygranosine to a final concentration of 1.25 mM.

2.3. Culture the isolated thymus for eight days, refreshing both the culture media and 2'-deoxygranosine every two days.

3. Establish the subcapsular space in the renal capsule

3.1. To prepare the needle and the clipped infusion tube (**Figure 2**), cut the scalp vein needle on the tube part at a 45° angle using scissors.

3.2. Weigh the nude mouse and then anesthetize it with a pentobarbital (1.5%) injection (75 µg/g body weight).

3.3. When no reflex following toe pinch is observed, place the mouse on the operating table in a right lateral position.

3.4. With a 0.5% povidone iodine swab, disinfect the skin twice in the surgical area from the inside to the outside of the body.

3.5. Using scissors, make a 5–9 mm skin incision parallel to the spine in the left renal area (between the last rib and the iliac crest). Next, open the abdominal cavity by cutting through the subcutaneous tissue and muscle and expose the kidney.

3.6. With the kidney exposed, use a pair of tweezers in one hand to lift the muscle and fat tissue off the spine-side incision edge. With the other hand, gently squeeze the kidney out (alternatively, the kidney can be squeezed out using fingers of both hands).

3.7. To ensure that the renal capsule is moist during the surgery, wet the surface of the kidney with saline (0.9% NaCl).

3.8. Create a nick in the kidney capsule, and gently scratch the renal capsule on the lower right side using the needle tip prepared in step 3.1. The size of the nick should be 1/2–2/3 widths of the kidney; do not scratch on the kidney.

3.9. Slide the infusion tube prepared in step 3.1 into the nick on the renal capsule. Gently dissociate the renal capsule with the kidney along the kidney's long side until reaching 3–4mm inside the kidney capsule. Draw back the infusion tube; the kidney subcapsular space is established.

4. Transplant the embryonic murine thymus

4.1. Wash the thymus cultured in step 2.3 twice in saline to deplete the culture media.

4.2. Connect the clipped infusion tube prepared in step 3.1 to a syringe at its syringe-connecting interface. Aspirate the prepared thymus into the infusion tube slowly.

4.3. Gently insert the clipped infusion tube into the renal capsule and reach the superior pole. Deliver the thymus into the renal capsule; retract the tube slowly while simultaneously pushing the plunger of the syringe gently.

4.4. Using an alcohol lamp, slightly heat the needle prepared in step 3.1. After ensuring that the whole thymus is inside the subcapsular space, use the heated needle to cauterize the nick.

4.5. After cauterization, restore the kidney in the abdominal cavity. Suture the peritoneum and muscle.

4.6. Using a modified interrupted vertical mattress suture, close the skin incision (tie at least three knots and cut away any excess thread).

4.7. Using a povidone iodine swab, disinfect the incision. To prevent infection and relieve pain, provide a subcutaneous injection of antibiotic (cefmetazole at 90 μg/g of body weight) and flunixin (2 μg/g of body weight).

4.8. Until fully recovered from anesthesia, keep the mouse warm under the infrared lamp.

4.9. Keep the thymus in the recipient's kidney capsule for 8 weeks before dissecting the transplanted thymus and conducting phenotypic analysis as previously described[5,8,9].

REPRESENTATIVE RESULTS:

Here we show the isolated E18.5 thymus containing two complete lobes (**Figure 1**). Additionally, we show the scalp vein needle that was clipped to form a bevel on the infusion tube (**Figure 2**). Next, we also show a representative image of the position of the thymus that was transplanted in the renal capsule (**Figure 3A**) and the thymus after 8 weeks of growth within the recipient mice (**Figure 3B**). To determine whether the T cells were produced in nude mice transplanted with a thymus, in both the transplanted thymus and the peripheral blood, we detected the cell populations using CD4 and CD8 antibody staining and flow cytometry analysis. The peripheral blood was collected from the retro-orbital sinus as previously described[12]. We found the T cells were produced in both the transplanted thymus and the peripheral blood of nude mice transplanted with a thymus. However, no T cells were detected in peripheral blood of non-transplanted nude mice (**Figure 4**). To determine the source of T cells, we checked *Insm1* and *lacZ* genes in the peripheral white blood cells using genotyping methods routinely used in our laboratory and described previously[13,14]. Since the donor embryo *Insm1* gene was replaced by the *lacZ* gene in one or both alleles, when the T cells were co-transplanted with thymus from donor, we could detect the *lacZ* gene in the genome of the T cells collected from peripheral blood of recipient, which indicates that they were produced by the donor thymus. Additionally, as no *lacZ* gene was present, *lacZ* would not be detected when the T cells were generated from recipient's hematopoietic cells. We did not detect *lacZ* gene in the peripheral T cells indicating that the T cells were generated from recipient (**Figure 5**).

FIGURE LEGENDS:

Figure 1: Thymus isolated from E18.5 embryos.

Figure 2: Thymus delivering tools made from the scalp vein needle. The scalp vein needle was cut at the infusion tube part close to the needle at an angle of 45° to create a bevel. Both the needle and the infusion tube were used in the procedure.

Figure 3: Thymus transplanted into the renal capsule. (**A**) Freshly transplanted E18.5 thymus in the renal capsule. (**B**) Thymus in the renal capsule after 8-week growth in recipient.

Figure 4: Flow cytometry analysis of the T cells isolated from the transplanted thymus, blood of thymus-transplanted and non-transplanted nude mice. CD4 and CD8α antibodies were used for

T cells staining. $CD4^+CD8^+$ double positive cells, $CD4^+$ single positive, $CD8^+$ single positive and $CD4^-$ $CD8^-$ double negative cells are shown in each of the quadrants as indicated.

Figure 5: Identifying the source of the peripheral blood T cells in thymus transplanted nude mice. Genotyping of *lacZ* gene and *Insm1* gene in peripheral blood white cells is shown. Ladder: DNA marker, + : positive control DNA, -: negative control DNA, Anim1: DNA from peripheral white blood cells of nude mouse transplanted with $Insm1^{lacZ/lacZ}$ thymus, Anim2: DNA from peripheral white blood cells of nude mouse transplanted with $Insm1^{+/lacZ}$ thymus.

DISCUSSION:

Renal subcapsular transplantation of embryonic thymus is an important method to study the thymic epithelial cells function and the process of T cells maturation in vivo. Although there are several experimental studies on embryonic thymus organ culture and transplantation[6,7], our protocol provides a simple alternative procedure on murine embryonic thymus culture and renal subcapsular transplantation for older thymus tissue.

Our protocol improves upon previous protocols by incorporating several different modifications [6,7,10,11]. First, instead of E14–E16 thymus, we utilized the thymus isolated from E18.5 for transplantation. The advantage is that thymus at this later developmental stage contains relatively mature thymic structures and epithelial cell populations. Although the newborn or adult mice are an alternative source of mature thymus, if perinatal lethal phenotype occurs as a result of gene manipulation, such as mutations in *Jmjd6* or *Insm1* genes[5,13], this method provides a viable alternative for the study of mature thymus. A second modification is the prevision of a culture method of E18 thymus before transplantation. Additionally, a third modification occurs in the transplantation procedure, in which we used the needle tip to create a nick on the renal capsule instead of picking and cutting the renal capsule with tweezers and scissors. This modification reduced both renal capsule damage and injury of the kidney. One final modification is in the suture step. The modified interrupted vertical mattress suture eliminates the outside suture line on the skin and therefore prevents the opening of the incision due to biting.

While this protocol is used for E18.5 thymus transplantation into the kidney capsule, it can be modified for transplantation of the thymus at other developmental stages or for other tissues with similar sizes. Additionally, the materials used can be modified accordingly by different users from different areas, especially with respect to anesthesia reagents which may be restricted by local laws. The dosage of pentobarbital used in our protocol is 75 µg/g body weight. However, the maximal dosage should be no more than 100 µg/g body weight to prevent death of the anesthetized animals. Although the transplantation of the thymus into the renal capsule is an efficient method for the functional study of the thymus in vivo, some limitations exist in the method presented above. These limitations include risk of the thymus dropping out of the kidney capsule during the 8 weeks in vivo growth period (1 in 12). Secondly another limitation is the death of mice after the surgery (6 in 30). However, this death is mainly caused by the overdose of the pentobarbital. As such, other allowed methods of anesthesia can be employed.

In summary, we provide a simple and efficient protocol to isolate and culture the E18.5 thymus and to then subsequently transplant the thymus into the renal capsule. This then allows for the analysis of the thymic epithelial cells function and the process of T cells maturation.

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DISCLOSURES:

No conflicts of interest declared.

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figure 1

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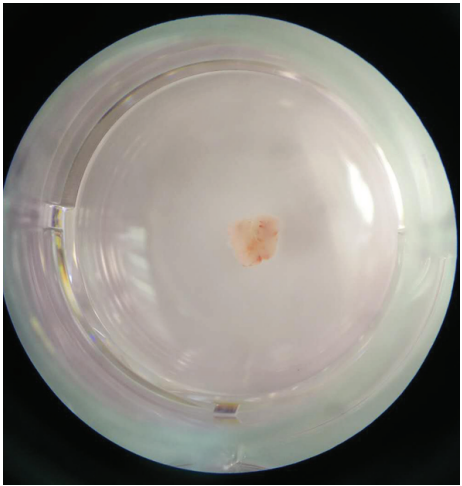

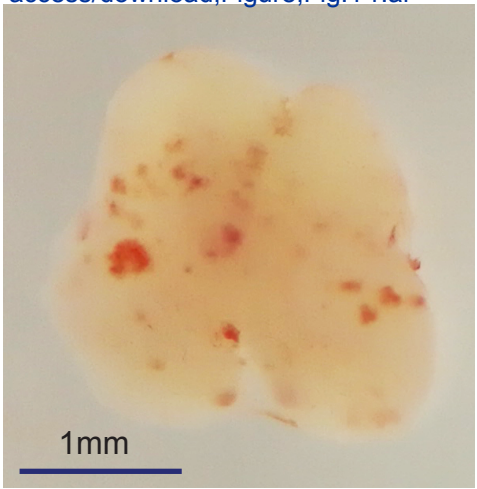

figure 2

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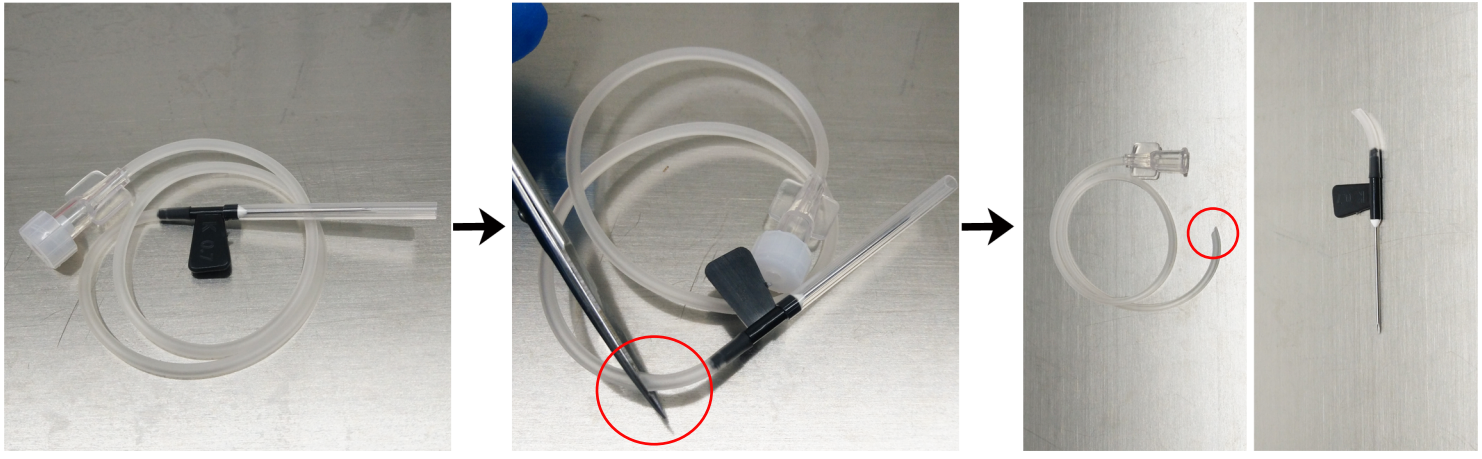

figure 3

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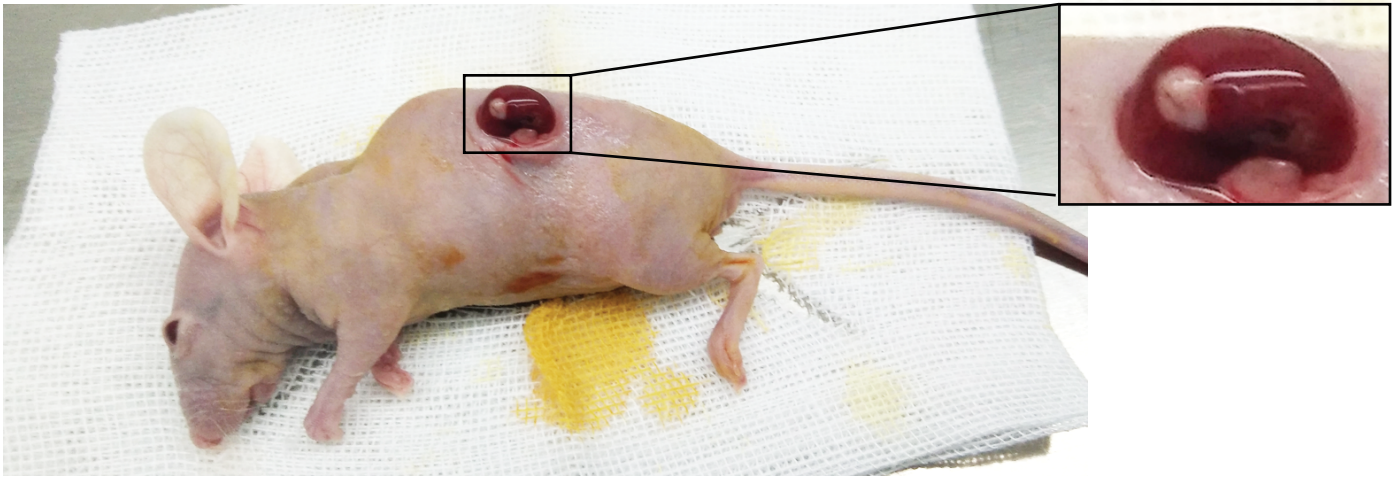

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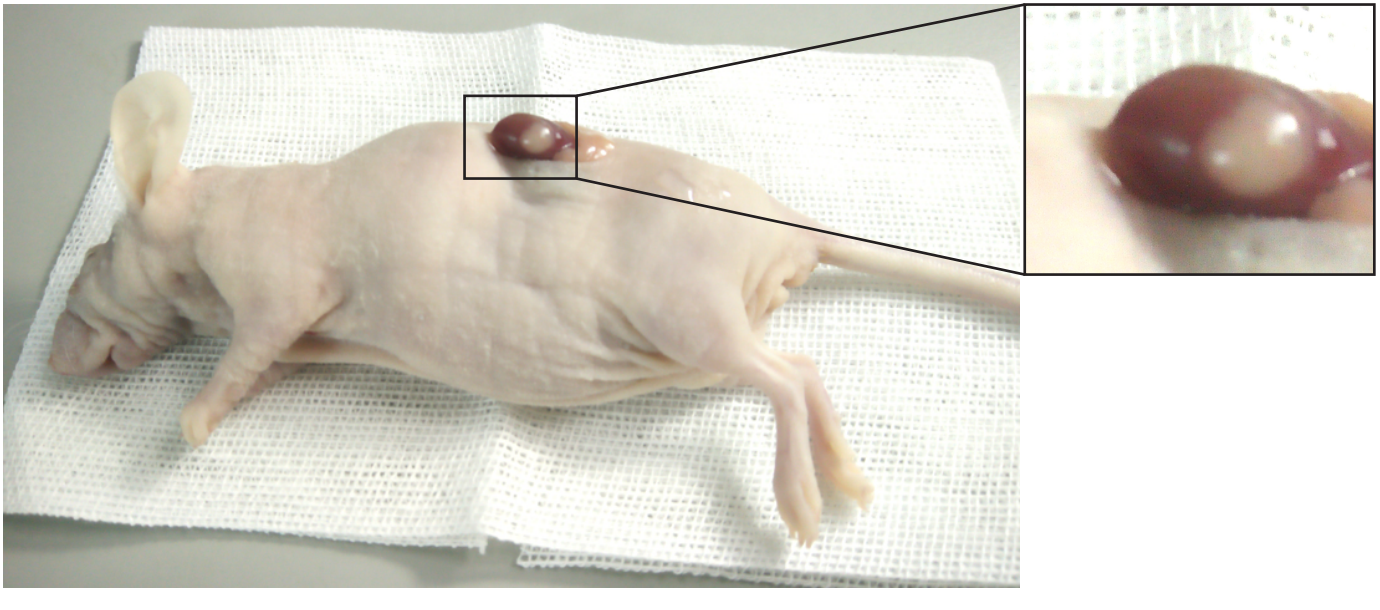

figure 4

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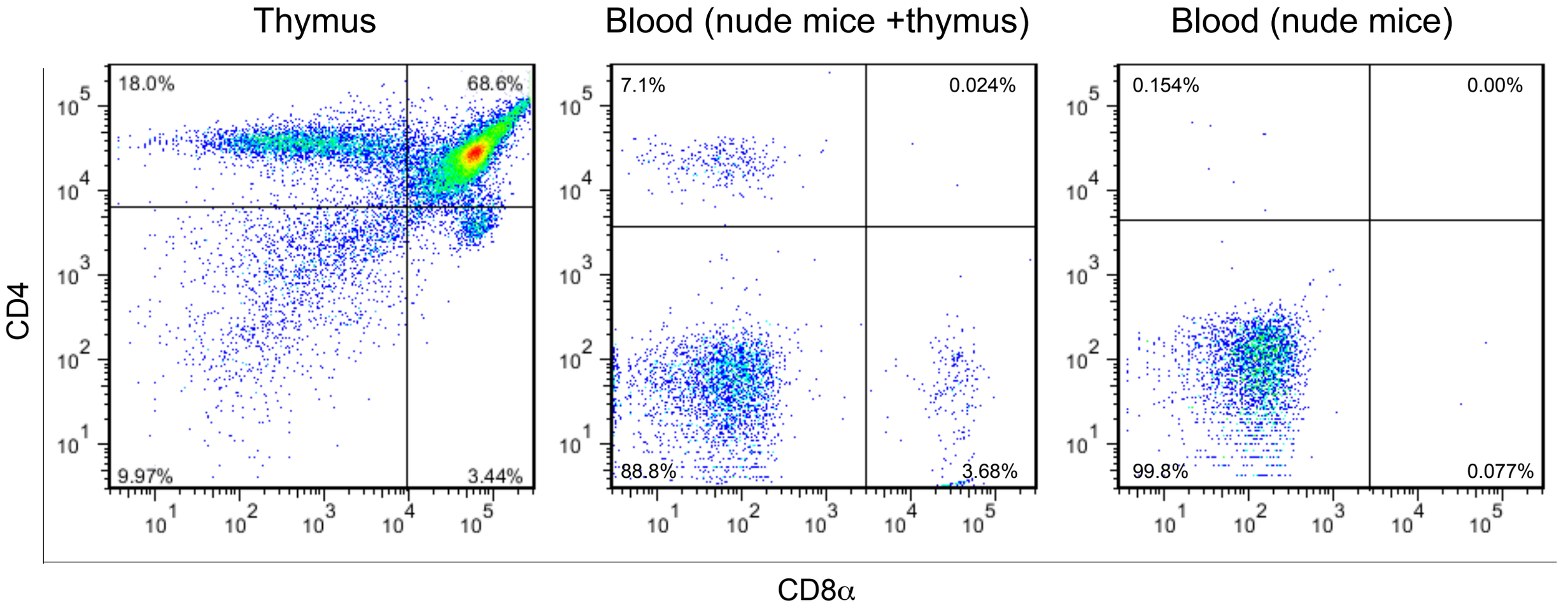

Figure 5

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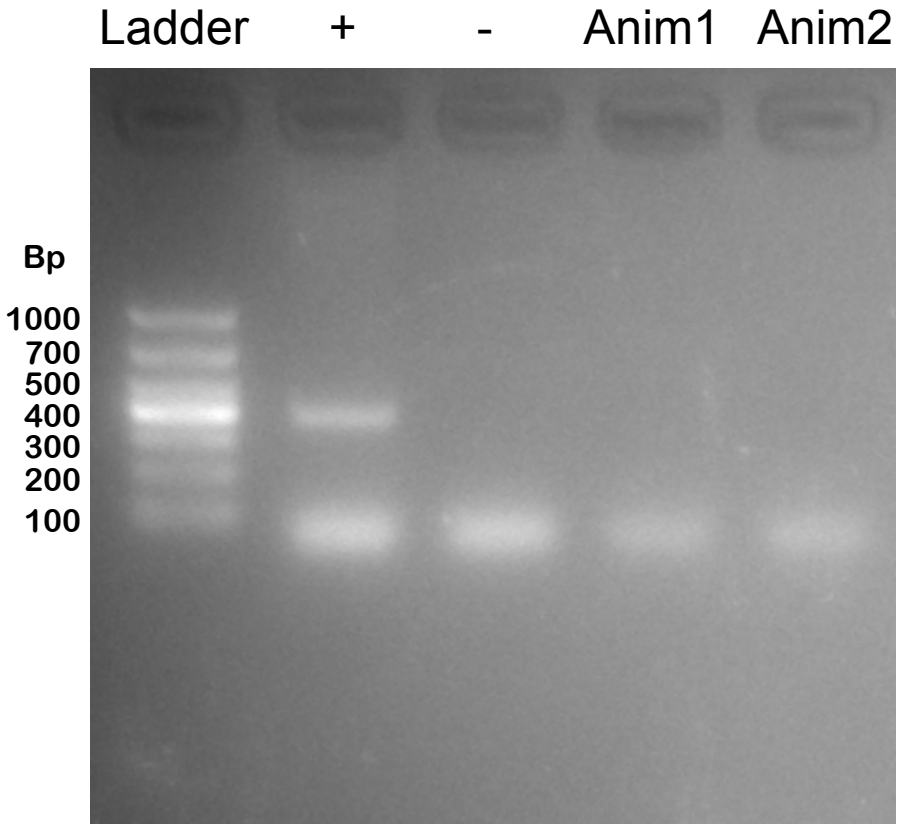
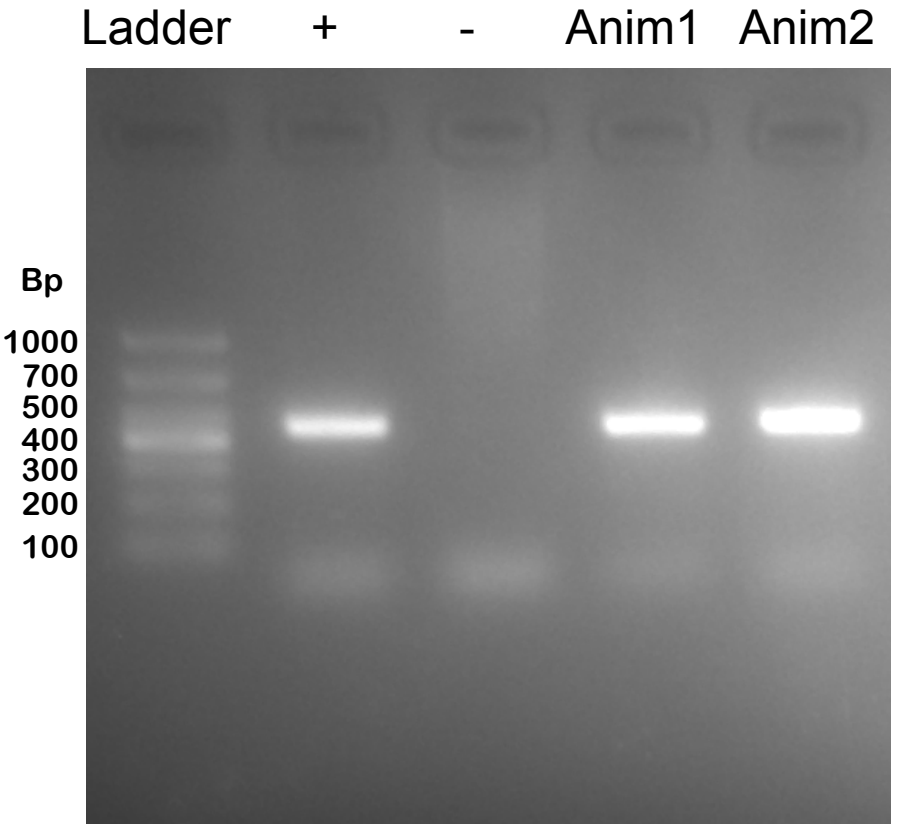


LacZ gene genotyping 380bp



Insm1 gene genotyping 450bp

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
0.5% Povidone iodine	Shanghai Likang Distinfectant Hi-Tech Co.Ltd	20171113
0.9% Sodium Chloride Injection	Shandong Qilu Pharmaceuyical Co.Ltd	2C17112101
1 mL Sterile syringe	Solarbio	YA1090
2'-Deoxyguanosine	MEC	HY-17563
24 Well Plate	Corning Incorporated	Costar 3524
4-0 Surgical suture needles with thread	NingBo Cheng-He Microsurgical Instruments Factory China	YY0166-2002
60mm Cell Culture Dish	Corning Incorporated	430166
70% ETOH	LIRCON	20181221
APC anti-mouse CD8a antibodies	Biolegend	100711
Bent-tip fine forceps, JZ 10 cm	Shanghai Medical Devices Group Co.,Ltd.	JD1060
Cefmetazole Sodium for Injection	Sichuan Hexin Pharmaceutical co,Ltd	17062111 079
Dissecting scissors, JZ 10 cm	Shanghai Medical Devices Group Co.,Ltd.	JC2303
Fetal bovine serum (FBS)	GIBCO	10270-106
Fine forceps, JZ 10 cm	Shanghai Medical Devices Group Co.,Ltd.	JD1050
Flow cytometry	BD	FACSCanto II
Flunixin meglumine	MACLIN	F810147
Forceps, Dumont#5	World Precision Instruments	14098
Infrared lamp	OTLAN	MT-810
Needle holder, JZ 14 cm	Shanghai Medical Devices Group Co.,Ltd.	J32010
PE anti-mouse CD4	Biolegend	100511
Penicillin-Streptomycin mixture	GIBCO	15140122
Pentobarbital sodium salt	Sigma	P3761
RPMI1640 Medium	GIBCO	C14-11875-093
Scalp vein needle	Shanghai Kindly Medical Instruments Co., Ltd	XC001
Spring scissors	VANNAS	S11014-12
stereomicroscope	OLYMPUS	SZ61
Sterile 15cm cotton swab	Guangzhou Haozheng	20150014
Sterile gauze 5 cm x 7 cm-8P	Guangzhou Haozheng	20172640868

Sterile PBS (1x)	GENOM	GNM20012
Tissue forceps, JZ 12.5 cm	Shanghai Medical Devices Group Co.,Ltd.	J41010

Comments/Description

1M in DMSO, 1:800 using (final 1.25mM)

1:100
To sterilize before use
6mg in 0.5ml 0.9% NaCl solution, 7.5ul/g body weight
To sterilize before use

To sterilize before use

1mg in 1ml 0.9% NaCl solution,2ul/g body weight
To sterilize before use

To sterilize before use
1:100
1:100
1.5% solution in PBS, 75ug/g body weight

To sterilize before use

To sterilize before use

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Cambridge, MA 02140
tel. 617.945.9051
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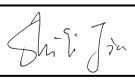
CORRESPONDING AUTHOR

Name:	**Shiqi Jia**
Department:	**The First Affiliated Hospital**
Institution:	**Jinan University, Guangzhou, China**
Title:	**Dr.**
Signature:	
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Dear Dr. Alisha DSouza

Many thanks for your help in editing the manuscript and providing the detailed comments! We followed the comments and revised the manuscript. Please see the responses below.

Best

Shiqi Jia

Comment 1 (line1): This article requires thorough language revisions. I have tried to fix most of the problems until the end of the introduction, but the rest of the article still requires language editing by a proficient English writer.

Response:

Many thanks for your help in the editing. We had the manuscript edited by a native English scientist.

Comment 2 (Line58-59): I cannot understand this sentence. Please revise to clarify.

Response:

We deleted this sentence.

Comment3 (Line 80): Male is also Insm1+/LacZ?

Response:

Yes, We added *Insm1*$^{+/LacZ}$ before male mice.

Comment 4 (Line 82): Using scalpel?

Response:

We added 'Using scissors'

Comment 5 (Line 85) Mention surgical tools used.

Response:

We added 'Using the scissors'

Comment 6 (Line 88-89) Release how?

Response:

We rewrote the sentence 'Using fine tweezers peel back the enveloped decidua tissues and cut the umbilical cord to release the embryos.'

Comment 7 (line 95) Jaw?

Response:

Yes, we corrected the wrong spelling.

Comment 8 (line 96) Supine?

Response:

Yes, we used 'supine'.

Comment 9 (line 99) Mention surgical tools used.

Response:

We added the tool used 'Using scissors'

Comment 10 (line 99-101) Unclear what is done here. Please revise the language for clarity.
Response:
We rewrote as 'Using scissors cut the lateral chest wall horizontally along the axillary front, and then cut the diaphragm to open the chest. The thymus should now be visible as two white lobes located in front of the trachea and adjacent to the heart.'

Comment 11 (line 100)?
Response:
'Two white lobes'

Comment 12 (line 99-101) Please clarify the language, this step is unclear.
Response:
We rewrote the step as 'Using scissors cut the lateral chest wall horizontally along the axillary front, and then cut the diaphragm to open the chest. The thymus should now be visible as two white lobes located in front of the trachea and adjacent to the heart.'

Comment 13 (line 103) You mean bent-tip forceps?
Response:
Yes, 'bent-tip forceps'

Comment 14 (line 111-112) Concentration?
Response:
We added '100u/ml penicillin and 100ug/ml streptomycin'

Comment 15 (line 115) These units are incorrect. Do you mean 1.25 mM? [M= mol/L]
Response:
Yes, Thanks. We deleted the '/L'

Comment 16 (line 128) How is the depth of anesthesia tested?
Response:
We rewrote the steps as 'When no reflex following toe pinch is observed, place the mouse on the operating table in a right lateral position.'

Comment 17 (line 131) %?
Response:
We added the concentration '0.5% povidone iodine'

Comment 18 (line 134) Using scalpel?
Response:
We added the tools used 'Using scissors'

Comment 19 (line 143) Sterile PBS? If not mention NaCl concentration
Response:
We added '0.9% NaCl'

Comment 20 (line 149-152) Unclear. Please revise for clarity.
Response:
We rewrote 'Slide the infusion tube prepared at step 3.1 into the nick on the renal capsule. Gently dissociate the renal capsule with the kidney along the kidney long side until reaching 3-4mm inside the kidney capsule. Draw back the infusion tube and the kidney subcapsular space is established.'

Comment 21 (line 149) Unclear when this was clipped.
Response:
We added step 3.1 and figure 2 to elucidate the preparation of the infusion tube. In step 3.9, we referred to step 3.1.

Comment 22 (line 149) Insert where?
Response:
We clarified as 'Slide the infusion tube prepared at step 3.1 into the nick on the renal capsule'

Comment 23 (line 157) I moved this here from the ends of the protocol but I'm not sure where it fits best. For how long should the thymi be kept in saline?
Response:
Thanks for the editing. We added one step to elucidate the procedure, '4.1 Wash the thymus cultured in step 2.3 twice in saline to deplete the culture media.'

Comment 24 (line 159-160) The infusion tube apparatus was not properly described. It is unclear at this point that one end is fitted with a syringe. Please describe the preparation properly in an earlier section before it was first used.
Response:
We added step 3.1 and figure 2 to describe the preparation.
We rewrote this step as 'Connect the clipped infusion tube prepared in step 3.1 to a syringe on its syringe-connecting Interface, aspire the prepared thymus into the infusion tube slowly.'

Comment 25 (line 163-164) Syringe plunger?
Response:
Yes, we used the word 'plunger of the syringe'

Comment 26 (line 166) What exactly was done to "prepare" this needle. Please ensure that the preparation is described appropriately before
Response:
We added step 3.1 and figure 2 to describe this.

Comment 27 (line 167)? I think this usage is incorrect. Please rewrite for clarity.
Response:
We changed to 'cauterize'

Comment 28 (line 167) What are the bleeding points?
Response:

We removed this describing

Comment 29 (line 172) Mention suture material used and add them to the table of materials.
Response:
We added the material in the table of materials.

Comment 30 (line 172) Mention suture material used and add them to the table of materials.
Response:
We added the material in the table of materials.

Comment 31 (line 176-177) Which antibiotics exactly? Mention dosage and also add the to the table of materials.
Response:
We added the name and dosage (Cefmetazole,90μg/g body weight) in the text and also in the table of materials

Comment 32 (line 182) How exactly is dissection performed? Mention briefly or cite a reference.
Response:
We cited the references (5,8,9).

Comment 33 (line 191) Add these to the table of materials along with concentrations and RRIDs
Response: We added the antibodies information into the table of materials.

Comment 34 (line 192-193) How was peripheral blood obtained? Please mention this briefly here or at the end of the protocol section.
Response:
We mentioned and added a reference.

Comment35 (line 197) Did you test genetic expression? If so how? What method was used.
Response:
We added the references for the genotyping method and described the method briefly in the results section. It a PCR method to detected whether the gene (*lacZ* and *Insm1*) is existed in the genome (reference 13 and 14 in the manuscript). For instance, if the mouse with *Insm1*$^{+/lacZ}$ genotype, we can detected both *Insm1* and *LacZ* genes in the genome by the PCR method. When the mouse is *Insm1*$^{lacZ/lacZ}$ genotype, then we can only detect *lacZ* gene but not the *Insm1* gene in the genome by the PCR method. The wild type mouse (*Insm1*$^{+/+}$) has *Insm1* gene on both alleles of the genome and the *Insm1* gene but not *lacZ* gene can be detected in the PCR amplification.
The nude mice have no genetic modification on *Insm1* locus, thus they contain only the wild type *Insm1* gene and without the *lacZ* gene, while the thymus that was transplanted into the nude mouse is isolated from the embryo which was genetic

modified on *Insm1* locus, the *Insm1* gene was replaced by a *lacZ* gene, and thus with a genotype of $Insm1^{+/lacZ}$ or $Insm1^{lacZ/lacZ}$. If a *lacZ* gene could be detected in the peripheral white blood cells of the nude mice, it is indicated that the peripheral T cells are generated from the donor. Otherwise, if only *Insm1* gene but not *lacZ* gene could be detected in peripheral white blood cells, we can conclude that the T cells are from recipients.

Comment36 (line 221-225) Is this best way to test for the expression/presence of this genes? Why not use qPCR instead to test expression?
Response:
Yes, it is the common method used to genotype a genetic modified animal. Since the result is yes or no for a certain gene and it is not necessary to quantify the dosage. The qPCR is not used in this case.

Comment37 (line 223) Please add the ladder markers to the figures
Response:
We added the ladder with bps numbers on the figures.

Comment38 (line 230) References??
Response:
We added the references

Comment39 (line 236-240) I cannot comprehend this sentence. It is still unclear why you choose E18.5 ,i.e., why do you claim it is better? Please support this claim with sufficient appropriate references.
Response:
We rewrote as 'The advantage is that thymus at this later developmental stage contains relatively mature thymic structures and epithelial cell populations. Although the newborn or adult mice are an alternative source of mature thymus, if perinatal lethal phenotype occurs as a result of gene manipulation, such as mutations in Jmjd6 or Insm1 genes 5,13, this method provides a viable alternative for the studying of mature thymus.'

Comment 40 (line 240-241) You do not present any tests to confirm that there were no LacZ+ lymphotcytes in the implanted thymus. Please cite a reference where you showed this or remove this claim.
Response:
We remove the inaccurate conclusion and changed to 'A second modification is the prevision of a culture method of E18 thymus before transplantation.'

Comment41 (line 254-255) Please revise the language
Response:
We revised it to 'the maximal dosage should be no more than 100 μg/g body weight to prevent death of the anesthetized animals'

Comment42 (line 255-260) Please add the limitations of your method here.

Response:

We added the limitation as ‘Although the transplantation of the thymus into the renal capsule is an efficient method for the functional study of the thymus in vivo some limitations exist in the method presented above. These limitations include risk of the thymus dropping out of the kidney capsule during the 8 weeks in vivo growth period (1 in 12). Secondly another limitation is death of mice after the surgery (6 in 30). However, this death is mainly caused by the overdose of the pentobarbital as such if allowable other methods of anesthesia should be employed.’