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Efficient Vascularization of Kidney Organoids through Intracelomic Transplantation in Chicken Embryos

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Abstract

Kidney organoids derived from human induced pluripotent stem cells contain nephron-like structures that resemble those in the adult kidney to a certain degree. Unfortunately, their clinical applicability is hampered by the lack of a functional vasculature and consequently limited maturation *in vitro*. The transplantation of kidney organoids in the celomic cavity of chicken embryos induces vascularization by perfused blood vessels, including the formation of glomerular capillaries, and enhances their maturation. This technique is very efficient, allowing for the transplantation and analysis of large numbers of organoids. This paper describes a detailed protocol for the intracelomic transplantation of kidney organoids in chicken embryos, followed by the injection of fluorescently labeled lectin to stain the perfused vasculature, and the collection of transplanted organoids for imaging analysis. This method can be used to induce and study organoid vascularization and maturation to find clues for enhancing these processes *in vitro* and improve disease modeling.

Introduction

Human induced pluripotent stem cell (hiPSC)-derived kidney organoids have been shown to have potential for developmental studies^{1,2,3,4}, toxicity screening^{5,6}, and disease modeling^{5,7,8,9,10,11,12,13}. However, their applicability for these and eventual clinical transplantation purposes is limited by the lack of a vascular network. During embryonic kidney development, podocytes, mesangial cells, and vascular endothelial cells (ECs) interact to form the intricate structure of the glomerulus. Without this interaction,

the glomerular filtration barrier, consisting of podocytes, the glomerular basement membrane (GBM), and ECs, cannot develop properly^{14,15,16}. Although kidney organoids *in vitro* do contain some ECs, these fail to form a proper vascular network and diminish over time¹⁷. It is therefore not surprising that the organoids remain immature. Transplantation in mice induces vascularization and maturation of the kidney organoids^{18,19,20,21}. Unfortunately, this is a labor-intensive

process that is unsuitable for the analysis of large numbers of organoids.

Chicken embryos have been used to study vascularization and development for over a century²². They are easily accessible, require low maintenance, lack a fully functional immune system, and can develop normally after opening the eggshell^{23,24,25,26}. The transplantation of organoids on their chorioallantoic membrane (CAM) has been shown to lead to vascularization²⁷. However, the duration of transplantation on the CAM, as well as the level of maturation of the graft, are limited by CAM formation, which takes until embryonic day 7 to complete. Therefore, a method was recently developed to efficiently vascularize and mature kidney organoids through intracelomic transplantation in chicken embryos²⁸. The celomic cavity of chicken embryos has been known since the 1930s to be a favorable environment for the differentiation of embryonic tissues^{29,30}. It can be accessed early in embryonic development and allows for relatively unlimited expansion of the graft in all directions.

This paper outlines a protocol for the transplantation of hiPSC-derived kidney organoids in the celomic cavity of day 4 chicken embryos. This method induces vascularization and enhanced maturation of the organoids within 8 days. Injection of fluorescently labeled lens culinaris agglutinin (LCA) prior to sacrificing the embryos enables visualization of perfused blood vessels within the organoids through confocal microscopy.

Protocol

In accordance with Dutch law, approval by the animal welfare committee was not required for this research.

1. Preparing hiPSC-derived kidney organoids for transplantation

1. Differentiate hiPSCs to kidney organoids using the protocol developed by Takasato et al.^{4,18,31}. Culture the organoids following this protocol on polyester cell culture inserts with 0.4 μm pores (cell culture inserts) until day 7 + 12 of differentiation. Each cell culture insert will contain three organoids.
2. Remove the organoids from the cell culture insert.
 1. Make a hole in the middle of the membrane of the cell culture insert with a pair of dissecting forceps. Using dissecting scissors, make three cuts in the membrane from the hole in the middle to the edge of the cell culture insert, cutting between the organoids. This will result in three pieces of membrane, each with one organoid attached, which are still connected to the cell culture insert at their outer edge.
 2. Take hold of one of the pieces of membrane close to its outer edge with a pair of forceps and tear it loose from the cell culture insert. Place the piece of membrane with the attached organoid in a Petri dish. Add a few drops of Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium (DPBS^{-/-}) to the organoid to avoid dehydration. Repeat for the other two organoids.
3. Bisect the organoids by holding their membrane in place with forceps and cutting them in half with a double-edge stainless steel razor blade (a whole organoid is too large for the celom to accommodate at this stage of development). Gently push the two organoid halves off the membrane with a dura dissector. Discard the

membrane and leave the organoids in DPBS^{-/-} until transplantation.

NOTE: Prepare a maximum of three organoids at a time for transplantation to avoid stress to the organoids prior to transplantation. Ideally, one person prepares the organoids while another performs the transplantation.

2. Preparing chicken embryos for transplantation

1. Incubate fertilized white leghorn eggs. Start the incubation of the eggs on kidney organoid differentiation day 7 + 8 to ensure the correct timing of the transplantation.

1. Place the fertilized white leghorn eggs (*Gallus domesticus*) horizontally on a holder (**Figure 1A; day 0**), marking the middle of the upward-facing side with a pencil.

NOTE: Either custom-made plastic holders or egg cartons can be used as holders to incubate the eggs.

2. Place the holder with the eggs in a humidified incubator at 38 ± 1 °C (**Figure 1A; day 0**).

3. Incubate the eggs for 3 days, keeping the water basin in the incubator filled.

2. Create a window in the eggshell on day 3 of incubation.

1. On day 3 of incubation, place a small piece (~1 cm x 0.5 cm) of transparent tape on the pointed tip of the egg (small end). Make a small hole in the eggshell in the middle of the transparent tape by tapping it with the sharp end of a pair of dissecting scissors.

2. Insert a 19 G needle on a 5 mL syringe into the hole at a 45° angle, avoiding damage to the yolk sac, and aspirate 2-3 mL of albumen from the egg to lower the embryo inside the egg. Seal the hole with a second piece (~1 cm x 0.5 cm) of transparent tape.

3. Place a large piece (~5 cm x 5 cm) of transparent tape on the pencil-marked, upward-facing side of the egg. Make a hole in the eggshell in the middle of the transparent tape by tapping it with the sharp end of a pair of dissecting scissors (**Figure 1A; day 3**).

4. Starting from this hole, cut a small circular window in the eggshell using curved dissecting scissors. Look through this window to locate the embryo, then enlarge the window to optimize access to the embryo (**Figure 1A; day 3**).

5. Remove any large pieces of eggshell that may have fallen on top of the embryo using forceps. Remove smaller pieces by placing a few drops of DPBS with calcium and magnesium (DPBS^{+/+}) on the embryo with a plastic transfer pipette, then aspirating the DPBS^{+/+} with the eggshell into the pipette.

6. Add three drops of DPBS^{+/+} supplemented with 0.5% penicillin/streptomycin to the egg using a plastic transfer pipette.

7. Carefully seal the window with a large piece (~5 cm x 5 cm) of transparent tape before placing the egg back in the incubator until day 4 of incubation, when the embryo is in Hamburger-Hamilton stage 23-24 (HH 23-24)³².

NOTE: Sealing the window is very important to avoid dehydration and death of the embryo.

8. Check the embryos daily for viability by looking at them through the tape (do not remove the tape to avoid dehydration). During these first days of incubation, the egg yolk color changes from bright to matte yellow upon embryo death. Discard deceased embryos.

9. Keep the water basin in the incubator filled.

3. Intracelomic transplantation on day 4 of incubation

1. Gaining access to the celomic cavity

1. Cut the tape from the window with curved dissecting scissors.
2. Place the egg under a dissecting microscope on a rubber holder or egg carton.
3. The chicken embryo is now in HH 23-24 and lying on its left side, with its right side facing the viewer (**Figure 1A; day 4**). Locate the right wing and leg bud of the embryo, as the celom will be accessed between these two limb buds. In the area between the right wing and leg bud, create an opening consecutively in the vitelline membrane, the chorion, and the amnion, by holding them with two pairs of dissecting forceps and gently pulling them in opposite directions.

NOTE: The vitelline membrane is the first membrane that is encountered after opening the egg, and in some cases, is already damaged after making the window on day 3. If the embryo is rotated, lying on its right side with its left side facing the viewer (**Figure 1A; day 4**), it is necessary to turn it around to enable transplantation. To do this, make a large opening in the vitelline and chorion membrane, then carefully turn the embryo around using forceps.

4. Check whether there is unobstructed access to the celomic cavity: gently take hold of the edge of the body wall between the wing and limb bud with a pair of dissecting forceps and pull it slightly toward the viewer. The celomic cavity must be clearly visible.

Carefully insert a blunt but slim instrument (e.g., a blunt tungsten wire in a microscalpel holder) into the celomic cavity.

NOTE: If insertion of the blunt instrument into the celomic cavity is not possible, it means one or more of the membranes have not been properly opened.

2. Transplantation

1. Place half an organoid inside the egg on top of the allantois using a dura dissector.
2. Using dissecting forceps, carefully take hold of the edge of the body wall and pull it slightly toward the viewer to make the opening to the celom visible.

NOTE: Avoid damaging the blood vessels in the body wall.

3. Gently move the organoid toward and through the opening in the body wall into the celom with a blunt Tungsten wire in a microscalpel holder. Push the organoid slightly cranially to lodge it inside the celom. It is now visible just behind the wing bud (**Figure 1A; day 4**).
 4. Add three drops of DPBS^{+/+} to the egg using a plastic transfer pipette.
 5. Carefully seal the window with a large piece (~5 cm x 5 cm) of transparent tape before placing the egg back in the incubator until day 12 of incubation (8 days after transplantation).
 6. Keep checking the embryos daily for viability by looking at them through the tape (do not remove the tape to avoid dehydration). Discard deceased embryos.
- NOTE:** As the embryos and their chorioallantoic membranes grow, they become more clearly visible through the tape. A lack of movement by the embryo

and collapsed or sparse blood vessels are a sign of embryo death.

7. Check the water basin in the incubator daily and keep it filled.

4. Injection of fluorescently labeled lectin

1. Preparing for injection

1. Make glass microinjection needles by pulling glass microcapillaries in a micropipette puller with the following settings: **Heat 533, Pull 60, Velocity 150, Time 200**. While looking through the dissecting microscope, carefully break the tips off the microinjection needles using dissecting forceps to create an opening.

NOTE: The required settings for the micropipette puller may differ depending on the machine.

2. Assemble the injection system. Take two pieces of 38 cm silicone tubing and connect them to each other by placing a 0.2 μm filter between them. Insert a mouthpiece into the end of the tube that is connected with the filter outlet (silicone tube 1), and a connector to the end of the tube that is connected with the filter inlet (silicone tube 2). Finally, insert the microinjection needle into the connector (**Figure 1B**).
3. Dilute fluorescently labeled lens culinaris agglutinin (LCA) with DPBS^{-/-} to a concentration of 2.5 mg mL⁻¹ in a 0.5 mL tube and spin down for ~30 s in a microcentrifuge to move the aggregates to the bottom of the tube.
4. Pipette 20 μL of LCA onto a piece of parafilm.

5. Aspirate the 20 μL of LCA from the parafilm into the microcapillary needle of the assembled injection system.

2. Injection

1. Cut the tape from the window with curved dissecting scissors. Place the egg under a dissecting microscope in a rubber holder.
2. Evaluate the vasculature and locate the veins, which can be distinguished from the arteries by their slightly brighter red color (**Figure 1A; day 12**). To improve access to the vasculature, carefully enlarge the window by cutting with curved dissecting scissors. Select a vein for injection based on accessibility and size.
3. Insert the tip of the microcapillary needle into the selected vein at a 0°-20° angle. Ensure the needle is in the vein by gently moving the tip from side to side. Gently and steadily blow into the injection system to inject the LCA (**Figure 1A; day 12**).

NOTE: If the needle in the vein is in the correct position, it should stay within the boundaries of the vein.

4. Place the egg back in the incubator for 10 min to let the LCA circulate.

NOTE: It is not necessary to seal the egg with tape during this time.

5. Collecting transplanted organoids on day 12 of incubation

1. Sacrificing the chicken embryo

1. Place the egg in a rubber holder on the bench. Cut the tape from the window with curved dissecting scissors. Then, cut through the

membranes surrounding the embryo with curved dissecting scissors.

NOTE: A dissecting microscope is not required for this step.

2. Scoop the embryo up from the egg with a perforated spoon and immediately decapitate the embryo using scissors. Place the body of the embryo in a Petri dish under the dissection microscope.

2. Locating and collecting organoids

1. Place the embryo on its back in the Petri dish and spread its limbs.
2. Carefully open the abdominal wall of the embryo along the longitudinal axis using forceps.
3. Locate the organoid inside the embryo. The organoid most frequently becomes attached to the right liver lobe, either at the caudal tip or cranially just below the rib cage (**Figure 1A; day 12**). It is therefore recommended to start by looking in these locations.
4. Once the organoid is located, remove it from the embryo by cutting around it with micro scissors. Place the organoid and the chicken tissue that is inevitably attached to it in a Petri dish under the dissecting microscope. Remove as much chicken tissue as possible with a double-edge stainless steel razor blade.
5. Process the organoid depending on the desired analysis.

6. Whole-mount immunofluorescence staining

1. Place a transplanted organoid in a 24-well plate and fix in 500 μ L of 4% paraformaldehyde (PFA) at 4 °C for 24 h. Wash 3x with DPBS^{-/-}.
2. Permeabilize and block the organoid in 300 μ L of blocking solution (0.3% Triton-X in DPBS^{-/-} containing 10% donkey serum) for 2 h at room temperature.
3. Prepare the primary antibody mix: for one organoid, dilute primary antibodies NPHS1 (sheep- α -human, dilution of 1:100), CD31 (mouse- α -human, dilution of 1:100), and LTL (biotin-conjugated, dilution of 1:300) in 300 μ L of blocking solution. Add the antibody mix to the organoid and incubate for 72 h at 4 °C.
4. Wash 3x with 0.3% TritonX in DPBS^{-/-}.
5. Prepare the secondary antibody mix: for one organoid, dilute secondary antibodies donkey- α -sheep Alexa Fluor 647 (dilution of 1:500), donkey- α -mouse Alexa Fluor 488 (dilution of 1:500), and streptavidin Alexa Fluor 405 (dilution of 1:200) in 300 μ L of blocking solution. Add the secondary antibody mix to the organoid and incubate for 2-4 h at room temperature. Cover with aluminum foil to avoid exposure of the secondary antibodies to light.
6. Wash 3x with DPBS^{-/-}.
7. Embed the organoid in ~30 μ L of mounting medium in a 35 mm glass bottom dish and allow to dry overnight at room temperature, covered with aluminum foil. Store at 4 °C.
8. Image using a confocal microscope.

Representative Results

The method and timeline for the differentiation of hiPSCs to kidney organoids, incubation of fertilized chicken eggs, transplantation of kidney organoids, injection of LCA, and collection of the organoids are summarized in **Figure 1A**. It is important to coordinate the timing of organoid differentiation and chicken egg incubation, starting differentiation 15 days before incubation. The actions on day 0, 3, 4, and 12 of incubation are illustrated by photographs below the timeline. Organoids are transplanted at day 7 + 12 of differentiation into day 4 (HH 23-24) chicken embryos. LCA is injected into the venous system of the embryo 8 days after transplantation to stain the perfused vasculature, before sacrificing the embryo

and retrieving the organoid. The assembled injection system is shown in **Figure 1B**.

Chicken embryos are sacrificed on day 12 of incubation (**Figure 1A**). Upon careful dissection of the embryo, the transplanted organoid can usually be found attached to the liver. Looking through a dissection microscope, it appears vascularized (**Figure 1A; day 12; step 5.2.3**). Confocal imaging of transplanted organoids injected with LCA and stained for nephron structures and human ECs confirms vascularization by perfused blood vessels (**Figure 2A**), which also invade glomerular structures (**Figure 2B**). The vasculature is chimeric: perfused human ECs (CD31+, LCA+), unperfused human ECs (CD31+, LCA-), and perfused chicken-derived ECs (CD31-, LCA+) can be distinguished (**Figure 2A, panels III, IV, V**).

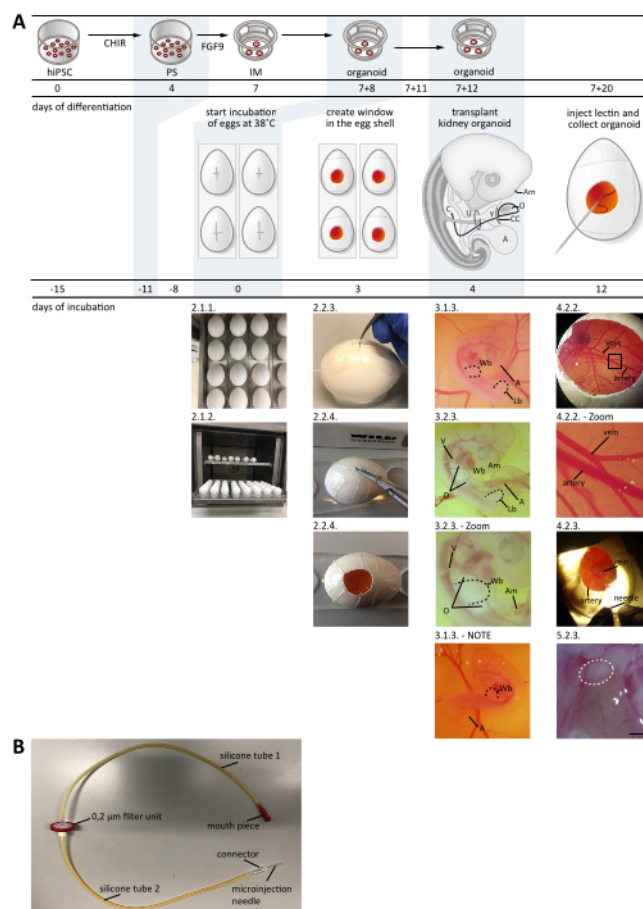


Figure 1: Intracelomic transplantation method. (A) Timeline of the differentiation of hiPSCs to kidney organoids, incubation of fertilized chicken eggs, and intracelomic transplantation of kidney organoids. Differentiation of hiPSCs to kidney organoids is initiated 15 days before incubation of the chicken eggs is started (differentiation day 0 = incubation day -15) to enable transplantation of the kidney organoids on day 7 + 12 of differentiation in chicken embryos on day 4 of incubation. Days of incubation: Day 0: Fertilized chicken eggs are positioned horizontally on holders (step 2.1.1), which are placed in an incubator at 38 °C ± 1 °C (protocol step 2.1.2). Day 3: A window is created in each egg by making a small hole in the upward-facing side of the egg with the sharp end of a pair of curved dissecting scissors (step 2.2.3) and cutting a circular window starting from this hole (step 2.2.4). The window is sealed with transparent tape before placing the egg back in the incubator. Day 4: Intracelomic transplantation of kidney organoids on day 7 + 12 of differentiation is performed. The embryo is in HH 23-24 and lying on its left side, with its right side facing the viewer (step 3.1.3). Between the wing and leg bud, an opening is made in the vitelline membrane, chorion, and amnion to obtain access to the celomic cavity, and the organoid is inserted through these openings into the celom. After transplantation, the organoid is visible as a white structure located just behind the wing bud. The edges of the opened vitelline and amnion membranes are visible (steps 3.2.3 and 3.2.3-Zoom). In some cases, the embryos are rotated, lying on their right instead of left side (3.1.3 NOTE), and must be turned

around prior to transplantation. Day 12: Fluorescently labeled lectin is injected intravenously. Veins are distinguished from arteries by their color; the blood in the veins is oxygen-rich, coming from the CAM, so they are a slightly brighter red than the arteries that are coming from the embryo (steps 4.2.2., 4.2.2-Zoom, and 4.2.3.). The embryo is sacrificed and the organoid retrieved. The organoid (circled) has become attached to the chicken liver and appears to be vascularized (step 5.2.3). Scale bar = 1 mm. The schematic image depicting the intracelomic transplantation image in the top panel was reprinted with permission from Koning et al.²⁸. **(B)** Image of the assembled injection system, consisting of a mouthpiece, two pieces of 38 cm silicone tubing, a 0.2 μ m filter, a connector, and a glass microinjection needle that was generated by pulling glass microcapillaries in a micropipette puller. Abbreviations: A = allantois; Am = amnion; C = celom; CC = cut chorion membrane; CHIR = CHIR99021; FGF9 = fibroblast growth factor 9; hiPSCs = human induced pluripotent stem cells; IM = intermediate mesoderm; Lb = leg bud; O = organoid; PS = primitive streak; U = umbilical ring; V = vitelline membrane; Wb = wing bud; Y = yolk stalk. [Please click here to view a larger version of this figure.](#)

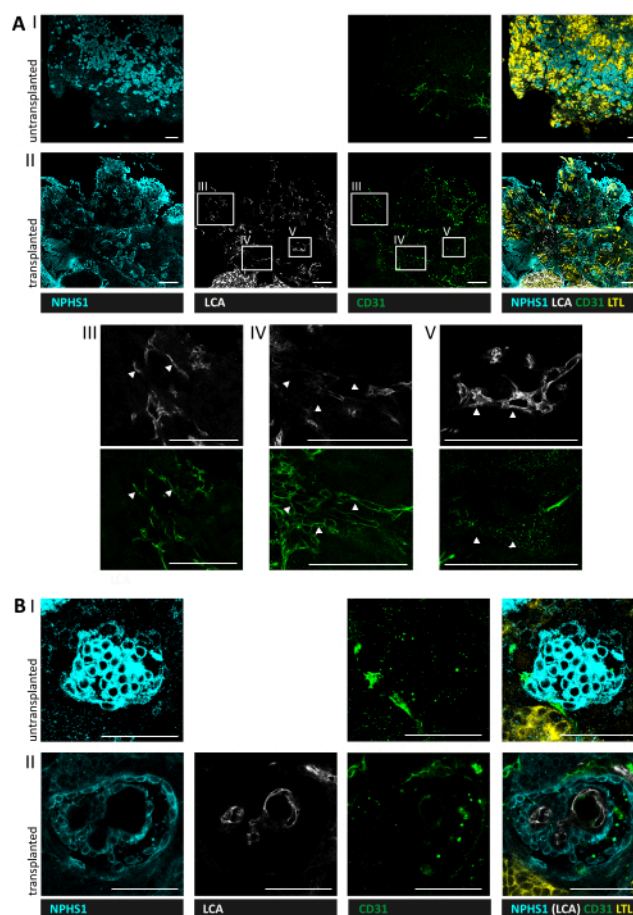


Figure 2: Vascularized transplanted kidney organoids. (A) Immunofluorescent images of (I) an untransplanted kidney organoid and (II) a transplanted kidney organoid. In both conditions, glomerular (NPHS1+, cyan) and tubular (LTL+, yellow) structures are visible. In the untransplanted organoids, some human ECs (CD31+, green) are present. In the transplanted organoid, a perfused vascular network (CD31+, green; injected rhodamine-labeled LCA+, white) is visible throughout the organoid. In panels III, IV, and V, magnifications of the boxed areas in panel II are shown, to demonstrate the three types of ECs that can be distinguished in transplanted organoids. Panel III contains perfused human ECs (CD31+, LCA+), marked with arrowheads. Panel IV contains unperfused human ECs (CD31+, LCA-), marked with arrowheads. Panel V contains perfused chicken-derived ECs (CD31-, LCA+), marked with arrowheads. Scale bar = 200 μm . (B) In untransplanted organoids (I), ECs (CD31+, green) surround glomerular structures (NPHS1+, cyan) but do not invade them. In transplanted organoids (II), glomerular structures (NPHS1+, blue) are vascularized by perfused capillaries (LCA+, white; CD31+, green). Scale bar = 50 μm . [Please click here to view a larger version of this figure.](#)

Discussion

In this manuscript, a protocol for intracelomic transplantation of hiPSC-derived kidney organoids in chicken embryos is demonstrated. Upon transplantation, organoids are vascularized by perfused blood vessels that consist of a combination of human organoid-derived and chicken-derived ECs. These are spread throughout the organoid and invade the glomerular structures, enabling interaction between the ECs and podocytes. It was previously shown that this leads to enhanced maturation of the organoid glomerular and tubular structures²⁸. The transplantation is very efficient, taking ~5 min per embryo, and the only maintenance that the embryos require is a regular refill of the water basin in the incubator. This method is therefore very suitable for the analysis of large numbers of organoids.

Vascularization and maturation of kidney organoids have been previously demonstrated through transplantation in mice^{18,20}. In these labor-intensive mouse models, organoids were transplanted for 2 to up to 12 weeks. In the intracelomic transplantation experiments shown here, the duration of transplantation was limited to 8 days. This allows for the sacrifice of the embryos before day 13 of incubation, when they are thought to start experiencing pain^{33,34}. Since chicken embryos hatch on day 21, the duration of transplantation could be extended to 15 days, sacrificing the embryos on day 19 to avoid hatching. This would, however, require the use of anesthetics. Despite the relatively short transplantation duration in this model, it induces extensive vascularization and significant maturation of organoid nephrons compared to *in vitro* organoids, including the formation of a GBM between organoid podocytes and the invading ECs²⁸.

When performing intracelomic transplantation experiments, it is important to consider that not all chicken embryos develop normally and survive. Usually, ~65% of the embryos placed in the incubator at day 0 reach the end point of the experiment. This is due to a combination of acute bleeding caused by vessel damage during transplantation (5%-10% in our hands) and the stress that is induced by the windowing and transplantation procedures. When embryos are in an earlier or later stage than HH 23-24, transplantation becomes complicated due to limited space and more extensive vasculature, respectively. If embryos are not in the correct stage at the expected time, this could be due to the temperature of the incubation, as a higher temperature generally leads to faster development.

Moreover, keeping fertilized eggs at room temperature for a prolonged period before incubation induces more variability in development. To avoid this, the temperature of the incubator must be kept stable throughout and between experiments, and incubation should be started within 3 days after delivery of the fertilized eggs. Unexpectedly high percentages of embryo death between procedures can be caused by dehydration. To avoid this, it is essential to add two or three drops of DPBS⁺ to each egg after opening it and after transplantation and seal the egg very carefully with tape, smoothing out creases in the tape as much as possible. Combining the windowing and transplantation steps to reduce the number of times the eggs are opened is not recommended, as windowing on day 4 considerably increases embryo death. This is the result of damage to blood vessels that have frequently become attached to the eggshell at this stage.

In conclusion, this method provides an efficient and powerful tool to induce vascularization and enhance maturation in kidney organoids. It can be used to study these processes in

large numbers of organoids and has potential for the modeling of renal diseases that require a higher level of maturation than can currently be acquired *in vitro*.

Disclosures

The authors have no conflicts of interest to disclose.

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