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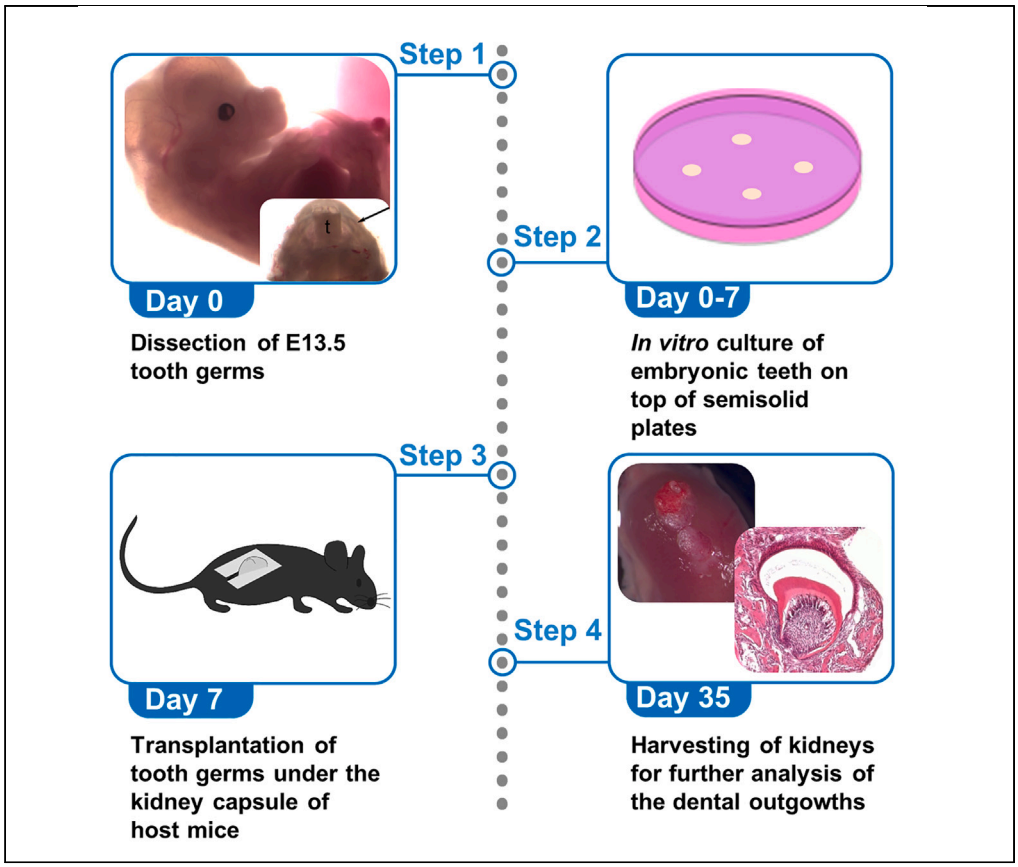
Kidney capsule transplantation of chemically treated embryonic murine tooth germs



Subcapsular transplantation of developing tissues and organs into the richly vascularized murine kidney provides the necessary trophic support, thus ensuring proper completion of their growth. Here, we provide a protocol for kidney capsule transplantation that allows the full differentiation of embryonic teeth previously exposed to chemicals. We describe steps for dissection and *in vitro* culture of embryonic teeth, followed by transplantation of tooth germs. We then detail harvesting of kidneys for further analysis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Dissection of mouse embryonic teeth

In vitro chemical treatment of embryonic tooth germs growing on top of semisolid plates

Transplantation of *in vitro* cultured teeth under the murine kidney capsule

Analysis of the transplanted teeth at terminal differentiation stages

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Kidney capsule transplantation of chemically treated embryonic murine tooth germs

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SUMMARY

Subcapsular transplantation of developing tissues and organs into the richly vascularized murine kidney provides the necessary trophic support, thus ensuring proper completion of their growth.[1–3] Here, we provide a protocol for kidney capsule transplantation that allows the full differentiation of embryonic teeth previously exposed to chemicals. We describe steps for dissection and *in vitro* culture of embryonic teeth, followed by transplantation of tooth germs. We then detail harvesting of kidneys for further analysis.

For complete details on the use and execution of this protocol, please refer to Mitsiadis et al.[4]

BEFORE YOU BEGIN

Institutional permissions (if applicable)

Before performing kidney capsule transplantation assays, permissions from the relevant institutions are required. In our case, all mice were maintained and handled according to the Swiss Animal Welfare Law and in compliance with the regulations of the Cantonal Veterinary office, Zurich (License for animal experimentation ZH151/2014; ZH146/17; ZH018/17). The animal facility provided standardized housing conditions, with a mean room temperature of 21 ± 1°C, relative humidity of 50% ± 5%, and 15 complete changes of filtered air per hour (HEPA H 14filter); air pressure was controlled at 50 Pa. The light/dark cycle in the animal rooms was set to a 12 h/12 h cycle (lights on at 07:00, lights off at 19:00) with artificial light of approximately 40 Lux in the cage. The animals had unrestricted access to sterilized drinking water, and ad libitum access to a pelleted and extruded mouse diet in the food hopper (Kliba No. 3436; Provimi Kliba / Granovit AG, Kaiseraugst, Switzerland). Mice were housed in a barrier-protected specific pathogen-free unit and were kept in groups of max. 5 adult mice per cage in standard IVC cages (Allentown Mouse 500; 194 mm × 181 mm × 398 mm, floor area 500 cm^2; Allentown, New Jersey, USA) with autoclaved dust-free poplar bedding (JRS GmbH + Co KG, Rosenberg, Germany). A standard cardboard house (Ketchum Manufacturing, Brockville, Canada) served as a shelter, and tissue papers were provided as nesting material. Additionally, crinklets (SAFE® crinklets natural, JRS GmbH + Co KG, Rosenberg, Germany) were provided as enrichment and further nesting material. The specific pathogen-free status of the animals was monitored frequently and confirmed according to FELASA guidelines by a sentinel program. The mice were free of all viral, bacterial, and parasitic pathogens listed in FELASA recommendations.[5]

Preparation of semisolid plates

Timing: 2–3 h

1. Prepare growth medium (DMEM/F12, 20% FBS, 1% penicillin/streptomycin, 1.8 mg/mL ascorbic acid). It is recommended to use fresh growth medium. It could be also stored at 4°C for up to one week.
2. Add 1.8 mL of growth medium to a plastic Petri dish (35 mm), and place it onto a hot plate (at around 55°C).
3. Prepare agarose at 5% by mixing 0.5 gr of agarose with 10 mL of distilled water in a previously sterilized 50 mL Erlenmeyer flask.
4. Heat the 5% agarose in the microwave until the solution is well dissolved.

Note: Make sure that the agarose solution does not overflow during the heating.

5. Add 200 μL of 5% agarose to the pre-warmed growth medium and mix well.

Note: The growth medium will be pre-warming from step 2 to step 5, which is estimated to take approximately 10–15 mins.

Note: When adding the dense agarose solution, cutting pipette tip is recommended.

Note: Mixing can be done with a cell spreader or a flame-bent glass pipette.

Scheduling the surgery

Timing: 3–4 h, 1 week

6. The day of the surgery should be scheduled based on the availability of:
   a. Host mice (RAG1$^{-/-}$ immunocompromised mice).
      i. Immunocompromised mice can be purchased or bred in the laboratory.

Note: It is recommended to perform the surgeries on host mice of the same gender. The election of the gender can have an impact of the successful growth of the graft if sex hormones influence the development of donor tissues.

   b. Pregnant mice from which embryos will be obtained (C57BL/6J mice).
      i. Breeding will be set up and once confirmed the presence of a plug, the day of the surgery can be scheduled.

Note: It is recommended to get a total of 2–3 plugged female mice on 2–3 consecutive days in order to ensure at least one litter to isolate tooth germs.

      ii. Given that tooth germs will be isolated from embryonic day 13.5 (E13.5) mouse embryos and then cultured for 7 days *in vitro*, the surgery can be performed 21 days after the day of the plug (E0.5).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
GI254023X	Sigma-Aldrich	Cat# SML0789
DMSO	Sigma-Aldrich	Cat# D2650

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Agarose D1 Low EEO	Condalab	Cat# 8010
L-Ascorbic acid	Sigma-Aldrich	Cat# A4544
L-Glutamine (200 mM)	Gibco	Cat# 25030081
Penicillin-Streptomycin	Gibco	Cat# 15140122
Fetal bovine serum	Gibco	Cat# 26140079
DMEM/F12, HEPES	Gibco	Cat# 31330038
PBS	Gibco	Cat# 20012027
Experimental models: Organisms/strains		
Mouse: C57BL/6J; 8–12 weeks old pregnant females; E13.5 embryos (both genders)	The Jackson Laboratory	RRID:IMSR_JAX:000664
Mouse: B6.129S7-*Rag1*^{tm1Mom}/J; 8-week-old males	The Jackson Laboratory	RRID:IMSR_JAX:002216

MATERIALS AND EQUIPMENT

Growth medium for semisolid plates		
Reagent	Final concentration	Amount
Vitamin C (L-ascorbic acid) (18 mg/mL)	0,18 mg/mL	100 µL
L-Glutamine (100×; 200 mM)	1×	100 µL
Penicillin-Streptomycin (100×; 10,000 units penicillin and 10 mg/mL streptomycin)	1×	100 µL
Fetal Bovine Serum	20%	2 mL
DMEM/F12	N/A	7 mL
Total	**N/A**	**10 mL**

Alternatives: DMEM/F12 can be substituted by other media such as RPMI 1640.

STEP-BY-STEP METHOD DETAILS

Dissection of embryonic mouse teeth from the lower jaw

Timing: 3–4 h

In this step, developing tooth germs will be dissected from E13.5 mouse embryos.

1. Sacrifice the pregnant mouse at embryonic day (E) 13.5.
2. Dissect out the uterus; place it in a plastic Petri dish with cold phosphate buffered saline (PBS) (Figure 1A).

Note: We strongly suggest to store the PBS solution that will be used in this step at 4°C (for up to one month) to slow down the degradation of the dissected tissues.

Note: Petri dish with the embryos should be kept on ice.

3. Separate the embryos by cutting the uterus between the implantation sites with scissors (Figure 1B).
4. Transfer one embryo into a Petri dish with cold PBS and remove the uterus and the decidua by using forceps (Figure 1C).
5. Using needles separate the head from the body (Figure 1D).
6. Transfer the isolated head to a glass Petri dish with cold PBS (Figure 1E).
7. Separate the upper and lower jaws (Figures 1F and 1G).

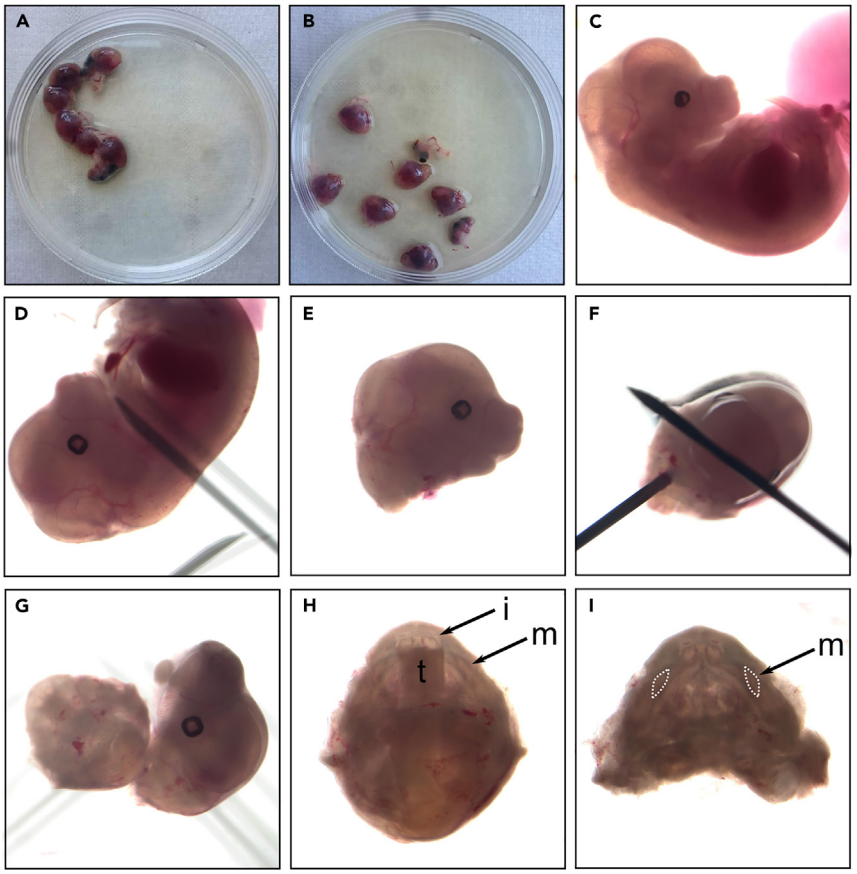

Figure 1. Dissection of tooth germs from the lower jaw of E13.5 embryos

(A-G) See description in dissection of embryonic mouse teeth from the lower jaw. m, molars (in H and I); t, tongue (in H).

8. Remove the tongue from the lower jaw in order to visualize tooth germs (Figure 1H).
9. Dissect developing tooth germs (Figure 1I; as described in Pagella et al.[6]) and place them in another glass Petri dish with cold PBS.

In vitro culture of embryonic tooth germs

Once dissected, embryonic teeth can be *in vitro* treated with the aim of studying the effect of the chemical disruption of a given molecule and/or signaling pathway on tooth development.[4] In our study, we aimed to study the effect of Adam10 chemical inhibition upon tooth development.[4] In order to do that, we placed dissected tooth germs on top of semisolid medium containing either the chemical inhibitor GI254023X (5μM) or vehicle (DMSO). At early developmental stages, at least 6 tooth germs can be placed in each Petri dish (plastic, 35 mm) (Figure 2A).

10. Transfer the embryonic teeth from the cold PBS in the glass Petri dish to the surface of the semisolid medium in the plastic Petri dish.
    a. With the help of a pipette (yellow tip) take one tooth at a time and place them on top of the semisolid medium.
    b. Once all the teeth are placed in the Petri dish, remove the remaining liquid around each of them.
    c. Incubate them at 37°C until they reach the desired developmental stage (7 days in our experiment).

Note: Developing teeth should be transferred to a fresh semisolid medium every 2–3 days.

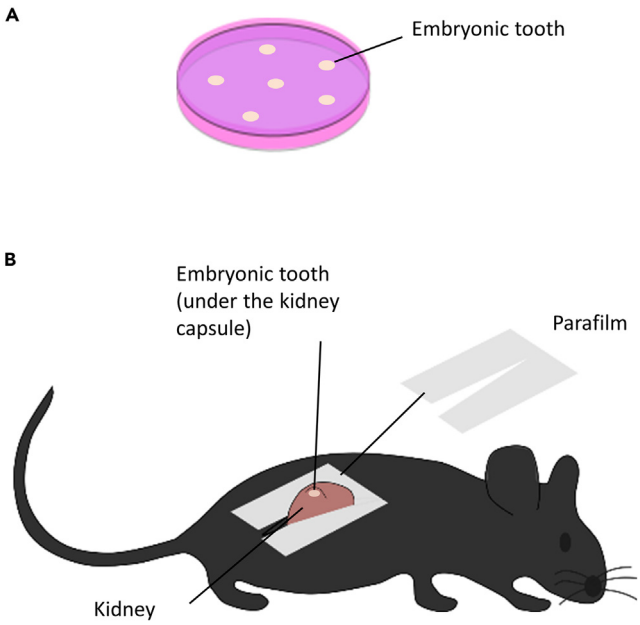

Figure 2. Schematic representation of the transplantation of embryonic teeth under the kidney capsule

Schematic representation of embryonic tooth germs growing on top of semisolid plates (A) and their subsequent transplantation under the murine kidney capsule (B).

Transplantation of embryonic teeth under the murine kidney capsule

Timing: 3–4 h

In this step, the *in vitro* cultured tooth germs will be transplanted under the kidney capsule of the immunocompromised host mice.

11. Inject buprenorphine (0.1 mg/kg bodyweight) subcutaneously to the recipient mouse 1 h before the surgery.
12. Anesthetize the mice with the inhalation anesthesia (isoflurane) (see Note).
13. Apply vitamin A ointment to the eyes to prevent them from drying.
14. Put the mouse on one side, and make an incision that exposes one kidney.
15. Make a small incision to the kidney capsule by using forceps.
16. The capsule will be then lifted, and the embryonic tooth will be placed under the capsule in a way that the capsule keeps the graft in place.
17. The animals will be first sutured with absorbable polyglycolic acid suture, and the skin incision will be closed with a metal clip.
18. Put the mouse onto a warming pad and keep it under observation until it reaches consciousness.
19. Buprenorphine treatment should be applied the day of the surgery and until the third day after the surgery. Posture, stiches, and wound healing should be controlled during the days that follow the surgery. If signs of distress become apparent (poor appearance, inflammation at site of graft), the animal should be sacrificed before reaching the experimental endpoint.

Note: Isoflurane will be used at 4%–5% for induction and 1%–3% for maintenance.

Note: A piece of parafilm can be used to stabilize the kidney (Figure 2B). With the help of laboratory scissors, a linear cut should be made in the middle of a rectangular-shaped piece of parafilm (Figure 2B). Then, the parafilm should be placed in a way that the kidney stays

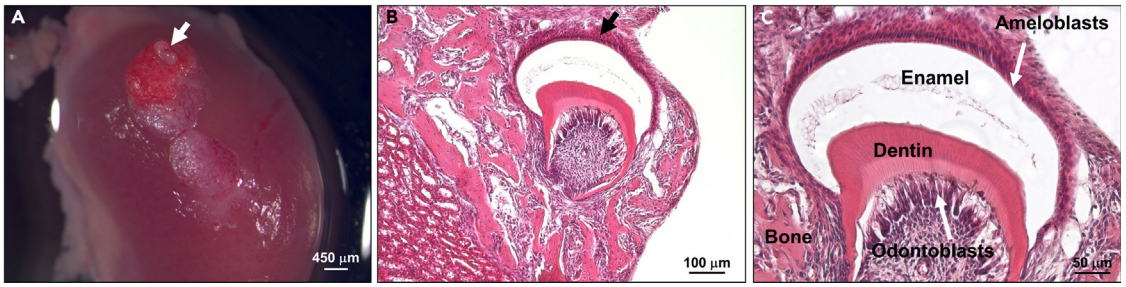

Figure 3. Development of embryonic tooth germs into mature teeth 4 weeks after being transplanted under the murine kidney capsule

(A) Analysis of the host kidney at the experimental termination point reveals the presence of a mineralized tooth (white arrow).

(B) Hematoxylin-eosin staining of histological sections after decalcification of the sample shows the presence of a tooth (black arrow) surrounded by trabecular bone.

(C) A magnification of the tooth shown in B reveals the presence of fully differentiated ameloblasts and odontoblast and the resulting mature enamel (observed mainly as a gap between dentin and ameloblasts due to the decalcification process) and dentin. Scale bars: 450 μm in A, 100 μm in B, 50 μm in C.

trapped in order to prevent that it moves back into the abdominal cavity during the transplantation.

Note: It is recommended to avoid drying of the surface of the kidney by adding sterile PBS.

EXPECTED OUTCOMES

Embryonically lethal mutations do not allow an extensive and comprehensive analysis of organs that have not yet completed their development. This stands true for the murine teeth that develop during the middle-late embryonic (tooth crown formation) and early postnatal stages (tooth root formation). Therefore, embryonically lethal mutations compromise the studies on the cellular and molecular effects caused by the mutated gene in dental tissues and, furthermore, restrict the thorough examination of the tooth-specific hard tissues (enamel, dentin, cementum). To overcome this problem, embryonic tooth germs extracted from mutant animals can be engrafted under the murine kidney capsule, where they can grow until the complete formation of both tooth crown and tooth root.

Indeed, transplantation of embryonic teeth under the murine kidney capsule allows them to complete their development. Terminal differentiation stages in which both odontoblasts and ameloblasts secrete dentin and enamel can be now achieved (Figure 3). The maturation stage of dental tissues at the time of the analysis depends on the *ex-vivo* culture time of the tooth explants. The experimental termination point is chosen based on the aim of the study. For example, short-term effects (cell proliferation, apoptosis, cell differentiation) upon drug or chemical treatment could be analyzed one to seven days post-transplantation.[4] Longer *ex-vivo* culture periods (4–8 weeks) are needed for the study of mature tissues and the thorough examination of dental hard tissues such as dentin and enamel. The regulatory effects of specific targeted molecules could thus be analyzed at a cellular (histology, electron microscopy) and molecular basis (immunohistochemistry, *in situ* hybridization, RT-PCR, bulk RNA sequencing).

LIMITATIONS

The successful rate of the kidney capsule transplantation of wild-type embryonic teeth depends on the experience of the researcher and the extension of the manipulation before the surgery. An experienced researcher can easily have a 100% successful rate. This rate may considerably decrease in cases of transplantation of tissues or organs that have been previously induced to pharmaceutical substances and/or extensively manipulated. For example, this can happen during tissue recombination experiments, dental tissue reconstruction experiments, etc. Concerning the present experimentation, the *in vitro* culture of the embryonic tooth germs, which proceeds their kidney capsule

transplantation (as showed in the present protocol), had not affected their successful outgrowth within the transplanted environment.

In addition, every operated animal was supervised and recorded in a post-operation animal health score sheet, which also included the inflammation signs during the wound healing process. We did not observe those type of complications after the kidney capsule experiment, most probably because mice were bred and operated in high hygienic standards established for the Animal Facility at the University of Zurich (UZH). It is worth noting that all mice with inflamed post-operatory wounds should be immediately sacrificed, according to the rules of the veterinary authorities of the UZH.

TROUBLESHOOTING

Problem 1

If the culture medium needed for the *in vitro* culture of embryonic teeth does not homogeneously solidify, teeth can sink during the incubation time instead of staying at the surface. This will result in the disrupted development or even degradation of the tissues/organs. To avoid this problem, preparation of semisolid plates (Step 5) will require special caution.

Potential solutions

- When adding the dense agarose solution, cutting pipette tip is recommended. This will ensure that the exact volume required is added.
- It is also crucial to spread the agarose well through the pre-warmed culture medium previously added to the plastic petri dish before the agarose starts to solidify.
- If the culture medium is not adequately pre-warmed, agarose will solidify immediately after being added and it will not be homogeneously spread.

Problem 2

The kidney moves back to the abdominal cavity during the surgery before the graft is correctly placed under the capsule (see Step 16 in Transplantation of embryonic teeth under the murine kidney capsule).

Potential solution

- A piece of parafilm can be used to stabilize the kidney (Figure 2B).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Thimios Mitsiadis (thimios.mitsiadis@zzm.uzh.ch).

Materials availability

This study did not use or generate new unique reagents.

Data and code availability

Data reported in this paper will be shared by the lead contact on request. This paper does not report original code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Performed experiments/data collection, L.J.R., Writing, Review, and Editing, L.J.R., T.A.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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