

Video Article

Isolation of Embryonic Tissues and Formation of Quail-Chicken Chimeric Organs Using The Thymus Example

Marta Figueiredo¹, Hélia Neves¹

¹Instituto de Histologia e Biologia do Desenvolvimento, Faculdade de Medicina, Universidade de Lisboa

Correspondence to: Hélia Neves at helia.neves@campus.ul.pt

URL: <https://www.jove.com/video/58965>

DOI: [doi:10.3791/58965](https://doi.org/10.3791/58965)

Keywords: Embryonic tissue isolation, 3D-preserved tissues, *in vitro* organotypic assay, quail-chicken chimeric organ, thymus

Date Published: 12/17/2018

Citation: Figueiredo, M., Neves, H. Isolation of Embryonic Tissues and Formation of Quail-Chicken Chimeric Organs Using The Thymus Example. *J. Vis. Exp.* (), e58965, doi:10.3791/58965 (2018).

Abstract

The capacity to isolate embryonic tissues was an essential step for establishing the quail-chicken chimera system, which in turn has provided undisputed contributions to unveiling key processes in developmental biology.

Herein is described an optimized method to isolate embryonic tissues from quail and chickens by microsurgery and enzymatic digestion while preserving its biological properties. After isolation, tissues from both species are associated in an *in vitro* organotypic assay for 48 h. Quail and chicken tissues can be discriminated by distinct nuclear features and molecular markers allowing the study of the cellular cross-talk between heterospecific association of tissues. This approach is, therefore, a useful tool for studying complex tissue interactions in developmental processes with highly dynamic spatial modifications, such as those occurring during pharyngeal morphogenesis and the formation of the foregut endoderm-derived organs. This experimental approach was first developed to study the epithelial-mesenchymal interactions during early-stages of thymus formation. In this, the endoderm-derived prospective thymic rudiment and mesoderm-derived mesenchyme, were isolated from quail and chicken embryos, respectively.

The capacity of the associated tissues to generate organs can be further tested by grafting them onto the chorioallantoic membrane (CAM) of a chicken embryo. The CAM provides nutrients and allows gas exchanges to the explanted tissues. After 10 days of *in ovo* development, the chimeric organs can be analyzed in the harvested explants by conventional morphological methods. This procedure also allows studying tissue-specific contributions during organ formation, from its initial development (*in vitro* development) to the final stages of organogenesis (*in ovo* development).

Finally, the improved isolation method also provides three-dimensionally (3D) preserved embryonic tissues, that can also be used for high-resolution topographical analysis of tissue-specific gene-expression patterns.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58965/>

Introduction

In the early 1970s, an elegant quail-chicken chimera system was developed by Le Douarin, opening new avenues to understand the role of cell migration and cellular interactions during development^{1,2}. The model was devised on the premise that cell exchange between the two species would not significantly disturb embryogenesis, later confirmed when used to study numerous developmental processes, including the formation of the nervous and the hematopoietic systems¹. Taking the latter as an example, the cyclic waves of hematopoietic progenitors colonizing the thymic epithelial rudiment was first observed using the quail-chicken chimera system³. For that, the prospective territory of the thymus, the endoderm of the third and fourth pharyngeal pouches (3/4PP), was mechanically and enzymatically isolated from quail (q) embryos at 15 to 30-somite stage [embryonic day (E) 1.5- E2.5]. These stages correspond to chicken Hamburger and Hamilton⁴ (HH) - stages 12-17. The isolation procedures started with the use of trypsin to enzymatically dissociate the endoderm from the attached mesenchyme. The isolated endoderm was grafted into the somatopleura region of a chicken (c) host embryos E3-E3.5 (HH-stages 20-21). This heterologous mesenchyme was considered "permissive" to thymic epithelium development contributing also to the organ formation³. Afterward, successive waves of chicken host blood-borne progenitor cells infiltrated the quail donor thymic epithelial counterpart contributing to thymus formation in the host embryo³.

More recently, a modified version of this approach was also proven to be important for studying epithelial-mesenchymal interaction during early-stages of thymus formation⁵. In this respect, the tissues involved in the formation of the ectopic thymus in chimeric embryos³ were isolated, both from donor and host embryos, and associated *ex vivo*. An improved protocol was used to isolate the quail 3/4PP endoderm (E2.5-E3) and the chicken somatopleura mesoderm (E2.5-E3). Briefly, embryonic tissues were isolated by microsurgery and subject to *in vitro* pancreatin digestion. Also, the conditions of enzymatic digestion, temperature and time of incubation were optimized according to tissue-type and developmental stage (Table 1).

Next, the isolated tissues were associated in an organotypic in vitro system for 48 h, as previously reported^{5,6}. The in vitro association of tissues mimics the local cellular interactions in the embryo, overcoming some restrictions of the in vivo manipulation. This system is particularly useful for study of the cellular interactions in complex morphogenic events, such as the development of the pharyngeal apparatus.

The contribution of each tissue in thymus histogenesis, as well as the ability of the heterospecific association to generate a thymus can be further explored using the CAM methodology, previously detailed^{5,7,8}. Succinctly, the cultured tissues were grafted onto the CAM of cE8 embryo and allowed to develop in ovo for 10 days. Then, thymus formation was evaluated by morphological analysis in the harvested explants. As in the classical quail-chicken studies³, the quail thymic epithelium was colonized by hematopoietic progenitor cells (HPCs) derived from the chicken embryo, which was later shown to contribute to organ development^{9,10}. The HPCs migrated from the embryo to the ectopic chimeric thymus through the highly vascularized CAM^{5,7,8}. Quail derived thymic epithelium can be identified by immunohistochemistry using species-specific antibodies (i.e., QCPN- MAb Quail PeriNuclear), overcoming the need for tissue-specific molecular markers.

This experimental method, as the two-step approach reported in previous publication⁸, allows the modulation of signalling pathways by regular administration of pharmacological agents during in vitro and in ovo development. Also, explants can be harvested at any time-point of the course of the experiment⁹.

Lastly, the isolation protocol here detailed allows the preservation of the natural properties and 3D-architecture of embryonic tissues, particularly useful for detailing in situ gene-expression patterns of embryonic territories otherwise inaccessible by conventional methods. In addition, transcriptome analysis approaches, including RNA-seq or microarrays, can also be applied in isolated tissues without requiring genetic markers while providing a tissue-specific high throughput "omics" analysis.

Protocol

All these experiments follow the animal care and ethical guidelines of the Centro Académico de Medicina de Lisboa.

1. Fertilized quail and chicken egg incubation

1. Place fertilized eggs of Japanese quail (*Coturnix coturnix japonica*) in a 38 °C humidified incubator for 3 days. Incubate the eggs (egg blunt end) facing up in the air chamber.
NOTE: The humidified environment is achieved by placing a water container at the bottom of the incubator.
2. Incubate fertilized eggs of chicken (*Gallus gallus*) for 2.5 days in a 38 °C humidified incubator. Incubate the eggs in a horizontal position and mark the upper side using a piece of charcoal to identify the embryo location.
NOTE: Start with 40 quail eggs and 60 chicken eggs when establishing this experiment.

2. Isolation of quail endoderm containing the prospective domain of the thymic rudiment

NOTE: Use a horizontal laminar flow hood and sterilized instruments and materials for egg manipulation procedures in sterile conditions.

1. Remove the embryonic region containing the presumptive territory of thymic rudiment, the pharyngeal arch region containing the 3rd and 4th arches (3/4PAR), as described^{7,8}.
 1. Fill a large borosilicate glass bowl (100 mm x 50 mm; 100 cm³) with 60 mL of cold phosphate-buffered saline solution (PBS).
 2. With the help of curved scissors, tap and cut a circular hole in the shell of a quail egg that has been incubated for 3 days. Make the hole on the opposite side of the egg blunt and transfer the yolk (with the embryo) to the bowl with cold PBS.
 3. Remove the embryo from the yolk by cutting the vitelline membrane externally to extra-embryonic vessels using curved scissors.
 4. With the help of thin forceps, transfer the embryo to a small bowl (60 mm x 30 mm; 15 cm³) filled with 10 mL of cold PBS.
 5. With a skimmer, move the embryo to a 100 mm Petri dish with a black base (see **Table of Materials**) containing 10 mL of cold PBS and place it under a stereomicroscope.
 6. Dissect the 3/4PAR, as previously described⁸.
 7. Aspirate the 3/4PAR and transfer to a glass dish three-quarters filled with cold PBS using a 2 mL sterile Pasteur pipette.
2. Isolate the endoderm containing the presumptive territory of thymic rudiment (the 3/4PP endoderm) by enzymatic digestion with pancreatin.
 1. With the help of spatula and thin forceps, transfer the 3/4PAR to a glass dish three-quarters filled with cold pancreatin (8 mg/mL; 1:3 dilution of 25 mg/mL with cold PBS).
 2. Incubate for 1 h on ice for enzymatic digestion.
NOTE: The time of enzymatic digestion depends of the stage of development (**Table 1**).
 3. Place the glass dish under the stereomicroscope (40x-60x magnification) to isolate the endoderm from the 3/4PAR.
NOTE: Keep all surfaces and solutions cold during this procedure. Change to a new cold pancreatin solution if taking a long time to dissect the tissues (>15 min). As an illumination source, use LED lights incorporated in the stereomicroscope or in the optic fibers, considering the limited heat load.
 4. To isolate the endoderm from the surrounding tissues, use two stainless steel microscalpels in pin holders.
NOTE: Use microscalpels with a diameter between 0.1 mm and 0.2 mm and nickel pin holders with a jaw opening diameter of 0 mm to 1 mm.
 1. First remove the neural tube and mesoderm attached to the dorsal surface of the pharyngeal endoderm.
 2. With the dorsal side up, carefully detach and remove the mesenchyme between the pharyngeal arches and expose the pharyngeal pouches. Perform this procedure on both sides of the 3/4PAR.
 3. Remove the heart tube and the mesenchyme surrounding the anterior pouches.
 4. With the ventral side up, cut the ectoderm of the 2nd and 3rd pharyngeal arches and carefully remove the mesenchyme attached to the pouches. Repeat this procedure on the other side of the 3/4PAR. At this stage the thyroid rudiment should be visible.

5. Remove any remaining mesenchymal cells attached to the pharyngeal endoderm with the two microscalpels.
 6. Make a transversal cut between the 2nd and 3rd PP, dissociating the pharyngeal endoderm containing the 3rd and 4th pouches from the anterior part of the endoderm having the thyroid rudiment and 2nd pharyngeal pouch.
 7. With the help of spatula and thin forceps, transfer the isolated 3/4PP endoderm to a glass dish three-quarters filled with 100% cold fetal bovine serum (FBS).
3. Keep the glass dish with the isolated tissues on ice during the preparation of in vitro assay. Alternatively, the isolated tissues can be three-dimensionally preserved and in situ analyzed for gene-expression.

3. Isolation of chicken somatopleura mesoderm

NOTE: Perform egg manipulation procedures in sterile conditions using a horizontal laminar flow hood and sterilized instruments and materials.

1. Remove the embryonic territory containing the somatopleura mesoderm at the level of somites 19-24 (ss19-24).
 1. Remove the chicken egg from the incubator after 2.5 days of incubation.
 2. With curved scissors, open a small hole in the shell. Insert a needle and aspirate 2 mL of albumin with a 10 mL syringe to lower albumin volume inside the egg and prevent damage of the embryo (located below the marked region of the shell). Discard the aspirated albumin.
 3. Cut a circular hole (up to two-thirds of the top surface area) in the marked region of the shell using curved scissors.
 4. Cut the vitelline membrane externally to the extraembryonic vessels while holding the embryo with thin forceps.
 5. Under a stereomicroscope, place the embryo in a 100 mm Petri dish with a black base containing 10 mL of cold PBS.
NOTE: Use a stereomicroscope from this point forward for progressive magnification of microsurgery procedures.
 6. Use four thin insect pins to hold the embryo to the bottom of the plate. Place the pins in the extraembryonic region forming a square shape.
 7. Perform two cuts between the somites 19 and 24 transversely to the embryo axis and crossing all embryo territory, using wecker eye scissors.
 8. Release the embryo section, ss19-24, by cutting marginal embryonic edges.
 9. Aspirate the ss19-24 tissues and transfer to a glass dish three-quarters filled with cold PBS using a 2 mL sterile Pasteur pipette.
2. Isolate the lateral mesoderm from somatopleura region (ss19-24) by enzymatic digestion with pancreatin (8 mg/mL; 1:3 dilution of 25 mg/mL with cold PBS).
 1. With the help of spatula and thin forceps, transfer the ss19-24 tissues to a glass dish three-quarters filled with cold pancreatin solution.
 2. Incubate for 30 min on ice for enzymatic digestion.
 3. Under the stereomicroscope, isolate the mesoderm from the surrounding tissues using two microscalpels in a holder.
NOTE: Keep all surfaces and solutions cold during this procedure. Change to a new cold pancreatin solution if taking a long time to dissect the tissues (>10 min.). As an illumination source, use LED lights incorporated in the stereomicroscope or in the optic fibers, considering the limited heat load.
 4. During mesoderm isolation, first remove the ectoderm at the surface followed by careful detachment of the ventrally located splanchnopleura tissues.
 5. Release the right lateral mesoderm of the somatopleura by cutting it in a parallel motion to the neural tube.
 6. Repeat the mesoderm separation of the left side of the embryo.
NOTE: Make slow microscalpel movements during this procedure. The exposed extra-cellular matrix proteins stick to tissues and instruments preventing fluid movements.
 7. With the help of spatula and thin forceps transfer the isolated mesoderm to a glass dish three-quarters filled with cold FBS.
3. Keep the glass dish with the isolated tissues on ice during the preparation of in vitro assay.

4. In vitro organotypic assay: heterospecific association of quail 3/4PP endoderm and chicken somatopleura mesoderm

1. Prepare the culture medium with RPMI-1640 medium supplemented with 10% FBS and 1% Pen/Strep^{3,5}.
2. Place a metal grid in a 35 mm Petri dish with 5 mL of culture medium.
NOTE: Remove the excess of liquid to level the medium surface with the top of the grid.
3. With the help of thin forceps, dip a membrane filter into the culture medium and then place it on the top of the grid to have one surface in contact with air.
NOTE: One-quarter of the membrane area (with 13 mm diameter) is adequate for the tissue association.
4. Under the stereomicroscope, associate the isolated tissues on the top of the membrane filter. First transfer the 3/4PP endoderm (step 2) from the glass dish by gentle sliding with the help of a transplantation spoon (or spatula) and thin forceps. Repeat this procedure for the isolated mesoderm (step 3).
NOTE: With the help of a microscalpel, mix the tissues to maximize its association.
5. Carefully place the associated tissues in a humidified incubator at 37 °C with 5% CO₂ for 48 h. Cultured tissues can be grafted onto the chorioallantoic membrane (CAM).
NOTE: Ectopic organ formation in the CAM was previously detailed⁸.

Representative Results

The protocol details a method to isolate avian embryonic tissues to be used in several cellular and developmental biology technical approaches. This method was previously employed to study epithelial-mesenchymal interaction during early stages of thymus formation⁵. Herein, new results are shown in **Figure 1** and **Figure 2**, using similar approaches.

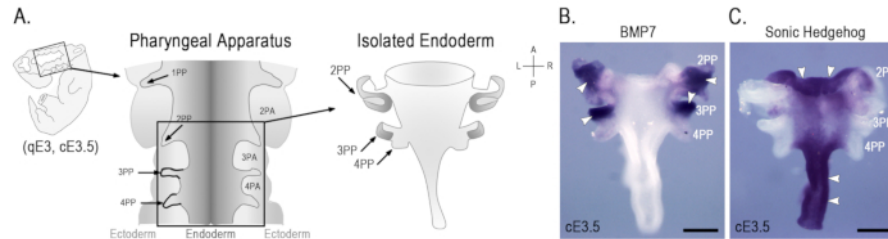


Figure 1. Representative results of gene-expression study of three-dimensionally preserved pharyngeal endoderm containing the presumptive territory of the thymus rudiment. Schematic representation of the pharyngeal apparatus and isolated endoderm containing the 2PP, 3PP and 4PP (at cE3.5 or qE3) (**A**). Whole-mount in situ hybridization with BMP7 (**B**) and Sonic Hedgehog (**C**) of isolated endoderm at cE3.5. Strong hybridization signals of BMP7 and Sonic Hedgehog pointed by white arrowheads in endoderm of the 2PP and 3PP (**B**) and central pharynx (**C**), respectively. A, anterior; cE, chicken embryonic day; D, dorsal; P, posterior; PP, pharyngeal pouch; qE, quail embryonic day; V, ventral. Scale bars, 50 μ m. [Please click here to view a larger version of this figure.](#)

Figure 1 is a schematic drawing of the endoderm isolated from the pharynx at qE3 (and cE3.5) (**Figure 1A**) and the in situ expression of two endoderm-related genes, *sonic hedgehog*^{11,12} and *BMP7*¹³ in the isolated tissue. The whole-mount in situ hybridization procedures were performed as previously described^{5,7}. The expression of BMP7 was expressed in the endoderm of the 2PP and 3PP and excluded from the central pharynx and 4PP (probe was kindly provided by Elisabeth Dupin) (**Figure 1B**). Conversely, *sonic hedgehog* was detected in the endoderm of the central pharynx and excluded from the pouches⁷ (**Figure 1C**).

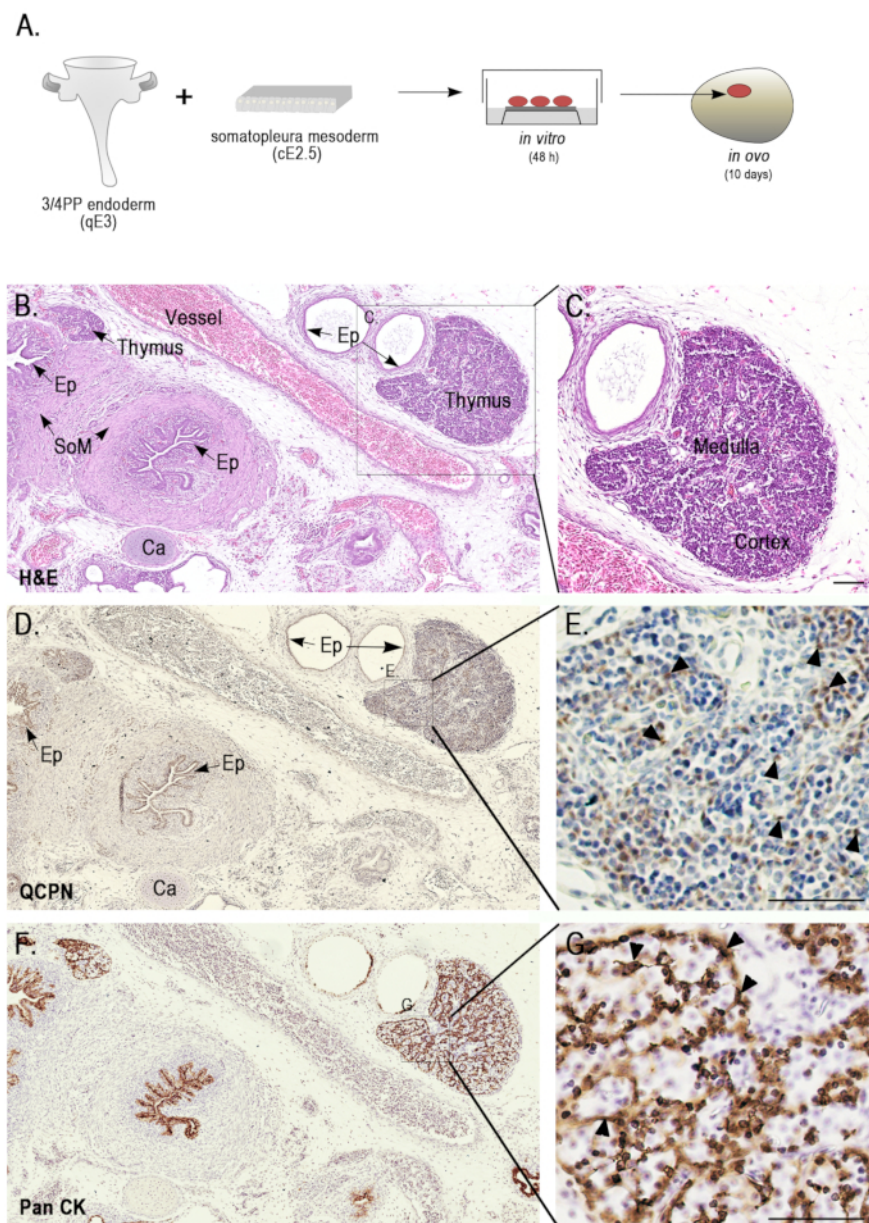


Figure 2. Representative results of ex vivo formation of chimeric organs. Schematic representation of the experimental approach used to develop quail-chicken chimeric thymi (A). Briefly, the isolated quail 3/4PP endoderm (qE3) was associated in vitro with chicken somatopleura mesoderm (cE2.5) for 48 h. The 48 h cultured tissues were then grafted onto the CAM (cE8) and allowed to develop in ovo for further 10 days. Serial sections of CAM-derived explants (B-G) were analyzed by conventional histology (B and C) and immunohistochemistry (D and G). In B and C (higher magnification of B), the slide was stained with H&E. In D and E (higher magnification of D) slide was immunodetected with QCPN antibody and counterstained with Gill's hematoxylin. In F and G (higher magnification of F) slide was immunodetected with anti-Pan CK antibody and counterstained with Gill's hematoxylin. Black arrow heads point to strong brown immunostaining of QCPN (E) and Pan CK (G). See [Table of Materials](#) for image acquisition details. Ca, cartilage; Ep, epithelium; PP, pharyngeal pouch; SoM, smooth muscle. Scale bars: 50 µm. [Please click here to view a larger version of this figure.](#)

Figure 2 depicts the experimental design used to develop ex vivo quail-chicken chimeric organs. The heterospecific association of tissues were grown in vitro for 48 h followed by in ovo development for 10 days (**Figure 2A**). Thymi formed in CAM-derived explants were identified by conventional histology. The thymus presented normal morphological features with well-developed medulla and cortex compartments (**Figure 2B,C**). Serial sections of the explants were further treated for immunocytochemistry (**Figure 2D-G**), as described^{5,7}. The QCPN- MAb Quail perinuclear (**Figure 2D,E**) and anti-pan cytokeratin (CK) (**Figure 2F,G**) antibodies were used as markers for quail (species-specific) and epithelial cells, respectively. The chimeric thymus showed QCPN⁺ thymic epithelial cells (**Figure 2D,E**), with reticular architecture (**Figure 2F,G**), and colonized by lymphoid cells (QCPN⁻) of donor origin (chicken).

Isolated tissue	Stage of development	Concentration	Temperature	Incubation period
Pharynx endoderm	q E2 - E2.5	8 mg/mL	On ice	45 – 60 min.
	c E2.5 - E3			
	q E3	8 mg/mL	On ice	60 - 90 min.
	c E3.5			
	q E4	8 mg/mL	On ice	90 min.
	c E4.5			
Somatopleura mesoderm	q E2 - E2.5	8 mg/mL	On ice	30 – 50 min.
	c E2.5 - E3			

c, chicken; E, Embryonic day; q, quail.

Table 1: Conditions of enzymatic digestion during embryonic tissues isolation.

Discussion

The embryonic tissue isolation procedure detailed here was improved from previous techniques to produce quail-chicken chimeric embryos in different biological contexts^{3,5,6}.

This approach is suitable to isolate pure embryonic tissues without requiring genetic manipulation or the use of tissue-specific markers, often limited in genetically modified animal models. It can be used to study epithelial-mesenchymal interactions during development, with the ability to isolate pure tissues being the limiting factor. For instance, as development progresses, tissues become thicker, more compact and attach to other neighboring tissues such that their separation is more difficult. This isolation procedure is, therefore, unsuitable for later stages of development, namely late-organogenesis.

This method is unique to study gene-expression in 3D-preserved embryonic tissues. To ensure the 3D-integrity of the isolated tissues, instruments, materials and solutions should be kept at low temperatures throughout the process.

The tissue microdissection procedure is also a critical step that relies, not only on the careful establishment of the experimental conditions (like temperature and duration of enzymatic digestion, as exemplified in **Table 1**), but also on the time-consuming hands-on training. This procedure requires patience and practice. If the operator loses the references of the region to be dissected, decreasing the stereoscope magnification (20x) will provide an overall observation that will help the next move decision.

The 48 h in vitro step was established to promote the cellular interactions between distinct embryonic tissues, while the in ovo tissue grown in the CAM supports the long-term development and chimeric organ formation of the heterospecific association of tissues⁵. The in vitro tissues associations may overcome some limitations of in vivo manipulations. For instance, local administration of drugs or growth-factors (using beads) in regions of the embryo otherwise inaccessible in vivo, can be easily performed using this in vitro approach. This has previously shown to mimic local tissue interactions during organ formation in the pharyngeal region⁵.

Harvesting explants growing in CAM^{5,7,8} is less time-consuming and is a simple method to track explants when compared to methods of collecting tissues grafted onto the body wall of chimeric embryos³. In addition, CAM can be transplanted with cells and tissues from other non-avian species, and it has been successfully used in several experimental contexts, from development to cancer^{14,15}. For example, the CAM assay was previously applied in mice-into-chicken xenografts studies¹⁶ and is frequently used to test the invasive capacity of human tumors cells¹⁵.

Recently, an elegant study with human-into-chicken xenograft has validated the chicken embryo as a model to test and explore early human development¹⁷. In the future, it will be interesting to explore the methodology herein described using interspecies association of tissues, which may provide additional approaches to the mouse and human developmental studies.

Disclosures

The authors have nothing to disclose.

Acknowledgements

The authors are grateful to Isabel Alcobia for the critical reading of the manuscript, to Mário Henriques for video narration and to Vítor Proa from the Histology Service of the Instituto de Histologia e Biologia do Desenvolvimento, Faculdade de Medicina de Lisboa, Universidade de Lisboa, for technical support. We are particularly indebted to Paulo Caeiro and Hugo Silva from the Unidade de audiovisuais (Audiovisual Unit), Faculdade de Medicina de Lisboa, Universidade de Lisboa for their outstanding commitment to the production of this video. We acknowledge Leica Microsystems for kindly providing a stereoscope equipped with a video system and to Interaves - Sociedade Agro-Pecuária, S.A for contributing with quail fertilized eggs. This work was supported by Faculdade de Medicina de Lisboa, Universidade de Lisboa (FMUL).

References

1. Le Douarin, N. The Nogent Institute--50 years of embryology. *The International Journal of Developmental Biology*. **49** (2–3), 85–103 (2005).
2. Le Douarin, N. M., Teillet, M. a The migration of neural crest cells to the wall of the digestive tract in avian embryo. *Journal of Embryology and Experimental Morphology*. **30** (1), 31–48 (1973).
3. Le Douarin, N. M., Jotereau, F. V. Tracing of cells of the avian thymus through embryonic life in interspecific chimeras. *Journal of Experimental Medicine*. **142** (1), 17–40 (1975).
4. Hamburger, V., Hamilton, H. L. A series of normal stages in the development of the chick embryo. 1951. *Developmental Dynamics*. **195** (4), 231–272 (1992).
5. Neves, H., Dupin, E., Parreira, L., Le Douarin, N. M. Modulation of Bmp4 signalling in the epithelial-mesenchymal interactions that take place in early thymus and parathyroid development in avian embryos. *Developmental Biology*. **361** (2), 208–219 (2012).
6. Takahashi, Y., Bontoux, M., Le Douarin, N. M. Epithelio–mesenchymal interactions are critical for Quox 7 expression and membrane bone differentiation in the neural crest derived mandibular mesenchyme. *Embo Journal*. **10** (9), 2387–2393 (1991).
7. Figueiredo, M. et al. Notch and Hedgehog in the thymus/parathyroid common primordium: Crosstalk in organ formation. *Developmental Biology*. **418** (2), 268–282 (2016).
8. Figueiredo, M., Neves, H. Two-step Approach to Explore Early-and Late-stages of Organ Formation in the Avian Model: The Thymus and Parathyroid Glands Organogenesis Paradigm Video Link. *Journal of Visualized Experiments*. (2018).
9. Nehls, M. et al. Two genetically separable steps in the differentiation of thymic epithelium. *Science (New York, N.Y.)*. **272** (5263), 886–889 (1996).
10. Morahan, G. et al. The nu gene acts cell-autonomously and is required for differentiation of thymic epithelial progenitors. *Proceedings of the National Academy of Sciences of the United States of America*. **93** (12), 5742–5746 (1996).
11. Jerome, L. A., Papaioannou, V. E. DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1. *Nature Genetics*. **27** (3), 286–291 (2001).
12. Nie, X., Brown, C. B., Wang, Q., Jiao, K. Inactivation of Bmp4 from the Tbx1 expression domain causes abnormal pharyngeal arch artery and cardiac outflow tract remodeling. *Cells Tissues Organs*. **193** (6), 393–403 (2011).
13. Zou, D. et al. Patterning of the third pharyngeal pouch into thymus/parathyroid by Six and Eya1. *Developmental Biology*. **293** (2), 499–512 (2006).
14. Davey, M. G., Tickle, C. The chicken as a model for embryonic development. *Cytogenetic and Genome Research*. **117** (1–4), 231–239 (2007).
15. Nowak-sliwinska, P., Segura, T., Iruela-arispe, M. L., Angeles, L. The chicken chorioallantoic membrane model in biology, medicine and bioengineering. *Angiogenesis*. **17** (4), 779–804 (2014).
16. Uematsu, E. et al. Use of in ovo chorioallantoic membrane engraftment to culture testes from neonatal mice. *Comparative Medicine*. **64** (4), 264–269 (2014).
17. Martyn, I., Kanno, T. Y., Ruzo, A., Siggia, E. D., Brivanlou, A. H. Self-organization of a human organizer by combined Wnt and Nodal signaling. *Nature*. **558** (7708), 132–135 (2018).