TITLE:
Production of Genetically Engineered Golden Syrian Hamsters by Pronuclear Injection of the CRISPR/Cas9 Complex

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Pronuclear injection, CRISPR/Cas9, gene editing, knockout, zygote, embryo transfer, animal model, golden Syrian hamster

SHORT ABSTRACT:
Pronuclear (PN) injection of the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein-9 nuclease (CRISPR/Cas9) system is a highly efficient method for producing genetically engineered golden Syrian hamsters. Herein, we describe the detailed PN injection protocol for the production of gene knockout hamsters with the CRISPR/Cas9 system.

LONG ABSTRACT:
The pronuclear (PN) injection technique was first established in mice to introduce foreign genetic materials into the pronuclei of one-cell stage embryos. The introduced genetic material may integrate into the embryonic genome and generate transgenic animals with foreign genetic information following transfer of the injected embryos to foster mothers. Following the success in mice, PN injection has been applied successfully in many other animal species. Recently, PN injection has been successfully employed to introduce reagents with gene-modifying activities, such as the CRISPR/Cas9 system, to achieve site-specific genetic modifications in several laboratory and farm animal species. In addition to mastering the special set of microinjection skills to produce genetically modified animals by PN injection, researchers must understand the reproduction physiology and behavior of the target species, because each species presents unique challenges. For example, golden Syrian hamster embryos have unique handling requirements *in vitro* such that PN injection techniques were not possible in this species until recent breakthroughs by our group. With our species-modified PN injection protocol, we have succeeded in producing several gene knockout (KO) and knockin (KI) hamsters, which have been used successfully to model human diseases. Here we describe the PN injection procedure for delivering the CRISPR/Cas9 complex to the zygotes of the hamster, the embryo handling conditions, embryo transfer procedures, and husbandry required to produce genetically modified hamsters.

INTRODUCTION:
The golden Syrian hamster (*Mesocricetus auratus*) is one of the most widely used rodents for biomedical research. According to the U.S. Department of Agriculture, approximately 100,000 hamsters were used in the United States in 2015, representing 13% of total laboratory animal

usage among the species covered by the Animal Welfare Act (http://www.aphis.usda.gov; accessed March 10, 2017).

The hamster offers several advantages over other rodents in the study of a number of human diseases. For example, the histopathology of N-nitrosobis(2-oxopropyl)amine (BOP) induced pancreatic ductal adenocarcinomas in hamsters is similar to human pancreatic tumors, while BOP treatment mainly induces thyroid gland tumors in rats and lung and liver tumors in mice[1]. Because hamsters are the only small rodent found to support the replication of adenoviruses, they are also the model of choice for testing adenovirus-based oncolytic vectors and anti-adenovirus drugs[2-4]. Another example wherein the hamster model offers an advantage over mice and rats is in the study of hyperlipidemia. Humans and hamsters exhibit great similarities in lipid metabolic pathways and both species carry the gene encoding cholesteryl ester transfer protein (CETP), which plays a central role in lipid metabolisms, while CETP is absent in mice and rats[5]. Additionally, hamsters develop hemorrhagic disease more representative of the human manifestation following exposure to Ebola virus[6]. Hamsters are also the models of choice for studying atherosclerosis[7], oral carcinomas[8], and inflammatory myopathies[9]. Recently, it has also been demonstrated that hamsters are highly susceptible to Andes virus infection and develop hantavirus pulmonary syndrome-like disease, providing the only rodent model of Andes virus infection[10].

To address the unmet need for novel genetic animal models to study the human diseases where no reliable small rodent model is available, we recently have succeeded in applying the CRISPR/Cas9 system to the hamster and have produced several lines of genetically engineered hamsters[11]. Hamster zygotes are highly sensitive to environmental milieus such that the PN injection protocols developed in other species are unsuitable. Therefore, we developed a PN injection protocol for the hamster that accommodates the special requirements for handling hamster embryos *in vitro*. Here, we describe the detailed PN injection procedure using the CRISPR/Cas9 system and the accompanying steps, from the preparation of single guide RNA (sgRNA) to the transfer of injected embryos into recipient females.

PROTOCOL:

The procedures described in this protocol were approved by the Institutional Animal Care and Use Committee (IACUC) of Utah State University (IACUC protocol: 2484). Hamsters used in this protocol are adult (6-10 weeks of age) LVG strain golden Syrian hamsters. All hamsters are housed in the vivarium at the Bioinnovation center, Utah State University. Room temperature is set at 23 ^{o}C, humidity is set at 40-50%, and light cycle is set 14L:10D (light:dark).

1. sgRNA and Cas9/sgRNA Ribonucleoproteins (RNP) Preparation

1.1) Synthesize sgRNAs following the *in vitro* transcription procedures described in the manual of synthesis kit (**Table 1, Supplement 1**).

1.2) To assemble ribonucleoproteins, mix 2 µg of Cas9 with 1 µg of sgRNA and incubate at room temperature for 10-15 min. For PN injection, make a working solution at the concentration of 100 ng/µL Cas9 and 50 ng/µL sgRNA with 10mM TE buffer (RNase free, **Table 1**).

2. Vasectomy Preparation

Note: Vasectomy is performed on male hamsters at 6-8 weeks of age. The surgery should be performed 10-14 days ahead of the first mating. Sterility is confirmed by failed pregnancies from mating with fertile females. Vasectomized males can normally be used for a year before they become less sexually active.

2.1) Anesthetize males by intraperitoneal (i.p.) injection of ketamine/xylazine with a dose of 40 mg/kg (ketamine) and 10 mg/kg (xylazine). Confirm anesthesia by lack of a pedal reflex (toe pinch).

2.2) Lay the sedated hamster in dorsal recumbency on a dry tissue paper. Rinse the abdomen with three rounds of antiseptic treatments by alternating surgical scrub solutions between 70% alcohol and povidone-iodine.

2.3) Make a vertical incision with surgical scissors starting 1.5 cm cranial to the prepuce extending 1 cm to each side from mid line (**Figure 1a**). Incise the skin, subcutaneous tissue, and linea alba to access the peritoneal space (**Figure 1b**).

2.4) Apply gentle pressure to the caudal aspect of the scrotum to force the testes and fat tissue out. Grasp the vas deferens gently with forceps and carefully separate the blood vessel from the vas with a second set of forceps (**Figure 1c**).

2.5) Hold the vas deferens with forceps to form a loop. Heat the tip of another forceps in flame until it is red, and then use it to remove the loop of the vas deferens. Insert fat tissue and testes back to abdomen. Remove the vas deferens on the opposing side with the same procedures.

2.6) Suture the musculature first followed by suturing the skin. Place the animal on a warm pad in a cage for 30 min until the animal recovers. Observe the animal until it resumes normal activity.

3. Donor/Recipient Hamster Preparation Schedule

3.1) Monitor and record estrous cycles.

Note: Female hamsters have a stable 4-days estrous cycle that is maintained immediately after parturition. Females on the first day of estrous can be identified by the opaque, yellowish and sticky vaginal discharge (**Figure 2a**). Females on Day 4 of estrous can be identified by the transparent, sticky mucus (**Figure 2b**).

3.2) For superovulation of donor hamster, superovulate the sexually mature females (>6 weeks) on the first day of the estrous cycle by IP injection of pregnant mare's serum gonadotropin (PMSG; dissolved in PBS as 50 IU/mL) (**Table 2**) between 9-12 AM.

Note: PMSG is for inducing superovulation but not for estrous cycle synchronization.

3.3) 80-84 h after injection of PMSG (Day 4, 6-8 PM), place the females in cages with males for mating. As hamster matings do not result in copulation plugs, ensure successful matings by watching the matings.

3.4) Prepare pseudopregnant females by mating sexually mature females on Day 4 of estrous with vasectomized males. The time schedule for preparing donor and recipient females are shown in **Table 3**.

Note: True pregnant females can also be used as recipients, i.e., females with a coat color distinguishable from the golden color matted with the same color fertile males. Pups derived from embryo transfer can be identified based on coat colors. A potential advantage in using true pregnant females is that endogenously produced embryos would ensure successful pregnancies and help PN injected embryos to implant; a potential drawback in using true pregnant females as recipients is that PN injected embryos need to compete with endogenously produced embryos for implantation.

4. Zygote Isolation

4.1) Prepare culture medium (HECM-9; recipe is described in Supplement 2), as described previously in McKiernan and Bavister[12].

4.2) One day prior to PN injection, prepare zygote handling dishes (25 µL/drop) and a PN injection drop (100 µL) in the lid of a 35 mm dish with HECM-9. Then cover the medium drops with mineral oil. Balance medium in an incubator overnight under the following conditions: 37.5 ^{o}C, 10% CO_2, 5% O_2, 85% N_2 and 100% humidity.

4.3) On the day scheduled for PN injection, prepare embryo flushing dishes (1 dish/donor hamster) with HECM-9 and store all dishes in the incubator until use.

4.4) Euthanize superovulated hamsters with CO_2.

4.5) Oviduct Isolation

4.5.1) Place a euthanized female hamster in dorsal recumbency on a surgical drape or tissue paper and prepare the abdomen with a 70% ethanol spray.

4.5.2) Firmly hold skin and abdominal muscle layer at the midline and make an incision. Incise the peritoneum to expose the abdominal organs.

4.5.3) Grasp one of the uterine horns with a fine forceps and retract the tissue from the abdomen. Separate the uterus from the mesometrium and fat tissue. Make a cut between the oviduct and ovary and a second cut adjacent to the junction of the oviduct and uterus (include a small part of uterus). Place the two oviducts in the same flush dish.

4.6) Flush and collect zygotes

4.6.1) Under the dissection microscope, grasp the side of uterine horn with a #5 Dumont forceps and insert a homemade needle (**Figure 3**) into the lumen of the oviduct from the infundibular end and flush the oviduct with 300-400 µL of HECM-9 medium.

4.6.2) Collect the zygotes immediately and wash them twice with HECM-9 medium in the handling dish. At this stage of development, all of the zygotes should be denuded from cumulus cells.

4.6.3) Place the zygotes in an incubator (37.5 ^{o}C, 10% CO_2, 5% O_2, 85% N_2 and 100% humidity) immediately after collection to minimize exposure to light .

5. PN Injection

5.1) Thaw the Cas9/sgRNA RNP on ice and maintain the complex on ice during the entire PN injection process.

5.2) Load the injection needles with Cas9/sgRNA RNP solution immediately before the injection experiments. Place the rear end of the injection needles into the tube containing the Cas9/sgRNA RNP solution and allow the Cas9/sgRNA RNP solution to fill the needle by capillary action.

5.3) Prepare the injection dish and needles. Fill the holder pipette with mineral oil to within ~3mm of the bend of the holder needle. Place the holder in the microinjector with the tip of the needle horizontal to the bottom of the dish and fill the tip of the needle with medium by suction. Set the injection needle at a 10-15^{o} angle opposite to holder.

5.4) PN injection

5.4.1) Identify a zygote and apply a fine suction with the holder needle. Ideally, the male and female pronuclei are within the same focal range and aligned parallel to holder.

5.4.2) Penetrate the zona and male pronuclei (3 o'clock position) with the injection needle.

5.4.3) Withdraw the injection needle quickly once the pronucleus swells to prevent the nucleus from adhering to the needle and to avoid damaging the pronucleus.

6. Zygotes Transfer to Pseudopregnant Hamsters

6.1) Anesthetize a pseudopregnant hamster (the same way as described in the vasectomy section above) and lay it on a surgical drape or dry tissue paper in ventral recumbency.

6.2) Cover the head with a cloth or paper to protect the eyes from light. Shave a proper skin area for incision on both sides. Prepare the lateral aspect of the body with 3 times of povidone-iodine scrubs and 3 times 70% ethanol scrubs. Make a 2 cm vertical incision 2 cm caudal to the last rib (as shown in **Figures 4a, 4b**) on each side of the back of the animal.

6.3) Grasp the fat pad with forceps and pull gently until the oviduct and uterus are visible. Clamp the fat pad with a hemostats and reflect the fat pad dorsally over the spine (**Figure 4c**).

Position the reproductive tract such that the uterine tube can be penetrated with a 30 gauge needle for embryo transfer (**Figure 4d**).

6.4) Wash the injected zygotes with HECM-9 in a new dish. Use a new glass pipette (diameter: ~150-200 µm) to load 10-15 zygotes. Arrange the zygotes as a chain of pearls followed by several air bubbles. Insert the pipette into the open penetrated tube from step 6.3 and gently blow into the pipette to transfer the zygotes to the oviduct. Continue blowing until the first air bubble is released into the oviduct tract (**Figure 4e**).

6.5) Release the fat pad and return the tissue to the abdomen. Transfer approximately the same number (10-15) of injected embryos to another oviduct tract by the same processes.

6.6) Suture the musculature and the skin in 2 layers. Return the hamster to a cage and place the cage on a warm pad for recovery. Return the cage to the animal room when the animal fully recovered from anesthesia.

REPRESENTATIVE RESULTS:

The efficiency of the described protocol in producing genetically modified hamsters depends on the outcomes of the following two critical steps: the live birth rate of recipient females and the number of live pups with the intended genetic modifications. The live birth rate is a direct results of the embryo quality and the skill of the individual performing the PN injection and embryo transfer procedures. To ensure that the developmental potential of manipulated embryos is not compromised, great care is necessary during the *in vitro* handling of the hamster embryos. We regularly achieve a live birth rate of 60-80% with pseudopregnant recipient females. **Table 4** illustrates live birth rates from the *RAG1* knockout experiment (pseudopregnant females were used) and the *STAT2* knockout experiment (true pregnant black females were used; live birth rate in this case was calculated as the percentage of litters produced golden pups).

The percentage of genetically modified pups produced from PN injected embryos depends on both the efficiency of sgRNA in introducing indels and the successful injection of the Cas9/sgRNA ribonucleoproteins into the pronuclei. We found that sgRNA efficiency varies among gene targets (unpublished observations). As shown in **Table 4**, the sgRNA designed for *STAT2* resulted in a gene targeting efficiency of 88.9%, while the sgRNA for *RAG1* was only 28.6% efficient. **Figure 5** provides an example of the genotyping results from a PCR restriction fragment length polymorphism (PCR-RFLP) assay of pups from *RAG1* gene targeting. It is important to note that some indels may occur outside of the restriction enzyme recognition sequence, such that PCR-RFLP may underestimate the gene targeting efficiency. Subcloning the PCR products into TA cloning vectors followed by Sanger sequencing of the PCR inserts are necessary to accurately measure gene targeting efficiency and to reveal the nature of indels.

Figure 1: Male hamster vasectomy.

a) and b) Make a 2 cm vertical incision beginning 1.5 cm cranial to the prepuce extending cranially; c) Separate the vas deference from the associated testicular vein and artery with two pairs of forceps; d) Grasp the vas deferens with a forceps to form a 1 cm loop. Heat a second set of forceps and excise the loop while cauterizing the ends concurrently.

Figure 2: Estrous cycle.
a) The vaginal discharge observed on Day 1 is opaque, yellowish, and sticky b) The vaginal discharge observable on Day 4 is clear and sticky.

Figure 3: Preparation of the needle for embryo isolation.
a) Fracture a 30 gauge needle along the red line and polish the tip until it is flat and smooth. b) make a 30-40^{o} angle at ~3 mm from the tip.

Figure 4: Zygote transfer.
a) and b) Make a 2 cm long vertical incision starting 2 cm caudal to the last rib (red line); c) Clamp the fat pad with a Hemostats and reflect the tissue dorsally; d) Adjust the position of oviduct tract and penetrate an open with a 30 gauge needle; e) Arrange the zygotes as a chain of pearls within the embryo transfer glass pipette and transfer them into the oviduct from the penetrated open. Bar=1000 µm.

Figure 5. PCR-RFLP assay of a single litter of founder animals with *RAG1* indels. M: 1 Kb Plus ladder. WT: wild type. ID 1, 3 and 7 show uncut band consistent with *RAG1* indels.

DISCUSSION:
To better exploit the potential of golden Syrian hamsters as models of human disease, we developed a PN injection protocol for delivering a CRISPR/Cas9 complex to target the hamster genome. The PN injection protocol optimizes several key variables including the embryo culture medium, temperature, and wavelengths of light[13]. There are also several hamster-specific animal handling procedures that need to follow for successfully conducting gene targeting in the hamster. For example, sexually matured female hamsters have stable 4-day estrous cycles that cannot be synchronized with exogenous hormones (e.g. PMSG). Thus accurately tracking the estrous cycles of each female is important for both superovulation and pseudopregnancy preparation. 30-60 zygotes can be produced from a female if she is successfully superovulated. To achieve successful superovulation, it is necessary to consider species-specific deposition of fat. Female hamsters, especially those over 12 weeks of age, tend to have excessive abdominal fat such that the syringe needle must be positioned correctly to fully penetrate the fat pad when performing hormone injections. We have found that penetration half-way into the peritoneal cavity achieves the appropriate distance for hormone delivery. We summarized the unique requirements for PN injection, hamster handling and husbandry in **Table 5**.

For PN injection, the number of embryos transferred to the injection dish for each round of injection must be determined experimentally to avoid extended injection time. The longer the time the embryos remain in the dish, the greater the chance that they experience overexposure to light. We recommend transferring approximately 15 embryos to the injection dish for each round of injections. This number achieves a good balance between the number of rounds of injections and the tolerable exposure of the embryos to light. As both of the cytoplasmic and pronuclear membranes of hamster embryos are quite flexible, substantial force must be applied to the injection needle to penetrate the membrane of the pronucleus. During this time, the pronuclei must remain within the focal range.

Researchers should consider the following surgical issues when performing embryo transfers. First, it is important that the incisions through the body wall correspond to the body size of the animal. If the incision is too small, it may result in extrusion of the zygotes from oviduct when returning the reproductive tract to the abdomen. In addition to incision size, the potential for extrusion of zygotes from the oviduct can be minimized by handling the associated fat pad rather than the reproductive tissue proper. In regard to incision size, it is also important to consider that larger incisions may cause more stress to the animal and exhibit a higher probability of complications (e.g. infection dehiscence). Second, to minimize bleeding, researchers should take care to avoid incising major blood vessels when accessing the abdomen because excess blood loss can increase surgery stress to reduce the success of embryo transfer, and extend the recovery time.

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DISCLOSURES:
ZW has financial interests in Auratus Bio, LLC., a biotechnology company specialized in creating genetically engineered animals for biomedical research and agricultural applications.

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Figure 1

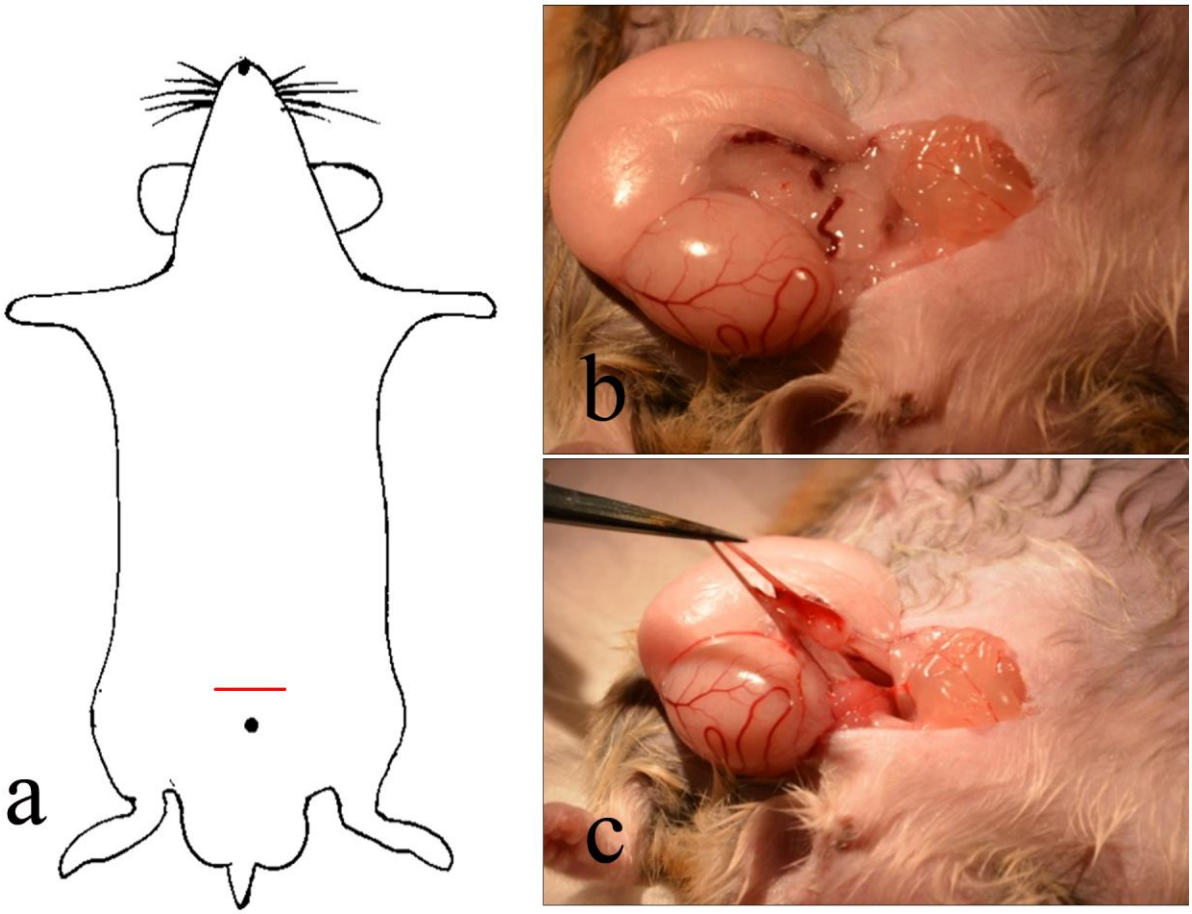

Figure 2

Figure 3

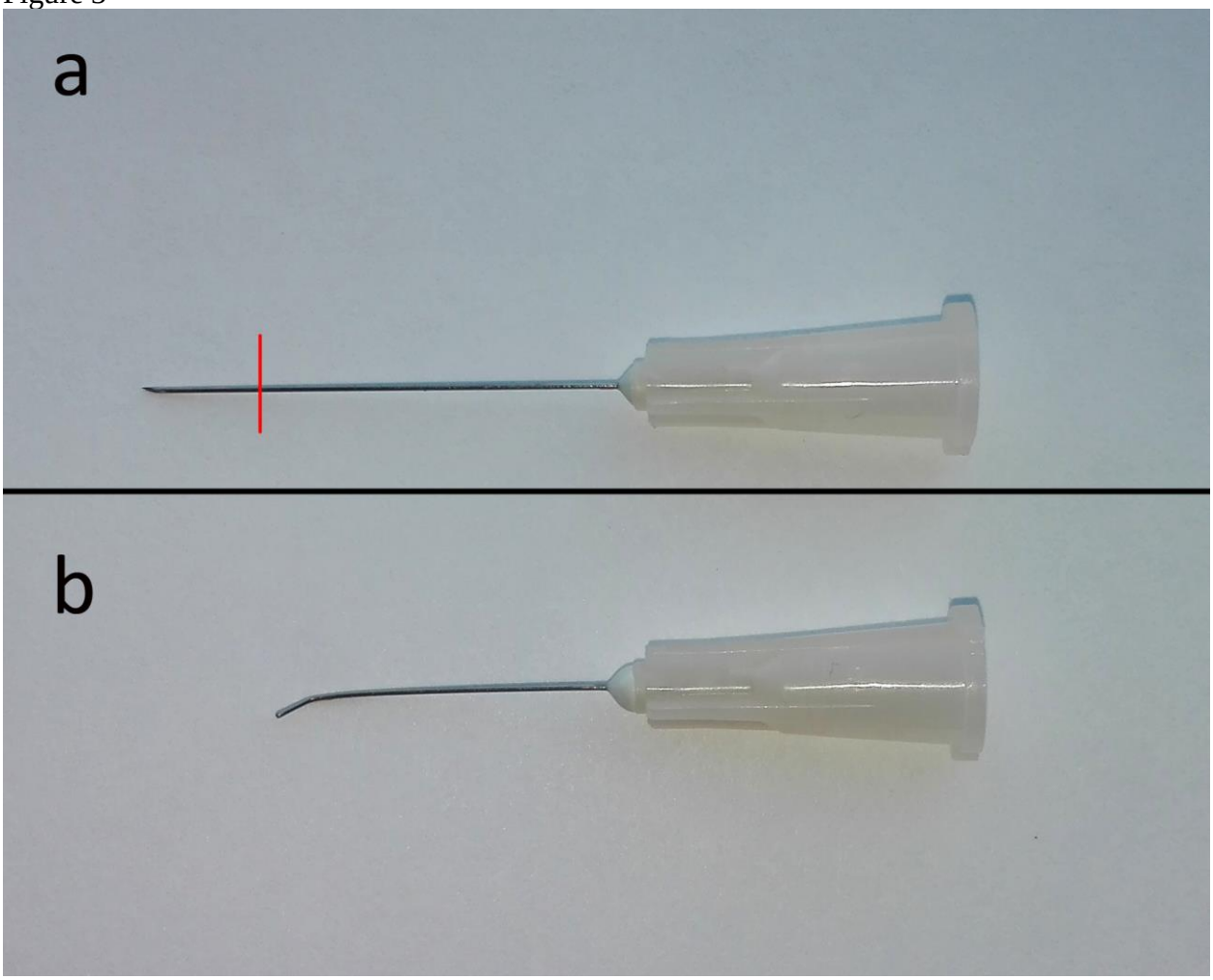

Figure 4

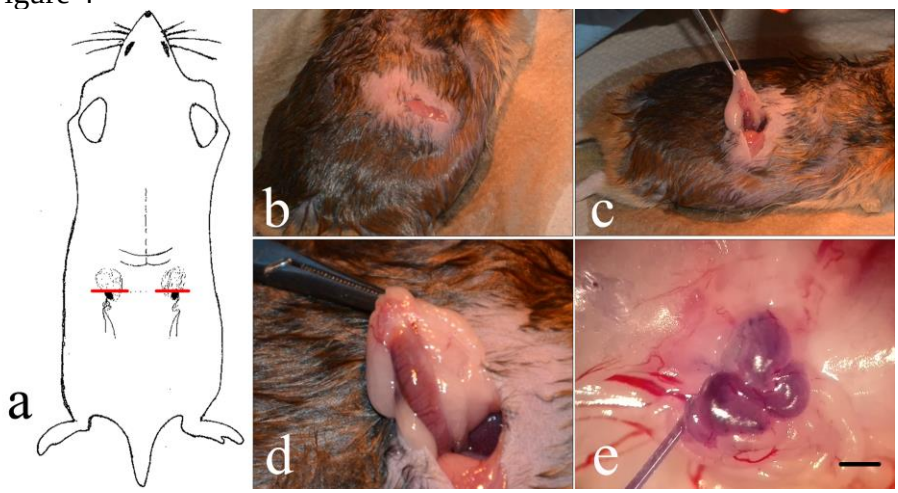

Figure 5

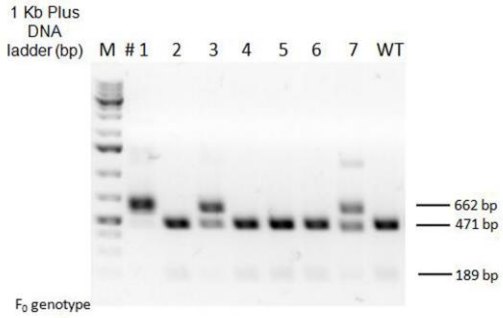
1 Kb Plus DNA ladder (bp)
M
#1
2
3
4
5
6
7
WT
662 bp
471 bp
189 bp
F0 genotype

Table 1. Key materials/equipments used in the protocol			
Materials/Equipments	**Company**	**Catalog number**	**Comments/Description**
Cas9	Invitrogen	B25640	1 ug/ul (~6.1 uM)
GeneArtTM Precision Synthesis Kit	Invitrogen	A29377	For sgRNA synthesis
Albumin from human serum	Sigma	A1653	For cultivation medium
Illuminator	Nikon	NI-150	For embryo transfer
Incubator	New Brunswick	Galaxy 14S	For embryo cultivation
Microforge	Narishige	PB-7	For making injection needles
Microscope	Nikon	ECLIPSE Ti-S	For microinjection
Microscope	invitrogen	SMZ745T	For embryo transfer
Mineral oil	Sigma	M1840	Keep in dark
PMSG	Sigma	G4877-2000IU	For superovulation

Table 2. PMSG does corresponding to body weights		
Body weight (g)	**PMSG (IU)**	**PMSG (ul)**
<110	10	200
110-135	15	300
135-160	20	400
160-185	25	500
>185	30	600
PMSG, pregnant mare’s serum gonadatropin		

Table 3. Time schedule of donor/recipient preparation					
Hamster	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Donor	PMSG (9-12 am)			Mating (6-8 pm)	Zygote isolation (12-1 pm) PN injection (1-3 pm)
Recipient				Mating (6-8 pm)	Embryo transfer (3-5 pm)

Table 4. Efficiency of gene targeting in golden Syrian hamster by the Cas9/sgRNA system			
Genes	**No. Embryos (Pups)**	**No. Litters (Recipients)**	**No. Positive pups (%)**
STAT2	229 (54)	14 (19)*	48 (88.9)
RAG1	77 (21)	3 (3)	6 (28.6)

* females matted with fertile males were used; the number of litters indicated is only those produced golden pups.

Table 5. Unique requirements on hamster embryo handling and husbandry		
Condition/Handling	**Requirements**	**Comments**
Ambient light	Turn off all ambient light	Hamster embryos are sensitive to light, especially to cool light
Light during in vitro handling	Less than 20 min for PN injection	Manipulate 15 embryos per round to reduce light exposure
Temperature	Ambient temperature: 28±0.5 ^{o}C Handling temperature: 37.5 ^{o}C	Hamster embryos are sensitive to temperature fluctuations
Medium	HECM-9	Do not store more than 3 days
Culture condition	37.5 ^{o}C, 10% CO_2, 5% O_2 and 100% humidity	Balanced overnight in incubator
Husbandry after embryo transfer	Don't change cage within 5 days before/after due date	Recipient will cannibalize pups if disturbed; provide enough feed/water

Supplementary 1

Synthesize sgRNA by GeneArt Precision Synthesis Kit

1. Design and synthesize a pair of target forward (F) and reverse (R) primers based on the USER GUIDE.
2. Prepare a 0.3 μM target oligonucleotide mix working solution in nuclease-free water.
3. Set up the PCR assembly reaction:

Phusion High-Fidelity PCR Master Mix (2X)	12.5 μL
Tracr Fragment + T7 Primer Mix	1 μL
0.3 μM Target F/R oligonucleotide mix	1 μL
Nuclease-free water	10.5 μL

4. Perform assembly PCR using the cycling parameters below.

Cycle step	Temperature	Time	Cycles
Initial denaturation	98°C	10 seconds	1X
Denaturation	98°C	5 seconds	32X
Annealing	55°C	15 seconds	
Final extension	72°C	1 minute	1X
Hold	4°C	Hold*	1X

Perform in vitro transcription

5. Set up the following in vitro transcription reaction, adding the reaction components in the order given.

NTP mix (100 mM each of ATP, GTP, CTP, UTP)	8 μL
gRNA DNA template (from PCR assembly, page 13)	6 μL
5X TranscriptAid™ Reaction Buffer	4 μL
TranscriptAid Enzyme Mix	2 μL

6. Incubate at 37°C for 2–3 hours.
7. Add 1 μL of DNase I into the reaction mix after the transcription reaction and incubate at 37°C for 15 minutes.

Purify in vitro transcribed (IVT) gRNA

8. Adjust the volume of the IVT reaction to 200 μL with nuclease-free water.
9. Add 100 μL of Binding Buffer. Mix thoroughly by pipetting.
10. Add 300 μL of ethanol (>96%) and mix by pipetting.
11. Transfer the mixture to the GeneJET RNA Purification Micro Column and centrifuge for 30–60 seconds at 14,000 × g. Discard the flow-through.

12. Add 700 µL Wash Buffer 1(diluted with 13 mL of >96% ethanol) and centrifuge for 30–60 seconds at 14,000 × g. Discard the flow-through.
13. Add 700 µL Wash Buffer 2 (diluted with 30 mL of >96% ethanol) centrifuge for 30–60 seconds at 14,000 × g. Discard the flow-through and repeat.
14. Centrifuge the empty purification column for an additional 60 seconds at 14,000 × g to completely remove any residual Wash Buffer and transfer the purification column to a clean 1.5-mL collection tube.
15. Add 10 µL of nuclease-free water to the center of the purification column filter, and centrifuge for 60 seconds at 14,000 × g to elute the gRNA.

Supplementary 2

Hamster Embryo culture Medium-9 (HECM-9) recipe

Components	mMol/L
Human Serum Albumin (HAS)	0.5mg/ml
NaCl	113.6
KCl	3.0
$CaCl_2$	1.9
$MgCl_2$	0.5
$NaHCO_3$	25.0
DL-Na-lactate	4.5
HCL 1mol/L	1.4 µl/ml
Taurine	0.50
Asparagine	0.01
Cysteine	0.01
Histidine	0.01
Lysine	0.01
Proline	0.01
Serine	0.01
Aspartic acid	0.01
Glycine	0.01
Glutamic acid	0.01
Glutamine	0.20
Pantothenate	0.003

Note: Inorganic salt solutions are prepared by dissolving the salts in Milli-Q water and can be kept at 4 ^oC for up to 3 days. Amino acids, vitamins and HSA are prepared as x100 stocks and are stored at -20 ^oC. HECM-9 is prepared by adding the stock solutions of amino acids, vitamins and HSA. HECM-9 should not be stored more than 3 days. The medium should be balanced in an atmosphere of 37 ^oC, 10% CO_2, 5% O_2, 85% N_2 overnight before use.